Universidade de Aveiro Departamento de Biologia
2011

ANDREIA DO CARMO MARTINS RODRIGUES

MERCURY TOXICITY AND BIOACCUMULATION: LAB & FIELD STUDIES

TOXICIDADE E BIOACUMULAÇÃO DE MERCÚRIO: ESTUDOS EM LABORATÓRIO E CAMPO

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, ramo de Toxicologia e Ecotoxicologia realizada sob a orientação científica do Professor Doutor Amadeu Soares, Professor catedrático do Departamento de Biologia da Universidade de Aveiro e co-orientação do Doutor Sizenando Nogueira de Abreu do Departamento de Biologia/CESAM - Centro de Estudos do Ambiente e do Mar, Universidade de Aveiro.

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Aos meus pais.

o júri

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palavras-chave

Mercúrio, Bioconcentração, Bioacumulação, Bioamplificação, Cadeias Tróficas, Ecossistemas Estuarinos, Organismos aquáticos

resumo

O objectivo deste trabalho é avaliar a toxicidade, a bioacumulação e a bioamplificação de mercúrio. O trabalho apresenta uma componente laboratorial e uma componente de campo. A componente laboratorial foi dividida em duas partes e a componente de campo foi realizada num ambiente estuarino, Ria de Aveiro, Portugal.

Na componente laboratorial, começou por se avaliar a toxicidade do mercúrio para diferentes organismos aquáticos, testando-se concentrações de mercúrio entre 0,5 µg/L e 2,4 mg/L. As espécies teste escolhidas para avaliar a toxicidade do mercúrio incluíram espécies modelo: *Pseudokirchneriella subcapitata, Daphnia magna* e *Chironomus riparius,* e espécies autóctones: *Chlorella vulgaris, Lemna minor* e *Daphnia longispina*. O mercúrio revelou ser tóxico para todas as espécies, obtendo-se valores de EC_{50} que variaram de 7.3 µg Hg/L (teste de imobilização de *D. longispina*) a 1,58 mg Hg/L (teste de imobilização das larvas de *C. riparius*). Este ensaio demonstrou que pequenas doses de mercúrio provocam efeitos consideráveis ao nível dos produtores primários, base das cadeias tróficas.

Num segundo procedimento experimental construiu-se uma cadeia trófica aquática, constituída pelo produtor primário *P. subcapitata,* pelo consumidor primário *D. magna* e o consumidor secundário *Danio rerio*. A contaminação iniciou-se pelo meio de cultura das algas com 10 µg Hg/L, do qual estas acumularam 70% do mercúrio disponível. Esta espécie foi usada como alimento para *D. magna*, que por sua vez, foi usada como alimento para o consumidor secundário *Danio rerio*. Após um período de 14 dias de teste *D. magna* acumulou 0,14 µg Hg/g. A concentração média obtida no músculo de *D. rerio*, após 21 dias de teste, foi de 0,27 µg Hg/g, peso fresco. Todos os organismos acumularam mercúrio ao longo do tempo de exposição, sendo que a maior bioamplificação de mercúrio ocorreu da microalga *P. subcapitata* para o microcrustáceo *D. magna*, reforçando assim o papel crucial dos produtores primários na bioconcentração de mercúrio da coluna de água para as cadeias tróficas.

O trabalho de campo foi realizado na Ria de Aveiro, em dois sítios específicos, cuja caracterização em termos de contaminação por mercúrio já estava descrita. Estudou-se a carga de mercúrio total na coluna de água, bem como o mercúrio total e orgânico nos sedimentos e a sua transferência e acumulação para peixes juvenis residentes na área, *Liza aurata*. O Cais do Bico, local mais próximo da fonte de contaminação apresentou os maiores valores de mercúrio total: 68 ng/L na coluna de água, 0,19 µg/g nos sedimentos e 0,07 µg/g nos peixes. O local mais distante da fonte de mercúrio, Barra, apresentou uma maior quantidade de mercúrio orgânico nos sedimentos (0,02 µg/g) e uma percentagem de mercúrio orgânico no músculo dos peixes igualmente superior, de 96%. Esta monitorização comprovou que, embora as descargas industriais de mercúrio já tenham sido interrompidas no final do século passado, o mercúrio armazenado nos sedimentos continua a ser ressuspendido para a coluna de água, ficando biodisponível para a biota. A utilização de organismos juvenis fornece informações sobre as variações a curto prazo das concentrações de mercúrio no ambiente.

keywords

Mercury, Bioconcentration, Bioaccumulation, Biomagnification, Trophic chains, Estuarine ecosystems, Aquatic organisms.

abstract

This work aims to evaluate the toxicity, bioaccumulation and biomagnification of mercury and it is divided into a laboratory and a field component. The laboratory component was divided into two parts and the field component was conducted into an estuarine environment in Ria de Aveiro, Portugal.
In the laboratory we started by evaluating the toxicity of mercury for different aquatic organisms, using mercury concentrations that ranged between 0.5 µg/L to 2.4 mg/L. The chosen species used in this assay to evaluate mercury toxicity were the models: *Pseudokirchneriella subcapitata*, *Daphnia magna* and *Chironomus riparius* and the autochthonous species: *Chlorella vulgaris, Lemna minor* and *Daphnia longispina*. Mercury showed to be toxic to all testes species, with EC_{50} values ranging from 7.3 µg Hg/L (immobilization test of *D. longispina*) to 1.58 mg Hg/L (immobilization test of the larvae of *C. riparius*). The assay showed that even low doses of mercury can cause significant effects at the levels of primary producers, the base of the trophic chain.
In the secondary laboratorial assay, an aquatic trophic chain was simulated using the primary producer *P. subcapitata*, the primary consumer *D. magna* and the secondary consumer *Danio rerio*. The trophic chain mercury contamination process was initiated exposing an algae culture to inorganic mercury (10 µg Hg/L), resulting in the accumulation of 70% of the available mercury in the primary producer. The contaminated algae were then used as food supply to the specie *D. magna* and subsequently *D. magna* specimens were used as food to the secondary consumer. After 14 days of exposure *D. magna* accumulates 0.14 µg Hg/g, whereas the final average concentration obtained in the muscle of the fish *D. rerio* after 21 days was 0.27 µg Hg/g (wet weight). All test species accumulate mercury along the time of exposure; the higher biomagnification occurred from the microalgae *P. subcapitata* to the mircrocrustacean *D. magna*, enhancing the crucial role of primary producers in the bioconcentration of mercury from the water column along the trophic chain.
Fieldwork was conducted in the Ria de Aveiro, in two specific sites (Cais do Bico and Barra) that were already characterized regarding dissimilar environmental mercury contamination levels. Mercury levels were evaluated in the water column (total mercury), sediments (total and organic mercury) and in juvenile fish *Liza aurata* inhabiting the area (total and organic mercury). Cais do Bico site, located near the source of contamination showed the highest values of total mercury: 68 ng/L in the water column, 0.19 µg/g in the sediments and 0.07 µg/g in fish. The site distant from the source of mercury (Barra) presented a great amount of organic mercury in the sediments (0.02 µg/g) and a higher percentage of organic mercury in fish muscle (96%). The study indicates that, although mercury discharges have already stopped in the end of the last century, mercury stored in sediments continues to be ressuspended to the water column, becoming bioavailable to biota. The adoption of juvenile specimens provides information on short-term variations of mercury concentrations in the environment.

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Chapter I: General Introduction

General Introduction

Mercury in the aquatic environment

Mercury (Hg) is a natural element that exists in the earth's crust and it can be released by volcanic eruptions, weathering of mercuriferous areas, fires and degassing of superficial areas (Green-Ruiz et al. 2005; Devlin 2006; Chen et al. 2011).

Mercury is ubiquitous and persistence in the environment. The metal occurs in three valence states (Hg^0, Hg^{1+}, Hg^{2+}), in several inorganic forms and in organomercury species (Ullrich et al. 2001). The species of Hg and subsequent speciation will determine the solubility, mobility, and toxicity of Hg in aquatic ecosystems, and the potential for methylation. Mercury will be permanently recycled through physical, chemical and biological processes, as it cannot be degraded into nontoxic products in the environment. Elemental mercury (Hg^0) is the most widespread form found in the environment (Burton et al. 2006; Ullrich et al. 2001). Elemental mercury represents 10 to 30% of the dissolved Hg in the ocean and freshwater. In surface waters, Hg^0 occurs mainly from de reduction of Hg (II) compounds by aquatic microorganisms and from abiotic reduction by humic substances of organic Hg forms (Ullrich et al. 2001; Morelli et al. 2009). The main input of Hg into the aquatic environment appears to be in the form of inorganic Hg compounds from either direct atmospheric deposition or terrestrial runoff (Nevado et al. 2011; Ullrich et al. 2001). In general, surface waters are saturated in Hg^0 comparative to the atmosphere, especially in summer (Ullrich et al. 2001; Morel et al. 1998). In aquatic ecosystems, Hg suffers a complex cycle with numerous Hg forms transforming into each other; the chemical form of Hg is highly dependent on variables such as salinity, dissolved oxygen and pH conditions as well as the concentrations of inorganic and organic complexing agents (Ullrich et al. 2001).

Mercury tends to be sorbed on surfaces, when Hg enters in the aquatic environment it firstly reacts with the different compounds in the water and the

remaining part of it is precipitated to the sediments, where will occur organic and inorganic reactions, dependents on variables such as pH and redox-potential (Green-Ruiz et al. 2005). Sediments can be considered as reservoirs for Hg, because Hg lifetime in sediments is very long, the mainly portion bound to organic matter and sulfur, the other fraction is associated with Mn and/or Fe-oxides near the redox boundary (Green-Ruiz et al. 2005; Ullrich et al. 2001). Mercury can be identified more readily by analyses of sediments than by the quantification of metal concentrations on water, because Hg concentrations in sediments usually exceed those from the overlying water column. In addition, sediments integrate the temporal variability that is characteristic of metals deposited from human sources (Ramalhosa et al. 2001).

Mercury interactions with natural organic matter affect the transport, transformation and bioavailability of Hg. The formation of strong ionic bonding between Hg and reduced sulfur sites in soil and aquatic organic matter is one of the central reactions, these strong complexations facilitates the mobility of Hg from contaminated soils and sediments into streams, lakes and groundwater, and also controls the partitioning of Hg to suspended particulate matter in the water column and the sequestration of Hg to sediments (Ravichandran 2004).

Several environmental and human health issues are associated with the geographically widespread prevalence of elevated levels of both inorganic and organic Hg compounds in freshwater and marine biota. An evident linkage between the bioaccumulation of methylmercury (MeHg) in aquatic systems and the atmospheric mobilization and deposition of mercury is known, from the local to the global components (Al-Majed and Preston 2000).

Mercury processes

The occurrence and behavior of Hg in aquatic environments is of great concern since it is possibly the only metal that bioaccumulates and biomagnifies through all levels of the trophic chain (Lawson and Mason 1998; Mathews and Fisher 2008).

In the water column, Hg can be present in the dissolved and particulate fractions. The operational definition for dissolved Hg is the fraction constituted by all the forms of the metal that pass through a 0.45 µm pore size filter. In other hand, the suspended particulate matter (SPM) is defined as the material retained by the same filter. Particulate Hg is constituted by Hg bound to inorganic particles and particulate organic matter, as well as biogenic particles such as bacteria, algae, and phytoplankton (Ullrich et al. 2001).

Several processes such as chemical oxidations and reductions, photochemical reactions and microbial transformations are responsible for converting elemental Hg into inorganic and organic forms (Devlin 2006). Methylmercury, the most toxic form of Hg, enters the aquatic environment mainly by the biomethylation of Hg compounds, but also as a result of human activity (Devlin 2006; Hope and Rubin 2005; Moreno et al. 2005). In estuarine and freshwater sediments, biomethylation occurs mostly by the activity of sulfate-reducing bacteria, as methylators of inorganic Hg (Benoit et al. 2000; Kim et al. 2008; Chen et al. 2011). The biomethylation process preferentially occurs under anoxic conditions, higher temperature, lower pH, higher organic matter content and sulphate concentrations around 200 to 500 µM (Baeyens et al. 2003). Estuarine and coastal sediments have the biogeochemical conditions for a great organic Hg production, mainly MeHg (Tavares et al. 2011). Therefore the inorganic mercury that has been discharge from industrial effluents (such as the chlor-alkali industry) is gradually transformed into organic mercury. Nowadays and after the “zero mercury discharge” policy, there are still large amounts of stored mercury in estuaries sediments that call for assessment and monitoring programs.

In aquatic environments, bioconcentration processes refers to Hg accumulation directly from the dissolved faction, without dietary pathways. This distribution between the organism and the environment depends on substance chemical properties, environmental conditions, and biological factors, so bioconcentration can be considered as the result of the balance between the chemical uptake and elimination rates (Mace 2002; Watras et al. 1998). Bioconcentration factor (BCF) in aquatic organisms is defined as the ratio of the substance concentration in the exposed organism to the concentration of the

dissolved substance in the surrounding environment, at equilibrium. So, BCFs are result from exposure to waterborne chemical, usually determined under laboratorial conditions (Meylan et al. 1999; DeForest et al. 2007).

Bioaccumulation may also occur in the aquatic environments and it is the process in which it is observed an increased chemical concentration in an organism with age, compared to that in the water, being this uptake by all exposure routes (dietary absorption, transport across respiratory surfaces and dermal absorption), the contaminants may be metabolized so the final concentration is a balance between intake, regulation and excretion (Fatemi and Baher 2009; Gray 2002). The bioaccumulation factor (BAF) is the ratio of a chemical concentration in an organism to the concentration in the water, but including all possible routes of exposure (DeForest et al. 2007). Biodilution may also occur mainly due to the organism growth factor leading to a decrease of mercury concentration in the tissues in the growing process (Stafford and Haines 2001).

Hazard identification determines the adverse effects that one substance can cause, based on its intrinsic proprieties (McGeer et al. 2003). Aquatic hazard identification uses bioaccumulation, persistence and acute toxicity in order to establish the potential for undesirable effects to biota (McGeer et al. 2003). Risk assessment integrates hazard identification, dose-response assessment and exposure assessment (McGeer et al. 2003). Bioaccumulation can also be part of other regulatory toolboxes and is used in many jurisdictions for prioritization and risk assessment, like the framework of the Organization for Economic Cooperation and Development (OECD) (McGeer et al. 2003).

The transfer of metals in trophic chains is described by BCFs and BAFs, also with the BCF/BAF criterion that is the threshold above which a substance is considered bioaccumulative and consequently possessing the potential for long-term environmental impacts (Vries et al. 2007; McGeer et al. 2003). Generally, BAF is easily derived from measurements in natural environments and BCF is more readily measured under laboratory conditions (McGeer et al. 2003; DeForest et al. 2007).

Biomagnification is a special case of bioaccumulation, in which the chemical is bioaccumulated up in the trophic chain, by transfer of residues of the chemical within the prey to the predator in the trophic chain, so it results in higher concentrations in organisms at the higher trophic levels (Fatemi and Baher 2009; Gray 2002). Biomagnification is a complex mechanism, as there are several factors that control the uptake and elimination of a chemical from the consumption of contaminated food, factors that are specifics to the chemical (solubility, molecular weight, etc.) and also factors specifics to the organism (feeding rate, egesting rate, growth rate). In the case of Hg, there are an increase of the Hg levels and the percent of MeHg through the trophic chain. (Fatemi and Baher 2009; Gray 2002; Watras et al. 1998). Biomagnification factor (BMF) is usually dimensionless and it is estimated as the concentration of chemical in the organism at steady state dividing for the concentration of chemical in the organism's diet (Pérez Cid et al. 2001).

The trophic transfer factor (TTF) consists in the ratio of a substance concentration in an organism tissue and the concentration in the organism food item (DeForest et al. 2007). This concept is most applied to laboratory studies in which the main or unique font of contaminant is the food. This term can also include biomagnification, when the concentration in the organism is greater than its diet, or biodilution, when the substance concentration in an organism is lower than in its diet (DeForest et al. 2007).

