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Mercury accumulation in *Liza aurata* from two southern European estuaries

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em 2008, realizada sob a orientação científica do Professor Doutor Miguel Pardal (Universidade de Coimbra) e da Professora Doutora Eduarda Pereira (Universidade de Aveiro)

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RESUMO

O principal objectivo deste estudo foi avaliar a acumulação de mercúrio em quatro tecidos de *Liza aurata* (músculo, brânquias, fígado e cérebro) em dois ecossistemas costeiros, um contaminado com o metal (Ria de Aveiro) e outro não contaminado (Estuário do Mondego). Foram capturados indivíduos de quatro classes de idade em dois locais de amostragem na Ria de Aveiro (Laranjo e Mira), um sistema historicamente contaminado por mercúrio com origem industrial, e no Estuário do Mondego, que é considerado um sistema de referência em termos de contaminação por metais pesados. Verificou-se que a concentração de mercúrio em todos os tecidos foi significativamente maior no Laranjo, em comparação com Mira e Mondego, o que está de acordo com os dados obtidos nas análises à água, sedimentos e SPM. O fígado foi o tecido que registou os níveis mais elevados de mercúrio em todos os locais de amostragem, seguido pelo músculo, cérebro e por último as brânquias. Estes resultados salientam o papel central do fígado no metabolismo dos metais pesados, e indicam que este órgão pode ser considerado adequado para reflectir contaminação do ambiente por mercúrio. *L. aurata* foi considerada uma boa espécie para ser utilizada como biomonitor neste tipo de investigações, devido às suas características. Os resultados obtidos sugerem que o consumo *L. aurata* capturada no Laranjo deve ser analisado com cautela, embora não tenha sido ultrapassada a concentração limite permitida para consumo humano.

CONTENTS

Mercury accumulation in *Liza aurata* from two southern European estuaries

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ABSTRACT

The main objective of this study was to assess mercury accumulation in four tissues of *Liza aurata* (muscle, gills, liver and brain) in two southern European coastal ecosystems, a contaminated one (Ria de Aveiro) and another no contaminated (Mondego estuary). Individuals from four age classes were captured in two sampling sites in Ria de Aveiro (Laranjo and Mira), a system historically contaminated by industrial mercury, and in Mondego estuary, which is considered to be a reference system in terms of heavy metal contamination. Mercury concentration in all tissues was found to be significantly higher in Laranjo site, compared to Mira and Mondego, what was in accordance with the data obtained in the water, sediments and SPM analyses for mercury. Liver was the tissue that registered the highest levels of mercury in all sampling sites, followed by muscle, brain, and finally gills. These results highlight the liver's central role in heavy metal metabolism, and make it suitable to reflect environmental contamination by mercury. *L. aurata* was considered to be a good species to be used as a biomonitor for this kind of investigations. Our

results suggest that the consumption of *L. aurata* captured in Laranjo must be analysed with caution, though it not exceeded the threshold concentration allowed for human consumption.

1. INTRODUCTION

Among the most important environments of the coastal zone are estuaries, which are subjected to several anthropogenic disturbances that lead to habitat modification and changes in the structure and dynamic of biotic communities (Kennish, 2002; Cardoso et al., 2008). One of the environmental problems in these aquatic systems is the contaminants that are either discharged directly into these unique environments or delivered by the rivers and streams.

The concern with contamination of aquatic systems arises as they represent unique habitats to a vast number of species, being an important support for commercial fishery and recreation activities. Coastal ecosystems very often support large urban and industrial areas and are, as a result, significantly affected by human activities (Martins, 2007).

Estuarine ecosystems are important sinks of pollutants (Amado et al., 2006), and among them metals have a special concern. The contamination of estuarine and coastal waters by metals and organometals derived from anthropogenic activities as long been considered a threat for some of the most productive and economically important ecosystems on earth (Coelho et al., 2006).

Elements like Fe, Mn, Co, Ni, Mo and Zn are considered to be essentials to the biological processes, but generally they are present only in trace

quantities, being toxic only in high concentrations (Miguel et al., 1999). Others, like Cd and Hg are considered to be non essential and toxic even at low concentrations (Joiris et al, 2000).

Among metals of environmental concern, mercury has received increasing attention due to its ubiquity, toxicity, and persistence in environment. It is a highly deleterious environment pollutant, a toxic element for all living organisms and its high mobility and lipophilicity justify the importance of environmental studies focused on it (Boening, 2000; Coelho et al, 2006). The high toxicity of mercury is related to its high affinity to the sulphide groups of host proteins. Organisms, therefore, tend to accumulate this toxic element at a higher rate than they eliminate it (Magalhães et al., 2007). The accumulation processes of these contaminants in aquatic organisms will determine, in part, the enhancement of their adverse effects on the biota (Coelho et al., 2005).

Mercury (Hg) is an element that occurs naturally in the earth's crust, through phenomena such as erosion and volcanic eruptions (Guilherme et al., 2008), but also has an origin in anthropogenic activities (Tchounwou et al., 2003). However, the anthropogenic sources of mercury are responsible for the highest environmental impact (Cardoso et al., 2008).

Mercury and its compounds are present in various common products like fluorescent lamps, thermometers, car batteries or amalgam material for dental restoration. It has been use over time in medicine, dentistry, cosmetics, but nowadays is used primarily for the manufacture of industrial chemicals or for electrical and electronic applications (Mason et al., 2000). The main sources of anthropogenic mercury emissions are coal-fired power plants, municipal and medical incinerators, commercial boilers, cement and paint production, chlor-

alkali plants, among others (EPA, 1997). Europe is a major global exporter of mercury (SEC, 2005).

Mercury is a volatile heavy metal and as such, can be re-emitted into the atmosphere from land and water surfaces repeatedly after its initial release into the environment. Consequently, much of the mercury circulating in the environment is mercury that was released decades or centuries ago, when mercury was commonly used in many industrial, commercial, and residential products and processes (EPA, 1997).

As a result of the concern around mercury pressures and impacts, it is considered a priority element in the environment protection policies. It is a priority hazardous pollutant under European Union regulations (including the Water Framework Directive; EC, 2000), under the OSPAR convention (Convention for the Protection of the Marine Environment of the North-East Atlantic) and environmental agencies across the world (Martins, 2007).

The ocean is an important sink in the global mercury cycle (Mason and Sheu, 2001). However, estuaries and coastal regions may be highly affected by anthropogenic mercury sources. These regions are directly affected by mercury transported from freshwater systems as well as direct oceanic discharges. These regions are also potentially affected to a greater degree by dissolved mercury species and particulate-bound mercury released to the atmosphere from nearby anthropogenic sources (EPA, 1997). Estuaries make a significant contribution to the mercury mass balance in local coastal environments (Laurier et al., 2003; Mason et al., 2006; Schäfer et al.,2006), being very important transition zones for the understanding of the global biogeochemical cycling of the metal.

Once released into the environment, the cycling of mercury (Figure 1) is complex and not well understood. Due to its chemical properties, environmental mercury is thought to move through various environmental compartments (atmospheric, terrestrial, aquatic and biotic), possibly changing form and species during this process (EPA, 1997). Most of the mercury discharges to environment are inorganic, in its elementary form Hg^{o} or in ionic form Hg^{2+}. In the atmosphere, generally, it is mostly in is elementary form, but the ionic form is the principal responsible for the flows between the terrestrial and aquatic compartments (Wiener et al., 2003).

Mercury, in Hg^{o} form, can stay in the atmosphere as long as one year, being able to travel large distances in the atmosphere before it is eventually deposited back to the earth in rainfall or in dry gaseous form (EPA, 1997; Wiener et al., 2003). Thus, mercury is a global problem that knows no national or continental boundaries (EPA, 1997).

In the terrestrial compartment Hg^{2+} is the dominant form, being associated with organic matter in solid matrices. In the aquatic systems, mercury can be found in the sediments, in the water column and in the organisms, existing in many forms that can be converted into each others (Wiener et al, 2003).

