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Trace metals (Cu, Zn, Cd and Pb) in juvenile fish from estuarine nurseries along the Portuguese coast

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SUMMARY: Organic and inorganic pollution can impact organisms directly and affect condition, growth and survival of juvenile fish which use estuaries as nurseries, and thereby affect marine adult populations quantitatively and qualitatively. Trace element contamination (Cu, Zn, Cd, Pb) in juveniles of commercial fish *Solea solea, Solea senegalensis, Platichthys flesus, Diplodus vulgaris* and *Dicentrarchus labrax* collected in putative nurseries of the main Portuguese estuaries (with diverse intensities and sources of anthropogenic pressures) was determined via atomic absorption spectrometry. Contamination was significantly different among species. Similar levels of contamination were found among estuaries, except for *D. vulgaris*. Cu and Zn concentrations ranged from 1.0 to 2.1 and 14 to 59 μg g^{-1} muscle dry weight respectively; while Cd and Pb concentrations were very low. The results indicate that juvenile migration to off-shore habitats is associated with low export of contamination, and no particular estuary increases the potential contamination of adult stocks. This knowledge is of the utmost importance in view of the ecological and economical value of these species and their use of estuarine areas as nurseries.

Keywords: juveniles, nursery grounds, populations, anthropogenic effects, heavy metals.

RESUMEN: METALES TRAZA (CU, ZN, CD Y PB) EN PECES JUVENILES DE LAS GUARDERÍAS ESTUÁRICAS A LO LARGO DE LA COSTA PORTUGUESA. – La contaminación orgánica e inorgánica puede afectar a los organismos, a saber al estado fisiológico, crecimiento y supervivencia de los peces juveniles los cuales utilizan los estuarios como guarderías; afectando de forma cuantitativa y cualitativa a la poblaciones marinas adultas. La contaminación de elementos traza (Cu, Zn, Cd, Pb) en las formas juveniles de los peces comerciales *Solea solea, Solea senegalensis, Platichthys flesus, Diplodus vulgaris* y *Dicentrarchus labrax* recolectados en guarderías putativas de los principales estuarios portugueses (con diversos tipos e intensidades de presiones antropogénicas) fueron determinados por espectrometría de absorción atómica. La contaminación era significativamente diferente entre especies. Sobre todo se encontraron niveles similares de contaminación entre estuarios, excepto para *D. vulgaris*. Las concentraciones de Cu y Zn oscilaban desde 1.0 a 2.1 y desde 14 a 59 μg g^{-1} peso seco muscular, respectivamente, mientras que las concentraciones de Cd y Pb eran muy bajas. Los resultados indican que la migración de los juveniles a mar abierto está asociada a la baja exportación de la contaminación y que los estuarios no amplifican particularmente el potencial de contaminación de los adultos. En vista del valor ecológico y económico de estas especies y del uso de las zonas estuáricas como guarderías, este conocimiento es de vital importancia.

Palabras clave: juveniles, guarderías, poblaciones, efectos antropogénicos, metales pesados.

INTRODUCTION

Individual contamination by inorganic and organic pollutants represents one of the most direct impacts on biota out of the multitude of anthropogenic disturbances in coastal and estuarine systems (Goldberg, 1995; Kennish, 2002; Vasconcelos *et al.*, 2007). Trace metals such as copper, zinc, cadmium and lead (amongst others, e.g. chromium, nickel, silver, mercury, arsenic and selenium) are persistent in aquatic environments and are likely to occur in increased concentrations due to anthropogenic actions (Neff, 2002). Moreover, metals are of great interest as they can result in direct energetic and physiological costs, expressed in decreased growth and low physiological condition, as well as indirect costs due to the many defence mechanisms triggered (Livingstone, 2001; Marchand *et al.*, 2003; van der Oost *et al.*, 2003; Fonseca *et al.*, 2009).

