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Cláudia Leopoldina de Brito e Veiga Rodrigues Mieiro

Mercury in feral fish: Distribution, Accumulation and Toxicity

Mercúrio em peixes: Distribuição, Acumulação e Toxicidade

Disssertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Química, realizada sob a orientação científica da Doutora Maria Eduarda Pereira, Professora Auxiliar do Departamento de Química da Universidade de Aveiro e do Professor Mário Guilherme Garcês Pacheco, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro.

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“ The error is the engine of knowledge”
(The truth is filled of corrected ideas)
G. Bachelard

o júri

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Investigador Coordenador do IPIMAR - Instituto Nacional de Investigação Agrária e das Pescas

Doutora Maria Eduarda da Cunha Pereira (Orientadora)
Professora Auxiliar da Universidade de Aveiro

Doutor Mário Guilherme Garcês Pacheco (Co-Orientador)
Professor Auxiliar da Universidade de Aveiro

Doutor Carlos Alexandre Sarabando Gravato
Investigador Auxiliar do CIIMAR – Centro Interdisciplinar de Investigação Marinha e Ambiental

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palavras-chave

Mercúrio, peixes, *Liza aurata*, *Dicentrarchus labrax*, bioacumulação, stress oxidativo.

resumo

O principal objectivo desta dissertação foi estudar a acumulação de mercúrio em vários tecidos de peixes marinhos, a sua relação com factores biológicos e as respectivas respostas bioquímicas. O trabalho realizado permitiu obter novos conhecimentos sobre a acumulação de mercúrio em peixes, possibilitando avaliar a influência da biodisponibilidade do elemento e as suas possíveis implicações no ambiente. O trabalho foi desenvolvido na Ria de Aveiro (Portugal), uma zona costeira onde existe um gradiente ambiental de mercúrio, o que oferece a oportunidade de estudar a sua acumulação e os seus efeitos tóxicos em condições realísticas. As amostragens foram efectuadas em dois locais considerados críticos em termos de contaminação por mercúrio – Largo do Laranjo (L1 e L2) e num local afastado da principal fonte de poluição, usado como termo de comparação (Referência; R); L1 e L2 corresponderam a locais moderadamente e altamente contaminados, respectivamente. Foram escolhidos juvenis de duas espécies ecologicamente diferentes e representativas da comunidade piscícola local, a tainha garrento (*Liza aurata*) e o robalo (*Dicentrarchus labrax*). Em cada local foram recolhidas amostras de água e de sedimento para determinação de mercúrio. Foram quantificadas as concentrações de mercúrio total (T-Hg) e orgânico (O-Hg) em vários tecidos dos peixes, escolhidos tendo em conta a sua função relativamente à toxicocinética e toxicodinâmica de metais. As respostas antioxidantes (Catalase- CAT, glutationa peroxidase- GPx, glutationa reductase- GR, glutationa –S-transferase- GST e conteúdo em glutationa total- GSHt), o dano peroxidativo (LPO) e o conteúdo em metalotioninas (MTs) foram também avaliados.

A acumulação de T-Hg foi semelhante para as duas espécies de peixes estudadas, embora *D. labrax* tenha apresentado concentrações tendencialmente maiores. Ambas as espécies demonstraram capacidade de reflectir o grau de contaminação ambiental existente, indicando claramente que a acumulação depende da concentração ambiental. A acumulação revelou-se específica de cada tecido. O padrão da acumulação em *L. aurata* foi rim > fígado > músculo > cérebro > guelras > sangue e em *D. labrax* foi fígado > rim > músculo > cérebro ≈ guelras > sangue. Relativamente à acumulação de O-Hg, verificou-se que *D. labrax* exibiu concentrações mais elevadas que *L. aurata*. Todos os tecidos foram capazes de reflectir diferenças entre R e L2. Os níveis de O-Hg no fígado, músculo e nos conteúdos intestinais foram diferentes entre espécies, sendo mais elevados para *D. labrax*. As guelras e o intestino foram os tecidos onde se obtiveram os valores mais baixos de O-Hg e observaram-se valores idênticos para as duas espécies.

Com excepção das guelras, as concentrações de O-Hg variaram em função do valor observado nos conteúdos intestinais, indicando que a alimentação é a via dominante da acumulação. As concentrações de O-Hg nos conteúdos intestinais revelaram ser uma informação relevante para prever a acumulação de O-Hg nos tecidos, pois verificou-se uma razão praticamente constante entre o teor de mercúrio no fígado, no músculo e nos conteúdos intestinais. A percentagem de O-Hg no músculo e no fígado variou de acordo com o grau de contaminação ambiental e com o tipo de assimilação preferencial do elemento (alimentação *vs.* água), sugerindo que o fígado exerce um papel protector em relação à acumulação de mercúrio nos outros órgãos. Ambas as espécies de peixes demonstraram ser boas sentinelas da contaminação ambiental com mercúrio (T-Hg e O-Hg), sendo o cérebro e o músculo os tecidos que melhor reflectiram o grau de acumulação com o elemento.

A análise conjunta dos dados de bioacumulação e de respostas ao stress oxidativo permitiram estabelecer uma relação entre as concentrações de mercúrio nas guelras, fígado, rim e cérebro e a sua toxicidade. As respostas do cérebro aos efeitos tóxicos do mercúrio revelaram ser específicas de cada espécie. Enquanto que para o cérebro de *L. aurata* se verificou um decréscimo de todos os parâmetros antioxidantes estudados nos locais contaminados, sem haver evidência de qualquer mecanismo compensatório, no *D. labrax* observaram-se respostas ambivalentes, que indicam por um lado a activação de mecanismos adaptativos e, por outro, o decréscimo das respostas antioxidantes, ou seja, sinais de toxicidade. Embora em ambas as espécies de peixe fosse evidente uma condição pró-oxidante, o cérebro parece possuir mecanismos compensatórios eficientes, uma vez que não se verificou peroxidação lipídica. As respostas antioxidantes do cérebro de *D. labrax* foram comparadas em diferentes períodos do ano - quente *vs.* frio. O período quente mostrou ser mais crítico, uma vez que no período frio não se verificaram diferenças nas respostas entre locais, ou seja, a capacidade antioxidante do cérebro parece ser influenciada pelos factores ambientais. As guelras revelaram susceptibilidade à contaminação por mercúrio, uma vez que se verificou uma tendência para o decréscimo da actividade de CAT em L2 e ausência de indução em L1. O fígado e o rim demonstraram mecanismos adaptativos face ao grau de contaminação moderada (L1), evidenciados pelo aumento de CAT. O rim também demonstrou adaptabilidade face ao grau elevado de contaminação (L2), uma vez que se verificou um aumento GST. Embora o grau de susceptibilidade tenha sido diferente entre os órgãos, não se verificou peroxidação lipídica em nenhum.

A determinação do conteúdo em MTs em *D. labrax* e em *L. aurata* revelou que este parâmetro depende não só da espécie, mas também do tecido em causa. Assim, em *D. labrax* foi observado um decréscimo de MTs no cérebro, bem como a incapacidade de síntese de MTs no sangue, guelras, fígado, rim e músculo. Em *L. aurata* observou-se um aumento do conteúdo em MTs no fígado e no músculo. Estes resultados indicam que a aplicabilidade das MTs como biomarcador de exposição ao mercúrio parece ser incerta, revelando limitações na capacidade de reflectir os níveis de exposição ao metal e por consequência o grau de acumulação.

Este trabalho comprova a necessidade de se integrarem estudos de bioacumulação com biomarcadores de efeitos, de modo a reduzir os riscos de interpretações erróneas, uma vez que as respostas nem sempre ocorrem para os níveis mais altos de contaminação ambiental com mercúrio.

keywords

Mercury, fish, *Liza aurata*, *Dicentrarchus labrax*, bioaccumulation, oxidative stress.

abstract

The main objective of this work was to study mercury accumulation in several tissues of marine fish, their relationship with biological factors and respective biochemical responses. This research brings a new viewpoint to the understanding of mercury burdens, helping to predict mercury bioavailability and its implications for ecosystem health. The work was carried out in the Ria de Aveiro costal lagoon (Portugal), where a well-established mercury environmental contamination gradient provides the opportunity to assess mercury accumulation and its toxic effects under realistic conditions. Samples were collected from two critical locations in terms of mercury occurrence – Laranjo basin (L1, L2), and compared with a reference area (R); L1 and L2 represent a moderately and a highly contaminated scenario, respectively. In order to fulfil the objective, juveniles of two representative and ecologically different fish species of the Ria de Aveiro, the golden grey mullet (*Liza aurata*) and the European sea bass (*Dicentrarchus labrax*), were chosen. At each location water and sediments were collected for mercury determinations. Total (T-Hg) and organic mercury (O-Hg) were determined in several tissues chosen according to their function in the context of metal toxicokinetics and toxicodynamics. Antioxidant responses (Catalase- CAT, glutathione peroxidase- GPx, glutathione reductase- GR, glutathione –S-transferase- GST and total glutathione content- GSHt), peroxidative damage (LPO) and metallothioneins contents (MTs) were also assessed.

T-Hg accumulation patterns were similar between the two species, although *D. labrax* showed a tendency to accumulate higher amounts of mercury. Both species were able to reflect the environmental contamination profile, clearly indicating that accumulation was related with environmental contamination. T-Hg accumulation revealed to be dependent on the specific tissue. Accordingly, the accumulation pattern in *L. aurata* was kidney > liver > muscle > brain > gills > blood and for *D. labrax* was liver > kidney > muscle > brain ≈ gills > blood. Regarding O-Hg accumulation, *D. labrax* revealed higher levels than *L. aurata*. All tissues exhibited differences between R and L2. The O-Hg levels of liver, muscle and intestinal contents were different between species, being higher for *D. labrax*. Gills and intestine showed similar low values for both species. In agreement, internal O-Hg concentrations, with the exception of gills, seemed to vary as a function of the intestinal content, suggesting that diet was the dominant pathway for metal uptake. Additionally, the O-Hg concentrations in the intestinal contents seemed to be a promising tool in predicting the O-Hg accumulation in the tissues, since a stable ratio was verified among liver, muscle and intestine burden increments in mercury.

The proportion of O-Hg in the muscle and liver can be dependent of the degree of contamination and of the type of uptake (food *vs*. water) and suggested that liver exerts a protective function relatively to mercury accumulation in other organs. Both species demonstrated to be good bio-sentinels of the environmental mercury contamination (T-Hg and O-Hg), being brain and muscle the best tissues to reflect the mercury accumulation extent.

The combination of bioaccumulation data with the oxidative stress responses allowed connecting the mercury concentration at gills, liver, kidney and brain with its intrinsic toxicity. Brain vulnerability to mercury toxicity was specific for each species. While *L. aurata*'s brain showed an overall depletion of the studied antioxidant defences at the mercury-contaminated areas, without showing any compensatory mechanisms, *D. labrax*'s brain had ambivalent responses, revealing a balance between adaptive mechanisms and signs of toxicity. Though a pro-oxidant status was evident, brain showed, in both species, to possess compensatory mechanisms able to avoid lipid peroxidative damage. *D. labrax*'s brain antioxidant responses were compared in two different year periods (warm *vs*. cold). The warm period revealed to be the most critical since no inter-site changes on oxidative stress endpoints occurred during the cold period. As a consequence, the brain antioxidant capacity seemed to be influenced by the environmental factors. Gills showed susceptibility to mercury toxicity by the tendency to deplete CAT activity at L2. Liver and kidney showed an adaptive capacity to the intermediate degree of contamination (L1) revealed by CAT increase. Kidney also revealed adaptability at L2, depicted in a GST activity increase. Although some organs seemed more susceptible than others, no peroxidative damage occurred in any of them.

The determination of MTs contents, both in *D. labrax* and *L. aurata*, indicated that it depends not only on fish species, but also on the specific tissue. Thus, in *D. labrax* was observed a depletion in MTs brain content, as well as the incapacity to induce MTs synthesis in gills, blood, liver, kidney and muscle. *L. aurata* showed the ability to increase MTs in liver and muscle. According to our results, the aplicability of MTs 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.

Overall, this research pointed out the need to combine the bioaccumulation and effect biomarkers approaches in order to avoid risk of misinterpretations, since responses did not always occur in the highest mercury concentrations

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CHAPTER 1

Introduction

1. General introduction

1.1 Background

Mercury is one of the priority hazardous substances all over the world. During centuries, mercury was an essential part of medicine, used mainly as preservative and anti-bacterial agent, and also largely used in industry. However, the problems associated with mercury poisoning were not recognized until the 20th century, given the occurrence of several incidents such as Minamata Bay (Japan, 1960), China ethylmercury contaminated rice (1971), methylmercury grain contamination in Iraq (1974), Amazonian methylmercury contamination and elemental spill at Catamarca in Peru (2000) (Gochfeld, 2003; Tchounwou et al., 2003).

The worldwide concern regarding its impacts in the environment and in human life has been emphasized by several organizations, especially concerning human health, such as: United States Environmental Protecting Agency (USEPA), Agency for Toxic Substances and Disease Registry (ATSDR), Canadian Environmental Protecting Agency (CEPA), Food Standards Australia New Zealand (FSANZ), United Nations Environment Program (UNEP), European Food Safety Authority (EFSA), Food Standards Agency Scotland (FSA Scotland), National Food Administration Sweden (NFA Sweden). In accordance, strict environmental policies have been defined in Europe concerning mercury discharges, such as the OSPAR Convention (Convention for the Protection of the Marine Environment of the North East Atlantic), the European Water Framework Directive (2000/60/EC), amended by Decision Nº 2455/2001/EC, Directive 2008/32/EC, and the directive 84/156/EEC amended by Directive 2008/105/EC (mercury directive on limit values and quality objectives for mercury discharges by sectors other than the chlor-alkali electrolysis industry).

Mercury releases occur both by natural and anthropogenic sources, and in Europe, the anthropogenic component of mercury deposition exceeds the natural one (SEC, 2005). In Portugal, both the Tagus Estuary and Ria de Aveiro coastal lagoon are referred as historically contaminated by mercury from industrial sources (Figuères et al., 1985; Pereira et al., 2009). The Ria de Aveiro is located on the Portuguese northwest coast and has an inner area (the Laranjo basin), which, during decades, has been subjected to effluent discharges from a chlor–alkali industry. These discharges resulted in the accumulation of about 25.4 tons of mercury in the Laranjo basin and its upstream channel. Although effluent releases stopped in 1994, high mercury concentrations are still found in

the surface sediments of this area, creating a contamination gradient (Coelho et al., 2005).

This area has been recurrently adopted as a “field laboratory” offering a unique opportunity to assess mercury accumulation and toxicity under realistic conditions (Lucas et al., 1986; Abreu et al., 2001; Coelho et al., 2005; Ramalhosa et al., 2005; Pereira et al., 2006; Coelho et al. 2007; Coelho et al., 2008; Guilherme et al., 2008a; Válega et al., 2008). Most of these studies focused on the abiotic and biotic compartments, although mercury in fish has scarcely been addressed.

Fish play a major ecological role in the aquatic food webs, due to their function as a carrier of energy from lower to higher trophic levels (Beyer, 1996). Regardless of their high mobility, fish are considered the most feasible organisms for monitoring the aquatic pollution (Van der Oost et al., 2003). Though several studies regarding metal contamination and its effects have been conducted, information on the mercury sequestration and detoxification mechanisms in wild fish is still scarce (Cidziel et al., 2002; Campbell et al., 2005). Additionally, most of the studies on mercury accumulation in fish were focused on repercussions to human health, resulting in little research directed towards the understanding of mercury contamination throughout the life cycles of diverse groups of fishes and its potential toxicological effects (Wiener and Spry, 1996). Thus, studies regarding mercury exposure and its toxicity in wild fish are of utmost relevance for assessing the impacts of such a harmful substance in ecosystems and humans.

1.2 Mercury

1.2.1 Mercury’s main features

Mercury is the only metal that is liquid at room temperature. Its elemental symbol, Hg, is derived from the Greek word *hydrargyrias,* meaning "water silver." There are three chemical forms of mercury: elemental, inorganic and organic and all forms exhibit toxicological characteristics for both wildlife and humans (SEC, 2005). Elemental mercury occurs naturally in the environment, where it can be found in three easily interconvertible oxidation states (0, +1, +2). Inorganic mercury is globally used in disinfectants and pesticides (Manahan, 1990). Organic mercury can be either chemically synthesized (e.g. for fungicides) or being converted from other mercury forms by bacteria (e.g. methylmercury). Methylmercury, in particular, can bioaccumulate and biomagnificate specially in aquatic food webs (SEC, 2005). In the aquatic ecosystems, organic mercury

cannot be degraded into harmless products, and will be permanently recycled through physical, chemical and biological processes in the environment (OSPAR, 2000).

1.2.2 Mercury in the environment

Sources, fate and transport

Mercury is released into the environment from a variety of sources, both natural and anthropogenic. Natural sources, primarily in the form of elemental mercury, include volcanic emissions, degassing from soils and volatilization from the ocean (Boening, 2000; EPA, 2001). On the other hand, anthropogenic sources are mainly derived from emissions of industrial processes and combustion sources, having significantly contributed to an increase in exposure and environmental deposition (EPA, 2001; SEC, 2005). It is recognized that after the industrialization period, the mercury levels in the atmosphere increased around 3-fold, being the average deposition also incremented by a factor of 1.5 to 3. Around industrialized areas, mercury deposition is thought to have increased 2- to 10-fold (SEC, 2005).

Mercury emissions, environmental fate and its effects are determined not only by the total amounts released or by the total concentration levels present in the environment, but also by the distribution of mercury between different chemical forms (speciation). Mercury speciation influences its mobility in various environmental compartments (air, water and soil); the methylmercury fraction, in particular, determines the uptake and negative health effects of mercury in humans and wildlife (Munthe et al., 2009).

Two cycles are believed to be involved in the environmental transport and distribution of mercury: one is global in scope and implies the atmospheric circulation of elemental mercury vapour from inland sources to the oceans, while the second cycle is local in scope and depends upon the methylation of inorganic mercury, mainly from anthropogenic sources. The steps in this cycle remain poorly understood, but it likely involves the atmospheric circulation of dimethylmercury formed by bacterial action (Boening, 2000).

Mercury in the atmosphere, terrestrial and aquatic systems

In the atmosphere, natural mercury emissions are essentially in the elemental form, while anthropogenic emissions are either vapour (elemental or oxidized mercury) or

as particles (oxidized compounds). Mercury may reside in the atmosphere for about one year, contributing to its global circulation and thus transporting mercury to regions far from its source, such as the Arctic, which has few or no mercury sources (EPA, 2001; SEC, 2005).

Mercury transference from the atmosphere to the surface soil occurs mainly by wet deposition, but also by dry deposition. Most of the mercury found in the soil is in the form of oxidized mercury complexes/compounds, being methylmercury and elemental mercury present in a small extent. Reduction of the oxidized complexes by humic and fulvic acids as well as photochemical reduction promotes the remobilization of gaseous mercury back into the atmosphere (Morel et al., 1998; Costa and Liss, 1999; Fitzgerald et al., 2007). Mercury binds to soil particles, being soil the major sink for mercury (Stein et al., 1996).

In water, mercury undergoes a set of chemical and biological transformations, with a part being reduced and volatized back into the atmosphere, while the other either enters the food chain or settles into sediments (Mason et al., 1994). This sedimentary compartment functions as an important mercury reservoir in aquatic systems (Pereira, 1996; Kim et al., 2004; Hung and Chmura, 2005; De Marco et al., 2006). Mercury entrance in food chain starts with bacterial methylation of the inorganic mercury in water (Spry and Wiener, 1991). This is the most important step in the environmental mercury cycle since it greatly increases mercury toxicity and bioaccumulation potential (Scudder et al., 2009). Methylmercury biotransfer will occur from sites of production to higher trophic levels, both via benthic and pelagic pathways (Chen et al., 2009) (Figure 1.1).

Mercury in biota

Mercury bioaccumulation in aquatic organisms occurs from the combination of two sources of contamination: direct exposure (in the water) and trophic exposure (in the diet) (Boudou and Ribeyre, 1985). The contribution of each route is species-specific and depends on the mercury bioavailability in water and diet (Rainbow, 2002), as well as on the chemical form of mercury, which can exhibit different abilities to cross the biological barriers (Boudou and Ribeyre, 1985). In this sense, some authors estimated the bioaccumulation potential for mercury forms, demonstrating that methylmercury's potential is 1000 times higher than that of the inorganic form (Scudder et al., 2009). Furthermore, the bioaccumulation factor from water to edible fish tissues is thought to exceed 10 million times, indicating that even small environmental levels have the potential to accumulate in harmful concentrations (Clarkson, 1992).

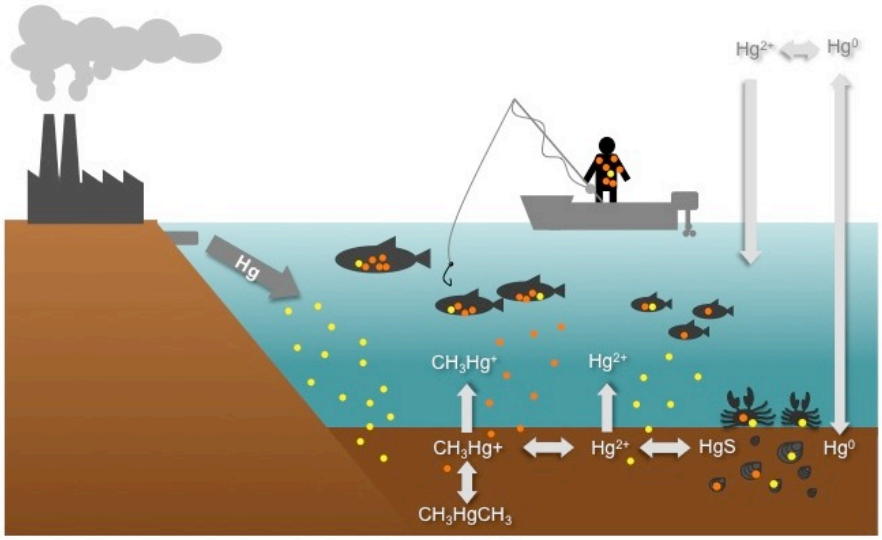

Figure 1.1 Conceptual model of the aquatic mercury cycle.

Mercury in biota have been extensively assessed, from primary producers to invertebrates, fish and birds (e. g. Abreu et al., 2000; Coelho et al., 2005; Coelho et al., 2007; Coelho et al., 2008; Tavares et al., 2008) with considerable amounts of mercury having being found in several aquatic ecosystems, with severe implications for the ecosystems' health.

Mercury in humans

Humans are susceptible to mercury contamination through several pathways, such as air, food, water and chemical products (e.g., cosmetics and vaccines) (Zahir et al., 2005). However, fish and seafood have been referred as the major source of methylmercury through ingestion, leading fish eating populations to an increased risk. Although all forms of mercury are highly toxic, elemental and organic mercury are more efficiently absorbed, the former by inhalation and the latter by ingestion. Chronic exposure to low mercury concentrations occurs either by fish consumption or occupational exposure and it is a matter of concern, having originated the development of several guidelines regarding mercury hazardous effects, as well as public awareness campaigns. Weiner and Nylander (1995) found that individuals occupationally exposed had high amounts of inorganic mercury in the brain, thyroid and pituitary gland, with biological half-lives of several years or decades.

The most common adverse health effects associated to mercury are: cardiovascular diseases, anemia, developmental abnormalities, neurobehavioral disorders, kidney and liver damage, and in some cases, cancer (Suton and Tchounwou, 2006). Mercury has also been recently linked to diseases like Alzeihemer's, Parkinson's, Autism, Lupus and Amyotrophic Lateral Sclerosis (Zahir et al., 2005). In humans, mercury seems to have particular toxicity in the first years of life. In fact, mercury is able to cross the placental barrier and cause damage in fetus' central nervous system (CNS). Moreover, Ramón et al. (2008) identified a high proportion of newborns at levels of exposure that could pose a risk of subtle adverse effects in neurodevelopment. This is particularly important since the CNS is not completely developed and damage seems to occur in an extensive and unspecific way, contrary to what is verified in adults. In addition, children exposed to mercury seem to develop acrodynia (peripheral neuropathy, skin discoloration, swelling and desquamation) and photophobia (excessive sensitivity to light) (ATSDR, 2003).

1.3 Mercury toxicity mechanisms

Oxidative stress

In general, the most important toxicological effects reported for mercury are neurotoxicity, nephrotoxicity and gastrointestinal toxicity, with ulceration and haemorrhage (Stohs and Bagchi, 1995). Once absorbed, mercury rapidly accumulates in all tissues and organs, but mainly in the brain, liver and kidney (Quig, 1998; Ercal et al., 2001). Although mercury pathologies are well recognized, their molecular mechanisms of action are not fully understood. However, its chemical and biological features suggest that oxidative stress might be involved in mercury-induced toxicity, demonstrated for both *in vivo* and *in vitro* models (Ercal et al., 2001; Crespo-López, 2007; Shanker and Aschner, 2003). Mercury is highly reactive with sulphydryl groups of proteins, forming covalent bonds with reduced glutathione (GSH) and cystein residues of proteins. GSH is the primary antioxidant and conjugating agent, being the first line of cellular defence against mercury. One single ion of mercury is able to bind up to two GSH molecules, causing their irreversible excretion (Quig, 1998); this conjugation is required in order to process mercury excretion into the bile. However, the binding of mercury to GSH or cystein, GSH's precursor, promotes the accumulation of reactive oxygen species (ROS), since they would normally be eliminated by GSH (Sarafian, 1999).

Oxidative stress occurs when ROS are not adequately removed. This can happen if antioxidants are depleted and/or if the formation of ROS is increased beyond the ability of the defences to cope with them (Sies, 1991). Under oxidative stress conditions, hydrogen peroxide is formed, enhancing the formation of lipid peroxides and the hydroxical radical, the most toxic ROS (Figure 1.2). Thus, it is expected that the activation of the antioxidant defence enzymes occurs, in order to compensate for the mercury-induced oxidative stress.

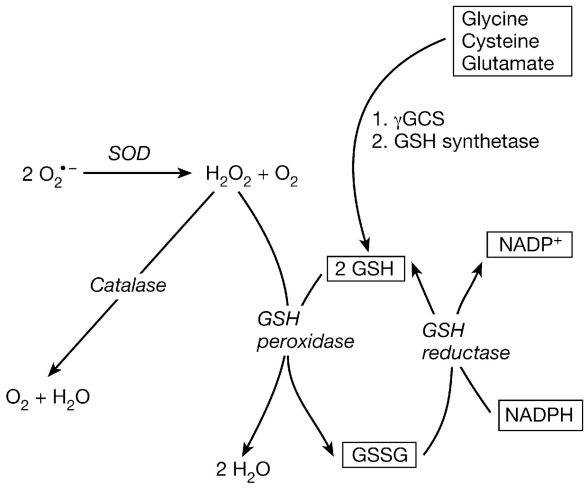

Figure 1.2 Diagram showing the interaction between intracellular antioxidants. $O_2^{\bullet -}$ = superoxide anion radical, SOD= superoxide dismutase, H_2O_2= hydrogen peroxide, GSH= reduced glutathione, GSSG= oxidized glutathione, γGCS= γ-glutamylcystein synthetase, $NADP^+$/NADPH= nicotinamide adenine dinucleotide phosphate. In Macdonald et al. (2003).

Superoxide dismutases (SODs) are a group of metalloenzymes that catalyse the dismutation of the superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), providing an important defence against the toxicity of the superoxide radical. SODs are regarded as a crucial part of cellular antioxidant defence system, exerting a pivotal antioxidant role (Malstrom et al., 1975). Their importance is confirmed by their presence in all studied aerobic organisms (Stegeman et al., 1992).

Catalases (CATs) are hematin-containing enzymes that facilitate the removal of H_2O_2, which is metabolized to O_2 and water. Contrarily to other peroxidases that can reduce various lipid peroxides as well as H_2O_2, CATs can only reduce H_2O_2 (Stegeman et al., 1992; Filho, 1996) and use H_2O_2 itself as electron donor. Glutathione peroxidase (GPx) catalyzes the reduction of peroxides, including hydrogen peroxide, using GSH as a

co-factor. It plays an essential role in protecting membranes from damage due to lipid peroxidation (LPO) and thus its major function is the termination of radical chain propagation by quick reduction to yield further radicals (Lauterburg et al., 1983).

Glutathione reductase (GR) is a flavoprotein that catalyzes the NADPH-depend reduction of the oxidized glutathione (GSSG) to GSH (Calberg and Mannervik, 1985). This enzyme is essential to maintain the adequate levels of cellular GSH, by maintaining a high GSH/GSSG ratio. Furthermore, it plays a major role in GPx and glutathione S transferases (GSTs) reactions as an adjunct in the control of peroxides and free radicals (Bompart et al., 1990). Elevated GR activity has been observed in organisms exposed to pro-oxidant stressors (Regoli et al., 2000, 2002).

Enhancement of GSH and the described antioxidant enzymes, both in mammals and fish, has been demonstrated to be an adaptative response to mercury environmental pollution (Sringari et al, 2007; Vieira et al., 2009; Huang et al., 2010; Oliveira et al., 2010). However, Elia et al. (2000, 2003) verified that the activation and inhibition of the enzymes by the action of mercury depends on its concentration. Furthermore, depletion of the antioxidant enzymes is often demonstrated under mercury exposure/contamination (Padmini et al., 2009; Rao and Chhunchha, 2009; Argawal et al., 2010; Chatziargyriou et al., 2010). Despite the diversity of responses according to the different mercury contamination degrees, the existing data suggests that mercury affects the antioxidant defense systems, exhibiting a pro-oxidative ability.

Peroxidative damage

Quantification of LPO is fundamental to assess the role of oxidative injuries (Cross et al., 1987; Porter et al., 1995; Halliwell, 1996). LPO is a consequence of the formation of highly reactive and unstable hydroperoxides, of both saturated and unsaturated lipids. The process of LPO occurs by a chain reaction, demonstrating the ability of a single radical species to propagate a number of deleterious biochemical reactions. The actual chemistry of LPO and associated production of various free-radical species is extremely complex (Kappus, 1987). LPO intensity is assessed as the levels of primary products, conjugated dienes and lipid peroxides, and/or end products of LPO such as malondialdehyde and other aldehydes, which are assayed with thiobarbituric acid and expressed as thiobarbituric acid reactive substances (TBARS) (Rice-Evans et al., 1991).

Metal toxicity exerted through lipid peroxidation is considered as a first step of cellular membrane damage (Viarengo, 1989). In agreement, the increase of LPO levels

with increasing mercury concentrations has been demonstrated in several *in vivo* and *in vitro* studies (Rao and Chhunchha, 2009; Vieira et al., 2009; Argawal et al., 2010).

Inhibition of Mitochondrial Oxidative Phosphorylation

Impair of the efficiency of oxidative phosphorylation and electron transport at the ubiquinone-cytochrome b5 step has also been pointed as one of the molecular adverse effects of mercury (Chavez and Holguin, 1988; Lund et al., 1991). It seems that mercury is responsible for accelerating electron transfer rates in the electron transport chain in mitochondria, causing premature shedding of electrons to O_2, which increases and generates O_2^- and H_2O_2 (Ercal et al., 2001). This has been pointed out as the mechanism that leads to nephrotoxicity; inorganic mercury seems to increase the H_2O_2 production, by impairing oxidative phosporylation and electron transport (Nath et al., 1996). Moreover, it is recognized that organic mercury can be an uncoupling agent, stimulating state IV respiration (Verity et al., 1975); its exposure induces a decrease in the activity of enzymes of the mitochondrial energy metabolism such as cytochrome C oxidase (CCO), SOD and succinate dehydrogenase (SDH) (Yoshino et al., 1966).

Effects on Calcium Homeostasis

Ca^{2+} increase beyond physiological levels activates hydrolytic enzymes such as phospholipases, proteases, and endonucleases, causes mitochondrial dysfunction and disturbs cytoskeletal organization (Nascimento et al., 2008). The increase of calcium is known to activate phospholipase A2 that induces the generation of arachidonic acid, which is recognized as an important target for ROS (Halliwell and Gutteridge, 1989). Arachidonic acid, on its turn, induces lipoxygenase and cyclooxygenase resulting in the production of O_2^- (Keyser and Alger, 1990). Conversion of xanthine dehydrogenase to xanthine oxidase is known to be another outcome of the calcium increase leading to the formation of O_2^- and H_2O_2 as by-products (Ercal et al., 2001).

The ability of organic and inorganic mercury to alter calcium homeostasis is recognized, although exhibiting different mechanisms of action (Tan et al., 1993). While organic mercury is thought to enhance intracellular calcium by both the influx of calcium from the extracellular medium and by mobilizing intracellular calcium stores, inorganic mercury is believed to increase intracellular calcium only by enhancing the influx from the extracellular medium (Tan et al., 1993). This effect was verified for mammals and has all the potential to disrupt the synaptic function and impair the neural development (Marty

and Atchison, 1998).

In vitro experiments demonstrated that Ca^{2+} channel blockers significantly delay organic mercury induced increase of Ca^{2+} levels (Marty and Atchison, 1997). Moreover, an experiment with rats subjected to organic mercury, demonstrated that the blockers of voltage-dependent Ca^{2+} channels avoid the development of neurological disorders (Sakamoto et al., 1998). Modification of calcium channels (depolarization or stimulation) by mercury activates apoptosis in cell lines (Sutton and Tchounwou, 2006). These findings indicate that alterations in Ca^{2+} homeostasis represent important cellular features in the organic mercury toxicity.

Effects on microtubules networks

Mercury's ability to react with sulfhydryl groups of proteins and enzymes contributes to its ability to disrupt cell cycle progression and/or apoptosis in various tissues (Sutton and Tchounwou, 2006). Mercury can interact with cytoplasmatic cytoskeletal components, including microtubules (Sager and Doherty, 1983). *In vitro* studies demonstrated that low levels of mercury present high affinity for tubulin sulphydryl groups, depolymerizing microtubules from brain cells and directly inhibiting their assembly (Leong et al., 2001). Additionally, it has been demonstrated that mercury disruption of microtubules occurs in several cell models like human fibroblasts, neuroblatoma and glioma cells (In Nascimento et al., 2008). The microtubule function is essential for the physiological development of CNS, including cell proliferation, migration of post-mitotic neurons to form the cortical layers of the cerebrum and cerebellum, extension and stabilization of neuritis, and axodendritic transport (Nascimento et al., 2008). Microtubule collapse is critical due to the changes in the neurons' cellular membrane, leading to disassemble of microtubules that maintain neurite structure. These alterations caused by mercury are consistent with neuropathologic findings in the brain of Alzheimer's patients (Leong et al., 2001).

Genotoxicity

Mercury genotoxicity has been considered one of the consequences of mercury's ability to react with the sulfhydryl groups of tubulin, impairing spindle function and leading to chromosomal aberrations and polyploidy (Figure 1.3). Organic mercury compounds have been demonstrated to be more active in terms of genotoxicity, than inorganic compounds (De Flora et al., 1994). Moreover, mercury genotoxicity can also be attributed to the production of free radicals that can cause DNA damage (Schurz et al., 2000).

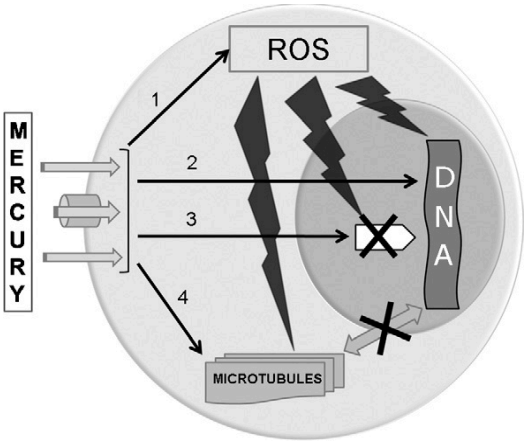

Figure 1.3. Possible mechanisms for mercury-induced toxicity. Molecular mechanisms of mercury genotoxicity. Mercury compounds enter the cell through plasmatic membrane or transport proteins (grey cylinder). (1) Inside the cell, they may produce reactive oxygen species (ROS) which react directly with DNA or, indirectly, induce conformational changes in proteins responsible for the formation and maintenance of DNA (DNA repair enzymes, proteins of microtubules). Mercury compounds may be also able to bind directly to: (2) DNA molecules, forming mercury species-DNA adducts, (3) "zinc fingers" core of DNA repair enzymes (white large arrow), affecting their activity and (4) microtubules, avoiding mitotic spindle formation and chromosome segregation. From Crespo-López et al., 2009.

Silva-Pereira et al. (2005) found a significant dose-related increase in the number of cells showing chromosome aberrations and increased incidence of polyploidy after treatment with organic mercury, confirming the effects of mercury on the mitotic spindle. A recent study demonstrated that very low organic mercury concentrations can initiate genotoxic processes in human cell lines of brain origin (Crespo-Lopez et al., 2007). Studies with fish revealed that both mercury compounds are able to induce genotoxicity by interactions with the functioning of motor proteins, leading to aneugenicity, and generation of reactive oxygen, leading to clastogenicity (Çavas, 2008; Guilherme et al., 2008b).

Studies with humans exposed to mercury, either by fish consumption or by occupational exposure, have also demonstrated a clastogenic effect (Al-Sabti et al., 1992; Amorim et al., 2002). These processes seem to be involved in spontaneous abortion, birth defects, cell transformation, and tumor progression (Kirsch-Volders et al., 2002).

Endocrine disruption

Endocrine effects of mercury are not fully explored, although there are sufficient

evidences supporting disruptive effects of mercury compounds on the functions of the thyroid and adrenal glands, ovaries, and testis (Zhu et al., 2000). The major mechanism is thought to be by broad enzyme inhibition and the influence on the combining of hormones and their receptors, as a consequence of mercury avidity for binding to sulphydryl groups. In addition, peroxidation and calcium homeostasis changes can also be involved (Zhu et al., 2000). Tan et al. (2009) reviewed data evidencing mercury endocrine effects in both wildlife and humans. The previous authors found that the main endocrine-related mechanisms of mercury are: (a) accumulation in the endocrine system; (b) specific cytotoxicity in endocrine tissues; (c) changes in hormone concentrations; (d) interactions with sex hormones; and (e) up-regulation or down-regulation of enzymes within the steroidogenesis pathway.

1.4 The Ria de Aveiro

The Ria de Aveiro is a partially mixed coastal lagoon located in the north-western coast of Portugal, that connects to the Atlantic Ocean by a single narrow deep channel (Barra de Aveiro) (Figure 1.4). Its geological formation started in the tenth century, and man first stabilized the connection to the sea in 1808 (Cunha, 1930). The lagoon has a complex morphology, consisting of many branching channels, being the four main channels the Ovar, Murtosa, Ílhavo and Mira channels (da Silva et al., 2004). It is 45 km long and in the largest part about 10 km wide (NNE–SSW), covering an area of approximately 83 km^2 of wetlands in high tide and 66 km^2 in low tide (Abrantes et al., 2006; Dias and Fernandes, 2006).

Characterized as a mesotidal system with predominantly semi-diurnal tides, the Ria de Aveiro has a mean tidal range of about 2.0 m. The minimum tidal range is 0.6 m (neap tides), and the maximum tidal range is about 3.2 m (spring tides), corresponding to a maximum and a minimum water level of 3.5 and 0.3 m, respectively (Dias et al., 2000). The fresh water contribution results from two major rivers, the Vouga and the Antuã, which outflow into the east side of the lagoon. Its hydrological circulation is dominated by seawater exchange due to the small freshwater input, comparing to the tidal prism at the entrance channel (Moreira et al., 1993). Accordingly, the Ria de Aveiro exhibits a well defined west-east salinity gradient from about less than 0.5 in the rivers to about 35 at the bar entrance, due to the dual effects of the freshwater discharge and tidal penetration (Lopes et al., 2001).

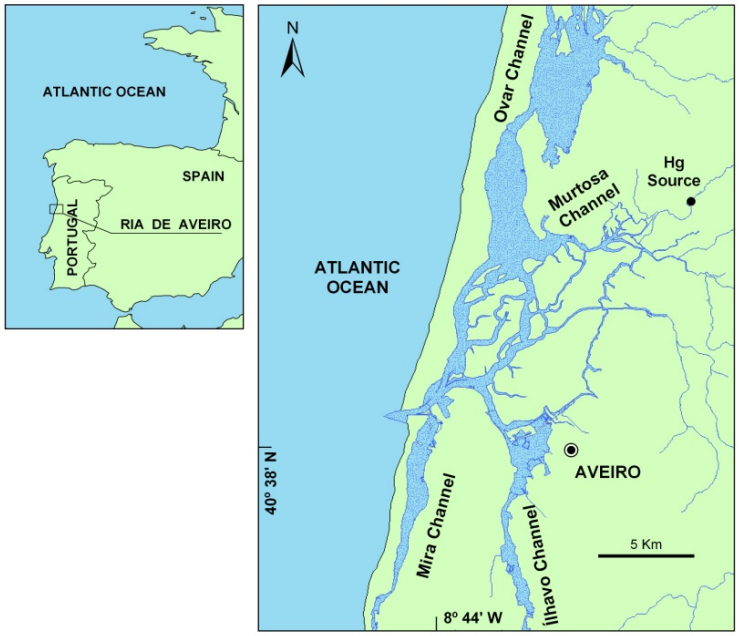

Figure 1.4 The Ria de Aveiro coastal lagoon (main channels and mercury source indicated).

The Ria de Aveiro has an inner area, the Laranjo Basin, that is considered a sub-system within the lagoon, as itself constitutes a lagoon due to its semi-enclosed characteristics. This inner basin is connected to the lagoon by a narrow channel and presents a well-defined salinity gradient, between saline water in the interior and freshwater from rivers, industrial and wastewaters (Lopes et al., 2001).