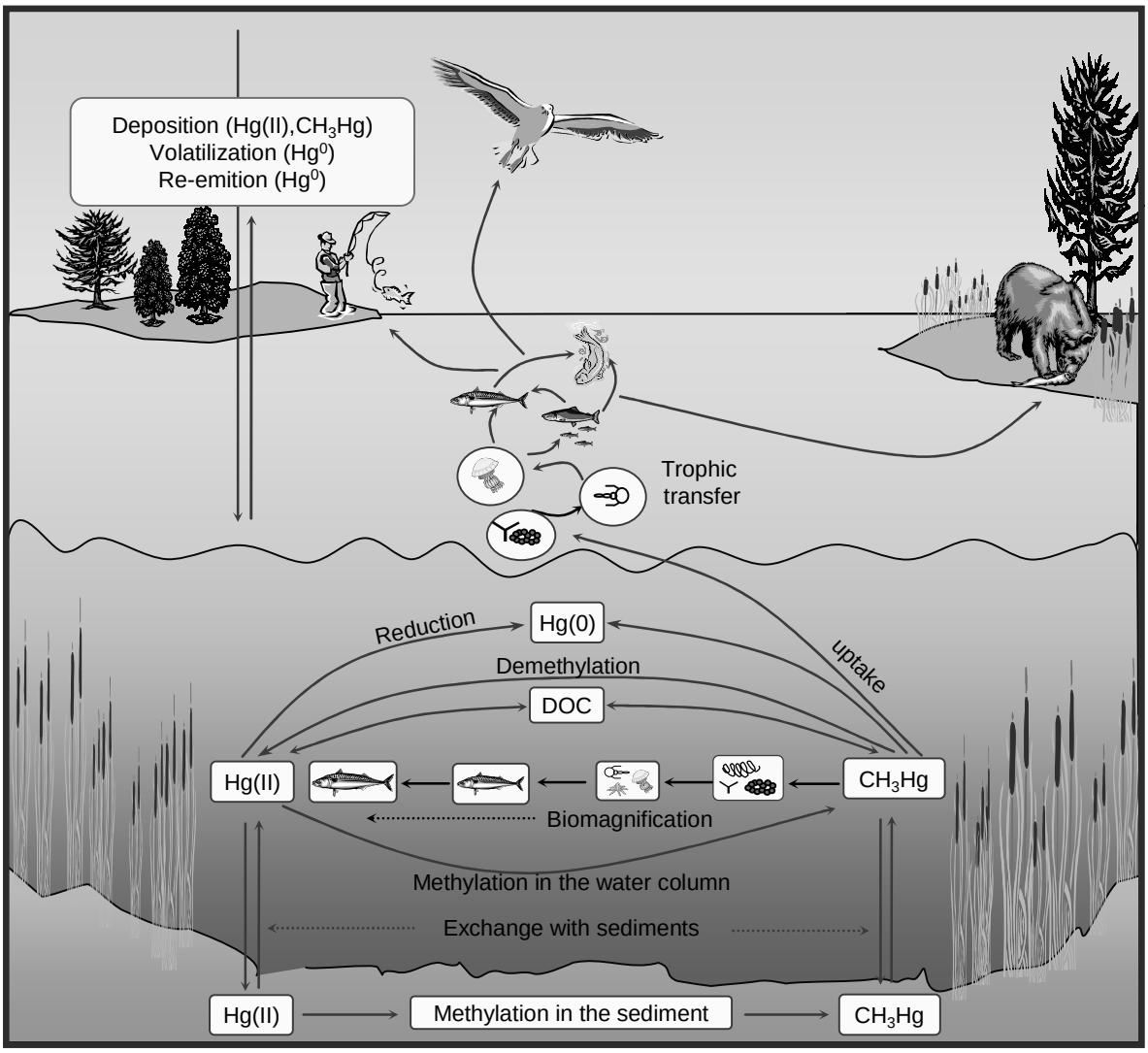

FIGURE 1: Simplified biogeochemical cycle of mercury in aquatic systems (adapted from Wiener et al., 2003)

In the water column mercury is preferentially associated to suspended particulate matter (Schäfer et al., 2006). Once the particles usually have negative charge at the surface, the adsorption of cations is favorable comparatively with the adsorption of anions, becoming a key process of mercury transport in this compartment (Pereira et al, 1998; Ramalhosa et al., 2001). Thus, over 90% of the total mercury transported from river systems is bound to particles. Much of this transported particle bound mercury appears to

be unreactive and is assumed to be buried in near shore sediments (Cossa et al., 1996).

However, sediments may act not only as sinks, but also as sources of contaminants in aquatic systems (Mucha et al., 2004) Despite of the recent settings of restrictions on anthropogenic emissions, mercury buried in sediments may be released into the water column trough disturbances inducing resuspension or changes in the physicochemical environment (redox potential, temperature, oxygen, salinity), making it available to aquatic organisms and also to transport (Martins, 2007). In addition, the transport of mercury may also occur via the activities of burrowing organisms or by higher trophic level organisms feeding on benthic invertebrates (Mason et al, 2006). The changes in mercury species resulting from sediments resuspension can also include methylmercury production (Bloom and Lasorsa, 1999).

The majority of mercury released into the marine environment is inorganic but can be converted on the methyl form by both aerobic and anaerobic bacteria (Dixon and Jones, 1994). The sulphate-reducing bacteria (anaerobic) are the primarily methylators of mercury in both lacustrine and estuarine sediments (Wiener et al., 2003). Methylmercury production also occurs at the sediment-water interface, potentially providing a way of entry to the water column and resulting in the exposure of organisms feeding at the sediment surface (Sunderland et al., 2004).

Of all the mercury compounds, methylmercury has a special interest, not only for its high toxicity, but because it also accumulates along the trophic chains (Martins, 2007), and organisms need considerably more time to eliminate methylated mercury (Rosa, 2006). The methylation occurring

preferentially in the sediments is a fundamental route in the bioaccumulation of mercury, since it results in the formation of the most rapidly accumulated species in the biota, resistant to environmental transformation processes (Martins, 2007).

Bioaccumulation involves two distinct processes. The first one, bioconcentration, corresponds to accumulation in aquatic organisms from mercury uptake from water (Rosa, 2006). The extent of bioconcentration is quantified by the bioconcentration factor (BCF) defined as the ratio between the mercury concentration in the organism tissue and the concentration in water, in equilibrium conditions (Martins, 2007). The second one is biomagnification, defined as the increase in mercury concentration caused by the transfer from a trophic level to a higher level. The biomagnification of mercury occurs even when the concentrations in the system, namely in the water, are low (Morel et al., 1998; Kehrig et al., 2002; Yang et al., 2002).

Bioaccumulation is balanced by other mechanisms, namely biotransformation and growth dilution (Rosa, 2006). Once inside the organism, mercury becomes available to biotransformation, and can be transformed into another chemical species. The biotransformation can promote the elimination, detoxification, isolation, redistribution or activation of the metal, so it can result in less toxic forms and in the case of elimination even in a decrease of mercury quantity in the organism (Martins, 2007). The concentration of mercury in an organism during its lifetime can also display a decrease known as growth dilution (Meilli, 1997), a consequence that is verified when an organism growth is faster than its uptake rate of the metal (Rosa, 2006).

The resultant concentrations of metals in biota arise from a series of complex interactions between several processes. In any particularly organism, tissue metal concentrations reflect the amount of metal taken up into the organism, the proportion of metal which is distributed in each tissue, and the extent to which the metal enters and is retained within each tissue (Brown and Depledge, 1998; Abreu et al., 2000)

Biota is an important indicator of heavy metal contamination. Fish are often used as indicators of marine pollution in coastal ecosystems since they are relatively large and easily identified (Blasco et al., 1998), having also a wide distribution and a high trophic position. Higher trophic level species assimilate mercury throughout their food webs and so reflect the environmental abundance and bioavailability of mercury, and its potential harm to ecosystems and humans (Monteiro et al., 1999).

Mercury contamination in fish is also a widespread problem, which generates important public health concerns (Lindqvist, 1991). Seafood consumption is, in fact, the main source of this toxin, which accumulates in the human body and causes damage in many of its basics systems, like cardiovascular and immune systems, and particularly in the nervous system (Dey et al. 1999). Methylmercury is a neurological poison, and its effects are particularly disturbing when exists a prenatal exposure in children, because of the high susceptibility of the developing tissues (Herreros et al., 2008).

