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Thicklip grey mullet (*Chelon labrosus*) as a bioindicator for mercury contamination: Results from Ria de Aveiro and Mondego estuary

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)

Helena Isabel Costa de Oliveira

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Resumo:

O trabalho realizado incidiu na análise do contaminante mercúrio em *Chelon labrosus*, de forma a avaliar o estado da qualidade ambiental dos ecossistemas aquáticos, bem como o risco do consumo desta espécie para os humanos.

Para tal, mediram-se as concentrações totais de mercúrio em amostras de fígado, músculo, guelras e cérebro de *C. labrosus*. Os peixes foram capturados em três locais de amostragem, dois deles na Ria de Aveiro (um em Mira, perto da embocadura do estuário e o outro no Laranjo, localizado perto de uma fonte de descarga de mercúrio), e o terceiro no estuário do Mondego, considerado um sistema não poluído. Em Mira e no Mondego as condições ambientais apresentavam baixa contaminação de mercúrio, já o Laranjo apresentou condições ambientais onde se pode encontrar contaminação de mercúrio proveniente de actividades antropogénicas. As escamas dos peixes foram também analisadas para determinar a idade de cada indivíduo e para verificar se houve bioacumulação de mercúrio ao longo da vida.

O Laranjo foi o local de amostragem onde se verificaram os valores mais elevados de mercúrio, em comparação com Mira e Mondego, apesar de não terem sido encontradas diferenças estatisticamente significativas entre os vários sistemas. Em todos os locais de amostragem, a concentração de mercúrio foi maior no fígado do que nos outros órgãos analisados. As concentrações de mercúrio variam de acordo com o tecido do peixe, do seguinte modo: fígado > músculo > cérebro > guelras. Os valores de mercúrio nos tecidos dos peixes variam de 0.0077 (guelras no Laranjo) a 2.1 $\mu g\ g^{-1}$ (fígado no Laranjo) (peso seco). A correlação entre a concentração de mercúrio

e o tamanho do peixe foi variável e dependeu do tecido analisado e do local de amostragem, não se verificando bioacumulação de mercúrio nos três locais de amostragem. No Laranjo observou-se uma redução da concentração de mercúrio com o aumento da idade, ao contrário dos outros dois sistemas, onde os níveis de mercúrio permaneceram quase constantes com o crescimento dos peixes.

Os resultados indicam que as concentrações de mercúrio total obtidas para os músculos de *C. labrosus* nos diferentes sistemas são inferiores ao limite estabelecido como seguro para o consumo humano equivalente a 0.5 µg g^{-1}.

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Thicklip grey mullet (*Chelon labrosus*) as a bioindicator for mercury contamination: Results from Ria de Aveiro and Mondego estuary

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Abstract

The focus of this work was to evaluate mercury contamination in *Chelon labrosus* as a tool to assess the environmental health status on aquatic ecosystems, as well as the evaluation of the risk to human of the consumption of these organisms.

Total mercury concentrations were measured in samples of liver, muscle, gills and brain from thicklip grey mullet (*Chelon labrosus*). Fish were collected in three different sampling sites: two of them in Ria de Aveiro (Mira, which is located close to the estuary mouth and Laranjo located near a mercury

discharge source); the third station is located in a non-polluted system, Mondego estuary. Mira and Mondego have environmental conditions of low contamination of mercury; in the other hand, Laranjo has high contamination with the metal. The scales were also analyzed to determine the age of each fish and to see if mercury bioaccumulates along the lifespan.

Clearly higher total mercury values were observed in Laranjo comparing to Mondego and Mira, in spite of no significant difference were found between different sampling sites. In all sampling sites, the concentration of mercury was higher in liver than in other three organs. Concentrations of mercury varied according to the fish tissue, in the following way: liver > muscle > brain > gills. The values of total mercury in fish tissues ranged from 0.0077 (gills at Laranjo) to 2.1 µg g^{-1} (liver at Laranjo) (dry weight). The correlation between mercury concentration and fish size was variable and depended upon the tissue analyzed and the sampling site. Mercury bioaccumulation with growth were not seen in the three sites. In Laranjo sampling site, it was observed a reduction of mercury concentration with age increase, different from the two other sites, where the levels of mercury remain almost constant along the lifespan.

The results showed that total mercury concentrations obtained for the muscles of *C. labrosus* in different systems were below the established limit for safe human consumption of 0.5 µg g^{-1}.

Key words: Mercury contamination, *C. labrosus*, bioindicator, Ria de Aveiro, Mondego estuary.

1. Introduction

The estuarine processes are extremely important from the biogeochemical, ecological and economical point of view, but also of public health, due to contamination of aquatic natural resources. Estuaries have been considered nurseries for several significant faunal species and they are mainly located in the biggest cities of the world (Raffaelli et al., 1998; Flindt et al., 1999; Coelho et al., 2004; Lillebø et al., 2004). Also, estuarine and coastal waters are some of the most productive and economically important ecosystems. Their contamination by organometals and metals resulting from anthropogenic actions (such as human population growth, intensive agricultural practice and progressive industrialization and urbanization) has long represented a threat to the environment (Coelho et al., 2006). So, there is still a tremendous need for more research in the field of aquatic sciences as an effort to preserve the biodiversity and ecology of these incomparable areas, i.e., to maintain the high quality status of these zones.

The processes of contaminants accumulation in aquatic organisms will determine, in part, the enhancement of their adverse effects on the biota. Most of the times, estuaries and coastal zones are analyzed in the scope of its environmental quality, when suffering the consequences of anthropogenic inputs. These kinds of inputs implicate deep studies and surveys on different aspects of the interactions between compartments and contaminants, as, for example, their chemical speciation (Coelho et al., 2006).

The use of bioindicators has been recommended as an efficient methodology (Saiz-Salinas et al., 1996; Liang et al., 2004; Ugoliniet et al., 2004;

Roméo et al., 2005) that could give useful monitoring data without requiring such a complex set of studies. Trace metal content of biota has been commonly used in biomonitoring programmes of metal pollution in the marine environment since is considered to give a time-integrated measure of metal availability (Saiz-Salinas et al., 1996; Coelho et al., 2006).

Mercury contamination of aquatic systems

Mercury (Hg) is an element that has both anthropogenic and natural sources (OSPAR, 2004). It is often assumed that, in Europe, the anthropogenic deposition of mercury exceeds the natural one, which means that Europe is a major global exporter of mercury (SEC, 2005).

Several point emissions of mercury from human activities are identified as more important, mainly chlor-alkali production using mercury as a cathode, mining activities, cement production, coal and oil combustion, waste incineration (medical and municipal) and lightning industry (OSPAR, 2000). With the growth of industry and intensive agriculture, mercury has been extensively used in the production of paper, pesticides, fungicides, electrical goods, batteries and other items, which has caused high amounts of mercury to be emitted to the environment (Jian-bo Shi, 2007). However, the knowledge of its toxicity and persistence in the environment led to the search for substitutes, which originated a decrease in its use in some sectors (Simon et al., 1998).

Having in mind the important impacts of mercury in the environment, this metal is considered an extremely dangerous pollutant with highest priority concerning environmental consequences and threat to human health. Mercury

has high mobility in aquatic systems, persists in the environment, posses lipophilicity, and ubiquity, which justifies the environmental study, as it is a toxic element to all living organisms (Boening, 2000) and ecosystems. Their high toxicity is related to its high affinity to the sulphide groups of host proteins (ATSDR, 1999). Mercury has mutagenic and teratogenic effects, even though the data on the mechanisms of those effects are very sparse and controversial in the available literature (Calderón et al., 2003; Tchounwou et al., 2003).

Mercury has high affinity to particulate material, namely suspended particles, conducting to its accumulation in sediments by deposition processes. Thus, the sedimentary compartment constitutes an important source and deposit of mercury to the pore water and biota (Ramalhosa, 2001). To become available to aquatic organisms and to be transported, the mercury buried in sediments has to be released to the water column through disturbances inducing resuspension or changes in the physicochemical environment (temperature, salinity, redox potential, oxygen) (Rajar et al.,1997; Ramalhosa, 2001; Hung and Chmura, 2006). The changes in mercury species resulting from sediments resuspension can also include methylmercury production (Bloom and Lasorsa, 1999).