Mercury in aquatic biota

All mercury compounds are toxic to living organisms, have no biological function and frequently create long-term contamination problems (Costa et al. 2011; Rattner et al. 2008; Vries et al. 2007). Mercury concentrations in biota arise from a series of complex interactions between several processes.

Water chemistry influences the bioaccumulation of Hg into primary producers, the basis of the trophic chain; it ultimately determines the resultant levels in higher trophic organisms. Lower trophic levels, like primary producers,

play a major function in Hg bioaccumulation as the highest bioconcentration occurs between the water and phytoplankton (Lawson and Mason 1998; Mason et al. 2000; Lawrence and Mason 2001). The adsorption of Hg to suspended particulate matter which includes plankton varies significantly in the first trophic levels; it can adsorb onto organic films or colloidal material at the surface or by crossing the plankton cell walls. Once aggregated or incorporated into plankton, Hg can be transferred along the trophic chain and transformed by the subsequent organisms increasing or lightening its toxicity (Monterroso et al. 2003; Pickhardt et al. 2002). As sediments are the principal sites for Hg methylation in estuaries, benthic and epibenthic organisms in coastal waters generally contain elevated concentrations of mercury, especially MeHg (Lawrence and Mason 2001).

Detectable quantities of Hg can be accumulated by freshwater biota even from natural sources (Ullrich et al. 2001). Methylmercury is readily up taken and bioaccumulated by aquatic organisms and biomagnification occurs along the trophic chain by a factor of ≥ 1 million (Devlin 2006; Hope and Rubin 2005; Vieira et al. 2009; Zhang et al. 2007). In consequence, higher trophic levels organisms will have bigger levels of MeHg. For example, piscivorous species usually have higher levels of Hg than invertivorous species (Hope and Rubin 2005). The positive relationship between trophic position and bioaccumulative Hg load is widely accepted (Piraino and Taylor 2009; Mason et al. 1995). Tissue Hg concentrations reveal the quantity of Hg taken up by organisms, the proportion of metal which is distributed to each tissue, and the extent to which the metal enters and is retained within each tissue. The uptake of Hg by fish is a cumulative process; it involves the bioaccumulation of mercury with age and also the biomagnification through the trophic chain (Tavares et al. 2011; Harmelin-Vivien et al. 2009). The uptake of MeHg by fish is mainly through their diet, direct uptake from the water is less significant (Ullrich et al. 2001). Methylmercury is the most toxic form and dominant in most species of fish, especially top predators, with MeHg corresponding to ≥ 95% of the Hg content (Moreno et al. 2005; Hope and Rubin 2005; Vieira et al. 2009; Carrasco et al. 2011; Cossa and Coquery 2005; Mason et al. 2000). Methylmercury

concentration in fish can be 1000 to 10 000 times higher than in other types of food like vegetables or meats (Mieiro et al. 2009).

Metal contamination in aquatic systems is of great concern due to their persistence, metal uptake and toxicity for many aquatic organisms, in addition to the possibility of trophic transfer along trophic chains, eventually reaching humans. Due to the great affinity of metals by particulate matter, they tend to settle and are thus stored in estuarine and marine ecosystems, so, point source pollution by metals have a great impact on marine ecosystems, affecting primarily intertidal and near shore ecosystems (Tavares et al. 2011).

The monitoring of Hg in water, sediment and biota is of great importance to assess the environmental impacts, their bioavailability, pharmacodynamic and potential exposure routes (Oliveira et al. 2010). The direct and indirect coupling between ichthyofaunal communities and human impact on estuaries, as well as fish wide distribution and trophic position reinforces the use of these organisms as a bioindicators (Mieiro et al. 2009; Tavares et al. 2011). Carnivorous fish, in the top of aquatic trophic chains, are valuable to use for monitoring Hg pollution and other species, with different trophic positions, should also be monitored for human health aspects (Al-Majed and Preston 2000). The assessment of mercury accumulation in fish is valuable in indicating the quality status of an aquatic environment, as well as a contribution to a better understanding of the ecological role of fish in Hg transfer between estuaries, coastal and open waters, and its crucial importance for public health (Tavares et al. 2011).

Mercury is one of the metals of higher concern and has received increasing attention from researchers and policy makers. Mercury is considered to be one of the most hazardous metals, along with lead (Pb) and cadmium (Cd) (Pérez Cid et al. 2001; Vries et al. 2007) and is one of the highest priority environmental pollutants in the scope of the European Water Framework Directive, Directive of Priority Substances 2008/105/EC and on global scale (US EPA, 2001; OSPAR, 2000).

Even with a significant amount of literature on the subject (Table 1), the behavior of Hg and many of the transformation and distribution mechanisms

operating in the natural aquatic environment are still poorly understood as well as the little knowledge of the impact of metal levels in biota.

Table 1 Total mercury ([THg]) and methylemercury ([MeHg]) concentrations (ng/g), in aquatic biota and sediments around the world.

Local	[THg]		[MeHg]	References
	In Biota	In sediments	In biota	
Hubei, China	37 to 159	-	-	(Zhang et al. 2007)
Texas, USA	30 to 1065 (ww)	-	-	(McClain et al. 2006)
Oregon, USA	19 to 294 (dw)	190 to 140 (dw)	13 to 210 (dw)	(Henny et al. 2005)
Ebro River, Spain	750 ± 710 (ww)	-	760 ± 660 (ww)	(Carrasco et al. 2011)
Kuwait Territorial Waters	4 to 3923 (dw)	-	1 to 3270 (dw)	(Al-Majed and Preston 2000)
Gulf of California, USA	63 to 230	300 to 2300	-	(Green-Ruiz et al. 2005)
Tagus River, Spain	159 to1057 (dw)	nondetectable values to 1200	970 to 440 (dw)	(Nevado et al. 2011)
Three estuaries from Argentina	110 to 530 (dw)	39 to 9500 (dw)	-	(De Marco et al. 2006)

Toxicity of mercury to wildlife

As mercury does not have any biological function, the presence of Hg in an organism' body may have several consequences. Its lipophilicity makes possible it to pass through lipid membranes of cells, which facilitates its distribution to all tissues. Consequently, organisms tend to accumulate Hg at a higher rate than they are capable of eliminate it (Tavares et al. 2011). The

primary mechanism of cellular toxicity of Hg consists in binging readily and directly to sulfhydryl (-SH) groups, forming disulfide bridges (-S-S-) in proteins, which may result in protein conformational changes, consequently changing their normal function (Guilherme et al. 2008a). Mercury can alter the activity of enzymes, binding to their functional groups or by displacing the metal associated with the enzyme. Mercury is recognized as a prooxidant that induces oxidative stress and modifies the cytochrome P450 function. This alteration has physiological implications that have not been fully established, but it is assumed that it may reduce the ability of fish to metabolize and excrete xenobiotics, provoking alterations at several biological levels (Guilherme et al. 2008a).

Exposure of aquatic organisms to Hg could stimulate a multiplicity of undesirable effects on respiratory, immune and/or reproductive systems (Huang et al. 2010). Mercury is a powerful neurotoxic substance for wildlife since, brain and its vital neurological functions are the primary targets for organic mercury compounds. The mechanisms of Hg toxicity remain uncertain, mainly in fish, but Hg neurotoxicity has been already associated to the excessive generation of reactive oxygen species (ROS) and lipid peroxidation (LPO) in teleost fish and mammalian systems (Mieiro et al. 2010). The available fish studies report neurodegenerative damage and necrotic lesions in brain, with consequences on fish sensory capacities and behavior (Mieiro et al. 2011).

Mercury compounds may also exert clastogenic effects in eukaryotes, causing aneuploidy and/or polyploidy and causing an indirect damage of DNA trough the ROS (Guilherme et al. 2008b).

Anthropogenic sources of mercury

Population growth and human needs on goods and services highly increased over the last century which has been associated with a significant increase of toxic contaminants daily discharge to aquatic ecosystems from industrial, agricultural and domestic activities (Oliveira et al. 2010).

Anthropogenic emissions account for 10 to 30% of the Hg emitted annually (Burton et al. 2006; Ciesielski et al. 2010).

The use of mercury by humans is reported over the history, its toxic proprieties have been used for therapeutic and disinfectant purposes, with applications on medicine, as fungicides and antiseptics (Clarkson et al. 2003). The proprieties of Hg, like its fluid of high density and uniform expansion, and the good conduction of electrical current, make Hg useful for different industries. So, with the Industrial Revolution, and in the posterior decades, the amount of Hg in environment was largely increased (Zhang and Wong 2007).

One of the most important sources of anthropogenic Hg contamination is artisanal gold mining that uses Hg amalgamation. As well as industrial mining of gold and/or cinnabar involves the extraction of Hg by the mineralization of cinnabar (HgS) or as Hg contaminants with other ore-bearing minerals (Lasut et al. 2010; Edinger et al. 2007; Henny et al. 2009). Mercury from both sources can be released into the atmosphere as a result of roasting, or into the water from the oxidation of Hg-bearing minerals, potentially being methylated at the sediment-water interface or at the surface layers of sediment and enters the trophic chain (Lasut et al. 2010; Hope and Rubin 2005).

Another important source of mercury to the environment is coal-burning power plants. Power plants release inorganic Hg into the atmosphere, which can then be deposited onto earth’s surface and also be methylated (McClain et al. 2006).

Other anthropogenic activities such as metallurgical processes, fossil fuel burning, wood pulping, paint industries, pharmaceutical industry, battery production, waste incineration and agricultural application of organomercurials can also release large amounts of Hg to the environment (Green-Ruiz et al. 2005; Costa et al. 2011; Devlin 2006; Ullrich et al. 2001), but the chlor-alkali industry is the most important source of anthropogenic Hg, being responsible for 90% of the European anthropogenic Hg emissions to the atmosphere (Hylander 2001). The traditional method of chlor-alkali industry to produce chlorinated solvents is based on Hg cell technology, which releases inorganic Hg into the aquatic environment (Pereira et al. 2009; Carrasco et al. 2011;

Ramalhosa et al. 2005). The large majority of these chlor-alkali plant industries have been discharging tons of Hg directly or indirectly to the estuarine ecosystems. One example, is the Ria de Aveiro, Portugal, an estuarine ecosystem (described below) that received highly contaminated effluent discharges from a chlor-alkali industry, resulting in an accumulation of about 33 t of Hg in the sediments (Pereira et al. 2009).

Over 90% of the Hg resultant from anthropogenic sources and released to the atmosphere in the last 100 years is still present in the terrestrial environment and it is slowly released to streams and rivers, and will continue to be for a considerable period after the end of the anthropogenic input (Mason et al. 2000). Once in aquatic environment, Hg can be transported far from the source, impacting the downstream environment. Mercury is transported through air, released into water or into the landscape. All these routes will finally lead to water supplies as for example rivers, and then bioaccumulated and biomagnified throughout all the food net and then uptake by Humans through the ingestion of the contaminated food (Fig. 1)

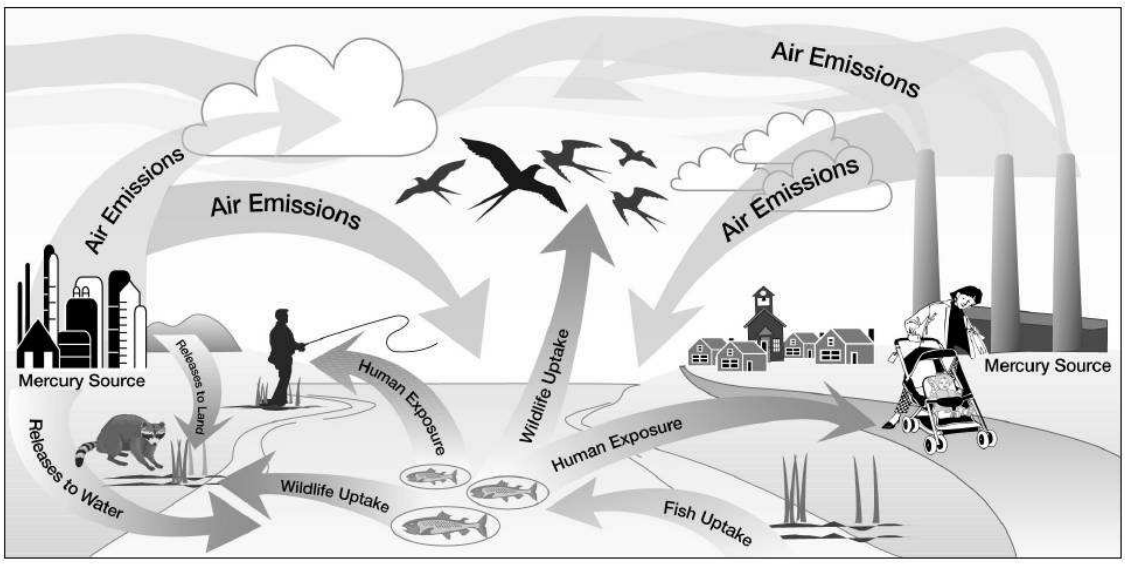

Fig. 1 Cycle of mercury from anthropogenic sources and mercury fish ingestion exposure pathway (adapted from USEPA, 2000).

Mercury and human health

Mercury reaches humans because it can be present in crops and animal products, as well as in drinking water and air quality (Vries et al. 2007). The main source of Hg to humans is the consumption of Hg-contaminated fish (McClain et al. 2006; Carrasco et al. 2011; Vries et al. 2007; Guilherme et al. 2008b).

Accumulation of Hg causes toxic effects on humans. The MeHg content in the ingested fish is almost totally absorbed from gastrointestinal tract, distributed throughout the body and easily penetrates blood-placental barrier in humans (Ciesielski et al. 2010). Fetuses are specially affected by Hg consumed by pregnant women, and that prenatal exposure to low levels of Hg may have developmental and cognitive problems (McClain et al. 2006; Shastri and Diwekar 2008). Even low doses of Hg can provoke damage to nervous and cardiovascular systems (Piraino and Taylor 2009). Teratogenic effects and irreversible neurological damage are some effects of mercury to humans (Guilherme et al. 2008a).

The World Health Organization (WHO) recommended a safety guideline of consume of fish with no more 0.5 µg MeHg/kg wet weight and 0.2 µg THg/g wet weight to that at-risk groups, as frequent fish consumers, pregnant women, and individuals under 15 years old.

Ria de Aveiro: a natural laboratory

Estuaries are transition environments characterized by a mixture of salt and fresh water, and are the end-point of fine sedimentary material. They represent a physiological challenge to biota being at the same time highly productive ecosystems (Green-Ruiz et al. 2005; Kim et al. 2008). Estuaries function as nurseries for several species (fish, crustaceans and mollusk species), are vital feeding areas for several birds species.

There is a human history of use of estuaries as they are strategic points as places of navigation, agricultural abundance, and locations of the biggest cities in the world. Consequently, they have been used as repositories of industrial and domestic effluents over the decades. Metals and mercury in particular are one of the contaminants that are released by these anthropogenic sources (Mieiro et al. 2009; Coelho et al. 2006).

The Ria de Aveiro is a coastal lagoon adjacent to the Atlantic Ocean, located at northwestern coast of Portugal, it has about 45km long and 8.5 km wide (Oliveira et al. 2009). It is a biologically productive system with major role in the life cycle of numerous organisms being used as nursery for several species. The coastal plain around the lagoon supports an intensive, diversified agriculture, a multiplicity of industries and other urban pressures. From 1950s until 1994, the inner bay, Laranjo bay, received, a highly contaminated effluent discharges from a mercury cell chlor-alkali plant located in Estarreja industrial complex. It is estimated that 33 t of mercury are accumulated in the lagoon, being about 27 t considered to be sediment-associated in an inner-basin called Laranjo close to the industrial effluent discharge (Pereira et al. 2009). Due to the inner-basin's morphology, mercury deposition occurred mainly in the entrance of Laranjo, decreasing within the distance to the contamination source, and lower mercury concentrations can be found throughout the rest of Ria de Aveiro lagoon, leading to a gradient of anthropogenic contamination. During spring tides, in which approximately 75 % of the water of the bay is renewed (Monterroso et al. 2003), and also during periods of stronger tidal currents and bottom resuspension, the anthropogenic contaminants are released and can became bioavailable.