Two main goals are usually pursued when analysing fish contaminant loads: to determine the health risk for humans, and to use fish as environmental indicators of aquatic system quality (Davis et al., 2002). Studies directed towards life span accumulation patterns, dietary effects on accumulation (Henry

et al., 2004; Magalhães et al., 2007) may grant valuable information on the bioaccumulation, biomagnification and toxicity of contaminants along aquatic food webs (Coelho et al., 2005).

Mercury uptake by marine fish is a cumulative process (Monteiro et al., 1999) resulting in higher concentrations of the metal with increasing trophic position, growth rate, fish age and food web complexity (Olsson, 1976; Lindqvist, 1991; Storelli et al., 1998; Brabo et al., 2000; Power et al., 2002). Moreover other studies show differences in mercury concentrations between pelagic and benthic species (Roméo et al. 1999; Bustamante et al. 2003; Henry et al. 2004). Animals living in close association with sediments in which they bury and from where they mainly feed are eventually more exposed to sediment-associated contamination than other fish (Storelli et al, 2007).

Fish populations can be adversely affected by the presence of mercury within their tissues, making it important to assess its distribution and subsequent retention. Mercury quantifications in tissues are, generally, the best way to gain a better understanding of the dynamics of this contaminant in the fish body (Guilherme et al., 2008). Nonessential elements like mercury are not associated with biochemical mechanisms and are not regulated within a particular tissue; therefore, concentrations of these elements within biological tissues tend to vary according to exposure (Turoczy et al, 2001).

The choice of not using a whole body perspective but studying various fish tissues is usually made because the former can provide information about contaminant bioavailability and bioaccumulation patterns but the origin and pathways of such accumulation are not easy to infer. Taking in consideration that organisms may accumulate contaminants from water, sediments or diet

(Rainbow and Phillips, 1993; Rainbow, 2002) studying their distribution and accumulation within organisms may confer an insight to specific bioaccumulation pathways. This information is essential for understanding the relative contribution of the various environmental compartments in accumulation and toxicity processes.

The chosen species of fish to use in this work is the golden grey mullet, *Liza aurata*, a member of the Mugilidae Family. The Mugilidae (Osteichthyes) is a widespread family of fish in estuaries, coastal waters and rivers (McDowall, 1988). They are very resistant to environmental stress and are found very often in impacted ecosystems. Its success is, in part, due to its food plasticity that allows them to subsist with the consumption of large variety of food items (Costa et al., 1993; Almeida, 2003). An extensive terminology to label its trophic behavior is used (Almeida 2003), like benthivorous for example, but more recently they are considered to be limnobenthofagous (Laffaille et al., 2002). Mullet are directly exposed to trace metal concentrations as a result of feeding and the ingestion of contaminated sediment and detritus (Licata et al., 2003).

Grey mullets are coastal marine and less frequently freshwater fish; some of them seem capable of spending a great part of their life in fresh or brackish water and others only some months of each year (Quignard and Farrugio, 1981). *L. aurata* thrive in a wide range of salinities, and in spite of staying in polyhaline sites during most of the year they are euryhaline (Cardona, 2006). This is a catadromous species, and always spawns on sea (McDowall, 1988).

Liza aurata is the most abundant and widespread of the four mullet species in Ria de Aveiro (Arruda et al., 1991), and is also very common in

Mondego estuary. It was considered to be a good bioindicator of mercury pollution in the Ria (Pacheco et al., 2005) considering their ecological relevance, abundance and distribution, as well as easy capture and handling. Despite the eventual seasonal migrations, these mullets present a reduced mobility radius, which also represents a favourable characteristic as a bio-indicator (Pacheco et al., 2005) and are well adapted to water salinities ranging from 0 up to 35‰. This attribute is very important when it is intended to biomonitor coastal ecosystems within a wide range of water salinity.

The main objectives of this study were: 1) to investigate the bioaccumulation pattern of mercury in *Liza aurata* tissues in a contaminated and in a non-contaminated site; 2) to select the better tissue to reflect the environmental health status of the studied ecosystems and to determine if this species is a suitable biomonitor of mercury contamination; 3) to assess if there is a human health risk due to the consumption of fish captured in the studied sites and; 4) to contribute to the assessment of the quality status of the two coastal systems studied.

2. MATERIAL AND METHODS

2.1 Study areas

This study was performed into two coastal ecosystems: Ria de Aveiro, a coastal lagoon located in Atlantic coast of Portugal (40º 38 N, 8º 44W), and Mondego estuary, 60 Km south to the Ria (40º 08 N, 8º 50 W).

Ria de Aveiro is a temperate shallow coastal lagoon (45 Km length, 10 Km wide) located on the north-western coast of Portugal. This system as an irregular and complex geometry, with four main narrow channels: Ovar, Mira,

Ílhavo and Murtosa. Water circulation is dependent on one single connection to the sea (outer boundary) and the freshwater inputs are from two major rivers, the Antuã and the Vouga. Concerning the hydrodynamic conditions the Ria de Aveiro is considered to be a mesotidal system where tides are semi-diurnal and propagate from the mouth to the lagoon's inner areas (Dias et al., 2000).

Ria de Aveiro received mercury rich effluents continuously for five decades, with origin in a chlor-alkali plant located in a chemical-complex near Estarreja. This effluents dispersed into the system and led to widespread contamination of sediments, water and biota of the area (Pereira et al., 1998a). The major impacted areas are Estarreja channel and Laranjo (a confined area of this lagoon), due to its proximity of the contamination source and semi-enclosed characteristics. Mercury storage in the system is estimated to be 33 x 10^3 kg, of which 77% are stored in these two areas (Pereira et al., 1998b). In Laranjo mercury in sediments buried at 30-40 cm depth can reach 35 μg g^{-1}, corresponding to the period of greatest industrial production.

In spite of the efficient retention of industrial mercury in sediments of Laranjo, and the fact that the industry improved the production processes (at approximately 15 years ago) leading to considerable decrease in mercury released, high mercury concentrations are still present in sediments, creating a well defined anthropogenic contamination gradient (Pereira et al, 1998a; Coelho et al., 2005). This contamination may be exported during periods of stronger tidal currents and bottom resuspension, phenomena that can increase mercury availability to organisms (Pereira et al., 1998a).

These high concentrations of mercury turned the Ria into a hotspot in terms of mercury contamination on the southwest Atlantic coast of Europe

(OSPAR, 2000). This fact and the absence of other important sources of contaminants in this area makes it a unique “field laboratory” that offers to researchers the opportunity to assess mercury toxicity under realistic conditions (Guilherme et al., 2008).

The impact of mercury contamination in Ria de Aveiro has been reported trough several studies in different compartments (biotic and abiotic). Mercury levels in water, sediments (Pereira et al., 1998b; Ramalhosa et al., 2005; Pato et al., 2008) and biota (Abreu et al., 2000; Monterroso et al., 2003; Coelho et al., 2005, 2006; Válega et al.,2008) has been assessed contributing to an integrated study of this area.

The Mondego River estuary consists of two channels with contrasting hydrographical characteristics (Marques et al., 2003). The south channel is shallower (max. 2-4 m deep, at high tide) and presents higher residence times (2-8 days). The discharge from the Pranto tributary is small and artificially regulated by a sluice according to water needs of the rice crop of the valley (Dolbeth et al., 2003). The north channel is deeper (max. 5-10 m, at high tide) and connects directly to the Mondego river, which drains a hydrological basin of approximately 6670 Km^2 with intensive agriculture activity in the lower section and large urbanized populated areas (e.g., Coimbra city) in the middle section (Pereira et al., 2005). It has lower residence times (< 1 day) and constitutes the main navigation channel. The Mondego estuary is considered to have pristine conditions referring to heavy metals (Vale et al., 2002).

Data obtained in this study were from samples collected in three different sampling sites, with different degree of contamination. In Ria de Aveiro were chosen two sampling sites: Laranjo, near the mercury source and with

recognized high contamination, and Mira channel, located in the opposite extreme of the lagoon and close to its mouth, distant from the mercury source and having supposedly a lower degree of contamination (Figure 2). The other sampling site is referred to the Mondego estuary south arm, used as a reference site for comparison purposes (Coelho et al., 2006) (Figure 2).