Along the Portuguese coast, the main estuarine systems (Douro, Ria de Aveiro, Mondego, Tejo, Sado, Mira, Ria Formosa and Guadiana) comprise a wide range of hydrological and geomorphologic features, as well as a plethora of anthropogenic activities and associated pressures, which lead to distinct pressure intensities and dominant sources with a wide range of detectable ecological impacts and endpoints (Vasconcelos *et al.*, 2007). These estuarine systems have been characterised as important nursery grounds for several fish species, namely common sole *Solea solea* (Linneaus, 1758), Senegalese sole *Solea senegalensis* Kaup, 1858, flounder *Platichthys flesus* (Linnaeus, 1758), sea breams, such as *Diplodus vulgaris* (Geoffroy Saint-Hilaire, 1817), and sea bass *Dicentrarchus labrax* (Linnaeus, 1758) (Cabral *et al.*, 2007; Vasconcelos *et al.*, 2010). Adults of these species live and spawn offshore whereas juveniles inhabit estuarine nursery areas. In this context, the two environments are linked through the transport of larvae from off-shore areas into estuaries and later through the migration of juveniles off-shore. Juveniles benefit from the favourable conditions (water temperature, food availability and refuge from predators) in estuarine nursery areas which allow for enhanced growth and survival (Gibson, 1994; Beck *et al.*, 2001). However, they also face demanding environmental conditions, both of natural origin, such as salinity variations and the consequent physiological demands, and human origin, such as pollution loads, which degrade habitat quality and may negatively affect individual fitness, maximum growth rates and ultimately survival (Meng *et al.*, 2001; Amara *et al.*, 2007; Le Pape *et al.*, 2007). Variations in habitat quality, growth and survival during early life history stages influence connectivity with adult sub-populations (Gibson, 1994; van der Veer *et al.*, 1994). Therefore, processes that occur in estuaries have consequences at a larger scale (Beck *et al.*, 2001; Able, 2005) and individual contamination becomes of major ecological relevance, as well as economic relevance

