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Metallothioneins failed to reflect mercury external levels of exposure and bioaccumulation in marine fish – Considerations on tissue and species specific responses

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ABSTRACT

The suitability of metallothioneins (MT) in fish as biomarker of exposure to mercury has been questioned. Therefore, this study aimed at investigating the relationship between external levels of exposure, mercury accumulation and MT content, assessing species and tissue specificities. Two ecologically different fish species – *Dicentrarchus labrax* and *Liza aurata* – were surveyed in an estuary historically affected by mercury discharges. Total mercury (T-Hg) and MT content were determined in gills, blood, liver, kidney, muscle and brain. All tissues reflected differences in T-Hg accumulation in both species, although *D. labrax* accumulated higher levels. Regarding MT, *D. labrax* revealed a depletion in brain MT content and an incapacity to induce MT synthesis in all the other tissues, whereas *L. aurata* showed the ability to increase MT in liver and muscle. Tissue-specificities were exhibited in the MT inducing potential and in the susceptibility to MT decrease. *L. aurata* results presented muscle as the most responsive tissue. None of the investigated tissues displayed significant correlations between T-Hg and MT levels. Overall, the applicability of MT content in fish tissues as biomarker of exposure to mercury was uncertain, reporting limitations in reflecting the metal exposure levels and the subsequent accumulation extent.

1. Introduction

Metallothioneins (MT) constitute a superfamily of ubiquitous low molecular weight proteins capable of binding metals (Romero-Isart and Vašák, 2002), whose behaviour is dominated by the chemistry of the thiol (–SH) group. While some controversy remains regarding the physiological roles of MT, it is recognized that they are primarily involved in the homeostasis of essential oligoelements such as copper and zinc (Cosson et al., 1991), and in cellular antioxidant functions (Sato and Bremner, 1993). MT also play a key role protecting cells against high levels of essential metals, as well as in detoxifying non-essential metals such as mercury and cadmium (Roesijadi, 1996; Viarengo et al., 2000). Concerning aquatic species, several laboratory and field studies have demonstrated an increase in MT concentrations under metal exposure (Fernandes et al., 2008; Ghedira et al., 2008; Costa et al., 2009; Falfushynska and Stoliar, 2009). Accordingly, measurement of MT in different tissues of aquatic organisms is part of the recommended biomarkers for heavy metals biomonitoring programs (Dragun et al., 2009). The relevance of MT as a biomarker in fish was related to their ability to signal sub-lethal concentrations of metal ions as well as to their biological significance (Chan, 1995). In this direction, several fish studies have focused on metal-binding properties of MT under field conditions (Rotchell et al., 2001; Marijić and Raspor, 2006; Fernandes et al., 2008; Dragun et al., 2009).

In vertebrates, and particularly in fish, trace metal detoxification processes depend mainly on metal binding to MT (Amiard et al., 2006). In addition, MT induction in fish is known to be high in tissues directly involved in metal uptake, storage and excretion, such as gills, liver, kidney, intestine (Hogstrand and Haux, 1991; Roesijadi and Robinson, 1994; Viarengo et al., 2007), muscle (Wang and Rainbow, 2010) and, in a lesser extent, blood (Kito et al., 1982b). Differences in metal accumulation and MT levels showed to vary with fish species and to depend on the organ/tissue as a function of its biochemical and physiological features (De Boeck et al., 2003). Moreover, MT induction is also dependent on the exposure duration and on the metal concentration (Hamza-Chaffai et al., 1995).

Mercury is one of the non-essential metals reported in the literature as being able to induce MT synthesis (Roméo et al., 2003; Amiard et al., 2006). It is recognized that sub-lethal concentrations

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of mercury are able to induce thionein synthesis and the binding of the apoprotein to the metal, thus forming metallothionein (Hamilton and Mehrle, 1986). Several laboratory and field studies were conducted focusing on MT response in fish under mercury exposure (Gonzalez et al., 2005; Bebianno et al., 2007; Jebali et al., 2008; Oliveira et al., 2010). However, while some studies revealed MT induction (e.g. Bebianno et al., 2007), others verified poor correlation between mercury levels and MT content (e.g. Rotchell et al., 2001). In accordance, some authors pointed the existence of conflicting reports of mercury's ability to induce MT formation in fish (Hamilton and Mehrle, 1986; Amiard et al., 2006). Whilst laboratory studies demonstrated MT induction with acute exposures to low concentrations, field studies reported the need of longer periods of exposure to stimulate MT synthesis. Furthermore, MT usefulness as biomarkers of metal exposure was questioned in the presence of high concentrations (Hamza-Chaffai et al., 1995). Accordingly, two main questions arise: Is mercury really able to induce MT in feral fish? Are MT a suitable biomarker of mercury exposure? More to the point is the fact that most fish studies concerning MT response to mercury addressed a single species and a limited number of target tissues. In this context, the present study brings a new perspective regarding the usefulness of MT as a protective mechanism for fish. Hence, it was aimed at exploring the causal relationships between external levels of exposure, mercury accumulation, and MT content, assessing tissue and species specificities. In conformity, six key organs/tissues (gills, blood, liver, kidney, muscle and brain) were analysed and two fish species (*Dicentrarchus labrax* and *Liza aurata*) were chosen in order to assess the influence of different feeding behaviours and habitats. This investigation was carried out in an estuarine area historically affected by discharges from a chlor-alkali industry – Ria de Aveiro, Portugal, which displays a well-established mercury contamination gradient (Coelho et al., 2005) and negligible levels of other contaminants.