Ria de Aveiro and Mondego Estuary ecosystems present the same climatic characteristics. The geographic proximity and the environmental similarities between them make the biological parameters of *Liza aurata* (life time, spawning season, population structure, growth rate, etc.) quite similar. This is important because allows a direct comparison between the data obtained in each location.

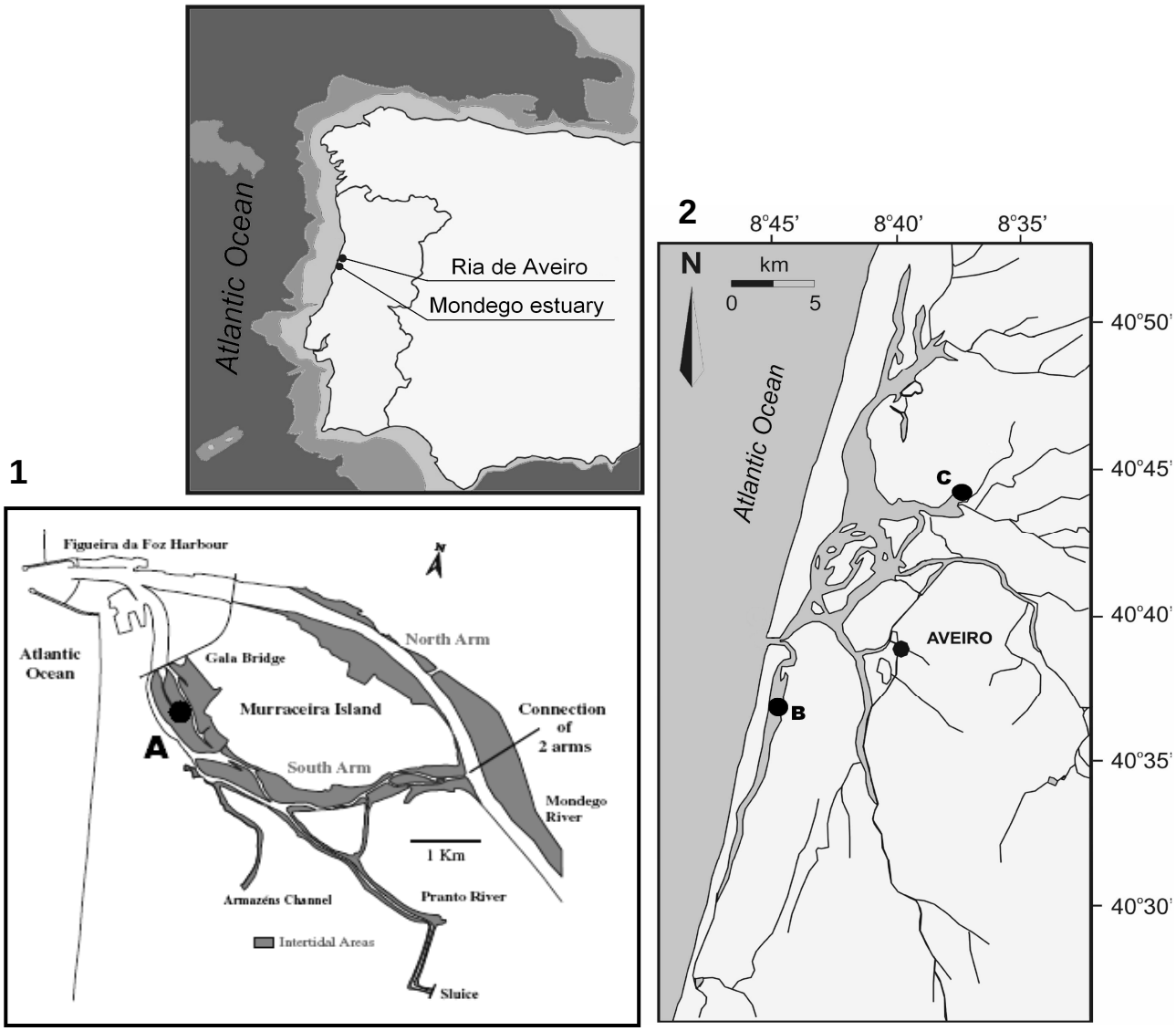

FIGURE 2: Location of the studied systems and sampling sites: (1) Mondego estuary; (2) Ria de Aveiro; (A) South arm; (B) Mira channel; (C) Laranjo.

2.2 Sampling procedures

2.2.1 *Fish samples*

Sampling occurred between September 2007 and April 2008 in two areas of the Ria de Aveiro (Laranjo and Mira channel) and in the south arm of Mondego estuary.

Fishes were captured at each sampling site during low tide, using a traditional beach-seine net named “chincha”, to catch the youngest, and a

trammel net, for older individuals. After capture samples were transported to the laboratory in a cool box, where they were measured (TL- total length; to the nearest 1mm) and weighted (total and eviscerated weight; to the nearest 0,01g). Samples were grouped in size classes, with range of 3 cm each, and dissected with tissue differentiation (muscle, gills, liver and brain), cleaned with distillate water and placed in bottles that were stored at -18º C. Sex was determined by macroscopic observation. All the captured individuals were immature.

When possible, were chosen randomly 5 specimens of each age class to store, from which 3 were analysed for mercury. 59 golden grey mullets were used in this experience, 18 collected in Mondego, 25 in Mira and 16 from Laranjo. Fish collected had a large range of sizes (10.8 to 22.5 cm in Mondego, 7.7 to 26.4 cm in Mira and 8.6 to 26.5 cm in Laranjo) and weights (11 to 22.5g in Mondego, 4 to 172g in Mira and 6 to 133.3g in Laranjo). After these samples were freeze-dried (Snijders scientific lyophilizator), homogenised, and stored until further analytical procedure. Scale samples for age determination were also collected, being removed from the flanks below the first dorsal fin.

A set of tissues from 15 individuals, covering all range of size, were weighted before being freeze-dried and weighted again before analysis to establish a latter comparison between the values of mercury concentration directly obtain in dry weight and the values established for safe consume in fresh weight. All efforts were made during the laboratory work to avoid mercury contamination between samples, what could have compromised all the work.

2.2.2 *Sediment, water and suspended particulate matter samples*

Sediment, water and suspended particulate matter (SPM) samples were collected in low tide. Sediment samples were oven-dried to constant weight at 60º C, homogenised and sieved trough a 1mm sieve before storage until analysis.

Water sample treatment and analysis were performed using ultra-clean protocols (adapted from Bloom, 1995). Ultra-pure water was obtained from a Millipore Milli-Q model 185 system. All glassware was previously soaked for at least 24 hours in a bath containing 5% Decon, then in 25% HNO_3 and finally thoroughly rinsed with ultra-pure water. After sampling, water samples were transported to the laboratory and processed within a few hours.

2.3 Analytical procedures

2.3.1 *Fish samples*

For the determination of total mercury in fish tissues, samples were directly weighted (18-120 ± 0,1 mg) into pre-cleaned boats, and mercury concentrations quantified by atomic absorption spectroscopy (AAS) following thermal decomposition of the sample, using an Advanced Mercury Analyser (LECO AMA-254). The sample is firstly dried at 120º C prior the combustion at 680-700º C in an oxygen atmosphere. The mercury vapour that is formed is collected in a gold amalgamator that after a pre-defined time (120-150 seconds) is heated at 900º C to quantitatively release the mercury. Mercury is transported to a heated cuvette (120º C) and then analysed by atomic absorption spectrometry using a silicon diode detector (more details of the methodology can be found in Costley et al., 2000). Operational conditions used included a

drying time (10 seconds), decomposition time (150 seconds) and waiting time (40 seconds).