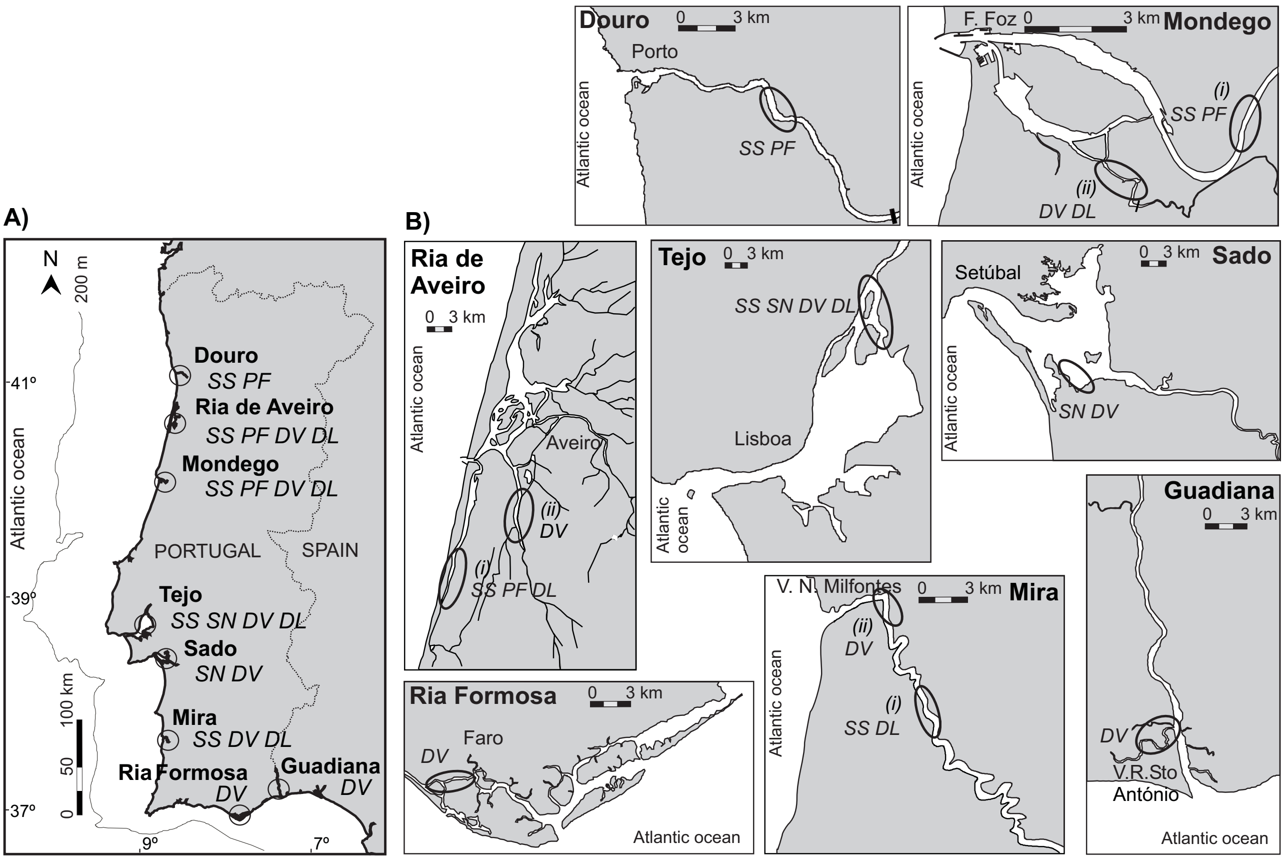

FIG. 1. – Map of Portugal and sampled estuarine nursery sites. A, location of estuarine systems sampled along the Portuguese coast and fish species sampled in each system: *Solea solea* (*SS*), *Solea senegalensis* (*SN*), *Platichthys flesus* (*PF*), *Diplodus vulgaris* (*DV*) and *Dicentrarchus labrax* (*DL*). B, location of sampled sites within each estuary - within the Ria de Aveiro, Mondego and Mira estuaries, distinct nursery areas were sampled for different species and are represented by *i* and *ii*.

TABLE 1. – Mean sediment contamination by trace metals (Cu, Zn, Cd and Pb expressed in µg g^{-1} DW) (and standard deviation, SD), in the fine sediment fraction (<63 µm) of sampled sites within eight estuarine systems located along the Portuguese coast. Sites with samples with concentrations above Effects Range Low (ERL) [i.e. concentrations at which occasional effects on resident organisms occur according to Long *et al*. (1995)] are identified with an asterisk.

Estuary	Cu (µg g^{-1} DW)			Zn (µg g^{-1} DW)			Cd (µg g^{-1} DW)			Pb (µg g^{-1} DW)		
	Mean	SD	ERL	Mean	SD	ERL	Mean	SD	ERL	Mean	SD	ERL
Douro[a, b]	29.7	27.3	*	110	100	*	0.2	0.1		43	42	*
Ria de Aveiro [a, c]	96.0	97.9	*	953	1700	*	2.5	3.7	*	45	26	*
Mondego [a, d]	23.0	20.7		94	82		0.2	0.2		28	17	
Tejo [a, e]	52.6	26.3	*	284	155	*	2.4	2.3	*	43	73	*
Sado [a, f]	117.8	46.5	*	401	172	*	0.5	0.2		401	28	*
Mira [a]	40.7	1.5		109	6		0.3	0.0		24	2	
Ria Formosa [a, g]	26.2	29.7	*	119	83	*	0.3	0.0		23	21	
Guadiana [a, h]	64.0	9.9	*	182	26	*	0.8	0.7	*	26	6	

Data sources: [a] INAG, unpublished data; [b] Vinagre *et al*., 2004; Mucha *et al*., 2005; [c] Monterroso *et al*., 2003; [d] Pereira *et al*., 2005; [e] França *et al*., 2005; Cardoso *et al*., 2008; [f] Caeiro *et al*., 2005; [g] Padinha *et al*., 2000; [h] Ruiz, 2001

since these species represent a high percentage of fish landings value.

The present paper aims to determine trace metal contamination (copper - Cu, zinc - Zn, cadmium - Cd and lead - Pb) in juveniles of five commercially important fish species which use the estuarine systems along the Portuguese coast as nurseries. The information obtained is a direct measure of trace metal pollution, a human generated environmental disturbance widespread in estuaries, in juvenile fish at an individual level. The results provide information on the variability of trace metal pollution amongst these estuarine nurseries. Moreover, present data expand knowledge on the degradation of habitat quality in these systems as well as the expected export of contamination from estuarine to marine habitats, due to the migration of juveniles to off-shore areas from age 0+ onwards.