2. Materials and methods

2.1. Study area

The study was carried out at Ria de Aveiro, a coastal lagoon located on the northwest coast of Portugal (Fig. 1). This estuarine system has an inner area–Laranjo Basin, which has persistently received effluents from chlor–alkali industry (1950–1994). Although discharges ceased, past releases resulted in an accumulation of high mercury concentrations in the sediments, with a maximum of about 300 mg kg^{-1} of total mercury in the most contaminated area (Pereira et al., 1998). These high concentrations found in the fine surface sediments of Laranjo Basin contributed to the formation of a contamination gradient (Coelho et al., 2005). The occurrence of other contaminants in this area was evaluated, revealing negligible levels of arsenic, cadmium, lead, copper and zinc in superficial sediments (2006 and 2009; unpublished data). In addition, the levels found for priority polycyclic aromatic hydrocarbons (PAHs) were considered low (Pacheco et al., 2005). In accordance, Laranjo Basin has been regularly adopted as a "field laboratory", offering the opportunity to assess mercury effects in natural conditions (Guilherme et al., 2008; Válega et al., 2009).

Sampling sites (R, L1 and L2) were selected in accordance to the existing contamination gradient (Fig. 1). Two sampling sites were selected at Laranjo Basin, i.e., L1 as the moderately contaminated site and L2 as the highly contaminated site. L2 was located closer to the mercury source and 2 km away from site L1. An area close to the lagoon entrance (S. Jacinto) and far from the main polluting sources was chosen as the reference site (R).

2.2. Sampling procedures

Dissolved oxygen (WTW–OXI 330i set), pH (WTW–pH 330i set), temperature (WTW–COND 330i) and salinity (WTW–COND 330i)

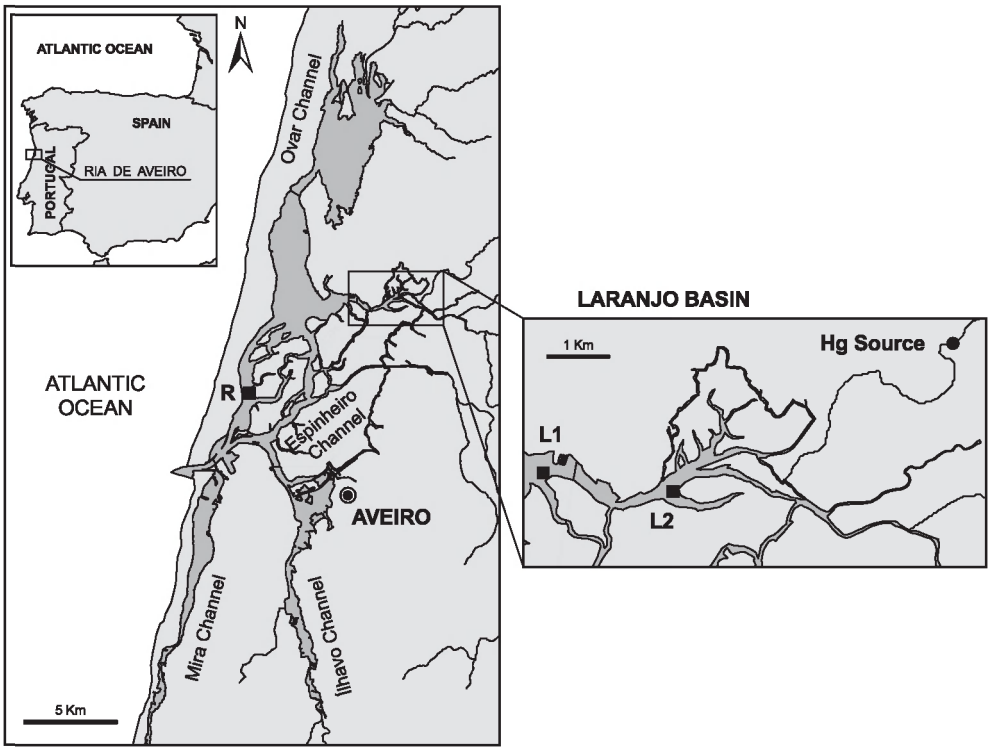

Fig. 1. Map of the sampling sites (■) in the Ria de Aveiro (Portugal): reference (R-40°41′00″N, 8°42′44″W), moderately (L1-40°43′34.46″N, 8°38′53.16″W) and highly contaminated (L2-40°43′28.98″N, 8°37′35.80″W) areas.