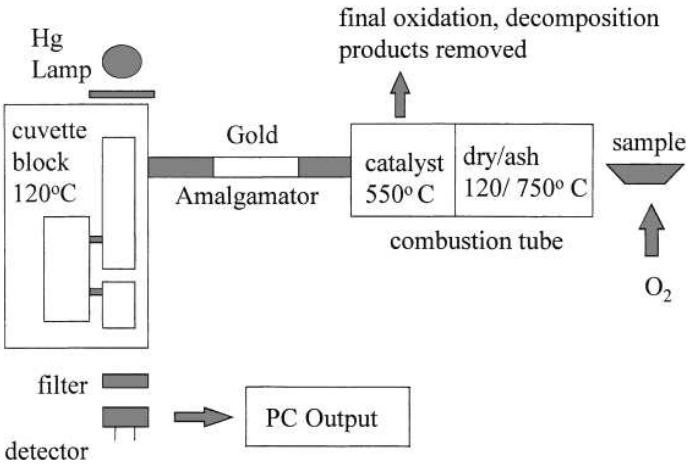

FIGURE 3: Diagram of the AMA-254 atomic absorption spectrometer (from Costley et al., 2000)

The equipment response was checked daily (in the beginning and at the end of the analysis) with analysis of a Certified Reference Material (CRM) of similar matrix of the samples, in this case lobster hepatopancreas TORT-2 obtained from the National Research Council of Canada. The results were corrected according to the daily recoveries obtained for TORT-2. This procedure intends to correct daily variation of equipment response and decay of accuracy due to poisoning of instrument catalyst. To control memory effect between samples, blank analyses were always performed between samples. Sample analyses were always performed in triplicate.

In 94% of the cases the coefficient of variation (defined as the ratio between the standard deviation and the mean) was lower than 10%, in 4% of the cases the coefficient of variation was between 10 to 20% and only in 2% of the times the coefficient of variation was higher than 20%. This latter situation represented generally low concentration or low biomass.

For fish age determination it was used the scale reading method. This is the most frequently used method for ageing Mugilidae, and consists on the examination of scales, interpreting and counting the annuli, that are defined as the boundaries between two successive growth zones and are interpreted as representing annual events (Arruda et al., 1991; Hotos, 2003).

A total of 40 individuals were analysed. For each one were chosen 10-20 scales. In the laboratory scales were placed in Petri dishes with distilled water for 20-30 minutes; the epidermis was then removed by finger rubbing. Scales were thereafter fixed between labelled glass plates for microscopical examination. For the reading it was used an optical microscope (Leitz diaplan).

In each set of scales were selected the best 6 ones, where the annuli were more clear, to read. Scales without annuli were assigned as 0+ age, those with one as 1+, with two as 2+ and so on in order to build a size-age key for the estimated ages. In order to minimize the error, all the readings were repeated twice by each reader, and each set of scales were analysed by two different readers. Only when the readings were in agreement were the counts recorded.

2.3.2 *Sediment, water and SPM samples*

Sediment quantification of total mercury was performed by atomic absorption spectroscopy following thermal decomposition of the sample, using an Advanced Mercury Analyser LECO AMA-254. The procedure is similar to the one described for fish samples. Analytical quality control for mercury quantification in sediments was performed by using the certified reference

material (CRM) IAEA-356. The values obtained for the CRM analysis were 98 ± 1%.

The water samples were filtered and the suspended particulate matter was collected on pre-weighted 0.45 µm pore size Millipore filters for mercury determinations. The variability of replicates for filtration was assessed through analysis of two replicates of each sample, analysed three to four times each; the coefficient of variation was in the range from 2 to 6%. Filters were dried at 60ºC and digested with HNO_3 4 mol L^{-1} for determination of the mercury concentration in suspended particulate matter (for detailed information on the method see Pereira et al., 1998). Dissolved mercury and suspended particulate matter mercury analyses were performed by cold-vapor atomic fluorescence spectrometry (CV-AFS) using a PSA model Merlin 10.023 equipped with a detector PSA model 10.003, with tin chloride as reducing agent (2% in 10% HCl). The method for mercury analysis in water and in SPM has a mean analytical detection limit (defined as three times the standard deviation of the blank signal) of 0.42 ng L^{-1} (n=10).

2.4 Statistical analysis

The statistical tests used were analysis of variance (ANOVA) followed by pairwise multiple comparison procedures (Newman-Keuls test) to detect differences between the three stations. Was also performed a Spearman rank correlation factor (r) for the total mercury concentrations between tissues.

Data analysis followed standard analytical procedures (Zar, 1999). All presupposes of the methods were assured (goodness of fit to normal

distribution and homogeneity of variances). Differences between means were considered significant at $p < 0.05$.

3. RESULTS

3.1 Mercury in the sediments, water and SPM

In table 1 are summarized the total mercury concentration in sediments, dissolved in the water and in the suspended particulate matter obtained for the three sampling sites.

TABLE I: Total mercury concentration in the sediment, dissolved in the water and in SPM for the three sampling sites.

Sampling site	Sediment Hg ($\mu g\ g^{-1}$)	Dissolved Hg ($ng\ L^{-1}$)	SPM Hg ($\mu g\ g^{-1}$)
Laranjo	5.2	97.8	9.0
Mira	0.2	1.0	0.6
Mondego	0.1	4.6	1.2

As we can see, Mira and Mondego have very similar values, presenting low concentrations of mercury in the sediment and in the water column. The most contaminated station is Laranjo, with higher values in these environmental compartments. Differences between Mira and Laranjo, that are located in the same coastal system, are a sign of the mercury contamination gradient present in Ria de Aveiro.

3.2 Mercury accumulation in *Liza aurata* tissues

Total mercury concentrations in fish, independently from its sampling site, varied according to the tissue in the following manner: liver > muscle >

brain > gills (Figure 4, 5 and 6). Globally, total mercury values ranged from 0.015 (correspondent to gills at Mondego) to 4.2 (liver at Laranjo) μg g^{-1} dry weight.

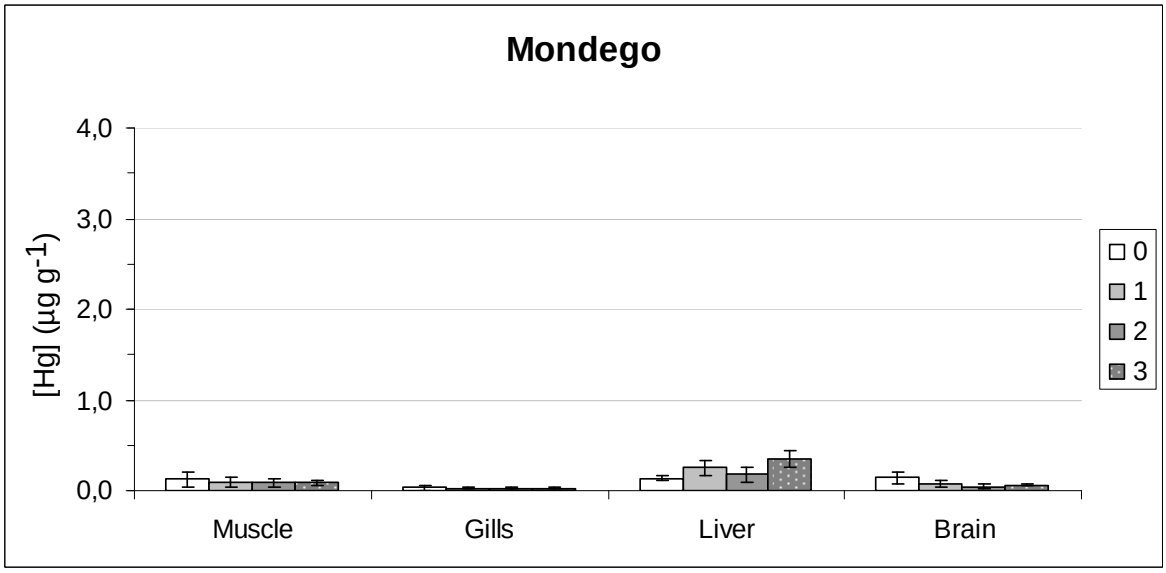

FIGURE 4: Average mercury concentration in *Liza aurata* tissues captured in Mondego (μg g^{-1} dry weight) for all age classes (indicated in the legend). Error bars represent standard deviation.