The watershed area of this complex system has a population of 250,000 inhabitants, with considerable regional importance since it supports activities intrinsically associated with the major population in coastal areas: port facilities, industries, aquaculture, salt-production and fishing (Lopes et al., 2008). The development of industry and agriculture has been pointed out as the main source of pollution, which has adversely affected the water quality.

1.4.1 The Laranjo Basin mercury problem

During four decades (1950 - 1994) the Laranjo area persistently received industrial effluents from a chlor-alkali chemical complex, located in Estarreja which represented the major contribution for the high accumulation of mercury in sediments (Pereira et al., 1997). The combined effects of the discharges and the cohesive nature of the sediments render this part of the lagoon more susceptible to contamination by metals (Marques et al., 1993). Mercury was one of the metals known to be present at high concentrations in the effluent discharges, inducing an environmental contamination gradient inside the lagoon (Pereira *et al.*, 1998a). Pereira et al. (1998b) estimated that about 33 tons of mercury are stored in the system, 77% of which are deposited in the Laranjo area. Mercury determinations performed during the chlor-alkali plant discharges, revealed concentrations ranging from 4 to 167 $\mu g\ L^{-1}$ in the dissolved fraction (Dis-Hg) and concentrations ranging from 141 to 3144 mg kg^{-1} in suspended particulate matter (SPM-Hg) (Pereira et al., 1995). The assessment of mercury contamination in the sediments (Sed-Hg) showed the existence of high mercury concentrations, ranging from 6.8 to 377 mg kg^{-1} (Lucas et al., 1986). Data from the same area reflected constant high mercury concentrations in the first 9 cm, and a vertical decline from 15 cm depth below (Hall et al., 1987).

In the last decade, the chlor-alkali industry changed the production technologies, leading to a considerable decrease of mercury discharges. Consequently, a decrease of mercury concentration in the surface sediments has been verified, with concentrations ranging from 2.3 and 63 mg kg^{-1} (Pereira et al., 1997). Since 2002 mercury emissions completely ceased, being verified an overall reduction of mercury levels in the estuary. Coelho et al. (2005) reported Dis-Hg levels around 0.34 to 2.8 µg L^{-1} and SPM-Hg levels ranging from 8.9 to 25.8 mg kg^{-1}, demonstrating a clear decrease when compared to previous studies. However, the same authors found that in the Laranjo area, Sed-Hg levels were between 6.2 and 51.6 mg kg^{-1}, corresponding to contaminated dredge material and to high contaminated sediments (class 4 and 5 of the Portaria nº1450/2007, Portuguese legislation).

These exceptionally high concentrations make the Ria de Aveiro a hotspot in terms of mercury contamination on the southwest Atlantic European coast (OSPAR, 2000; Pato, 2007). In this sense, efforts to understand the mercury processes in the environmental compartment and in biota resulted in several studies, ranging from sediment analysis to mercury speciation (Pereira et al., 1998; Abreu et al., 2000; Ramalhosa et al., 2005; Coelho et al., 2006; Guilherme et al., 2008a; Pato et al., 2008; Válega et al., 2009).

1.5 Gaps in knowledge

Mercury is a priority hazardous substance and a global problem being subject of numerous studies, strategies and policies. Despite the large number of studies regarding mercury, knowledge concerning accumulation and toxicity in feral marine fish are still scarce.

To better predict the environmental dangers of mercury, it is necessary to understand its fate and accumulation in the ecosystems, extrapolating beyond laboratory. Moreover, body bioaccumulation is difficult to predict, leading to the need of ascertain accumulation at the tissue level. Thus, to better acknowledge mercury accumulation and distribution processes within the body, it is necessary to study a wide range of tissues, rather than only muscle and liver that are commonly used as body burdens. Bioaccumulation studies in fish are important to clarify the aquatic behaviour of environmental contaminants. However, it is essential to establish the potential relationship between the mercury accumulation and the fish health status in order to detect early-warning signals of environmental risk. In agreement, the combined use of bioaccumulation markers (body burdens) and effect biomarkers is essential and has a great potential to integrate environmental monitoring studies. Additionally, this combined perspective should also integrate the understanding of sediment/biota accumulation factors, and the influence of environmental parameters and mercury speciation. This perspective engages several advantages by avoiding confounding factors and thus providing information regarding mercury levels able to induce toxic effects.

In accordance several gaps were identified and addressed in this research, contributing with new knowledge of mercury fate and effects in the environment. Those gaps were:

1. The mercury whole-fish picture was not fully investigated in marine species under realistic conditions;
2. The knowledge of the mercury burden and distribution among a wide and representative variety of key tissues of fish is scarce;
3. Mercury speciation in fish tissues is not common, despite its relevance for understanding and predicting organic mercury bioavailability;
4. The mechanisms of mercury-induced toxicity in fish are still unclear;
5. Scarce information regarding mercury levels able to induce toxic effects.

1.6 General Aims and Thesis Outline

The general goal of this work was to contribute to the understanding of the environmental mercury problem by addressing questions such as mercury accumulation in several fish tissues and their interdependence with biological factors and biochemical responses. To accomplish this, two representative fish species of the Ria de Aveiro, with different feeding strategies, were chosen - the golden grey mullet (*Liza aurata*) and the European sea bass (*Dicentrarchus labrax*). Moreover, the study relied on juveniles in order to avoid misinterpretations regarding the interference of variables such as, gender and reproductive processes, as well as the potential occurrence of a growth dilution effect, relatively to mercury accumulation.

The general aim was accomplished by addressing the following specific objectives, which are in agreement with the identified gaps in knowledge:

1. To evaluate the mercury loads in the different tissues (gills, blood, liver, kidney, intestine, brain and muscle) and its relation with mercury abiotic concentrations (water, sediment and suspended particulate matter);
2. To compare mercury distribution in several tissues of different fish species, identifying the species and tissue that best reflect the mercury environmental levels;
3. To assess mercury speciation in fish tissues by determining the organic mercury concentrations;
4. To evaluate the interdependency between accumulated levels in the different tissues and factors such as feeding ecology and other non contamination-related factors;
5. To assess the mercury-induced toxicity, by evaluating antioxidant defences, membrane damage, and metallothioneins content. It was also intended to establish a causal relationship between mercury burdens and these previous responses, as well as to evaluate the influence of seasonal variations of mercury availability and the tissue-specific biomarker responses.

These main questions are addressed in the seven chapters (Chapters 2 to 8) that constitute the focal point of this thesis and corresponded to scientific articles accepted or submitted to peer-review journals. In the end, an overview of the results is presented,

integrating the response to the previous questions, main conclusions and future research areas (Chapter 9).

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CHAPTER 2

Mercury distribution in key tissues of fish (*Liza aurata*) inhabiting a contaminated estuary – Implications for human and ecosystem health risk assessment

Mercury distribution in key tissues of fish (*Liza aurata*) inhabiting a contaminated estuary – Implications for human and ecosystem health risk assessment

Abstract

This study brings a new viewpoint based on multiple-tissue analyses to form the basis of a predictive mode of mercury accumulation dynamics in fish body under field conditions. Total mercury (T-Hg) was determined in key tissues of *Liza aurata* captured along an estuarine contamination gradient, displaying the following hierarchy: kidney > liver > muscle > brain > gills > blood. Brain was the tissue that better reflected the mercury contamination extent, closely followed by liver and muscle. Organic mercury (O-Hg) measured in muscle and liver, represented more than 85% and less than 30% of T-Hg, respectively. The lowest O-Hg percentage was found in the most contaminated area, for both muscle and liver. Mercury distribution and accumulation patterns showed to depend on the specific tissue. The high mercury levels found in organs involved in vital physiological processes point out the risk to autochthonous fish fauna. Human risk associated to the ingestion of fish living in the surveyed areas cannot be excluded.

Keywords: Mercury; accumulation dynamics; tissue distribution; Ria de Aveiro; *Liza aurata*.

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2.1 Introduction

Estuarine habitats are potentially impacted by many anthropogenic influences, being important sinks of pollutants (Amado et al., 2006) where metals represent a particular threat for both aquatic wildlife and humans. Among metals of environmental concern, mercury has deserved increasing attention due to its ubiquity, persistence and toxicity. Mercury has high affinity for suspended particles, which conducts to its removal from the water column and accumulation in sediments. Thus, sediments function as deposit and as source of mercury to the pore water and biota (Ramalhosa et al., 2001). It is also known that methylation processes mediated by bacteria occur in sediments, converting inorganic mercury into methylmercury, the most toxic form. Both methylmercury and inorganic mercury are present in the organisms associated to sulphydryl groups thereby disturbing almost any function where critical or non-protected proteins are involved (Guzzi and La Porta, 2008).

The direct and indirect coupling between ichthyofaunal communities and human impacts on estuaries reinforces the choice of this taxonomic group as a biological indicator that can assist in the formulation of environmental and ecological quality objectives, and in the setting of quality standards (Whitfield and Elliott, 2002). Fish is the main route of environmental exposure to mercury and thus the main source of methylmercury in human's diet (Shimshack et al., 2007). Methylmercury concentrations in fish are approximately 1,000 to 10,000 times greater than in other food (such as cereals, vegetables, meats, eggs and milk) (EPA, 2001), presenting a risk of negative impacts on human health, affecting the central nervous (CNS), cardiovascular and immune systems (EPA, 2001; Jewett and Duffy, 2007). On the other hand, due to its wide distribution and trophic position, fish are particularly able to reflect aquatic contamination by metals, being thus desirable components of biomonitoring programs. Therefore, from the standpoint of both human and ecosystem health risk assessment, fish emerge as a suitable choice.

The prediction of the fate of metals with simple models is virtually impossible. Hence, according to Van der Oost et al. (2003), bioaccumulation should be addressed including toxicokinetics, metabolism, biota-sediment accumulation factors and organ-specific bioaccumulation. While considerable work has focused on mercury accumulation in fish liver and muscle, the most common body burdens (Afonso et al., 2007; Agusa et al., 2007), relatively little attention has been devoted to the distribution in other important target tissues. Moreover, the majority of available literature, though sporadically addressing other tissues, concerns laboratory approaches (Berntssen et al., 2003; Mela et

al., 2007). The significance of fish laboratory exposures is often compromised by the use of environmentally unrealistic concentrations, as well as by artificial modes of exposure such as a single exposure route. Additionally, previous field studies didn't take into account an extensive range of tissues, and their main purpose was to assess the human risk through fish consumption (Storelli et al., 2005; Burger and Gochfeld, 2007). The field works carried out by Cizdziel et al. (2003) and Maury-Brachet et al. (2006) constitute an exception since a wide set of tissues was evaluated, but it concerned only freshwater species.

In the light of the previous statements, it is manifest that the whole-fish picture was not fully explored on marine species under realistic conditions and further information is still needed concerning a wide and representative variety of key tissues. This integrated and multi-compartment approach is essential to predicted mercury bioavailability to fish as well as to meaningful risk assessment. In this perspective, the present study brings a new viewpoint in the distribution of total mercury in six tissues (gills, blood, brain, liver, kidney and muscle) evaluated in feral golden grey mullet (*Liza aurata*) captured along a mercury-contaminated area (Laranjo Basin, Ria de Aveiro – Portugal). The study area was selected on the basis of an identified mercury gradient, resulting from five decades of continuous discharges from a chlor-alkali plant (Pereira et al., 1998). This confined mercury gradient and the absence of other important sources of contamination offer a unique opportunity for the assessment of mercury accumulation dynamics under natural conditions. Thus, the main objectives of this study were: i) to investigate the tissue-specific total mercury loads in *Liza aurata* and their relation to abiotic concentrations (water, sediment and suspended particulate matter - SPM); ii) to improve the knowledge on mercury uptake, distribution and retention, and select the tissue that better reflects the metal contamination degree; iii) to evaluate the environmental risk to the autochthonous fish fauna; and iv) to measure total mercury and methylmercury concentrations in the edible tissue in order to estimate the risk for human health resulting of the consumption of fish inhabiting the study area.

2.2 Material and methods

2.2.1 Study area

Ria de Aveiro is a lagoon adjacent to the Atlantic Ocean, presenting an inner area (Laranjo Basin) (Fig. 2.1), which has persistently received mercury-containing effluents

from a chlor–alkali plant since 1950s until 1994. The discharges resulted in an accumulation of about $27x10^3$ kg of mercury in the lagoon, mostly (about 74%) associated to the sediment in the Laranjo Basin (Pereira et al., 1998). Due to the basin's morphology, mercury deposition occurred mainly in the entrance of the basin, decreasing farther from the contamination source, and low mercury concentrations can be found throughout the Ria de Aveiro lagoon (Ramalhosa et al., 2001; Ramalhosa et al., 2005).

The field campaign took place in March 2007 at three different locations, chosen according to the distance to the mercury source. Two sampling sites (L1 and L2) were chosen at Laranjo Basin, separated by a 2 km distance: L2 located closer to the mercury source and identified as a highly contaminated area and L1, downstream L2, as a moderately contaminated. A reference area (R) located in S. Jacinto was selected for comparison purposes due to its proximity to the lagoon entrance and the distance to the main polluting sources (pacheco et al., 2005).

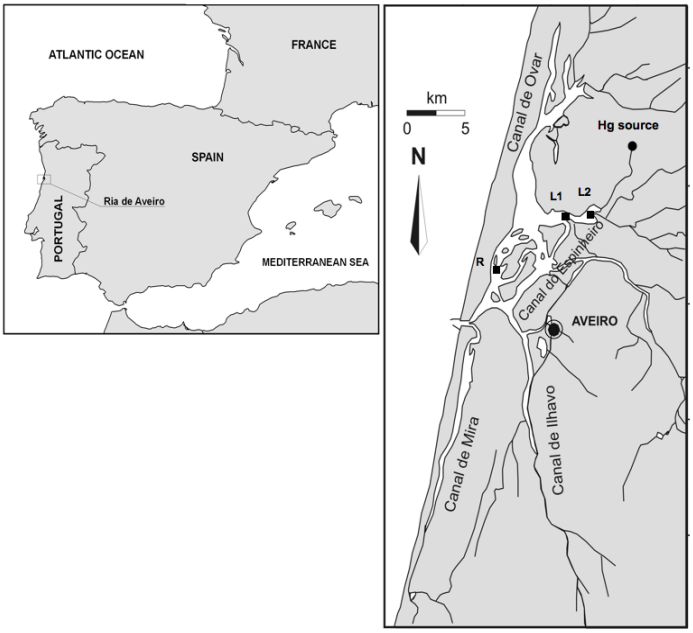

Figure 2.1. Map of the sampling stations (■) in the Ria de Aveiro (Portugal): reference (R - 40º41'00'' N, 8º42'44'' W), moderately (L1 - 40º43'34.46'' N, 8º38'53.16'' W) and highly contaminated (L2 - 40º43'28.98'' N, 8º37'35.80'' W) areas.

2.2.2 Sampling procedures

Fifteen juvenile golden grey mullets (*Liza aurata*) from the same-size group, i.e. with a total length of 11.6 ±1.25 cm and wet weight (w wt) of 14.6 ± 5.47 g (average ±

standard deviation), were collected at each sampling site during low tide, using a beach-seine net named “chincha”. Immediately after being caught, fish were sacrificed according to ethical recommendations and blood, brain, kidney, liver, gills and muscle (lateral dorsal) were sampled and kept cold. Blood was collected from the posterior cardinal vein by using heparinised Pasteur pipettes.

At the laboratory, tissue samples were freeze-dried, homogenized, weighted for mercury fresh weight calculations and total (T-Hg) and organic (O-Hg) mercury (only for muscle and liver) analyses were performed.

Water physico-chemical parameters such as pH (WTW-pH 330i), dissolved oxygen (WTW- oxi 330i), temperature and salinity were measured at sub-surface level, in low and high tide conditions. Turbidity was measured using a 20 cm black and white Secchi disc and water column depth was also evaluated. Sub-surface water samples were collected in acid-washed plastic bottles (one sample per site and tide conditions), kept cold 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 to pH <2 and stored at 4ºC until analysis. Filters were re-weighed after drying overnight at 60ºC and stored for determination of mercury in suspended particulate matter (SPM-Hg). Five replicates of surface sediments were taken in each sampled area. At the laboratory, sediment samples were freeze-dried, homogenized and sieved through a 1 mm sieve and stored for mercury determination.

2.2.3 Mercury analysis

Reactive (R-Hg) and total dissolved mercury (Dis-Hg) in water 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 Dis-Hg analysis, 50 mL of each sample was oxidized with 500 µL of a saturated solution of potassium persulfate and by irradiation with a UV lamp (1000 W) for 30 min; following irradiation, the excess of oxidant was reduced with 37.5 mL of hydroxylamine solution 12% (w/v) (Mucci et al., 1995). For determination in SPM (SPM-Hg), the same equipment was used after digestion of filters with HNO_3 4 mol L^{-1} (Pereira et al., 1998).

Sediments (Sed-Hg) and *L. aurata* tissues (T-Hg) samples were analyzed for T-Hg by atomic absorption spectrometry (AAS) with thermal decomposition and 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), namely 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 92-103%.

Organic mercury (O-Hg) determination was performed according to Válega et al. (2006), through digestion of the sample with a mixture of 18% KBr in 5% H_2SO_4, followed by extraction into toluene. Extractions were performed in duplicates and the aqueous fraction resulting from the addition of $Na_2S_2O_3$ solution was analyzed using an AMA - LECO 254 as referred for total mercury. Since this method requires a high sample amount (0.05–0.2 g), it was only possible to perform the analyses in muscle and liver samples. For the same reason, liver composite samples of three fish were prepared. Due to the lack of sufficient sample, the analysis was not performed in R fish. To validate O-Hg analyses, reference material TORT–2 was used. Precision of the method ranged between 0 and 5.9%, with a median extraction efficiency of 101.3%.

2.2.4 Statistical analysis

Data analysis followed standard statistical procedures (Zar, 1999). Data were tested for goodness of fit to normal distribution and requirements of homogeneity of variances were also determined. Analysis of variance (ANOVA) on ranks were 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 test). Spearman rank correlation factor (r) was determined for the total mercury concentration between the different tissues. Differences between means were considered significant at $p<0.05$.

2.3 Results

2.3.1 Environment characterization

Physico-chemical parameters of water are summarized in Table 2.1 In general, the three sampling stations were similar regarding environmental characterization with the exception of salinity during low tide, which ranged from 13 in L2 to 34 in R station. SPM in low tide also exhibited differences, i.e., L2 levels were 2.2 and 1.3 times higher in relation

to R and L1, respectively. Parameters such as temperature, pH, dissolved oxygen, water depth and turbidity were in the same range.

Table 2.1 Hydrological characteristics on reference (R), moderately (L1) and highly mercury contaminated (L2) areas at Ria de Aveiro: water temperature (T), dissolved oxygen (DO), pH, salinity, suspended particulate matter (SPM), turbidity and water depth.

Sampling station	Tide	T (ºC)	DO (mgL^{-1})	pH	Salinity	SPM (mgL^{-1})	Turbidity (m)	Depth (m)
R	high	16.4	10.8	8.4	34	45.1	1.2	5.4
	low	15.3	10.9	8.4	34	31.7	0.5	1.6
L1	high	15.3	10.6	8.2	28	40.5	0.9	3.1
	low	15.0	10.8	8.1	15	53.2	0.5	2.8
L2	high	12.5	8.7	8.3	32	m.v.	1.2	2.3
	low	12.2	8.9	7.8	13	70.0	0.3	1.0

n.d. – not determined

2.3.2 Mercury in water (dissolved and in SPM) and in sediment

Mercury concentrations in water column were, in general, low in the three sampling stations (Table 2.2). At high tide, only SPM-Hg showed higher values in Laranjo area (L1) in relation to R. Differently, during low tide conditions, R-Hg concentrations were similar in all the stations, while Dis-Hg concentrations doubled in L2 comparing to R and L1. The SPM-Hg concentration was almost ten times higher in L2 than in R.

Table 2.2 Concentrations of reactive mercury (R-Hg), total dissolved mercury (Dis-Hg) ($ng\ L^{-1}$), total mercury in suspended particulate matter (SPM-Hg) ($mg\ Kg^{-1}$) in water, and Sed-Hg in sediment ($mg\ Kg^{-1}$ dry weight) (average ± standard deviation) at each sampling station at Ria de Aveiro: reference (R), moderately (L1) and highly mercury contaminated (L2) areas.

		Water			Sediment
Sampling station	Tide	R-Hg ($ng\ L^{-1}$)	Dis-Hg ($ng\ L^{-1}$)	SPM-Hg ($mg\ Kg^{-1}$)	Sed-Hg ($mg\ kg^{-1}dw$)
R	high	5.8 ± 1.0	19 ± 4.5	0.6 ± 0.08	0.01 ± 0.001
	low	4.4 ± 1.6	10.3 ± 1.1	0.84 ± 0.12	
L1	high	3.0 ± 1.4	8.3 ± 0.64	1.2 ± 0.72	0.08 ± 0.006
	low	2.7 ± 0.75	10.6 ± 0.91	1.60 ± 0.71	
L2	high	3.0 ± 0.64	10.2 ± 1.2	n.d.	6.8 ± 0.16
	low	4.9 ± 1.8	20.8 ± 2.4	8.0 ± 0.61	

n.d. – not determined

Relevant differences between tides, at each sampling station, were observed mainly for T-Hg, showing clearly higher levels in low tide at L2 and the opposite at R.

Total mercury concentrations in sediments (Sed-Hg) increased 8 times from the reference station (R) to L1 and 85 times from L1 to L2, displaying the environmental contamination gradient (Table 2.2). L2 presented an increment of 680 times in relation to R.

2.3.3 Mercury accumulation in fish tissues

T-Hg concentrations, either in R or contaminated stations (L1 and L2), varied according to the tissue in the following manner: kidney > liver > muscle > brain > gills > blood (Fig. 2.2). Globally, T-Hg values ranged from 0.008 (blood at R) to 1.19 (kidney at L2) mg kg^{-1} (w wt).

The inter-stations statistical comparisons carried out for each individual tissue demonstrated that only brain and muscle displayed significantly higher T-Hg levels in L1. Though no statistically significant, clearly higher T-Hg values were observed in liver (2 times) comparing L1 with R. On the other hand, in L2 all the tissues, with the exception of kidney, showed significant differences to R. The brain was the tissue that revealed the greater T-Hg increase, followed by the liver, when compared L2 with R. Thus, when the L2 data were analysed in terms of T-Hg increment degree in relation to R, the tissues appeared ordered as follows: brain (4.8x) > liver (4.0x) > muscle (3.8x) > blood (2.8x) > kidney (2.4x) > gills (2.0x). Despite the lower increase magnitude, the same tissue ordination was observed in L1. Statistical significant increments from L1 to L2 were only verified for gills and muscle (1.8 and 2 times, respectively).

Tissue-to-tissue T-Hg ratios were calculated for the combination of all the assessed tissues (Table 2.3). The highest values were determined for tissue/blood ratios, being the maximum value found for kidney/blood, followed by the liver/blood ratio. Comparing each ratio in the three sampling stations, no statistically significant differences were found.

The Spearman rank correlation (r) analysis revealed significant positive correlations between T-Hg in muscle and in all the other tissues (r=0.565, r= 0.692, r= 0.947, r=0.555 and r=0.807 for gills, blood, liver, kidney and brain, respectively). Beside the mentioned correlation with muscle, T-Hg in blood was also positively correlated with gills, liver and brain (r=0.580, r=0.573 and r=0.748, respectively). In addition, significant

correlations were found between T-Hg in liver and brain (r=0.745), as well as between gills and kidney (r=0.621).

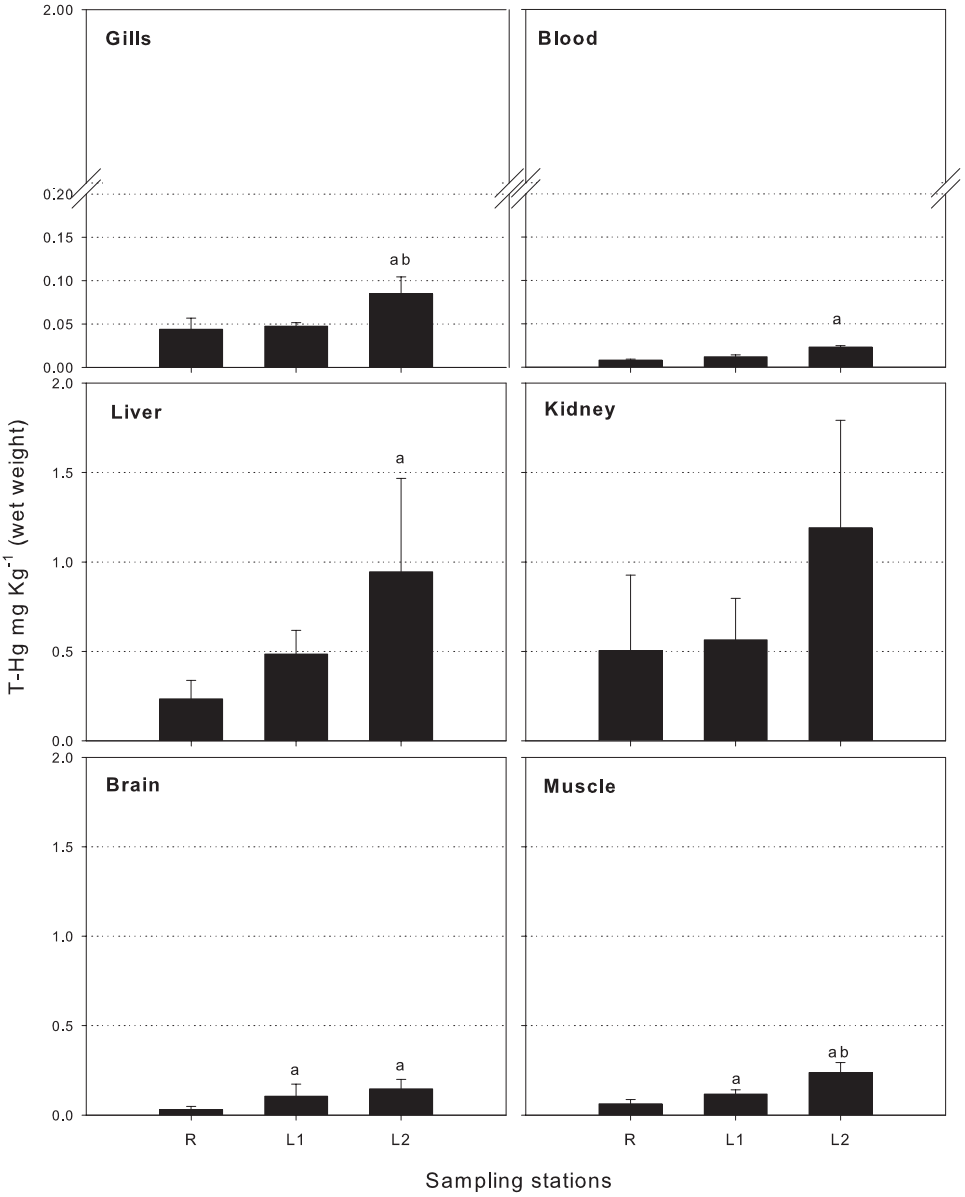

Figure 2.2 Total mercury (T-Hg) average concentration (mg Kg^{-1}w wt) in each sampling station at Ria de Aveiro: reference (R), moderately (L1) and highly mercury contaminated (L2) areas. The letters denote statistically significant differences ($p<0.05$): (a) *versus* R and (b) *versus* L1. Error bars represent the standard deviation.

Table 2.3 Inter-tissue ratios (average ± standard deviation) for the three sampling stations at Ria de Aveiro: reference (R), moderately (L1) and highly mercury contaminated (L2) areas.

	Sampling station	Inter-Tissue Ratio					
		Muscle	Blood	Liver	Kidney	Gills	Brain
Tissue/Muscle	R		0.13 ± 0.06	3.7 ± 0.62	7.5 ± 12	0.58 ± 0.22	0.51 ± 0.20
	L1		0.12 ± 0.03	4.0 ± 0.36	5.5 ± 1.2	0.4 ± 0.09	0.51 ± 0.64
	L2		0.10 ± 0.04	3.3 ± 2.0	3.3 ± 3.0	0.40 ± 0.09	0.70 ± 0.30
Tissue/Blood	R	7.5 ± 2.5		27 ± 11.0	65 ± 0.42	5.0 ± 1.3	4.4 ± 2.1
	L1	8.5 ± 3.2		32 ± 13	32 ± 0.23	4.0 ± 0.90	9.5 ± 7.5
	L2	9.9 ± 0.4		34 ± 24	47 ± 0.82	3.8 ± 0.78	5.8 ± 2.5
Tissue/Liver	R	0.28 ± 0.04	0.04 ± 0.01		1.7 ± 2.9	0.18 ± 0.13	0.13 ± 0.07
	L1	0.25 ± 0.02	0.03 ± 0.08		1.0 ± 0.24	0.01 ± 0.03	0.12 ± 0.18
	L2	0.27 ± 0.09	0.03 ± 0.01		1.2 ± 0.45	0.11 ± 0.05	0.18 ± 0.10
Tissue/Kidney	R	0.28 ± 0.27	0.03 ± 0.03	0.60 ± 0.9		0.51 ± 0.42	0.17 ± 0.13
	L1	0.24 ± 0.07	0.028 ± 0.01	0.98 ± 0.20		0.56 ± 0.23	0.26 ± 0.22
	L2	0.25 ± 0.12	0.023 ± 0.01	0.87 ± 0.31		1.2 ± 0.82	0.18 ± 0.13
Tissue/Gills	R	1.6 ± 0.85	0.2 ± 0.05	6.1 ± 3.9	10.4 ± 8.1		0.70 ± 0.5
	L1	2.5 ± 0.52	0.26 ± 0.05	10 ± 3.0	12.0 ± 5.1		1.4 ± 1.2
	L2	2.8 ± 0.82	0.30 ± 0.08	11 ± 5.6	11.3 ± 7.8		1.8 ± 1.0
Tissue/Brain	R	1.9 ± 0.21	0.22 ± 0.045	6.8 ± 1.6	9.1 ± 6.1	1.4 ± 9.3	
	L1	1.5 ± 0.80	0.16 ± 0.12	6.3 ± 3.6	6.4 ± 4.5	0.71 ± 0.30	
	L2	1.7 ± 0.85	0.20 ± 0.10	7.1 ± 4.03	9.7 ± 7.2	0.58 ± 0.41	

The determination of O-Hg revealed high percentage values (> 85%) in muscle with a concentration range (absolute values) of 0.065 - 0.20 mg Kg^{-1} w wt, whilst hepatic O-Hg was lower than 30% with a concentration range of 0.16 - 0.25 mg Kg^{-1} (Table 2.4). The lowest percentage of O-Hg was found in the most contaminated station (L2), both for muscle and liver. For muscle, significant differences ($p<0.05$) were found on O-Hg between L2 and R and between the two contaminated stations (L2 and L1). For liver, no significant difference on O-Hg was found between L1 and L2 (comparisons with R are not feasible). A positive correlation was found between T-Hg and O-Hg in muscle ($r=0.987$; $p<0.05$).

Table 2.4 Total (T-Hg) and organic mercury (O-Hg) (average ± standard deviation) concentrations (mg Kg^{-1} dry weight) and percentage of O-Hg relative to T-Hg in muscle and liver of *L. aurata* at each sampling station at Ria de Aveiro: 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. % O-Hg was calculated as the average of individual values of O-Hg/T-Hg.

Sampling station	Tissue	T-Hg (mg Kg^{-1})	O-Hg (mg Kg^{-1})	% O-Hg
R	Muscle	0.063 ± 0.023	0.07 ± 0.02	94.0 ± 0.034
	Liver	0.23 ± 0.10	n.d.	n.d.
L1	Muscle	0.12 ± 0.023 [a]	0.11 ± 0.027	97.0 ± 0.058
	Liver	0.51 ± 0.11	0.16 ± 0.060	30.0 ± 0.051
L2	Muscle	0.24 ± 0.055 [a,b]	0.20 ± 0.042 [a,b]	85.0 ± 0.082 [b]
	Liver	1.1 ± 0.46 [a]	0.25 ± 0.025	24.0 ± 0.077

n.d.- not determined

2.4 Discussion

Mercury, as a non-essential element, is not expected to have its uptake/elimination actively regulated and subsequently its tissue concentrations can vary in a wide range, reflecting exposure to environmental levels and feeding behaviour (Capelli et al., 2008). Hence, mercury body burdens in bioindicator species provide sensitive indications of aquatic pollution as well as on the potential impact on organism health (Kotze et al, 1999). However, the metal distribution within the body depends both on the fish species and the metal's properties (Gaspić et al., 2002). Additionally, biotopes' physico-chemical characteristics and the dominant uptake route are important factors to determine the bioavailability and accumulation patterns. As mentioned in the literature, fish tissues have high mercury bioaccumulation capacity for both organic and inorganic forms (Gochfeld, 2003); moreover, the mercury accumulation in different fish tissues is to a large extent dependent on their physiological role and regulatory ability. Therefore, in order to have a full insight of accumulation/detoxification mechanisms, several tissues/organs should be addressed.

The selection of key tissues/organs in the present study was carried out on the basis of their structural and functional properties, and subsequent association with the main processes that determine the mercury kinetics in fish body - uptake, distribution, biotransformation, storage, and depuration/excretion. Gills, due to their wide surface area and continuous contact with the external medium, are considered the main route for

uptake of mercury present on aqueous phase (Chen and Chen, 1999). In addition, their role on bioconcentration and excretion of toxicants can not be overlooked. Blood was selected as it is the vehicle for mercury distribution and can reflect current body burdens (Choi and Cech, 1998). Kidney and liver, besides their central role in basic physiology, are the main target organs since they are actively involved in the metabolism of heavy metals (Elia et al., 2003), acting as detoxification and storage organs (Filipović and Raspor, 2003). Beyond its neurological functions essential for survival, brain is of interest because it is a target organ for methylmercury, which is able to react directly with important receptors (Berntssen et al., 2003). Skeletal muscle is essential on mercury accumulation assessment as it constitutes more than 60% of the fish's body mass and a significant amount of tissue can be used for analytical purposes. Furthermore, it is well known that mercury accumulates on muscle mainly in the methylated form (Storelli et al., 2005; Magalhães et al., 2007), which is highly relevant regarding bioamagnification along food chains and also the risk to human health.

2.4.1 Relationships between environmental and tissue-specific mercury loads

The physico-chemical environmental parameters were similar along the three sampling stations and thus, not affecting determinately either the mercury bioavailability or the fish condition. Nonetheless, an exception should be made for SPM levels, which were found to be higher in Laranjo stations, namely at L2 in low tide (around 2 times the R levels). This difference is probably affecting the mercury bioavailability to fish, as discussed bellow.

Analysing the mercury levels in the different environmental compartments along the surveyed area, it is pertinent to stress that both Dis-Hg and R-Hg were not regularly higher at Laranjo stations (L1 and L2) in relation to R. The importance to ascertain R-Hg results from the fact that it is an easily reducible mercury species, representing the pool of mercury in the dissolved fraction that is bioavailable for the marine food web (Mason et al., 1995; Mason et al. 1996). Nevertheless, no clear differences on this mercury source are perceptible among sampling areas.

Contrarily, mercury in the sediment revealed great increments at Laranjo stations relatively to R (e.g. 680 times from R to L2). In the same way, SPM-Hg showed an increasing pattern towards the metal source. Considering the previously mentioned SPM increase in L2, the mercury bioavailability rise through this fraction can be estimated at around 21 times, i.e. 9.5 (for SPM-Hg) x 2.2 (for SPM) = 21.

The hierarchy of the assessed tissues on the basis of the T-Hg was kidney > liver > muscle > brain > gills > blood. The few available field studies concerning the determination of mercury in different fish tissues provide heterogeneous accumulation patterns depending on the species. Maury-Brachet et al. (2006) found the highest T-Hg either in kidney or in liver depending on the species, while muscle and gills presented substantial lower levels. In another study (Cizdziel et al., 2003), a larger set of tissues was analyzed displaying the order liver > muscle > brain > gill > blood, which completely agrees with our results found for *L. aurata*. Overall, the present results are consistent with the dominant idea proclaimed in the literature that liver and kidney are typically important organs for metal accumulation and storage in fish, presenting the highest mercury loads.

Comparing the tissue-specific T-Hg between the sampling stations, it is noteworthy that all the assessed tissues, with the exception of kidney, were able to signal the mercury contamination at L2. On the other hand, only brain and muscle showed the ability to reflect a moderate contamination status occurring at L1. The absence of statistically significant increases in kidney was related to high inter-individual variance, probably a consequence of limited amount of tissue available for chemical analysis.

According to Spry and Wiener (Spry and Wiener, 1991), concentrations higher than 5 μg g^{-1} T-Hg in brain and muscle are generally needed to exhibit symptoms of toxicity in fish. Therefore, the levels measured in these tissues in *L. aurata*, though significantly elevated, are below that limit.

An attempt to select the tissue that better reflects the mercury contamination extent should consider the following aspects: 1) the adoption of a tissue with high mercury loads may increase the assessment efficacy and minimize problems associated with the detection limits of the analytical methods; 2) a high increasing rate, measured in relation to reference conditions, improves the discriminatory power of a given tissue; 3) the capacity to point out from low to high environmental mercury levels expands its applicability to different contamination scenarios. In view of point 1, the liver appears as the best candidate followed by the muscle, since the usefulness of kidney seems to be compromised by the absence of statistically significant differences between R and L1 or L2 levels. Keeping in view the increments in environmental mercury levels from R to L2, namely on sediment (680 times) and SPM (9.5 times), and owing to the point 2, the brain would be the first choice (4.8 times increment), followed by the liver and muscle (4 and 3.8 times increment, respectively). Liver and kidney displayed the higher T-Hg basal levels in R; even so, liver demonstrated the ability to elevate T-Hg almost as distinctively as the

brain. In view of point 3, brain and muscle appear as appropriate tissues since both were able to signal mercury contamination at L1 and L2. Moreover, muscle was capable to distinguish between these two sites. Hence, a selection based on the joint analysis of the three criteria points out the brain as the most suitable tissue, closely followed by liver and muscle (brain > liver ≈ muscle).

Though gills and blood displayed less mercury loads, they can be particularly recommendable for species with high mobility or in migratory stages, since they usually reflect current exposures while more quiescent tissues/organs with high storage propensity can reflect past exposure and, thus, increasing the risk of misinterpretations.
The lowest percentage of O-Hg was found in the most contaminated area (L2), for both muscle and liver. Similar results were previously reported (Kannan et al., 1998; Coelho et al., 2006) and described as the "mercury accumulation paradox", being associated to the induction of mer-encoded enzymes, responsible for the degradation of organic mercury (Schaefer et al., 2004). The induction of these enzymes is proportional to the mercury in the environment; high levels induced the mer-encoded system that promotes the demethylation of mercury, leading to low O-Hg accumulation rates in biota (Schaefer et al., 2004).

2.4.2 Mercury accumulation dynamics

In this point, the results are discussed with the purpose to form the basis of a predictive mode of mercury accumulation dynamics, mainly on account of T-Hg inter-tissue ratios and correlations. The T-Hg determination on the selected tissues compared to skeletal muscle (considered as the reference tissue for biomagnification effects) has been used to study the uptake, retention, and elimination of this metal in fish (Cizdziel et al., 2003). Data from the literature indicate that when T-Hg in fish muscle is relatively low (less than 0.5 mg Kg^{-1} w wt), the corresponding levels in the liver are less than the muscle (Goldstein et al., 1996). Considering that current T-Hg in *L. aurata* muscle are of that magnitude (<0.24 mg Kg^{-1} w wt), lower levels would be expectable for the liver. However, liver displayed T-Hg (<1.1 mg Kg^{-1} w wt) around 4 times higher than the muscle, corresponding to high liver/muscle ratios, which constitutes an apparent divergence with the statements of Goldstein et al. (1996). In our opinion, this does not represent a disagreement with the functional explanations presented by Goldstein et al. (1996) but a need to redefine the point where this ratio is reversed: Goldstein et al. (1996) set that limit at 1 $\mu g\ g^{-1}$ and the present results point to a four times lower level.

The occurrence of high liver/muscle ratios was previously reported in other fish species environmentally exposed (Abreu et al., 2000; Raldúa et al., 2007). This fact, allied with the possibility of a reversion on the ratio to values < 1, can be regarded as evidence that liver has a central function in mercury accumulation, playing a buffering role, i.e., after liver retention capacity exhaustion, mercury is able to bypass to muscle and consequently, its accumulation in muscle starts increasing. Furthermore, the same type of action can be stated in relation with the other studied tissues (with the exception of kidney) seeing that the respective liver/tissue ratios were also > 1.

The explanation presented by Henny et al. (2002) for the occurrence of high liver/muscle ratios is that as methylmercury exposure increases the percentage of inorganic mercury in the liver increases, indicating greater hepatic demethylation. Subsequent binding and immobilization of inorganic mercury to metallothioneins, preferentially produced in the liver (Hogstrand and Haux, 1990), could result in augmented liver concentrations relative to muscle (Cizdziel et al., 2003). This theory is supported by current mercury speciation analyses, showing a considerable prevalence of inorganic mercury (70 – 76% of the total) in the liver, in contrast with muscle where organic mercury was the dominant form (85 – 97 % of the total).

Taking into account that liver/kidney ratios were close to 1 (from 0.6 in R to 0.98 in L1) and all the kidney/tissue ratios were largely greater than 1, the buffering role referred for the liver should also be attributed to the kidney. Furthermore, in view of the feeding behaviour of *L. aurata*, the present results are in agreement with Maury-Brachet et al. (2006) who stated that high liver–kidney/muscle ratios are typically found in benthivorous fish species.