Many studies have examined sources, environmental transport, accumulation and biological effects of mercury (Coelho et al. 2008; Abreu et al. 2000; Ramalhosa et al. 2001). Although this progress, many significant questions remain concerning its exposure and toxicological effects.

Research objectives and thesis outline

The main goal of this work was to evaluate the toxicity, bioaccumulation and biomagnification of mercury using bioassays under laboratory conditions (Chapter II and III) and the field work in an estuarine ecosystem (Chapter IV).

Chapter II describes laboratorial experiments in which we started by evaluating the toxicity of mercury for several aquatic organisms representing different trophic levels. The toxicity was evaluated using model species chosen species used, were the models (*Pseudokirchneriella subcapitata*, *Daphnia magna* and *Chironomus riparius*) and the autochthonous species (*Chlorella vulgaris, Lemna minor* and *Daphnia longispina*) having immobilization or growth inhibition as endpoints.

In Chapter III, the bioconcentration and biomagnification of mercury is evaluated in an aquatic trophic chain. The aquatic trophic chain was simulated using the primary producer and representative of phytoplankton: *P. subcapitata*, the primary consumer and representative of zooplankton: *D. magna* and the secondary consumer *Danio rerio.*

Chapter IV describes the evaluation of bioaccumulation and biomagnification of mercury in a real scenario, conducted in an estuarine ecosystem (Ria de Aveiro), comparing two specific sites (Cais do Bico and Barra) known to show dissimilar mercury contamination. The load of total mercury was studied in the water column, as well as total and organic mercury in sediments and its transfer and accumulation in juvenile fish, *Liza aurata*.

Chapter V encloses final remarks and general conclusions.

References

Abreu SN, Pereira E, Vale C, Duarte AC (2000) Accumulation of Mercury in Sea Bass from a Contaminated Lagoon (Ria de Aveiro, Portugal). Mar Pollut Bull 40 (4):293-297

Al-Majed NB, Preston MR (2000) An Assessment of the Total and Methyl Mercury Content of Zooplankton and Fish Tissue Collected from Kuwait Territorial Waters. Mar Pollut Bull 40 (4):298-307

Baeyens W, Leermakers M, Papina T, Saprykin A, Brion N, Noyen J, De Gieter M, Elskens M, Goeyens L (2003) Bioconcentration and Biomagnification of Mercury and Methylmercury in North Sea and Scheldt Estuary Fish. Archives of Environmental Contamination and Toxicology 45 (4):498-508. doi:10.1007/s00244-003-2136-4

Benoit JM, Gilmour CC, Mason RP (2000) The Influence of Sulfide on Solid-Phase Mercury Bioavailability for Methylation by Pure Cultures of Desulfobulbus propionicus (1pr3). Environ Sci Technol 35 (1):127-132. doi:10.1021/es001415n

Burton D, Turley S, Fisher D, Green D, Shedd T (2006) Bioaccumulation of Total Mercury and Monomethylmercury in the Earthworm *Eisenia Fetida*. Water, Air, & Soil Pollution 170 (1):37-54. doi:10.1007/s11270-006-3113-0

Carrasco L, Barata C, García-Berthou E, Tobias A, Bayona JM, Díez S (2011) Patterns of mercury and methylmercury bioaccumulation in fish species downstream of a long-term mercury-contaminated site in the lower Ebro River (NE Spain). Chemosphere

Chen MH, Teng PY, Chen CY, Hsu CC (2011) Organic and total mercury levels in bigeye tuna, *Thunnus obesus*, harvested by Taiwanese fishing vessels in the Atlantic and Indian Oceans. Food Additives and Contaminants: Part B 4 (1):15-21. doi:10.1080/19393210.2010.535908

Ciesielski T, Pastukhov MV, Szefer P, Jenssen BM (2010) Bioaccumulation of mercury in the pelagic food chain of the Lake Baikal. Chemosphere 78 (11):1378-1384

Clarkson TW, Magos L, Myers GJ (2003) Human exposure to mercury: The three modern dilemmas. The Journal of Trace Elements in Experimental Medicine 16 (4):321-343. doi:10.1002/jtra.10050

Coelho JP, Pereira ME, Duarte A, Pardal MA (2005) Macroalgae response to a mercury contamination gradient in a temperate coastal lagoon (Ria de Aveiro, Portugal). Estuarine, Coastal and Shelf Science 65 (3):492-500

Coelho JP, Rosa M, Pereira E, Duarte A, Pardal MA (2006) Pattern and annual rates of *Scrobicularia plana* mercury bioaccumulation in a human induced mercury gradient (Ria de Aveiro, Portugal). Estuarine, Coastal and Shelf Science 69 (3-4):629-635

Cossa D, Coquery M (2005) The Mediterranean Mercury Anomaly, a Geochemical or a Biologocal Issue. In: The Mediterranean Sea, vol 5K. The Handbook of Environmental Chemistry. Springer Berlin / Heidelberg, pp 121-130. doi:10.1007/b107147

Costa S, Crespo D, Henriques B, Pereira E, Duarte A, Pardal M (2011) Kinetics of Mercury Accumulation and Its Effects on *Ulva lactuca* Growth Rate at Two Salinities and Exposure Conditions. Water, Air, & Soil Pollution 217 (1):689-699. doi:10.1007/s11270-010-0620-9

De Marco SG, Botté SE, Marcovecchio JE (2006) Mercury distribution in abiotic and biological compartments within several estuarine systems from Argentina: 1980-2005 period. Chemosphere 65 (2):213-223

DeForest DK, Brix KV, Adams WJ (2007) Assessing metal bioaccumulation in aquatic environments: The inverse relationship between bioaccumulation factors, trophic transfer factors and exposure concentration. Aquatic Toxicology 84 (2):236-246

Devlin E (2006) Acute Toxicity, Uptake and Histopathology of Aqueous Methyl Mercury to Fathead Minnow Embryos. Ecotoxicology 15 (1):97-110. doi:10.1007/s10646-005-0051-3

Edinger E, Siregar P, Blackwood G (2007) Heavy metal concentrations in shallow marine sediments affected by submarine tailings disposal and artisanal gold mining, Buyat-Ratototok district, North Sulawesi, Indonesia. Environmental Geology 52 (4):701-714. doi:10.1007/s00254-006-0506-8

Fatemi M, Baher E (2009) A novel quantitative structure–activity relationship model for prediction of biomagnification factor of some organochlorine pollutants. Molecular Diversity 13 (3):343-352. doi:10.1007/s11030-009-9121-4

Gray JS (2002) Biomagnification in marine systems: the perspective of an ecologist. Mar Pollut Bull 45 (1-12):46-52

Green-Ruiz C, Ruelas-Inzunza J, Páez-Osuna F (2005) Mercury in surface sediments and benthic organisms from Guaymas Bay, east coast of the Gulf of California. Environmental Geochemistry and Health 27 (4):321-329. doi:10.1007/s10653-004-5741-x

Guilherme S, Válega M, Pereira ME, Santos MA, Pacheco M (2008a) Antioxidant and biotransformation responses in *Liza aurata* under environmental mercury

exposure - Relationship with mercury accumulation and implications for public health. Mar Pollut Bull 56 (5):845-859

Guilherme S, Válega M, Pereira ME, Santos MA, Pacheco M (2008b) Erythrocytic nuclear abnormalities in wild and caged fish (*Liza aurata*) along an environmental mercury contamination gradient. Ecotoxicology and Environmental Safety 70 (3):411-421

Harmelin-Vivien M, Cossa D, Crochet S, Bănaru D, Letourneur Y, Mellon-Duval C (2009) Difference of mercury bioaccumulation in red mullets from the north-western Mediterranean and Black seas. Mar Pollut Bull 58 (5):679-685. doi:10.1016/j.marpolbul.2009.01.004

Henny C, Kaiser J, Grove R (2009) PCDDs, PCDFs, PCBs, OC pesticides and mercury in fish and osprey eggs from Willamette River, Oregon (1993, 2001 and 2006) with calculated biomagnification factors. Ecotoxicology 18 (2):151-173. doi:10.1007/s10646-008-0268-z

Henny C, Kaiser J, Packard H, Grove R, Taft M (2005) Assessing Mercury Exposure and Effects to American Dippers in Headwater Streams near Mining Sites. Ecotoxicology 14 (7):709-725. doi:10.1007/s10646-005-0023-7

Hope BK, Rubin JR (2005) Mercury Levels and Relationships in Water, Sediment, and Fish Tissue in the Willamette Basin, Oregon. Archives of Environmental Contamination and Toxicology 48 (3):367-380. doi:10.1007/s00244-004-0096-y

Huang W, Cao L, Liu J, Lin L, Dou S (2010) Short-term mercury exposure affecting the development and antioxidant biomarkers of Japanese flounder embryos and larvae. Ecotoxicology and Environmental Safety 73 (8):1875-1883

Hylander LD (2001) Global Mercury Pollution and its Expected Decrease after a Mercury Trade Ban. Water, Air, & Soil Pollution 125 (1):331-344. doi:10.1023/a:1005231017807

Kim E, Mason RP, Bergeron CM (2008) A modeling study on methylmercury bioaccumulation and its controlling factors. Ecological Modelling 218 (3-4):267-289

Lasut M, Yasuda Y, Edinger E, Pangemanan J (2010) Distribution and Accumulation of Mercury Derived from Gold Mining in Marine Environment and Its Impact on Residents of Buyat Bay, North Sulawesi, Indonesia. Water, Air, & Soil Pollution 208 (1):153-164. doi:10.1007/s11270-009-0155-0

Lawrence AL, Mason RP (2001) Factors controlling the bioaccumulation of mercury and methylmercury by the estuarine amphipod Leptocheirus plumulosus. Environmental Pollution 111 (2):217-231. doi:10.1016/s0269-7491(00)00072-5

Lawson N, Mason R (1998) Accumulation of mercury in estuarine food chains. Biogeochemistry 40 (2):235-247. doi:10.1023/a:1005959211768

Mace B (2002) Bioaccumulation And Bioconcentration In Aquatic Organisms. In: Handbook of Ecotoxicology, Second Edition. CRC Press. doi:doi:10.1201/9781420032505.ch32

Mason RP, Laporte JM, Andres S (2000) Factors Controlling the Bioaccumulation of Mercury, Methylmercury, Arsenic, Selenium, and Cadmium by Freshwater Invertebrates and Fish. Archives of Environmental Contamination and Toxicology 38 (3):283-297. doi:10.1007/s002449910038

Mathews T, Fisher NS (2008) Evaluating the trophic transfer of cadmium, polonium, and methylmercury in an estuarine food chain. Environmental Toxicology and Chemistry 27 (5):1093-1101. doi:10.1897/07-318.1

McClain W, Chumchal M, Drenner R, Newland L (2006) Mercury Concentrations in Fish from Lake Meredith, Texas: Implications for the Issuance of Fish Consumption Advisories. Environmental Monitoring and Assessment 123 (1):249-258. doi:10.1007/s10661-006-9194-9

McGeer JC, Brix KV, Skeaff JM, DeForest DK, Brigham SI, Adams WJ, Green A (2003) Inverse relationship between bioconcentration factor and exposure concentration for metals: Implications for hazard assessment of metals in the aquatic environment. Environmental Toxicology and Chemistry 22 (5):1017-1037. doi:10.1002/etc.5620220509

Meylan WM, Howard PH, Boethling RS, Aronson D, Printup H, Gouchie S (1999) Improved method for estimating bioconcentration/bioaccumulation factor from octanol/water partition coefficient. Environmental Toxicology and Chemistry 18 (4):664-672. doi:10.1002/etc.5620180412

Mieiro C, Ahmad I, Pereira M, Duarte A, Pacheco M (2010) Antioxidant system breakdown in brain of feral golden grey mullet (*Liza aurata*) as an effect of mercury exposure. Ecotoxicology 19 (6):1034-1045. doi:10.1007/s10646-010-0485-0

Mieiro CL, Pacheco M, Pereira ME, Duarte AC (2009) Mercury distribution in key tissues of fish (*Liza aurata*) inhabiting a contaminated estuary-implications for human and ecosystem health risk assessment. Journal of Environmental Monitoring 11 (5):1004-1012

Mieiro CL, Pereira ME, Duarte AC, Pacheco M (2011) Brain as a critical target of mercury in environmentally exposed fish (Dicentrarchus labrax)--Bioaccumulation and oxidative stress profiles. Aquatic Toxicology 103 (3-4):233-240

Monterroso P, Abreu SN, Pereira E, Vale C, Duarte AC (2003) Estimation of Cu, Cd and Hg transported by plankton from a contaminated area (Ria de Aveiro). Acta Oecologica 24 (Supplement 1):S351-S357

Morel FMM, Kraepiel AML, Amyot M (1998) The Chemical Cycle and Bioaccumulation of Mercury. Annual Review of Ecology and Systematics 29 (ArticleType: research-article / Full publication date: 1998 / Copyright © 1998 Annual Reviews):543-566

Morelli E, Ferrara R, Bellini B, Dini F, Di Giuseppe G, Fantozzi L (2009) Changes in the non-protein thiol pool and production of dissolved gaseous mercury in the marine diatom *Thalassiosira weissflogii* under mercury exposure. Science of The Total Environment 408 (2):286-293

Moreno F, Anderson C, Stewart R, Robinson B, Nomura R, Ghomshei M, Meech JA (2005) Effect of Thioligands on Plant-Hg Accumulation and Volatilisation from Mercury-contaminated Mine Tailings. Plant and Soil 275 (1):233-246. doi:10.1007/s11104-005-1755-0

Nevado J, Martín-Doimeadios R, Bernardo F, Moreno M, Ropero M, de Marcos Serrano A (2011) Mercury Speciation in Fish Tissues from a Mediterranean River Basin: The Tagus River (Central Spain) as a Case Study. Archives of Environmental Contamination and Toxicology:1-11. doi:10.1007/s00244-011-9666-y

Oliveira M, Ahmad I, Maria V, Pacheco M, Santos M (2010) Monitoring pollution of coastal lagoon using *Liza aurata* kidney oxidative stress and genetic endpoints: an integrated biomarker approach. Ecotoxicology 19 (4):643-653. doi:10.1007/s10646-009-0436-9

Oliveira M, Maria VL, Ahmad I, Serafim A, Bebianno MJ, Pacheco M, Santos MA (2009) Contamination assessment of a coastal lagoon (Ria de Aveiro, Portugal) using defence and damage biochemical indicators in gill of *Liza aurata* - An integrated biomarker approach. Environmental Pollution 157 (3):959-967

Pereira M, Lillebø A, Pato P, Válega M, Coelho J, Lopes C, Rodrigues S, Cachada A, Otero M, Pardal M, Duarte A (2009) Mercury pollution in Ria de Aveiro (Portugal): a review of the system assessment. Environmental Monitoring and Assessment 155 (1):39-49. doi:10.1007/s10661-008-0416-1

Pérez Cid B, Boia C, Pombo L, Rebelo E (2001) Determination of trace metals in fish species of the Ria de Aveiro (Portugal) by electrothermal atomic absorption spectrometry. Food Chemistry 75 (1):93-100

Pickhardt PC, Folt CL, Chen CY, Klaue B, Blum JD (2002) Algal blooms reduce the uptake of toxic methylmercury in freshwater food webs. Proceedings of the National Academy of Sciences 99 (7):4419-4423. doi:10.1073/pnas.072531099

Piraino MN, Taylor DL (2009) Bioaccumulation and trophic transfer of mercury in striped bass (Morone saxatilis) and tautog (Tautoga onitis) from the Narragansett Bay (Rhode Island, USA). Marine Environmental Research 67 (3):117-128

Ramalhosa E, Monterroso P, Abreu S, Pereira E, Vale C, Duarte A (2001) Storage and export of mercury from a contaminated bay (Ria de Aveiro, Portugal). Wetlands Ecology and Management 9 (4):311-316. doi:10.1023/a:1011864702531

Ramalhosa E, Pereira E, Vale C, Válega M, Duarte AC (2005) Distribution of mercury in the upper sediments from a polluted area (Ria de aveiro, Portugal). Pollut Bull 50 (6):682-686. doi:10.1016/j.marpolbul.2005.02.043