MATERIALS AND METHODS

Fish collection

Juvenile specimens of *S. solea* (common sole), *S. senegalensis* (Senegalese sole), *P. flesus* (flounder), *D. vulgaris* (common two banded sea bream) and *D. labrax* (sea bass) were collected during July 2005 in eight estuarine systems of the Portuguese coast: Douro, Ria de Aveiro, Mondego, Tejo, Sado, Mira, Ria Formosa and Guadiana (Fig. 1A). Specimens of each species were collected in the estuaries in which they occur (Fig. 1a); specifically, in the main nursery ground for their juveniles as previously determined (Vasconcelos *et al*. 2010) (Fig. 1B). A beam trawl was used to collect fish and ten replicate tows were made per site (tow duration ca. 10 min, trawled area ca. 900 m^2). Sampled estuarine systems differ in their hydrologic and geomorphologic features as well as in anthropogenic pressures (see Vasconcelos *et al.*, 2007) including contamination levels of the sediment (Table 1). Sampling in all estuaries was carried out in the shortest time frame possible (a fortnight) in order to avoid temporal variation in environmental and muscle contamination as well as in the length/age composition of juvenile samples. Upon collection, fish were stored and transported on ice to the laboratory and preserved frozen until dissection.

TABLE 2. – Number (n) and mean total length (mm) (and standard deviation, SD) of juvenile *Solea solea*, *Solea senegalensis*, *Platichthys flesus*, *Diplodus vulgaris* and *Dicentrarchus labrax* sampled in estuaries along the Portuguese coast, and used for trace metal analysis.

Species	n	Length (mm)	
		Mean	SD
Solea solea	46	86.4	5.6
Solea senegalensis	28	83.9	5.5
Platichthys flesus	27	83.5	7.0
Diplodus vulgaris	83	68.9	5.8
Dicentrarchus labrax	68	98.4	20.2

Trace element analysis

In each site ca. 10 individuals of each species were selected for trace metal analysis. These individuals were representative of the length frequency distributions of age-0 juveniles. The total length of each fish was measured (Table 2). For trace element analysis, a muscle sample from each individual, preferably from the dorsal anterior region, was extracted using plastic forceps and stored in a microcentrifuge tube at -20°C. All plasticware, containers and implements used were decontaminated with an acid wash in 10% analytical grade nitric acid (HNO_3) (Merck) for 24 h, rinsed with Normapur water and dried in a laminar flow positive pressure fume hood.

Muscle samples were dried in an oven at 60°C for a minimum period of 24 h, until they reached constant weight. Dry muscle samples were ground manually using a mortar and pestle. A sub-sample of dry muscle of each individual (ca. 0.1 g weighed to the nearest 0.1 mg) was then separated in new microcentrifuge tubes. Whenever the muscle sample of one individual was insufficient, a pooled sample of individuals of similar length was used. Muscle samples were digested with 2 ml of a mixture of nitric and percloric ($HClO_4$) acids (9:1) (suprapure quality, Merck) in individual Teflon reactors in an oven at 110°C during 2 h (adapted from Julshamn *et al.*, 1982). Following a cooling period at room temperature, the extract was filtered (ashless filter papers Whatman 42) and diluted to a total volume of 10 ml with distilled water.

The concentrations of four trace elements (Cu, Zn, Cd and Pb) (expressed in μg element g^{-1} muscle dry weight, DW) were determined with a GBC 932 Plus atomic absorption spectrometer with a GBC GF3000 graphite oven. A reference material for trace metals was analysed for quality control: TORT-2 (Lobster Hepatopancreas - National Research Council Canada, Institute for National Measurement Standards, Ottawa, ON, Canada). The long-term precision results for this material were: Cu (17% RSD - relative standard deviation), Zn (20% RSD), Cd (13% RSD), Pb (6% RSD). The limits of detection for Cu, Zn, Cd and Pb were 0.5 μg g^{-1} DW, 0.05 μg g^{-1} DW, 0.1 μg g^{-1} DW and 0.6 μg g^{-1} DW respectively. Samples were read in triplicate and random order. Standard curves were used to determine Cu and Zn, whereas standard addition procedures were employed to calculate Cd and Pb. Procedural blanks were run for all samples.

Data analysis

Variations in the concentration of the analysed trace elements in juveniles of the five species collected in the eight estuarine systems were analysed using univariate statistical techniques. Raw data for each element were checked for normality and homogeneity of variances, and both assumptions were met. Differences in element concentrations amongst juveniles of different species were tested with analysis of variance (ANOVA), using the overall data set for all the estuaries and data for each estuarine site individually. Differences in juvenile contamination amongst the estuaries were also tested using ANOVA, considering the five species simultaneously and also each species individually. Separate one-way ANOVA, instead of two-way ANOVA, were applied due to inherent species-specific patterns in element contamination and differences in species occurrence amongst the estuaries.