Most of available data on tissue-to-tissue relations has been focused on tissue/muscle ratios mainly because it is closely associated with the risk of human contamination via fish consumption (Maury-Brachet et al., 2006). However, the computation of all the possible tissue-to-tissue relations can provide new information on mercury inter-tissues or tissue–blood exchange.

Data from literature indicates that mercury uptake from food is the predominant accumulation pathway (Andres et al., 2002; Laporte et al., 2002). However, in the present study, the importance of direct uptake via gills was ascertained. Furthermore, the relevance of aqueous uptake via gills on mercury toxicity was demonstrated in a previous study with *L. aurata* caged in Laranjo Basin, as the dietary uptake was almost completely restricted by caging (Guilherme et al., 2008). On the other hand, gills are between the

venous and arterial circulation, receiving nearly all of the cardiac output and, thus, is predisposed to accumulate chemicals taken up by other exposure routes. In this context, the present data showed that gills/tissue ratios reach the maximum for blood and the minimum for the liver, which can be an indication of a low relocation of mercury stored in the liver. The gills aptitude to maintain a high T-Hg differential to blood is also apparent. It was established an association between the higher intake of inorganic mercury (the most water soluble form) and the gills close contact with the dissolved and particulate metal species in water (Laporte et al., 2002). Moreover, Maury-Brachet et al. (2006) demonstrated that benthivorous species absorb the metal principally in the inorganic form (48% to 72%). Therefore, the high T-Hg differential from gills to its internal interface (blood) gives support to the idea that gills provide a rapid and significant storage compartment for inorganic mercury (Oliveira Ribeiro et al., 2002). This aspect can assume an augmented significance if we consider the high renewal rate of branchial tissue as an unfavourable factor to bioaconcentration; gills' epithelium is regularly subject to exfoliation and erosion, which is counteracted by an intense cell division rate (Pacheco et al., 1993).

The tendency of the gills/kidney ratios to be nearer to 1, namely at L2, associated with the significant correlation observed between T-Hg in gills and kidney, corroborates the idea that kidney is preferentially targeted by chemicals when taken up through the gills (Pritchard and Bend, 1984). It is also well known that kidney is quite susceptible to water-borne inorganic mercury exposure (Oliveira Ribeiro et al., 2002).

The role of blood on the transportation and redistribution of mercury can be better understood by analysing blood/tissue ratios, namely for internal tissues not directly involved in the absorption. Thus, it is perceptible that the lowest blood/tissue values were obtained for the liver and kidney (<0.04), which can be regarded as an additional indication that mercury is accumulated in these organs under stable and chelated forms. The highest ratios (still <1) were found for muscle and brain (around 0.1 and 0.2, respectively). Current measurements showed a high prevalence of organic mercury in muscle, and the same was previously demonstrated for the brain (Zheng et al., 2003). In view of the elevated stability of organic mercurials depots due to a strong affinity for thiol groups of certain proteins (Bustamante et al., 2006), both tissues should be regarded as end of the line for mercury distribution. Furthermore, muscle tissues have been suggested to act as a sink for methylmercury (Leaner and Mason, 2004). Methylmercury is incorporated in fish muscle and brain tissue, most likely by forming a methylmercury-cysteine complex (Harris et al., 2003). This mechanism is particularly determinant in the

brain since this complex mimics the behaviour of normal endogenous substrates, utilizing transport systems inherent to the blood-brain barrier (BBB) to gain access to the central nervous system (CNS) (Zheng et al., 2003). The similarity between blood/muscle and blood/brain ratios is a symptom of an equivalent mercury uptake in the two tissues and, subsequently, an evidence of the inefficacy of the BBB in reducing the rate of mercury transport into the CNS parenchyma.

A lack of significant differences in each tissue-to-tissue T-Hg ratio was observed when the three stations were compared. This indicates that mercury organotropism is not markedly affected by the environmental levels or by the subsequent body burdens extent.

2.4.3 Suitability of *L. aurata* as bioindicator for mercury contamination

L. aurata was selected in the present work because it is one of the dominant species in the surveyed lagoon (Ria de Aveiro), being easy to identify and capture in both pristine and metal-contaminated environments (Pacheco et al., 2005). As a benthopelagic species, its feeding behaviour (detritivore) and its life history make it particularly appropriate to the current goals. In fact, *L. aurata* showed the ability to detect inter-sites differences in relation to L1 and L2. This is a particularly interesting finding, considering the proximity of these two sampling stations (2 km) and the mobility usually attributed to fish species and invoked as a liming factor for its application as bioindicator. Hence, the current results indicate *L. aurata* as a relatively sedentary species, making it a good candidate as bioindicator in the context of fish species. Additionally, the adoption of juvenile specimens provides information on short-term variations of mercury concentrations in the environment, which can be compromised by using adult specimens.

2.4.4 Human health implications

The presence in fish muscle of high T-Hg, where large proportions are organometallic mercury, in combination with the fact that seafood consumption is the main source of mercury intake in people not occupationally exposed amplifies the need for preventive measures to safeguard public health (Storelli et al., 2005). The official regulatory agencies have set limits for mercury concentrations above which the fish is considered unsuitable for human consumption. The European Commission decision 93/351 established this limit at 0.5 μg g^{-1} of w wt (1994). In view of that, the T-Hg measured in the current study in *L. aurata* muscle at Laranjo basin should not be regarded as unsafe for consumption as did not exceed the previous regulatory limits.

However, the previous assumption should be regarded with some criticism since it has been increasingly assumed that the regulatory thresholds should take into consideration the fish consumption rate of each particular population. The Portuguese population is the major seafood consumer in the EU, with a weekly consumption average of 1192 g and an annual rate of 62 kg per person (Lourenço et al., 2006). Estimation of the weekly fish intake for T-Hg and O-Hg was calculated and compared with the Provisional Tolerable Weekly Intake (PTWI) recommended by the Joint FAO/WHO Expert Committee on Food Additives (5.0 and 1.6 µg Kg^{-1} body weight for T-Hg and O-Hg, respectively) (WHO, 2007). Calculations were done adopting the fish weekly consumption of the Portuguese population using a body weight of 60 kg (Figure 2.3).

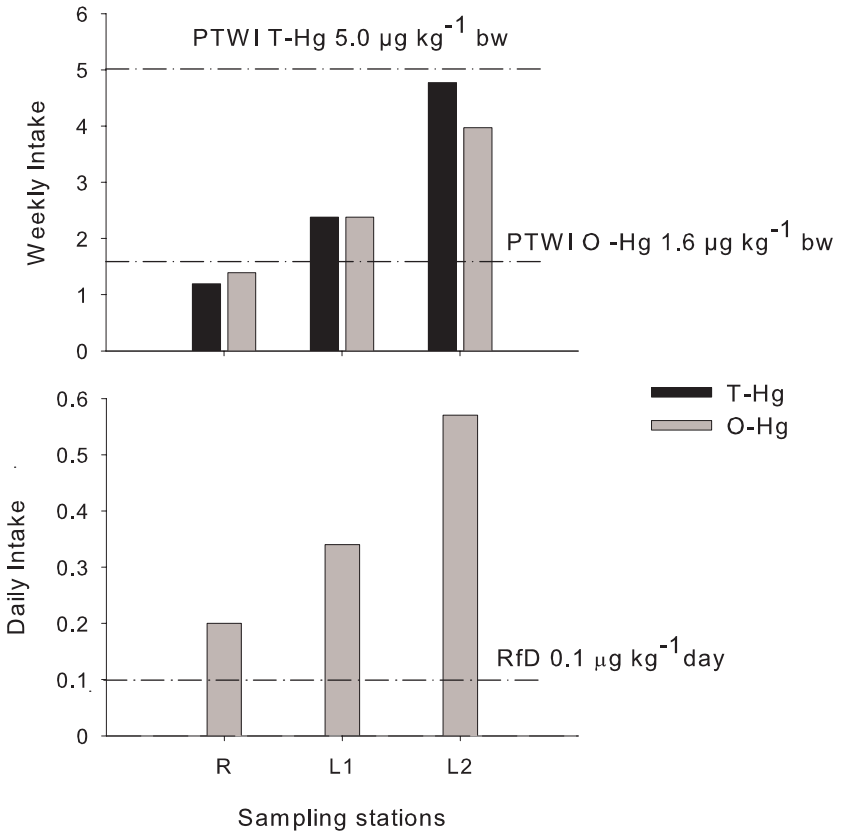

Figure 2.3 The estimated weekly intake for total (T-Hg) and organic (O-Hg) mercury and daily intake for O-Hg (lines) in *L. aurata* muscle for each sampling station, compared to the WHO Provisional Tolerable Weekly Intake (PTWI) limits and EPA Reference Dose (RfD), respectively. Sampling stations at Ria de Aveiro are: reference (R), moderately (L1) and highly contaminated (L2) areas. PTWI values are 5.0 and 1.6 µg Kg^{-1} body weight (bw) for T-Hg and O-Hg respectively. RfD is 0.1 µg kg^{-1} per day. Calculations considered 60 Kg body weight.

Concerning T-Hg, the estimated weekly intake is below the established PTWI in R and L1, but in L2 reaches the advised limit. On the other hand, weekly intake estimated for O-Hg clearly exceeds the safety PTWI limit in the contaminated stations L1 (1.5-fold) and L2 (2.5-fold). Additionally, the daily fish intake dose was calculated and compared with the EPA reference dose (RfD = 0.1 μg Kg^{-1} per day) (EPA, 2001). Fish from all the sampling stations were above this limit, reaching in L2 a level 6 times higher than the imposed RfD (Figure 2.3).

The present results raise a question concerning the relevance of mercury quantification in kidney when the risk to humans is under analysis. Despite the substantially low mass of kidney in relation to muscle, this aspect must be carefully considered taking into account the high levels likely to be found in this organ, as well as because it is not removed by the common evisceration procedures.

2.5 Conclusions

The results of this work demonstrated that:

1) The determination of mercury accumulation in *L. aurata* key tissues reflected inter-sites differences, strengthening its suitability as indicators of metal contamination. Besides the tissues commonly mentioned in the literature (liver and muscle), the brain showed a promising ability in order to reveal the environmental mercury contamination extent. Moreover, brain and kidney can play a relevant role on biomagnification processes in top consumers, highlighting their importance on environmental risk assessment;

2) The mode of mercury distribution and deposition showed to depend on the specific tissue and thus, clear differences were observed resulting in the pattern kidney > liver > muscle > brain > gills > blood. The evaluation of mercury load in a large set of tissues/organs, as well as the computation of tissue-to-tissue relations, can provide new information contributing to the knowledge on mercury organotropism;

3) The high mercury levels found in organs involved in vital physiological processes, namely the brain, pointed out the risk to autochthonous fish fauna;

4) The risk to humans can not be excluded in relation with the consumption of fish living in Laranjo basin; it was reinforced the importance to define the regulatory thresholds taking into consideration the fish consumption rate in order to efficiently protect against hazardous exposure.

Acknowledgments

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CHAPTER 3

Mercury organotropism in feral European sea bass (*Dicentrarchus labrax)*

Mercury organotropism in feral European sea bass (*Dicentrarchus labrax)*

Abstract

The knowledge of mercury (Hg) burdens in a wide set of tissues and organs of exposed fish is crucial to understand the internal distribution dynamics and thus predict Hg bioavailability and implications for ecosystem and human health. Total Hg was measured in six tissues of Dicentrarchus labrax captured along an estuarine contamination gradient, revealing the following pattern: liver > kidney > muscle > brain ≈ gills > blood. All of the tissues displayed intersite differences, although brain and muscle seemed to better reflect the extent of contamination. Hg speciation showed that liver presented higher concentration than muscle for both organic and inorganic forms. Furthermore, liver seemed to exert a protective action in relation to Hg accumulation in the other tissues and organs. This protection seems to be particularly marked in relation to the brain, whereas liver is assisted in that action by kidney and muscle.

Key words: Mercury; European sea bass; tissue distribution; accumulation; field conditions

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3.1 Introduction

Mercury (Hg) is a toxic and ubiquitous metal with no known essential functions in cells. Its toxicity depends on the chemical form, amount, pathways of exposure, and vulnerability of the target exposed. Hg under the lipophilic form of methylmercury can bioaccumulate and biomagnificate, especially in aquatic food chains. Although Hg accumulation in fish has been widely studied, most of the work deals either with laboratory approaches or with field studies regarding only a few tissues (Riisgård and Hansen 1990; Berntssen et al. 2003; Afonso et al. 2007; Burger and Gochfeld 2007). In agreement, the majority of the existing field works have focused mainly on liver, the major organ regarding metal detoxification, and muscle, related to human risk from fish consumption (Afonso et al. 2007; Chien et al. 2007; Herreros et al. 2008). However, Cizdziel et al. (2003) and Maury-Brachet et al. (2006) evaluated several fish tissues for Hg accumulation, but only in freshwater species. The knowledge of the Hg burdens among the tissues and organs of exposed fish is required to understand the internal distribution dynamics and thus predict Hg bioavailability and risk for fish. Consequently, an effort should be made to quantify Hg in marine fish species covering a wide set of tissues having a key role in determining the toxicologic effects both at the individual and the population levels.

The European sea bass (Dicentrarchus labrax) is a commercially valuable and representative species of the Ria de Aveiro lagoon (Portugal) ichthyofauna, which use this estuary as a nursery area. Hence, using D. labrax as bioindicator of environmental Hg contamination, the following specific objectives were pursued: (1) to evaluate the total Hg tissue-specific loads and demonstrate their relation to Hg abiotic concentrations; (2) to better understand Hg uptake, distribution, and retention processes; (3) to select the tissue that best reflects Hg environmental contamination; (4) to determine Hg accumulation status by measuring total and organic Hg in liver and muscle. In this work, the selected tissues included gills, blood, kidney, liver, brain, and muscle, which were all chosen on the basis of their functional and anatomic properties. Effects on major Hg-accumulation processes, such as distribution, demethylation, and storage, were also measured.

3.2 Experimental Methods

3.2.1 Brief description of the sampling area

The study was performed at Ria de Aveiro, a coastal lagoon located on the northwest coast of Portugal (Fig. 3.1). This aquatic system contains an inner area (Laranjo

Basin) that has been subjected to Hg effluents from a chlor-alkali plant for almost five decades (1950–1994). Due to the continuous discharges, high Hg concentrations are still associated to sediments, generating a contamination gradient with increased concentrations near to the source (Coelho et al. 2005).

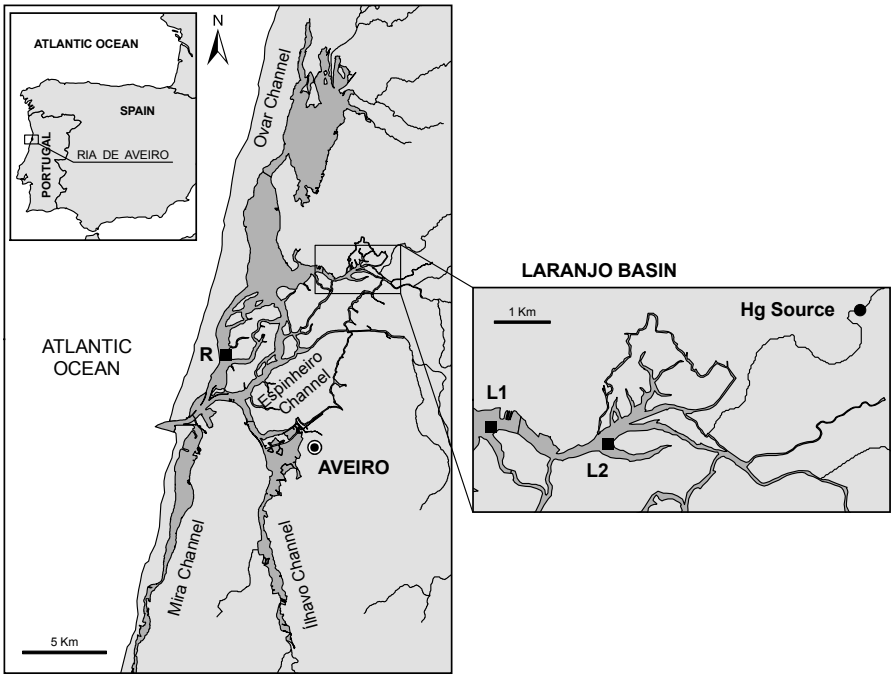

Figure 3.1 Map of the sampling stations (■) in the Ria de Aveiro (Portugal): reference (R - 40º41'00'' N, 8º42'44'' W), moderately (L1 - 40º43'34.46'' N,8º38'53.16'' W) and highly contaminated (L2 - 40º43'28.98'' N, 8º37'35.80'' W) areas.

Sampling was performed in July 2007 at three different locations, which were chosen according to their distance from the Hg source. Two sampling stations were chosen at Laranjo Basin: L2, near the Hg source and recognized as a highly contaminated area, and L1 (downstream L2), which was assumed to be a moderately contaminated area. The two sampling stations are 2 km apart from one another. A third sampling station (R), near the lagoon entrance and 10 km away from the polluting source (Pacheco et al. 2005), was adopted as a reference area.

3.2.2 Procedures for sampling and samples treatment

Fifteen juvenile European sea bass (D. labrax) were caught at each sampling station using a fishing rod. The arithmetic mean for total length and weight at each

location was 15.8 ± 4.8 cm and 46.9 ± 44.3 g at R; 14.7 ± 1.6 cm and 23.0 ± 8.8 g at L1; and 14.1 ± 3.0 cm and 25.0 ± 23.8 g at L2. All fish belonged to age class 1^+ according to Dolbeth et al. (2008) and Martinho et al. (2008). Immediately after being caught, fish were killed by cervical transection, and blood, brain, kidney, liver, gills, and dorsal muscle were sampled and stored in liquid nitrogen. Blood was collected from the posterior cardinal vein using heparinised Pasteur pipettes.

Dissolved oxygen, pH, temperature, and salinity were measured in the water column at the subsurface level under low and high tide conditions. Turbidity and water-column depth were also evaluated. At both low and high tide, subsurface water samples for Hg analyses were collected in acid-washed plastic bottles and kept in an ice box during transportation to the laboratory, where they were immediately filtered through pre weighed 0.45-lm Millipore cellulose acetate membrane filters, acidified with ''Hg-free'' HNO3 (Merck) to pH< 2, and stored at 4ºC until analysis. Filters were reweighed after drying overnight at 60ºC and stored for determination of Hg in suspended particulate matter (SPM-Hg). Five replicates of sediments were taken from the surface sediment layer (1 to 2 cm depth) in each sampled area. At the laboratory, sediment samples were freeze-dried, well mixed, sieved through a 1-mm sieve, and stored for total Hg determination (Sed-Hg). Tissue samples were freeze-dried and well mixed before analysis for total (T-Hg) and organic (O-Hg) Hg.

3.2.3 Methodology and analytical quality control of mercury determinations

All glassware and plastic implements were cleaned in accordance with Monterroso et al. (2003), and ultraclean laboratory procedures were performed during sample manipulations. Hg in water was determined by cold-vapour atomic fluorescence spectrometry using a PSA model Merlin 10.023 equipped with a detector PSA model 10.003 using SnCl2 decrease. The detection limit (±SD) based on procedural blanks was 1.2 ± 0.3 ng L^{-1}. The procedure and reagent contamination was followed by analysis of filtrate blanks and ultrapure water. For determination of SPM-Hg, filters were digested with HNO3 4 mol L^{-1} (Pereira et al. 1998), and the previous equipment was used. Blank filters were used to examine any possible contamination and revealed Hg levels between 3.5 and 9.4% of the typical content in the sample filters. Total Sed-Hg and in D. labrax tissues (T-Hg) was determined by atomic absorption spectrometry with thermal decomposition and gold amalgamation using an Advanced Mercury Analyser (AMA) LECO 254 (Costley et al. 2000). The accuracy and precision of the analytic methodology for T-Hg

determinations were assessed by replicate analysis of certified reference materials (CRM), namely MESS-3 and PACS-2 (marine sediments) for sediments and TORT-2 (lobster hepatopancreas) for biologic samples. Precision of the method was better than 9% ($n > 5$), with recovery efficiency between 83 and 102%. Blanks evaluated between each sample were always < 1% of the T-Hg.

O-Hg determination in fish tissues was performed through triplicate digestion of the sample with a mixture of 18% KBr in 5% H_2SO_4, followed by extraction into toluene (for further details see Válega et al. 2006). O-Hg was determined using an AMA-LECO 254 as previously described for T-Hg. Because this method requires a large amount of sample (0.2 g), determination of O-Hg was only possible in muscle and liver. For the same reason, samples of randomly chosen three fish were needed to prepare composite samples of liver. Analytic validations of the O-Hg determinations were performed with reference material TORT-2. The precision of the method ranged between 0 and 10%, with an extraction efficiency between 75 and 91%. Levels of inorganic Hg (I-Hg) were determined by subtracting O-Hg from T-Hg.

3.2.4 Statistical data analysis

Data were tested for goodness of fit to normal distribution; requirements of homogeneity of variances were also determined. Analyses of variance were performed, followed by pairwise multiple comparison procedures (Tukey test). Whenever the assumptions for parametric statistics failed, the nonparametric correspondent test (Kruskall–Wallis) was performed, followed by nonparametric pairwise multiple comparison procedure (Dunn's test). Differences between means were considered significant at $p < 0.05$. Spearman rank correlation factor (r) was determined for T-Hg between the different tissues ($p<0.05$). One gill sample from R showed a surprisingly high T-Hg level, attributable to external contamination. Hence, this value was assumed to be an outlier and was removed from statistical analysis.

3.3 Results

3.3.1 General physicochemical characterization

Table 3.1 lists the general physicochemical characteristics of the water column in the three sampling stations. Although temperature, water depth, turbidity, and pH showed no clear trend, salinity and dissolved oxygen (DO) decreased toward the contamination

source. SPM showed increments from R to the contaminated areas during low tide and the opposite during high tide.

Table 3.1 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.

	Sampling station					
	R		L1		L2	
	High Tide	Low Tide	High Tide	Low Tide	High Tide	Low Tide
Temperature (ºC)	20.7	22.1	22.1	22.0	22.2	22.0
Dissolved Oxygen (mg L^{-1})	8.0	8.1	4.7	7.1	5.7	5.0
Depth (m)	2.7	1.9	3.8	2.5	2.3	1.0
Turbidity (m)	1.2	0.8	0.7	0.3	1.0	0.3
pH	8.1	8.2	8.0	7.8	7.9	7.6
Salinity	30	30	20	10	18	5
Suspended Particulate Matter (mg L^{-1})	211.0	40.3	35.5	82.1	n.d.	78.7

n.d. not determined

The concentration of total dissolved Hg (Dis-Hg) increased toward the contamination source (Table 3.2). Concentrations of SPM-Hg also increased toward the Hg source during low tide. The percentage of Hg associated to particulate matter in the water column (% Hg particulate) was always > 90%, without clear differences between R and the contaminated stations.

Table 3.2 Concentrations of total dissolved mercury (Dis-Hg) (ng L^{-1}), percentage of mercury associated to the particulate matter (% Hg particulate), total mercury in suspended particulate matter (SPM-Hg) (mg Kg^{-1} dry weight) measured, at high and low tide and total mercury in sediment (Sed-Hg) (mg Kg^{-1} dry weight) on reference (R), moderately contaminated (L1) and highly contaminated (L2) areas at Ria de Aveiro.

Sampling station	Tide	Dis-Hg (ngL^{-1})	% Hg particulate	SPM-Hg ($mgKg^{-1}$)	Sed-Hg ($mgKg^{-1}dw$)
R	High	2.1	96.4	0.26	0.01
	Low	2.2	90.9	0.55	
L1	High	3.5	92.6	1.24	0.08
	Low	6.8	97.7	3.6	
L2	High	n.d.	n.d.	n.d.	6.8
	Low	11.4	97.6	6.0	

n.d. not determined

Sed-Hg showed an 8-fold increase from the reference station (R) to L1 and an 85-fold increase from L1 to L2, thus highlighting the existence of the reported contamination gradient. Moreover, L2 showed a 680-fold increase compared with R.

3.3.2 Accumulation of mercury in fish tissues

T-Hg in D. labrax from R and the contaminated stations L1 and L2 depends on the specific fish tissue under consideration as shown in Fig. 3.2 Thus, T-Hg accumulation varied as follows: liver > kidney > muscle > brain ≈ gills > blood (Fig. 3.2). The concentrations ranged from0.006 (blood at R) to 1.12 (liver at L2) mg kg^{-1} (wet weight).
All of the tissues exhibited significant ($p < 0.05$) increases from R to L2. Taking into account the T-Hg accumulation increment from R to L2, tissues were ordered as follows: kidney (11x) ≈ brain (11x) ≈ muscle (11x) > gills (9x) > blood (8x) > liver (3x).

Moreover, blood, brain and muscle also showed significantly ($p<0.05$) higher T-Hg levels in L1 in relation to R. Comparing the magnitude of T-Hg increase at L1 relative to R, tissues were order as follows: muscle (7x) > blood (5x) ≈ kidney (5x) > gills (4x) ≈ brain (4x) > liver (2x). Significant differences ($p<0.05$) of T-Hg between L1 and L2 were only observed in liver, showing a 2.5 times increase at L2.

Tissue-to-tissue ratios of T-Hg are listed in Table 3.3. The highest ratios were observed for tissue/blood ratios, being the maximum value for liver/blood ratios, followed by kidney/blood and muscle/blood ratios. The highest tissue/ blood ratios were always found for L2, with the exception of muscle. Comparing each ratio in the three sampling stations, statistically significant ($p < 0.05$) differences between L2 and R were found for brain/muscle, brain/liver, and brain/gills ratios, whereas brain/muscle ratio also displayed differences between L1 and L2.

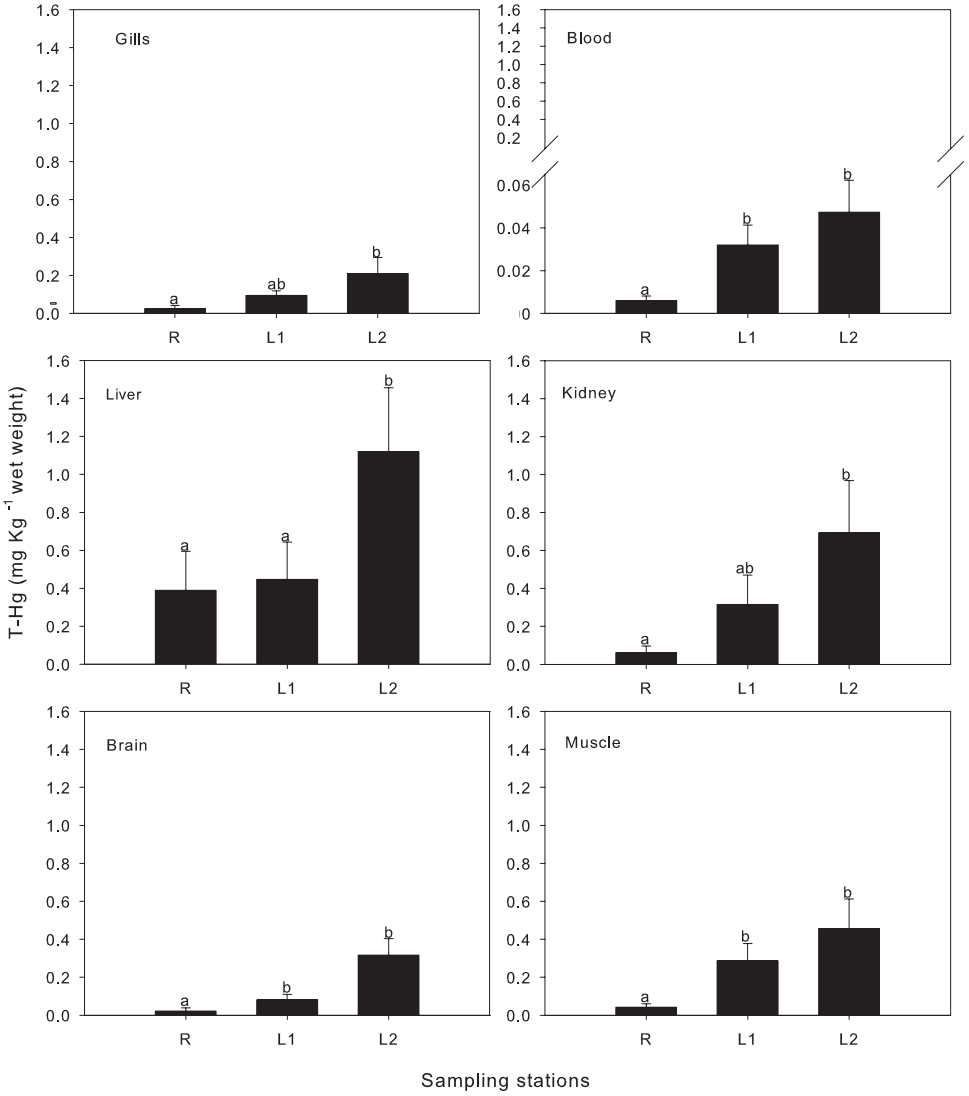

Figure 3.2 Total mercury (T-Hg) mean concentration (mg Kg-1 wet weight) in tissues of *D. labrax* caught on reference (R), moderately (L1) and highly mercury contaminated (L2) areas at Ria de Aveiro. Different letters (a, b) denote significant differences and the same letters denote non-significant differences ($p<0.05$). Error bars represent the standard deviation.

Table 3.3 Inter-tissue ratios (arithmetic mean ± standard deviation) for total mercury estimated for reference (R), moderately (L1) and highly mercury contaminated (L2) areas at Ria de Aveiro. The letters denote statistically significant differences ($p<0.05$): [a] *versus* R, [b] *versus* L1.

Inter-Tissue ratio	Sampling station	Muscle	Blood	Liver	Kidney	Gills	Brain
	R						
Muscle	**L1**						
	L2						
	R	0.11 ± 0.04					
Blood	**L1**	0.12 ± 0.04					
	L2	0.12 ± 0.03					
	R	2.2± 0.7	23.8 ± 9.1				
Liver	**L1**	1.8 ± 0.4	16.8 ± 7.5				
	L2	2.6 ± 0.7	24.3 ± 1.2				
	R	1.1 ± 0.4	10.1 ± 2.7	0.43± 0.28			
Kidney	**L1**	1.1 ± 0.2	7.8 ± 5.2	0.32 ± 0.26			
	L2	1.8 ± 0.5	14.4 ± 3.2	0.61 ± 0.11			
	R	0.35 ± 0.09	3.7 ± 1.2	0.21± 0.11	0.33 ± 0.09		
Gills	**L1**	0.35 ± 0.09	3.1 ± 0.9	0.23 ± 0.05	0.30 ± 0.19		
	L2	0.47 ± 0.11	4.2 ± 0.8	0.18 ± 0.03	0.31 ± 0.04		
	R	0.29 ± 0.08	3.0 ± 1.4	0.11±0.04	0.31 ± 0.12	0.64 ± 0.39	
Brain	**L1**	0.28 ± 0.05	2.6 ± 1.1	0.16 ± 0.02	0.27 ± 0.07	0.84 ± 0.22	
	L2	0.51 ± 0.12 [a,b]	4.6 ± 0.9	0.20 ± 0.04 [a]	0.34 ± 0.06	1.1 ± 0.07 [a]	

Spearman rank correlation (r) analysis showed significant positive correlations between T-Hg in blood and all of the other tissues (Fig. 3.3). T-Hg in muscle was also positively correlated with all of the other tissues. Significant correlations were found between T-Hg in gills and brain, in gills and kidney, and in brain and kidney.

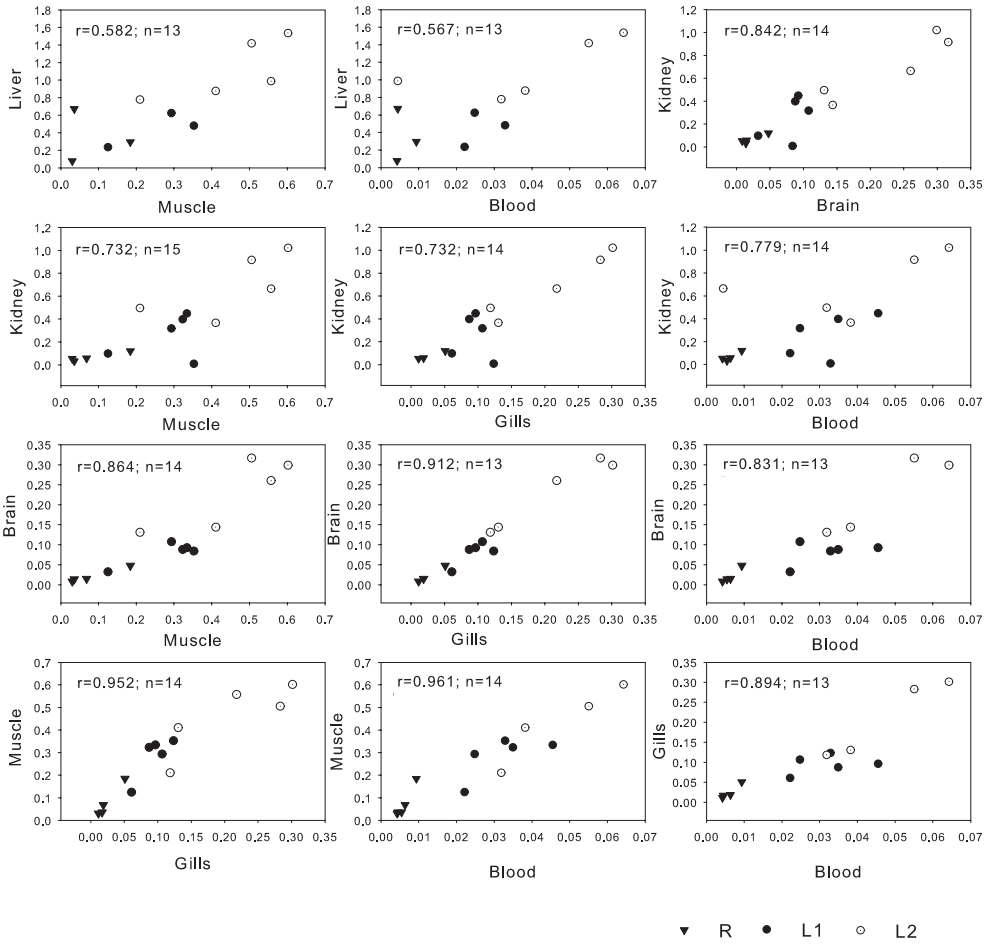

Figure 3.3 Spearman rank correlations between total mercury concentrations (T-Hg; mg Kg-1 wet weight) in the studied fish tissues. Non-significant correlations are not presented.

The concentration of O-Hg in muscle and liver showed significantly higher values at L2 compared with R (Table 3.4). In addition, liver O-Hg also showed significant differences between L1 and L2. O-Hg concentration was always greater in liver than in muscle, although the increment at L2 in relation to R was higher for muscle than for liver. O-Hg percentage in muscle decreased from R toward the contaminated area (L1 and L2), whereas in liver no spatial differences were observed for this percentage. Positive Spearman rank correlations were found between T-Hg and O-Hg levels for muscle ($r = 0.979$; $p < 0.05$) and for liver ($r = 0.988$; $p < 0.05$). Liver/muscle ratios for O-Hg and I-Hg were calculated, revealing the highest values at R and the lowest at L1 both metal forms.

Table 3.4 Organic (O-Hg) and inorganic mercury (I-Hg) (arithmetic mean ± standard deviation) concentrations (mg Kg^{-1} dry weight) and percentage of O-Hg relative to T-Hg in muscle and liver of *D. labrax* caught on reference (R), moderately (L1) and highly mercury contaminated (L2) areas at Ria de Aveiro. The ratio between liver and muscle both for O-Hg and I-Hg are also represented. The letters denote significant differences ($p<0.05$): [a] *versus* R, [b] *versus* L1. % O-Hg was calculated as the mean of individual values of O-Hg/T-Hg.

Sampling station	Tissue	O-Hg (mg Kg^{-1})	I-Hg (mg Kg^{-1})	O-Hg %	Ratios	
					Liver/Muscle O-Hg	Liver/Muscle I-Hg
R	Muscle	0.06 ± 0.06	0.01±0.01	90.5 ± 5.6	6.0	12.0
	Liver	0.36 ± 0.18	0.12±0.01	73.6 ± 8.9		
L1	Muscle	0.18 ± 0.08	0.08±0.04[a]	70.1 ± 6.3[a]	1.8	1.4
	Liver	0.33 ± 0.15	0.11±0.05	75.4 ± 2.8		
L2	Muscle	0.34 ± 0.13[a]	0.11±0.03[a]	73.8 ± 5.2[a]	2.8	2.0
	Liver	0.96 ± 0.29[a,b]	0.22±0.26	83.3 ± 15.9		

3.4 Discussion

3.4.1 Relationships between environmental data and tissue-specific mercury loads

The physicochemical characteristics of the water-column influence Hg bioavailability. However, only salinity, DO, and SPM showed differences among stations. It is knownthat Hg is able to form strong inorganic complexes with chloride in saline and oxygen-rich waters (Conaway et al. 2003). Nonetheless, the influence of salinity and DO on T-Hg in fish tissue and organs could not be confirmed by the present data because Dis-Hg in water was low and thus not relevantly affected by these parameters. Furthermore, interstation variations in SPM have important repercussions on Hg availability because particulate Hg content tends to be higher in conjunction with increased SPM, especially in shallow regions of estuaries where resuspension is easily enhanced (Coquery et al. 1997; Domagalski 2001). Water column Hg was almost 100% associated with SPM, reinforcing the significant role of SPM in Hg water-column dynamics. In agreement, this could be relevant when comparing areas with Hg-enriched sediments with nonpolluted areas.

Previous field studies concerning Hg in different fish tissues demonstrated that the accumulation pattern depends on the species (Cizdziel et al. 2003; Maury-Brachet et al. 2006). According to the present D. labrax data, the hierarchy of the assessed tissues on the basis of their T-Hg levels was liver > kidney >muscle > brain ≈ gills > blood. The higher Hg loads registered in liver and kidney reinforces the idea that these organs play a

central role in metal accumulation and transformation in fish (Elia et al. 2003; Drevnick et al. 2008). The comparison of tissue specific T-Hg between sampling stations demonstrated that all of the assessed tissues were able to signal Hg contamination at L2. In addition, blood, brain, and muscle were able to signal the moderate state of contamination (L1) as well. Nonetheless, only liver was able to detect difference between the two levels of contamination, i.e., between L1 and L2.

Current data on Hg accumulation show that liver always presented higher concentrations than muscle for both O-Hg and I-Hg, highlighting the importance of liver in the protection of the other organs and tissues, including muscle. This protective action can be exerted by the liver, mainly through the demethylation of O-Hg into I-Hg (Riisgård and Hansen 1990), and the induction of metallothionein synthesis, both of which are well known to play roles in binding, detoxifying, and storing Hg, thus preventing Hg reactions with other cellular targets (Viarengo and Nott 1993; Bebianno et al. 2007). The observation that liver/ muscle ratios clearly decreased from R to contaminated stations (for both Hg forms) may indicate that uptake exceeded the hepatic-retaining capacity (Table 4). This pattern is probably associated with the preponderance of each exposure pathway (direct by way of water or by trophic transfer) in reference versus contaminated areas. Hence, it is suggested that Hg uptake directly from water (I-Hg) had increased importance in Laranjo stations compared with the reference station, where the diet represents almost the only pathway. In agreement with Pato et al. (2008), no temporal variations concerning Hg levels in the water column occurred, thereby allowing the previous explanation. Nevertheless, it is important to keep in mind that changes in Hg distribution might be induced by seasonal differences in feeding patterns and fish migration (Schmitt and Brumbaugh 2007).

3.4.2 Selecting the tissue that best reflects mercury environmental contamination

Several criteria should be considered to select the tissue that best reflects the extent of Hg contamination. First, to avoid analytic problems associated with the detection limit, the chosen tissue should have a relatively high concentration of Hg. Second, to enhance its discriminatory power, a tissue must present significant Hg increments relative to reference conditions. Third, to extend its applicability to distinct contamination scenarios, the tissue must have the capacity to highlight both low and high environmental levels. Consequently, liver is likely to be the best choice concerning the first criterion, followed by kidney. However, based on the increment relatively to reference, kidney,

brain, and muscle (11x) appear to be the tissues that best meet the second requirement. Taking into account the environmental Hg increments from R to L2 (particularly SPM-Hg with a 10.9X increase), gills (9x) are a relevant choice, specifically regarding Hg uptake through water. Taking into account the third criterion, blood, brain, and muscle are suitable choices because they were able to reflect both moderate (L1) and high (L2) degrees of contamination. Nonetheless, only liver was able to detect differences between the two levels of contamination, i.e., between L1 and L2. Synthesizing then, the selection based on the three criteria considers brain and muscle as the most suitable tissues. Furthermore, it must be pointed out that muscle presents advantages in relation to brain because it can provide greater amounts of tissue for analysis and offers the possibility to be collected using minimally invasive and nonlethal methods.