Rattner B, Golden N, Toschik P, McGowan P, Custer T (2008) Concentrations of Metals in Blood and Feathers of Nestling Ospreys (*Pandion haliaetus*) in Chesapeake and Delaware Bays. Archives of Environmental Contamination and Toxicology 54 (1):114-122. doi:10.1007/s00244-007-9004-6

Ravichandran M (2004) Interactions between mercury and dissolved organic matter--a review. Chemosphere 55 (3):319-331

Shastri Y, Diwekar U (2008) Optimal control of lake pH for mercury bioaccumulation control. Ecological Modelling 216 (1):1-17

Stafford CP, Haines TA (2001) Mercury contamination and growth rate in two piscivore populations. Environmental Toxicology and Chemistry 20 (9):2099-2101. doi:10.1002/etc.5620200933

Tavares S, Oliveira H, Coelho JP, Pereira ME, Duarte AC, Pardal MA (2011) Lifespan mercury accumulation pattern in Liza aurata: Evidence from two southern European estuaries. Estuarine, Coastal and Shelf Science 94 (4):315-321

Ullrich SM, Tanton TW, Abdrashitova SA (2001) Mercury in the Aquatic Environment: A Review of Factors Affecting Methylation. Critical Reviews in Environmental Science and Technology 31 (3):241-293. doi:10.1080/20016491089226

Vieira LR, Gravato C, Soares AMVM, Morgado F, Guilhermino L (2009) Acute effects of copper and mercury on the estuarine fish Pomatoschistus microps: Linking biomarkers to behaviour. Chemosphere 76 (10):1416-1427

Vries Wd, Romkens PFAM, Schutze G (2007) Critical soil concentrations of cadmium, lead and mercury in view of health effects on humans and animals. Reviews of Environmental Contamination and Toxicology 191:91-130

Watras CJ, Back RC, Halvorsen S, Hudson RJM, Morrison KA, Wente SP (1998) Bioaccumulation of mercury in pelagic freshwater food webs. Science of The Total Environment 219 (2-3):183-208

Zhang L, Wong MH (2007) Environmental mercury contamination in China: Sources and impacts. Environment International 33 (1):108-121. doi:10.1016/j.envint.2006.06.022

Zhang L, Zang X, Xu J, Xie P, Zhu Z, Su J (2007) Mercury Bioaccumulation in Fishes of Three Gorges Reservoir After Impoundment. B Environ Contam Tox 78 (3):262-264. doi:10.1007/s00128-007-9117-7

Chapter II: Mercury toxicity assessment to aquatic biota

Mercury toxicity assessment on different trophic levels

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Abstract

Mercury (Hg) is a global pollutant highly persistent in the environment and is one of the most toxic metals to aquatic organisms. In this study, we intended to assess the toxicity of Hg to aquatic biota of several trophic levels. The Hg concentrations tested varying between 0.5 µg/L and 2.4 mg/L. Test species include microalgae, plants, microcrustaceans and larvae of insects, representing model organisms or autochthonous species. Mercury was highly toxic to the aquatic biota, with EC_{50} values ranging from 7.3 µg/L (immobilization of *Daphnia longispina*, 48 h of exposure) to 1.578 mg/L (immobilization of larvae of I instar of *Chironomus riparius*, 48 h exposure). This paper also shows that the maximum legal concentration for superficial water affect by discharges (1 µg Hg/L) established by the Directive 2000/60/EC will potentially affect the microalgae. Being algae the basis of aquatic food chains, mercury may potentially cause deleterious effects to upper trophic levels. The general increase of Hg tolerance in organisms as higher is their trophic position and complexity suggests a natural selection or adaptability to higher levels of Hg exposure along trophic chains.

Key-words: Mercury, Aquatic toxicity, *Pseudokirchneriella subcapitata, Chlorella vulgaris, Daphnia magna, Daphnia longispina, Chironomus riparius, Lemna minor*

Introduction

Water pollution by metals is an issue of great concern as metals do not break down in aquatic environments and, thus, they persist in the environment (Azevedo-Pereira and Soares 2010; Devlin 2006), mostly aggregated to sediments, ressuspended particles and organic matter (Pereira et al. 2005; Azevedo-Pereira and Soares 2010). Mercury (Hg) is one of the most toxic metals (Dirilgen 2011; Vieira et al. 2009) and a priority substance listed in the European Community directive 2000/60/EC (2000). It is naturally present in the earth's crust, but its concentration in the aquatic environment was highly increased in the last decades due to anthropogenic activities, mainly industry (Verlecar et al. 2008). Mercury has no essential function to organisms (Verlecar et al. 2008), and is considered a neurotoxic contaminant (Mousavi et al. 2011) that can bring deficiencies of essential elements because it can compete for active sites of biologically important molecules for the organisms (Azevedo-Pereira and Soares 2010). Moreover, Hg may bioaccumulate and biomagnify in all levels of the trophic chains (Dirilgen 2011; Biesinger et al. 1982; Vieira et al. 2009).

Mercury concentrations in aquatic environments are usually low. Values between 1 and 5 ng Hg/L were reported in natural waters in South and North America (Lechler et al. 2000; Hope and Rubin 2005) and values between 0.001 and 118 ng Hg/L were reported in Europe (European Community 2000). In order to protect surface waters from the toxicity of Hg the European Community established a maximum of 1 µg Hg/L in continental surface water affected by industrial discharges (European Community 2000).

Aquatic environments are favorable to Hg accumulation and speciation, so Hg persists and interact with the nearby biota. Despite Hg toxicity, persistence in aquatic environments and potential to bioaccumulate along trophic chains, few studies addressed Hg toxicity to aquatic biota belonging to different trophic levels. In aquatic environments, microalgae and vascular plants are the main primary producers, thus essential to the whole ecosystem maintenance (Zhou et al. 2008; Feng et al. 2005). They accumulate high concentrations of metals, allowing them

to enter the trophic chains which causes serious threaten to animals and human health through biomagnification (Zhou et al. 2008; Lin et al. 2005; Källqvist et al. 2008).

The assessment of Hg toxicity to aquatic biota using reference organisms intends to be a valuable tool to evaluate toxicity to various biological important species and subsequent risks to human health (Khangarot and Das 2009), increasing the understanding of the Hg dynamic behavior in aquatic environments.

In this study we intended to assess the toxicity of Hg to aquatic biota of several trophic levels. We focused on low Hg concentrations varying between 0.5 µg/L and 2.4 mg/L. Additionally, we compared our results with the maximum legal limits established by European legislation. We assessed Hg toxicity to two microalgae: the model species *Pseudokirchneriella subcapitata* and the autochthonous species *Chlorella vulgaris* and to the autochthonous vascular plant *Lemna minor*. Filter-feeding zooplankton, commonly represented by Daphniidae, are ecologically relevant as they are often the primary grazers of phytoplankton, and the primary forage for planktivorous fish. Additionally, they are worldwide distributed (Zhou et al. 2008; Guan and Wang 2006; Freitas and Rocha 2011). In this study we used two species: *Daphnia magna* and *Daphnia longispina* representing, respectively, a model species widely used in ecotoxicological testing and an autochthonous species in Portugal (Antunes et al. 2003). A variety of insects living in close proximity to the aquatic environment can be used for the biomonitoring of metals pollution (Zhou et al. 2008). These insects make the connection between the aquatic and terrestrial ecosystems, and promote the transport of the accumulated metals. The life cycle of the insect *Chironomus riparius* includes both an aquatic phase (as larvae) and a terrestrial phase (as an insect), which makes this species exposed to Hg toxicity in the aquatic environment.

Material & Methods

Metal preparation and chemical analysis

Mercury was tested as mercury (II) chloride ($HgCl_2$, Sigma-Aldrich, p. a. ≤ 99.0%). A stock solution of mercury salt was prepared with Milli-Q water and kept in the dark. Serial dilutions were prepared from the stock solution to the desired concentrations by diluting with the appropriate test medium for each species. The concentration of the stock solution and tested metal concentrations were certified with analysis by atomic absorption using the mercury analyzer AMA-254 (Altec, Czech Republic).

Test species and experimental conditions

All test species were obtained from laboratorial cultures at the Department of Biology of the University of Aveiro.

Toxicity tests were carried out in accordance with guidelines of the Organization for Economic Cooperation Development (OECD). A summary of main experimental conditions for each test species is presented in Table 2.

Microalgae

The unicellular green algae *Pseudokirchneriella subcapitata* and *Chlorella vulgaris,* were maintained in unialgal batch cultures with sterilized MBL medium at 20ºC and continuous light and aeration.

Growth inhibition tests for each species followed the OECD guideline 201 (OECD 2006a), using three replicates per treatment. Tests were initiated with $1x10^5$ cells/ml in the exponential growth phase. Algae were grown in Erlenmeyer flasks sealed with cotton bungs and containing 200 mL of sterilized MBL medium with the desired Hg concentration. Algae were incubated in a controlled temperature chamber at 20 ± 2ºC, under continuous light (white fluorescent light,

3,000-4,000 lux) and continuous gentle agitation (Table 2). After 72h of exposure the optical density (at 440 nm) was determined with a spectrophotometer (Jenway 6505 UV/Visible, UK). The optical density was converted to number of cells employing a regression model previously developed for each species. Growth rates for each species were determined as recommended by the OECD guideline 201 (OECD 2006a).

Macrophyte

Cultures of *Lemna minor* were maintained in Steinberg culture medium (OECD 2006b) at 20ºC ± 1ºC and 16 h light : 8 h dark photoperiod with light intensity of about 6500 lux.

Growth inhibition tests followed the OECD guideline 221 (OECD 2006b), and were carried out in triplicate, under static conditions. Experimental conditions are summarized in Table 2. Three colonies with three visible fronds each were randomly assigned to each treatment, in 100 mL of Steinberg medium with the desired Hg concentration. Thus, tests were started with 9 fronds per vessel. Hg effects were assessed based on the number of fronds and dry weight after 7 days of exposure.

Microcrustaceans

Stock cultures of *Daphnia magna* [clone F, sensu Baird et al. (1990)] and *Daphnia longispina* (clone EV20, sensu Antunes et al. (2003)] were maintained in ASTM hard water (ASTM 2004) with a standard organic additive (Marinure seaweed extract, supplied by Glenside Organics Ltd.) and fed *Pseudokirchneriella subcapitata* (5×10^5 cells/ml). Culture medium was renewed every other day. Temperature was 20 ± 1ºC and 16h:8h (light:dark) photoperiod.

Acute immobilization toxicity tests with neonates (≤ 24h old) of *D. magna* and *D. longispina* were carried out in accordance with OECD guideline 202

(OECD 2004), with 5 replicates per treatment. Each replicate consisted of 5 organisms exposed to 50 ml of ASTM hard water (OECD 2004) with the desired Hg concentration and no food. Experimental conditions are summarized in Table 2. The number of immobilized daphnids was recorded after 24 h and 48 h exposure (immobilization was defined as the inability to swim or move within 15 s of gentle agitation, and was taken to indicate lethality).

Insects

Chironomus riparius cultures were kept in a 4L aquaria containing a layer of inorganic acid-washed fine sediment (≤1 mm) as substrate and ASTM hard water (ASTM 2004), provided with aeration. Organisms were fed twice a week *ad libiditum* with macerated fish flakes, (Tetramin, Tetrawerke, Germany). Prior to the experiment two egg ropes were removed from the culture, transferred to a crystallizing dish with ASTM hard water, and kept at 20ºC until eclosion.

Acute immobilization tests followed the draft of OECD guideline for *Chironomus* sp. (OECD 2010), with 5 replicates per treatment. *C. riparius* larvae (≤24 h old, instar I, 5 larvae per replicate) were exposed to Hg in Petri dishes containing 40 ml of ASTM (ASTM 2004) with the desired Hg concentration and no food. Experimental conditions are summarized in Table 2. The number of immobilized larvae was recorded after 24h and 48h of exposure.

Statistical analysis

SigmaPlot statistical package (SigmaPlot, v. 10, Systat Software Inc.) was used for statistical analyses. All statistical analyses were based on 0.05 significance level. A one-way analysis of variance (ANOVA) followed by a Dunnett test were pursued to test whether Hg caused a significant change in the response of organisms compared with the appropriate control. Non-normally distributed or heteroscedastic data sets were analyzed with the nonparametric test Kruskal-Wallis followed by the Dunn's post-hoc test. EC_{50} values (the effective

concentration that causes 50% effect) for continuous responses (microalgae and plant growth) were calculated with SigmaPlot software package. Whenever possible, we also determined LOEC (lowest observed effect concentration) which is the lowest tested concentration at which the substance is observed to have statistically significant reducing effect when compared with the control. EC_{50} values for binary responses (microcrustaceans and larvae of insects) were calculated with PriProbit software package (Masayuki 1998).

Table 2 Summary of main experimental conditions for each tests species.

Species	Trophic level	Test medium	Temperature (ºC)	Photoperiod (light:dark)	Duration of exposure	[Hg]
Pseudokirchneriella subcapitata	Primary producer	MBL	20 ± 2	24h:0h	72h	0, 2.5 to 60 µg/L
Chlorella vulgaris	Primary producer	MBL	20 ± 2	24h:0h	72h	0, 1 to 75 µg/L
Lemna minor	Primary producer	Steinberg	20 ± 1	16h:8h	7 days	0, 0.4 to 2.4 mg/L
Daphnia longispina	Primary consumer	ASTM	22 ± 1	16h:8h	48h	0, 0.5 to 36 µg/L
Daphnia magna	Primary consumer	ASTM	22 ± 1	16h:8h	48h	0, 5 to 160 µg/L
Chironomus riparius	Detritivore	ASTM	20 ± 1	16h:8h	48h	0, 0.4 to 2.4 mg/L

Results

The measured concentrations of freshly prepared test solutions were in good agreement with the nominal concentrations, as the measured concentrations did not differ more than 10% from the nominal concentrations.

Microalgae

Growth rate of both microalgae species after 72h exposure to Hg is represented in Fig. 2. Algae were highly sensitive to Hg, with Hg concentrations as low as 1 µg Hg/L causing a significant reduction in the growth rate of *C. vulgaris* (Fig. 2; Table 3). However, the lowest EC_{50} growth inhibition value was registered for *P. subcapitata*: 19.1 µg Hg/L, whereas the value for *C. vulgaris* was 47.5 µg Hg/L (Table 3).

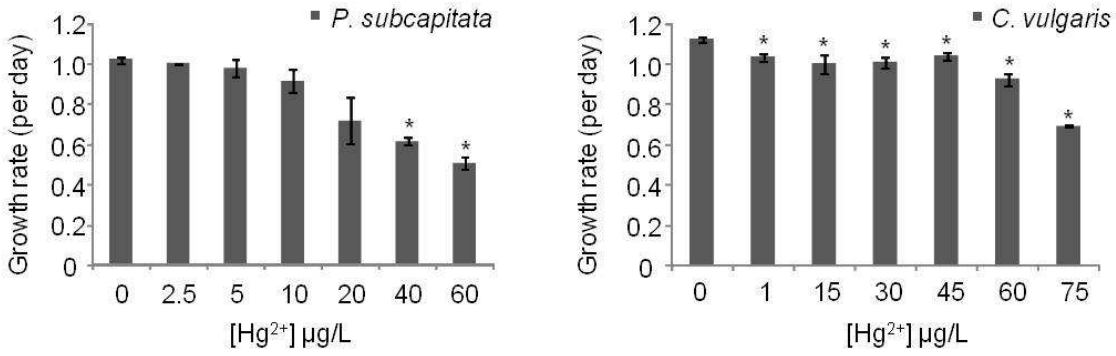

Fig. 2 Growth rate (per day) of the algae *P. subcapitata* and *C. vulgaris* when exposed to increasing concentrations of Hg (µg/L), * = denotes a significant difference when compared with the control.

Macrophyte

Figure 3 shows the growth rate of *L. minor* after 7 days of exposure to Hg. Mercury concentrations above 0.4 mg/L (LOEC) caused a significant decrease in growth rates compared to the control. Growth rates decrease most pronouncedly for concentrations below 0.8 mg/L (corresponding to the EC_{50} growth inhibition); an increase of Hg concentration above this value caused only a slight decrease in growth rates.