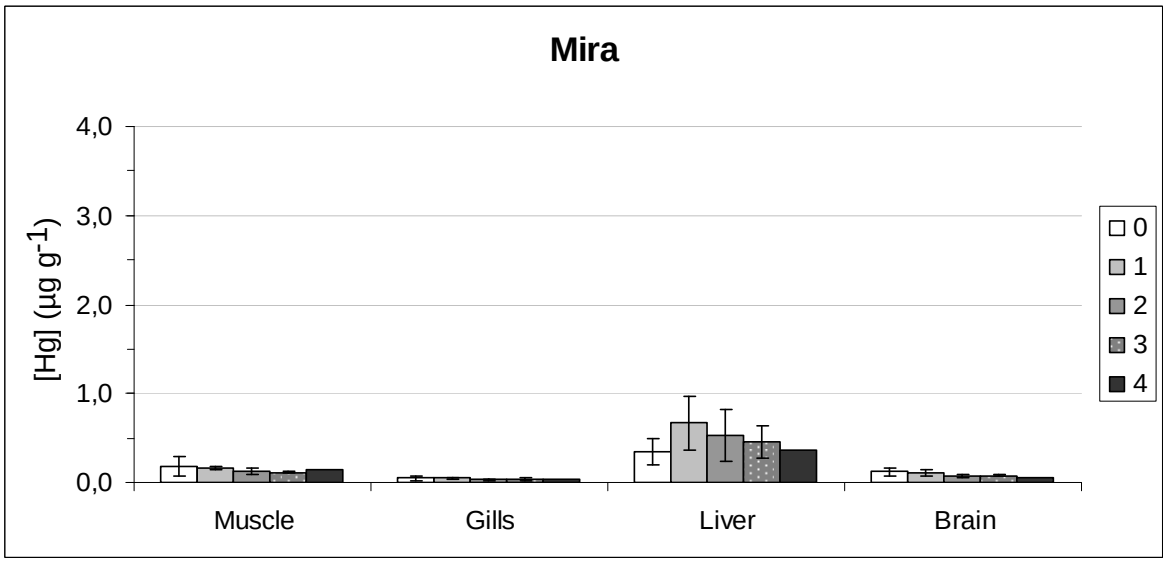

FIGURE 5: Average mercury concentration in *Liza aurata* tissues captured in Mira (μg g^{-1} dry weight) for all age classes (indicated in the legend). Error bars represent standard deviation.

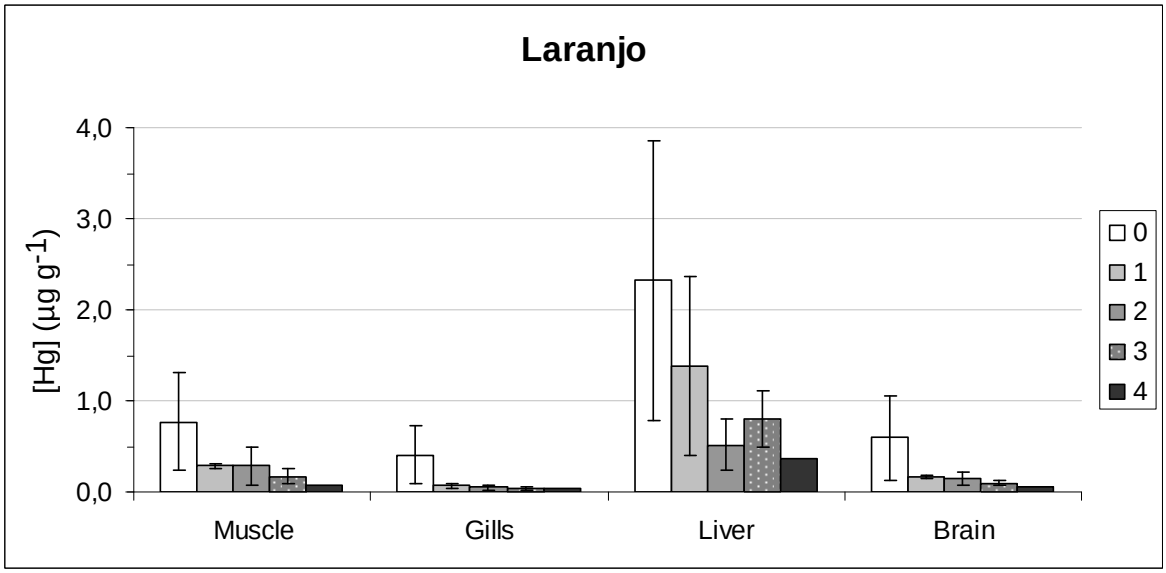

FIGURE 6: Average mercury concentration in *Liza aurata* tissues captured in Laranjo (µg g^{-1} dry weight) for all age classes (indicated in the legend). Error bars represent standard deviation.

Statistical comparisons between sampling sites made for each tissue showed significant differences between Laranjo and the other two sampling sites in all analysed tissues, muscle ($F_{2.52}$=7.619, p=0.001), gills ($F_{2.52}$=5.213, p=0.009), liver ($F_{2.47}$=10.76, p=0.001) and brain ($F_{2.49}$=4.609, p=0.014). So, in *L. aurata* from Laranjo the total mercury concentrations were higher than in the samples from Mira and Mondego, being this last the one that revealed lower contamination in tissues, but without significant differences to Mira. The existence of differences in total mercury concentrations between the age classes for each system was also tested, but no significant differences were detected.

Tissue-to-tissue ratios were achieved for all possible combinations of the analysed tissues (Table II).

TABLE II: Tissue-to-tissue ratios calculated for the combination of all tissues.

Ratio (a/b)	Sampling site	Liver [b]	Muscle [b]	Brain [b]	Gills [b]
Liver [a]	Mondego	----	2.99	4.31	10.7
	Mira		3.39	5.06	12.5
	Laranjo		4.08	6.39	15.7
Muscle [a]	Mondego	0.46	----	1.47	3.91
	Mira	0.34		1.56	3.80
	Laranjo	0.36		1.62	3.86
Brain [a]	Mondego	0.44	0.99	----	3.76
	Mira	0.27	0.73		2.62
	Laranjo	0.22	0.66		2.40
Gills [a]	Mondego	0.12	0.27	0.40	----
	Mira	0.09	0.27	0.42	
	Laranjo	0.11	0.31	0.47	

No significant differences were found between these ratios in the three sampling sites. The highest values were found for [Hg]liver/[Hg]gills followed by [Hg]liver/[Hg]brain, what reinforces the idea that the highest mercury concentration of all tissues is found in the liver and the lowest in the gills.

The performed Spearman correlation analysis showed significant positive correlations between the total mercury concentrations of all the tissues. Muscle is positively correlated with gills ($r=0.912$, $p=0.000$), liver ($r=0.715$, $p=0.000$) and brain ($r=0.689$, $p=0.000$). Gills are also correlated to liver ($r=0.747$, $p=0.000$) and brain ($r=0.687$, $p=0.000$), and liver is correlated to brain ($r=0.425$, $p=0.003$).

4. DISCUSSION

It has been found that metal accumulation in fish depends on several factors which include nature and activity of organisms, biochemical reactions of mercury inside the organisms and physicochemical variables (Abreu et al., 2000). These variables determine mercury bioavailability by controlling the

speciation, binding, release, distribution and biogeochemical pathways of mercury in the environment (Driscoll et al., 1995; Jackson, 1998). So, for the same species in different systems, even with similar properties, we can have different patterns of metal accumulation; the same happens when the studied system is the same but the fish species is different. The wide variety of biological and environmental factors has prevented that results of studies held in different contaminated areas may be extrapolated and applied in other places (Abreu et al., 2000).

Because mercury compounds are toxic and mercury is not known to perform any essential biochemical function (Bowen, 1966) its accumulation is of great concern in aquatic organisms. Non-essential elements like mercury are not expected to have its uptake and elimination actively regulated, so their tissue concentrations can vary reflecting exposure to environmental levels and feeding behaviour (Capelli et al., 2008). The absorption of this element may occur mainly through the gills by exchange with water and by ingestion (Dixon and Jones, 1994). Uptake from the diet is generally the principal route of mercury bioaccumulation in several organisms (Tremblay, 1999), and the feeding behaviour is also very important, namely if it includes contact with contaminants stored in the sediments (Chen and Chen, 1999).