3.4.3 Mercury accumulation dynamics

Tissue-to-tissue ratios are important tools in predicting Hg organotropism (Maury-Brachet et al. 2006). Muscle is considered a reference tissue regarding biomagnification processes (Wiener et al. 2003); hence, tissue/muscle ratios are a valuable tool for the evaluation of Hg accumulation. Furthermore, after uptake Hg is distributed throughout body tissues and bounds to sites with higher affinity, generating tissue-specific Hg loads (Ruelas-Inzunza et al. 2008). The highest intertissue ratios were found for tissue/ blood ratios, confirming the importance of blood in Hg transport and redistribution rather than in its accumulation. This is in accordance with the positive correlations found between blood and all of the other tissues. In addition, liver/blood ratio achieved the highest value, clearly highlighting the role played by liver in storage and detoxification of Hg. The similar values of kidney/blood and muscle/ blood ratios are indicative of comparable Hg relocation from blood to these tissues. Taking into account low brain/blood ratio compared with liver/blood, kidney/blood, and muscle/blood ratios, it may be suggested the existence of a protective mechanism in relation to brain undertaken by the other tissues. Several studies (Wiener and Spry 1996; Leaner and Mason 2004) showed that muscle is the major tissue retaining O-Hg associated to thiol groups of proteins, thus preventing accumulation in other tissues, such as the brain, where the repercussions could be more severe.

Brain/muscle ratio showed significant differences between R and L2 as well as between L1 and L2, revealing that the described muscle protection (sink) decreased with increased environmental contamination. Although the results are indicative that liver and kidney share this protective capacity, the role of muscle in this action will always be more

decisive due to its relative amount in body mass of fish (> 60%). In the present study, tissue/muscle ratios for T-Hg were found to be low (< 1) (except for liver and kidney), which, in accordance with Maury-Brachet et al. (2006), permits to ascertain that the major source of Hg was trophic uptake.

The estimation of tissue/gills ratios is particularly relevant when evaluating direct Hg uptake from water (Dis-Hg and SPM-Hg), especially due to the high contact of gills with particulate matter, to which Hg has high affinity. Still, gill epithelium has a high rate of renewal due to frequent exfoliation and erosion, thus providing indication of recent exposures, namely to I-Hg, the most water-soluble form. A previous work described I-Hg uptake in freshwater fish species preferentially through gills (Oliveira Ribeiro et al. 2000).

From the tissue-to-tissue T-Hg ratio comparisons among the three sampling stations, it is important to highlight that only brain/tissue ratio showed significant differences (for all tissues except kidney and blood), indicating that Hg organotropism is affected by the extent of contamination, with particular impact on brain load. Moreover, the higher brain/muscle and brain/liver ratios estimated for L2 compared with R may indicate that the muscle- and liver-protective capacity was surmounted, thus showing increased susceptibility of brain toward Hg accumulation in highly contaminated areas.

3.5 Conclusions

The current study demonstrated that T-Hg distribution and accumulation has tissue-specific patterns, resulting in the following hierarchy: liver > kidney > muscle > brain ≈ gills > blood. In addition, all of the selected tissues were able to reflect intersite differences regarding Hg accumulation, although brain and muscle seem to better reflect the extent of environmental contamination.

Tissue-to-tissue analysis evidenced the importance of blood in the transport and redistribution of Hg rather than in its accumulation, whereas muscle appears to act as an Hg depositor. Liver seemed to exert a protective action in relation to the other tissues/organ ratios. This protective mechanism seems to be particularly marked in relation to brain, assisted in that action by kidney and muscle. Furthermore, the results might suggest different preponderance of the exposure pathways (water versus diet) according to the degree of contamination. In accordance, D. labrax seems to be appropriate for biomonitoring purposes because it was possible to detect intersite variations, particularly between different degrees of contamination.

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CHAPTER 4

Diet as the main factor for mercury accumulation in fish: comparison between *Dicentrarchus labrax* and *Liza aurata*

Diet as the main factor for mercury accumulation in fish: comparison between *Dicentrarchus labrax* and *Liza aurata*

Abstract

The main aim of this research was to compare the mercury accumulation and tissue distribution in two estuarine fish species with contrasting feeding tactics. In accordance, mercury accumulation and tissue distribution in European sea bass and Golden grey mullet were compared. Preferential accumulation was observed in liver, followed by muscle and intestine, while gills and brain presented the lower levels observed in both species. Results suggest fish feeding tactics as the dominant pathway for metal accumulation, especially in target tissues for mercury sequestration such as liver and muscle. *D. labrax*, with a higher trophic position, generally evidenced a higher proportion of organic mercury, and significant differences were observed between species in liver and muscle, especially regarding organic mercury, which seems to vary as a function of intestinal content contamination. For the two species, a very stable ratio was observed between mercury increments from reference to contaminated area, which suggest that the organic mercury content of diet regulates the internal levels of this contaminant, and may prove to be a useful contamination predictor.

Keywords: Mercury; accumulation; feeding strategies; trophic level

Estuarie Coastal and Shelf Science
Submitted

4.1 Introduction

Fish are useful as sentinel species and bioindicators since they can help to understand the risk to the organisms themselves, to the ecosystem and to humans (Peakall and Burger, 2003). The major limitation associated with the use of pelagic fish for monitoring studies is the increased mobility of these organisms, through tidal and seasonal migrations within aquatic systems, when compared to benthic mollusks and crustaceans (Coelho et al., 2009). Despite this disadvantage, fish are generally considered to be the most feasible organisms for pollution monitoring in aquatic systems (van der Oost et al., 2003). Moreover, fish studies on contaminant bioaccumulation are valuable in different research fields: being a major part of the human diet, most research focuses on health risk assessment, with contaminants mostly quantified in muscular tissue of edible species (Alonso et al., 2000; Marcovecchio, 2004). Such studies typically assess the risks associated with fish consumption, but do not provide a great deal of environmental information (Coelho et al., 2009). Inversely, studies directed towards lifespan accumulation patterns, contaminant differential distribution and dietary effects on accumulation (de Pinho et al., 2002; Henry et al., 2004) may grant invaluable information on the bioaccumulation, biomagnification and toxicity of contaminants along aquatic food webs, since recent studies indicated that the transfer of certain metals along the food chain is controlled by their internal distribution within the prey (Bebianno et al., 2007).

Feeding tactics have been reported to play a major role in the differential mercury bioaccumulation of co-existing species within ecological niches (Coelho et al., 2008a). Little information exists, however, regarding the effects of distinct diets on fish tissue mercury distribution in contaminated estuaries, especially in benthic feeding species that can represent an important trophic and contaminant link between invertebrates and larger predatory fish.

European sea bass (*Dicentrarchus labrax*) is an abundant, ecologically and economically relevant species in the European context, which uses estuaries as nursery areas; as an euryhaline demersal species with benthonic feeding habits (Cabral and Costa, 2001; Martinho et al., 2008) and an opportunistic diet including benthic invertebrates and small fish (Martinho et al., 2008), *D. labrax* may play an essential role in the mercury biomagnification processes in estuarine trophic webs. Golden grey mullet (*Liza aurata*), on the other hand, is a ubiquitous European mugilidae distributed in both Atlantic and Mediterranean coastal waters, which has been chosen by several authors as a biosentinel for metal contamination. This specie is commonly found in both unpolluted

and metal contaminated environments (Guilherme et al., 2008), and feeds essentially on planctonic communities and decaying organic matter together with associated bacteria and fungi. There is no interspecific competition between these species given their distinct feeding tactics, but both co-exist in most western European estuaries. Therefore, they were considered suitable candidates to assess if different feeding strategies are responsible for distinct Hg distribution pattern in the tissues, the main goal of this research.

To pursue this goal, immature individuals from both species, which commonly have a limited geographic range and thus will better reflect local contamination, were collected, dissected with tissue differentiation and analysed for total and organic mercury content.

4.2 Experimental Methods

4.2.1 Brief description of the sampling area

The study was carried out at Ria de Aveiro coastal lagoon, located on the northwest coast of Portugal (40º38'N, 8º44'W) (Fig. 4.1). Over five decades, this system, particularly its inner bay - Laranjo Basin – received a highly mercury contaminated effluent from a chlor-alkali plant. Although the discharges ceased in 1994, high amounts of mercury are still buried in the sediments, in a well-established mercury gradient with distance to source, possibly available trough ressuspension and bioturbation (Coelho et al., 2005).

Field campaign was performed in September 2007, at two different locations. One sampling site was chosen at Laranjo Basin (L) near the mercury source and recognized as a highly contaminated area; the other sampling site (R), was selected close to the lagoon entrance and 10 km away from the polluting sources (Pacheco *et al*., 2005), and was adopted as a reference area.

4.2.2 Procedures for sampling and samples treatment

L. aurata with a total length of 11.7 ± 1.4 cm and *D. labrax* with a total length of 16.1 ± 5.4 cm (mean ± standard deviation) were caught (n=20 for each species) at each sampling site using a beach seine net and a fishing rod, respectively for *L. aurata* and for *D. labrax*. Immediately after being caught, fish were sacrificed by cervical transection and gills, liver, brain, intestines and dorsal muscle were sampled and kept in liquid nitrogen. At the laboratory, the intestinal contents (referred as contents from this point forward) were

removed and stored at -20ºC until analysis. Tissue samples and the contents were freeze-dried, homogenized and well mixed previously to analyses for total (T-Hg) and organic (O-Hg) mercury.

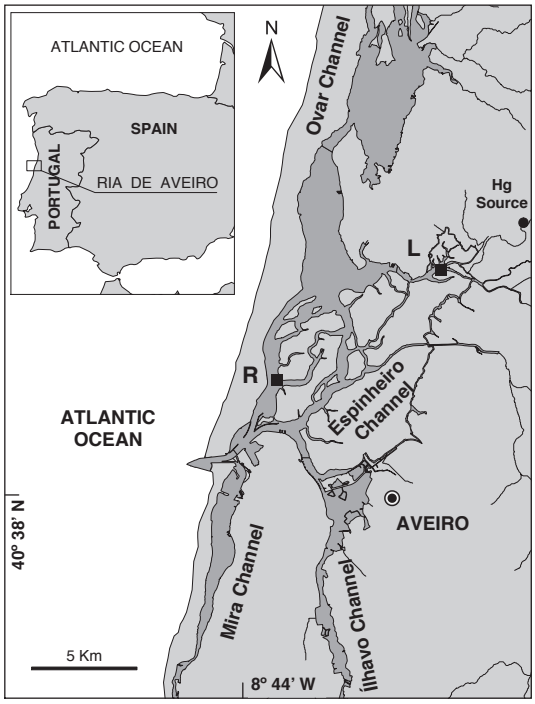

Figure 4.1 Map of the sampling sites (■) in the Ria de Aveiro (Portugal): reference (**R** - 40º41'00'' N, 8º42'44'' W) and contaminated (**L2** - 40º43'28.98'' N, 8º37'35.80'' W) areas.

Sub-surface water samples for mercury analyses were collected in acid-washed PET 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 4ºC until analysis. Filters were re-weighed after drying overnight at 60ºC and stored for determination of mercury in suspended particulate matter (SPM-Hg). Five replicates of sediment were taken from the surface layer (2 cm depth) in each sampled area. Once at the laboratory, sediment samples were freeze-dried, well mixed, sieved through a 1 mm sieve and stored for total mercury determination (Sed-Hg).

4.2.3 Methodology and analytical quality control of mercury determination

Mercury dissolved in water (reactive (R-Hg) and total dissolved (Dis-Hg)) was determined 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 Dis-Hg, 50 mL of each sample was oxidized with 500 μL of a saturated solution of potassium persulfate and by irradiation with a UV lamp (1000 W) for 30 minutes; following irradiation, the excess of oxidant was reduced with 37.5 mL of hydroxylamine solution 12% (w/v) (Mucci et al., 1995). For determination of SPM-Hg, filters were digested with HNO_3 4 mol L^{-1} (Pereira et al., 1998) and the previous equipment was used. The accuracy of the methods for mercury quantification was tested by fortification of samples (at two concentration levels within the range found in samples), showing recovery efficiencies always between 90 and 100%.

Total mercury in sediments (Sed-Hg), in fish tissues (T-Hg) and in contents was determined by atomic absorption spectrometry (AAS) with thermal decomposition and gold amalgamation, using an LECO Advanced Mercury Analyser AMA-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), namely MESS-3 and PACS-2 (marine sediments) for sediments and TORT-2 (lobster hepatopancreas) for biological samples. Precision of the method was better than 9% (n>5), with recovery efficiency between 86-102%.

Organic mercury (O-Hg) determination in fish tissues and contents was performed according to Válega et al. (2006), through digestion of the sample with a mixture of 18% KBr in 5% H_2SO_4, followed by extraction into toluene. Extractions were performed in duplicates and the aqueous fraction resulting from the addition of $Na_2S_2O_3$ solution was analyzed using an LECO AMA-254 as referred for T-Hg. The analytical validations of the O-Hg determinations were performed with reference material TORT–2. The precision of the method ranged between 0 and 10%, with an extraction efficiency between 80-100%. O-Hg determination was not carried in the brain, since this method requires a high amount of sample (0.2 g) and the mean of brain weight was 0.3 g wet weight (wwt) and 0.04 g wwt for *D. labrax* and *L. aurata* respectively. Levels of inorganic mercury (I-Hg) were determined by subtracting O-Hg to T-Hg.

4.2.4 Relationship between fish and contamination load

In order to report metal contamination for both studied fish species, tissue toxic units

(TU_t) were calculated. By definition TU_t is a measure of the enrichment of the tissues with the measured metals, compared to those in fish from unpolluted/reference sites. The calculations were carried using the following formula, adapted by Bervoets and Blust (2003):

$TU_t = Cij / CRi$

where TU_t is the metal load in the tissues of *L. aurata* and *D. labrax* at a site, Cij is the concentration of metal i at site j and CRi is the concentration of metal i at the reference site. When Cij < CRi, the Cij/CRi was considered 1. Accordingly, when no enrichment occurred, TU_t=1 (Bervoets and Blust, 2003).

The relative mercury body burdens were ascertained by calculating the percentage of T-Hg within the percent proportional tissue relative to the total body weight (Pethybridge *et al.*, 2010):

Relative body burden % = (100 x % T-Hgi) / total body weight; where T-Hgi is the total mercury concentration in the tissue i.

4.2.5 Feeding ecology

The feeding ecology of *D. labrax* and *L. aurata* was studied based only on the intestinal contents, which were identified to the lowest possible taxonomical level. Each content was counted and relative importance of prey was assessed using the occurrence index (OI) (Hyslop, 1980).

OI = (Ni/Np), where Ni is the number of the stomachs in which the i food item was found and Np is the number of non-empty stomachs.

4.2.6 Statistical data analysis

Data were tested for goodness of fit to normal distribution and requirements of homogeneity of variances were also determined. A two-way ANOVA was carried out in order to ascertain differences in the accumulation patterns between the two studied species and the sampling sites. Differences between means were considered significant at $p<0.05$. Pearson's correlation coefficient (r) was determined for T-Hg and O-Hg TU_t ($p<0.05$). A Principal Components Analysis (PCA) was used to compare the tissue mercury concentrations (T-Hg and O-Hg) of each fish species within the selected sampling sites. This analysis aimed to outline the organ contamination levels of the two studied species according to their feeding strategy and metal contamination gradient. This analysis was performed using Canoco (Ter Braak, 1995).

4.3 Results

4.3.1 Environmental mercury contamination

Mercury contamination levels in the water column revealed an increase of 3 to 5-fold in dissolved mercury concentrations, from the reference to the contaminated site. Despite this, dissolved mercury concentrations were comparable to non contaminated systems (Coelho et al., 2005) (5.2 ng L^{-1} of reactive dissolved mercury (R-Hg), 11.4 ng L^{-1} of total dissolved mercury (Dis-Hg) in L, 1.6 ng L^{-1} and 2.2 ng L^{-1} respectively in R, Table 4.1). The sedimentary compartment, on the other hand, given its role as a contaminant repository, reflects the historical contamination of the Laranjo Basin, with Sed-Hg concentrations close to 7 mg Kg^{-1}, a 700x increment from reference conditions (0.01 mg Kg^{-1}, Table I). Suspended particulate matter showed evidence of seaward transport from the most impacted area, since while in L SPM-Hg was similar to Sed-Hg (6 and 6.8 mg Kg^{-1}, respectively), in the reference site it was 55x higher than Sed-Hg (0.55 and 0.01 mg Kg^{-1}, respectively).

Table 4.1 Mercury concentrations in the dissolved (reactive (R-Hg) and total (Dis-Hg)), suspended particulate matter (SPM-Hg) and sedimentary (Sed-Hg) compartments of the sampling sites (neap tide situation).

	Water			Sediment
Site	**R-Hg (ng L^{-1})**	**Dis-Hg (ng L^{-1})**	**SPM-Hg (mg Kg^{-1})**	**Sed-Hg (mg Kg^{-1})**
R	1.6	2.2	0.55	0.01 ± 0.0009
L	5.2	11.4	6.0	6.8 ± 0.16

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

4.3.2 *D. labrax* and *L. aurata* feeding ecology

The OI revealed that the feeding habits of *D. labrax* and *L. aurata* are distinct (Table 4.2). The diet of *D. labrax* is more diversified and constituted in high amounts by crustaceans and polychaetes and in a lesser extent by non-identified material (NIM), fish, algae, mollusks and echinoderms. On the other hand, *L. aurata*'s diet is mainly composed by NIM, sediments and polychaetes, being also found crustaceans and algae.

Table 4.2 Intestinal contents of *D. labrax* and *L. aurata* collected from the Ria de Aveiro. Values represent the percent of *D. labrax* and *L. aurata* intestines that contained a given prey item (Ocurrence index, OI).

	Ocurrence index (%)	
Contents	***D. labrax***	***L. aurata***
Mollusca		
Bivalvia ni	3.8	_
Polychaeta		
Nereis sp	46.2	29.7
Polychaeta ni	7.7	
Crustacea		
Isopoda		
Gnathidae	7.7	_
Sphaeromatidae	7.7	_
Isopoda ni	23.1	_
Amphipoda		
Amphipoda ni	7.7	_
Decapoda		
Carcinus sp	15.4	_
Crangonidae	23.1	_
Mysidacea	7.7	_
Decapoda ni	26.9	_
Ostracoda	_	2.7
Crustacea ni	76.9	2.7
Echinodermata		
Echinodermata ni	3.8	_
Teleostei		
Teleostei ni	15.4	_
Algae		
Algae ni	11.5	2.7
Non Identified	23.1	78.4
Sediments	_	43.2

ni- non identified

4.3.3 Biological mercury contamination

Total mercury

Mercury bioaccumulation and distribution in *L. aurata* and *D. labrax* tissues is represented in Figure 4.2. A similar distribution pattern was observed for both species, despite a tendency for higher mercury loads in *D. labrax*. The brain and gills were consistently the least contaminated tissues, regardless of species or sampling site, ranging from 0.03 mg Kg^{-1} in R to 0.12 mg Kg^{-1} in L in brain and 0.03 to 0.19 mg Kg^{-1} in

gills. Significant differences were found between the two study areas, but not between species neither for brain (Two Way ANOVA, F=2.495, p=0.120 for species, F=43.711 p<0.001 for site) nor gills (Two Way ANOVA, F=1.222, p=0.291 for species, F=27.646, p<0.001 for site). Regarding the most contaminated tissues (liver and intestine), and the contents, whilst *L. aurata* retained the same distribution pattern from the reference site to the impacted area (still presented the highest concentrations), an inversion was observed for *D. labrax*. While in R the tissues with the highest contamination load where muscle and liver, in L the metal distribution showed a similar pattern to that of *L. aurata*, with intestinal content, intestine and liver with higher levels than muscle tissue.

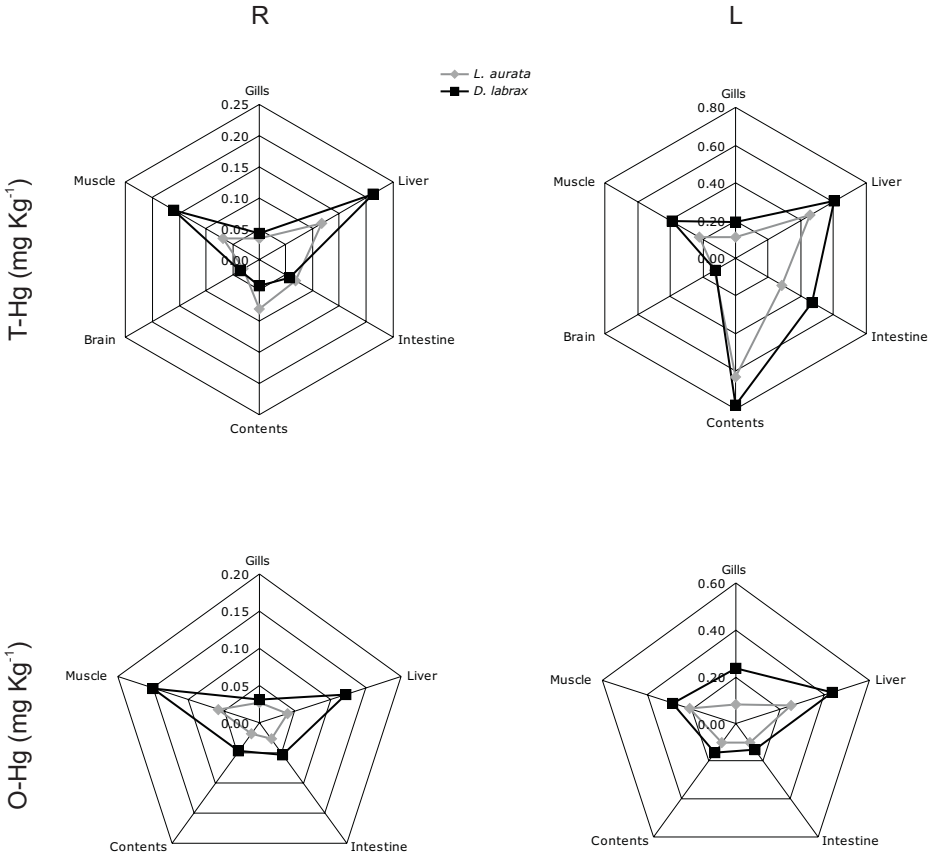

Figure 4.2 Total (T-Hg) and organic (O-Hg) (mg Kg^{-1}wet weight) mercury distribution in the tissues and intestinal contents of *D. labrax* and *L. aurata* in each sampling site at Ria de Aveiro.

Relative mercury body burdens (Table 4.3) revealed that for both species, muscle tissue accounts only for less than 30% of the mercury load, and tends to decrease

inversely to environmental contamination, with liver, intestine and contents making up for more than 70% mercury body burden.

Table 4.3 Relative mercury body burdens of *D. labrax* and *L. aurata* tissues and intestinal contents (Contents) in the Ria de Aveiro.

	Site	Gills	Intestine	Liver	Brain	Muscle	Contents
L. aurata	R	8.7	17.1	29.6	7.5	16.9	20.2
	L	6.2	15.3	25.0	6.9	12.1	34.5
D. labrax	R	7.6	10.3	38.2	6.6	29.4	7.8
	L	7.8	18.3	23.5	4.9	15.1	30.4

These differences between species were reflected in the PCA performed (Figure 4.3).

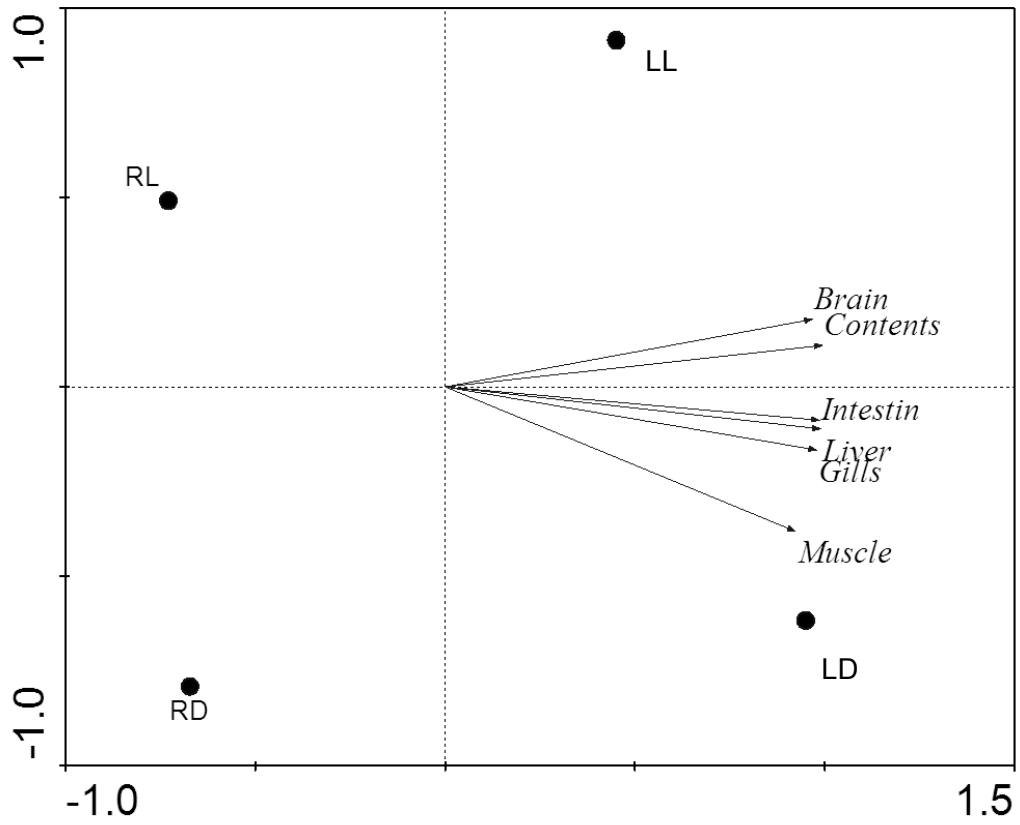

Figure 4.3 Principal Components Analysis (PCA) for total mercury contents (T-Hg) in tissues and intestinal contents (Contents) of *D. labrax* and *L. aurata* in the Ria de Aveiro. RD- Reference *D. labrax*; RL- Reference *L. aurata*; LD- Laranjo *D. labrax*; LL- Laranjo *L. aurata*.

For T-Hg, the first two axes of this ordination analysis accounted for 92.3 % of the total variance, and accumulation patterns in the different tissues and contents where higher in the contaminated area. As an example, *D. labrax* muscle accumulated higher amounts of mercury at L. Comparing both species, T-Hg accumulation in the intestine, liver, muscle and gills seemed to be higher in *D. labrax* than in *L. aurata*, opposite to what was observed for the contents mercury levels.

Organic mercury

O-Hg levels were almost always higher in *D. labrax*, consistent to what was observed for T-Hg (Figure 4.2). Gills, intestine and contents presented comparable, low organic mercury levels in both species, only contents being significantly different between species (Two Way ANOVA, F= 3.784, p=0.076, 2.527, p=0.140, F=11.181, p=0.004, gills, intestine and contents, respectively), while all showed differences between sites (F= 25.468, p<0.001, F=24.713, p<0.001, F=44.753, p<0.001, gills, intestine and contents, respectively). Muscle and liver are highlighted as the privileged organs for organic mercury bioaccumulation. Significant differences between species were found for both tissues (Two Way ANOVA, F=21.427, p<0.001 for liver, F=64.080, p<0.001 for muscle) and a shift in dominance was observed from reference conditions to L, from muscle tissue to liver, in both species. These tissues also revealed significant differences between sampling sites (Two Way ANOVA, F=71.978, p<0.001 for liver, F=151.201, p<0.001 for muscle).

The PCA performed with O-Hg tissue concentrations, for *D. labrax* and *L. aurata*, showed inter-species and inter-sites variability, the first two axes of this ordination analysis explaining 99.8% of the variance. Similar to T-Hg, O-Hg accumulation was higher at the contaminated site L (Figure 4.4). O-Hg accumulation in all the tissues and in contents occurred in a higher extent for *D. labrax* than for *L. aurata*.

The comparison of organic/inorganic mercury tissue contents (Figure 4.5) revealed different mercury species distribution between the two species. In gills and muscle tissue the organic fractions were similar between species for both sampling sites, ranging from 70 to >90% of the total mercury body burden; liver, intestine and contents of *D. labrax* consistently presented a considerable higher proportion of organic mercury (2 to 4x the organic mercury fraction of the respective tissue in *L. aurata*). The most visible example was in contents, in which O-Hg ranged from 10 to 20% in *L. aurata*, while in *D. labrax* varied from 40 to 80% depending on location.

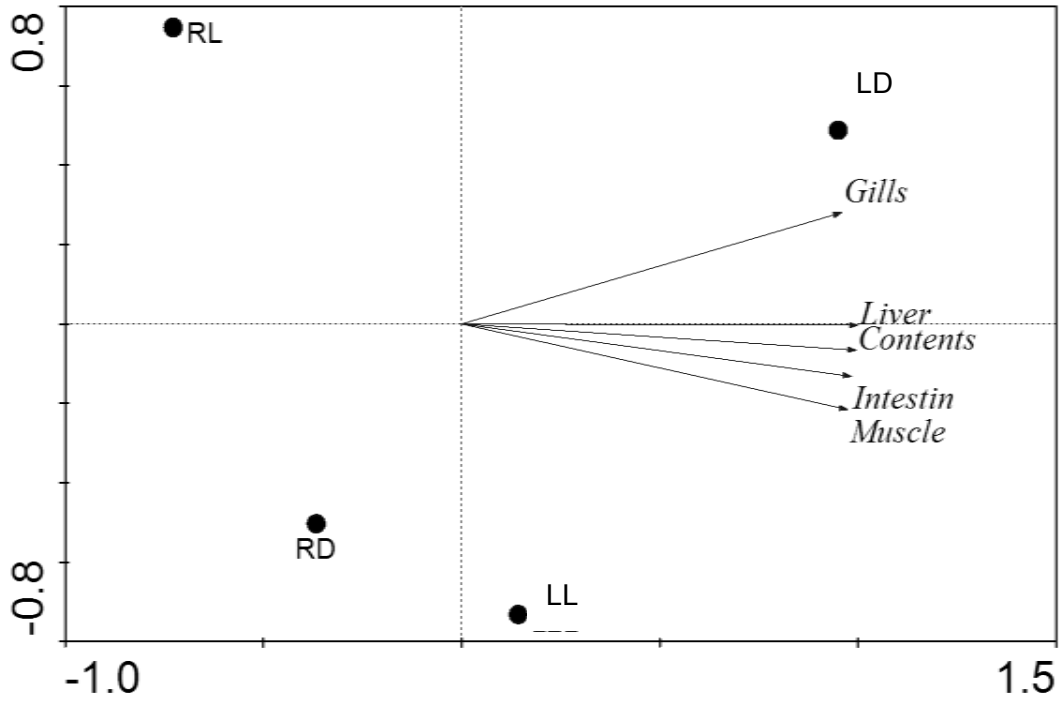

Figure 4.4 Principal Components Analysis (PCA) for organic mercury contents (O-Hg) in tissues intestinal contents (Contents) of *D. labrax* and *L. aurata* in the Ria de Aveiro. RD- Reference *D. labrax*; RL- Reference *L. aurata*; LD- Laranjo *D. labrax*; LL- Laranjo *L. aurata*.

TU_t were calculated for both T-Hg and O-Hg, and reflected the increment of a selected contaminant from reference conditions to the contaminated site (Figure 4.6A and 4.6B). For total mercury, no regular pattern was observed, and toxic units reached as high as 18 in intestinal contents (*D. labrax*). TU_t for *D. labrax* intestine varied accordingly, as did gills tissue in close contact with the environment, while for *L. aurata* the increment of T-Hg was higher in internal organs (liver, brain and muscle tissue).

For O-Hg, on the contrary, and excluding gills tissue, TU_t for each species varied proportionally among them (Figure 4.6B) with very strong correlation coefficient ($r=0.96$, $p=0.0419$) (Figure 4.6C), which may suggest intestinal content to be regulating organic O-Hg levels in tissues of both species. Considering only muscle and liver tissue, together with intestinal contents, the correlation coefficient increases even further ($r=1.00$, $p=0.0097$), which enforces the role of intestinal contents on the O-Hg bioaccumulation in these two tissues.

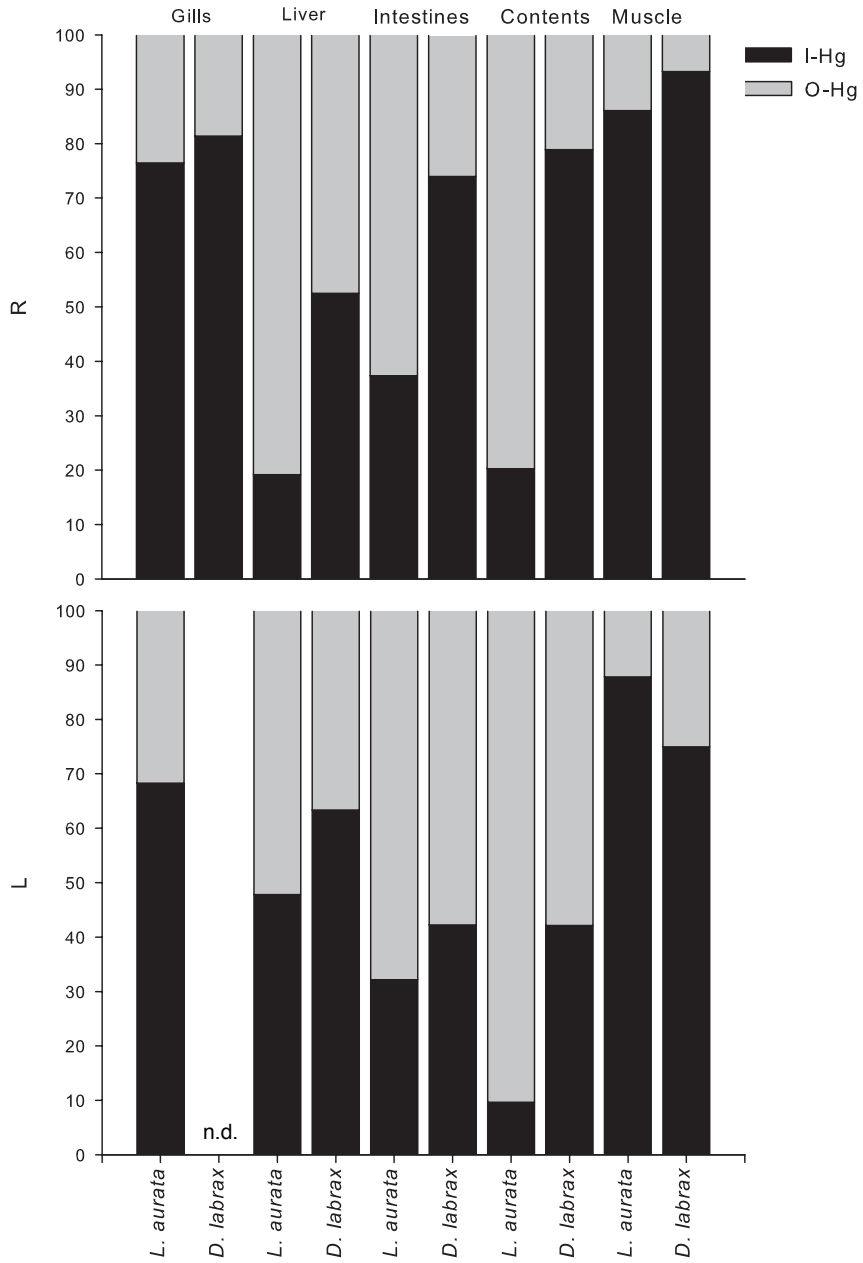

Figure 4.5 Percentages of inorganic and organic mercury in the tissues and intestinal contents (Contents) of *D. labrax* and *L. aurata* in the Ria de Aveiro. n.d. – not determined.

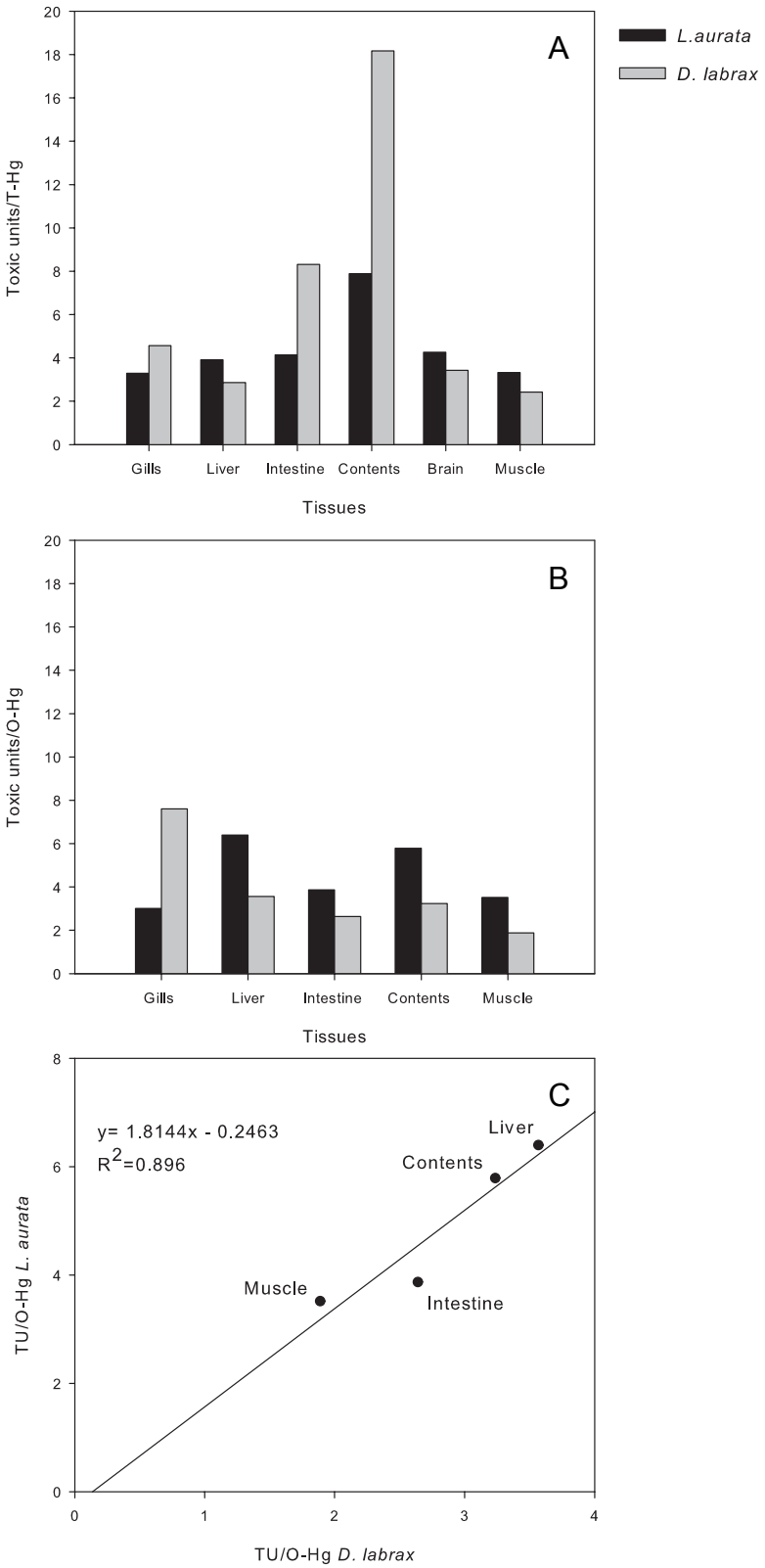

Figure 4.6 Toxic Units (TU_t) for total and organic mercury bioaccumulation in *D. labrax* and *L. aurata* in the Ria de Aveiro (A and B). Pearson correlation between TU and the O-Hg (C).

4.4 Discussion

4.4.1 Mercury environmental availability

A recent review of the historical mercury pollution problem in the Ria de Aveiro reported the reduction of surface sediment mercury levels in the system, and suggested that, presently, contamination problems are restrict to Laranjo Basin, where mercury is mainly associated with sediments (Pereira et al., 2009). Our environmental results are in agreement with these findings since, despite the contamination gradient being perceptible in all compartments, mercury is stored mostly in the sedimentary fraction, and dissolved mercury concentrations are low in both sampling sites. Pereira and co-authors (2009) also proposed re-suspension of mercury rich particles by tidal movements and high-energy phenomena as the most likely events to increase water column mercury levels, consistent with the observed mercury transport in suspended particulate matter (SPM) from the Laranjo area seawards, and visible in R SPM-Hg levels, higher than expected. As previously stated, mercury accumulation in Ria de Aveiro system seems to be mainly associated to sediment contamination. In accordance, Chen and Chen (1999) considered three major pathways for sediment - bound contamination; via re-suspended fine particles in the water column taken up via the gills and digestive tract; via leached sedimentary contaminants accumulated via respiration, and direct contact and sediment consumption by bottom-dwellers through the skin and intestine. Having in mind that *D. labrax* is a benthonic feeder and *L. aurata* both herbivorous and detritivorous, it is possible to hypothesise that in the reference site, given the low water column Hg levels, the dominant pathway is the dietary in opposition to the contaminated site, where a combination of all pathways may occur.

4.4.2 Mercury loads in tissues and in contents

Considering fish contamination, information for this system is scarce. In 1986 Lucas and co-workers (1986) reported fresh weight Hg concentrations in sea bass (*D. labrax*) not to exceed 0.92 mg kg^{-1}, but more than a decade later, Abreu and colleagues (2000) observed mercury concentrations ranging from 0.03 to 1.7 mg kg^{-1} (fresh weight) in muscle tissue from juveniles of the same species, suggesting that despite the significant decrease in surface sediment contamination, estuarine biota are still subject to mercury bioaccumulation. Our results suggest an improvement in the condition of the estuary, given that the maximum observed T-Hg concentrations in *D. labrax* muscle never exceeded 0.47 mg kg^{-1}, a 3-fold reduction in less than a decade. Regarding *L. aurata*,

studies in the system are recent (Guilherme et al., 2008; Mieiro et al., 2009) and reported concentrations, in juvenile specimens, were in the same range as reported here, for gills, liver, brain and muscle, suggesting the area may have attained a steady state concerning mercury contamination in the last few years.