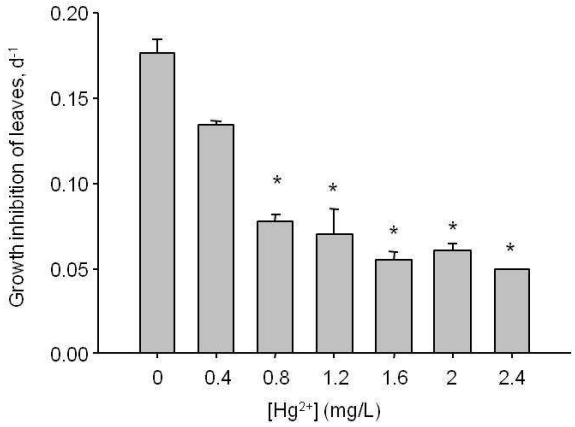

Fig. 3 Growth rate (per day) of *Lemna minor* when exposed to increasing concentrations of Hg (mg/L). *= denotes a significant difference when compared with the control.

Microcrustaceans

Among the microcladocerans, immobilization increased with increasing Hg concentration and exposure period (Fig. 4). Comparing both species, we observed that *D. longispina* was more sensitive to Hg than *D. magna*. After 48h exposure the EC_{50} for *D. longispina* was 7.3 µg/L, whereas for *D. magna* it was almost 5x higher: 34.8 µg/L (Table 3).

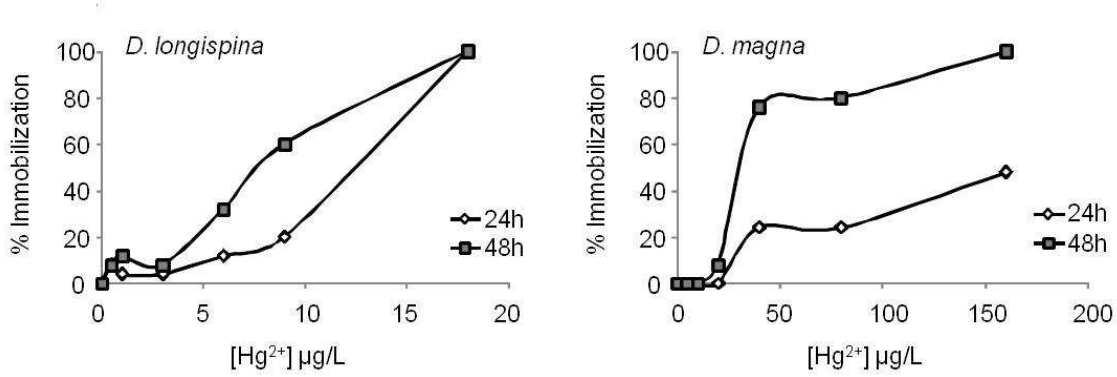

Fig. 4 Percentage of immobilization of *D. magna* and *D. longispina* after 24 and 48h of exposure to increasing concentrations of Hg (µg/L).

Insects

The immobilization of *C. riparius* larvae (I instar) after 24h and 48h of exposure to Hg is shown in Fig. 5. After 24h of exposure the EC_{50} was 2.311 mg/L and after 48h of exposure the EC_{50} was 1.578 mg/L (Table 3).

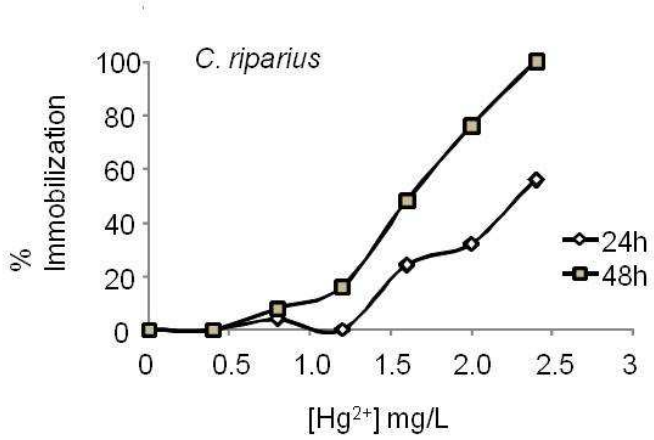

Fig. 5 Percentage of immobilization of *C. riparius* larvae after 24h and 48h of exposure to increasing concentrations of Hg (mg/L).

Table 3 Toxicity values (EC_{50} and LOEC) of Hg to the test species.

Test species	Endpoint	LOEC	EC_{50} (95% confidence interval) (µg Hg/L)			
			24h	48h	72h	7 days
Pseudokirchneriella subcapitata	Growth inhibition	5 µg/L	-	-	19.1 µg/L (14.1 – 24.1)	-
Chlorella vulgaris	Growth inhibition	1 µg/L	-	-	47.5 µg/L (34.5 – 60.5)	-
Lemna minor	Growth inhibition	0.4 mg/L	-	-	-	0.8 mg/L (0.6 – 1.0)
Daphnia longispina	Immobilization	-	10.5 µg/L (9.4, 12.4)	7.3 µg/L (6.2 – 8.7)	-	-
Daphnia magna	Immobilization	-	15.7 µg/L (10.7 – 32.8)	34.9 µg/L (29.4 – 40.3)	-	-
Chironomus riparius	Immobilization	-	2.3 mg/L (2.1- 2.7)	1.6 mg/L (1.4 – 1.7)	-	-

Discussion

Among primary producers, the tested microalgae showed to be most sensitive to Hg than the tested vascular plant. Microalgae were very sensitive to Hg, showing LOEC growth inhibition values < 10 µg Hg/L. The extreme toxicity of Hg to microalgae species has been reported as a result of photosynthetic inhibition (Pereira et al. 2005; Zhou et al. 2008; Juneau et al. 2001) and altered enzymatic activities (Jonsson and Aoyama 2009).

Lemna minor was less sensitive to the tested Hg concentrations than microalgae, although organisms were exposed for a longer period (7 days). The EC_{50} value obtained in this study (0.8 mg/L) is in good agreement with two other studies that report 7 days EC_{50} value of 0.48 mg/L (Dirilgen 2011) and 0.68 mg/L (Naumann et al. 2007) for *L. minor*. It is known that specific metals, as Hg, have specific effects on the photosynthetic machinery, on the formation of reactive oxygen species, on the integrity of cell membranes or enzymatic activity (Naumann et al. 2007).

Among primary consumers, we found that Hg concentration higher than 5 µg/L caused effects, supporting previous studies which refer that mercury is one of the most toxic metals for *Daphnia* (Khangarot and Ray 1987, 1989). Comparing both daphnid species, *D. longispina* was more sensitive to Hg with an EC_{50} of 7.3 µg/L almost five times lower that the EC_{50} of *D. magna* (34.9 µg/L). This difference can be explained by the smaller size of *D. longispina*, and the consequent greater surface to volume ratio, which lead to increased exposure to the organism to mercury. This pattern has been reported in other works comparing acute toxicity of other compounds to the two daphnids (Ventura et al. 2010). *D. longispina* showed to be the most sensitive species tested fact that can have consequences to the equilibrium of the ecosystem as it would affect organisms of higher levels, such as fish, that feed on daphnids, and it would increase populations of organisms of lower levels like mircroalgae, on which daphnids feed. For our knowledge, there are no values in the literature for *D. longispina,* which is probably due to the fact this species is not very common for ecotoxicological studies. However, this

species can be found in aquatic ecosystems throughout the world (Taylor and Hebert 1994; Petrusek et al. 2008). In other hand, several studies report Hg toxicity to *D. magna*, since this species is frequently used as test organism. Reported 48h EC_{50} immobilization values for *D. magna* varied: EC_{50} of 24.8 µg/L for neonates (Tsui and Wang 2006) and EC_{50} values of 22 µg/L (Khangarot and Das 2009), or 0.093 µg/L to adults (Khangarot and Ray 1987). The differences between these results can be explained by multiple factors, mainly genetic variability and temperature (Tsui and Wang 2006).

Larvae (I instar) of *C. riparius* were the least sensitive to Hg (48h EC_{50}= 1.6 mg/L, immobilization effect). Previous studies report LC_{50} values of 3.26 mg/L (Azevedo-Pereira and Soares 2010), 0.75 and 1.8 mg/L (Rossaro et al. 1986), but refer to IV instar larvae. It is also known that Hg affects the development and behavior of the larvae of *C. riparius* (Azevedo-Pereira and Soares 2010).

Comparing EC_{50} among tested species and corresponding level in the trophic chain, data suggests a natural predisposition to an increasing tolerance to Hg increment along the trophic chain. This is consistent with the Hg bioaccumulation and bioamplification processes. That can be supported by different mechanisms of toxicity and different mechanisms of tolerance developed by each species.

The high sensivity of microalgae and *D. longispina* to Hg enhances the impact that Hg contamination can have in the base of a trophic chain, which can have serious repercussions along superior levels of trophic chain. In the aquatic environment, larvae of *C. riparius* develop in the sediment, thus they will be exposed to higher Hg concentrations, since Hg tends to adsorb to sediments (Hope and Rubin 2005). As a consequence, they are exposed to another uptake route, which can increase Hg toxicity to the larval phases of this insect.

Conclusions

Mercury was very toxic to aquatic biota. Overall data indicates an increasing Hg tolerance along trophic chain, suggesting a natural selection or adaptability to higher levels of Hg exposure along trophic chains. Indeed, microalgae and *D. longispina* were the most sensitive to Hg, whereas larvae of *C. riparius* were the least sensitive. This paper also shows that an Hg concentration of 1 µg/L will likely affect significantly the basis of trophic chain. The data obtained with the present study shows that is necessary a toxicity characterization performed for different aquatic organisms, including autochthon species, as they have different sensibility to the pollutant, in order to have legislation that preserve the ecosystems.

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References

Antunes SC, Castro BB, Gonçalves F (2003) Chronic responses of different clones of Daphnia longispina (field and ephippia) to different food levels. Acta Oecologica 24 (Supplement 1):S325-S332

ASTM (2004) Standard Guide for Conducting *Daphnia magna* Life-cycle Toxicity Tests. ASTM E1193 - 97(2004). American Society for Testing and Materials, Pennsylvania

Azevedo-Pereira H, Soares A (2010) Effects of Mercury on Growth, Emergence, and Behavior of *Chironomus riparius* Meigen (Diptera: Chironomidae). Archives of

Environmental Contamination and Toxicology 59 (2):216-224. doi:10.1007/s00244-010-9482-9

Baird DJ, Barber I, Calow P (1990) Clonal variation in general responses of *Daphnia magna* Straus to toxic stress. 1. Chronic life-history effects. Functional Ecology 4 (3):399-407

Biesinger KE, Anderson LE, Eaton JG (1982) Chronic effects of inorganic and organic mercury on *Daphnia magna*: Toxicity, accumulation, and loss. Archives of Environmental Contamination and Toxicology 11 (6):769-774. doi:10.1007/bf01059166

Devlin E (2006) Acute Toxicity, Uptake and Histopathology of Aqueous Methyl Mercury to Fathead Minnow Embryos. Ecotoxicology 15 (1):97-110. doi:10.1007/s10646-005-0051-3

Dirilgen N (2011) Mercury and lead: Assessing the toxic effects on growth and metal accumulation by Lemna minor. Ecotoxicology and Environmental Safety 74 (1):48-54

European Community (2000) Directive 2000/60/EC of the European Parliament and of the Council establishing a framework for Community action in the field of water policy. Official Journal of the European Communities. doi:10.1007/s10661-008-0416-1

Feng F-Y, Yang W, Jiang G-Z, Xu Y-N, Kuang T-Y (2005) Enhancement of fatty acid production of Chlorella sp. (Chlorophyceae) by addition of glucose and sodium thiosulphate to culture medium. Process Biochemistry 40 (3-4):1315-1318

Freitas E, Rocha O (2011) Acute Toxicity Tests with the Tropical Cladoceran *Pseudosida ramosa*;: The Importance of Using Native Species as Test Organisms. Archives of Environmental Contamination and Toxicology 60 (2):241-249. doi:10.1007/s00244-010-9541-2

Guan R, Wang W-X (2006) Comparison between two clones of *Daphnia magna*: Effects of multigenerational cadmium exposure on toxicity, individual fitness, and biokinetics. Aquatic Toxicology 76 (3-4):217-229

Hope BK, Rubin JR (2005) Mercury Levels and Relationships in Water, Sediment, and Fish Tissue in the Willamette Basin, Oregon. Archives of Environmental Contamination and Toxicology 48 (3):367-380. doi:10.1007/s00244-004-0096-y

Jonsson CM, Aoyama H (2009) Extraction, partial characterization and susceptibility to Hg2+ of acid phosphatase from the microalgae Pseudokirchneriella subcapitata. Scientia Agricola 66:634-642

Juneau P, Dewez D, Matsui S, Kim S-G, Popovic R (2001) Evaluation of different algal species sensitivity to mercury and metolachlor by PAM-fluorometry. Chemosphere 45 (4-5):589-598

Källqvist T, Milacic R, Smital T, Thomas KV, Vranes S, Tollefsen K-E (2008) Chronic toxicity of the Sava River (SE Europe) sediments and river water to the algae Pseudokirchneriella subcapitata. Water Research 42 (8-9):2146-2156

Khangarot BS, Das S (2009) Acute toxicity of metals and reference toxicants to a freshwater ostracod, *Cypris subglobosa* Sowerby, 1840 and correlation to EC50 values of other test models. Journal of Hazardous Materials 172 (2-3):641-649

Khangarot BS, Ray PK (1987) Correlation between heavy metal acute toxicity values in *Daphnia magna* and fish. B Environ Contam Tox 38 (4):722-726. doi:10.1007/bf01608609

Khangarot BS, Ray PK (1989) Sensitivity of midge larvae of *Chironomus tentans*, Fabricius (Diptera chironomidae) to heavy metals. B Environ Contam Tox 42 (3):325-330. doi:10.1007/bf01699956

Lechler PJ, Miller JR, Lacerda LD, Vinson D, Bonzongo JC, Lyons WB, Warwick JJ (2000) Elevated mercury concentrations in soils, sediments, water, and fish of the Madeira River basin, Brazilian Amazon: a function of natural enrichments? The Science of The Total Environment 260 (1-3):87-96

Lin J-H, Kao W-C, Tsai K-P, Chen C-Y (2005) A novel algal toxicity testing technique for assessing the toxicity of both metallic and organic toxicants. Water Research 39 (9):1869-1877

Masayuki S (1998) Probit analysis of preference data. Applied entomology and zoology 33 (3):339-347

Mousavi A, Chávez RD, Ali A-MS, Cabaniss SE (2011) Mercury in Natural Waters: A Mini-Review. Environmental Forensics 12 (1):14-18. doi:10.1080/15275922.2010.547549

Naumann B, Eberius M, Appenroth K-J (2007) Growth rate based dose-response relationships and EC-values of ten heavy metals using the duckweed growth inhibition test (ISO 20079) with Lemna minor L. clone St. Journal of Plant Physiology 164 (12):1656-1664

OECD (2004) Guidelines for the Testing of Chemicals. Test No. 202: *Daphnia* sp., Acute Immobilisation Test. Organisation for Economic Co-operation and Development, Paris

OECD (2006a) Guidelines for the Testing of Chemicals. Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test. Organisation for Economic Co-operation and Development, Paris

OECD (2006b) Guidelines for the Testing of Chemicals. Test No. 221: *Lemna* sp., Growth Inhibition Test. Organisation for Economic Co-operation and Development, Paris

OECD (2010) Guidelines for the Testing of Chemicals. Draft: *Chironomus* sp., Acute Immobilisation Test. Organisation for Economic Co-operation and Development, Paris

Pereira MJ, Resende P, Azeiteiro UM, Oliveira J, Figueiredo DRd (2005) Differences in the Effects of Metals on Growth of Two Freshwater Green Algae (*Pseudokirchneriella subcapitata* (Korshikov) Hindak and *Gonium pectorale* Müller). B Environ Contam Tox 75 (3):515-522. doi:10.1007/s00128-005-0782-0