RESULTS

Juveniles of the five analysed fish species showed detectable contamination levels of Cu and Zn in the muscle, and Zn had the highest mean concentrations (Table 3). Cd and Pb concentrations were below detection limits, except in one individual (0.7 μg g^{-1} DW Pb in *S. solea* from the Mira estuary). The concentrations of Cu and Zn differed significantly amongst the analysed species collected in the estuaries: *D. vulgaris* and *D. labrax* showed significantly higher Cu concentrations than *S. solea,* whereas *P. flesus* and *D. labrax* had significantly higher concentrations of Zn than the remaining three species (Tables 3 and 4). Considering each estuarine site individually, significant differences in element concentrations amongst species occurred in some estuaries, namely for Cu in the Mira estuary (site *i*), Zn in Ria de Aveiro (*i*) and Tejo, and for both elements in Douro and Mondego (*i*) (Tables 3 and 5).

Table 3. – Mean concentrations of Cu and Zn (μg element g^{-1} muscle dry weight) (and 95% confidence intervals, CI) in muscle of juvenile *Solea solea*, *Solea senegalensis*, *Platichthys flesus*, *Diplodus vulgaris* and *Dicentrarchus labrax* collected in estuaries along the Portuguese coast: Douro, Ria de Aveiro, Mondego, Tejo, Sado, Mira, Ria Formosa and Guadiana.

Species	Estuary	Cu Mean±CI	Zn Mean±CI
Solea solea	Douro	1.1±0.3	28±2
	Ria de Aveiro	1.1±0.2	25±3
	Mondego	1.0±0.2	26±4
	Tejo	1.2±0.3	24±1
	Mira	1.1±0.3	29±6
Solea senegalensis	Tejo	1.6±0.5	28±3
	Sado	1.4±0.4	27±8
Platichthys flesus	Douro	1.7±0.5	50±12
	Ria de Aveiro	1.3±0.7	59±19
	Mondego	1.6±0.1	44±21
Diplodus vulgaris	Ria de Aveiro	2.0±0.7	14±2
	Mondego	1.5±0.1	34±3
	Tejo	2.0±0.3	34±2
	Sado	1.4±0.3	30±2
	Mira	2.0±0.4	14±2
	Ria Formosa	1.8±0.8	44±5
	Guadiana	1.7±0.2	39±3
Dicentrarchus labrax	Ria de Aveiro	2.1±0.8	33±1
	Mondego	1.8±0.7	35±3
	Tejo	1.7±0.2	34±2
	Mira	1.8±0.4	32±4

Table 4. – Inter-specific variation of trace metal concentration in muscle of juveniles. Results of ANOVA comparisons and respective Tukey post-hoc multiple comparisons tests for Cu and Zn concentrations in muscle of juveniles of five fish species (*SS*, *Solea solea*; *SN*, *Solea senegalensis*; *PF*, *Platichthys flesus*; *DV*, *Diplodus vulgaris;* and *DL*, *Dicentrarchus labrax*) collected in eight estuaries along the Portuguese coast (Douro, Ria de Aveiro, Mondego, Tejo, Sado, Mira, Ria Formosa and Guadiana). * $P<0.05$; *ns*, non-significant; different numbers (superscript) represent significant differences among species at $P<0.05$ in Tukey post-hoc tests.

Element	ANOVA df	*F*	*P*	Tukey post-hoc tests				
Cu	4	6.00	*	SS^{1}	-	-	DV^{2}	DL^{2}
Zn	4	38.45	*	SS^{1}	$SN^{1,3}$	PF^{2}	DV^{1}	DL^{3}

Only *D. vulgaris* had significant variations in element concentrations amongst juveniles collected in different estuaries (Tables 3 and 6). In this species, Cu concentrations were higher in Mira and lower in Sado, whereas Zn concentrations were higher in Ria Formosa and Guadiana and lower in Mira and Ria Aveiro. Considering the five species simultaneously, significant variation in the individual element concentrations amongst estuaries was only observed for Zn, with the highest values in juveniles from the Douro estuary and the lowest in the Mira estuary (Tables 3 and 6).