Studying contaminant distribution and accumulation within organisms may confer an insight to specific bioaccumulation pathways, fundamental to assess the relative input of the various environmental compartments in accumulation and toxicity processes, as well as to define appropriate quality guidelines for water and sediments (Wang and Fisher, 1999). All tissues highlighted the difference between the reference condition and the contaminated area, suggesting both species to be suitable bioindicators of mercury contamination. Taking into consideration the high mobility of pelagic species and the possible intermittent exposure to point source contamination, the use of immature individuals, commonly with limited geographic range, may have minimized bias and permitted a better reflection of local contaminant stress (Coelho et al., 2009).

Despite that *D. labrax* and *L. aurata* have different biological and ecological traits, no significant differences were observed between the two species in gills, for both T-Hg and O-Hg bioaccumulation, confirming this tissue to reflect the metal bioavailability in the water column, with which is in close and permanent contact. Gills tissue was one of the least contaminated tissues, a consequence of the frequent renewal through exfoliation and erosion to which it is subject, as observed earlier (Mieiro *et al.*, 2009). A caging experiment with *L. aurata* highlighted the relevance of this tissue in bioaccumulation processes through the restriction of foraging activities (Guilherme et al., 2008), although the season in which it was performed (winter) may have originated an overestimation of the gills function in Hg bioaccumulation processes. According to Post and co-workers (1996), at suboptimal temperatures respiration Hg uptake increases relative to dietary uptake because respiration rates are a larger component of the energy budget than at optimal temperatures.

In addition to gills tissue, no significant differences between species were observed in brain and intestine mercury levels. While only fragmentary information exists in literature concerning mercury accumulation in fish brain tissue, the low levels observed suggest some degree of protection from metal toxicity, possibly through transport limitation at the brain-blood barrier (Zheng et al., 2003) and through a buffering action exerted by other organs/tissues, such as muscle, liver and kidney, protecting brain especially for organic mercury (Mieiro et al., 2009). Our results are in agreement with

previous research, suggesting that uptake rates of both organic and inorganic mercury are particularly low in the brain compared with other organs (Niimi and Kissoon, 1994). However, Berntssen and co-workers (2003) reported for Atlantic salmon that despite liver and kidney accumulated most methylmercury, oxidative injury occurred in the brain and not in other tissues, highlighting the sensitivity of brain tissue to Hg toxicity.

Regarding Hg accumulation in intestine, studies performed on invertebrates reported that both inorganic and organic Hg uptake across intestinal tissues is rapid and at equivalent rates, due to the relatively unspecific nature of the uptake (Andres et al., 2002; Laporte et al., 2002). On the other hand, studies on mercury distribution kinetics reported metal flux from intestinal tissue to blood (Leaner and Mason, 2002, 2004), suggesting this tissue not to be a target accumulator organ, but rather a pathway for mercury uptake, which can explain the absence of significant differences between species in intestinal tissue. The same authors refer that despite the rapid mercury flux to the intestine, low mobilization rates of mercury were found from intestine to blood, suggesting that the limiting step to mercury distribution is the digestion process instead of the transport. This could explain the significant differences observed from the reference site to the contaminated area.

Diet has been widely reported as a primordial source for Hg accumulation in heterotrophic organisms, from plankton (Lawson and Mason, 1998), marine invertebrates (Coelho et al., 2006) to fish (de Pinho et al., 2002; Zhou and Wong, 2000), birds (Tavares et al., 2008) and humans (Calderón et al., 2003). The metal distribution in tissues has been found to reflect the relative contribution of diet and environmental Hg levels to body burdens in estuarine invertebrate species (Coelho et al., 2008b; Laporte et al., 1997), albeit the balance between Hg sources is seasonal and dependent on variables such as temperature, diet and growth efficiency (Post et al., 1996). It would therefore be expectable that two species with distinct feeding strategies show specific mercury distribution patterns. In fact, while in general T-Hg levels where higher in *D. labrax* tissues, significant differences to *L. aurata* were only observed in muscle and contents. Similarly, both muscle and contents evidenced significant differences in O-Hg, as did liver tissue. The main distinction between the two sampling sites, for both species, was the boost in the relative mercury body burden associated with intestinal contents, confirmed by the superior toxic units, and suggesting the dietary pathway as the dominant process regulating mercury uptake for both species. PCA analysis confirmed the existence of an environmental pattern in mercury accumulation. Moreover, in general *D. labrax* exhibited

higher mercury levels and the O-Hg accumulation pattern reinforced the importance of the food items as the major source of mercury, especially O-Hg.

A recent study focussing on trophic web structure reported the trophic level of *D. labrax* juveniles (trophic level of 3.43), with a diet composed mostly of invertebrates (70%) and fish (16.67%) and the closely related to *L. aurata*, *Liza ramada* (trophic level of 2.87), whose stomach contents consisted of organic debris (Pasquaud et al., 2010). The intestinal contents of the studied species in the present work revealed to be in accordance to the previous authors. *D. labrax* contents were dominated by crustaceans and polychaets, with fish also present, while *L. aurata* contents revealed to be mainly composed by non - identified material and sediments, with polychaets also present. The diversity of the dietary items found between the species reinforces the idea that these two species have a distinct feeding behaviour, which may justify the observed differences in the contents between the two species, both for T-Hg and O-Hg. It was also evident an increase in both T-Hg and O-Hg accumulation from R to L, indicating a boost of mercury concentration in the consumed items. O-Hg is efficiently absorbed at the intestinal level (Andres et al., 2002; Laporte et al., 2002), and this “excess” mercury will be transported by the blood to liver, a target organ for mercury bioaccumulation (Leaner and Mason, 2004). Following the collapse of liver retention capacity, mercury will redistribute to the rest of the body and, consequently, its accumulation in muscle will increase (Mieiro et al., 2009).

Noteworthy was the high correlation coefficient observed between organic mercury toxic units for both species. Apart from gills tissue, which appears to be independent, the linear correlation with other tissues indicates co-variation of O-Hg increments from reference conditions to contaminated areas, most certainly as a vector of intestinal contents O-Hg levels. The ratio TU_{labrax}/TU_{aurata} was 0.56, 0.56 and 0.54 respectively for intestinal content, liver and muscle, suggesting the increment of organic mercury content of both liver and muscle to have a stable ratio to the O-Hg increment in dietary items, independently of dietary preferences of target species. These stable ratios, if verified repeatedly and elsewhere, may prove to be a useful management tool to predict O-Hg bioaccumulation in contaminated estuarine systems, based on dietary O-Hg content and regardless of trophic level.

4.5 Conclusions

In summary, dietary uptake emerges as the main pathway for mercury bioaccumulation in the two fish species, and intestinal mercury content the forcing function regulating internal tissue body burden, especially of preferential metal sequestrating tissues such as liver and muscle. Moreover, the stable ratio observed in mercury increments from reference conditions to the contaminated area, especially for muscle and liver and regardless of trophic position, may be a useful predictor for mercury accumulation, and justifies further investigation.

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CHAPTER 5

Antioxidant system breakdown in brain of feral golden grey mullet (*Liza aurata*) as an effect of mercury exposure

Antioxidant system breakdown in brain of feral golden grey mullet (*Liza aurata*) as an effect of mercury exposure

Abstract

Although brain has been recognized as a primary target for mercury toxicity in mammals, the effects of this metal in fish brain are scarcely described. Thus, the main objective of this study was to assess the mercury threat to feral fish (*Liza aurata*) by estimating the antioxidant defenses and peroxidative damage in brain, keeping in mind the association with mercury accumulation. Sampling was carried out in an estuarine area historically affected by discharges from a chlor-alkali industry - Laranjo Basin (Ria de Aveiro, Portugal). Total mercury (T-Hg) in brain increased towards the contamination source, clearly indicating mercury exposure. An overall antioxidant depletion was verified in brain of fish collected at the mercury-contaminated stations, since total glutathione content and the studied antioxidant enzymes (catalase – CAT, glutathione peroxidase – GPx, glutathione S-transferase – GST and glutathione reductase - GR) significantly decreased. In addition, this breakdown of the redox-defense system was significantly correlated with the accumulated T-Hg levels. Unexpectedly, fish exhibited unaltered lipid peroxidation levels, pointing out a higher propensity of mercury to inhibit enzymes than to oxidatively damage lipids in the brain. Nevertheless, an increased susceptibility of the fish's brain was identified, leaving the organ more vulnerable to oxidative stress-related challenges. Overall, the current findings provide information to better understand mechanisms of mercury neurotoxicity in fish.

Keywords: Brain; *L. aurata*; mercury; antioxidant responses; environmental exposure

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5.1 Introduction

Mercury has been recognized as a strong neurotoxic in humans and wildlife (Franco et al., 2006; Díez, 2008). As a primary target for organic mercury compounds, namely methylmercury (MeHg), the brain and its vital neurological functions have been widely studied in humans and rodents (e.g. Aschner et al., 2007; Ferraro et al., 2009). In contrast, the neurotoxicity of mercury to fish remains scarcely described, though both inorganic and organic forms have been pointed out as damaging agents to the central nervous system (CNS). Hence, several mercury-induced disturbances have been found on fish sensory capacities (Baatrup et al., 1990; Oliveira Ribeiro et al., 1995), as well as brain lesions and behaviour changes (Berntssen et al., 2003), being also hypothesized an association with reduced locomotor (Zhou and Weis, 1998) and predator avoidance capacities (Fjeld et al., 1998).

Though mercury has been involved in neurodegeneration (Leong et al., 2001), the mechanisms underlying its toxicity are still unclear, mainly in fish. In this context, it is particularly relevant to research the role of oxidative stress phenomena since the mercury neurotoxicity has been associated to the excessive generation of reactive oxygen species (ROS) and lipid peroxidation (LPO) both in mammalian systems (Huang et al., 2008) and in teleost fish (Berntssen et al., 2003). When compared to other organs, the brain is especially endangered due to the high potential to generate ROS, related to its high oxygen consumption and iron content, as well as the abundance of readily oxidizable substrates (e.g. unsaturated fatty acids) (Dringen, 2000). Subsequently, the induction of enzymatic (Berntssen et al., 2003) and non enzymatic antioxidants can occur as an adaptive onset of the redox defense system (Yee and Choi, 1994), linked to a neuroprotective action. On the other hand, antioxidants depletion/inhibition is thought to contribute to oxidative stress in mouse brain following mercury intoxication (Yee and Choi, 1994). Mercury, as a redox-inactive metal, does not undergo redox cycling and, thus, depletion of cells' major antioxidants, particularly thiol-containing antioxidants and enzymes, seems to be an important indirect mechanism for the oxidative stress induced by this metal (Stohs and Bagchi, 1995). Within this framework, the knowledge of the antioxidant system responses in brain cells of fish, as well as its capacity to prevent mercury-induced oxidative injury and the corresponding threshold limits is a challenging and timely issue for ecotoxicologists. The observation that fish brain can accumulate substantial levels of mercury (Mieiro et al., 2009) reinforces the previous statements.

Most studies on mercury-induced brain disorders carried out in fish concern freshwater species (Larose et al., 2008) and they are limited to laboratory approaches where the uptake routes are alternatively restricted to waterborne or dietary exposure (Berntssen et al., 2003; Keyvanshokooh et al., 2009). The processes on the basis of neurotoxicity have not yet been explored before in feral marine fish. Accordingly, the main objective of this work was to assess the mercury threat to fish by evaluating the antioxidant defenses modulation and peroxidative damage specifically in the brain of feral golden grey mullet (*Liza aurata*). In order to achieve this goal, three tasks were carried out: i) to determine mercury accumulation in the brain; ii) to evaluate brain antioxidant defenses (enzymatic and non-enzymatic) simultaneously with LPO; iii) to establish a causal relationship between organ burden and the previous oxidative stress responses in order to assess the risk towards neurotoxicity in fish inhabiting mercury contaminated areas. The samples for this study were from a historically impacted estuarine area - Laranjo Basin (Ria de Aveiro, Portugal) - receptor of a chlor-alkali plant effluent, which display a well-established mercury contamination gradient (Coelho et al., 2005).

5.2 Materials and Methods

5.2.1 General description of the study area

The study was carried out at Ria de Aveiro, a coastal lagoon located on the northwest coast of Portugal (Fig. 5.1). This aquatic system has an inner basin (Laranjo Basin), which has persistently received effluents from chlor–alkali industry (1950-1994). The discharges resulted in an accumulation of about 27 tons of mercury in the Laranjo Basin and its upstream channel. Although effluent discharge ceased by 1994, high mercury concentrations can still be found in the fine surface sediments of this basin (Coelho et al., 2005). The eventual presence of other contaminants in this area was assessed, pointing out negligible levels of arsenic, cadmium, lead, copper and zinc in superficial sediments (2006 and 2009; unpublished data). Moreover, Pacheco et al. (2005) assessed the levels of priority polycyclic aromatic hydrocarbons (PAHs) also reporting low levels. Accordingly, Laranjo Basin has been recurrently adopted as a “field laboratory”, offering a unique opportunity to assess mercury toxicity (Guilherme et al., 2008; Válega et al., 2009).

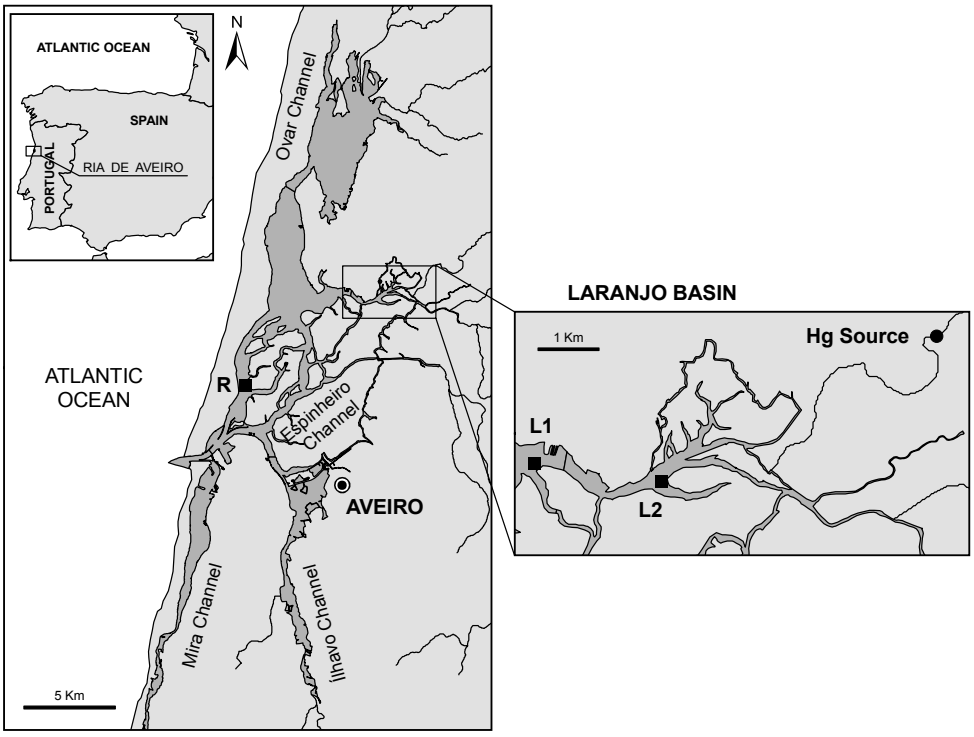

Figure 5.1 Map of the sampling stations (■) in the Ria de Aveiro (Portugal): reference (R - 40º41'00'' N, 8º42'44'' W), moderately (L1 - 40º43'34.46'' N, 8º38'53.16'' W) and highly contaminated (L2 - 40º43'28.98'' N, 8º37'35.80'' W) areas.

Three locations (R, L1 and L2) were chosen according to the existing contamination gradient (Fig. 5.1). Two sampling stations were located at Laranjo Basin, i.e. L1 as the moderately contaminated station and L2 as the highly contaminated station. The sampling site L2 was located closer to the mercury source and 2 Km away from L1. An area close to the lagoon entrance (S. Jacinto) characterized for a low contamination load and far away from the main polluting sources, was chosen as the reference station (R).

5.2.2 Sampling procedures

Water physico-chemical parameters such as pH, dissolved oxygen, temperature and salinity were measured at sub-surface level, during high and low tide. Turbidity was also measured as well as water-column depth. Sub-surface water samples were collected together with five replicates from the surface sediment layer (approximately 2 cm depth) for mercury measurements.

Fifteen juveniles of *Liza aurata* - golden grey mullet - (mean total length of 11.6 cm ± 1.25; mean wet weight of 14.6 g ± 5.47) were collected at each sampling station during low tide, in March 2007, using a beach-seine net. Juvenile specimens were selected due to their prevalence in the estuary and owing to their ability to reflect mercury contamination over the period of estuarine residency (Mieiro et al., 2009). Furthermore, the use of juveniles minimizes the interference of variables such as gender and reproductive processes, as well as the potential occurrence of a growth dilution effect in relation to mercury accumulation. Immediately after being caught, fish were sacrificed by cervical transection and the entire brain was excised, and instantly frozen in liquid nitrogen. In the laboratory, samples were preserved until further processing at -80ºC and -20°C for oxidative stress and mercury determinations, respectively.

5.2.3 Determination of total mercury

Mercury in water

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 4ºC until analysis. Filters were re-weighed after heating overnight at 60ºC and stored at 4ºC for suspended particulate matter (SPM) determinations. Total dissolved mercury (Dis-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 (see Mucci et al., 1995). For determination of SPM-Hg, filters were digested with HNO_3 4 mol L^{-1} and analyzed also by CV-AFS in the above mentioned equipment (Pereira et al., 1998). Water column mercury (W-Hg) was estimated as the sum of Dis-Hg and SPM-Hg and the percentage of mercury associated to the particulate matter (% Hg particulate) was also estimated by calculation. The accuracy of the methods for mercury quantification was tested by fortification of samples (at two concentration levels within the range found in samples), showing recovery efficiencies always between 90-100%.

Mercury in samples of sediment and in fish brain

At the laboratory, sediment samples were freeze-dried, well mixed, sieved through a 1 mm sieve and stored for total mercury determination (Sed-Hg). Brain samples were freeze-dried, homogenized, weighted for fresh weight determination and finally used for determination of tissue total mercury (T-Hg).

Sediment and brain 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 better than 9% (n>5), with recovery efficiency between 92-103%.

5.2.4 Biochemical analyses

Fish brain was homogenized in a 1:15 ratio (1 g tissue:15 mL buffer), using a Potter-Elvehjem homogenizer, in chilled phosphate buffer (0.1 M, pH 7.4). The homogenate was then divided in aliquots for LPO and total glutathione (GSHt) quantification, as well as for post-mitochondrial supernatant (PMS) preparation. The PMS fraction was obtained by centrifugation in a refrigerated centrifuge (Eppendorf 5415R) at 13,400 g for 20 min at 4ºC. Aliquots of PMS were stored at -80ºC until analysis.

Catalase (CAT) activity was assayed in PMS (at 25ºC) by the method of Claiborne (1985) as described by Giri *et al*. (1996). Briefly, the assay mixture consisted of 1.99 mL phosphate buffer (0.05 M, pH 7.0), 1 mL hydrogen peroxide (0.030 M) and 0.01 mL of sample in a final volume of 3 mL. Change in absorbance was recorded spectrophotometrically (Jasco UV/VIS, V-530) at 240 nm. CAT activity was calculated in terms of μmol H_2O_2 consumed/min/mg protein (ε = 43.5 M^{-1} cm^{-1}).

Glutathione peroxidase (GPx) activity was assayed (at 25ºC) according to the method described by Mohandas et al. (1984) as modified by Athar and Iqbal (1998). The assay mixture consisted of 0.09 mL phosphate buffer (100 mM, pH 7.0), 0.03 mL EDTA (10 mM), 0.03 mL sodium azide (1 mM), 0.03 mL glutathione reductase (GR; 2.4 U/mL), 0.03 mL reduced glutathione (GSH; 10 mM), 0.03 mL NADPH (1.5 mM), 0.03 mL H_2O_2 (1.5 mM) and 0.03 mL of PMS in a total volume of 0.3 mL. GPx activity was determined monitoring the oxidation of NADPH to $NADP^+$, resulting in an absorbance decrease at 340 nm. The absorbance was read every 30 seconds for a period of 3 minutes using a SpectraMax 190 microplate reader. The enzyme activity was calculated as nmol NADPH oxidized/min/mg of protein ($\varepsilon = 6.22\times10^3$ M^{-1} cm^{-1}).

Glutathione reductase (GR) activity was measured (at 25ºC) according to the method of Carlberg and Mannervik (1975). Briefly, the reaction medium consisted of 0.1 M

phosphate buffer (pH 7.0), DTPA 0.5 mM, NADPH 0.2 mM and GSSG 1 mM. In a quartz cuvette, 0.025 mL of PMS was added to 0.975 mL of reaction medium. Enzyme activity was spectrophotometrically measured (Jasco UV/VIS, V-530) by assessing NADPH disappearance at 340 nm and expressed as nmol of NADPH oxidised/minute/protein ($\varepsilon = 6.22\times10^{3}\ M^{-1}\ cm^{-1}$).

Glutathione-S-transferase (GST) activity was determined using CDNB (1-chloro-2,4-dinitrobenzene) as substrate, according to the method of Habig *et al*. (1974). The assay was carried out (at 25 ºC) in a quartz cuvette with a 2 mL mixture of 0.2 M phosphate buffer (pH 7.4), 0.2 mM CDNB and 0.2 mM GSH. The reaction was initiated by the addition of 0.01 mL PMS, and the increase in absorbance was recorded spectrophotometrically also in a Jasco UV/VIS, V-530 at 340 nm during 3 min. The enzyme activity was calculated as nmol CDNB conjugate formed/min/mg protein ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$).

Total glutathione (GSHt) content was determined (in deproteinated PMS, at 25ºC) adopting the enzymatic recycling method using GR excess, whereby the sulfhydryl group of GSH reacts with 5,5, dithiobis-tetranitrobenzoic acid (DTNB) producing a yellow colored 5-thio-2-nitrobenzoic acid (TNB) (Tietze, 1969; Baker *et al*., 1990). Formation of TNB was measured by spectrophotometry (Jasco UV/VIS, V-530) at 412 nm. The results were expressed as nmol TNB formed/min/mg protein ($\varepsilon = 14.1\times10^{3}\ M^{-1}\ cm^{-1}$).

Estimation of lipid peroxidation (LPO) was performed in the tissue homogenate, based in the thiobarbituric acid reactive substances (TBARS) measurement, according to the procedures of Ohkawa (1979) and Bird and Draper (1984), as adapted by Filho et al. (2001). Briefly, to 0.15 mL of homogenate, 0.01 mL of 1-1 butylated hydroxytoluene (4% in methanol) was added to prevent oxidation. To this aliquot, 0.5 mL of 12% TCA, 0.45 mL Tris–HCl (60 mM, pH 7.4; and 0.1 mM DTPA) and 0.5 mL 0.73% TBA were added and mixed well. The mixture was heated for 1 hour in a water bath set at boiling temperature and then cooled to room temperature, decanted into 2 mL microtubes and centrifuged at 13,400 g for 5 min. Absorbance was measured at 535 nm, using a SpectraMax 190 microplate reader, and LPO was expressed as nmol of TBARS formed per milligram of fresh tissue ($\varepsilon = 1.56\times10^{5}\ M^{-1}\ cm^{-1}$).

Total protein contents were determined according to the Biuret method (Gornall et al., 1949), using bovine serum albumin (E. Merck-Darmstadt, Germany) as a standard. Absorbance was measured at 550 nm using a SpectraMax 190 microplate reader.

5.2.5 Statistical analysis

Data were tested for goodness of fit to normal distribution and requirements of homogeneity of variances were also determined. Analysis of variance (ANOVA) were 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, antioxidant defenses and LPO. A significance level of 0.05 was considered in all test procedures.

5.3 Results

5.3.1 Environment characterization

In general, the three sampling stations were analogous in terms of hydrological parameters, with the exception of salinity, which ranged from 13 (L2) to 34 (R), and SPM that was higher in the contaminated areas, especially in L2 (two-fold higher than R). Parameters such as temperature, pH, dissolved oxygen, depth and turbidity were in the same range (Table 5.1).

Table 5.1 Environmental characteristics measured in high and low tide on reference (R), moderately (L1) and highly mercury contaminated (L2) areas at Ria de Aveiro: water temperature (T), dissolved oxygen (DO), water-column depth, turbidity, pH, salinity, and suspended particulate matter (SPM).

	Sampling station					
	R		L1		L2	
	High Tide	Low Tide	High Tide	Low Tide	High Tide	Low Tide
T (ºC)	16.4	15.3	15.3	15.0	12.5	12.2
DO (mg L^{-1})	10.8	10.9	10.6	10.8	8.5	8.9
Depth (m)	5.4	1.6	3.1	2.8	2.3	1.0
Turbidity (m)	1.2	0.5	0.9	0.5	1.2	0.3
pH	8.4	8.4	8.2	8.1	8.3	7.8
Salinity	34	34	28	15	32	13
SPM (mg L^{-1})	45.1	31.7	40.5	53.2	n.d.	70.0

n.d. – not determined

The Dis-Hg concentrations were, in general, low (Table 5.2). Low tide Dis-Hg concentration doubled in L2 comparing to R and L1. Contrastingly, high tide reflected half

Dis-Hg levels at L2 and L1 in comparison to R. The SPM-Hg concentrations increased towards the contamination source, showing increments of 3.2 (L1) and 21.3 (L2) times comparing to R during low tide. Also at low tide, both W-Hg and % Hg particulate showed the same variation profile as SPM-Hg; thus, comparing to R values, W-Hg was 2.6-fold higher at L1 and 15.7-fold higher at L2, while the % Hg particulate was always higher than 70% in all the sampling stations, increasing 1.2-fold at L1 and 1.3-fold at L2. Sed-Hg increased 8 times at L1 and 680 times at L2, when compared to R, displaying a clear contamination gradient.

Table 5.2 Total mercury concentrations in dissolved fraction (Dis-Hg) (ng L^{-1}), suspended particulate matter (SPM-Hg) (ng L^{-1}), water column (W-Hg) (ng L^{-1}), and sediment (Sed-Hg) (mg Kg^{-1} dry weight) (average), as well as percentage of mercury associated to the particulate matter (% Hg particulate) measured at each Ria de Aveiro sampling stations: reference (R), moderately (L1) and highly mercury contaminated (L2) areas. Dis-Hg, SPM-Hg and W-Hg were analyzed in several aliquots from one sample, with a coefficient of variation <10%.

		Water				Sediment
Sampling station	**Tide**	**Dis-Hg** (ng L^{-1})	**SPM-Hg** (ng L^{-1})	**W-Hg** (ng L^{-1})	**% Hg particulate**	**Sed-Hg** (mg Kg^{-1}dw)
R	High	18.8	26.7	45.5	58.6	0.01± 0.0009
	Low	10.3	26.7	37.0	72.1	
L1	High	8.3	49.7	58.0	85.7	0.08±0.006
	Low	10.6	85.2	95.8	89.0	
L2	High	10.2	n.d.	n.d.	n.d.	6.8±0.16
	Low	20.8	561.0	581.8	96.4	

n.d. – not determined

5.3.2 Mercury levels in *L. aurata* brain

Total mercury in brain (T-Hg) showed significantly higher levels in fish captured in contaminated stations, both L1 and L2, when compared to reference (R) station (Fig. 5.2). Moreover, T-Hg increased from R towards the contamination source reflecting a positive relationship with mercury levels measured in the environment. Thus, a 3.6- and 5-fold T-Hg increment was observed at the moderately (L1) and highly contaminated (L2) areas in comparison with R, respectively.

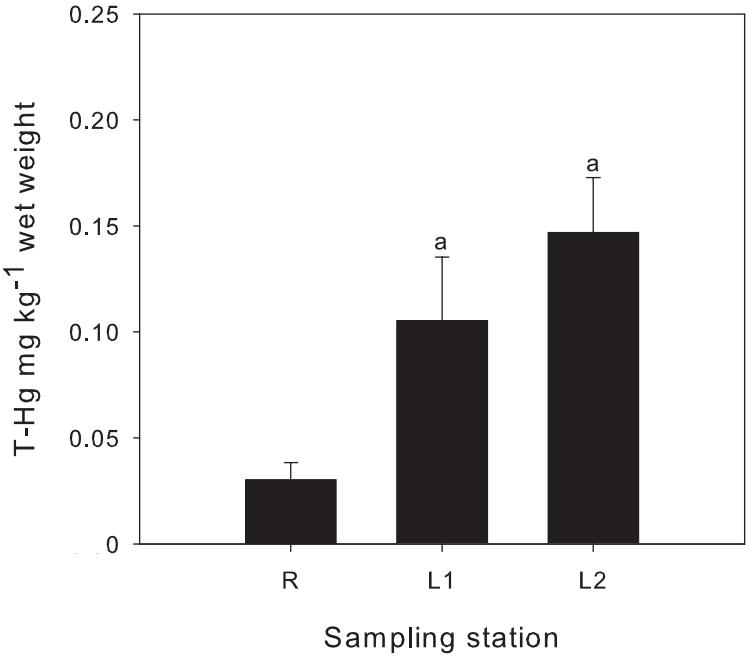

Figure 5.2 Total mercury (T-Hg) average concentration (mg Kg^{-1} wet weight) in brain of *L. aurata* captured at each Ria de Aveiro sampling station: reference (R), moderately (L1) and highly mercury contaminated (L2) areas. The letter (a) denotes statistically significant differences ($p<0.05$) *versus* R. Bars represent the standard error.

5.3.3 Oxidative stress profile

Antioxidant responses

Significant decreases in the activity of antioxidant enzymes CAT, GPx, GR, and GST were observed in the brain of fish collected at L1 and L2 when compared to R (Fig. 5.3). However, no significant changes were observed in the enzyme activities when the two contaminated stations (L1 and L2) were compared. Thus, samples of brain from fish captured at these stations displayed similar alteration extents, i.e., CAT activity decreased around 9 and 11 times, GPx activity 6.5 and 6 times, GR activity 7 and 5 times, and GST activity 8 and 7 times, respectively for L1 and L2 in comparison to R. Regarding GSHt content, it was verified that the brain from fish collected at L1 and L2 had significantly decreased levels when compared to R station. This decline in GSHt levels was almost 10 times at L1 and 11 times at L2.

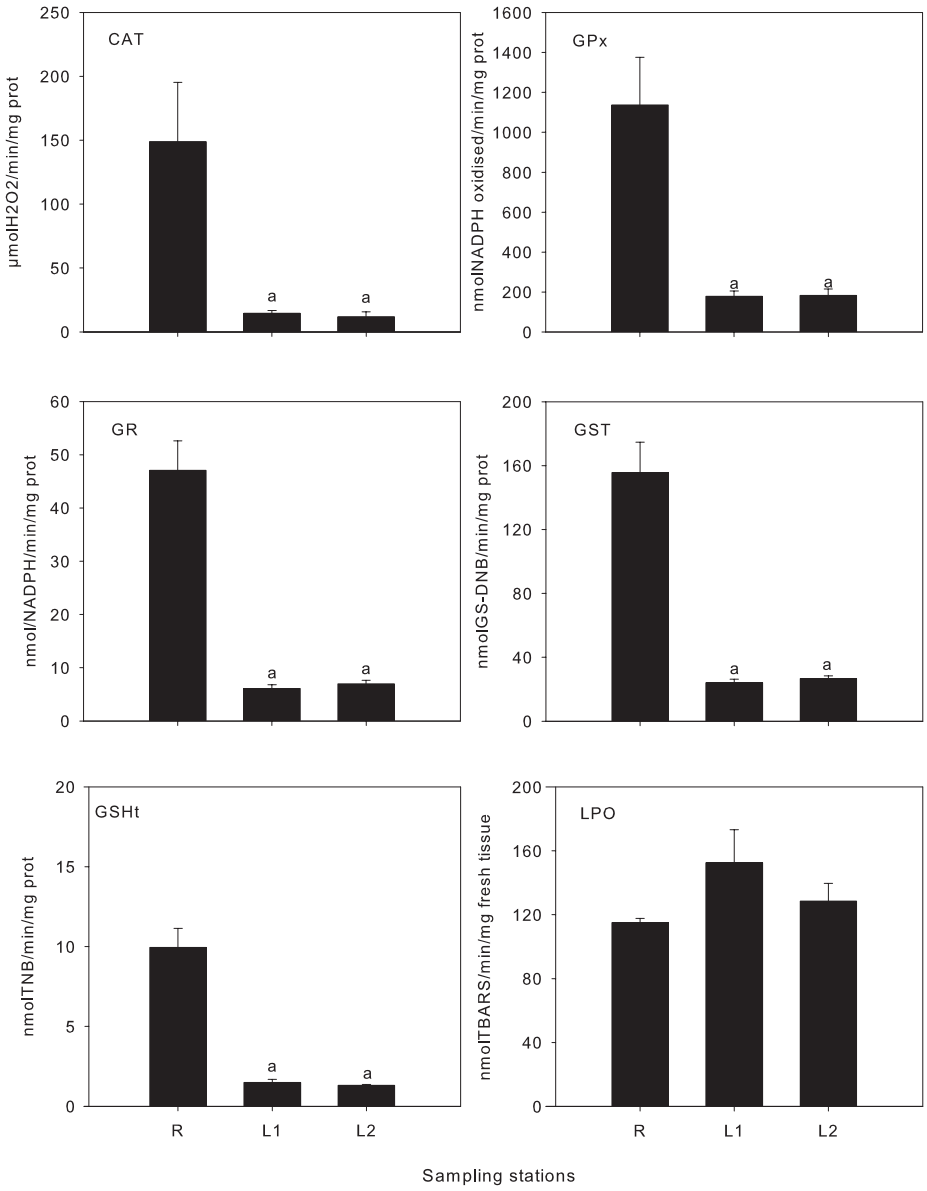

Figure 5.3 Oxidative stress responses in brain of *L. aurata* captured at each Ria de Aveiro sampling station: reference (R), moderately (L1) and highly mercury contaminated (L2) areas. The letter (a) denotes statistically significant differences ($p<0.05$) *versus* R. Bars represent the standard error.

According to the Spearman rank correlations (Fig. 5.4; only significant correlations are presented), GPx was positively correlated with GR and GST, as well as GR with GST. Moreover, GPx, GR and GST were also positively correlated with GSHt (Fig. 5.4A). The studied enzymatic antioxidants and GSHt content were all negatively correlated with T-Hg concentrations in brain (Fig. 5.4B).

Peroxidative damage (LPO)

LPO levels in *L. aurata* brain showed no significant differences among the surveyed stations (Fig. 5.3). Though statistically insignificant, the highest LPO level was measured in brain of fish from L1. No significant Spearman rank correlations were found between T-Hg and LPO, neither between LPO and the studied antioxidant defenses (Fig. 5.4).

5.4 Discussion

5.4.1 Mercury accumulation in brain and relationship with environmental parameters

The current results revealed high total mercury (T-Hg) levels accumulated in brain of fish inhabiting the contaminated areas (L1 and L2), clearly evidencing mercury exposure. Moreover, brain T-Hg levels reflected, in some extent, the levels measured in the environment. These results are in agreement with a previous study where brain was shown as the organ that best reflects the variations in environmental mercury levels (Mieiro et al., 2009). On the other hand, it is reinforced the assertion of Guzzi et al. (2008) that lipid-rich compartments such as the brain are good targets for mercury accumulation due to its lipid solubility.

Though mercury accumulation in the brain is well reported in vertebrates (Feng et al., 2004; Kenow et al., 2008), including fish (Berntssen et al., 2003), some uncertainties persist in relation to the mechanisms by which mercury can reach the brain. It has been demonstrated that mercury passes through the blood–brain barrier (BBB), and reaches either cellular or nuclear components (Boening, 2000; Zheng et al., 2003). In opposition, Rouleau et al. (1999) found that BBB is impervious to mercury in plasma, suggesting that waterborne inorganic mercury is taken up by water-exposed receptor cells of sensory nerves and subsequently transferred toward the brain by axonal transport. Although accumulation of inorganic mercury in brain has been reported (Rouleau et al., 1999), its uptake rate is known as 200 times lower than for MeHg (Oliveira Ribeiro et al., 2000). On the other hand, it was suggested that some of the MeHg in brain (3 to 6%) is converted into inorganic mercury (Hg^{2+}) (Ercal et al., 2001). Hence, though mercury speciation (organic *versus* inorganic) was not carried out in the present study due to limitations on the amount of tissue available, in light of the previous statements it can be hypothesized that mercury found in the *L. aurata* brain is predominantly in the organic form.

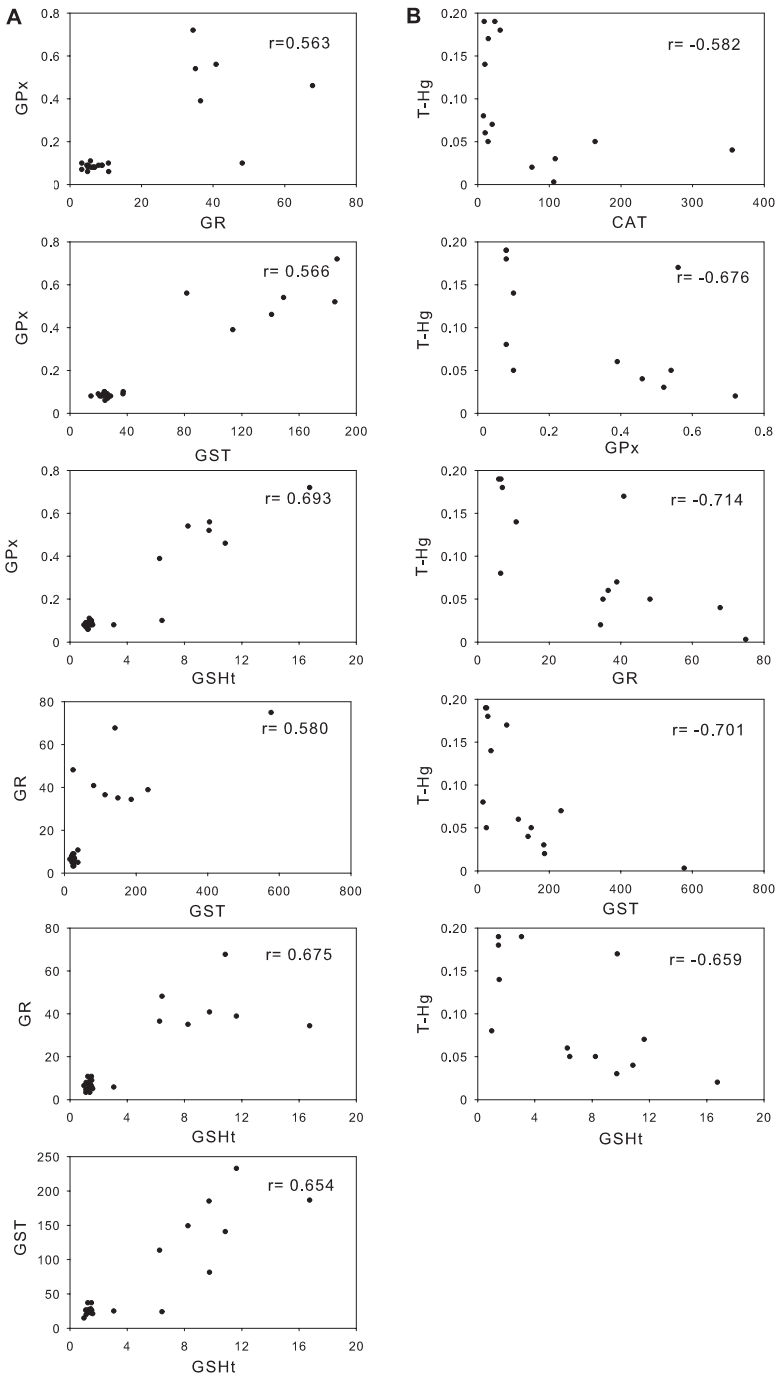

Figure 5.4 Significant Spearman rank correlations between the studied antioxidant responses (**A**) and correlations between the total mercury in brain (T-Hg) and the studied antioxidant responses (**B**). (CAT - catalase, GPx - glutathione peroxidase, GR - glutathione reductase, GST - glutathione S-transferase and GSHt - total glutathione content). r- Spearman rank, $p<0.05$.

The dominant uptake route and the biotope's physico-chemical characteristics are important factors to determine the mercury bioavailability to fish and accumulation pattern. Keeping in mind that dietary uptake (of great importance in the context mercury accumulation) was not assessed in the present study, it was though evident a consistency between T-Hg in the brain and the levels measured in the water column (W-Hg and SPM-Hg) in the moderately contaminated station (L1). Under higher contamination load (L2), the association between environmental and accumulated levels was less obvious, suggesting a tendency to reach the brain limit to accumulate mercury and/or a higher preponderance of a buffering action exerted by other organs/tissues (e.g. muscle, liver and kidney), protecting brain, as pointed out by Mieiro et al. (2009) especially for organic mercury.

The assessed water physico-chemical characteristics did not substantially differ between the surveyed stations, with the exception of salinity and SPM. Differences on these parameters could affect mercury bioavailability. It is known that mercury is able to form strong inorganic complexes with chloride in saline and oxygen rich waters (Conaway et al., 2003). Monserrat et al. (2007) related the increase of bioavailability and uptake rate of metals with the decrease in salinity. Nonetheless, the influence of salinity on T-Hg in the fish brain could not be confirmed by the present data, since mercury levels in water are low and thus not relevantly affected by salinity. Differently, the currently observed inter-station variations in SPM probably played an important role on mercury bioavailability, since the metal has high affinity for SPM. Particulate mercury content tends to be higher in conjunction with elevated SPM, especially in shallow regions of estuaries where resuspension is easily enhanced (Domagalski et al., 2001). Mercury speciation in environmental matrices would be also useful for interpreting the present accumulation dynamics.