Petrusek A, Hobæk A, Nilssen JP, Skage M, ČErný M, Brede N, Schwenk K (2008) A taxonomic reappraisal of the European Daphnia longispina complex (Crustacea, Cladocera, Anomopoda). Zoologica Scripta 37 (5):507-519. doi:10.1111/j.1463-6409.2008.00336.x

Rossaro B, Gaggino GF, Marchetti R (1986) Accumulation of mercury in larvae and adults *Chironomus riparius* (Meigen). B Environ Contam Tox 37 (1):402-406. doi:10.1007/bf01607780

Taylor DJ, Hebert PDN (1994) Genetic assessment of species boundaries in the North American Daphnia longispina complex (Crustacea: Daphniidae). Zoological Journal of the Linnean Society 110 (1):27-40. doi:10.1111/j.1096-3642.1994.tb01469.x

Tsui MTK, Wang W-X (2006) Acute Toxicity of Mercury to Daphnia magna under Different Conditions. Environ Sci Technol 40 (12):4025-4030. doi:10.1021/es052377g

Ventura SPM, Gonçalves AMM, Gonçalves F, Coutinhoa JAP (2010) Assessing the toxicity on $[C_3mim][Tf_2N]$ to aquatic organisms of different trophic levels. Aquatic Toxicology 96:290–297

Verlecar XN, Jena KB, Chainy GBN (2008) Modulation of antioxidant defences in digestive gland of Perna viridis (L.), on mercury exposures. Chemosphere 71 (10):1977-1985

Vieira LR, Gravato C, Soares AMVM, Morgado F, Guilhermino L (2009) Acute effects of copper and mercury on the estuarine fish Pomatoschistus microps: Linking biomarkers to behaviour. Chemosphere 76 (10):1416-1427

Zhou Q, Zhang J, Fu J, Shi J, Jiang G (2008) Biomonitoring: An appealing tool for assessment of metal pollution in the aquatic ecosystem. Analytica Chimica Acta 606 (2):135-150

Chapter III: Trophic transfer of mercury: starting from water and reaching secondary consumers

Trophic transfer of mercury: starting from water and reaching secondary consumers

Andreia C. M. Rodrigues, Fátima T. Jesus, Fernando Morgado, Amadeu M. V. M. Soares, Sizenando N. Abreu

Abstract

To better understand the mercury pathway and its distribution within the principal secondary consumers tissues, we simulated a sequential transfer of mercury from water to a producer (*Pseudokirchneriella subcapitata*), from a producer to a primary consumer (*Daphnia magna*) and from this primary consumer to a secondary consumer (*Danio rerio*). The algae *P. subcapitata* were exposed to 10 µg Hg/L for a 5-days exposure period, accumulating 0.007 µg/g ww. These algae were then fed to D. magna for a 14-days exposure period and accumulated 0.144 µg Hg/g. The BCF values (water-algae) and BMF (algae-daphnids-fish) found within the transfer pathway were respectively 0.7; 20.7 and 1.9.

The final exposure lasted for a 28-days period where *D. rerio* was fed with the contaminated daphnids and the content of Hg in liver, muscle and stomach tissues was sampled every 7-days. Total mercury content was always higher in muscle tissues, and no significant differences between the control and the Hg concentration in tissues were found for all sampling time.

Key-words: Mercury, aquatic trophic chain, bioconcentration factor, biomagnification factor

Introduction

Mercury (Hg) is the only known metal that is biomagnified along trophic chains in marine and freshwater environments. The majority of Hg enters the

aquatic environments in the inorganic form (Leady and Gottgens 2001; Baeyens et al. 2003).

Bioaccumulation in aquatic organisms is the sum of bioconcentration, the uptake from surrounding water, and trophic transfer or dietary uptake, being food the dominant uptake pathway in the upper trophic levels (McIntyre and Beauchamp 2007).

Transfer along trophic chains is responsible for the accumulation of Hg to highest concentrations in the longer-lived species in upper trophic levels (Leady and Gottgens 2001). So, quantifying Hg dynamics and understanding the mechanisms of bioaccumulation in lower and intermediate trophic levels is particularly important to clarify and predict the efficiency of Hg biomagnification and which trophic chain is at risk for higher rates of bioaccumulation and biomagnification, which is, therefore, determinant for the health of upper-trophic predators, including humans (McIntyre and Beauchamp 2007).

The greatest bioconcentration occurs between the water and the phytoplankton, so lower trophic levels play a major role in Hg bioaccumulation (Lawson and Mason 1998; Mason et al. 2000; Coelho et al. 2009). For instance, algae can concentrate Hg from the water column 10^4 to 10^6 times providing the greatest inputs of Hg into the trophic chain (Pickhardt et al. 2002, 2005; García and Reyes 2001). Previous studies demonstrated that the phytoplankton uptake of inorganic Hg (Hg (II)) in estuarine environments involved passive diffusion from water on neutral Hg species across the membrane (Lawson and Mason 1998; Zhou and Wong 2000; Schmitt et al. 2001).

Bioaccumulation of persistent contaminants, like Hg, is a complex phenomenon, potentially controlled by several physiological and environmental factors. Water chemistry influences the bioaccumulation of Hg into primary producers, but the effect of water chemistry is not significant in comparison to the magnitude of the subsequent trophic transfer that occur at the higher trophic levels (Mason et al. 2000). There are few models that relate ecological factors like primary production and plankton density with Hg bioaccumulation within aquatic environments (Kim et al. 2008).

Zooplankton has a important trophic position in aquatic environments and plays an important role on Hg transfer as it is the link between phytoplankton and fish (Tsui and Wang 2004). Mercury accumulation in zooplankton occurs from the ingestion of phytoplankton and suspended particles with Hg associated (Zhou and Wong 2000; Pickhardt et al. 2002).

Mercury accumulation in fish constitutes a global public health concern, as fish are the principal source of Hg to humans (Pickhardt et al. 2002). In fish high Hg concentrations result from the producers uptake efficiency that are the basis of the trophic chain, and also from their cellular retention and the subsequent transfer to their respective predators (Morel et al. 1998; Power et al. 2002). Fish from the pelagic trophic chain feeds on zooplankton and usually presents high values of Hg (Pickhardt et al. 2002; Power et al. 2002).

Bioaccumulation potential in aquatic trophic chains is usually expressed using ratios of chemical concentrations in organism tissue relative to chemical exposure concentrations, such as bioconcentration factors (BCFs) and biomagnification factors (BMFs), depending if the source of chemical is from water exposure or food intake, respectively (Meylan et al. 1999; DeForest et al. 2007). Previous reviews of metal BCFs, water-only exposure, have shown that BCFs and BMFs are highly variable between organisms and frequently inversely related to exposure concentration, so the concentration of exposure may be taken always in consideration (DeForest et al. 2007). These inverse relationships have important implications for hazard classification and environmental and human health risk assessments.

In this study, we choose the model green algae *Pseudokirchneriella subcapitata*, as representative of phytoplankton. Phytoplankton is for the daphnids in the natural environment one of food sources (Guan and Wang 2004). We also choose the model freshwater zooplankton *Daphnia magna* which is considered a “keystone” species for understanding Hg transfer. *Daphnia* are widely distributed in freshwater temperate ecosystems and are representative of other zooplankton groups. Additionally, *D. magna* is able to accumulate more Hg than other *taxa*. Because zooplankton constitutes one of the major food for planktivorous fish (Pickhardt et al. 2002), they have been

used as predictors of Hg concentration in fish in different lakes, showing a positive relationship among them (Tsui and Wang 2004).

The main goal of the present study is to evaluate Hg accumulation in algae (*Pseudokirchneriella subcapitata*) and to observe the trophic transfer to a primary consumer (*Daphnia magna*) and to a secondary consumer (*Danio rerio*). In contrast with other studies that used commercial food products, the present study was based on a trophic relationship. The secondary goal of this study is to analyse the distribution of total mercury within three different tissues (liver, muscle and stomach).

Material & Methods

Experimental conditions

Mercury was tested as mercury (II) chloride ($HgCl_2$, Sigma-Aldrich, p. a. ≤ 99.0%). A stock solution was prepared with Milli-Q water and kept in the dark, during all the assay. The concentration of the stock solution and tested metal concentration were certified by analysis by atomic absorption in the mercury analyzer AMA-254 (Altec, Czech Republic).

Test organisms

Tested organisms, belong to the species *Pseudokirchneriella subcapitata* as a representative of phytoplankton, primary producers, *Daphnia magna* (clone F sensu Baird et al (1990)) as a representative of zooplankton, primary consumers and *Danio rerio* as a representative of fish, secondary consumers.

The unicellular green algae *P. subcapitata,* were maintained in unialgal batch cultures with sterilized MBL medium at 20ºC with continuous light and aeration.

Stock culture of *D. magna* were maintained in ASTM hard water (ASTM 2004) with a standard organic additive (Marinure seaweed extract, supplied by Glenside Organics Ltd.) and fed with *P. subcapitata* (5×10^5 cells/ml). Culture

medium was renewed every even day. Cultures were maintained at 20 ± 1ºC with a 16h:8h light:dark photoperiod.

Zebrafish, *D. rerio,* was obtained from Zebrafish Laboratory, Biology Department of University of Aveiro. They were maintained at 26 ± 2ºC, pH 7.5 ± 0.5, conductivity 750 ± 50 µS/cm and with a 12h:12h light:dark photoperiod.

Mercury accumulation in algae

Assays were initiated with $1x10^5$ cells/ml in the exponential growth phase. Algae were grown in glass flasks containing 1000 mL of sterilized MBL medium. After sterilization to one treatment was added 10 µg Hg/L and the other was treated as a control (Hg-free). Mercury concentration tested was chose based on the previous growth tests described in Chapter II. Algae were incubated in a controlled temperature chamber at 20 ± 2ºC, under continuous aeration and light (white fluorescent light, 3,000-4,000 lux). After five days of exposure a 50 ml subsample per replicate was filtered with a 0.45 µm Millipore filter. Filters were dried at 40ºC until constant weight and its mercury content analyzed. The remaining volume was centrifuged at 2500 rpm (4ºC), ressuspended in ASTM hard water, and stored at 4ºC for the feeding experiments with *D. magna* individuals.

Bioconcentration factor (BCF) for algae were determined as the ratio between metal concentration in algae and the metal concentration in test media (DeForest et al. 2007).

Trophic transfer

The conditions used in the cultures of *D. magna* were maintained for the assays. Neonates (<24h old) of *D. magna* were fed daily with contaminated *P. subcapitata* for 14 days, and a control group were fed daily with Hg-free algae. After the exposure period a few daphnids were selected for Hg analyses, and the other ones were frozen at -20ºC and used as food for *D. rerio*.

Biomagnification factor (BMF) was calculated as the ratio between the mercury concentration in *D. magna* and the mercury concentration in *P. subcapitata* (Fatemi and Baher 2009).

Adult zebrafish (body wet weight: 994.9 ± 280.3 mg; standard length: 44.5 ± 3.9 mm) were individually placed in 0.4L tanks to provide equal amounts of food during the study and avoid food competition, with permanently oxygenated chlorine-free water. Each fish was individually fed twice a day (for 28 days) with *D. magna* fed with Hg or Hg-free food. The daily amount of food ingested, corresponds approximately to 2% of the fish wet weight (aprox. 20mg). Seven fish per treatment were removed at 7-days intervals, until day 28, killed and dissected on ice. Zebrafish skeletal muscle, liver and stomach were collected for total Hg analyses (THg).

The biomagnification factor (BMF) was calculated as the ratio between mercury concentration in muscle tissue of *D. rerio* and the mercury concentration in *D. magna* (Fatemi and Baher 2009).

Mercury analysis

Total mercury tissue concentrations were determined with a Mercury Analyser (Leco AMA 254), by Atomic Absorption Spectrometry (AAS) after sample thermal decomposition (Costley et al. 2000). The entire analytical procedure was validated by analysing two standard biological certified reference materials (CRM's) DORM-3 and TORT-2 at the beginning and at the end of each set, to ensure data quality. The results TORT-2 (0.217±0.007 µg/g) and DORM-3 (0.330±0.010 µg/g) were always within the certified values.

Statistical analyses

SigmaPlot statistical package (SigmaPlot, v. 12, Systat Software Inc.) was used for statistical analyses. Data were tested for goodness of fit to normal distribution and requirements of homogeneity of variances. All data suffered a *ln* transformation prior to analysis. Student's *t*-test (α=0.05) was used to detect the differences between contaminated algae, daphnids and their respective

controls. Two way analysis of variance (ANOVA) was performed to analyze the significance between mercury concentrations in fish tissues and time exposure. The Spearman rank correlation factor (*r*) was determined for the total mercury concentration between the different tissues.

Results

Mercury accumulation in algae

Statistical differences between the contaminated algae and the control were found (t_4=-58.540, $p \leq 0.001$). After 5 days of exposure the algae *P. subcapitata* bioaccumulated Hg to a final concentration of 0.007 µg Hg/g wet weight. The bioconcentration factor (BCF) obtained was 0.7 (Table 4).

Trophic transfer of mercury

During the assay no mortality was observed for *D. magna*, as for the *D. rerio* assay, mortality below 5% was observed in treatment with the contaminated food.

The quantities of algae consumed by the daphnids can be considered equal because of the homogeneity of the daphnia used. Statistical differences were found between the daphnids fed with contaminated food and the control (t_4=-16.784, $p \leq 0.001$). Seven *D. magna* that corresponds to the weekly dose for each fish presented a concentration of 0.144 ± 0.005 µg Hg/g wet weight.

Biomagnification factor of *D. magna* feed with contaminated algae was 20.7 and, BMF of fish, based on muscle Hg content, feed with contaminated daphnia was 1.9 (Table 4).

The control treatment showed no statistical differences between the beginning of the assay and the last sampling time for liver (t_{10}=-0.225, p=0.826), muscle (t_{11}=1.308, p=0.217) and stomach (t_9=-0.820, p=0.433). Since no

differences were found within the control, the two sampling times were joined and used as comparison for the treatments with Hg.

When reporting to the liver tissues, the comparison showed no significant differences between control and treatment (p=0.878), time of exposure (p=0.184), and control and treatment vs time of exposure (p=0.184). As previous the same happen for muscle tissues, the comparison showed no significant differences between control and treatment (p=0.378), time of exposure (p=0.051), and control and treatment vs time of exposure (p=0.051). The stomach tissues showed as the other two tissues no significant differences, between control and treatment (p=0.982), time of exposure (p=0.121), and control and treatment vs time of exposure (p=0.162).

The Spearman rank correlation (r) analysis revealed significant positive correlations between Hg in muscle and liver (r=0.410, p=0.0248), and no correlations between liver and stomach (r=0.0323, p=0.0868) and between muscle and stomach (r=0.0593, p=0.785).

The increment obtained from the linear regression shows the following sequence from higher to lower: liver tissues (r=0.198; df=37; p=0.233) > muscle (r=0.249; df=31; p=0.177) > stomach (r=0.168; df=29; p=0.385 – Fig. 6).

Muscle shows during the exposure period the higher Hg concentrations, with a maximum of 0.266 µg/g after 21 days of exposure. A clear increase pattern in the concentration of Hg in liver tissues was observed during the first 21-days of exposure (Fig. 6).