Total contaminant concentrations in sediments do not reflect their bioavailability to organisms, which can be affected by some variables such as particle size, sediment geochemistry or organic matter content (Rainbow, 1995; Luoma, 1996). In addition, the activities of burrowing organisms or higher trophic level organisms feeding on benthic invertebrates may be the way of bringing the mercury to the water column from the sediments (Mason et al., 2006).

The ocean is an important sink in the global mercury cycle. Recently, the biogeochemistry of mercury (Figure 1) in coastal and estuarine environments has received particular notice (Mason et al., 1996; Horvat et al., 1999; Hines et al., 2000; Conaway et al., 2003) for being one of the most complex and attractive elemental cycles, due to its influence in significant processes (Fitzgerald and Mason, 1997; Mason and Sheu, 2002). Mercury is chiefly reactive in the environment, shifting fast between the four interconnected compartments (atmospheric, terrestrial, aquatic and biotic) (Fitzgerald and Mason, 1997).

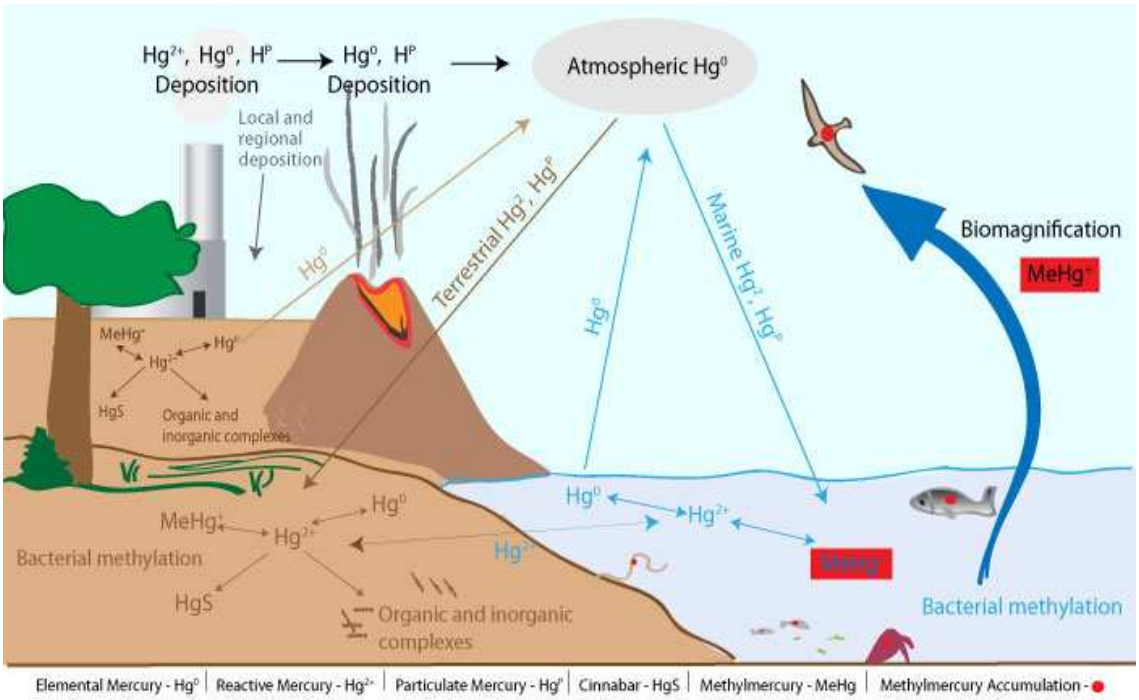

Figure 1 – Conceptual biogeochemical cycling of mercury (adapted from www.learner.org/.../vis_bytype.php?type=graphic).

The global cycle of mercury (Figure 1) is dominated by anthropogenic and natural emissions of gaseous mercury to the atmosphere subjected to long-

range atmospheric transport (Mason et al., 1994). The great part of mercury brought to the marine environment, through different pathways (as for example waste-water discharges and atmospheric deposition), is inorganic but can be converted to the methyl-form by both aerobic and anaerobic bacteria (Dixon and Jones, 1994). So, in coastal environments, the atmosphere is the main pathway for the transport of mercury between the land and the oceans, while riverine inputs, globally, are comparatively small (Cossa et al., 1996; Fitzgerald and Mason, 1997). Nevertheless, estuaries make an important contribution to the mercury mass balance in local coastal environments (Laurier et al., 2003; Mason et al., 2006; Schäfer et al., 2006). Consequently, estuarine systems are crucial transition zones for the understanding of the behaviour and destiny of mercury, with potential implications for the global biogeochemical cycle of the metal (Pato et al., 2008). Moreover, its presence and behaviour in aquatic systems is extremely relevant since it is the metal that have a higher capacity to accumulate and to increase its concentration along the trophic chain (Kehring et al., 2001).

The quantity of mercury accumulated in an organism is a function of the exposure route, availability of mercury and physico-chemical and environmental factors (e.g temperature, pH and concentration of dissolved organic carbon) (Watras et al., 1998). The bioaccumulation process consists in the sorption of contaminants in the organisms faster than its elimination. The ratios of mercury sorption or elimination are specific for each organism (Rosa, 2006).

Bioaccumulation includes two distinct processes, bioconcentration and biomagnification. The first is the accumulation in aquatic organisms by mercury uptake from water alone and the second is defined as the increase in mercury

concentration caused by the transfer from a trophic level to a higher level which, thus, amplifies the rates of bioaccumulation at the top of the chain (Rosa, 2006). The biomagnification of mercury occurs even in system with low concentrations of the metal (water for example) (Morel et al., 1998; Kehrig et al., 2002).

The requests for biomagnification include an effective uptake of contaminant by microorganisms at the bottom of the trophic chain, the retention in these organisms and finally the transfer to their predators. Decisive to the biomagnification behaviour of mercury is the fact that elementar mercury (Hg^0), Hg^{2+} and dimethylmercury are not so extensively bioaccumulated, in contrast with methylmercury, which is (Morel et al., 1998).

Summarizing, two important processes are involved in the cycle of mercury in coastal and ocean environments (Jian-bo Shi, 2007). The first is the methylation process mediated by bacteria which convert inorganic mercury into methylmercury in water and sediment systems (Tchounwou et al., 2003). This will affect the toxicity and bioavailability of the metal (Jian-bo Shi, 2007). The second one is the bioaccumulation of mercury in aquatic organisms through the food chain (Jian-bo Shi, 2007), which brings problems that come from higher methylmercury concentrations in seafood. Methylmercury is the most toxic form of mercury and is subject to high biotic bioaccumulation and biomagnification (Mason et al., 1995; Tchounwou et al., 2003). Through the processes of biomethylation and bioaccumulation through estuarine food webs, methylmercury finds its way to species usually consumed by humans (Clarkson et al., 2003; Coelho et al., 2005). Therefore mercury represents a particular threat for both aquatic wildlife and human health (OSPAR, 2000).

Examples of toxicological effects of mercury are neurological damages, growth inhibition, reproduction capacity reduction, development abnormalities and changes of responses in terms of behaviour (Wiener et al., 2003). In relation to the exposure of adult fish to methylmercury, the neurotoxicity appears to be the main effect, taking into account the observed coordination faults, feeding incapacity, lack of appetite, diminished response capacity, lethargy, abnormal movements and brain lesions (Wiener and Spry, 1996). The health effects associated to the exposure to mercury is related to the period of exposure, mercury form and exposure route (Clarkson et al., 2003). Nevertheless, studies specifically focused on mercury contamination are limited, despite being one of the most hazardous elements.

Hotspots of mercury contamination are recognized in the Portuguese coast, mainly in estuarine ecosystems such as the Tagus (Canário et al., 2003, 2005) and the Ria de Aveiro (Pereira et al., 1998; Ramalhosa et al., 2005a), while other systems keep near pristine conditions when referring to this metal, namely the Douro (Ramalhosa et al., 2005b) or the Mondego (Vale et al., 2002).