The uptake of sediment-associated contaminants by fish may occur through three pathways; (1) fine particles re-suspended to water column which is uptaken by filter-feeders via gill and digestive tract, (2) leaching of sedimentary contaminants to water which can be accumulated in fish body via respiration, (3) direct contact and consumption of the sediment by bottom-dwellers/mud-eaters via skin and intestine. Owing to the feeding habit and life

style of *Liza* fish, it is reasonable to assume that they might accumulate the sediment-associated contaminants through all this three routes (Chen and Chen, 1999).

The majority of the studies carried out to determine mercury concentration in fish tissues focus only in the inorganic, and above all the organic content of the muscle, outstanding to its importance in bioaccumulation through food chains what constitutes a risk to human health (Storelli et al., 2007; Magalhães et al., 2007).

However, the study of other tissues is also important to assess fish and ecosystem health, and to provide a better understanding in mercury dynamics inside the organisms. For this reason it were selected for this study key tissues on the basis of their structural and functional properties, associated to the main processes that determine the mercury kinetics in fish body (uptake, distribution, excretion, storage and biotransformation). Besides muscle, that constitutes more than 60% of fish body, is relatively easy to obtain in significant amounts to analytical procedures and has an enhanced importance due to accumulate mercury mostly in its methylated form, were also selected the gills, the liver and the brain. Gills are considered to be the main route for uptake of mercury present in the water column (dissolved on the aqueous phase and in suspended particulate matter). Its large surface area and continuous contact with the external medium, as well as their role in excretion of toxic substances make it an organ to explore in this kind of contamination. As a neurological poison, mercury can react directly with important receptors in the brain (Berntssen et al., 2003), what makes it also an important organ to evaluate mercury accumulation. The liver is targeted due to its role in detoxification and

importance to individual fish health (Cizdziel et al., 2003), and mercury as been seen to accumulate mostly in this organ (Blasco et al., 1998; Chen and Chen, 1999; Licata et al., 2003; Usero et al., 2003; Guilherme et al., 2008). This accumulation can be due to the great tendency of mercury to associate with metallothioneines (low-weight proteins involved in trace metals metabolism) (Licata et al., 2003), whose concentration is highest in the liver (Al-Yosuf et al., 2000).

4.1 Mercury levels in the environment

As expected, the highest concentrations of mercury in sediments, water and SPM were obtained in Laranjo. The obtained values are consistent to an entrance spot of contaminated industrial effluents (Fowler, 1990). These higher values affect the mercury bioavailability to fish that is higher in Laranjo than in the other sampling sites.

In Mira the obtained values for the environmental compartments studied were much lower, what is justified by the distance to the contamination source and the proximity of marine water entrance. Mira, in fact, has been considered in several studies as a reference site for mercury contamination (Coelho et al., 2006; Rosa, 2006; Cardoso et al., 2008).

Concerning the Mondego, the total mercury concentration determined in sediments, water and SPM showed low values, what is quite understandable because no direct sources of mercury contamination are known in this system. Water levels of mercury in Mondego estuary are similar to those found in non-polluted estuaries (Davis et al., 2004).

Despite the values detected in Laranjo area, all values found were lower than 1 µg/L, the value permitted by law for mercury concentrations in the water column of aquatic systems (Guilherme et al., 2008).

4.2 Mercury accumulation in *Liza aurata* tissues

Mercury levels in all analysed tissues were significantly higher in Laranjo than in Mira and Mondego. These results are in accordance with the contamination showed in the sediments, water column and SPM of Laranjo site and definitely reveal a contamination by mercury of this fish, as a consequence of the incessant discharges into the system for long time (Pereira et al., 1998a). Previous studies have shown that several fish species captured in Laranjo are contaminated by the industrial mercury (Lima, 1986; Lucas et al., 1986; Abreu et al., 2000; Guilherme et al., 2008).

Fishes captured in Mira and Mondego showed lower mercury levels in its tissues, what is also in agreement with the lower mercury contents presented by these systems. The obtained values for all the analysed tissues in these two sites are similar, and come as a result of an inexistence of a direct mercury contamination source in both areas. These results prove than both stations can be used as reference sites regarding to mercury contamination, as it already happen in previous studies (Vale et al., 2002; Coelho et al., 2006; Rosa, 2006; Cardoso et al., 2008).

There is no evidence of mercury bioaccumulation with fish age, in any analysed tissue, on the contrary of what we would have expected. In fact, in all sampling areas was found a decrease of mercury levels with age class, in all tissues. The only exception was liver in Mondego. Also Blasco et al., (1998)

studied heavy metal accumulation in some fishes of the Mugilidae family in Cádiz Bay and verified that were not found a significant relationship between the concentration of metal in muscle and liver, and fish size.

The negative relation between mercury concentration in tissues and age class could be explained by growth dilution phenomena, or even by a possible mechanism of biotransformation or excretion. Growth dilution happens when the organism's growth is faster than its rate of absorption (Scott and Amstrong, 1972). In that case is verified a decrease of mercury concentration in fish body, not because there was a stop in mercury entry to the organism, but because the fish growth occurs at a higher rate than mercury uptake and so growth "dilutes it". Another possible explanation is related to this species life cycle. After spawning at the sea, between August and October (Almeida, 1996), the juveniles of this species begin entering into these coastal systems (Arruda, 1991). After this entry they stay inside estuaries and lagoons for a while, taking benefit of these productive ecosystems. Still, *Liza aurata* prefers polyhaline and euryhaline habitats (Cardona, 2006) and so older individuals migrate occasionally to the sea, where there is no direct source of mercury, and return to estuaries latter. This fact, allied to growth dilution, can possibly explain the decrease of mercury with the age classes in the sampling sites, because the older individuals have a low permanence these areas on the contrary of the juveniles, who spend more time in this area. Additionally, juveniles seem to accumulate mercury at a relatively rapid rate (like it was already seen in sea bass (Abreu et al., 2002)).

Independently from the sampling site, were found differences in the mercury content of the four analysed tissues, being liver > muscle > brain >

gills. Studies carried out related to heavy metal contamination in different tissues provide different accumulation patterns depending on the species and site. A study carried out by Abreu et al., (2000) also in Ria de Aveiro but using a different species, *Dicentrarchus labrax* (sea bass), revealed the same hierarchy liver > muscle > gills, and values in the liver almost doubled those in muscle. Cizdiel et al., (2003) found similar results for one of the 5 species that was used, striped bass (liver > muscle > brain > gills), which is in complete conformity with the results presented in this work. Usero et al., (2003) studied the accumulation of heavy metal in three fish species, including *Liza aurata*, and also confirmed that metal content in fish livers was considerably higher than in muscle, and grey mullet was the fish that accumulated the most metal in the liver and the least in the muscle. Guilherme et al., (2008) also found higher levels of mercury in liver (approximately 5 to 10 fold) than in muscle from *Liza aurata* captured in Laranjo.

The overall results for liver emphasize the effect of mercury exposure. This differential tissue accumulation is related to its physiological role, with liver being a main target and muscle remaining in a second line. In fact, higher accumulation of mercury in liver may be considered the primarily signal of metal exposure (Olsson et al., 1998), because it is actively involved in metabolism of heavy metals (Elia et al., 2003) acting as a storage organ (Filipović and Raspor, 2003). Looking at the tissue-to-tissue ratios we can see that in Laranjo liver displayed values around 4 times higher than the muscle, corresponding to high [Hg]liver/[Hg]muscle ratios. These results can indicate that liver is the preferential organ to accumulate mercury, and only when its retention capacity is exhausted mercury starts increasing it accumulation rate in muscle, and

possibly also in all the other tissues, seeing that all [Hg]liver/[Hg]tissue were >1. Another possible explanation for this high [Hg]liver/[Hg]muscle ratios is based in the increase of inorganic mercury content of the hepatic tissue when occurs an increase in the methylmercury exposure (Henny et al., 2002). This would indicate a higher demethylation on the liver, as well as the binding of the inorganic mercury produced to metallothionines preferentially produced in the liver (Hogstrand and Haux, 1990), and could result in high [Hg]liver/[Hg]muscle ratios (Cizdziel et al., 2003). These results are also in agreement with the statement of Maury-Brachet et al., (2006) that high [Hg]liver/[Hg]muscle ratios are typically found in benthivorous species, like *L. aurata*.