were measured in the water column at sub-surface level, in both low and high tide conditions. Turbidity was also measured, as well as water-column depth. At each sampled area, sub-surface water samples were collected in acid-washed plastic bottles kept in an ice box during transportation to the laboratory, where they were immediately filtered through pre-weighed 0.45 μm Millipore cellulose acetate membrane filters, acidified with "mercury-free" HNO_3 (Merck) to pH < 2 and stored at 4 °C until mercury measurements. Additionally, five replicates from the surface sediment layer (approximately 2 cm depth) were collected.

Sampling took place in July 2009 and, at each sampling site, twenty juvenile European sea bass (*D. labrax*) were caught using a fishing rod and twenty juvenile golden grey mullet (*L. aurata*) were captured using a beach-seine net. *D. labrax* and *L. aurata* specimens, selected on the basis of their size, had, respectively, a mean total length of 19.3 ± 2.9 and 13.1 ± 1.1 cm and a mean wet weight (wwt) of 61.6 ± 9.3 and 23.1 ± 5.0 g. The chosen species are euryhaline, abundant, ubiquitous, and use the estuary as a nursery area, but with different feeding ecology: *D. labrax* has benthonic feeding habits, including benthic invertebrates and small fish (Martinho et al., 2008a), and is usually found in shallow waters, on various substrates (Rogdakis et al., 2010). *L. aurata*, on the other hand, has an omnivorous diet, feeding essentially on planktonic communities and detritus (Martinho et al., 2008b; França et al., 2009), and is commonly found in sheltered mud sediments (Richard et al., 2010). Accordingly, and despite that they can co-exist in the same areas, competition for food resources is scarce. Moreover, the use of juvenile specimens better reflects confined contamination, since they commonly have a limited geographic range within estuarine areas.

Immediately after being caught, fish were sacrificed by severing their spinal cord, and blood, brain, kidney, liver, gills and dorsal muscle were sampled, kept in liquid nitrogen and transported to the laboratory. Blood was collected from the posterior cardinal vein using heparinised Pasteur pipettes. Two sets of samples were obtained: one for MT determination (n = 10) and another for T-Hg determination (n = 10). In the laboratory, tissue samples were preserved until further processing at −80 °C for MT determination and at −20 °C for mercury quantification.

This study was conducted in accordance with national guidelines (Portaria no. 1005/92 de 23 Outubro) for the protection of human subjects and animal welfare.

2.3. Total mercury determinations

2.3.1. Mercury in water

Sub-surface water samples were filtered through pre-weighed 0.45 μm Millipore cellulose acetate membrane filters, acidified with mercury-free HNO_3 (Merck) to pH < 2 and stored at 4 °C until analysis. Before the digestion process, the filters were re-weighed after heating overnight at 60 °C and stored at 4 °C for suspended particulate matter (SPM) determinations.

Reactive mercury (R-Hg) and mercury in suspended particulate matter (SPM-Hg) were analyzed by cold-vapour atomic fluorescence spectrometry (CV-AFS) with a PSA model Merlin 10.023 equipped with a detector PSA model 10.003 using $SnCl_2$ reduction. For determination of SPM-Hg, filters were digested with HNO_3 4 mol L^{-1} (for detailed description, see Monterroso et al., 2003) and the previous equipment was used (Pereira et al., 1998).

2.3.2. Mercury in sediment and in fish tissues

At the laboratory, sediment samples were freeze-dried, well mixed, manually sieved through a 1 mm mesh nylon sieve and stored for total mercury determination (Sed-Hg). Fish tissues were freeze-dried, well mixed, fresh weight determined, and finally used for determination of total mercury (T-Hg).

Sediment and tissue samples were analyzed for total mercury determination by atomic absorption spectrometry (AAS) with thermal decomposition with gold amalgamation, using an Advanced Mercury Analyser (AMA) LECO 254 (Costley et al., 2000). The accuracy and precision of the analytical methodology for total mercury determinations were assessed by replicate analysis of certified reference materials (CRM). The CRM used were MESS-3 and PACS-2 (marine sediments) for sediments and TORT-2 (lobster hepatopancreas) for biological samples. Precision of the method was always better than 9% (n > 3), with recovery efficiency between 101% and 110%.