The Mugilidae (Osteichthyes) is a widespread family of fish in estuaries, coastal waters and rivers of tropical, subtropical and temperate zones (McDowall, 1988; Almeida, 1995). In contrast with other teleosteans occurring in the estuaries of temperate regions, mugilids have the benefit of using directly the food resource provided by the primary producers contributing decisively to the energy and organic matter flux in the estuarine ecosystems (Almeida, 2003).

Mugilids are resistant to environmental stress. Its success, in areas with high intervention, is in great part, due to food plasticity that allows the

consumption of large variety of food items and the subsistence at the expense of low quality food, not usable by other fish (Costa, 1993). The combination of these two conditions allows thus the reduction of intra and interspecific food competition (Costa, 1993). Therefore and despite its great capacity to adapt to polluted environments, mugilids present with other species, a critical period of their life cycle – the breeding season – during which a disturbance may cause an unbalance in estuarine ecosystems (Costa, 1993).

In this work a fish (*Chelon labrosus* from Mugilidae family) was chosen as bioindicator for mercury contamination because is relatively large, easily identified, sampled and capture in pristine and metal contaminated environments (Pacheco et al., 2005). Another reason was the trophic position and its extensive range, which makes it able to reflect aquatic contamination by persistent metals. These points and the point that are a relation (direct and indirect) among ichthyofaunal communities and human impacts on estuaries are the main reasons for the selection of this taxonomic group as a bioindicator.

As we have already seen, fish is a major pathway of environmental exposure to mercury and, therefore, the main source of methylmercury in human diet (Shimshack et al., 2007) causing negative impacts in human health, by damaging the central nervous (CNS), cardiovascular and immune systems (EPA, 2001; SEC, 2005; Jewett and Duffy, 2007; Guzzi and La Porta, 2008). So, fish are a good choice for the development of this research work, also from the point of view of human and ecosystem health risk assessment.

The main goals of this work are:

a) To study the accumulation of total mercury in different tissues (liver, muscle, gills, brain) of *Chelon labrosus* captured in different systems, with different mercury contamination (Mira channel in Ria de Aveiro and Mondego estuary which have low contamination and Laranjo area of Ria de Aveiro which has high metal levels);

b) To evaluate the value of *C. labrosus* as bioindicator of mercury pollution;

c) To determine which tissue best reflects the concentration of mercury and why;

d) To determine mercury bioaccumulation along the life of the fish;

e) To assess the environmental health status of the Mondego estuary and Ria de Aveiro;

f) To assess the risk for human health due to the consumption of fish inhabiting the studied areas.

2. Material and Methods

2.1 Study area

The study was conducted in two different aquatic systems, the Ria de Aveiro and Mondego River estuary.

Ria de Aveiro (Figure 2) was choose because it is a temperate shallow coastal lagoon (45 Km-length; 10 Km-wide), so are among the most productive ecosystems, with a diversity of habitats that must be preserved. The Ria de Aveiro is adjacent to the Atlantic Ocean and located on the north-western coast

of Portugal (40º 38´N, 8º 45´w), covering an area of approximately 83 Km^2 of wetland at high tide to 66 Km^2 at low tide (Dias et al., 2001).

The system is characterized by an irregular and complex geometry, with four main narrow channels and a significant area of intertidal zones. The channel of Ovar (25 Km) turned to northeast, which is the most extensive and profound channel, and the channel of Mira with orientation to southwest are the two major channels. The smallest channels are channel of Murtosa (8 Km) turned to east and Ílhavo (7 Km) with orientation to south (Monterroso, 2004). Water circulation in Ria de Aveiro depends exclusively on a single narrow opening in the sea and the freshwater inputs come from two major rivers, Antuã and Vouga (Dias et al., 2000). Circulation of water inside the lagoon is complex and difficult due to an extensive web of channels and islands, so it is possible that any conservative contaminants spread inside the system before they are discharged into the coastal waters through the single sea mouth (Ramalhosa, 2005).

The Vouga is the major river discharging into the Ria and has a drainage area of 2425 km^2 (Dias et al., 2000). It has an average flow of 25 $m^3 s^{-1}$, which corresponds to 60% of the freshwater discharged into the lagoon (Dias et al., 2000). Nevertheless, the freshwater contribution is small comparing to the tidal prism at the sea boundary (Dias et al., 2000). Concerning hydrodynamic conditions, the Ria de Aveiro is considered a mesotidal system where tides are semi-diurnal and propagate from the mouth to the lagoon's inner areas. The minimum tidal range is 0.6 m (neap tides) and the maximum tidal range is about 3.2 m (spring tides) (Dias et al., 2000).

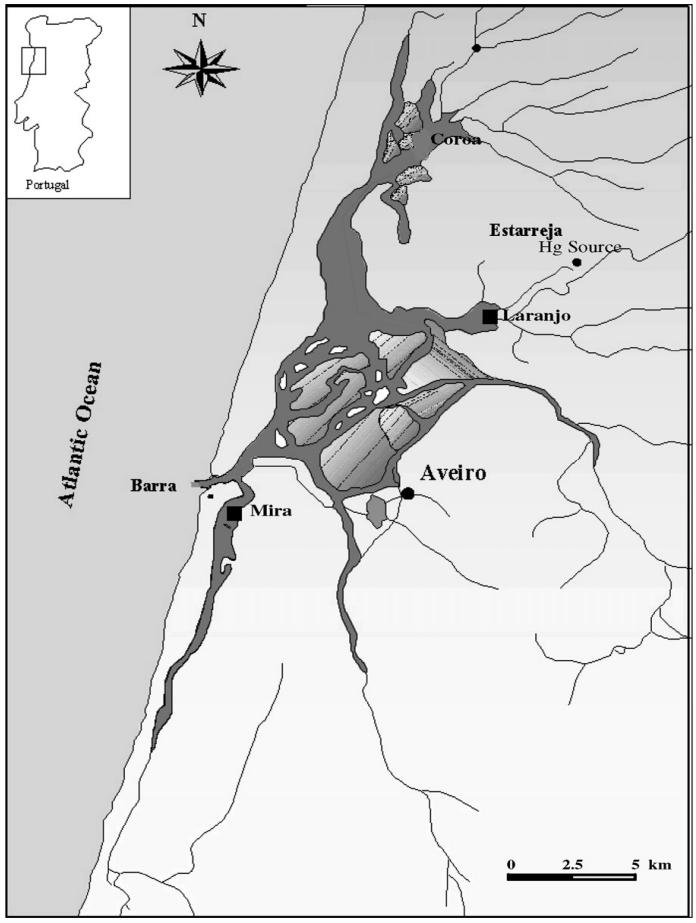

Figure 2 – The Ria de Aveiro with sampling sites (Laranjo and Mira) indicated by the square sign.

In the coastal plain around the lagoon are located a wide range industries, an exhaustive and diversified agriculture, and a population of about half a million people. Part of this population discharge their untreated or partially treated sewage into the lagoon (Lucas et al., 1986), causing pollution problems and affecting water quality. In the past five decades, Ria de Aveiro has received, in a distant branch (Estarreja channel, Figure 2) of the lagoon that ends in an inner bay of 1,5 km^2, Laranjo basin (Figure 2), incessant discharges of an effluent rich in mercury from a chlor-alkali industry. As a consequence, tons of mercury were deposited in the water, sediments and biota of the area, causing a well defined anthropogenic mercury gradient in the system (Coelho et

al., 2007). The discharges began in 1950s and in 1994 the effluent stop released. Nevertheless, high mercury concentration is still present in sediments (Pereira et al., 1998; Coelho et al., 2005). A great part of the discharged mercury is still present in the Laranjo basin, with high mercury concentrations in sediments (maximum of 35 μg g^{-1}) buried at 30–40 cm depth (Pereira et al., 1998a), corresponding to the period of greatest industrial production. The storage of mercury in the lagoon is estimated to be 33 x 10^3 kg, of which 77% are stored in the Estarreja channel and Laranjo basin (associated to the sediments) (Pereira et al., 1998). Probably decades are needed for a full system recover (Pereira et al., 1998b; Abreu et al., 1998). These high concentrations turned the Ria into a hotspot in terms of mercury contamination on the southwest Atlantic coast of Europe (OSPAR, 2000). In different compartments (biotic and abiotic), Ria de Aveiro has been reported the impact of mercury contamination (e.g. Ramalhosa et al., 2001; Coelho et al., 2005).