Gills were the tissue that presented lower concentration of mercury, possibly due to the high renewal rate of the branchial tissue. In fact, this epithelium is regularly subjected to exfoliation and erosion, what is counteracted by an intense cell division rate (Pacheco et al., 1993). However the importance of this organ in mercury uptake from the environment was already seen in some studies, like the one from Guilherme et al., (2008) that restricted dietary uptake of mercury in *L. aurata* by caging. The minimum value of tissue-to-tissue ratios was encountered for [Hg]gills/[Hg]liver, which can be an indication that an possible relocation of mercury stored in the liver take place at a low rate.

The fact that both [Hg]liver/[Hg]brain and [Hg]muscle/[Hg]brain were > 1 can be a sign that preferential accumulation in liver and muscle may act like a protection mechanism to the brain in reducing it exposure, since brain is the organ with higher sensibility to the adverse effects of methylmercury (Wiener and Spry, 1996).

Spry and Wiener (1991) stated that to exhibit symptoms of toxicity fish would have total mercury concentration in muscle and brain above 5 μg g^{-1}. All the measured values in *L. aurata* are very distant from that limit, so captured fish do not exhibited symptoms of toxicity.

To select the better tissue to reflect environmental exposure to mercury some aspects should be considered, like the fact that a tissue with high mercury loads amplify the evaluation efficacy and minimize the possible error associated with the detection limits of the analytical procedures. Taking this statement into account the liver seems to be the best choice, since it was the organ that exhibited higher levels of mercury, making possible a clear assessment of site contamination. On the other hand, liver is an organ with high storage propensity, and so can reflect past exposures and lead to misinterpretations. The gills, for instance, are a tissue that has a higher renewal rate and is always in direct contact to the environment, being more suitable to reflect current exposures.

An additional aspect to keep in mind on tissue selection is, according to Cizdziel et al., (2003), the possibility to be collected using minimally invasive and non-lethal methods. Therefore, muscle also emerges as a potential choice, joined to the fact that besides liver, muscle is the tissue that revealed more mercury accumulation in this study, and is available in large quantity in each sample which is important to analytical procedures.

4.3 Suitability of *Liza aurata* as a biomonitor for mercury contamination

In this study was seen that *L. aurata* was able to detect inter-sites differences in mercury contamination. Also the differences between mercury tissues loads were evident for each sampling site.

This species, as already seen, is one of the most abundant fish species in Ria de Aveiro and Mondego estuary, and are easy to catch and identify in both pristine and metal contaminated environments (Pacheco et al., 2005). Besides, its feeding behaviour (filter and mud feeder) makes it particularly adequate to study trace metals contamination in aquatic systems, because it implies a close contact and ingestion of the most contaminated components of these systems, the sediments, detritus and SPM in the water column.

Even so, it revealed to be ineffective in detecting the bioaccumulation of mercury in fish tissues with age, what can be related both with growth dilution phenomena and its life cycle. Indeed, the major limitation in the use of pelagic species for monitoring is the increased mobility of these organisms being tidal and seasonal migrations within aquatic systems common (Usero et al., 2003).

4.4 Human health risk due to *L. aurata* intake

Today there is a high concern with the risks associated with mercury exposure, especially by the health authorities, mainly due to its teratogenic effects and ability to cause irreversible neurological damage to humans (De Flora et al., 1994; Gilbertson and Carpenter, 2004). It has been so well established that most (> 95%) of the total mercury content of fresh and saltwater fish is methylmercury (Bloom, 1992). Thus, seafood consumption is a key route of methylmercury exposure for humans, depending on the diet habits

and geographical location of the populations. High intakes of fish and seafood are traditional components of the diet in different human populations worldwide, specifically in the Mediterranean diet (Herreros et al., 2008). Portugal is the European country that presents the higher fish consumption rate in European Union, and the second one in the world, after Japan (Herreros et al., 2008).

Several official regulatory agencies throughout the world have set limits for mercury concentrations in edible tissues above which fish is considered to be unsuitable for human consumption. In Europe, the European Commission set this limit at 0.5 μg g^{-1} of wet weight (decision 93/351, Official Journal of the European Communities, 1994), except for some particular species, for which it raised to 1 μg g^{-1} wet weight. The US Food and Drug Administration (FDA), for instance, set a level of 1 μg g^{-1} wet weight, while in Japan this limit is 0.4 μg g^{-1} wet weight (Storelli et al., 2005). The mercury concentration in the muscle obtained in this work varied from a minimum of 0.01 μg g^{-1} wet weight in Mondego to a maximum of 0.29 μg g^{-1} wet weight in Laranjo. Hence, the mercury values registered in the current study in *L. aurata* muscle did not exceed these limits, and therefore these fish should not be regarded as unsafe for consumption.

Nevertheless, the joint FAO/WHO expert committee on Food Additives recommends a provisional tolerable weekly intake (PTWI) of 300 μg of total mercury per person, an amount equivalent to 5 μg/kg of body weight (WHO, 2003). Considering the weekly fish consumption of the Portuguese population, an average of 1250 g (Lourenço et al., 2006), using a human body weight of 60 Kg and the mean mercury concentrations in *L. aurata* from Laranjo (0.083 μg g^{-1} wet weight), the estimated weekly intake for the Portuguese population would

be 1.729 µg/kg of body weight of total mercury, corresponding to an intake of 103.75 µg for person in a week. Subsequently, the estimated weekly intake does not exceed the established PTWI. However, some juveniles from Laranjo showed higher concentrations of mercury in muscle than the average, and if the consume are directed to these individuals the PTWI can be exceeded. In two individuals were found concentrations of 0.249 and 0.293 µg g^{-1} wet weight, and if we calculate the estimated weekly intake based in this values and in the previous assumptions, the results are 5.188 and 6.104 µg/kg of body weight of total mercury, respectively, higher than the established PTWI.

Moreover, US EPA (NRC, 2000) also defined a reference dose (RfD), the highest possible level of daily oral mercury exposure, as 0.1 µg/person kg/day (Guilherme et al., 2008). Considering an mean concentration of mercury in muscle of 0.083 µg g^{-1} wet weight), the RfD calculated for *L. aurata* from Laranjo would be 0.247 µg/person kg/day, which is more than two times higher than the EPA safe limit.

Consequently, the assumption that fish from Laranjo are suitable for human consumption must be regarded carefully, and the set limits in edible tissues above which fish represents a threat to human health must be used with caution as they not take into account the differences between the fish intake of the various populations, in order to assess a correct evaluation of the real hazard in seafood consumption.

5. CONCLUSIONS

The results of this study demonstrated that:

1) *L. aurata* tissues mercury content analysis revealed inter-sites differences, showing higher concentrations in the site historically contaminated by mercury, Laranjo, significantly different from those reported in the two reference sites, Mira and Mondego;

2) The mercury accumulation is tissue-specific and a clear hierarchy between the studied tissues was noted: liver > muscle > brain > gills. This hierarchy was similar for the three sampling sites, and the ratios between tissues are additional information that can contribute for a better knowledge of mercury dynamics in the ecosystems;

3) Liver central role in mercury metabolism in biota was reinforced, and its high mercury load and storage make it a good tissue to reflect fish and ecosystem contamination; muscle is also a good choice due to its availability in large quantity in a fish body, its high content of methylmercury and the implications in human health;

4) In spite of do not have reflected differences in mercury concentration within is tissues with age, *L. aurata* seems to be a good biomonitor of mercury contamination in aquatic systems due to its ecological relevance, abundance and distribution, easy handling and capture and resistance to environmental stress, just like its ability to indicate mercury contaminated and non-contaminated habitats;

5) The risk to human health associated with consumption of fish from Laranjo cannot be excluded, and to define set limits of mercury concentration in edible tissues taking into consideration the fish consumption rate of the populations is necessary to avoid misinterpretations.

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