2.4. Metallothioneins determination

Fish tissue samples were homogenized on ice in three volumes (w/v) of 10 mM Tris–HCl buffer (pH 7.4), containing 85 mM NaCl with a Potter–Elvehjem homogenizer. Tissue homogenates were centrifuged at 16,000*g* for 20 min at 4 °C (5415R centrifuge, Eppendorf). The supernatant was stored at −80 °C until MT measurement. The MT concentration was measured using the Cd saturation thiomolybdate assay, according to Klein et al. (1994). This protocol was in accordance with the method described by Bartsch et al. (1990), through the addition of an extra step in which ammonium tetrathiomolybdate and DEAE-Sephacel are added, in order to remove all MT-bound to metals and to subsequently saturate the MT molecules with the ^{109}Cd isotope. This method allows the quantification of total MT in biological materials, including the oxidized and aggregated MT, since particularly Cu-containing MT seems to have the tendency to polymerize. The main features of the procedure are: that oxidized MT is converted into native MT with 2-mercaptoethanol as a reducing agent and Zn^{2+} as a metal donor, and MT are subsequently quantified via Cd saturation. High molecular weight Cd-binding compounds are denatured with acetonitrile, while Cu and other metals bound to MT are removed with ammonium tetrathiomolybdate. The excessive tetrathiomolybdate and its metal complexes are removed with DEAE-Sephacel. Finally, ^{109}Cd isotopes are quantified using a Minaxi-Autogamma 5530 counter (Canberra Packard). For Cd-MT concentration calculations, a ratio of 7 mol Cd/mol MT was assumed (Kito et al., 1982a).

2.5. Statistical analysis

Data was tested for goodness of fit to normal distribution and requirements of homogeneity of variances were also determined. Analysis of variance (ANOVA) was performed, followed by all pairwise multiple comparison procedures (Tukey test). Whenever the assumptions for parametric statistics failed, the non-parametric correspondent test (Kruskall Wallis) was performed followed by the non-parametric all pairwise multiple comparison procedure (Dunn's method). Spearman rank correlation factor (r) was used to test significant relations between T-Hg and MT contents. A significant level of 0.05 was considered in all test procedures.

3. Results

3.1. Mercury in the environment

3.1.1. General physico-chemical characterization

Physico-chemical parameters of the water are summarized in Table 1. In general, the three sampling sites were analogous regarding environmental characterization with the exception of SPM level at R, during low tide.

3.1.2. Mercury in water and sediments

R-Hg concentrations in the water column increased toward the contamination source, and from high to low tide. Although R-Hg

Table 1
General physico-chemical characterization of the water column at high tide and low tide on reference (R), moderately contaminated (L1) and highly mercury contaminated (L2) areas at Ria de Aveiro: Temperature (T), dissolved oxygen (DO), water-column depth, turbidity, pH, salinity and suspended particulate matter (SPM).

Site	High tide–low tide						
	T (°C)	DO (mg L^{-1})	Depth (m)	Turbidity (m)	pH	Salinity	SPM (mg L^{-1})
R	20.00–20-50	8.80–8.80	5.40–1.90	1.20–0.80	8.20–8.20	34.00–34.00	20.60–59.20
L1	21.60–21.80	9.00–6.80	4.50–3.10	0.50–0.50	7.90–7.70	33.00–28.00	28.50–27.60
L2	21.90–22.00	9.20–6.10	3.70–1.00	0.90–0.20	8.00–7.40	32.00–25.00	30.50–35.00

measurements have reflected the environmental gradient, values were low in the three sampling sites (Table 2). SPM-Hg concentrations also demonstrated the environmental contamination pattern. This pattern was more relevant during low tide conditions, when L2 levels were 42.7 times higher than R and 10.7 higher than L1. The contamination pattern verified for the water column was also demonstrated for sediments. Total mercury in sediments (Sed-Hg) increased 17 times from R to L1 and 301 times from R to L2. In addition, Sed-Hg increased 18 times from L1 to L2. Hence, Sed-Hg concentrations were found to be in accordance with contamination levels formerly assessed in the estuary, although a moderate decline occurred from previous to current data.

3.2. Mercury bioaccumulation in fish tissues

Arithmetic mean of T-Hg in the tissues of the two fish species are depicted in Fig. 2. T-Hg concentrations ranged from 0.02 in blood (R) to 1.01 mg kg^{-1} wwt in liver (L2) for *D. labrax* and from 0.005 in blood (R) to 1.51 mg kg^{-1} wwt in liver (L2) for *L. aurata*. Concentrations were in general higher for *D. labrax* with the exceptions of liver (L2) and kidney (L1 and L2). However, both species reflected the exposures levels, clearly indicating to be related with the environmental contamination pattern, also showing tissue specificity. Hence, for *D. labrax* T-Hg varied according to the pattern liver > muscle ≈ kidney > brain ≈ gills > blood; in the case of *L. aurata*, the pattern was liver > kidney > brain ≈ muscle > gills > blood.