Two sampling sites were selected in Ria de Aveiro, as shown in Figure 2. The Laranjo basin, near Estarreja, in channel of Murtosa, corresponds to a high contaminated area, located near the mercury discharge source. Mira is located close to the estuary mouth at one opposite extreme of the lagoon (Coelho et al., 2007).

Other sampling station was selected in the Mondego estuary, 60 km south from the Ria de Aveiro. Both systems studied in this work have, in the present, the same climatic characteristics (interface among Mediterranean and Atlantic climate), which are characterized by hot summers and cold and rainy winters.

Mondego estuary was considered to have pristine conditions referring to heavy metals (Vale et al., 2002; Coelho et al., 2005) and the determined values

in terms of sediments and suspended particulate matter in previous studies enable us to use this estuary as a reference to the present study. So, this reference site was used for comparison purposes.

Mondego River estuary (Figure 3), located on the Atlantic coast of Portugal (40º 08' N, 8º 50' W) consists of two separate channels, northern and southern, separated by Murraceira Island (Marques et al., 2003).

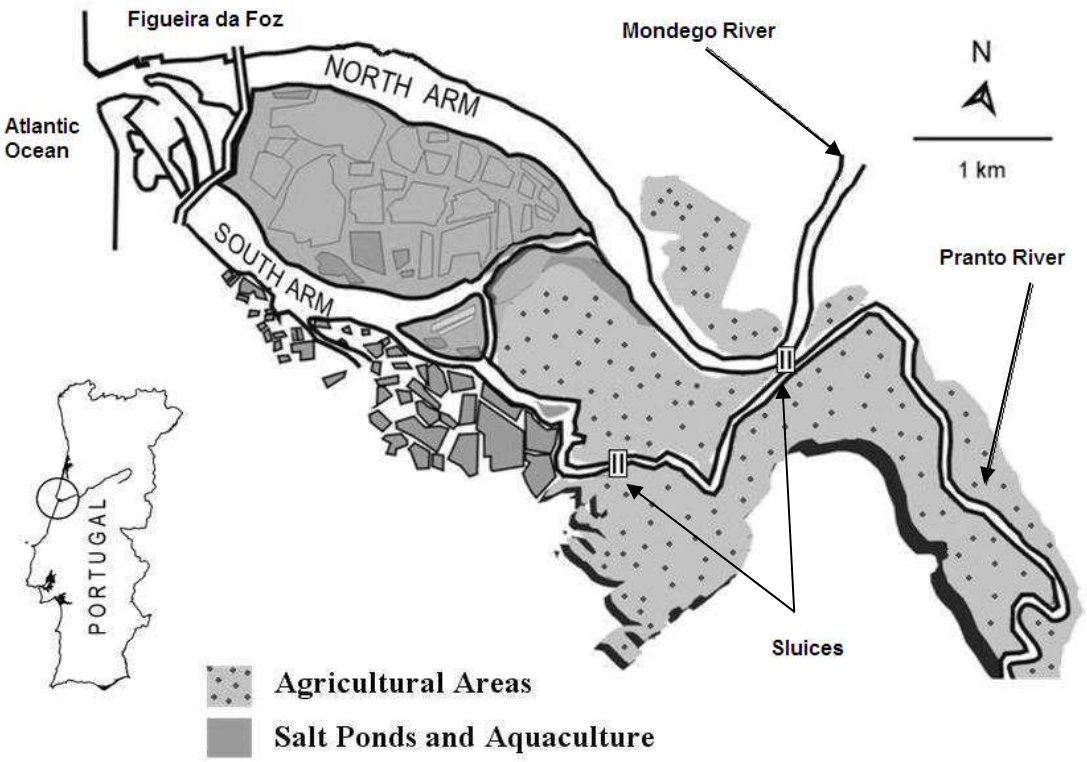

Figure 3 – Mondego estuary located on the Atlantic coast of Portugal.

The two arms show different hydrological characteristics (Marques et al., 2003). The south channel is shallower (max. 2–4 m deep, at high tide), has higher residence times (2-8 days) and is almost silted up in the upstream areas,

being the water circulation mostly driven by the tidal excursion. 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) has lower residence times (<1 day) and constitutes the main navigation channel. The northern channel is in direct connection 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. The shorelines of the north channel were artificially elevated and covered with rocks, eliminating most of the intertidal soft sediment areas. The wet area was reduced and inundation of agricultural areas by river runoff minimized. Life in this channel became, however, more sensitive to the sharp increase of river discharges occurring in spring and winter (Marques et al., 2003). Previous works (Marques et al., 1993) revealed that the bottom of the north channel consists of a mixing of coarser material transported by the river and marine sands, while fine-grain particles are deposited in the south channel.

2.2 Sampling procedures

2.2.1 Biota

83 individuals of thicklip grey mullet (*Chelon labrosus*) were collected, during low tide, in the three sampling sites (56 fishes in Mondego, 13 in Mira and 14 in Laranjo). The fish caught had different sizes that was between 14 to 57 cm in Mondego, 19 to 33 cm in Mira and 17 to 37 cm in Laranjo, and different weight wet which vary from 25 to 2296 g in Mondego; 83 to 398 g in Mira and 46 to 490 g in Laranjo.

A beach-seine net named “chincha” was used to catch smaller individuals and networks of trammel to catch older individuals. All samples were collected during the autumn of year 2007 and April 2008. After being caught, the fish were brought into the laboratory, where each individual was weighed, measured and dissected. Through the length, the individuals were separated into classes of size. Each class differed from the next class by 3 cm. Whenever possible, we store five individuals of each class for later analysis of the levels of mercury.

Liver, gills, brain and muscle of selected individuals were removed from each fish. All samples were washed with distilled water and placed in bottles of flicker. The sex was determined macroscopically by examination of the gonads after dissection. A set of 15-20 scales were taken from each fish to determine the age, observing the number of annulus. The scales were frozen.

The samples were then freeze-dried, homogenized, and weighted again for mercury fresh weight calculations. Analyses for determination of total mercury were performed.

Efforts were made during the laboratory work to keep all the material used clear enough to try to avoid the contamination of the samples that can irreversibly compromise all the work.

2.2.2 Sediments, suspended particulate matter and water

In the field, samples of sediments, suspended particulate matter (SPM) and water were also collected. Sampling was conducted in low tide situation. Sediment samples were oven-dried to constant weight at 60ºC, homogenized and sieved through a 1 mm 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 24h 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. The water samples were filtered and the suspended particulate matter was collected on pre-weighed, 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, analyzed three to four times each; the coefficient of variation (defined as the ratio between standard deviation and the mean) 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., 1998b).

2.3 Mercury analysis

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).

Total mercury levels were quantified in solid samples (biological material and sediments) by thermal decomposition atomic absorption spectrometry (AAS) with gold amalgamation, using an Advanced Mercury Analyzer (LECO

AMA-254). This methodology is simple and based on a thermal decomposition of the sample, which replaces all the delicate stage of digestion of sample (Hintelman, 1999), and based on collection of the mercury vapour on a gold amalgamator. The homogenized tissues samples (20-160 mg) were directly weighed, then placed into a nickel boat and located in a quartz combustion tube, containing a catalyst (Figure 4).