5.4.2 Oxidative stress profile

Alterations on redox-defense system

Despite the limited information on biochemical mechanisms of mercury toxicity, there is some evidence pointing out the association with ROS generation (Berntssen et al., 2003; Huang et al., 2008). In view of this assumption, it is commonly hypothesized the occurrence of an adaptive response expressed by an activation of the redox-defense system (Franco et al., 2006; Stringari et al., 2008). However, in the present work a general breakdown of this system was observed, as GSHt content and the studied antioxidant

enzymes (CAT, GPx, GR and GST) decreased in brain of fish collected at the mercury contaminated area (Laranjo basin; L1 and L2). A glutathione decrease has been previously found in mouse CNS after mercury exposure (Franco et al., 2006); nevertheless, the explanation for this alteration is not completely established. It is known that mercury forms covalent bonds with GSH and a single mercury ion can bind to and cause irreversible excretion of two GSH molecules (Franco et al., 2009). The releasing of GSH-mercury conjugates results in greater activity of the free mercury ions disturbing GSH metabolism and damaging cells (Franco et al., 2009). Hence, given that the lower GSHt levels currently observed in *L. aurata* exposed to mercury cannot be justified by its use on the GSH-dependent enzymes activity (both GST and GPx were inhibited), the explanation for that decrease is probably the release of GSH-mercury conjugates (mercury elimination) and a concomitant obstruction of GSH reposition by *de novo* synthesis. Considering the generalized inhibition observed for the assessed enzymes (CAT, GPx, GST and GR), the inhibition of the enzymes involved on GSH synthesis pathway (gamma-glutamylcysteine synthetase and GSH synthetase) can also be suggested. This hypothesis is supported by Zalups and Lash (1996) who described a mercury-induced inhibition of GSH synthetase. Astrocytes play a major role in the brain GSH metabolism, determining the neuronal GSH content through the exportation of GSH or its precursors (e.g. glutamine) to neurons (Minich et al., 2006). Thus, the astroglial glutathione system could have been particularly targeted by mercury in *L. aurata* brain, compromising its function.

Beyond the extensively recognized limitation of defense against oxidative stress, GSH depletion has also been proposed as one of the early biochemical events associated with neuronal apoptosis (Merad-Boudia et al., 1998). Accordingly, Gatti et al. (2004) demonstrated that GSH depletion is the main step in the redox regulation of MeHg-induced apoptosis.

In agreement with the present results, depletion on the antioxidant enzymatic defenses (SOD and GPx) was previously found in brain of Atlantic salmon after dietary MeHg exposures (Berntssen et al., 2003). Furthermore, this pattern of response showed to be organ-specific since both SOD and GPx activities increased in the liver and kidney. This organ specificity, pointing to higher vulnerability of brain towards enzymatic inhibition, was also evident in *L. aurata* as no signs of CAT or GST activity decline were detected in liver of specimens captured in the same area and season by Guilherme et al. (2008).

The general decrease of enzymatic antioxidants represents an increased *L. aurata*

brain's vulnerability to any sort of oxidative stress-related challenges. Furthermore, considering that dietary MeHg demonstrated a potential to inhibit simultaneously antioxidant enzymes (SOD and GPx) and key enzymes for neurological functions (e.g. monoamine oxidase) in brain of fish (Berntssen et al., 2003), a disturbance of the monoaminergic system may also be hypothesized as affecting *L. aurata* brain.

The positive correlation found in the current study between the GSHt content and GSH-related enzymes (GPx, GST and GR) was expectable taking into account their well-known interdependence. Thus, besides a direct interference of the metal or the effect of ROS on the enzyme structure (e.g. protein oxidation), it cannot be overlooked that the observed GPx and GST activities depletion can in part be explained by the low GSH availability.

Peroxidative damage and its association with antioxidant defenses

Lipid peroxidative stress has been suggested as an additional mechanism by which mercury exerts initial neurotoxic effects in mammals (Yee and Choi, 1994) and fish (Berntssen et al., 2003). Some authors acknowledged that pro-oxidant properties of metals, including mercury, are intensified by their inhibitory effects on antioxidant processes (Stohs and Bagchi, 1995; Doyotte et al., 1997), thereby potentiating the risk of LPO induction. Keeping in view the general breakdown of the redox-defense system currently observed in the brain of *L. aurata* inhabiting Laranjo area (L1 and L2), the inexistence of a subsequent LPO increase is intriguing. Brain LPO measurements revealed that fish were able to cope with the mercury peroxidative potential, despite the depletion on the measured antioxidants. One explanation for this resistance may be related to the action of other non-enzymatic antioxidants, such as cystein, alpha tocopherol and ascorbic acid, whose protective role against metals has been already demonstrated in the brain of rats (Patra et al., 2001). An alternative explanation concerns the selective inhibition of specific enzymatic antioxidants while others can keep their functionality. This idea is supported by Keyvanshokooh et al. (2009) who investigated changes in the brain tissue's proteome of fish (*Huso huso*) in response to MeHg, reporting on one hand, an over-expression of α/β hydrolase superfamily (e.g. epoxide hydrolase), and, on the other, an inhibited expression of AKR superfamily that includes antioxidative enzymes involved in the protection against aldehydes.

The binding of toxic metals, including mercury (Navarro et al., 2009), to metallothioneins (MT) represents a sequestration function that renders them unable to

interact with cellular key molecules, and thereby is known to play an important protective role. Recent determinations of MT levels in the brain of *L. aurata* captured in the stations surveyed in the present study showed no increase on the levels of these metalloproteins (Mieiro, C.L., unpublished data). Therefore, an increased protection provided by brain MT cannot be invoked in the current context.

Overall, the present results are in conformity with Ahmad et al. (2006) who stated that LPO increase cannot be predicted only on the basis of antioxidants depletion. Though the peroxidation of lipids seems to have been avoided in brain cells, the occurrence of mercury-induced oxidative damage in other crucial macromolecules like DNA and protein cannot be excluded. In fact, the observed enzymatic inhibitions can be regarded as a higher vulnerability of brain proteins to mercury toxicity comparing to lipids, which may be explained by the mercury avidity to SH groups.

Oxidative stress responses and association with mercury accumulation

The analysis of the response profiles in parallel with the mercury burden may provide interesting information on the organ-specific threshold limits to express signs of toxicity. In this perspective, the present data revealed an association between mercury load in the fish brain and the vulnerability towards the breakdown of the redox-defense system, since T-Hg concentrations were negatively correlated with all the antioxidants assessed. The results indicated that the T-Hg levels measured in the *L. aurata* brain, including in the moderately contaminated station (L1), exceeded the threshold with respect to antioxidative defense impairment, setting the safe limit to keep redox-defense system active bellow 0.11 mg Kg^{-1} wet weight. Moreover, it is likely that the concentration range able to induce antioxidants activation was also surpassed. In line with that, Elia et al. (2003) also found that moderate doses of mercury were able to increase GST and GPx activities in *Ictalurus melas*, whereas high concentrations inhibited those enzymes. Similarly, Wolf and Baynes (2007) have described that in mammals high concentrations of mercury (>3–5 μM) induced GSH depletion and inhibition of thiol enzymes activity, whereas low concentrations (1–2 μM) increased those parameters.

Differently, lipids integrity (measured as TBARS levels) was not affected in fish exposed to mercury, indicating that the threshold limit for accumulated T-Hg after which LPO increases in the brain is beyond 0.15 mg Kg^{-1} wet weight. To some extent, this finding may be considered to be in accordance with Wiener and Spry (1996), who

reported that toxic effects in the brain arise for mercury concentrations around 7 mg Kg^{-1} in adult freshwater fish and around 3 mg Kg^{-1} in particularly sensitive species.

5.5 Conclusions

The brain of *L. aurata* revealed a mercury-induced depletion of the assessed antioxidant defenses, enzymatic (CAT, GPx, GST and GR) and non-enzymatic (GSHt), which was significantly correlated with accumulated T-Hg concentrations. This inability to set up adequate antioxidant defenses represents an increased susceptibility of brain towards oxidative damage. In addition, current data confirms that the antioxidant enzymes, usually responsive to pro-oxidant environments, may also display suppressed activities due to oxidative damage and a loss in compensatory mechanisms.

No LPO increase was associated to mercury exposure, indicating that fish were able to cope with the mercury potential to oxidatively degrade lipids, despite the detected breakdown of redox-defense system. Moreover, the results suggested that mercury interacts with brain proteins more critically than with lipids, highlighting that the definition of critical-tissue-concentrations depends of the biochemical endpoint addressed (e.g. enzymes inhibition *versus* lipid peroxidation).

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CHAPTER 6

Brain as a critical target of mercury in fish (*Dicentrarchus labrax*) environmentally exposed – bioaccumulation and oxidative stress profiles

Brain as a critical target of mercury in fish (*Dicentrarchus labrax*) environmentally exposed – bioaccumulation and oxidative stress profiles

Abstract

Although mercury is recognized as a potent neurotoxicant, information regarding its threat to fish brain and underlying mechanisms is still scarce. In accordance, the objective of this work was to assess fish vulnerability to mercury neurotoxicity by evaluating brain pro-oxidant status in wild European sea bass (*Dicentrarchus labrax*) captured in an estuarine area affected by chlor-alkali industry discharges (Laranjo Basin, Ria de Aveiro, Portugal). To achieve this goal, brain antioxidant responses such as catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST) and total glutathione (GSHt) were measured. Additionally, damage was determined as lipid peroxidation. To ascertain the influence of seasonal variables on both mercury accumulation and oxidative stress profiles, surveys were conducted in contrasting conditions - warm and cold periods. In the warm period, brain of fish from mercury contaminated sites exhibited ambivalent antioxidant responses, *viz*. GR activity increase as an adaptive mechanism and CAT inhibition as a sign of toxicity. Though a pro-oxidant status was evident, brain showed to possess compensatory mechanisms able to avoid lipid peroxidative damage. The warm period revealed to be the most critical as no inter-site alterations on oxidative stress endpoints were detected in the cold period. Environmental factors played a crucial role in regulating the antioxidant capacity of brain rather than on mercury accumulation. This work contributes to improve the knowledge on mercury neurotoxicity in feral fish, highlighting that the definition of critical tissue concentrations depends on environmental variables.

Keywords: Brain, *D. labrax*, mercury, oxidative stress, environmental exposure, temporal variability

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6.1 Introduction

Mercury is a widespread natural and anthropogenic contaminant with no biological function. Since methylation processes can occur in aquatic environments, all sources of mercury can be potentially harmful to humans and aquatic life (Clarkson et al., 2003; Aschner et al., 2007). Methylmercury (MeHg), in particular, is able to conduct damage in a number of organs, especially in the central nervous system (Holmes et al., 2009).

Notwithstanding that mercury neurotoxicity have been well reported in both humans and mammalian models (Stohs and Bagchi, 1995; Clarkson e al., 2003; Aschner et al., 2007; Stringari et al., 2008), the knowledge regarding mercury threat to fish brain is still limited to a small number of published works. The available fish studies reported neurodegenerative damage, including disturbances on sensory capacities (Baatrup et al., 1990; Oliveira Ribeiro et al., 1995), necrotic lesions in brain and behaviour changes (Berntessen et al., 2003). In addition, Mieiro et al. (2009) found that fish brain can have an important role in biomagnification processes, highlighting its importance in environmental risk assessment.

Even though the mechanisms underlying mercury toxicity are still unclear, its ability to react with and deplete free sulphydryl groups as well as to disrupt cell cycle progression and/or induce apoptosis in several tissues is well recognized (Stohs and Bagchi, 1995; Sutton and Tchounwou, 2006). Moreover, mercury-induced neurotoxicity is known to be mediated by reactive oxygen species (ROS) in both *in vivo* and *in vitro* mammalian models (Shanker and Aschener, 2003). Studies with cell lines and mammals addressed the possible mechanisms by which mercury induces ROS formation in brain (Kaur et al., 2009; Roos et al., 2009). In accordance, disruption of the glutathione system, leading to the depletion of total glutathione (GSHt), glutathione reductase (GR) and glutathione peroxidase (GPx), is frequently suggested to be an expression of mercury neurotoxicity (Stringari et al., 2008; Roos et al., 2009). Furthermore, mercury-induced damage has also been pointed as conducting to an increase in lipid peroxidation (LPO) (Berntssen et al., 2003; Stringari et al., 2008). Accordingly, brain seems to be particularly sensitive to oxidative stress and, comparing with other organs, it denotes some weakness regarding the generation and the detoxification of ROS (Dringen, 2000).

In this context, further information on the antioxidant system in fish brain is required in order to assess the susceptibility towards mercury-induced neurotoxicity and the subsequent threshold limits. To accomplish this purpose, in the present study a set of pro-oxidant status indicators was assessed in brain of European sea bass (*Dicentrarchus*

labrax) - a key species for ecotoxicological evaluations – environmentally exposed to mercury. The selected parameters were GSHt content as non-enzymatic antioxidant, catalase (CAT), GPx, GR and gluthatione S-transferase (GST) as antioxidant enzymes, and LPO as a measure of oxidative damage. It was also intended to establish an association between brain mercury accumulation and the previous oxidative stress responses, addressing seasonal variations. This study was carried out in an estuary (Ria de Aveiro, Portugal) historically impacted by a chlor-alkali plant effluent.

6.2 Materials and Methods

6.2.1 Study area and sampling procedures

The study was carried out at Ria de Aveiro, a coastal lagoon in the Portuguese northwest coast (Fig. 6.1). This aquatic system has an inner area (Laranjo basin), which, during decades, has been subjected to effluent discharges from a chlor–alkali industry. These discharges resulted in the accumulation of about 25 tons of mercury in the Laranjo basin and its upstream channel. Although effluent releases stopped in 1994, high mercury concentrations are still found in the fine surface sediments of this area, creating a contamination gradient (Coelho et al., 2005).

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 distant from L1. An area close to the lagoon entrance (S. Jacinto) and far from the main polluting sources was chosen as the reference site (R).

In order to assess the influence of contrasting environmental conditions, sampling was carried out in warm (July 2007) and cold (December 2007) periods. Water physico-chemical parameters such as water temperature, dissolved oxygen (DO), pH, salinity and suspended particulate matter (SPM) were measured at sub-surface level, as well as water-column depth and turbidity, during high and low tide. For mercury measurements, sub-surface water samples (high and low tide) and five replicates from the surface sediment layer (approximately 2 cm depth) were collected at each sampled site.

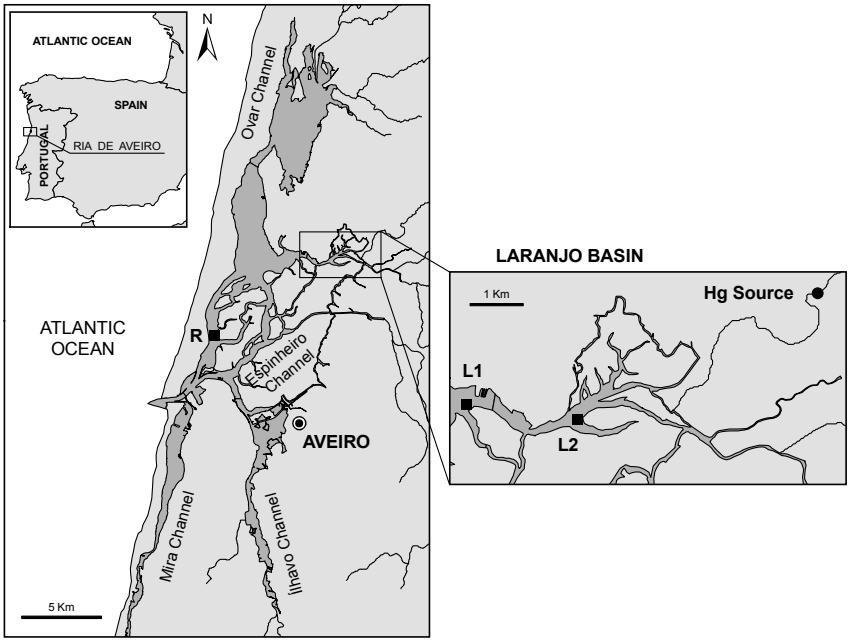

Figure 6.1 Map of the sampling sites (■) in the Ria de Aveiro (Portugal): reference (R - 40º41'00'' N, 8º42'44'' W), moderately (L1 - 40º43'34.46'' N, 8º38'53.16'' W) and highly contaminated (L2 - 40º43'28.98'' N, 8º37'35.80'' W) areas.

Twenty juvenile specimens of *D. labrax* – European sea bass – were caught at each sampling site using a fishing rod. Fish had an average total length (TL) of 15.4 ± 3.5 cm in the warm period, and 14.6 ± 1.4 cm TL in the cold period. Immediately after being caught, fish were sacrificed by cervical transection and the entire brain was excised, and instantly frozen in liquid nitrogen. Two sets of samples were obtained: one for oxidative stress assessment (n=10) and another for T-Hg determination (n=10). In the laboratory, samples were preserved until further processing at -80ºC and -20°C for oxidative stress and mercury determinations, respectively. The use of juvenile specimens minimizes the interference of variables such as gender and reproductive processes, as well as the potential occurrence of a growth dilution effect in relation to mercury accumulation, as defined by Meili (1997).

In the cold period, it was not possible to catch fish at L2 and thus, the corresponding abiotic parameters were not determined.

6.2.2 Total mercury determinations

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 4ºC until analysis. Filters were re-weighed after heating overnight at 60ºC and stored at 4ºC for suspended particulate matter (SPM) determinations.

Total dissolved mercury (Dis-Hg) and mercury in SPM (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 (see Mucci et al., 1995). For determination of SPM-Hg, filters were digested with HNO_3 4 mol L^{-1} and the previous equipment was used (Pereira et al., 1998). Water column mercury (W-Hg) was estimated by the sum of Dis-Hg and SPM-Hg. The percentage of mercury associated to the particulate matter (% Hg particulate) was also calculated. The accuracy of the methods for mercury was tested by fortification of samples (at two concentration levels within the range found in samples), with recovery efficiencies always between 90-100%.

Mercury in sediment and in brain

At the laboratory, sediment samples were freeze-dried, well mixed, sieved through a 1 mm sieve and stored for total mercury determination (Sed-Hg). For total mercury (T-Hg) analysis in *D. labrax* brain, each organ was freeze-dried, homogenised and weighted for fresh weight determination.

Sediment and brain 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>5), with recovery efficiency between 83-102%.

6.2.3 Biochemical analyses

Each brain was homogenized in a 1:15 ratio (1 g tissue:15 mL buffer), using a Potter-Elvehjem homogenizer, in chilled phosphate buffer (0.1 M, pH 7.4). This homogenate was then divided in three aliquots, for LPO and total glutathione (GSHt) quantification, as well as for post-mitochondrial supernatant (PMS) preparation. The PMS fraction was obtained by centrifugation in a refrigerated centrifuge (Eppendorf 5415R) at 13,400 g for 20 min at 4ºC. Aliquots of PMS were stored in microtubes at -80ºC until enzymatic antioxidants analyses.

CAT activity was assayed (at 25ºC) by the method of Claiborne (1985) as described by Giri *et al*. (1996). Briefly, the assay mixture consisted of 1.99 mL phosphate buffer (0.05 M, pH 7.0), 1 mL hydrogen peroxide (0.030 M) and 0.01 mL of sample in a final volume of 3 mL. Change in absorbance was recorded spectrophotometrically (Jasco UV/VIS, V-530) at 240 nm and CAT activity was calculated in terms of μmol H_2O_2 consumed/min/mg protein ($\varepsilon = 43.5\ M^{-1}\ cm^{-1}$).

GPx activity was assayed (at 25ºC) according to the method described by Mohandas et al. (1984) as modified by Athar and Iqbal (1998). The assay mixture consisted of 0.09 mL phosphate buffer (100 mM, pH 7.0), 0.03 mL EDTA (10 mM), 0.03 mL sodium azide (1 mM), 0.03 mL glutathione reductase (GR; 2.4 U/mL), 0.03 mL reduced glutathione (GSH; 10 mM), 0.03 mL NADPH (1.5 mM), 0.03 mL H_2O_2 (1.5 mM) and 0.03 mL of PMS in a total volume of 0.3 mL. GPx activity was determined monitoring the oxidation of NADPH to $NADP^+$, resulting in an absorbance decrease at 340 nm. The absorbance was read every 30 seconds for a period of 3 minutes using a SpectraMax 190 microplate reader. The enzyme activity was calculated as nmol $NADP^+$/min/mg of protein ($\varepsilon = 6.22\times10^3\ M^{-1}\ cm^{-1}$).

GR activity was measured (at 25ºC) according to the method of Carlberg and Mannervik (1975). Briefly, the reaction medium consisted in 0.1 M phosphate buffer (pH 7.0), DTPA 0.5 mM, NADPH 0.2 mM and GSSG 1 mM. In a quartz cuvette, 0.025 mL of PMS was added to 0.975 mL of reaction medium. Enzyme activity was spectrophotometrically (Jasco UV/VIS, V-530) quantified by measuring NADPH disappearance at 340 nm and expressed as nmol of $NADP^+$/minute/protein ($\varepsilon = 6.22\times10^3\ M^{-1}\ cm^{-1}$).

GST activity was determined using CDNB (1-chloro-2,4-dinitrobenzene) as substrate, according to the method of Habig *et al*. (1974). The assay was carried out (at 25 ºC) in a quartz cuvette with a 2 mL mixture of 0.2 M phosphate buffer (pH 7.4), 0.2 mM CDNB and 0.2 mM GSH. The reaction was initiated by the addition of 0.01 mL PMS, and

the increase in absorbance was recorded spectrophotometrically (Jasco UV/VIS, V-530) at 340 nm during 3 min. The enzyme activity was calculated as nmol GS-DNB conjugate min/mg protein (ε = 9.6 mM^{-1} cm^{-1}).

For GSHt measurement, protein content in the tissue homogenate was precipitated with trichloro acetic acid (TCA 12%) for 1 hour and then centrifuged at 12,000 g for 5 min. at 4ºC. GSHt was determined (in deproteinated PMS, at 25ºC) adopting the enzymatic recycling method using GR excess, whereby the sulfhydryl group of GSH reacts with DTNB (5,5'-dithiobis-2-nitrobenzoic acid) producing a yellow coloured 5-thio-2-nitrobenzoic acid (TNB) (Tietze, 1969; Baker *et al.*, 1990). Formation of TNB was measured by spectrophotometry (Jasco UV/VIS, V-530) at 412 nm. The results were expressed as nmol TNB formed/min/mg protein (ε = 14.1×10^3 M^{-1} cm^{-1}).

The determination of LPO was performed in the tissue homogenate, according to the procedure of Ohkawa (1979) and Bird and Draper (1984), as adapted by Filho et al. (2001). Briefly, to 0.15 mL of homogenate, 0.01 mL of 1-1 butylated hydroxytoluene (4% in methanol) was added and mixed well. To this aliquot, 0.5 mL of 12% TCA in aqueous solution, 0.45 mL Tris–HCl (60 mM, pH 7.4; and 0.1 mM DTPA) and 0.5 mL 0.73% TBA were added and mixed well. The mixture was heated for 1 hour in a water bath set at boiling temperature and then cooled to room temperature, decanted into 2 mL microtubes and centrifuged at 13,400 g for 5 min. Absorbance was measured at 535 nm, using a SpectraMax 190 microplate reader and LPO was expressed as nmol of thiobarbituric acid reactive substances (TBARS) formed per milligram of fresh tissue (ε = 1.56×10^5 M^{-1} cm^{-1}).

Total protein contents were determined according to the Biuret method (Gornall et al., 1949), using bovine serum albumin (E. Merck-Darmstadt, Germany) as a standard. Absorbance was measured at 550nm using a SpectraMax 190 microplate reader.

6.2.4 Statistical analysis

Data were tested for goodness of fit to normal distribution and requirements of homogeneity of variances were also determined. One-way Analysis of Variance (ANOVA) followed by all pairwise multiple comparison procedures (Tukey test) were performed to test differences in brain T-Hg concentrations and biochemical responses among sampling sites within the same period. 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). A T-test was

also performed in order to assess differences between periods (warm vs. cold) for the same site. A significance level of 0.05 was considered in all test procedures.

6.3 Results

6.3.1 Environmental characterization

Water physico-chemical parameters

Table 6.1 shows the general physico-chemical characteristics of the water column in the different sampling sites in warm and cold periods. During the warm period, water parameters showed no relevant dissimilarities among sites, with the exception of DO, salinity and SPM. While DO and salinity decreased towards the mercury source, SPM showed increments from R to the contaminated areas during low tide and the opposite during high tide. In addition, depth, salinity and SPM revealed tidal variations. During the cold period, DO, salinity and SPM revealed to be higher in the reference area and to diverge between tides. Seasonal differences were evidenced by the higher water temperature in warm period, while salinity and DO increased during cold period. SPM concentrations were generally higher during the warm period.

Table 6.1 Environmental characteristics corresponding to high and low tide, measured in warm and cold periods on reference (R), moderately (L1) and highly mercury contaminated (L2) areas at Ria de Aveiro: water temperature (T), dissolved oxygen (DO), water-column depth, turbidity, pH, salinity, and suspended particulate matter (SPM)

		High tide - Low tide						
Period	**Site**	**T** (ºC)	**DO** (mg L^{-1})	**Depth** (m)	**Turbidity** (m)	**pH**	**Salinity**	**SPM** (mg L^{-1})
Warm	**R**	20.7 -22.1	8.0 - 8.1	2.7 - 1.9	1.2 - 0.8	8.1 -8.2	30.0-30.0	211.0 - 40.3
	L1	22.1 -22.0	4.7 - 7.1	3.8 - 2.5	0.7 - 0.3	8.0 -7.8	20.0-10.0	35.5 - 82.1
	L2	22.1 -22.0	5.7 - 5.0	2.3 - 1.0	1.0 - 0.3	7.9 -7.6	18.0 - 5.0	n.d. - 78.7
Cold	**R**	15.7 -15.3	10.9 -10.8	2.7 - 1.9	1.2 - 0.5	8.4 -8.4	34.0-34.0	53.2 - 36.7
	L1	11.5 -13.2	5.8 - 5.1	3.9 - 2.1	0.7 - 0.8	8.0 -7.9	31.0-25.0	30.5 - 23.6
	L2	n.d. - n.d.	n.d. - n.d.	n.d.-n.d.	n.d. - n.d.	n.d.-n.d.	n.d - n.d	n.d. - n.d.

n.d. - not determined

Mercury in environmental matrices

In the warm period, Dis-Hg was comparable to values in non- contaminated systems (Coelho et al., 2005), being higher at L1 during low tide (Table 6.2). SPM-Hg, W-Hg and the % of particulate Hg increased towards the mercury source during low tide. In this period, more than 90% of the mercury in the water column was in the particulate form. During the cold period, Dis-Hg was also low for all areas, and SPM-Hg, W-Hg and the % of particulate Hg were higher at L1, mainly during low tide. For both periods, Dis-Hg values showed to be equally low, being higher during the cold period. In opposition, SPM-Hg, W-Hg and the % of particulate Hg were higher during the warm period.

Table 6.2 Concentrations of total mercury in water and sediment (Sed-Hg) matrices measured in warm and cold periods on reference (R), moderately contaminated (L1) and highly contaminated (L2) areas at Ria de Aveiro. Water values determined in high and low tide represent dissolved mercury (Dis-Hg), mercury in suspended particulate matter (SPM-Hg), mercury in water column (W-Hg), and percentage of mercury associated to the particulate matter (% Hg particulate).

		Water (High tide - Low tide)				Sediment
Period	**Site**	**Dis-Hg** (ng L^{-1})	**SPM-Hg** (ng L^{-1})	**W-Hg** (ng L^{-1})	**% Hg particulate**	Sed-Hg (mg Kg^{-1}dw)
Warm	**R**	2.2 – 2.2	54.5 - 22.2	56.5 – 24.4	96.4 - 90.9	0.01±0.0009
	L1	3.5 – 6.8	44.0 – 293.8	47.6 – 300.6	92.6 – 97.7	0.08 ±0.006
	L2	n.d.- 1.4	n.d. – 472.7	n.d. – 474.1	n.d. – 97.6	6.8 ±0.16
Cold	**R**	9.1 - 8.6	4.6 - 4.1	13.7 - 12.7	33.5 - 32.5	0.007 ±0.001
	L1	11.4 - 11.9	6.6 - 15.8	18.0 - 27.7	36.7 - 56.9	0.05 ±0.003
	L2	n.d. - n.d.	n.d. - n.d.	n.d. - n.d.	n.d. - n.d.	n. d.

Dis-Hg and SPM-Hg were analyzed in three aliquots from each sample, with a coefficient of variation <10%.
n.d. - not determined

Sed-Hg in the warm period increased 8 times from the reference site (R) to L1 and 85 times from L1 to L2, highlighting the existence of the already known contamination gradient. Moreover L2 showed an increment of 680 times in relation to R. During the cold period, and in accordance with the warm period data, Sed-Hg increased 7 times from R to L1. No relevant differences in the Sed-Hg concentrations occurred between the two periods.

6.3.2 Mercury levels in *D. labrax* brain

In the warm period, brain T-Hg exhibited a significant increase from R to L2, which corresponded to a 11-fold increment (Fig. 6.2). The difference between contaminated sites

was also significant, being L2 3-fold higher than L1. Taking into account the T-Hg accumulation during the cold period, L1 revealed a significant increase (11-fold) in relation to R. In both periods, T-Hg increments from reference to contaminated areas reflected the increments in the environmental mercury levels. No significant differences were found between sampling periods.

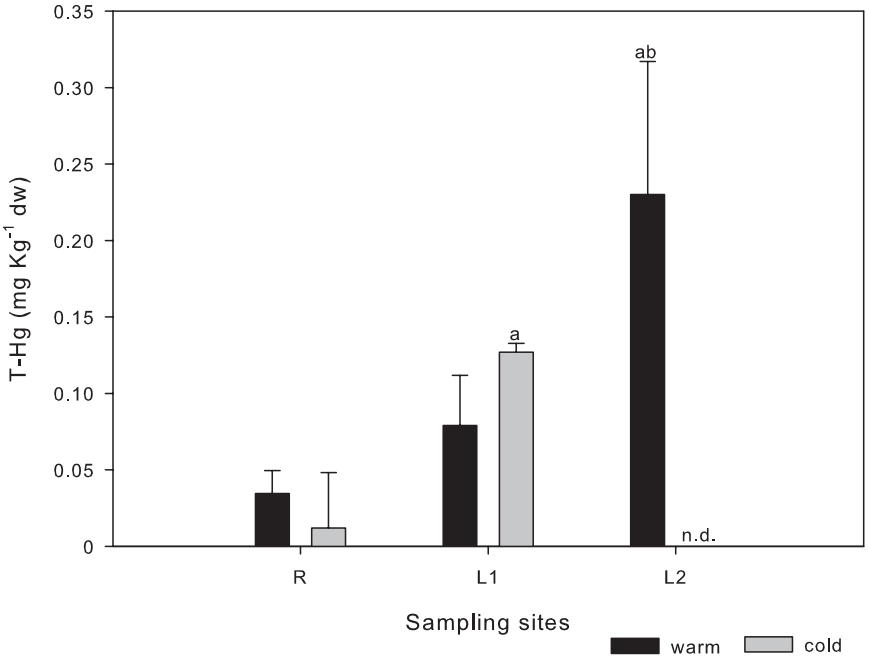

Figure 6.2 Total mercury (T-Hg) mean concentration (mg Kg^{-1} wet weight) in the brain of *D. labrax* captured during warm and cold periods at each Ria de Aveiro sampling site: reference (R), moderately (L1) and highly mercury contaminated (L2) areas. The letters denote statistically significant differences ($p<0.05$): (a) *versus* R and (b) *versus* L1. Bars represent the standard error.

6.3.3 Oxidative stress profile

During the warm period, significant differences in the activity of the antioxidant enzymes were observed for CAT and GR (Fig. 6.3). Thus, CAT activity decreased at L1 (2-fold) and L2 (1.5-fold) when compared to R, along with a significant increase from L1 to L2 (2-fold). Contrastingly, GR activity increased from R to L1 (3-fold) and L2 (4-fold). In addition, a significant difference was also found between L1 and L2 (1.4-fold). During the cold period, no significant inter-site differences were found for any of the antioxidant responses.

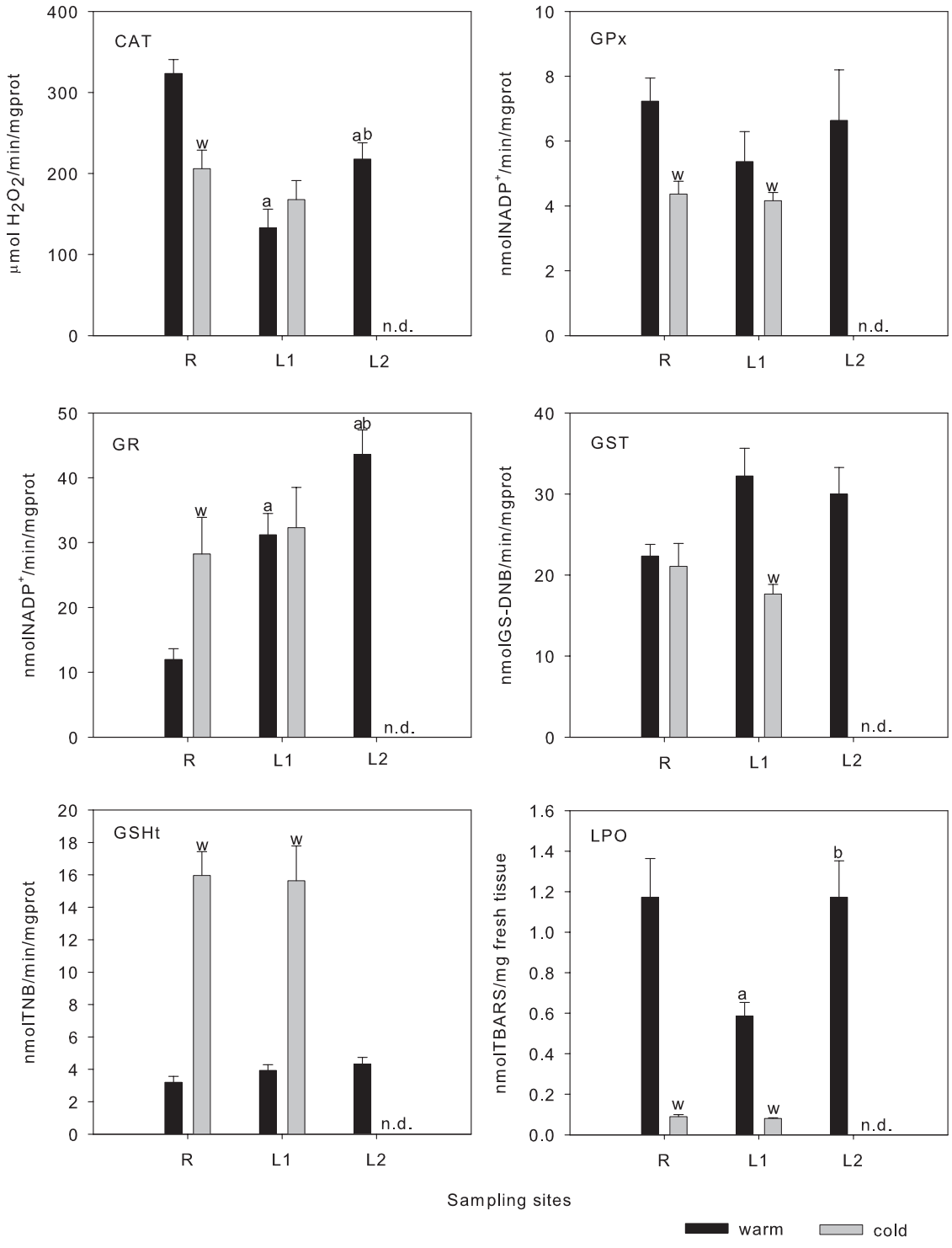

Figure 6.3 Oxidative stress responses in the brain of *D. labrax* captured during warm and cold periods at each Ria de Aveiro sampling site: reference (R), moderately (L1) and highly mercury contaminated (L2) areas. The letters denote statistically significant differences ($p<0.05$): (a) *versus* R, (b) versus L1 within the same period; (w) *versus* warm period for the same site. Bars represent the standard error.

All the antioxidant responses exhibited differences between warm and cold periods. Overall, CAT (R), GPx (R and L1) and GST (L1) exhibited higher activities in the warm period, whereas GR activity (R) and GSHt content (R and L1) were higher in the cold period.

Peroxidative damage in the warm period revealed significant differences between L1 and both R and L2. Thus, fish at L1 showed to have a 2–fold reduction relatively to R and L2 (Fig. 6.3). In opposition, no alterations in LPO were verified during cold period. Differences between sampling periods are reflected in the lower LPO levels observed in the cold period for both R and L1 sites.

6.4 Discussion

Measurements of bioaccumulation and response biomarkers in fish from contaminated sites have good potential to integrate environmental monitoring programs (van der Oost et al., 2003). Moreover, attention should be addressed to variables not related to contamination, such as abiotic and biotic factors, since they can vary throughout the year on the basis of seasonal cycles (Bodin et al., 2004; Pain et al., 2007). Accordingly, the present study surveyed contrasting environmental conditions keeping in mind that toxicity mechanisms are better understood integrating information on external levels of exposure, organ burden and biological responses.

6.4.1 Mercury accumulation in brain and its relationship with environmental parameters

Differences in water physico-chemical characteristics are able to influence mercury bioavailability and thus, its incorporation in fish (Driscoll et al., 1995). However, with the exception of DO, salinity and SPM, the studied parameters did not substantially differ between the surveyed sites.

Despite DO and salinity are known to affect mercury availability, Dis-Hg found in water column was in general low for all sampling sites, and thus not notably affected by those parameters. In the water column, mercury is preferentially associated with SPM (Coquery et al., 1997). Accordingly, differences in SPM concentration are extremely significant in determining mercury availability when comparing areas where sediments are mercury enriched with non-polluted areas.

Brain is of high concern due to the importance of its neurological functions crucial

for survival. In fact, both organic and inorganic mercury accumulation has been reported in fish brain (Boudou and Ribeyre, 1985; Rouleau et al., 1999; Berntssen et al., 2003). In the current study, it was possible to recognize a mercury accumulation pattern in brain, parallel to the environmental contamination gradient.

Similar results were found for another fish species, *L. aurata*, revealing that brain was one of the organs that better reflected environmental mercury contamination (Mieiro et al., 2009).

Among year periods, both environmental parameters and organisms' physiology can greatly vary, being a possible source of variation in mercury accumulation in fish. Some studies revealed differences in tissue mercury accumulation with season, although with different patterns ((Ward and Neumann 1999). Despite this, mercury accumulation in *D. labrax* brain showed no variation among seasons, even in the presence of differences between reference and contaminated conditions both for warm and cold periods. In warm period, brain was able to distinguish R from L2 (the most contaminated site) as well as the two degrees of contamination, i.e. L1 (intermediate) from L2, separated by only 2 km. This reinforces the feasibility of *D. labrax* use as bioindicator, regardless of the mobility often invoked as limitation associated to fish species.

Once absorbed, mercury is rapidly distributed to all tissues. It is believed that MeHg is widely distributed when comparing to inorganic mercury, using neutral amino acids carriers to cross the blood brain barrier (BBB) (Holmes et al., 2009). In humans, about 10% of the body burden of MeHg was found in the brain, where it is slowly demethylated to inorganic mercuric (Clarkson et al., 2003). As a result, large amounts of mercury, derived both by metallic or organic forms, are believed to remain immobilized in the brain (ATSDR, 1999). Notwithstanding, the toxicological role of organic and inorganic mercury remains a matter of debate. While some authors state that the different forms of mercury have the same toxic entity, being toxicity dependent mainly on a differential bioavailability (De Flora et al., 1994), others stated that each mercury form has different physicochemical properties and toxicity profiles (Clarkson, 1997). Nevertheless, both forms can induce a wide range of toxic effects (Crespo-López et al., 2007). Recent findings of Hu et al. (2010) revealed that MeHg could pass through the BBB in a dose-dependent manner and thus, total mercury accumulation in three different regions of the brain of neonatal rats occurred. These authors also demonstrated that pos-natal prolongation of exposure induces redistribution of mercury in the brain, being hippocampus the main target.

6.4.2 Mercury-induced oxidative stress responses

Enzymatic and non-enzymatic antioxidant responses

Formation of ROS has been pointed as a key mechanism in mercury induced toxicity (Shanker and Aschner, 2003; Roos et al., 2009). Depletion of GSH and antioxidant enzymes has also been referred as a consequence of the pro-oxidative ability of mercury (Stringari et al., 2008; Roos et al., 2009). In accordance, CAT activity depletion was detected in *D. labrax* from both contaminated sites (warm period). This inhibitory effect on a critical antioxidant process potentially exacerbates the pro-oxidant properties of mercury, increasing H_2O_2 cellular levels and thereby promoting oxidative stress conditions.

The primary mechanisms by which sulfhydryl-reactive metals, which include mercury, elicit their toxic effects involve the disruption of biological activities of many proteins, namely antioxidant enzymes, due to their high affinity for free sulfhydryl groups (Quig, 1998). On the other hand, ROS overproduction itself can also be responsible for CAT depletion. Recent studies on *D. labrax* antioxidant responses indicated that CAT is a very sensitive parameter concerning ROS formation (Ahmad et al., 2009; Maria et al., 2009). Although various fish studies demonstrated CAT depletion under exposure to contaminants (Padmini and Rani, 2009), only Bagnyukova et al. (2005a) referred it for brain. Besides this, in the previous studies CAT decrease was normally associated with an overall decrease in the antioxidant defences, which was not confirmed in the current study.