Inter-site comparisons were carried out for each tissue, in both species, revealing that all tissues were able to reflect differences in T-Hg accumulation. Thus, all the tissues were able to reflect significant differences between R and L2, in both species. For *D. labrax*, brain was also able to signal differences between R and L1, as well as kidney, brain and muscle in *L. aurata*. Differences between L1 and L2 were detected in *D. labrax* gills and brain, as well as in *L. aurata* liver, brain and muscle.

3.3. Metallothioneins content in fish tissues

MT concentrations ranged from below the detection limit (muscle at L1) to 20 nmol g^{-1} (liver at R) for *D. labrax*, and from 0.17 (muscle at R) to 48 nmol g^{-1} (liver at L2) for *L. aurata* (Fig. 3). In *D. labrax*, inter-site differences were only found for brain that displayed MT levels reduction at L2 in relation to R and L1. In *L. aurata*, significant MT elevation was detected at L2 in liver (versus R and L1) and muscle (versus R). In addition, *L. aurata* muscle was able to reflect differences between L1 and R. No significant differences were found for the other tissues.

No significant correlations were verified between T-Hg and MT levels in the different tissues for both species. Nevertheless, in *L. aurata*, MT content in the muscle matches with accumulated

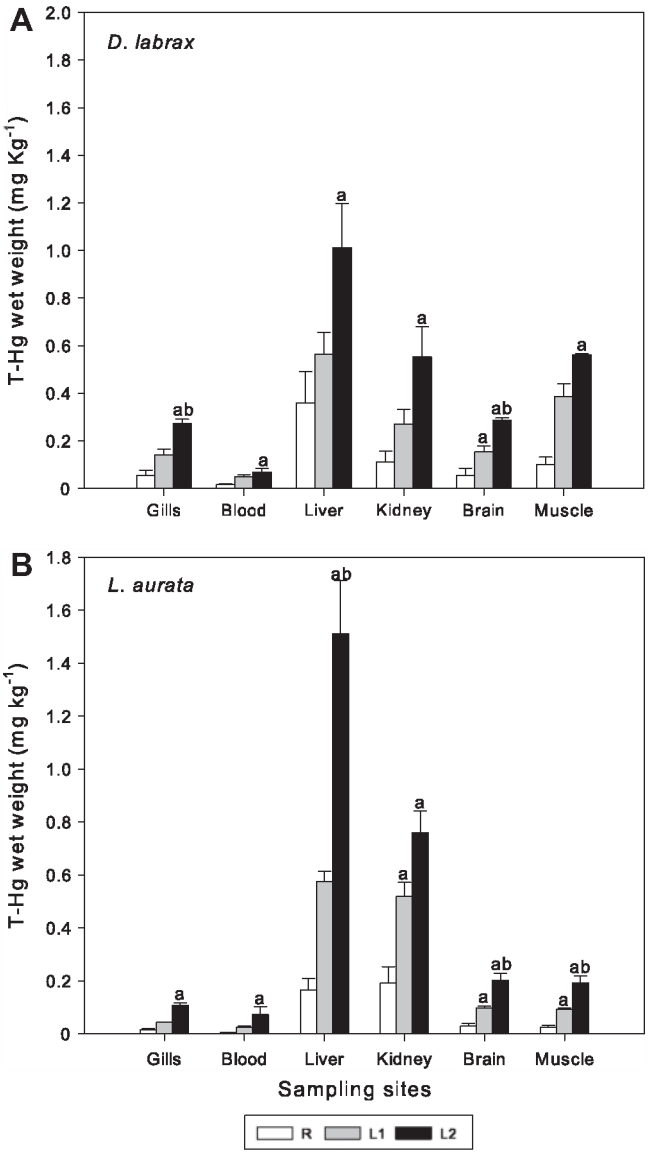

Fig. 2. Total mercury (T-Hg) mean concentration (mg kg^{-1} wet weight) in the tissues of *D. labrax* (A) and *L. aurata* (B) captured at each Ria de Aveiro sampling station: reference (R), moderately (L1) and highly mercury contaminated (L2) areas. The letters denote statistically significant differences ($p < 0.05$): (a) versus R and (b) versus L1. Bars represent the standard error.

Table 2
Concentrations of total mercury in water and sediment (Sed-Hg) on reference (R), moderately contaminated (L1) and highly contaminated (L2) areas at Ria de Aveiro. Water values determined in high and low tide represent reactive dissolved mercury (R-Hg) and mercury in suspended particulate matter (SPM-Hg).

Site	Water (High tide–low tide)		Sediment Sed-Hg (mg L^{-1})
	R-Hg (ng L^{-1})	SPM-Hg (ng L^{-1})	
R	3.00–6.30	0.23–0.26	0.005
L1	2.90–4.00	0.58–1.02	0.080
L2	4.20–9.50	0.84–10.90	1.390

R-Hg and SPM-Hg were analyzed in three aliquots from each sample, with a coefficient of variation <10%.