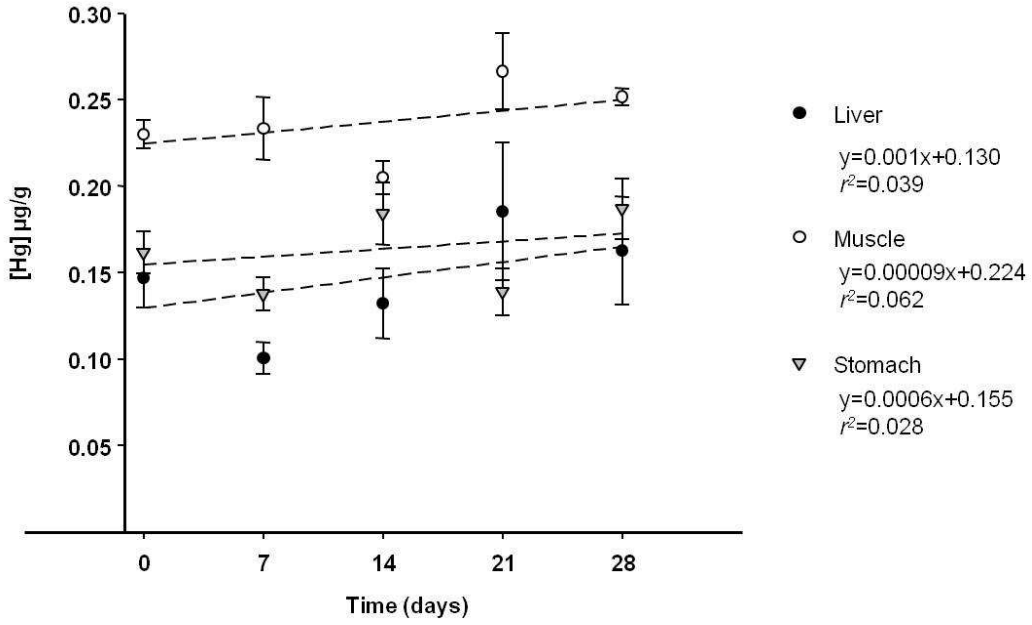

Fig. 6 Mean mercury concentration and respective *SE* in *D. rerio* tissues (liver, muscle and stomach) during exposure period (0, 7-days, 14-days, 21-days and 28-days) and respective linear regressions lines.

Table 4 Aquatic organisms and respective test conditions for obtain biocentration factors (BCF) and biomagnification factors (BMF) found in the literature. *****= Values are described in literature as trophic transfer factors (TTF).

Trophic pathway	Type of Hg exposure	Concentration	Time of exposure	BCF	BMF	References
Water to *P. subcapitata*	$HgCl_2$	10 µg/L	5 days	0.7	-	Our work
P. subcapitata to *D. magna*			14 days	-	20.7	Our work
D. magna to *D. rerio*			28 days	-	1.9	Our work
Macroalgae to *Haliotis diversicolor*	$^{203}HgCl_2$	0.25 - 100 µg/L	3 days	-	>1*****	(Huang et al. 2008)
Water to *H. diversicolor*	$^{203}HgCl_2$	0.01 to10 µg/L	3 h	10^3 - $2.5x10^4$	-	(Huang et al. 2008)
Water to *Acetabularia calyculus*	$HgCl_2$	1 mg/L	7 days	37.5 ± 1.25	-	(García and Reyes 2001)
Water to *Viviparus georgianus*	$Hg(NO_3)_2$	100 µg/L	20 days	85.0	-	(Tessier et al. 1994)
Water to *Elliptio complanata*	$Hg(NO_3)_2$	100 µg/L	20 days	16.2	-	(Tessier et al. 1994)
Water to *Chlamdomonas reinhardtii*	$Me^{203}Hg$	2.5 nmol/L	24 h	$20x10^4$	-	(Mathews and Fisher 2008)

Chlamdomonas reinhardtii to *Daphnia pulex*			2 days	-	26 to 40*****	(Mathews and Fisher 2008)
Daphnia pulex to *Fundulus heteroclitus*			2 h	-	26 to 40*****	(Mathews and Fisher 2008)
Callinectes sapidus fed with *Pogonias cromis*	THg	> 1 µg/g ww	28 days	-	3.0	(Evans et al. 2000)
Penaeus duorarum fed with *Pogonias cromis*	THg	> 1 µg/g ww	28 days	-	2.5	(Evans et al. 2000)

Discussion

Our results show a great capacity of the green algae *P. subcapitata* to bioaccumulate Hg from the aquatic environment with an accumulation of 70% of the Hg available in the water. The fast and successful uptake of mercury by other algae was reported in other studies as presented in Table 4, and although our BCF values were bellow 1, it can be explained by starting the assay with the algae in the exponential growth phase. As algae begin to growth, a bloom dilution effect is observed. The Hg already accumulated within their cells not only was transferred to the new divided ones, but also its concentration was lowering since they gained more body mass. As algal biomass increases, the concentration of mercury per cell decreases, resulting in a low dietary input to secondary consumers and reduced bioaccumulation in algae-rich eutrophic systems (Pickhardt et al. 2002). The capacity of *P. subcapitata* to mobilize Hg from the environment may be harmful for several herbivores that feed on them.

Within this study the trophic transfer of mercury from primary producers to primary consumers and secondary consumers was analysed for its BMFs. There is a direct relationship between the dissolved and particulate fraction of an element in phytoplankton and the efficiency of its assimilation by herbivorous zooplankton (Mason et al. 1996). Although a direct comparison to the work of Mathews and Fisher (2008) cannot be made, since they used organic mercury which is more persistent within organisms tissues, the BMF obtained between primary producers and primary consumers (20.7) in our study is similar to the one found in their work. This value corroborates the great biomagnification of mercury in the lower trophic levels, although values falling in the range between 2.5 and 3.4 (Gorski et al. 2003) or 0.3 to 10 (Back and Watras 1995) were found for freshwater lakes studies.

The BMF value obtained for fish (1.9) is smaller than the value for *D. magna*. Nevertheless as this BMF value is higher than 1, suggesting biomagnification of Hg between *D. magna* and *D. rerio*. A fieldwork in Lake Baikal

reported a similar BMF value of 1.4 for other species of secondary consumer (Ciesielski et al. 2010).

Biomagnification factors are useful tools to assess the biomagnification of metals from one trophic level to another. Mercury BMF obtained from transfer within primary producers to primary consumers and from them to secondary consumers were always > 1, so we can extrapolate that Hg biomagnifies through all the dietary exposures (DeForest et al. 2007).

The average Hg concentrations ratios between trophic levels, related to freshwater environments are typically 2-5, and similar to both marine and estuarine environments (Ciesielski et al. 2010; Evans et al. 2000).

Throughout our study we can exclude the interference of methylation process in the bioavailability of Hg, as our experimental conditions did not contain sediments, anoxia or suspected of high concentration of sulphate reducing bacteria. This particular condition can explain some of the extreme values found in other studies.

Regarding the tissues Hg content, several conclusions can be made. The primary consideration to be made is the fact that the amount of food given to *D. rerio* during the exposure period may not be enough to simulate environmental conditions. In fact as presented in the Chapter IV of this Thesis high concentrations of Hg were found within liver tissues and lower ones in muscle tissues. These differences in tissues concentration may also be important information regarding the detoxification process, that tends to be more delayed and persistent in muscle tissues and faster in liver tissues.

Another consideration that must be taken in consideration is the differences between Hg content at the start of the exposure period and the first 7-days of exposure. Despite the fact that no differences were found between this two sampling periods, a decreasing trend can be observed for all tissues after 7-days of exposure, after which tends to increase. This can be explained by the type of food given to the culture of *D. rerio*, since some commercialized fish food present

lower concentrations of mercury (personal unreported data showed that ZM400 ® presented 0.004 µg Hg/ g food).

As for the mercury total concentration in the different tissues, although not presenting statistical differences to the control, patterns can be observed, for example, in liver tissues an increase pattern is observed until 21 days of exposure, for muscle tissues, a smaller increase pattern is also observed. The stomach is the only tissue sampled where no patterns were found, which can be explained by several conditions. One of these explanations can be the amount of food content, although the organisms were left around 12 hours without supplying any food the stomach content was higher or lower due to each organism digestive velocity. The same condition described before can influence the time that the prey was within the stomach and absorption that could occur within it.

The final remark within the Hg tissues concentrations goes to the linear regressions for the Hg increment within each tissue. As expected a similar slope was obtained for all the tissues, but despite this fact different elevations were observed as the liver tissues tend to accumulate more Hg than the other two tissues. This result was in accordance once to the results observed in Chapter IV, as described before, where liver Hg concentration is higher. In the same way, muscle tissues showed the second lower accumulation, leaving the stomach in the last place.

Conclusion

This study demonstrates the high potential of mercury to be bioaccumulated by the green algae *P. subcapitata* from the aquatic medium. Additionally, mercury accumulated in algae cell is available for trophic transfer and has high potential to be bioaccumulated and biomagnified to *D. magna* and from this primary consumer, to a secondary consumer (*D. rerio*). This high accumulation of mercury confirms that algae can be important as mercury bioremovers, but also indicates their roles as mercury introducers in the trophic chains.

The BCF and BMF values from this work can be an important information to understand also in which step of the trophic chain the bioaccumulation and biomagnification can be more prejudicial. For example although the algae accumulated 70% of the total mercury presented in water, higher damage within the biota may be found not between water-algae (BCF=0.7), but within the daphnids population (BMF=20.7). In a more severe and general way, this trophic transfer of mercury through all trophic levels may threaten biodiversity as well as human health.

References

ASTM (2004) Standard Guide for Conducting *Daphnia magna* Life-cycle Toxicity Tests. ASTM E1193 - 97(2004). American Society for Testing and Materials, Pennsylvania

Back RC, Watras CJ (1995) Mercury in zooplankton of Northern Wisconsin Lakes: taxonomic and site-specific trends. Water, Air, & Soil Pollution 80 (1):931-938. doi:10.1007/bf01189747

Baeyens W, Leermakers M, Papina T, Saprykin A, Brion N, Noyen J, De Gieter M, Elskens M, Goeyens L (2003) Bioconcentration and Biomagnification of Mercury and Methylmercury in North Sea and Scheldt Estuary Fish. Archives of Environmental Contamination and Toxicology 45 (4):498-508. doi:10.1007/s00244-003-2136-4

Baird DJ, Barber I, Calow P (1990) Clonal variation in general responses of *Daphnia magna* Straus to toxic stress. 1. Chronic life-history effects. Functional Ecology 4 (3):399-407

Ciesielski T, Pastukhov MV, Szefer P, Jenssen BM (2010) Bioaccumulation of mercury in the pelagic food chain of the Lake Baikal. Chemosphere 78 (11):1378-1384

Coelho JP, Pereira ME, Duarte AC, Pardal MA (2009) Contribution of primary producers to mercury trophic transfer in estuarine ecosystems: Possible effects of eutrophication. Mar Pollut Bull 58 (3):358-365

Costley CT, Mossop KF, Dean JR, Garden LM, Marshall J, Carroll J (2000) Determination of mercury in environmental and biological samples using pyrolysis atomic absorption spectrometry with gold amalgamation. Analytica Chimica Acta 405 (1-2):179-183. doi:10.1016/s0003-2670(99)00742-4

DeForest DK, Brix KV, Adams WJ (2007) Assessing metal bioaccumulation in aquatic environments: The inverse relationship between bioaccumulation factors, trophic transfer factors and exposure concentration. Aquatic Toxicology 84 (2):236-246

Evans DW, Kathman RD, Walker WW (2000) Trophic accumulation and depuration of mercury by blue crabs (*Callinectes sapidus*) and pink shrimp (*Penaeus duorarum*). Marine Environmental Research 49 (5):419-434. doi:10.1016/s0141-1136(99)00083-5

Fatemi M, Baher E (2009) A novel quantitative structure–activity relationship model for prediction of biomagnification factor of some organochlorine pollutants. Molecular Diversity 13 (3):343-352. doi:10.1007/s11030-009-9121-4

García EM, Reyes RE (2001) Synthesis Pattern of an Hg-Binding Protein in *Acetabularia calyculus* During Short-Term Exposure to Mercury. B Environ Contam Tox 66 (3):357-364. doi:10.1007/s001280013

Gorski PR, Cleckner LB, Hurley JP, Sierszen ME, Armstrong DE (2003) Factors affecting enhanced mercury bioaccumulation in inland lakes of Isle Royale National Park, USA. The Science of The Total Environment 304 (1-3):327-348

Guan R, Wang W-X (2004) Dietary assimilation and elimination of Cd, Se, and Zn by Daphnia magna at different metal concentrations. Environmental Toxicology and Chemistry 23 (11):2689-2698. doi:10.1897/03-503

Huang X, Ke C, Wang W-X (2008) Bioaccumulation of silver, cadmium and mercury in the abalone *Haliotis diversicolor* from water and food sources. Aquaculture 283 (1-4):194-202

Kim E, Mason RP, Bergeron CM (2008) A modeling study on methylmercury bioaccumulation and its controlling factors. Ecological Modelling 218 (3-4):267-289

Lawson N, Mason R (1998) Accumulation of mercury in estuarine food chains. Biogeochemistry 40 (2):235-247. doi:10.1023/a:1005959211768

Leady BS, Gottgens JF (2001) Mercury accumulation in sediment cores and along food chains in two regions of the Brazilian Pantanal. Wetlands Ecology and Management 9 (4):349-361. doi:10.1023/a:1011856517552

Mason RP, Laporte JM, Andres S (2000) Factors Controlling the Bioaccumulation of Mercury, Methylmercury, Arsenic, Selenium, and Cadmium by Freshwater Invertebrates and Fish. Archives of Environmental Contamination and Toxicology 38 (3):283-297. doi:10.1007/s002449910038

Mason RP, Reinfelder JR, Morel FMM (1996) Uptake, Toxicity, and Trophic Transfer of Mercury in a Coastal Diatom. Environ Sci Technol 30 (6):1835-1845. doi:10.1021/es950373d

Mathews T, Fisher NS (2008) Evaluating the trophic transfer of cadmium, polonium, and methylmercury in an estuarine food chain. Environmental Toxicology and Chemistry 27 (5):1093-1101. doi:10.1897/07-318.1

McIntyre JK, Beauchamp DA (2007) Age and trophic position dominate bioaccumulation of mercury and organochlorines in the food web of Lake Washington. Science of The Total Environment 372 (2-3):571-584

Meylan WM, Howard PH, Boethling RS, Aronson D, Printup H, Gouchie S (1999) Improved method for estimating bioconcentration/bioaccumulation factor from octanol/water partition coefficient. Environmental Toxicology and Chemistry 18 (4):664-672. doi:10.1002/etc.5620180412

Morel FMM, Kraepiel AML, Amyot M (1998) The Chemical Cycle and Bioaccumulation of Mercury. Annual Review of Ecology and Systematics 29 (ArticleType: research-article / Full publication date: 1998 / Copyright © 1998 Annual Reviews):543-566

Pickhardt PC, Folt CL, Chen CY, Klaue B, Blum JD (2002) Algal blooms reduce the uptake of toxic methylmercury in freshwater food webs. Proceedings of the National Academy of Sciences 99 (7):4419-4423. doi:10.1073/pnas.072531099

Pickhardt PC, Folt CL, Chen CY, Klaue B, Blum JD (2005) Impacts of zooplankton composition and algal enrichment on the accumulation of mercury in an experimental freshwater food web. Science of The Total Environment 339 (1-3):89-101. doi:10.1016/j.scitotenv.2004.07.025

Power M, Klein GM, Guiguer KRRA, Kwan MKH (2002) Mercury accumulation in the fish community of a sub-Arctic lake in relation to trophic position and carbon sources. Journal of Applied Ecology 39 (5):819-830. doi:10.1046/j.1365-2664.2002.00758.x

Schmitt D, Müller A, Csögör Z, Frimmel FH, Posten C (2001) The adsorption kinetics of metal ions onto different microalgae and siliceous earth. Water Research 35 (3):779-785

Tessier L, Vaillancourt G, Pazdernik L (1994) Temperature effects on cadmium and mercury kinetics in freshwater molluscs under laboratory conditions. Archives of Environmental Contamination and Toxicology 26 (2):179-184. doi:10.1007/bf00224802

Tsui MTK, Wang W-X (2004) Temperature influences on the accumulation and elimination of mercury in a freshwater cladoceran, *Daphnia magna*. Aquatic Toxicology 70 (3):245-256

Zhou HY, Wong MH (2000) Mercury accumulation in freshwater fish with emphasis on the dietary influence. Water Research 34 (17):4234-4242

Chapter IV: Assessment of mercury contamination in a coastal lagoon (Ria de Aveiro, Portugal) using juvenile *Liza aurata*

Assessment of mercury contamination in a coastal lagoon (Ria de Aveiro, Portugal) using juvenile *Liza aurata*

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Abstract

Mercury concentrations (total and organic mercury) were determined in sediments and fish samples (*Liza aurata*) from two sites in Ria de Aveiro, Portugal. Total and organic mercury increments transferred from sediments into fish were assessed in both sites. The mean concentration of total mercury in liver (0.28 µg/g) and muscle (0.07 µg/g) of fish in the contaminated site (Cais do Bico) were higher than the total mercury mean concentrations in liver (0.04 µg/g) and muscle (0.02 µg/g) from the reference site (Barra). This supports the lower contamination gradient found with the distance to the mercury source. The ratio values >1 between total mercury in liver and muscle tissues from fish from the both sites reveals a bioavailability increment in the environment; in spite of that, all mercury data were considerably below those recommended by specific legislation levels for aquatic ecosystems.