DISCUSSION

Contamination by trace elements (Cu, Zn, Cd and Pb) in juveniles of five fish species in estuarine nursery areas along the Portuguese coast showed: (1) similar

Table 5. – Inter-specific variation in trace metal concentration in muscle of juveniles within each estuarine site. Results of ANOVA comparisons and Tukey post-hoc multiple comparison tests for Cu and Zn concentrations in muscle of juveniles of five fish species (*SS*, *Solea solea*; *SN*, *Solea senegalensis*; *PF*, *Platichthys flesus*; *DV*, *Diplodus vulgaris;* and *DL*, *Dicentrarchus labrax*) collected at the same site within eight estuaries along the Portuguese coast (Douro, Ria de Aveiro, Mondego, Tejo, Sado, Mira, Ria Formosa and Guadiana). * $P<0.05$; *ns*, non-significant; different numbers (superscript) represent significant differences among species at $P<0.05$ in Tukey post-hoc tests; *na*, post-hoc tests not applicable.

Estuary	Site	Species	Element	ANOVA			Tukey post-hoc
				df	*F*	*P*	
Douro		*SS PF*	Cu	1	8.71	*	*na*
			Zn	1	39.98	*	*na*
R. Aveiro	*i*	*SS PF DL*	Cu	2	1.97	*ns*	*na*
			Zn	2	55.09	*	*SS*^{1} *PF*^{2} *DL*^{3}
Mondego	*i*	*SS PF*	Cu	1	29.18	*	*na*
			Zn	1	8.96	*	*na*
	ii	*DV DL*	Cu	1	0.56	*ns*	*na*
			Zn	1	0.24	*ns*	*na*
Tejo		*SS SN DV DL*	Cu	3	1.94	*ns*	*na*
			Zn	3	13.31	*	*SS*^{1} *SN*^{1} *DV*^{2} *DL*^{2}
Sado		*SN DV*	Cu	2	0.00	*ns*	*na*
			Zn	2	0.49	*ns*	*na*
Mira	*i*	*SS DL*	Cu	1	9.94	*	*na*
			Zn	1	0.60	*ns*	*na*

Table 6. – Inter-estuarine variation in the trace metal concentration in muscle of juveniles. Results of ANOVA comparisons and respective Tukey post-hoc multiple comparisons tests for Cu and Zn concentrations in muscle of juvenile *Solea solea*, *Solea senegalensis*, *Platichthys flesus*, *Diplodus vulgaris* and *Dicentrarchus labrax* among estuaries along the Portuguese coast (D, Douro; RA, Ria de Aveiro; Mo, Mondego; T, Tejo; S, Sado; Mir, Mira; RF, Ria Formosa; and G, Guadiana). * $P<0.05$; *ns*, non-significant; different numbers (superscript) represent significant differences among estuaries at $P<0.05$ in Tukey post-hoc tests; *na*, post-hoc tests not applicable.

Species	Element	ANOVA			Tukey post-hoc tests
		df	*F*	*P*	
All species	Cu	7	0.57	*ns*	*na*
	Zn	7	7.35	*	D^1 RA^{1,2} Mo^{1,2} T^2 S^{2,4} Mir^{3,4} RF^{1,2} G^{1,2}
S. solea	Cu	4	0.51	*ns*	*na*
	Zn	4	1.63	*ns*	*na*
S. senegalensis	Cu	1	0.27	*ns*	*na*
	Zn	1	0.09	*ns*	*na*
P. flesus	Cu	2	1.87	*ns*	*na*
	Zn	2	2.90	*ns*	*na*
D. vulgaris	Cu	6	2.95	*	- - - S^1 Mir^2 - -
	Zn	6	26.13	*	RA^1 Mo^{2,3} T^{2,3} S^2 Mir^1 RF^3 G^3
D. labrax	Cu	3	0.18	*ns*	*na*
	Zn	3	1.03	*ns*	*na*

levels of contamination in juveniles amongst all analysed estuaries, with the exception of *D. vulgaris*; (2) low concentrations of Cu and Zn; and (3) very low concentrations of Cd and Pb, at this early life stage, even though, for the majority of the estuaries, moderate levels of anthropogenic pressure and water/sediment or biota contamination by these elements have been previously reported.