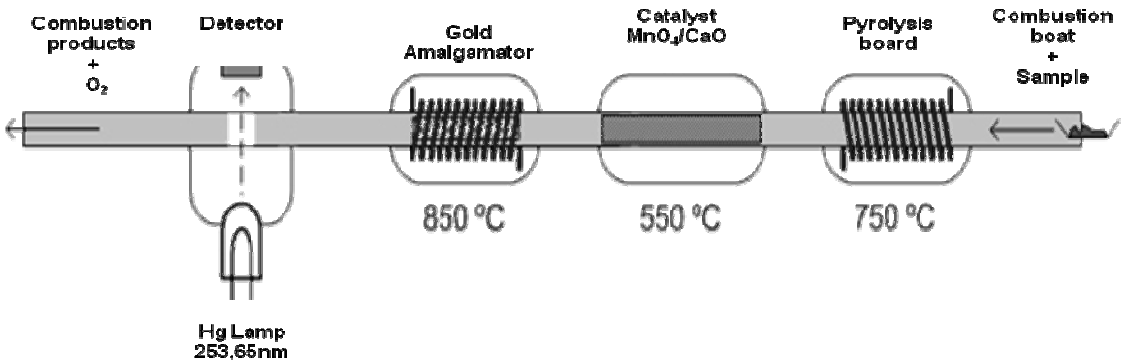

Figure 4 – Schematic representation of the methodology used in equipment LECO AMA-254 for mercury determinations.

The sample is firstly dried at 120℃, prior the combustion at 680-700℃, in an oxygen (200 mL min^{-1}) atmosphere. The gases advenient from the combustion (nitrogen and sulphur oxides, as well as halogens) are removed in a "second" oven to 550℃ (Costley et al., 2000), in a column formed by a catalytic mixture based on MnO4 e CaO. The mercury vapour is collected in a gold amalgamator and after a pre-defined time (120–150 seconds) the gold amalgamator is heated at 900℃. The released mercury is taken to a heated cuvette (120℃) and then analyzed by atomic absorption spectrometry (AAS) using a silicon UV diode detector (more details on the methodology in Costley et al. (2000), Cizdziel et al. (2002) and Haynes et al. (2006)). Operational

conditions used included a drying time: 10 seconds; decomposition time: 150 seconds; waiting time: 45 seconds. The methodology allows rapid quantification of mercury in a sample (usually a time less than 5 minutes).

The evaluation of the accuracy of the analytical methodology for total mercury determinations were made by replicate analysis of Certified Reference Materials (CRM), namely TORT-2 (lobster hepatopancreas) for biological samples and MESS-2 (marine sediment) for sediments, in parallel with samples and procedure blanks. The recovery efficiency of the analysis varied between 97-116% (defined as the difference between the certified mercury concentration and the obtained value).

Analyses were always performed in triplicate. In 91% of the cases the coefficient of variation (defined as the ratio between standard deviation and the mean) was less than 10%, 6% of the times the error was between 10 to 20% and only in 3% of the times the error was superior to 20%. This latter situation represents generally low concentration or low biomass.

2.4 Statistical analysis

Mondego was the only site in this study where immature individuals were caught, males and females. So, to test if there were significant differences between sexes, in different tissues and also to compare the concentration of mercury in different tissues and different places one way ANOVA (analyses of variance) were performed followed by the parametric Newman-Keuls test to confirmed the results. Differences between means were considered significant at $p<0.05$.

Normality and homogeneity variance tests were carried out before the

application of the one-way ANOVA test and Newman-Keuls test. Therefore, data analysis followed standard statistical procedures (Zar, 1999).

3. Results

Concentration of mercury in the environment

From the results shown in Table I, it is possible to see that mercury in the sediments, dissolved in water and associated with SPM, is similar in Mira and Mondego estuary, presenting the two sampling sites low levels of mercury on these three environmental compartments.

Table I – Mercury concentrations in sediments and water column (dissolved and associated with SPM) of 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

In Laranjo, the results show a quite different situation from the two other sites, with values up to 97.8 ng/L of mercury dissolved in water. Also samples of sediments and SPM collected in the Laranjo present higher levels of mercury comparing with Mira and Mondego.

Concentration of mercury in fish tissues

The results (Figures 5, 6, 7) showed that in the different studied sites (Mondego, Mira and Laranjo) the concentrations of mercury varied according to the fish tissue, in the following way: liver > muscle > brain > gills.

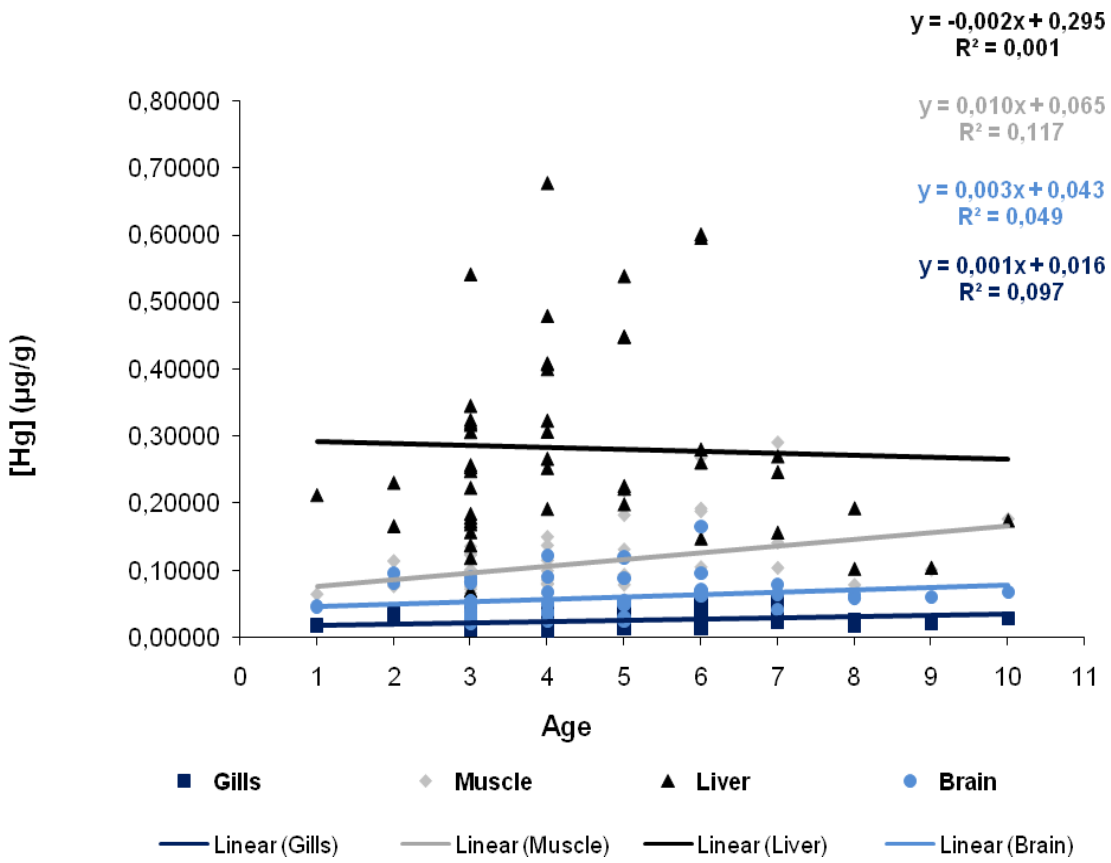

Figure 5 – Mercury concentrations (µg/g) in tissues in fishes collected in the Mondego estuary.