Albeit each particular enzyme accomplishes a specific function, its action can be partly replaced by the actions of other antioxidants (Bagnyukova et al., 2005b). In this perspective, the first option to compensate CAT inhibition would be an increment in GPx activity as both catabolise H_2O_2. However, GPx didn't show any increase tendency in brain of fish from contaminated sites. Alternatively, it can be suggested that CAT depletion was counterbalanced by the reinforcement of defence mechanisms involving GSH, which is supported by the concomitant increase in GR activity. Similarly, Lopez-Torres et al. (1993) found that long-term inhibition of CAT led to an induction of GR in frog liver and kidney.

Anyhow, GR induction signalled a pro-oxidant challenge in fish from L1 and L2, following a pattern parallel to T-Hg accumulation in the brain. This response is indicative of higher glutathione recycling in order to maintain the proper GSH redox status and avoid its depletion. The restoration of GSH pools is responsible for continuously providing GSH

as substrate for GPx and GST, and thus indicating GR high precocicity and sensitivity to pollutants (Doyotte et al., 1997; Padmini and Rani, 2009). Nevertheless, taking into account that neither GPx nor GST exhibited significant elevations, probably GSH has been scavenging ROS directly, acting spontaneously through non-enzymatic reactions. The Hg-GSH conjugation may be a pathway for metal elimination from the cell; however, this process can deplete the cell of GSH, which didn't occur in *D. labrax* brain, making this GSH use improbable. Unlike CAT, GR (and, in some extent, the other assessed enzymatic antioxidants) didn't reveal susceptibility towards inhibition induced by mercury. Furthermore, for the same T-Hg accumulated levels, i.e. between 0.08 (L1) and 0.23 $mg.kg^{-1}$ (L2), CAT was inhibited and GR was induced, depicting different threshold limits for their modulation.

In cold period, the studied antioxidant responses showed no differences among sampling sites, indicating that, in the presence of winter conditions, the observed T-Hg concentrations didn't reach the threshold levels to induce neither adaptive responses nor to evidence toxicity (as observed in warm period trough enzyme inhibition). In general, the influence of environmental/seasonal factors (regarded as non-contaminant related factors) is best-assessed considering fish responses at the reference site, where the potential interference of seasonality in the contaminants bioavailability is diminished in comparison with contaminated sites. Nevertheless, in the present study the seasonal differences observed in antioxidant endpoints at L1 coincided with those from R, indicating that seasonal variables determinately affected fish physiology, superimposing to mercury bioavailability and brain accumulation (when time-related variations are addressed). Hence, two patterns of seasonal variation was perceived, i.e., higher CAT, GPx and GST activities in the warm period and the opposite pattern for GR and GSHt.

The increase of antioxidant levels during summer conditions appears as expectable since it is assumed that biomarker responses are usually higher during warm periods (Pain et al., 2007). Such condition is related with an array of environmental variables, with emphasis to increased temperature and decreased oxygenation, leading to the activation of biomarkers of stress (Pain et al., 2007). ROS generation, oxidation rates and antioxidant status in fish, as poikilothermic organisms, are directly related to ambient temperature and metabolic activity (Filho et al., 2000). In this perspective, the increase of brain GR activity and GSHt content in the cold period seems less understandable. Moreover, high GSH content presupposes high cysteine accessibility (needed for its synthesis), which is less likely during wintertime when *D. labrax* can experience a period

of food deprivation. Though most of the studies reported GSH increase as an adaptive response to pro-oxidant status, earlier findings also suggested that the presence of high GSH content in red blood cells (Filho, 1996) and in gills (Marcon and Filho, 1999) of fish are associated with attenuation of oxidative stress.

Antioxidant defences can be altered as a consequence of the alternation of periods of normal feeding and fasting during the annual cycle. This phenomenon has not been extensively studied in fish and the available reports provided inconclusive results, since increased and decreased enzymatic antioxidants were observed in association with food deprivation, also showing enzyme-specific and species-specific profiles (Blom et al., 2000; Pascual et al., 2003; Morales et al., 2004; Nam et al., 2005). In this context, Pascual et al. (2003) found that prolonged starvation enhanced GR activity in the liver of *Sparus aurata*. Therefore, the influence of a feeding decline experienced by *D. labrax* during colder periods cannot be overlooked on explaining brain antioxidant levels, mainly GR and GSHt.

Overall, the present results suggest that normal redox homeostasis in brain may be achieved by the integrated action of all components of the antioxidant defence system notwithstanding the modulation of individual components can follow different trends.

Peroxidative damage (LPO)

The oxidation of polyunsaturated fatty acids is a very important consequence of the oxidative stress and has been pointed as a high predictive biomarker of effect (Van der Oost et al., 2003; Guilherme et al., 2008). Brain as a fatty rich compartment is a potential target for lipid peroxidation. In agreement, LPO has been proposed as an additional mechanism of mercury induced neurotoxicity (Yee and Choi, 1996). Furthermore, LPO can provide information on the organ threshold limits, at which toxicity is expressed. Some fish studies reported increase in brain LPO under mercury exposure (Berntssen et al., 2003; Bagnyukova et al., 2005a). According to the present data, no LPO increases were verified in brain of fish captured in the contaminated sites. Moreover, the moderately contaminated site (L1) demonstrated a significant decrease in LPO levels, comparing to the reference and to the most contaminated site. This prominent decrease in LPO occurred along with a decrease in CAT activity. Surprisingly, an insufficiency in the antioxidant defences showed no increased susceptibility towards lipid peroxidation. Beyond the protective role of GSH previously invoked (denunciated by the GR induction), two further explanations can be presented. First, the increment of other antioxidant defences, such as non-enzymatic antioxidants, may have a protective effect against LPO.

Patra et al. (2001) demonstrated that cystein, alpha tocopherol and ascorbic acid exert a protective role against metals in the brain of rats. The second explanation is related to the dual role of H_2O_2 in the living organisms. Brain is an organ with a high dependence on oxidative phosphorylation, which means that a certain level of H_2O_2 is needed as electron donor and also as signalling molecules responsible for its normal functioning (Bagnyukova et al., 2005a). Hence, the levels of ROS present in L1 fish brain were great enough to induce changes in the antioxidant responses, although part of that amount were employed for the normal functioning of the brain. The possibility of metallothioneins (MTs) as a protecting mechanism is not hypothesized since *D. labrax* captured in the same area exhibited decreased MTs levels in the brain (Mieiro et al., unpublished data).

These results corroborate the idea that LPO levels cannot be predicted only on the basis of antioxidants performance. In this direction, Dotan et al. (2004) stated that the relation between the LPO and antioxidants, both enzymatic and non-enzymatic, can present divergent patterns, as elevated levels of LPO were accompanied either by elevation or reduction on antioxidant levels.

The response profile registered for LPO in warm period as a function of accumulated T-Hg can be explained in the light of the principles underlying the ecological intermediate disturbance hypothesis (intermediate levels of disturbance correspond to high levels of diversity/adaptation) (Connell, 1975). Though this theory is relative to biodiversity, similarities can be perceived with the net of processes on the basis of LPO expression (e.g. ROS generation, oxidative damage of lipids, lipids turnover, and antioxidants modulation). Accordingly, membrane integrity in brain cells seems to be maximized when pro-oxidant stimulus is neither too weak nor too intense.

The significantly lower LPO levels measured in the cold period comparing with warm period reinforces the idea that winter conditions represent a lower pro-oxidative risk to cerebral tissue, as it was above mentioned on antioxidants discussion.

6.5 Conclusions

According to the results, it can be concluded that:

D. labrax brain accumulated T-Hg levels reflecting environmental levels. Brain of fish from mercury contaminated sites (warm period) exhibited ambivalent antioxidant responses, *viz*. GR activity increase as an adaptive mechanism and CAT inhibition as a sign of toxicity. Though a pro-oxidant status was evident, brain showed to possess

compensatory mechanisms able to avoid lipid peroxidative damage. Hence, mercury showed to interfere in a lesser extent with brain lipids than with proteins.

The warm period revealed to be the most critical since no inter-site differences were detected in oxidative stress endpoints in the cold period. It was demonstrated that seasonal changes in environmental factors play a crucial role in regulating the antioxidant capacity of brain of *D. labrax*. Moreover, the T-Hg threshold limits in brain for enzyme activation/inhibition strongly depends on the environmental conditions. Overall, it was confirmed the need to assess different seasons to achieve a better knowledge of mercury-induced pro-oxidant status in brain.

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CHAPTER 7

Mercury accumulation patterns and biochemical endpoints in feral fish (*Liza aurata*): a multi-organ approach

Mercury accumulation patterns and biochemical endpoints in feral fish (*Liza aurata*): a multi-organ approach

Abstract

The integration of bioaccumulation and effect biomarkers in fish has been proposed for risk evaluation of aquatic pollutants. However, this approach is still uncommon, namely in the context of mercury contamination. Furthermore, a multi-organ evaluation allows an overall account of the organisms' condition. Having in mind the organs' role on metal toxicokinetics and toxicodynamics, gills, liver and kidney of *Liza aurata* were selected and mercury accumulation, antioxidant responses and peroxidative damage were assessed. Two critical locations in terms of mercury occurrence were selected from an impacted area of the Ria de Aveiro, Portugal (L1, L2), and compared with a reference area. Although kidney was the organ with the highest mercury load, only gills and liver were able to distinguish mercury accumulation between reference and contaminated stations. Each organ demonstrated different mercury burdens, whereas antioxidant responses followed similar patterns. Liver and kidney showed an adaptive capacity to the intermediate degree of contamination/accumulation (L1) depicted in a catalase activity increase. In contrast, none of the antioxidants was induced under higher contamination/accumulation (L2) in any organ, with the exception of renal GST. The lack of lipid peroxidation increase observed in the three organs denunciates the existence of an efficient antioxidant system. However, the evidences of enzymatic inhibition in gills and kidney at L2 cannot be overlooked as an indication of mercury-induced toxicity. Overall, organ-specific mercury burdens were unable to distinguish the intermediate degree of contamination, while antioxidant responses revealed limitations on signalizing the worst scenario, reinforcing the need to their combined use.

Keywords: mercury bioaccumulation; oxidative stress, organ specificities

Ecotoxicology
Submited

7.1 Introduction

Fishes are generally acknowledged as a worthy model for assessing aquatic contamination and to be used as environmental sentinels (Padmini and Rani, 2009). The main advantages of using fishes in the assessment of various effects of contaminants present in the aquatic environment include their ability to metabolize, concentrate and accumulate pollutants. Furthermore, fish responses to toxicants are similar to those of higher vertebrates. Hence, they are also useful tools to screen for chemicals that are potentially harmful for humans (El-Shehawi et al., 2007).

Mercury is one of the most dangerous contaminants, known by its ability to generate reactive oxygen species (ROS), resulting in lipid peroxidation (LPO), DNA damage, depletion of sulphidryl groups and altered calcium homeostasis (Stohs and Bagchi, 1995). Traditionally, liver and kidney are the preferentially studied organs due to their capacity to accumulate and store metals in marine fish, being the organs with the highest metal loads (Mieiro et al., 2009). Nevertheless, gills are directly involved in metal uptake, and their ability to reflect environmental status through metal bioaccumulation and oxidative stress responses has been shown (Pereira et al., 2010). Additionally, Ahmad et al. (2004) emphasised gills higher susceptibility concerning pollutant-induced oxidative stress, when comparing with liver and kidney. In agreement, and having in mind the organs' function in the context of metal toxicokinetics and toxicodynamics, gills (uptake), liver (accumulation and detoxification) and kidney (detoxification and excretion) were selected to describe the accumulation patterns and biochemical responses in a mercury contaminated estuarine area. Moreover, knowing the responses of different organs will improve the possibility to acquire a better perspective of the overall status of the organisms. In accordance, and taking into account that this kind of approach is still scarce, a combined use of chemical analysis (mercury body burdens) and biochemical biomarkers was adopted to ascertain the biological effects of mercury exposure in Golden grey mullet (*Liza aurata*) from an estuarine impacted area, Laranjo Basin (Ria de Aveiro, Portugal). This area was selected on the basis of an identified mercury gradient, resulting from five decades of continuous discharges from a chlor-alkali plant (Coelho et al., 2005). This restrained mercury gradient and the lack of other important sources of contamination provides an exceptional opportunity for the evaluation of mercury toxicity under natural conditions (Ramalhosa et al., 2005; Coelho et al., 2007; Guilherme et al., 2008; Mieiro et al., 2009). Therefore, the main goals of this study were: i) to assess the antioxidant

defences and peroxidative damage in gills, liver and kidney of *L. aurata*, ii) to establish organ-specific associations between the mercury burdens and the mentioned effects.

7.2 Material and methods

7.2.1 Characterization of the studied area

The Ria de Aveiro is a coastal lagoon adjacent to the Atlantic Ocean which has an inner basin (Laranjo Basin) (Fig. 7.1) that persistently received mercury-containing effluents from a chlor–alkali plant since 1950s until 1994. The discharges resulted in an accumulation of about $33x10^3$ kg of mercury in the lagoon, mostly (about 77%) associated to the sediment in the Laranjo Basin (Pereira et al., 1998). Despite the end of effluent releases, high mercury concentrations are still present in sediments, creating a contamination gradient (Pereira et al., 1998; Coelho et al., 2005).

Three stations were surveyed in March 2007, selected according to the distance to the mercury source. At Laranjo Basin, two sampling stations (L1 and L2) were chosen, separated by a 2 km distance: L2 located closer to the mercury source and identified as a highly contaminated area and L1, downstream L2, as a moderately contaminated area. For comparison purposes, a reference area (R) was selected near to the lagoon entrance and far from the main polluting sources (Pacheco et al., 2005).

7.2.2 Sampling procedures

Water environmental parameters such as pH, dissolved oxygen, temperature and salinity were measured at sub-surface level, in low and high tide conditions. Turbidity was measured using a 20 cm black and white Secchi disc and water column depth was also evaluated. At each station, sub-surface water samples were collected in acid-washed plastic bottles and kept cold during transportation to the laboratory. Five replicates of sediments were taken from the surface sediment layer (about 2cm depth) in each sampled site.

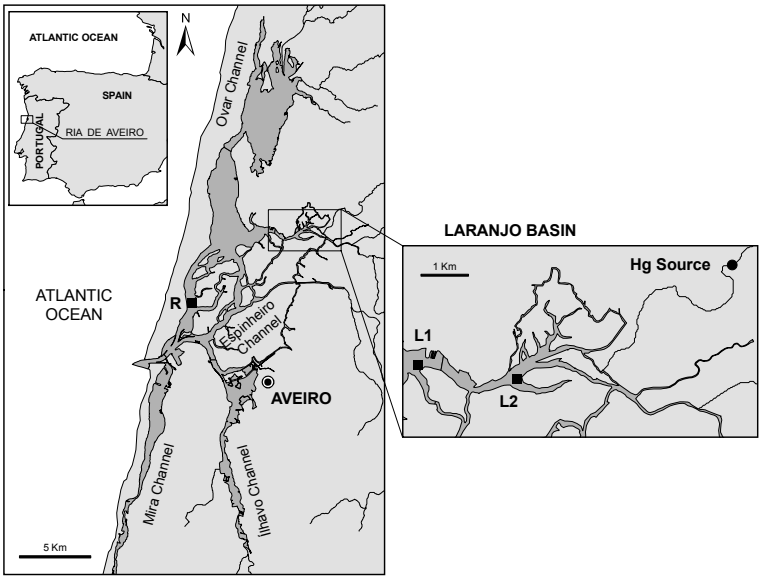

Figure 7.1 Map of the sampling sites (■) in the Ria de Aveiro (Portugal): reference (R - 40º41'00'' N, 8º42'44'' W), moderately (L1 - 40º43'34.46'' N, 8º38'53.16'' W) and highly contaminated (L2 - 40º43'28.98'' N, 8º37'35.80'' W) areas.

Fifteen Golden grey mullets (*Liza aurata*) were collected at each sampling site during low tide, using a beach-seine net. Juvenile specimens were selected on the basis of their size, having an average length of 11.6 ±1.25 cm and an average wet weight of 14.6 ± 5.47 g. The election of juveniles relied on the need to avoid gender effects and growth dilution factor relatively to mercury accumulation. Immediately after being caught, fish were sacrificed according to ethical recommendations and gills, liver and kidney were sampled and instantly frozen in liquid nitrogen. Two sets of samples were taken for each organ: one set for oxidative stress quantifications (n=10) and the other for mercury determinations (n=5). In the laboratory, samples were preserved until further processing at -80 and -20°C for oxidative stress and mercury determinations, respectively.

7.2.3 Analytical procedures and quality control

Mercury analysis in the environmental compartment

At the laboratory, water samples were immediately filtered through pre-weighed 0.45 µm Millipore cellulose acetate membrane filters, acidified with "mercury-free" HNO_3 to pH <2 and stored at 4ºC until analysis. Filters were re-weighed after drying overnight at 60ºC and stored for determination of mercury in suspended particulate matter (SPM-Hg).

Reactive (R-Hg) and total dissolved mercury in water (Dis-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 Dis-Hg analysis, 50 mL of each sample was oxidized with 500 μL of a saturated solution of potassium persulfate and by irradiation with a UV lamp (1000 W) for 30 min; following irradiation, the excess of oxidant was reduced with 37.5 mL of hydroxylamine solution 12% (w/v) (Mucci et al., 1995). The detection limit based on procedural blanks was 1.2 ± 0.3 ng L^{-1} (± standard deviation). The procedure and reagent contamination was followed by analysis of filtrate blanks and ultra-pure water. For SPM determination, the same equipment was used after digestion of filters with HNO_3 4 mol L^{-1} (Pereira et al., 1998). Blank filters were used to examine any possible contamination, revealing mercury levels between 3.5 and 9.4%. The accuracy of the methods for mercury quantification was tested by fortification of samples (at two concentration levels within the range found in samples), showing recovery efficiencies always between 90 and 100%.

Sediment samples were freeze-dried, homogenized and sieved through a 1 mm sieve and stored for mercury determination. Total mercury in the sediments (Sed-Hg) was determined by atomic absorption spectrometry (AAS) with thermal decomposition and 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) for marine sediments, namely MESS-3 and PACS-2. Precision of the method was always better than 9% (n>5), with recovery efficiency around 92%.

Mercury analysis in the fish organs

Gills, liver and kidney samples were freeze-dried, homogenized, weighted for fresh weight calculations and total mercury (T-Hg) analyses were performed. T-Hg determination was carried out by AAS as described for Sed-Hg determination. The accuracy and precision of the analytical methodology was assessed by replicate analysis CRM - TORT-2 (lobster hepatopancreas). Precision of the method was always better than 9% (n>3), with recovery efficiency between 92-103%.

7.2.4 Biochemical analyses

Each tissue sample was homogenized, using a Potter-Elvehjem homogenizer, in chilled phosphate buffer (0.1 M, pH 7.4). The ratio of homogenization [tissue mass

(g)/buffer volume (mL)] was 1/10 for gills, 1/15 for liver and 1/20 for kidney. This homogenate was then divided in three aliquots, for LPO and total glutathione (GSHt) quantification, as well as for post-mitochondrial supernatant (PMS) preparation. The PMS fraction was obtained by centrifugation in a refrigerated centrifuge (Eppendorf 5415R) at 13,400 g for 20 min at 4ºC. Aliquots of PMS were stored in microtubes at -80ºC until analyses.

CAT activity was assayed in PMS (at 25ºC) by the method of Claiborne (1985) as described by Giri *et al*. (1996). Briefly, the assay mixture consisted of 1.99 mL phosphate buffer (0.05 M, pH 7.0), 1 mL hydrogen peroxide (0.030 M) and 0.01 mL of sample in a final volume of 3 mL. Change in absorbance was recorded spectrophotometrically (Jasco UV/VIS, V-530) at 240 nm and CAT activity was calculated in terms of μmol H_2O_2 consumed/min/mg protein ($\varepsilon = 43.5\ M^{-1}\ cm^{-1}$).

GPx activity was assayed (at 25ºC) in accordance to the method described by Mohandas et al. (1984), as modified by Athar and Iqbal (1998). The assay mixture consisted of 0.09 mL phosphate buffer (100 mM, pH 7.0), 0.03 mL EDTA (10 mM), 0.03 mL sodium azide (1 mM), 0.03 mL glutathione reductase (GR; 2.4 U/mL), 0.03 mL reduced glutathione (GSH; 10 mM), 0.03 mL NADPH (1.5 mM), 0.03 mL H_2O_2 (1.5 mM) and 0.03 mL of PMS in a total volume of 0.3 mL. GPx activity was determined monitoring the oxidation of NADPH to $NADP^+$, resulting in an absorbance decrease at 340 nm. The absorbance was read every 30 seconds for a period of 3 minutes using a SpectraMax 190 microplate reader. The enzyme activity was calculated as nmol NADPH oxidized/min/mg protein ($\varepsilon = 6.22\times10^3\ M^{-1}\ cm^{-1}$).

GR activity was measured (at 25ºC) according to the method of Carlberg and Mannervik (1975). Briefly, the reaction medium consisted in 0.1 M phosphate buffer (pH 7.0), diethylenetriaminepenta-acetic acid (DTPA) 0.5 mM, NADPH 0.2 mM and oxidised glutathione (GSSG) 1 mM. In a quartz cuvette, 0.025 mL of PMS was added to 0.975 mL of reaction medium. Enzyme activity was spectrophotometrically (Jasco UV/VIS, V-530) quantified by measuring NADPH disappearance at 340 nm and expressed as nmol NADPH oxidised/minute/mg protein ($\varepsilon = 6.22\times10^3\ M^{-1}\ cm^{-1}$).

GST activity was determined using CDNB (1-chloro-2,4-dinitrobenzene) as substrate, according to the method of Habig *et al*. (1974). The assay was carried out (at 25 ºC) in a quartz cuvette with a 2 mL mixture of 0.2 M phosphate buffer (pH 7.4), 0.2 mM CDNB and 0.2 mM GSH. The reaction was initiated by the addition of 0.01 mL PMS, and the increase in absorbance was recorded spectrophotometrically (Jasco UV/VIS, V-530)

at 340 nm during 3 min. The enzyme activity was calculated as nmol GS-DNB formed/min/mg protein (ε = 9.6 mM^{-1} cm^{-1}).

For GSHt measurement, protein content in the tissue homogenate was precipitated with trichloro acetic acid (TCA 12 %) for 1 hour and then centrifuged at 12,000 g for 5 min. at 4ºC. GSHt was determined (in deproteinated PMS, at 25ºC) adopting the enzymatic recycling method using GR excess, whereby the sulfhydryl group of GSH reacts with DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) producing a yellow coloured 5-thio-2-nitrobenzoic acid (TNB) (Tietze, 1969; Baker *et al*., 1990). Formation of TNB was measured by spectrophotometry (Jasco UV/VIS, V-530) at 412 nm. The results were expressed as nmol TNB conjugated/min/mg protein (ε = 14.1×10^3 M^{-1} cm^{-1}).

The determination of LPO was performed in the tissue homogenate, according to the procedure of Ohkawa (1979) and Bird and Draper (1984), as adapted by Filho et al. (2001). Briefly, to 0.15 mL of homogenate, 0.01 mL of 1-1 butylated hydroxytoluene (4% in methanol) was added and mixed well. To this aliquot, 0.5 mL of 12% TCA in aqueous solution, 0.45 mL Tris–HCl (60 mM, pH 7.4; and 0.1 mM DTPA) and 0.5 mL 0.73% TBA were added and mixed well. The mixture was heated for 1 hour in a water bath set at boiling temperature and then cooled to room temperature, decanted into 2 mL microtubes and centrifuged at 13,400 g for 5 min. Absorbance was measured at 535 nm, using a SpectraMax 190 microplate reader and LPO was expressed as nmol of TBARS formed per milligram of fresh tissue (ε = 1.56×10^5 M^{-1} cm^{-1}).

Total protein contents were determined according to the Biuret method (Gornall et al., 1949), using bovine serum albumin (E. Merck-Darmstadt, Germany) as a standard. Absorbance was measured at 550nm using a SpectraMax 190 microplate reader.

7.2.5. Statistical analysis

Data analysis followed standard statistical procedures. Data were tested for goodness of fit to normal distribution and requirements of homogeneity of variances were also determined. The 2-way Analysis of variance (ANOVA) were performed, followed by all pairwise multiple comparison procedures (Holm - Sidak). Whenever the assumptions for parametric statistics failed, the ANOVA on ranks (Kruskall Wallis) was performed followed by the non-parametric all pairwise multiple comparison procedure (Dunn's test). Differences between means were considered significant at $p<0.05$.

7.3 Results

7.3.1 Environmental conditions

Water environmental parameters are described on Table 7.1. Whilst parameters such as temperature, pH, dissolved oxygen, depth and turbidity were in the same range, SPM and salinity varied among stations, showing respectively an increase and decrease tendency towards the contamination source, namely in low tide.

Table 7.1 Hydrological parameters on reference (R), moderately (L1) and highly mercury contaminated (L2) areas at Ria de Aveiro: water temperature (T), dissolved oxygen (DO), pH, salinity, suspended particulate matter (SPM), turbidity and water depth.

	High tide - Low tide						
Site	**T** (ºC)	**DO** (mg L^{-1})	**Depth** (m)	**Turbidity** (m)	**pH**	**Salinity**	**SPM** (mg L^{-1})
R	16.4 – 15.3	10.8 – 10.9	5.4 - 1.6	1.2 - 0.5	8.4 - 8.4	34.0 - 34.0	45.1 – 31.7
L1	15.3 – 15.0	10.6 – 10.8	3.1 - 2.8	0.9 - 0.5	8.2 – 8.1	28.0 - 15.0	40.5 -53.2
L2	12.5 – 12.2	8.5 - 8.9	2.3 - 1.0	1.2 - 0.3	8.3 - 7.8	32.0 - 13.0	n.d. – 70.0

7.3.2 Mercury environmental contamination

The assessment of mercury contamination in the environmental compartments (Table 7.2) confirmed the previously described gradient. Regarding mercury in the water column, the exhibited R-Hg and Dis-Hg values were consistently low. Dis-Hg concentration increased towards the mercury source in low tide, while in high tide that pattern was not apparent. SPM-Hg and the % of particulate Hg also increased towards the mercury source. Sediment reflected the decades of mercury discharges into the lagoon better than water compartment. As a result, Sed-Hg increases were 8-, 85- and 680-fold, respectively for L1 in relation to R, for L2 in relation to L1, and for L2 in relation R. According to the Portuguese legislation (Portaria nº1450/2007), sediments from L2 were classified as contaminated dredged material.

Table 7.2 Concentrations of reactive mercury (R-Hg), total dissolved mercury (Dis-Hg) (ng L^{-1}), % of particulate mercury and total mercury in suspended particulate matter (SPM-Hg) (mg Kg^{-1} dry weight) in the water column in high and low tide conditions, and total mercury in sediment (Sed-Hg) (mg Kg^{-1} dry weight) (mean ± standard deviation) at each sampling station at Ria de Aveiro: reference (R), moderately (L1) and highly mercury contaminated (L2) areas.

	Water (High tide - Low tide)				Sediment
Site	**R-Hg** (ng L^{-1})	**Dis-Hg** (ng L^{-1})	**% Hg particulate**	**SPM-Hg** (mg Kg^{-1}dw)	**Sed-Hg** (mg Kg^{-1}dw)
R	5.8 - 4.4	18.8 - 10.3	58.6 - 72.1	0.6 - 0.8	0.01 ± 0.0009
L1	3.0 - 2.7	8.3 - 10.6	85.7 - 89.0	1.2 - 1.6	0.08 ± 0.006
L2	3.9 - 4.9	10.2 - 20.8	n.d. - 96.4	n.d - 8.0	6.8 ± 0.16

n.d. – not determined

7.3.3 Mercury levels in *L. aurata* organs

T-Hg in the assessed organs increased with increasing environmental mercury contamination. Moreover, gills and liver were able to reflect significant differences between the reference station (R) and the most contaminated area (L2) (Fig. 7. 2).

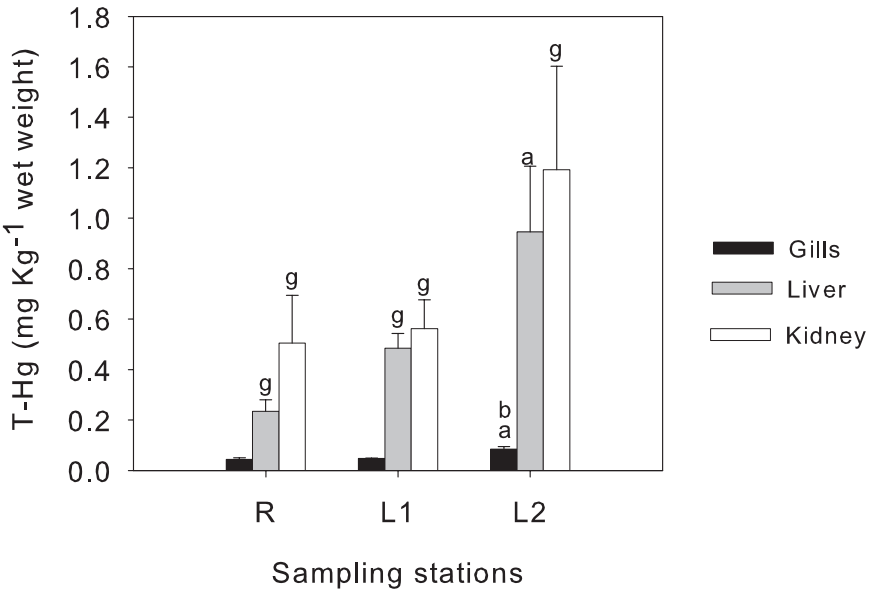

Figure 7.2 Total mercury (T-Hg) mean concentration (mg Kg^{-1} wet weight) in gills, liver and kidney of *L. aurata* captured at each Ria de Aveiro sampling station: reference (R), moderately (L1) and highly mercury contaminated (L2) areas. The letters denote statistically significant differences ($p<0.05$): (a) *versus* R and (b) *versus* L1 within the same organ, (g) *versus* gills within the same site. Bars represent the standard error.

T-Hg in gills also showed differences between the two contaminated stations (L1 *versus* L2). On the other hand, gills revealed significantly lower T-Hg levels than liver and kidney at R and L1. At L2, T-Hg in gills was lower than kidney. Liver and kidney didn't show differences in T-Hg levels.

7.3.4 *L. aurata* organ-specific oxidative stress responses

Oxidative stress responses in the different organs are shown in figure 7.3. In gills, antioxidant defences as well as LPO levels, in general, remained unaltered. It is only noticeable the observation of a significantly lower CAT activity at the most contaminated site (L2) when compared with the intermediate site (L1).

Liver oxidative stress responses reflected an induction of CAT activity at L1 relatively to R. GPx, GR and GST activities didn't vary between the three sampling sites, as well as the GSHt content. Hepatic LPO levels also showed no differences between the sampling stations.

In relation to kidney, CAT activity increased at L1 relatively to R and L2, whereas GST activity increased at L2 relatively to R and L1. All the other antioxidants showed no inter-station differences, as well as LPO levels.

Comparing the three organs (Fig. 7.3), it is interesting to point out that liver presented clearly higher basal levels (assumed as the values measured at R) of CAT, GST and GSHt. Subsequently, liver also presented the highest levels of CAT and GST at the contaminated stations and GSHt at L2. Gills and liver displayed comparable levels of GPx and GR activity at R as well as high GR levels at the contaminated stations. GSHt levels at L1 along with GPx at L1 and L2 were similar among the three organs. In relation to LPO, the three organs displayed comparable levels at both R and L1, whereas at L2, kidney's LPO levels were significantly lower than liver and gills. The differences between organs are supported by the statistical analysis.

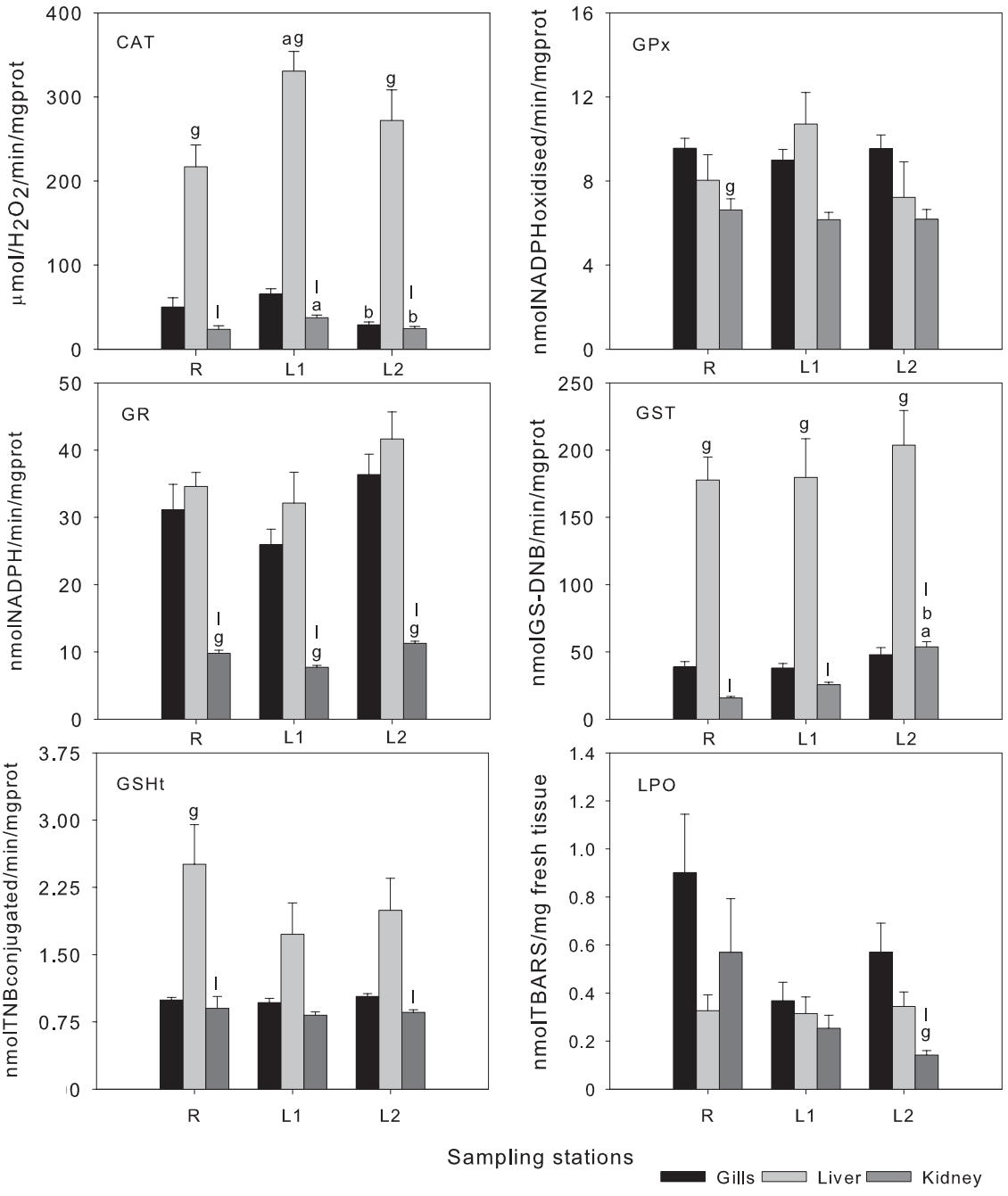

Figure 7.3 Oxidative stress responses in gills, liver and kidney of *L. aurata* captured at each Ria de Aveiro sampling station: reference (R), moderately (L1) and highly mercury contaminated (L2) areas. The letters denote statistically significant differences ($p<0.05$): (a) *versus* R, (b) versus L1 within the same organ, (g) *versus* gills and (l) *versus* liver within the same site. Bars represent the standard error.

7.4 Discussion

The mercury levels detected in the environment confirmed the existent contamination gradient, though it was clearer in the sediments than in the water column, where mercury levels were low. Surface sediments have shown to be the main reservoir of mercury among the studied compartments, which is in agreement with Pereira et al. (2009), who suggested that mercury contamination in Ria de Aveiro (Portugal) is mainly associated with the sediments and confined to the Laranjo area. The water physicochemical parameters did not diverge considerably among the sampling stations, thereby not influencing greatly the mercury bioavailability and fish biochemical responses. Nevertheless, the differences in salinity and SPM among sampling stations should be considered. Although it has been recognized that salinity can affect metals bioavailability (Monserrat et al., 2007), under the present conditions its influence should be undervalued since the mercury levels found in the water column were low. In opposition, SPM could play an important role in the dispersion of mercury due to the mercury's high affinity for particulate matter.

Mercury has been reported to be able to induce changes in the antioxidant enzyme activities, leading to the generation of ROS, depleting the glutathione system and causing peroxidative damage (Berntssen et al., 2003; Elia et al., 2003). A previous study with *L. aurata*'s brain revealed a generalized antioxidant system breakdown along with mercury exposure, although no peroxidative damage was observed (Mieiro et al., 2010). In the current work, changes in the antioxidant system of gills, liver and kidney also occurred under mercury contamination.

Gills have been referred as an important organ regarding mercury uptake from water compartment and thus, generally reflecting current exposures (Guilherme et al., 2008). This seems in accordance with the present results, demonstrating the ability of T-Hg in gills to reflect mercury increments towards the proximity to the mercury source, namely in terms of SPM-Hg. Consequently, gills antioxidant responses were expected to increase in response to the T-Hg increments at L1 and, mainly, at L2. However, the measured antioxidant parameters in gills revealed to be unaltered, with the exception of CAT activity, showing a predisposition to decrease at the highest T-Hg load (L2). Accordingly, Regoli and Principato (1995) suggested that CAT inhibition is a temporary response to acute pollution. Thus, it seems that CAT activity is a very sensitive parameter regarding ROS, possibly being inactivated by its over-production (Bagnyuokova et al., 2006; Maria et al., 2009). LPO has been pointed out as a consequence of oxidative

stress, being used as a measure of damage. Consequently, increasing of mercury accumulation and oxidative stress accelerates the damage. In agreement, several studies regarding mercury toxicity revealed an increase of LPO in fish tissues (Berntssen et al., 2003; Vieira et al., 2009; Huang et al., 2010). However, no LPO induction was currently observed in the gills of *L. aurata*.

Liver is often described as the organ that greatly accumulates mercury (Berntssen et al., 2003; Maury-Brachet et al., 2006) as it was observed in the current study. Matching with the increased T-Hg levels at L1 (though not statistically significant), liver demonstrated the ability to increase CAT activity. In agreement, CAT increments with mercury exposure have also been reported in previous studies (Berntssen et al., 2003; Huang et al., 2010). High CAT activity reflects more ability to destroy H_2O_2, the major cellular precursor of the hydroxyl radical, which is the most toxic ROS. The ability of liver to induce CAT activity in response to intermediate mercury exposure/accumulation evidenced the elevated antioxidant protection characteristic of this organ, which is in line with its detoxifying ability. On the other hand, liver was unable to induce CAT at high mercury levels, which can corroborate the assumption that CAT is a very sensitive parameter, inhibited by ROS overproduction, as previously referred for gills. CAT induction in liver was not followed by any other enzymatic or non-enzymatic antioxidant response, demonstrating the effectiveness of this enzyme in respond to the H_2O_2 challenge, possibly being the first enzymatic defence against this ROS (Lima et al., 2007; Oliveira et al., 2010). As previously observed in gills and in accordance with Guilherme et al. (2008), no peroxidative damage was verified for liver.

Kidney is also often referred as one of the organs that preferentially accumulates mercury (Zalups, 2000). Accordingly, kidney was the organ with the highest T-Hg loads, both as basal levels (R) and at the contaminated stations (L1 and L2); however, no significant T-Hg increments were verified from R to L1 and L2, probably resulting from the high individual variability associated to these particular data. Nonetheless, the exhibited T-Hg levels seemed to induce CAT activity at L1, evidencing an adaptability to mercury pro-oxidative action. In contrast, the CAT activity depletion observed at L2 relatively to L1 may be regarded as a sign of an inhibition tendency, as verified for gills. Kidney GST activity increased at L2, when compared with both R and L1. In fact, GST was the only antioxidant that showed induction at L2, reinforcing the idea that GST is a good indicator of the presence of contaminants in the aquatic environment (Elia et al., 2003). Moreover, the current results support the suggestion of Tuvikene et al. (1999) that GST is less

vulnerable to the inhibitory action of some aquatic contaminants in comparison with other enzymatic activities commonly used as contamination biomarkers. GSTs function both as a conjugation agent of electrophilic compounds with GSH (Van der Oost et al., 2003) and as a direct antioxidant, by catalyzing the reduction of organic hydroperoxides by GSH (Wang and Ballatori, 1998). According to Ahmad et al. (2008), GST induction in the presence of mercury suggests its antioxidant action against metals, rather than its role at phase II detoxification, since metals have not been reported as substrate to GSTs. In accordance with gills and liver, no damage was observed in kidney.

Overall, antioxidant responses revealed do not vary linearly with T-Hg accumulation in the three studied organs, which can be explained mainly by the propensity to inhibition (evident for CAT) in the presence of high T-Hg levels. Nonetheless, CAT activity showed to be the most responsive parameter.

Oliveira et al. (2008) stated that the susceptibility to oxidative stress of a given organ depends on several factors, such as the propensity to accumulate the xenobiotic, the specific antioxidants basal levels, the ability to adapt and thus, to activate antioxidant defences, and the organ's metabolic rates. Therefore, due to the high levels of mercury accumulated, namely at L2 (4 times higher than at R), and the inherent elevated metabolic rates, liver was expected to be the most susceptible organ in relation to oxidative damage. However, these aspects were counteracted by elevated basal levels of antioxidants (CAT, GR, GST and GSHt) along with the ability to adapt facing an increase in the T-Hg loads, as demonstrated by the induction of CAT activity at L1. From this balance resulted no peroxidative damage in the liver.