Metal uptake in fish is influenced by multiple factors and has been shown to result from several sources (water, particulate material, sediment and food) (Livingstone, 2001). In comparison with pollution levels in other estuaries worldwide and considering guidelines for sediment quality classification (DelValls *et al.*, 1998; Miramand *et al.*, 1998; Ruiz, 2001), the estuaries along the Portuguese coast generally have low to moderate pollution levels of the analysed trace metals (with the highest levels found in the Tejo and Sado estuaries and the lowest in the Mira and Mondego estuaries). Despite the registered contamination levels, the bioavailability of these metals in the sediment has been suggested to be low (Monterroso *et al.*, 2007). Mining and industrial activities have been identified as the main sources of these metals entering the drainage basins or directly into these estuaries (Cortesão and Vale, 1995). However, in the last decade, a small decreasing trend in the contamination of recent sediment layers has been found in some of these systems, most probably as a result of decrease in contaminant inputs (Monterroso *et al.*, 2007).

The role of metal uptake via bio-transference from prey and the bio-magnification of metal contamination through food webs are still not fully resolved (Barwick and Maher, 2003) and should therefore be considered as potential sources of contamination. Benthic invertebrates are the main prey for the analysed fish species (see Reis-Santos *et al.*, 2008) and their contamination has been explored in the Douro and Tejo estuaries and reported to vary among sites and species (França *et al.*, 2005; Mucha *et al.*, 2005; Cardoso *et al.*, 2008). For instance, Cu, Cd, Pb and Zn in benthic invertebrate species in the Tejo estuary ranged from ca. 4 to 67 $\mu g\ g^{-1}$ DW, 0.1 to 4 $\mu g\ g^{-1}$ DW, 1 to 73 $\mu g\ g^{-1}$ DW and 80 to 900 $\mu g\ g^{-1}$ DW respectively.

All the analysed elements have toxic effects on biota above certain concentrations. They differ fundamentally in function and regulation in aquatic organisms. Cu and Zn are essential elements (i.e. micronutrients necessary for body functioning which cannot be synthesised and are obtained externally via diet) as Cu is a component of numerous oxidation-reduction enzyme systems and Zn is a component or cofactor in many important enzyme systems. Both are efficiently regulated by aquatic organisms, particularly fish (Amiard *et al.,* 1987; Vallee and Auld, 1990; Beinert, 1996). However, Cd and Pb have no organic functions and are not regulated by aquatic organisms (Amiard *et al.*, 1987).

High energy requirements, metabolism, growth and ingestion rates of juveniles as well as metal uptake rate are responsible for the observed levels of Cu and Zn (Henry *et al.*, 2004; Zhang and Wang, 2007). The very low contamination by non-essential trace elements Cd and Pb, less abundant and/or bioavailable in estuarine systems, is in agreement with the low uptake and accumulation of metals observed in flatfish species (namely *S. solea* and *P. flesus*) in short time period trials (1-3 months) at estuarine sites with heavily contaminated sediments, specifically a slight increase in Cu and Zn but no increase in Cd and Pb (Berge and Brevik, 1996). Despite the contaminated sediment and prey at the sampled sites, the limited time spent by these juvenile fish in the estuaries also probably accounted for their low contamination. In addition, the low bioavailability of trace metals in sediment or prey is suggested to play a role in the variability of metal uptake (Beyer *et al.*, 1996; Bragigand *et al.*, 2004). Data from studies on similar early life history stages of fish are not available, and therefore comparisons are not possible.

Low concentrations are usually measured in muscle tissue but higher concentrations are generally reported in liver, and there is normally a positive correlation between the concentrations in the tissues (Usero *et al.*, 2003; Henry *et al.*, 2004; Vinagre *et al.*, 2004). Differences can surpass one or two orders of magnitude (Usero *et al.*, 2003; Henry *et al.*, 2004) and originate from the distinct physiological functions of muscle and liver. For instance, the accumulation of Cu can be explained by its relation to low-molecular weight proteins (metallothionein-like) which are abundant in hepatic tissue. In the present study, liver tissue could not be analysed due to the difficulty in obtaining and preserving liver samples in such small juveniles, even if fish were preserved at -20°C immediately after capture. Therefore, it remains to be investigated if the observed lack of differences in contamination among systems is confirmed in other biological tissues. Nevertheless, Henry *et al.* (2004) stated that when there are inter-site differences in liver they are also found in the corresponding muscle, which reflects the positive correlation between metal contents in liver and muscle tissues.