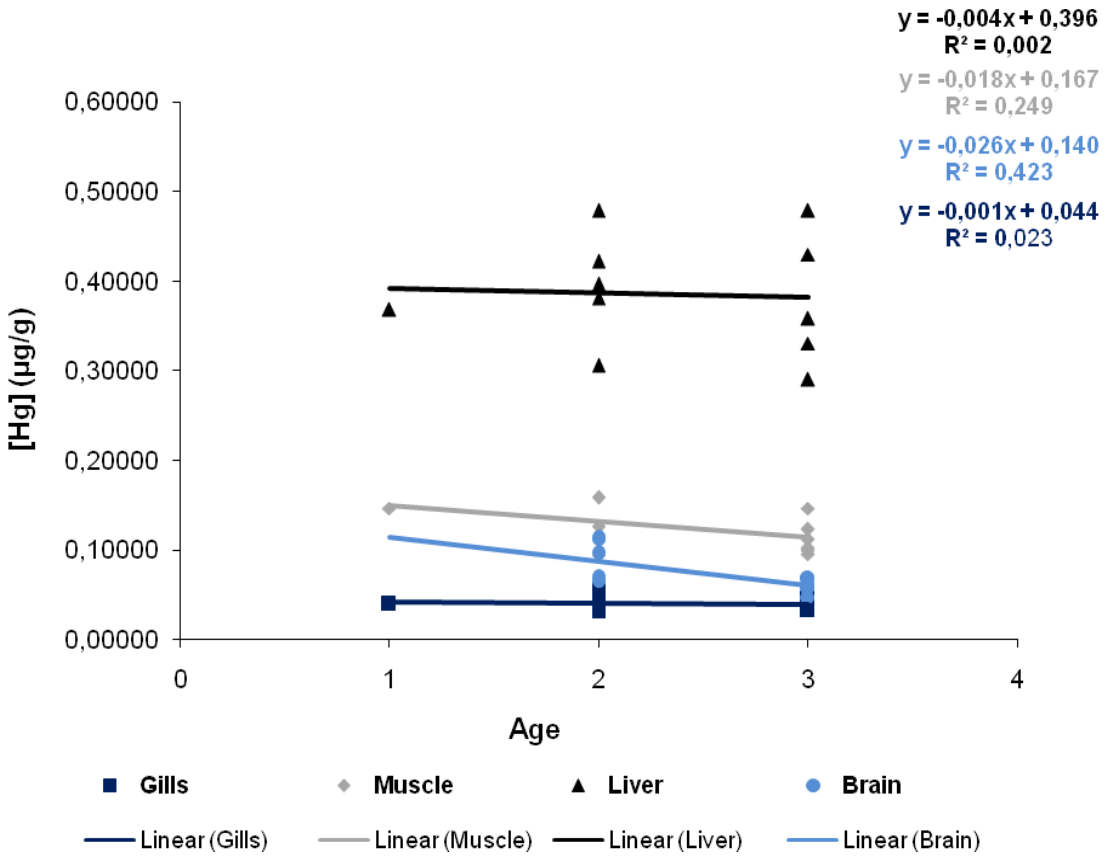

Figure 6 – Mercury concentrations (µg/g) in tissues in fishes collected in Mira.

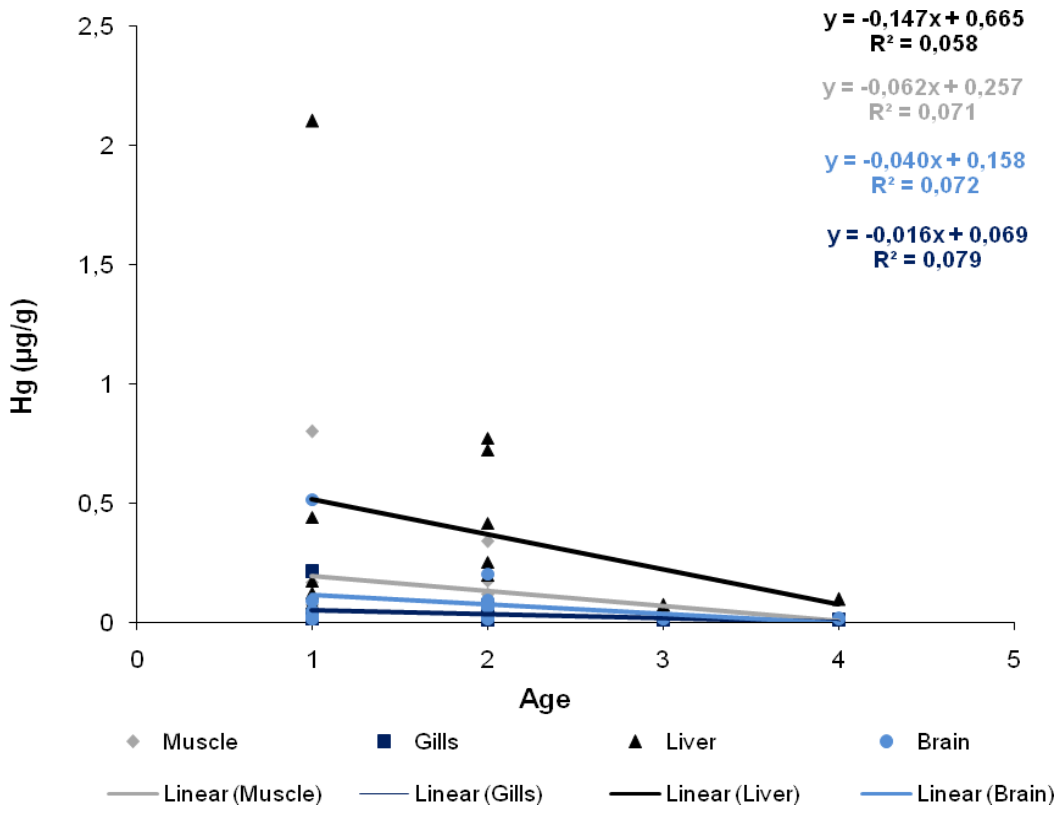

Figure 7 – Mercury concentrations (µg/g) in tissues in fishes collected in Laranjo.

The values of total mercury in fish tissues ranged from 0.0077 (Gills at Laranjo) to 2.1 µg g^{-1} (Liver at Laranjo) (dry weight). Figures 5, 6 and 7 show that there are not bioaccumulation of mercury with age (along the life span).

Comparing mercury concentration in different tissues in both sexes for samples collected in Mondego estuary, we can see that males have higher concentrations of mercury than females and immature fishes, in the muscle ($F_{2,0}$=2.79; p=0.0713), gills ($F_{2,0}$=2.38; p=0.102), liver ($F_{2,0}$=1.33; p=0.272) and brain ($F_{2,0}$=3.0059 p=0.0588). However these differences are not statistically significant.

Afterwards, we compared statistically the mercury concentration in different

tissues in the different systems and we found that Laranjo is the sampling site where the muscles ($F_{2,74}$=0.790; p=0.457), gills ($F_{2,74}$=3.575; p=0.03295), liver ($F_{2,71}$=1.540; p=0.221) and brain ($F_{2,74}$=1.318; p=0.273) of *C.labrosus* have higher concentrations of mercury with respect to the other sites, followed by Mira and finally Mondego. However, the statistical tests showed that no significant differences were found in mercury concentration in different tissues in the different sampling sites. ANOVA of one way showed, in the case of the gills, significant differences in mercury concentration in the different sampling sites. So Newman-keuls test were developed (Table II) and comproved that no differences were found in mercury concentration in gills in the different systems.

Table II – Results of Newman-Keuls test, in the case of Gills.

	Laranjo	**Mondego**	**Mira**
Laranjo	----	0.0591	0.754
Mondego	0.0591	---	0.0725
Mira	0.754	0.0725	---

Tissue-to-tissue mercury concentrations ratios were calculated for the combination of all the evaluated tissues. The average for each combination and each system was also calculated. These results are summarized in Table III and IV.

Table III – Ratios between [Hg]muscle/[Hg]tissue and ratios [Hg]gills/[Hg]tissue for the three sampling sites (values showed are an average of all values of the each combination).

	Muscle/Gills	Muscle/Liver	Muscle/Brain	Gills/Muscle	Gills/Liver	Gills/Brain
Mondego	4.55	0.45	2.02	0.24	0.10	0.45
Mira	3.24	0.33	1.64	0.32	0.10	0.54
Laranjo	3.29	0.37	1.91	0.33	0.12	0.62

Table IV – Ratios [Hg]liver/[Hg]tissue and ratios [Hg]brain/[Hg]tissue in the three sampling sites (values showed are an average of all values of the each combination).