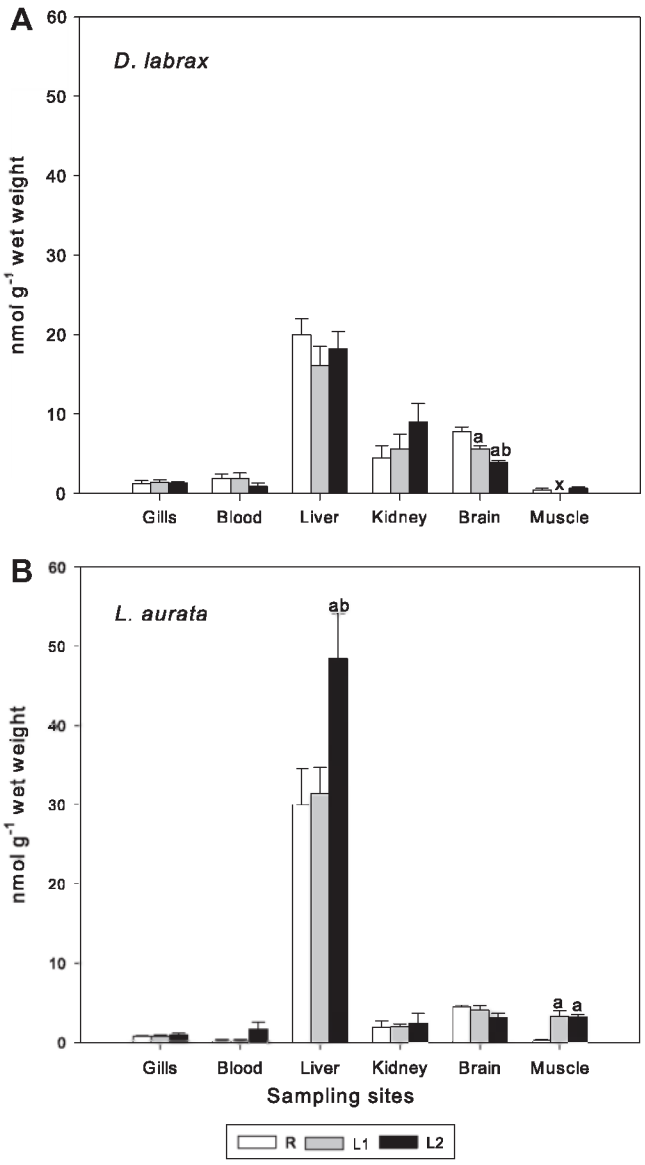

Fig. 3. Metallothioneins (MTs) mean concentration (nmol g^{-1} wet weight) in the tissues of *D. labrax* (A) and *L. aurata* (B) captured at each Ria de Aveiro sampling station: reference (R), moderately (L1) and highly mercury contaminated (L2) areas. The letters denote statistically significant differences ($p < 0.05$): (a) versus R and (b) versus L1. *x* – muscle levels below detection limit. Bars represent the standard error.

T-Hg while in the liver this association was only found for L2. Furthermore, in *D. labrax* brain the MT content varied inversely with T-Hg accumulation (and with exposure levels as well).

4. Discussion

MT induction by metals has been reported in several marine species (Cajaraville et al., 2000; Amiard et al., 2006), but its synthesis showed to be influenced by several factors. Estuaries are characterized by intense variation of water physicochemical parameters able to produce changes in pollutants bioavailability and, consequently, in their toxicity. In the present study, we compared the response of fish exposed to mercury in a well-contrasted environmental gradient regarding mercury availability, although avoiding, as much as possible, inter-site differences in physicochemical parameters such as water temperature, dissolved oxygen and salinity.

Fish biometric parameters such as total length, body weight and condition, as well as other biological factors such as age, sex, reproductive status and hormone levels may influence MT induction (Hamza-Chaffai et al., 1995; Filipović and Raspor, 2003). In order to reduce the interference of the described confounding factors, fish specimens used in this study were reproductively immature, belonging to the same age class.

Since MT are involved in metal storage and detoxification processes, it is relevant to compare populations chronically exposed to metal contamination in their environment with populations living in sites considered to be uncontaminated (Amiard et al., 2006). As stated previously, the applicability of MT as a biomarker of metal exposure requires a suitable selection of the indicator species and target tissues/organs (Marijić and Raspor, 2006).

4.1. Tissue-specificities on MT content and association with T-Hg accumulation

The present study reflected an increasing trend in mercury accumulation in the studied tissues/organs relatively to the contamination source. All tissues, for both species, were able to significantly differentiate the most contaminated area from the reference area. European regulatory guidelines for mercury levels in marketed fish tissues are limited to muscle – 0.5 mg kg^{-1} wwt (EC No 78/2005). However, whenever the whole fish is to be consumed, the established limit must be applied to the whole body. In accordance, *D. labrax* muscle exceeded this value, as well as liver and kidney of both species.