In opposition to liver, gills were the organs that demonstrated to accumulate a lesser amount of T-Hg. Moreover, gills showed to have the highest basal values of GPx and intermediate values for all the other antioxidants assessed. Under moderate degrees of both mercury contamination and accumulation, gills appear more vulnerable than liver and kidney due to their incapacity to significantly increase CAT activity. Under a more severe scenario (L2), gills displayed a predisposition to CAT inhibition, and thus indicating a certain degree of susceptibility to mercury-induced toxicity, although no peroxidative damage was observed.

Kidney, due to the high amounts of mercury accumulated and the low basal levels of antioxidant defences, could also be considered a susceptible organ. Nevertheless, it showed ambivalent responses at L2, translated into CAT inhibition and GST induction, indicating on one hand toxicity signs, while on the other showing an adaptive process.

From the balance between pro- and antioxidant conditions affecting renal cells in fish from L2, kidney exhibited the lowest LPO levels among the studied organs.

In general, the oxidative stress parameters in gills, kidney and liver followed similar patterns of variation in the presence of the two degrees of contamination/accumulation assessed. In addition, the differences on the levels of antioxidants measured in each organ were predominantly determined by organ-specific basal levels, rather than by an organ-specific capacity to react to mercury challenge.

7.5 Conclusion

The current results demonstrated that:

- The integration of data regarding mercury accumulation and oxidative stress responses of *L. aurata* organs were able to detect inter-station differences. However, organ-specific mercury burdens were unable to distinguish the intermediate degree of contamination (R *vs*. L1), while antioxidant responses revealed limitations on signalizing the highest degree of contamination (R *vs*. L2). This observation clearly pointed out the risk of misinterpretations resulting from the adoption of these two approaches separately, being advised their combined use on monitoring aquatic contamination by mercury.

- Organ-specificities were more obvious for T-Hg accumulation than for oxidative stress responses. In accordance, antioxidant responses revealed do not vary linearly with T-Hg accumulation. Overall, oxidative stress responses of gills, kidney and liver followed similar patterns, depicted in a CAT activity increase at L1 and a tendency to reduction at L2. None of the antioxidants revealed induction at L2 in any organ, with the exception of renal GST. The general lack of LPO increase observed in the three organs denunciates the existence of an efficient antioxidant system. However, the evidences of enzymatic inhibition reported for gills and kidney at L2 cannot be overlooked as an indication of mercury-induced toxicity.

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CHAPTER 8

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

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

Abstract

The suitability of metallothioneins (MTs) measurement in fish as biomarker of exposure to mercury has been questioned. Therefore, this study was designed to investigate the causal relationship between external levels of exposure, mercury accumulation and MTs 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 (Ria de Aveiro, Portugal). Total mercury (T-Hg) and MTs content were determined in gills, blood, liver, kidney, muscle and brain, as representative of fish tissue diversity. All tissues reflected differences in T-Hg accumulation in both species, although *D. labrax* demonstrated to accumulate higher levels. Regarding MTs, *D. labrax* revealed an uncapacity to induce MTs synthesis in all the tissues and a depletion in brain content, whereas *L. aurata* showed the ability to increase MTs in liver and muscle. Tissue-specificities were clearly evidenced in the MTs inducing potential and in the susceptibility to MTs decrease. *L. aurata* results showed muscle as the most responsive tissue, responding to moderate and high contamination degrees, while liver only responded to the worst scenario. No correlations between T-Hg and MTs levels were found. Overall, the aplicability of MTs content in fish tissues as biomarker of exposure to mercury was called into question, denunciating important limitations on reflecting the metal exposure levels and the subsequent accumalation extent.

Keywords: mercury, metallothioneins, marine fish, tissues

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8.1 Introduction

Metallothioneins (MTs) constitute a superfamily of ubiquitous low molecular weight proteins capable of binding metals (Romero-Isart and Vasak, 2002), whose behaviour is dominated by the chemistry of the thiol (-SH) group. While some controversy remains regarding the physiological roles of MTs, 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). MTs play also a key role protecting cell 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, in several laboratory and field studies an increase in MT concentrations under metal exposure has been demonstrated (Fernandes et al., 2008; Ghedira et al., 2008; Costa et al., 2009; Falfushynska and Stoliar, 2009). Accordingly, measurement of MTs in different tissues of aquatic organisms is part of the recommended biomarkers for heavy metals biomonitoring programs (Dragun et al., 2009). The relevance of MTs 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 MTs 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 MTs (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 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 MTs 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 MTs synthesis. Furthermore, MTs usefulness as biomarkers of exposure was questioned in the presence of high metal concentrations (Hamza-Chaffai et al. 1995). Accordingly, two main questions arise: Is mercury really able to induce MTs in feral fish? Are MTs really suitable biomarkers 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 MTs 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.

8.2 Materials and Methods

8.2.1 Study area

The study was carried out at Ria de Aveiro, a coastal lagoon located on the northwest coast of Portugal (Fig. 8.1). 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 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).

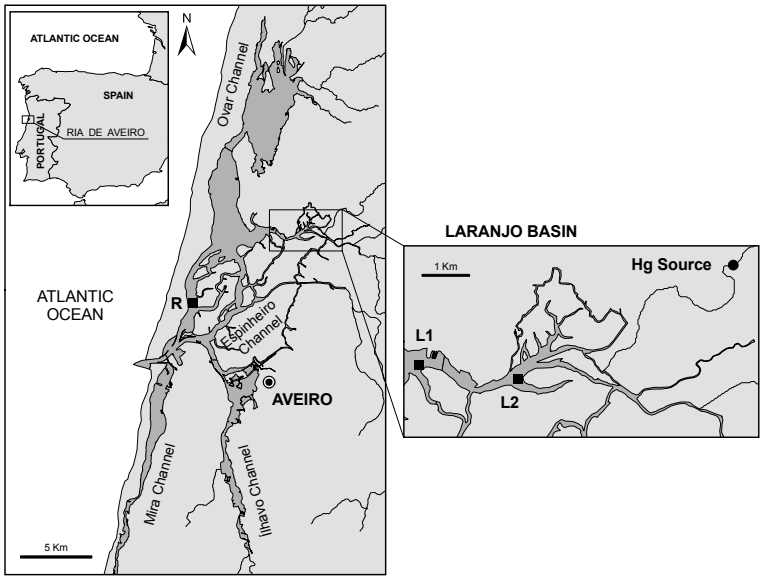

Figure 8.1 Map of the sampling sites (■) in the Ria de Aveiro (Portugal): reference (R - 40º41'00'' N, 8º42'44'' W), moderately (L1 - 40º43'34.46'' N, 8º38'53.16'' W) and highly contaminated (L2 - 40º43'28.98'' N, 8º37'35.80'' W) areas.

Sampling sites (R, L1 and L2) were selected in accordance to the existing contamination gradient (Fig. 8.1). 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 distant from L1. An area close to the lagoon entrance (S. Jacinto) and far from the main polluting sources was chosen as the reference site (R).

8.2.2 Sampling procedures

Dissolved oxygen (WTW–OXI 330i set), pH (WTW–pH 330i set), temperature (WTW–COND 330i) and salinity (WTW–COND 330i) 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 4ºC 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 of 61.6±9.3 and 23.1±5.0 g. Immediately after being caught, fish were sacrificed by cervical transection, 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 ºC for MT determination and at -20 ºC 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.

8.2.3 Total mercury determinations

Mercury in water

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

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 101-110%.

8.2.4 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 4ºC (5415R centrifuge, Eppendorf). The supernatant was stored at –80ºC until MTs measurement. The MTs concentration was measured using the Cd saturation thiomolybdate assay, according to Klein et al. (1994). This protocol was in agreement 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 seem to have the tendency to polymerize. The main features of the procedure are that oxidized MT are 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).

8.2.5 Statistical analysis

Data were 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 MTs contents. A significance level of 0.05 was considered in all test procedures.

8.3 Results

8.3.1 Mercury in the environment

General physico-chemical characterization

Physico-chemical parameters of the water are summarized in Table 8.1. In general, the three sampling sites were analogous regarding environmental characterization with the exception of SPM level at R, during low tide.

Table 8.1 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).

	High tide - Low tide						
Site	**T** (ºC)	**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

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 measurements have reflected the environmental gradient, values were low in the three sampling sites (Table 8.2). 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.

Table 8.2 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).

	Water (High tide - Low tide)		**Sediment**
Site	**R-Hg** (ng L^{-1})	**SPM-Hg** (ng $L^{-1)}$	**Sed-Hg** (mg 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%.

8.3.2 Mercury bioaccumulation in fish tissues

The arithmetic mean of T-Hg in the tissues of the two fish species is depicted in figure 8.2. T-Hg concentrations ranged from 0.02 in blood (R) to 1.01 $mgKg^{-1}$ wwt in liver (L2) for *D. labrax* and from 0.005 in blood (R) to 1.51 $mgKg^{-1}$ 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.

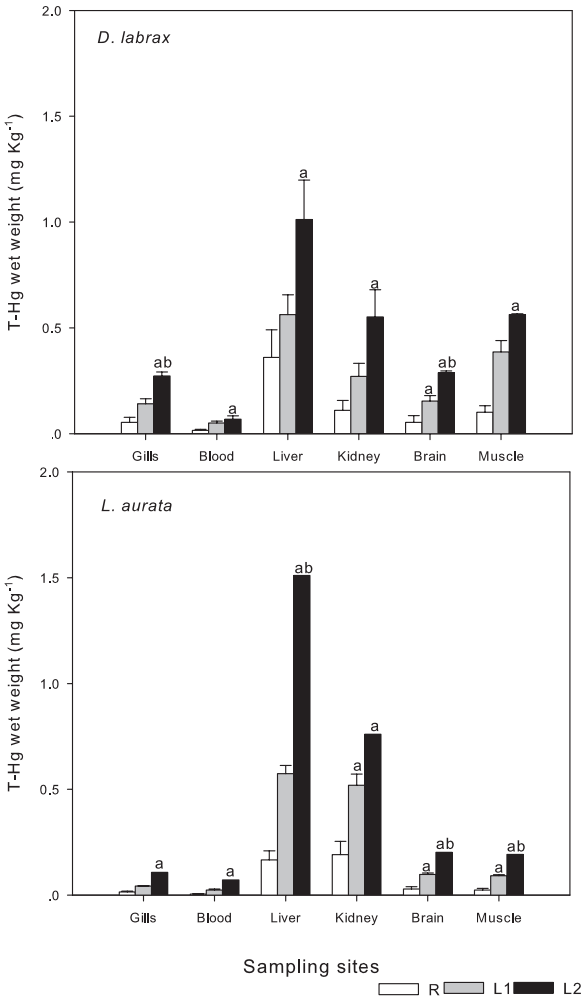

Figure 8.2 Total mercury (T-Hg) mean concentration (mg Kg^{-1} wet weight) in the tissues of *D. labrax* and *L. aurata* captured at each Ria de Aveiro sampling station: reference (R), moderately (L1) and highly mercury contaminated (L2) areas. The letters denote statistically significant differences ($p<0.05$): (a) versus R and (b) versus L1. Bars represent the standard error.

8.3.3 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. 8.3). 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.

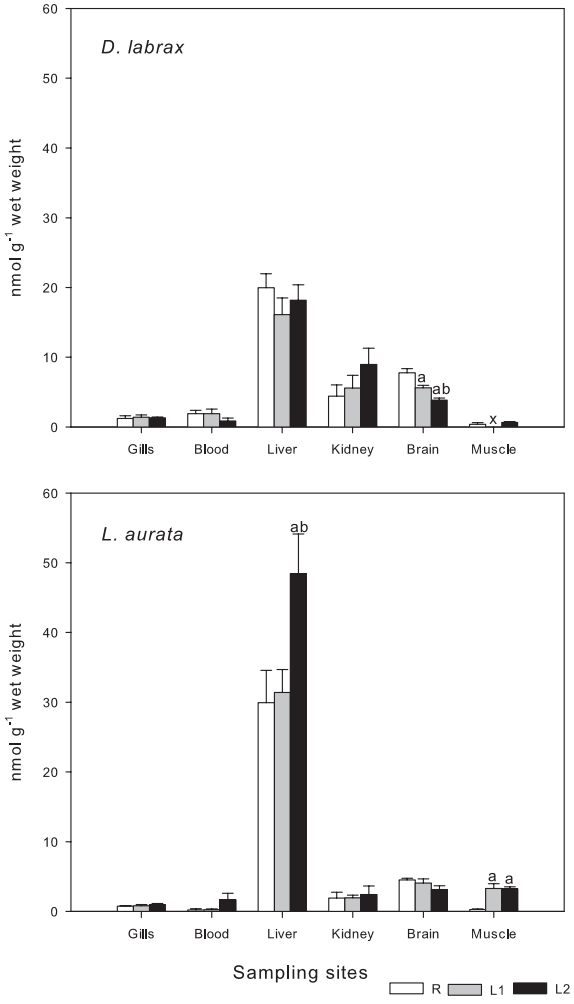

Figure 8.3 Metallothioneins (MTs) mean concentration (nmolg^{-1} wet weight) in the tissues of *D. labrax* and *L. aurata* captured at each Ria de Aveiro sampling station: reference (R), moderately (L1) and highly mercury contaminated (L2) areas. The letters denote statistically significant differences ($p<0.05$): (a) versus R and (b) versus L1. x – levels below detection limit. Bars represent the standard error.

No significant correlations were verified between T-Hg and MT levels in the different tissues for both species. Nevertheless, in *L. aurata*, MTs content in the muscle matches with accumulated T-Hg while in the liver this association was only found for L2. Furthermore, in *D. labrax* brain, the MT content varyed inversely with T-Hg accumulation (and with exposure levels as well).

8.4 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 physico-chemical parameters able to produce changes in pollutants bioavailability and, consequently, in its 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 physico-chemical 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 MTs 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 MTs as a biomarker of exposure requires a suitable selection of the indicator species and target tissues/organs (Marijić and Raspor, 2006).

8.4.1 Tissue-specificities on MTs 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 significant distinguish 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} wet weight (EC Nº 78/2005). However, whenever the whole fish is to be consumed, the established limit must be applied to the whole fish. In accordance, *D. labrax* muscle exceeded this value, as well as liver and kidney of both species.

D. labrax response profile

MTs 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 MTs content. This is corroborated by the absence of significant correlations between T-Hg levels and MTs 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 was coincident 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 MTs synthesis modulation for each tissue, which showed to present different threshold limits. Moreover, Shimada et al. (2005) suggested that MTs 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 MTs 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 MTs 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 MTs 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 MTs. In accordance with Mieiro et al. (submitted), 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 MTs content also found no induction in *D. labrax* 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 MTs 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.

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} ww) with the highest MT content (48.4 nmol g^{-1} ww). 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., 2009), pointing out the role of hepatic MTs 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 MTs (though no significant correlation was found). This is contrary to previous findings on fish muscle that demonstrated unaltered MT gene expression or inversely proportionality 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 MTs induction was found for kidney. Navarro et al. (2009) found contrasting results, since they found induction in kidney and no induction in carp liver. These authors suggested that MT genes are constitutively transcribed in carp liver at relatively high levels and that these 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 weren't able to demonstrate that MTs bind mercury as a sequestration function, and thus, not indicating their ability in protecting against mercury toxicity.

8.4.2 Species-specificities on MTs 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 MTs 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. Despite that 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, but 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 MTs 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 presented recurrently (except for kidney) higher T-Hg loads in comparison to *L. aurata*, in the 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.

It could 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 MTs.

The understanding of the differences among species in terms of mercury toxicodynamics and subsequent response as MT induction is a difficult task, since it depends on a number of factors besides the invoked difference on feeding habits (e.g. variations in morphology, physiology, metabolism and life cycle). In this direction, a toxicogenomics approach emerges as valuable tool to achieve a more consistent

knowledge.

8.5 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 an uncapacity to induce MT synthesis in all the tissues and a depletion in brain MT content, whereas *L. aurata* showed the ability to increase MT content in liver and muscle. In agreement, *L. aurata* seemed to be more efficient than *D. labrax* in protecting tissues from mercury adverse effects and thus, a better bioindicator of mercury contamination on the basis of MT response.

- Within each species, tissue-specificities were clearly evidenced in the MT inducing potential and in the susceptibility to MT decrease, as well as in the respective T-Hg threshould limits. *L. aurata* results showed muscle as the most responsive tissue, responding to moderate (L1) and high contamination (L2) degrees, while liver only responded to high mercury contamination (L2).

- Overall, the suitability and aplicability of MT content in fish tissues as biomarker of exposure to mercury was called into question, denunciating important limitations on reflecting the metal exposure levels and the subsequent accumalation extent. Moreover, it was pointed out the incapacity of using only MT contents as a monitoring tool for assessing the environmental mercury contamination.

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CHAPTER 9

Final Considerations

9. Overview

This chapter presents an overview of the various topics dealt with in the chapters 2 to 8, carrying out an integrative discussion of the main outcomes, while their linkage to the general and specific objectives of the thesis and the contribution to the scientific knowledge in the area are also highlighted.

First of all, it is important to point out that, though the present research has filled gaps on the understanding of mercury organotropism in fish (as discussed below), its originality is mainly related with the adoption of a combined use of bioaccumulation markers (mercury organ burdens) and effect biomarkers (oxidative stress and stress proteins), together with a chemical characterization of the abiotic compartment. This approach has been proposed by Van der Oost et al. (2003) as a monitoring tool for the evaluation of the risk of contaminants for aquatic ecosystems, allowing to establish a potential causal relationship between the levels of mercury accumulated in fish tissues/organs and their ability to induce toxic effects. Nonetheless, this combined strategy is still scarcely present in the literature. In accordance, this study was performed at two intercrossed levels: 1) tissue-specific accumulation, by focusing on mercury distribution and speciation (total and organic contents) in two fish species with different feeding strategies, and 2) tissue-specific biochemical responses, by evaluating oxidative stress endpoints (enzymatic and non-enzymatic antioxidants modulation and the occurrence of peroxidative damage) and the suitability of metallothioneins (MT) in reflecting the mercury exposure levels and accumulation extent.

This research was carried out on three sampling sites with different environmental mercury loads (R - reference, L1 – moderate contamination and L2 – high contamination, Laranjo basin, Ria de Aveiro coastal lagoon), corresponding to a typical mercury contamination gradient. The environmental characterization carried out in the different survey periods throughout the investigation, consistently indicated that the mercury levels (total and reactive contents) in the water column were low in all sites and almost comparable to non-contaminated systems. Mercury associated to suspended particulate matter revealed to be the major metal portion present in the water column. Mercury contamination of the sediment compartment clearly showed the existence of an environmental contamination gradient, thereby reinforcing the assertiveness of the selected study area taking into account the stipulated goals.

Since the water physicochemical parameters can influence mercury availability and, subsequently, mercury bioaccumulation, environmental parameters like dissolved

oxygen, salinity and SPM were also evaluated and allowed to observe some differences among sites. Nevertheless, no relevant influences in the mercury accumulation at each site could be established, since mercury levels in the water column were low and thus not strongly affected by dissolved oxygen and salinity. On the other hand, mercury confirmed to have a propensity to be associated with SPM, affecting mercury dynamics in water-column.

The evaluation of mercury accumulation in several tissues of feral fish in field samples is crucial in environmental impact assessment, since it allows acknowledging, under realistic conditions, which tissues are the major targets for mercury accumulation, as well as the mercury distribution between tissues and the impact of the overall accumulation at the organismic level. Mercury bioaccumulation is firstly influenced by its availability in the environment and mercury speciation (organic and inorganic contents) is mandatory to predict the biomagnification potential, as well as the risk for both fish and humans. Mercury accumulation within a tissue not only depends on the mercury speciation, but also on a range of environmental (mercury availability), ecological (food, habitat) and biological (age, size, sex, trophic position) factors (e.g. Gaspić et al., 2000; Kehrig *et al*., 2002; Harmelin-Vivien et al., 2009; Pirraino et al., 2009). In accordance, the combination of the first four objectives of this research embodies the action line above mentioned as level 1. Consequently, the following discussion will be done in the light of those objectives regarded either individually or jointly, due to their conceptual proximity and interception.

The first two objectives of this work (considering the timeline and the rationale of the thesis) were to evaluate, in two marine fish species (*D. labrax* and *L. aurata*), whether a set of tissues (gills, blood, liver, kidney, intestine, brain and muscle) were able to reflect the degree of mercury environmental contamination, as well as to compare the mercury distribution in both species tissues, identifying the tissue and species with the best capacity to reflect the environmental contamination levels (chapters 2 to 4). The studied tissues reflected the mercury environmental contamination, since all, with the exception of *L. aurata*'s kidney, were able to reflect differences between the reference (R) and the most contaminated site (L2). That's to say, to increasing environmental concentrations corresponded increasing total Hg concentrations in the tissues. When comparing both fish species, the pattern of total Hg accumulation was similar between them, being liver and kidney the tissues with the higher total Hg loads, reinforcing the idea sustained in literature that these organs are crucial for metal accumulation and detoxification. Intestine

also revealed to accumulate high total Hg levels, confirming its function as a pathway for mercury uptake, as observed by Riisgård and Hansen (1990). Gills and blood were generally the organs/tissues with the lower amounts of total Hg. Moreover, gills revealed to be an important pathway for mercury uptake through water (low mercury levels in the water column corresponded to low levels of mercury accumulation). Muscle and brain seemed to accumulate "moderate" levels of total Hg.

Data from the literature suggest that the accumulation pattern is dependent on the tissues and on the degree of contamination (Cizdziel et al., 2003; Maury-Brachet et al., 2006; Havelková et al., 2008). Accordingly, not all tissues were able to evidence the moderate degree of environmental contamination by mercury of the sampling site L1. In both fish species, brain and muscle were able to distinguish L1 and R sites, as well as *D. labrax*'s blood. Differences between the two degrees of contamination (L1 and L2) were only reflected by *D. labrax*'s liver and *L. aurata*'s gills. These results emphasised the importance of studying several tissues and how fundamental is to compare different degrees of environmental contamination, since each tissue respond differently in accordance to the extent of contamination. These results contributed to the election of brain and muscle as the best tissues in reflecting the environmental mercury levels and thus, are recommended as an alternative to the organs/tissues traditionally used when studying mercury accumulation. The advantages of choosing these tissues relatively to liver, for instance, are the discriminatory capacity and the ability to reflect differences between reference and contaminated locations. Furthermore, brain, as a vital organ, can give an insight of the probability of risk for the studied organisms and populations.

In view of a clarification of the mercury organotropism, inter-tissue ratios were evaluated (chapters 2 and 3). These ratios are useful to compare the uptake, retention and elimination differences between species and trophic levels (Cizdziel et al., 2003). For both species, the highest ratios were tissue/blood, indicating the role of blood in transport and redistribution of mercury throughout the body. In addition, and having in mind the role of muscle in mercury biomagnification, the use of the ratios tissue/muscle, in particular for organic Hg, showed to be useful in predicting the risk to human health. The liver/muscle ratio was always greater than 1 for both species, which, in agreement with Goldstein et al. (1996), means that mercury uptake is still occurring. This outcome is related with the assumption that during environmental exposure, mercury accumulates firstly in liver than in muscle (Olsson, 1976). In addition, *D. labrax* liver/muscle ratios were lower than in *L. aurata*, possibly being closer to an inversion of the accumulation capacity, translated by

the exhaustion of liver retention capacity. This points out the liver's protective role relatively to other tissues/organs, as well as the muscle's function as mercury sink since, as stated in Wiener and Spry (1996), much of the mercury in the body occurs in the skeletal muscle, where it accumulates bound to sulphydryl groups of proteins.

In *D. labrax*, the brain/muscle and brain/liver ratios increased from the reference to the highly contaminated site, which can indicate a less protective ability of its organs relatively to brain. In *L. aurata*, the protective effect was maintained even in the contaminated sites. These results contributed to the understanding of the mercury distribution processes among tissues and, as far as our knowledge, is the first time that they have been described for marine fish.

With the previous results in mind, the next step encompassed the evaluation of the main bioaccumulation pathways, possibly responsible for the observed species-to-species differences (chapters 2 to 4). This goal was attained with the aid of organic Hg determinations, the most toxic and bioaccumulative mercury form, accomplishing the third objective of this research. In aquatic ecosystems, it is crucial to evaluate organic levels in the top consumers, as this form accumulation has been referred to depend on the food regime (Maury-Brachet et al., 2006). Organic Hg lipophilicity and its strong ability to react with the sulphydryl groups of the proteins facilitate its uptake and accumulation, by using a neutral transporter system carrier inside cells (Zheng et al., 2003). Once inside the cells, organic Hg is hardly eliminated, but it is believed that demethylation can occur mainly in the liver, and also in the brain and intestine (Riisgård and Hansen, 1990). Therefore, mercury speciation in several tissues (such as gills, liver, intestine and muscle) and in the fish intestinal contents may provide an insight of the main bioaccumulation pathways, essential for the overall evaluation of *D. labrax* and *L. aurata* as bioindicators for mercury contamination. Similarly to total Hg, organic Hg levels found in the present study were higher in *D. labrax* than in *L. aurata*. In addition, all tissues (gills, liver, intestine and muscle) and intestinal contents from both species revealed increased organic Hg concentrations towards the contamination source, corresponding to higher organic Hg burdens incorporated by the uptake routes (water and food). Liver, muscle and intestinal contents exhibited different organic Hg accumulated levels between species, with higher values in *D. labrax*. Accordingly, the accumulation of organic Hg demonstrated to be species- and site-specific (chapter 4), reinforcing the primordial importance of diet in mercury accumulation. Organic Hg in gills was low in both species, indicating that this organ acts as an interface with the water column, providing also a fast and significant

route for inorganic Hg forms, more soluble in water. The analysis of organic Hg/inorganic Hg bioaccumulation ratio also corroborated the idea that different mercury chemical forms exhibited different distribution patterns between tissues. The values of organic Hg/inorganic found in the tissues, for both species, changed from reference to the contaminated sites, indicating that, besides diet (mainly for organic Hg), an extra source of mercury contamination existed (inorganic Hg).

In pursuit of the fourth objective, the influence of factors such as seasonal variations and feeding habits, on the levels of mercury accumulated in the tissues was evaluated in both species (chapters 2 to 4 and 6). Total Hg accumulation in *D. labrax* brain showed no differences between cold and warm periods. The total Hg accumulation pattern of *L. aurata* in two distinct seasonal periods exhibited the same hierarchical ordination: liver > muscle > brain > gills, indicating that the accumulation pattern is constant throughout the year. The absolute values for each *L. aurata* tissue showed that total Hg accumulation was approximately two times higher during the warm period. This evidence strengthened the importance of seasonal variations in the mercury accumulation capacity of fish. The influence of the diet composition in the mercury accumulation pattern was also evaluated in both species. As previously stated, the diet composition and the inherent mercury load are crucial for transferring mercury from the environment to the tissues/organs. The mercury levels in intestinal contents (total and organic) were different between sites as well as between species, being higher in *D. labrax*, demonstrating that diverse feeding habits (benthonic or detritivorous) contributed to different mercury loads in tissues. When calculating the tissues' enrichment in organic Hg, defined as toxic units (TUt), it was verified that the ratio of TUt among tissues was constant between the two species (TUt *D. labrax*/TUt *L. aurata*), with the exception of gills. Liver, muscle and intestinal contents revealed a stable ratio (≈0.56), suggesting the increments of organic Hg in both liver and muscle to be in accordance with the organic Hg in the diet. This ratio demonstrated to be a promising tool, given that by analysing the dietary items it would be possible to predict organic Hg bioaccumulation (chapter 4). This outcome should be further evaluated, both under field and laboratory conditions, in order to test a set of variables and to verify if it occurs in adult stages, as well as in other species.

In accordance with the previous findings, the objective of determining which species and tissues are the better indicators of mercury contamination was addressed. Hence, it was possible to determine that both species are good indicators of mercury environmental contamination in terms of tissue burdens assessment. Nevertheless, *D.*

labrax seemed to be a better indicator, since all the assessed tissues were able to distinguish the reference from the most contaminated site, and a higher number of tissues were able to detect the intermediate degree of contamination. In addition, both the increments from R to L2 and the mercury loads in tissues were higher in *D. labrax*. The study of mercury accumulation in fish tissues under laboratory conditions is relatively well documented in the literature; in opposition, little information existed regarding mercury accumulation in feral fish. Hence, the present findings concerning mercury accumulation in *D. labrax* and *L. aurata* caught at the Ria de Aveiro contributed to fill this gap in knowledge. Moreover, the evaluation of mercury accumulation in a wide range of tissues not only indicated muscle and brain as the best indicator tissues for the assessment of mercury accumulation, but also pointed out brain as an alternative tool. Data on mercury speciation analysis revealed that muscle exerts a protective function to vital organs such as brain, and that the proportion of organic Hg in the tissues is a function of the diet, independently of the specific feeding behaviour of each species. More research should be directed towards the understanding of this process in a diverse group of tissues, namely brain and kidney.

This research contributed to better understand the mercury distribution and accumulation processes in key tissues of two fish species, helping to predict the mercury fate in fish body and, in a broader scale, its fate in food chains. However, without knowing the mechanisms of mercury induced toxicity, it is not possible to relate mercury accumulation with the fish health status, and thus to have the perception of the mercury concentrations able to induce toxic effects. In agreement, the fifth objective of this research was addressed, representing the action line previously mentioned as level 2. Therefore, reference tissues in terms of metal detoxification, such as liver and kidney, were selected for assessing the possible mercury induced toxicity. Taking into account the main findings from the precedent component (level 1), gills were also selected since data on mercury accumulation indicated that this organ, as an important pathway for mercury uptake through water, was able to reflect the environmental status and thus, was regarded as a candidate to exhibit alterations of the antioxidant/pro-oxidant status. Moreover, the finding that the brain is a valuable indicator due to its ability to reflect different degrees of contamination and its vital functions, comprised the basis for the subsequent research on the oxidative stress responses under mercury exposure.

Mercury is a redox inactive metal that challenges antioxidant defences by reacting with proteins' sulphydryl groups (Patnaik et al., 2010). Its capability of eliciting toxicity is

mainly dependent on: a) the ability to induce the formation of hydrogen peroxide, which in turn, induces the production of lipid peroxides and the highly reactive hydroxyl radicals (Lund et al., 1991); b) the reduction of the antioxidant defences due to the ability of one mercury molecule to bind irreversibly to two or more GSH molecules, which causes the depletion of GSH levels (Quig, 1998); c) the ability to inhibit key enzymes in the metabolism of GSH (GSH synthetase and GR) (Zalups and Lash, 1996) and enzymes such as CAT, SOD and GPx (Benov et al.,1990). Therefore, fish susceptibility to mercury pro-oxidative action was evaluated by studying *L. aurata*'s organ-specific responses (in gills, liver, kidney and brain) and *D. labrax*'s brain responses in two different year periods, corresponding to chapters 5 to 7.

The responses of *L. aurata* demonstrated differences among the studied organs, being representative of different sensitivities to mercury pro-oxidant action, as well as indicating tissue-specific threshold limits for mercury toxicity. In agreement, two different patterns of response were recognized, one for brain and another for gills, liver and kidney. Brain revealed susceptibility to mercury expressed as a general breakdown of all the assessed antioxidant defences in fish from both moderately and highly contaminated sites (chapter 5). Differently, liver and kidney demonstrated an adaptability of the antioxidant system (depicted on CAT activity increase) under moderate levels of mercury contamination, whereas under high mercury levels gills and kidney showed vulnerability to mercury-induced toxicity (tendency to CAT inhibition) (chapter 7). However, kidney revealed ambivalent responses, since adaptability was also demonstrated at high mercury environmental levels, expressed as GST induction. This emphasized that the studied antioxidant defences exhibited a dependency relationship from the mercury burdens that can vary with the organ. In this direction, *L. aurata*'s gills and brain, the organs with lower total Hg levels revealed higher susceptibility to mercury as observed by the decreased antioxidant levels and the absence of induction. On the other hand, liver and kidney, the organs that accumulated higher amounts of total Hg, responded by increasing the protective responses against mercury. Although the specific antioxidants profiles could be interpreted as indicative of different vulnerability of the studied organs towards oxidative damage, no LPO induction occurred in any of them, demonstrating that the total Hg levels found in the studied organs were still below the threshold limit able to induce membrane damage. In addition, an effective action of the overall antioxidant defences could have occurred involving other antioxidants besides those currently evaluated. In fact, Argawal et al. (2010) demonstrated that α-tocopherol has a protective effect against mercury toxicity,

while Patra et al. (2001) demonstrated that cystein, ascorbic acid and α-tocopherol have a protective role against metals.

These different biomarker responses observed (induction, depletion or no alteration) reinforced the need to carefully choose the set of biomarkers as monitoring tools, as well as the importance in studying several biomarkers simultaneously. This is particularly important for LPO, which was demonstrated not to be a suitable indicator when used independently.

The susceptibility of *D. labrax* to mercury toxic effects was only evaluated in the brain (chapter 6). Since seasonal non contamination-related variables, such as temperature and metabolic rates, could also affect biochemical responses, brain antioxidants and damage were evaluated during warm and cold periods. According to the obtained results, seasonal variations played an important role in regulating the antioxidant capacity of *D. labrax*'s brain. Additionally, and in accordance with the literature (Pain et al., 2007), the warm period showed to be the most critical since the brain's responses were contradictory, showing both adaptive mechanisms and signs of toxicity. During cold period, no alterations in the oxidative stress parameters were observed. The different response patterns, according to the year period, also revealed that the environmental conditions influenced the total Hg threshold able to induce or inhibit the antioxidant defences, highlighting the importance in evaluating the fish antioxidant responses at different seasonal periods.

Having in mind both species and the studied organs (gills, liver, kidney and brain), CAT revealed to be the most responsive parameter to mercury pro-oxidant challenge. This evidence confirmed CAT's activity as a very sensitive parameter against the formation of ROS (Regoli et al., 2002; Maria et al., 2009) being considered the first enzymatic defence to control H_2O_2, as previously reported for *L. aurata*'s kidney (Oliveira et al., 2010).

The identification of species-specificities in terms of antioxidants response to mercury exposure is only feasible for brain. Hence, while *L. aurata* revealed a general breakdown of the brain antioxidant responses, *D. labrax* demonstrated a more complex profile marked by toxicity signs (as CAT inhibition) and adaptability (as GR induction), indicating different toxicity thresholds, depending on the measured parameter. Taking into account the main findings on mercury toxic effects, the risk to cerebral impairments seems to be higher in *L. aurata*. Moreover, *L. aurata* seemed a better bioindicator species than *D. labrax* as the former reflected a more consistent brain response under mercury exposure and thus more trustworthy findings.

Focusing on which tissue could be the most informative, gills revealed good representativeness of the environmental status, being a good indicator to assess acute mercury contamination through water. However, brain demonstrated to be both the most susceptible and the most responsive tissue, showing a wider array of responses to mercury exposure. This is particularly relevant, since brain is the primary target for mercury toxic effects (Clarkson, 1992). Overall, the differences in tissues sensitivity corresponded to different threshold limits in which antioxidants are able to respond, reinforcing the need to establish fish tissue residues of mercury associated to toxic effects. Information regarding this subject is still scarce and limited to adult freshwater species, such as salmonids (Wiener and Spry, 1996). Moreover, the existing critical mercury levels are quite higher comparing to those found in this research. Despite that this issue is not straightforward, since critical concentrations depend on each organ's sensitivity to mercury, accumulation rates, exposure pathways and time (Wiener and Spry, 1996), this research contributed to the understanding of the harmless total Hg levels in terms of potential to induce membrane damage in *D. labrax* and *L. aurata*, as well as the levels able to inhibit key enzymes. In conformity, *L. aurata*'s kidney that exhibited the highest total Hg concentration (1.12 mg Kg^{-1} wet weight) didn't show signs of membrane damage. Another interesting finding relatively to the critical concentrations of mercury was that, for *D. labrax*, the concentrations able to induce signs of toxicity were different depending on the year period. Thus, during warm season, toxicity signs were detected with 0.08 mg Kg^{-1} wet weight total Hg, whereas during cold period no alterations were observed at 0.13 mg Kg^{-1} wet weight total Hg.

Metallothioneins (MTs) have been proposed as a specific bioindicator of metal exposure (Roesijadi, 1987). In particular, mercury has been referred as the metal with the strongest binding affinity to MTs' thiols (Elinder and Nordberg, 1985). However, the knowledge of the value of MTs induction as a monitoring tool for fish exposure to mercury is still scarce and controversial. In this research, the MTs response modulation and its suitability as an effective biomarker of mercury exposure were evaluated in both species and respective tissues (blood, gills, liver, kidney, brain and muscle), corresponding to chapter 8. *D. labrax* and *L. aurata*, as species with different taxonomic relationships and possessing diverse ecological needs, diet and metabolic activity, demonstrated differences in MTs levels. Accordingly, *D. labrax*'s brain showed a decrease in MTs levels and the remaining tissues were unable to induce MTs with the increasing mercury bioaccumulation. In opposition, *L. aurata*'s liver and muscle were able to induce MTs with

increasing mercury levels. Muscle demonstrated to be a better indicator than liver, since it distinguished both intermediate and highly contaminated sites. As a consequence, MTs content in *L. aurata* revealed to be more suitable as an indicator of mercury exposure than in *D. labrax*. Furthermore, according to the present data, the role of MTs as a monitoring tool for fish exposure to mercury is doubtful, given the inconsistent association found between the accumulated levels of mercury and the MTs content. In addition, the different species exhibited different organ thresholds in which MTs can be induced, revealing a highly specific mechanism of response. Since the use of MTs as a suitable biomarker of mercury exposure could not be demonstrated, it is highly recommended to further investigate this crucial question. In particular, the mechanisms of MTs induction by mercury and the involved signalling pathways should be better understood, since MTs induction in vertebrates can occur via differing signalling pathways, which are still unknown for mercury in marine fish. Moreover, the organic fraction present in the tissues should be simultaneously determined, since organic Hg is acknowledged as not being able to induce MTs, as well as the use of other methodologies with different quantification techniques that could offer information regarding both the metal bound to MTs and the specific isoforms.

MTs are known to be involved in many biochemical pathways, such as detoxification of xenobiotics and metals, homeostasis of essential metal ions, cell proliferation, reservoirs of essential metals for other heavy-metal binding proteins (Adams et al., 2010), as well as acting as a potent free radical scavenger (Kimura and Itoh, 2008; Adams et al., 2010). In addition, and having in mind the existent MTs data on mammals, the induction of MTs by metals can occur via several differing signaling pathways. While, for instance, Cd and Zn may induce MTs mainly via a direct MTF-1 pathway (Haq et al., 2003), some other metals seem to induce MTs indirectly, either via the pathway of radical stress, or even via pathways which are not linked neither to the direct nor to the radical stress response (Dallinger, personal communication). Therefore, as long as there is no knowledge regarding the mechanisms of MTs induction by mercury, and consequently which pathways govern MTs induction by mercury in fish, it is difficult to deduce which role MTs were playing in *D. labrax* and *L. aurata* response to mercury. Though the specificity of MTs as biomarker of exposure to metals strongly depends on the clarification of their action and subjacent induction pathways, their suitability and usefulness do not strictly rely on the identification of MTs role (e.g. binding/sequestering mercury or as ROS scavenger). In this context, the limitations pointed out by the present research in relation

to the MTs applicability relied on the limited responsiveness observed in several tissues, as well as on the difficulty to establish a causal relation between increased metal concentrations in tissues and MTs induction.

This work presented a broad approach on the mercury environmental problem in fish. The performed research contributed with new knowledge to better understand mercury accumulation and its hazardous effects in fish, and thus, to better predict the risk to fish and to the entire ecosystem. As major outcomes, the mercury accumulation in different fish species, as well as the mercury toxicological effects, were all influenced in critical aspects by the environmental mercury contamination extent. Ultimately, this work also strengthened the viewpoint that the combined use of chemical and biological measurements is a practical and suitable approach to provide information on the steady-state mercury concentrations that are able to induce toxic effects in fish. Presently, the existing regulations only refer the safety limits for human consumption; data on the levels able to harm fish and wildlife are almost nonexistent. A definition of mercury critical tissue concentrations for marine fish is then mandatory, in order to assess the individuals' health status.

Within this framework, brain came out as a highly indicative organ to study mercury contamination in fish, being highly recommended for mercury surveillance programs. The present results indicated that juvenile fish are susceptible to mercury effects, suggesting that the adults could be at risk. Ultimately, mercury surveillance programs should be undertaken in other estuarine areas subjected to mercury contamination, whose function as a nursery area for marine fish as well as several other goods and services provided by those areas, might be compromised.

9.1 Future perspectives

This work contributed to a better knowledge of mercury accumulation and distribution in tissues of marine fish. However, to improve the understanding of mercury fate inside the body (absorption, distribution, storage, biotransformation and elimination), further toxicokinetic studies are imperative and should be carried out. Furthermore, mercury speciation plays a crucial role in determining the fate and effects of mercury in the fish body. Although brain mercury speciation was unviable to be determined, efforts to fulfil this issue would be essential. Moreover, brain proteins seemed to be more susceptible to mercury toxicity than lipids; thus, more attention should be devoted to mercury neurotoxic effects.

Mercury has also been reported as an anti-mitotic xenobiotic. Thus, its effects in DNA and RNA would be added value to the perception of toxic effects of mercury in fish populations, and in a broader scale, in marine and transitional ecosystems.

Having in mind the present results regarding MTs and since the mechanisms of induction by mercury and the involved signaling pathways are not known this subject should further be evaluated.

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