	Liver/Muscle	Liver/Gills	Liver/Brain	Brain/Muscle	Brain/Gills	Brain/Liver
Mondego	3.00	13.36	5.59	0.58	2.45	0.24
Mira	3.51	10.94	5.81	0.69	2.20	0.22
Laranjo	2.93	9.47	5.36	0.57	1.85	0.20

Tables III and IV allow to verify that the average of the ratios between tissues is similar in the three sampling sites, and in fact no significant differences were found. The ratios highest values were determined for [Hg]liver/[Hg]tissue, being the higher value found for the ratios [Hg]liver/[Hg]gills, followed by the ratios [Hg]liver/[Hg]brain.

4. Discussion

Decision 2455/2001/EC, published in 2001, November the 20th, in order to correct directive 2000/60/EC, classifies mercury and its compounds as priority hazardous substances (Rosa, 2006). This metal is not known for having any function on metabolic processes (Wiener and Spry, 1996). So, mercury for being a non-essential element, is not supposed to have its uptake/elimination actively regulated (Capelli et al., 2008). Therefore, concentration found in different tissues can vary greatly and in some organisms may reach high values. This level of concentration reflects exposure to environmental levels and feeding behaviour (Capelli et al., 2008). However, the uptake from the diet is generally the principal route of mercury bioaccumulation in several organisms, being the elimination rates from tissues slow (Tremblay, 1999).

Accumulation of mercury is of great concern in aquatic organisms and its quantity in the body of individuals give indications of aquatic pollution, as well as indications of its potential impact on organism health (Kotze et al., 1999). But, the metal distribution in body depends, not only from the fish species, but also from metal properties (Gaspic et al., 2002).

Many studies were carried out to analyze mercury concentrations in fishes, but most of them only reviewed one tissue of fish. In this work we selected more than one organ (four tissues), once they provide additional information on accumulation and detoxification mechanisms on a broader view and to try to understand the dynamics of mercury in fish body under field conditions. The anatomical structure of organs and its functional properties and subsequent association to processes like uptake, storage, depuration/excretion and biotransformation, main processes that determine the mercury kinetics in fish

body, are the main reasons for the choice of different organs selected for the study.

The statistical tests allow to see that in Mondego there aren't significant differences regarding the concentration of mercury found in different tissues studied when comparing different sexes. So, no differences were found between males, females and immatures in terms of quantity of mercury. Thus, mercury uptake seems to be independent of differential physiological responses between sexes and reproductory condition, and if bioaccumulation had happened, it would probably follow a similar pattern irrespective of gender.

Though no statistically significant were found between sampling sites regarding mercury concentration in different tissues, clearly higher total mercury values were observed in Laranjo. These higher values in different tissues in Laranjo are related to the particularly high total mercury concentration in the entrance spots of contaminated industrial effluents as we can see in Laranjo (Fowler, 1990). Just gills presented levels of contamination lower probably due to depuration process (explained below). Previous studies have shown that several fish species captured in Laranjo are contaminated by the industrial mercury (Lima, 1986; Lucas et al., 1986). It is known that concentrations of elements like mercury within biological tissues tend to vary according to exposure (Turoczy et al., 2001) and that the increase of body size is associated to the age increase and thus more exposure (Rosa, 2006).

As Laranjo was the station which presented more concentrated fish tissues and received incessant discharges for long time from an effluent rich in mercury from a chlor-alkali industry, we expected, that with fish growth, the mercury levels would increase. However, our results showed that with the growth of

individuals, the levels of mercury decreased. Thus, there was not bioaccumulation of mercury with age. Also, Blasco et al. (1998) when studied heavy metal in some fishes of the Mugilidae family in Cádiz Bay, showed that, in all cases, concentrations falls as fish size increased. So, the correlation between metal concentration and fish size was variable and depended upon the species and tissue analyzed (Blasco et al., 1998).

Probably, a response to these results can be an effect of dilution of mercury concentration with age. Scott and Armstrong (1972) defined growth dilution as a phenomenon that happens when the organisms' growth is faster than its rate of sorption. Therefore, mercury concentration in organisms is counterbalanced by the growth dilution (Scott and Armstrong, 1972).

It is known that a number of factors affect the susceptibility of aquatic organisms to mercury. One of them is life-cycle stage (Boening, 2000). This factor can also be responsible for the decreased mercury concentration with age in Laranjo. According to its life cycle, the juvenile fishes of *C. labrosus* initially go to estuary, where they stay a while, then start migration to the sea to reproduce, between December and February (Almeida, 1996). Bograd (1961) also said that *C. labrosus* support fresh water during its juvenile state but the older preferred brackish water or sea. So, *C. labrosus* prefers more saline areas of the estuary and adjacent coastal zones (Almeida, 1996). Thus, the permanence of *C. labrosus* in coastal zones during adult state, contribute for fish not to stay too far away from estuary, having the chance to occasionally return to it (Almeida, 1996). Probably, it was in this time that we captured the few fish with bigger size in Laranjo, which in turn, had their tissues less concentrated, possibly due to a lower permanence in Laranjo than the juveniles,

which had a time of greater permanence. In fact, when migrated to sea - where there isn´t a source of contamination like in Laranjo – fish deviate from the source of contamination, and when they enter the Ria they are less contaminated. So, the phenomena of growth dilution, the life cycle of *C. labrosus,* as well as a possible mechanism of excretion, are all possible responses to reduction of mercury with age increase in Laranjo sampling site.

Mercury concentration found in different tissues of fish from Mondego estuary sampling site demonstrated low values and the tendency of statistical tests showed that this place is the less contaminated one, comparing the three sampling sites. It is understood because there aren´t known direct sources of entry of this contaminant in the Mondego estuary and waters from this estuary have total mercury concentration from the same order of magnitude from the concentrations found in not polluted estuaries (Davis et al., 2004). This is probably the reason for the absence of mercury bioaccumulation with organism growth in different tissues. Thus, mercury concentration in different tissues was almost similar in both juvenile and older individuals.

The values found in Mira in Ria de Aveiro are also low and very similar to those found in Mondego. These results are also easy to understand because Mira sampling site is also referred as reference site (Rosa, 2006) and also in this case none direct source of contamination is known to be close to the sampling site. In fact, the reason for the low values and absence of bioaccumulation with growth are the same in Mira and Mondego.

Also Blasco et al. (1998) showed that no significant relationship was found between the concentration of metals in muscle or liver, and fish size. Similar results have been reported by other authors (Phillips, 1980). Our results

showed that a single tendency for mercury was not seen in the three sampling sites.

From the analyses of the total mercury concentration in different environmental compartments along different sampling sites, we could see that mercury in the sediments, dissolved in water and associated with SPM in Laranjo present higher levels of mercury comparing with Mira and Mondego. This difference probably affected the mercury bioavailability to fish. Thus, the great availability of mercury for fish was found in Laranjo, which is in accordance with our results. Though no significant differences were seen in different sampling sites, we observed differences between tissues. The hierarchy of the analysed tissues, in the three sampling sites, according to total mercury concentration, was liver > muscle > brain > gills, as already seen. The few field studies that determined the concentration of mercury in different fish organs showed heterogeneous accumulation patterns, which depend on species. Cizdziel et al. (2003) analyzed five species and in one of them (striped bass), the same tissues were analyzed showing the same order found in our results.

Levels in liver were much higher than values recorded in the other fish parts that have a direct influence from the environment. These high levels of mercury in liver, in three sampling sites, happen because liver is the major target organ of accumulation of mercury which occurs because it is actively involved in metabolism of heavy metals (Elia et al., 2003) acting as a storage organ (Filipović and Raspor, 2003) and, in this case, in storage of mercury in fish. Liver also has an important role in basic physiology (Elia et al., 2003).

Gills exhibited less mercury concentration in the three sampling sites. Gills analysis can be mainly recommendable for species in migratory stages or with high mobility, since gills usually reflect current exposures. Moreover, more quiescent organs with high storage tendency can reflect past exposure and, therefore, increase the risk of misinterpretations.

Concentrations higher than 5 µg g^{-1} in brain and muscle generally show symptoms of toxicity in fish, according to Spry and Wiener (1991). In fact, the levels analyzed in the tissues of *C. labrosus*, are below that limit in the three sampling sites studied.