Analysis of trace metals in muscle tissue is of great interest, particularly in commercial species and those integrated in health risk assessments, since muscle tissue is usually the part humans consume. The concentrations of trace metals were well below the European standards and guidelines for trace metals in fish and shellfish (Commission Regulation EC N. 1881/2006) (4-fold below for Zn and Cd, 13-fold below for Pb, 40-fold below for Cu) and represent no concern in terms of a human health risk due to consumption. This is particularly relevant for common sole and Senegalese sole which, although they have a minimum landing size, are still commonly targeted as juveniles in estuarine fisheries [e.g. in Tejo estuary (Gamito and Cabral, 2003)].

Juvenile fish leaving the estuaries to join off-shore stocks during the first year of life export low levels of contamination, which is a result of the low levels of Cd and Pb that accumulate in the course of their first months of life in estuarine nurseries. Nevertheless, many juveniles spend increased periods in estuaries and are exposed to pollution loads for longer periods, potentially increasing their contamination levels, as observed for older (> age-1) *P. flesus* in the Douro estuary (Vinagre *et al.*, 2004) and *S. senegalensis* in the Tejo estuary (França *et al.*, 2005). These individuals had Cu and Zn concentrations similar to the ones reported in the current study but higher levels of contamination by Cd and Pb (respectively 0.4 μg g^{-1} DW and 3 μg g^{-1} DW in *P. flesus* and 0.5 μg g^{-1} DW and 2 μg g^{-1} DW in *S. senegalensis*). Higher concentrations were also reported for *S. solea* in estuaries in Spain (Usero *et al.*, 2003). Moreover, juveniles of marine species that use estuaries as nursery grounds have lower contamination levels than resident or long-term inhabitants (e.g. *Pomatoschistus minutus*, *Liza ramada*, *Anguilla anguilla*), emphasising the combined role of residence time and diet in individual contamination (Usero *et al.*, 2003; Durrieu *et al.*, 2005; França *et al.*, 2005). Despite variation in sediment contamination at the analysed sites, metal contamination in juveniles did not differ amongst estuaries, with the exception of *D. vulgaris.* Variability in body loads of Cu and Zn in *D. vulgaris* may be due to the larger set of collection estuaries for this species. In addition, distribution patterns within estuaries differ with species and *D. vulgaris* was mostly found in the lower estuarine areas, in contrast with the remaining species; therefore, its analysis comprised sites not included for the remaining species.

Regarding inter-species differences, higher Cu concentrations were observed in the Perciformes species (*D. vulgaris* and *D. labrax*) and higher Zn concentrations in the flatfish *P. flesus*. These results are in agreement with previous data in other estuarine and coastal areas (Henry *et al.*, 2004; Durrieu *et al.*, 2005) and can be attributed to both physiological and ecological factors (e.g. variations in metabolism and physiological regulation of metal uptake, differences in habitat use, foraging habitat and diet). Similar factors were suggested to account for inter-specific variability in otolith elemental composition in juveniles within estuarine systems (Reis Santos *et al.*, 2008). Overall, though flatfish species are considered to be particularly exposed to sediment metal contamination, since they live in close association with the sediment (Johnson *et al.*, 1998; Bolton *et al.*, 2004), other species which also depend directly on the bottom for feeding, such as *D. labrax* and *D. vulgaris*, are also vulnerable.

A differential contribution of estuarine nurseries to marine adult subpopulations (i.e. number of recruited juveniles) is generally expected (Beck *et al.*, 2001) and was observed, through otolith elemental fingerprints, for these estuaries and species, with the exception of *D. vulgaris* (Vasconcelos *et al.*, 2008). Considering the homogeneously low contamination of juveniles amongst source nurseries for *S. solea*, *S. senegalensis*, *P. flesus* and *D. labrax*, the differential connectivity of these estuaries with adult habitats does not imply differences in terms of contamination of exported fish to the coast. No particular source estuary potentially increases the contamination of adult stocks. On the Portuguese coast, trace metal contamination in adult *S. solea* has been reported to be very low (Carvalho *et al.*, 2005); however, for the remaining species no data are available.

Although body metal loads provide a simplistic indicator of toxic effects, they yield valuable information on potential exposure that can be used to assess trends in the health of aquatic ecosystems and provide baseline information for risk assessment regarding the disruption of specific ecological functions. This knowledge is of the utmost importance in view of the ecological and economic value of these species and their use of estuarine areas as nurseries. Future assessments should focus on other contaminant types and individual biomarker responses to further investigate the different habitat quality of these estuarine nurseries.

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