4.1.1. labrax response profile

MT normally occur in tissues in trace amounts; however, exposure to metals induces its formation (Hamilton and Mehrle, 1986). Based on this assumption, it would be expected that tissues displaying increased T-Hg levels exhibit augmented MT contents. Surprisingly, though all the *D. labrax* tissues analyzed displayed significantly increased T-Hg levels (namely at L2), none of them showed the ability to respond by elevating the respective MT content. This is corroborated by the absence of significant correlations between T-Hg levels and MT contents in the different tissues.

Brain was the only organ/tissue able to reflect differences among sites, though revealing a decrease in MT content with an increasing T-Hg level. The MT decrease observed in brain coincided with a T-Hg level of 0.15 mg kg^{-1} (L1); however, in all the other tissues, with the exception of blood, equivalent or higher mercury concentrations were not accompanied by such inhibitory effect. This is indicative of a specificity of MT synthesis modulation for each tissue, which showed to present different threshold limits. Moreover, Shimada et al. (2005) suggested that MT in rat brain might not be involved in the uptake and transport of mercury. In addition, Uchida et al. (1991) referred the existence of a brain-specific form of MT, probably not affected by metal exposure. It is known that brain has an additional MT isoform (MT-III) that, contrarily to the ubiquity and high inducibility of the other isoforms (MT-I and MT-II), is specific of this organ and constitutively expressed Aschner et al. (2006). The occurrence of MT with high constitutive expression in the brain helps to understand its higher vulnerability towards mercury-induced reduction currently observed in *D. labrax*. Further studies pointed out that the chelating capacity of MT is dependent on the metal form (Gonzalez et al., 2005). In accordance Yasutake et al. (1998, 2003) stated that organic mercury (O-Hg) is not able to induce MT biosynthesis while mercury vapour (Hg^0) could enhance MT in the brain.

Since MT induction was not verified under T-Hg increase in *D. labrax* tissues, one can assume that this was related with the increasing levels of O-Hg from R to the contaminated areas, given the lower affinity of this mercury form to MT. In accordance with

Mieiro et al. (submitted for publication), this could be an explanation for the liver response, owing that its O-Hg percentage increased towards the contamination source. However, this cannot be applied to muscle, that in the cited study revealed a higher contribute of inorganic mercury (I-Hg) in the contaminated area. The inability to increase MT synthesis may also be associated with increased demand of cysteine residues for GSH synthesis during mercury detoxification, since the Hg-GSH conjugation may be a pathway for metal elimination from the cell (Franco et al., 2009).

MT responsiveness in gills is controversial and it has been stated that gills do not constitute a promising organ for MT quantification in fish (Hamza-Chaffai et al., 1997; Olsvik et al., 2001). In this perspective, the absence of MT induction in *D. labrax* gills was expected. Similarly, previous studies on muscle MT content also found no induction in fish exposed to mercury (Gonzalez et al., 2005; Bebianno et al., 2007). Bebianno et al. (2007) stated that mercury in muscle is not always trapped or detoxified by MT or by other cytosolic ligands, but can be present in insoluble forms. The mercury-MT complex formed may precipitate and therefore decrease MT concentrations in the cytosol.

4.1.2. L. aurata response profile

In *L. aurata*, the liver displayed significantly higher MT content in specimens captured at L2, revealing also a coincidence of the highest level of T-Hg accumulation (1.5 mg kg^{-1} wwt) with the highest MT content (48.4 nmol g^{-1} wwt). This is in agreement with previous studies in liver of fish (Bebianno et al., 2007; Fernandes et al., 2008), including *L. aurata* (Oliveira et al., 2010), pointing out the role of hepatic MT in mercury detoxification. Nevertheless, no statistical correlations were found between the two parameters.

L. aurata muscle revealed T-Hg accumulation increments in fish from both contaminated sites along with induction of MT (though no significant correlation was found). This is contrary to previous findings on fish muscle that demonstrated unaltered MT gene expression or inversely proportional to mercury (Gonzalez et al., 2005; Bebianno et al., 2007).

Liver and kidney showed similar levels of mercury accumulation; nevertheless, liver showed considerably higher MT levels than kidney (16 times for R and L1 and 20 times for L2). Moreover, no MT induction was found for kidney. Navarro et al. (2009) reported contrasting results, since they found induction in kidney and no induction in liver of carp. These authors suggested that MT genes are constitutively transcribed in carp liver at relatively high levels and that those levels remain essentially unchanged upon mercury injection. However, MT basal transcription in kidney was low but strongly activated as a response to external inputs. A similar pattern of response to mercury in both tissues has been reported in zebrafish (Gonzalez et al., 2005). Kidney also showed higher mercury levels than muscle (R-9.5 times; L1-6 times and L2-4 times), although only muscle revealed MT induction with mercury contamination. This can demonstrate that different tissues may have different activation thresholds. According to the present data, with the exception of liver and muscle, all the other tissues were not able to demonstrate that MT bind mercury as a sequestration function, and thus, not indicating their ability in protecting against mercury toxicity.