Comparing all ratio [Hg]tissue/[Hg]tissue, we verified that all cases are different from 1, from which we also concluded that different tissues accumulate mercury in different ways and loads.

The process of uptake, retention and elimination of mercury in fish has been studied by determination of mercury concentration on analyzed tissues compared with muscle (Cizdziel et al., 2003). This [Hg]tissue/[Hg]muscle ratios are very important because muscle is the largest compartment, representing 60% of fish body mass. So, it is an important tool concerning mercury accumulation assessment. Muscle is also the easiest tissue to access for sampling purposes, where a significant amount of tissue can be used. In addition, it is known that mercury accumulates on muscle, mainly in the methylated form (Storelli et al., 2005; Magalhães et al., 2007), which is highly relevant regarding bioaccumulation along food chains and the risk to human health. So, this organ is considered as the reference tissue for biomagnification effects (Cizdziel et al., 2003).

When we compared mercury levels in liver with different muscle analyzed, we verified that liver presented levels of mercury (< 0.45 µg g^{-1}) around 3 times higher than the muscle (< 0.15 µg g^{-1}), corresponding to high [Hg]liver/[Hg]muscle ratios. The high [Hg]liver/[Hg]muscle ratios were previously reported in other fish species environmentally exposed (Abreu et al., 2000; Raldúa et al., 2007). This can be explain taking into account that liver has a central role in mercury accumulation, playing a buffering role, i.e., when retention capacity of liver is exhausted, this metal is able to bypass to skeletal muscle and, as a result, the accumulation rate of mercury in this organ starts increasing. Moreover, the same type of action takes place in other studied tissues where the respective [Hg]liver/[Hg]tissue ratios were also > 1.

But a second explanation for this high [Hg]liver/[Hg]muscle ratios was proposed by Henny et al. (2002) who defend the theory that, as methylmercury exposure increases, the percentage of inorganic mercury in the liver also increases, indicating greater hepatic demethylation. Subsequent binding and immobilization of inorganic mercury to metallothionines (and other proteins containing sulphydryl groups), preferentially produced in the liver (Hogstrand and Haux, 1990), could give origin to increased liver concentrations relative to muscle (Cizdziel et al., 2003). The synthesis of this metallothionines is accepted, generally, to be induced under conditions of high metal concentration (Hg in this case) providing more sites for binding metal ions and limits possible damage to tissue (Monserrat et al., 2007). Maury-Brachet et al. (2006) said that high [Hg]liver/[Hg]muscle ratios are characteristically found in benthivorous fish species, depending on the feeding behaviour. Therefore, our specie stays in accordance with this statement.

Gills are an important organ due to their role on gaseous exchange, osmotic and ionic regulation, as well as on bioconcentration and excretion of toxicants. The accumulation in the gills has been associated with a higher intake of inorganic mercury (the most water soluble form). Thus, gills are considered the main route for uptake of mercury present on aqueous phase, due to their wide surface area and continuous contact mostly with the dissolved and particulate metal species in water (Laporte et al., 1997). Nevertheless, our results showed low [Hg]gills/[Hg]tissue (<1) ratios. This probably happens due to the phenomenon of depuration which occurred in the gills, mainly by the flow through dissolved and particulate inorganic mercury; other responses possible to low values in gills are the high renewal rate of branchial tissue as an unfavorable factor to bioconcentration or the fact that gills epithelium is regularly subject to exfoliation and erosion, which is counteracted by an intense cell division rate (Pacheco et al., 1993). We saw that [Hg]gills/[Hg]tissue ratios were minimum for the liver (<1), which can be an indication of a low relocation of mercury stored in the liver. Therefore, the high value for mean [Hg]muscle/[Hg]gills ratio might have resulted from the depuration, as already seen. And the higher values of mercury in muscle of *C. labrosus* than in gills suggested that diet may be the route of mercury incorporation and reveal an efficient sequestering of mercury in muscle tissues. When Abreu et al. (2000) studied the accumulation of mercury in Sea Bass from Ria de Aveiro, they concluded the same.

Brain is of an extreme interest, not only due to its neurological functions essential for survival, but also because it is a target organ for methylmercury which is able to react directly with important receptors (Berntssen et al., 2003).

Methylmercury is incorporated in fish muscle and brain tissue, most likely by forming a methylmercury-cysteine complex (Harris et al., 2003). [Hg]brain/[Hg]muscle ratio was lower than 1. One possible justification is the association of methylmercury to proteins of the skeletal muscle and this is an advantage of reducing the exposure of the brain, which is the organ more sensible to the adverse effects of methylmercury (Wiener and Spry, 1996). Thus, levels in muscle are more concentrated in mercury than in brain of *C. labrosus* and the muscles tissues have been suggested to act as a sink for methylmercury (Leaner and Mason, 2004).

When we compared the three sampling sites we verified that there is no significant differences in each [Hg]tissue/[Hg]tissue ratio. This fact denotes that mercury in fish is not clearly affected by the environmental levels or by the subsequent body burdens extent.

Consequences to human health

Mercury contamination in fish is a widespread problem, which generates important public health concerns (Lindqvist, 1991). As fish have great nutritious value and are an important alternative to other food sources, the main concern regarding mercury environmental health risks is associated to the consumption of aquatic organisms, particularly fish, with elevated levels of methylmercury in muscles. So, seafood consumption is, in fact, the principal source of mercury and thus, mercury accumulates in the human body and causes damage in many of its basic systems, particularly to the nervous system (Dey et al., 1999).

This argument, adequately underlines the need to develop preventive measures to protect public health (Storelli et al., 2005).

Thus, it is important assess the quantity of mercury in edible tissues of fish species included in human diet. The official regulatory agencies have put limits for mercury concentrations above which the fish is considered inappropriate for human consumption. The European Commission decision 93/351 recognized this limit at 0.5 µg g^{-1} of wet weight (Official Journal of the European Communities, 1994). In the present study, we registered values of mercury concentration in muscle from 0,004 µg g-1 (minimum) wet weight in Laranjo to 0,175 µg g-1 (maximum) wet weight in Laranjo. Hence, the total mercury concentrations obtained for the muscles of *C. labrosus* in different systems were below the established limit for safe human consumption of 0.5 µg g^{-1}. Thus, in references and contaminated sampling site, the values of environmental contamination do not determine the contamination of muscles of *C. labrosus* to make them unfit for consumption.

5. Conclusion

With this work, we were able to conclude that mercury concentration in the different tissues of *C. labrosus* does not reflect significant differences among the sampling sites that we studied. Nevertheless, Laranjo is a more contaminated site. We also concluded that mercury distribution and accumulation was dependent of the specific tissue. Therefore, clear differences result in the next pattern: liver > muscle > brain > gills. So, liver, like the major target organ for mercury accumulation, was the tissue that best reflects the environmental mercury contamination degree. Thus, the use of biota as a

source of environmental information provides this way, invaluable data on the health status of aquatic ecosystems, which reinforced the choice of *C. labrosus* as bioindicator of metal contamination.

The determination of mercury quantity in more than one tissue, as well as all ratio [Hg]tissue/[Hg]tissue, can give additional information on accumulation pathways and specific toxicity mechanisms of mercury. In spite of this, tissue discrimination is more labour intensive and time demanding, not to mention difficulties related with sample mass in smaller organisms.

We could see that a single tendency for mercury was not seen and mercury bioaccumulation along the life span in the three sampling sites was not verified. The reason for the low values and the absence of bioaccumulation with growth are the same in Mira and Mondego. Phenomena of growth dilution, the life cycle of *C. labrosus,* as well as a possible mechanism of excretion, are all possible responses for the reduction of mercury with age in Laranjo sampling site.

In the three sampling sites, the values of environmental contamination do not determine the contamination of muscles of *C. labrosus* to make them unfit for consumption. Anyway, in spite of this, we saw that levels in Laranjo were higher than the other sampling sites, thus, the threat to humans about the consumption of fish that lives in this site can not be ignored.

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