4.2. Species-specificities on MT levels modulation

D. labrax demonstrated higher T-Hg accumulation in all tissues comparing to *L. aurata*, with the exception of kidney. Despite this evidence, no MT induction was found for any of the studied tissues in *D. labrax*. Moreover, *D. labrax* brain revealed a significant MT depletion following a pattern parallel to the T-Hg levels, while in *L. aurata* brain that profile was not apparent.

MT basal levels (assumed as the values recorded at R site) showed to greatly differ among the assessed tissues and species. For both species, MT basal levels in liver and muscle revealed similar values. Notwithstanding liver exhibited the highest MT levels for both species, only *L. aurata* showed significant MT induction in the mercury-contaminated area. Muscle MT basal levels were also similar in both species; however, only *L. aurata* demonstrated MT inducibility in the presence of mercury challenge. Hence, although MT basal levels can be important as a defence strategy in a specific tissue, it does not seem determinant on the inherent MT synthesis inducing potential.

Roméo et al. (1997) proposed an explanation for the no inducibility of liver MT by copper, which can be extrapolated to other metals and other tissues. It was stated that too high metal concentrations ("critical concentrations") interfere with the MT synthesis, either directly on protein synthesis, or, indirectly, affecting some underlying metabolic processes. Taking into account that *D. labrax* tissues (except for kidney) repeatedly presented higher T-Hg loads in comparison to *L. aurata*, in light of the previous theory, it can be hypothesised that the critical concentrations were reached in the former species, justifying the species differences either on MT inducibility or on susceptibility to MT depletion.

The species-specificities could be strongly related to the taxonomic relationships. In this sense, both Mugiliformes (*L. aurata*, Mugilidae) and Perciformes (*D. labrax*, Moronidae) are considered to be phylogenetic related, and some authors have even considered that the Mugilidae family belongs to the order Perciformes (Aurelle et al., 2008). In accordance, since phylogenetic related species may share certain characteristics as a consequence of their common ancestry (Scudiero et al., 2005), similar response patterns should be expected. It could also be hypothesized that these species-specificities are partially due to different ecological features and feeding habits of the two species. *D. labrax* is a demersal species with benthonic feeding habits, whereas *L. aurata* is a pelagic species with detritophagus habits. Hence, one should point out differences regarding the preponderance of the two routes of metal uptake – water and diet. However, Duquesne and Richard (1994) demonstrated, for both wild fish or injected with cadmium, that the route of metal uptake does not affect the nature of the induced MT.

The studied species demonstrated that MT induction due to mercury exposure was species-specific. Moreover, the two species demonstrated different organ thresholds able to induce MT, indicating a highly specific mechanism of response. Nevertheless, the mechanisms of MT induction by mercury and the involved signalling pathways are still not known for fish, limiting the understanding of MT's role in *D. labrax* and *L. aurata*'s response to mercury. Though the specificity of MT as biomarker of exposure to metals strongly depends on the clarification of their action and subjacent induction pathways, their suitability and usefulness does not strictly rely on the identification of MT's role. In this context, the limitations pointed out by the present research in relation to the applicability of MT relied on the limited responsiveness observed in several tissues, as well as on the difficulty to establish a causal relationship between increased metal concentrations in tissues and MT induction.

Due to the controversial information regarding this subject, it is highly recommended to further investigate the suitability of MT as biomarker of mercury exposure in fish.

5. Conclusions

The results of the present investigation provided these main findings:

- The studied fish species displayed distinct patterns of MT modulation in response to mercury accumulation; thus, *D. labrax* revealed a depletion in brain MT content and an incapacity to induce MT synthesis in all the other tissues, whereas *L. aurata* showed the ability to increase MT content in liver and muscle. In agreement, *L. aurata* appeared as a more responsive species in this context and thus, a better bioindicator of mercury contamination on the basis of MT response.
- Within each species, tissue-specificities were clearly exibited in the MT inducing potential and in the susceptibility to MT decrease, as well as in the respective T-Hg threshold limits. *L. aurata* results showed muscle as the most responsive tissue, responding to moderate (L1) and high contamination (L2), while liver only responded to high mercury contamination (L2).
- Overall, the suitability and aplicability of MT content in fish tissues as a biomarker of exposure to mercury was called into question, indicating important limitations in reflecting the metal exposure levels and the subsequent accumulation extent. Moreover, it was pointed out that using only MT contents as a monitoring tool for assessing the environmental mercury contamination is inadequate.

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