Key-words: Total Mercury, Organic Mercury, Sediments, *Liza aurata*, Bioconcentration factor, Biota-Sediment bioamplification factor

Introduction

Mercury (Hg) is a widespread natural element in the environment, but anthropogenic activities that discharge Hg to the environment highly increase the amount presently cycled in the biosphere (Nevado et al. 2011; Mieiro et al. 2011b;

Guilherme et al. 2008b). Anthropogenic release of Hg is the main factor responsible for environmental impacts, that causes harmful effects on biota, at level of ecosystem functions and Human health (Pereira et al. 2009). Environmental studies about Hg interactions are of great importance, as Hg is persistent in the environment and is a non-essential element without biochemical or nutritional function to organisms, being toxic to all living organisms (Guilherme et al. 2008b; Abreu et al. 2000; Mieiro et al. 2009; Pereira et al. 1995).

Estuaries and coastal waters constitute the link between the terrestrial environment and open water. It is known that only a small fraction of Hg transported in rivers is exported to open waters due to the high retention of Hg in estuaries and coastal waters (Green-Ruiz et al. 2005). Mercury mobility and reactivity in estuaries is highly influenced by organic matter content, salinity, redox conditions and pH (Oliveira et al. 2009).

The particulate suspended matter (SPM) includes a nonbiogenic fraction but also a biogenic fraction, constituted for small living organisms and detritus (Baeyens et al. 2003). Generally, in the aquatic environment, Hg tends to be associated with suspended particles, however, the majority of Hg is removed from the water column to bottom sediments. Thus, the sediments play an important double role: initially they behave as depository of Hg and, in a posterior phase, as an internal source releasing Hg to water column, playing an significant role in the aquatic cycle of Hg (Ramalhosa et al. 2001; Ramalhosa et al. 2005; Mieiro et al. 2009; Green-Ruiz et al. 2005; Ullrich et al. 2001). The ressuspension of Hg occurs mainly during periods of stronger tidal currents, being responsible for increasing Hg bioavailability and for the transport of Hg over long distances (Guilherme et al. 2008b; Oliveira et al. 2009; Pereira et al. 1995). High Hg concentrations have been found in sediments, comparative to those found in water column (Ramalhosa et al. 2005), in addition, Hg of contaminated environments may be transferred from the abiotic to the biotic compartment (Pereira et al. 2009). Bacteria existing in sediments can convert several mercury compounds into a more toxic and water-soluble form, methylmercury (MeHg), through a process named biomethylation, being bioavailable and readily uptake by other aquatic organisms (Green-Ruiz et

al. 2005; Mieiro et al. 2009; Nevado et al. 2011). Biomethylation process is particularly harmful in estuaries because of their high bioproductivity (Green-Ruiz et al. 2005). All forms of Hg are extremely toxic but MeHg is of most concern especially in the aquatic environment because of its lipophilicity (Guilherme et al. 2008b). Methylmercury can cause damage in several organs, especially in the central nervous system (Mieiro et al. 2011b).

High loads of Hg in the aquatic environment have resulted in high accumulation levels of this metal in fish tissues and ultimately, in fish consumers, so, Hg readily enters the food chain, in inorganic or organic forms, being the predominant pathway of human exposure to MeHg (Guilherme et al. 2008a; Coelho et al. 2005).

Non-essential metals, as Hg, are not required to the normal fish cellular metabolism, but they can be taken up from water, food and/or sediments, and may also be absorbed and accumulated in tissues (Oliveira et al. 2010). The accumulation of Hg in fish tissues can adversely affect fish populations, so is important to evaluate its distribution and accumulation in fish tissues, in order to gain a better understanding of the dynamics of this pollutant in the fish body. The liver is a target to inorganic and metallic Hg due to its role in detoxification and fish metabolism. The fish muscle constitutes more than 60% of the fish's body mass and acts as a reservoir of Hg, mainly in most toxic form MeHg, and is recognized as the major route of human MeHg exposure (Carrasco et al. 2011; Guilherme et al. 2008a; Mieiro et al. 2009). Almost 100% of the Hg content in fish muscle tissue is methylated (Ciesielski et al. 2010).

Mercury accumulation in fish depends on several factors: fish characteristics, as trophic position, age and size, which limit nature and activity of organisms and biochemical reactions of Hg inside the organism. Mercury accumulation also depends on physicochemical variables (speciation, binding, release, distribution) and biogeochemical pathways of Hg in the environment that establish its bioavailability (Abreu et al. 2000; Tavares et al. 2011). Even in areas with tolerable aquatic Hg concentration, fish tend to bioconcentrate Hg by a factor of 10^5-10^7 (Nevado et al. 2011). It is widely accepted that there is a positive

relationship between trophic position and bioaccumulative contamination load. Mercury can biomagnify up 1000-fold through trophic chains (Zhang et al. 2007). It has been estimated that the half-life of total Hg in fish is approximately 5 days to 5 months, and the half-life for MeHg approximately 1 to more than 3 years (Hope 2003).

It has been recognized that fish and shell-fish consumption are the major route of Hg consumption by Humans, but the direct exposure to areas with high mercury levels in multiple environmental compartments may also represent a risk to human health (Pereira et al. 2009; Nevado et al. 2011; Tavares et al. 2011; Mieiro et al. 2009).

Results from several European Union research programs reinforce the great difference between the mercury levels found in water column and in biota (Cossa and Coquery 2005), which justify the need of using bioindicator species. The estimation of a target mercury water concentration requires a biomagnification factor (BMF) and a fish tissue criterion protective of human health. It is understood that such factors are habitually influenced by local conditions, so the U.S.EPA recommends the use of BMFs derived from data collected at the site of concern instead of default values (Hope 2003).

The golden grey mullet (*Liza aurata*) is a pelagic species that has a complex lifecycle, regularly contacts with sediments being often extensive to the whole water column; in a fry stage it has a zooplanktivorous behavior and detritivorous/herbivorous in a post-fry stage (Guilherme et al. 2008b; Pacheco et al. 2005; Oliveira et al. 2010). So, *L. aurata* juveniles have a more predatory behavior, that includes higher organic mercury concentrations dietary items than the diet of adults (Tavares et al. 2011). *L. aurata* appears as an appropriate bioindicator of Hg contamination due to its larger geographical distribution and abundance during whole year (Pacheco et al. 2005; Guilherme et al. 2008b). *L. aurata* is used for human consumption (Pérez Cid et al. 2001). Juvenile specimens were used due to their prevalence in the estuary, and in order to mitigate or avoid the interference of variables such as gender, reproductive processes, male/female

metabolism differentiation, as well as the potential occurrence of a growth dilution effect in relation to mercury accumulation.

This study was conducted in the Ria de Aveiro coastal lagoon, northwestern coast of Portugal (40º 38N, 8º 44W), with 43km^2 wet area with a single, artificially maintained connection to the sea. The existent web of islands and channels inside the lagoon make water circulation difficult and complex, allowing the spread of any conservative contaminants discharged into the coastal waters through the single sea mouth (Ramalhosa et al. 2005). Ria de Aveiro is a biologically productive system with a significant role in the life cycle of several organisms being used as nursery for many species, namely invertebrates and fish, many of which used for human consumption (Oliveira et al. 2009; Pérez Cid et al. 2001). This coastal lagoon and its surroundings are also essential for social, economic, community health and recreational reasons. Professional fishing and aquaculture are important activities in the lagoon (Pérez Cid et al. 2001) and around the lagoon there are an intensive, diversified agriculture, several types of industries and a population of about half a million people, a part of which discharges their untreated or partially treated sewage into the lagoon (Ramalhosa et al. 2005).

Cais do Bico is located immediately after an inner bay (Laranjo) highly contaminated by effluent discharges by a mercury cell chlor-alkali plant from the 1950s until 1994 (Pereira et al. 2009; Pacheco et al. 2005). Although effluent releases stopped, fine surface sediments of this basin still present high Hg concentrations, creating a contamination gradient (Mieiro et al. 2011b). Mercury storage is estimated to be 33x10^3 kg, of which 77% are as sediment-associated in the channel (Esteiro de Estarreja) that receives the effluent discharge (Pereira et al. 2009; Abreu et al. 2000). During spring tides, approximately 75% of the water in the inner basin is renewed, implying the resuspension of contaminated sediments by the tidal currents (Monterroso et al. 2003).

Concentrations of mercury in sediments and in biota, have been reported in several works, but most of them focus on the Laranjo area (Ramalhosa et al. 2005; Pereira et al. 1995; Guilherme et al. 2008a; Mieiro et al. 2011a; Abreu et al. 2000).

Within this context, the present study aims to evaluate the bioaccumulation of Hg (total and organic Hg) in juvenile fish populations of the species *Liza aurata* inhabiting the Hg contaminated site (Cais do Bico) and the downstream point (Barra) with focus on the liver and muscle tissue, total mercury loads and their relation to abiotic concentrations: water, suspended particulate matter (SPM) and sediment. This study also aims to improve the knowledge on mercury bioamplification, to evaluate the environmental risk using autochthonous fish fauna and to provide data on total mercury and organic mercury concentrations in muscle, which could prove to be useful when evaluating human health risk.

Material and Methods

Study area

Two locations (Cais do Bico and Barra) were chosen as sampling sites according to the existing Hg contamination gradient (Fig.7). The Cais do Bico site is located immediately after the pollution source, its Hg contamination results mainly from the ressuspended matter created by the tides. The Barra site was selected as a reference for comparison purposes due to the distance from the main polluting sources and the lagoon entrance proximity.

This study was carried out during the summer of 2011 (July), water, sediments and fish samples were collected and processed as described bellow.

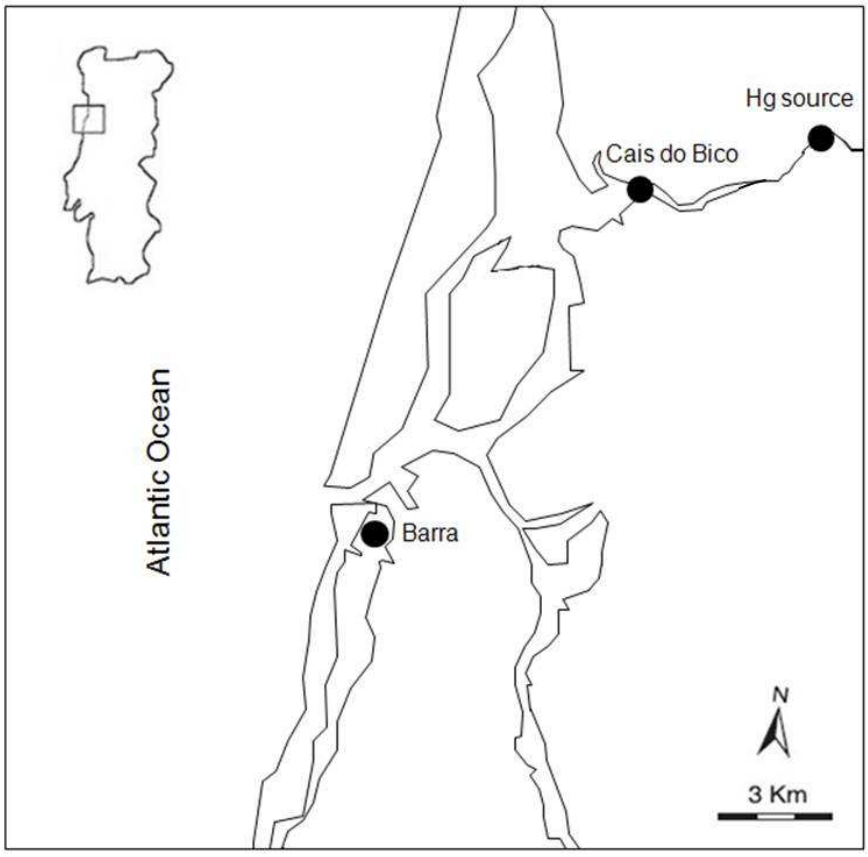

Fig. 7 Map of Ria de Aveiro (Portugal) with the two sampling sites (Cais do Bico and Barra) and the approximated location of the chlor-alkali plant (Hg source).

Water samples

Water physico-chemicals parameters: temperature, pH, dissolved oxygen and salinity were measured *in situ* using VWR Symphony™ meter.

Waters samples were filtered with 0.45 µm Millipore membranes. The filtrate was then acidified with HNO_3 (Merck, mercury-free) to pH<2 and stored at 4ºC until dissolved Hg analysis. The filters were oven dried at 40ºC until a constant weight and used to measure the suspended particulate matter (SPM). SPM filters were then used to analyze particulate Hg. Procedure blanks were always run with samples and replicates (three replicates per site).

Sediments

Superficial sediments (~2 cm depth) were collected for mercury analysis. Sediments were oven dried at 40ºC in order to remove humidity and total and organic Hg concentrations in the sediments were determined in the <63 µm fraction, to minimize the effect of grain size on metal distribution. Two replicates were used for each type of Hg sampling.

Fish collection and procedure

Juvenile specimens of *Liza aurata* (n=18, average weight of 5.2 ± 1.1 g and length of 9.2 ± 1.9 cm), age 1+, were captured during the low tide using a traditional manually operated trawl net “chincha”.

Upon arrival to the lab, fishes were weighted and their total body length was measured. Then, specimens were dissected on ice and tissue samples separated in white muscle tissue and liver tissue, and stored at -20ºC until further analysis.

The fish condition was calculated using morphometric data and the Fulton’s condition factor K determined following the formula: $K=100.W_t/L_t^2$, where W_t is total wet weight (g) and L_t total length (mm) (Vasconcelos et al. 2009).

The biota-sediment accumulation factor (BSAF) was calculated following the formula: BSAF = [Hg] in organism / [Hg] in associated sediment (Green-Ruiz et al. 2005).

Total mercury was determined using nine replicates and organic Hg with five replicates. Organic mercury was only sampled for the muscle tissue.

Mercury analyses

Total mercury (THg) concentrations in water (dissolved and particulate), and sediments were quantified by atomic absorption spectroscopy following

thermal decomposition of the sample, using an Advanced Mercury Analyzer (LECO AMA-254). The accuracy of the equipment was checked daily (in the beginning and at the end of the analyses) with two Certified Reference Material (CRM) of similar matrix of the samples: PACS-2 (marine sediments) for sediments, TORT-2 (lobster hepatopancreas) and DORM-3 (fish protein) for biological samples, obtained from the National Research Council of Canada. The results for PACS-2 (2.90±0.10 µg/g), TORT-2 (0.217±0.007 µg/g) and DORM-3 (0.330±0.010 µg/g) were always within the certified values.

Organic mercury (OHg) in sediments and fish muscle were determined through digestion of samples with a mixture of 18% KBr in 5%H_2SO_4, followed by extraction of organic mercury into toluene (Válega et al. 2006). The organic mercury compounds were back extracted into a $Na_2S_2O_3$ solution, and quantified in liquid aliquots (1 ml) of the resulting aqueous medium with LECO AMA-254. The analyses were validated using certified reference material TORT-2 and DORM-3. The results from TORT-2 (0.141 ± 0.001 µg/g), DOLT-3 (0.302 ±0.003 µg/g) were within the certified values.

Statistical analyses

SigmaPlot statistical package (SigmaPlot, v. 12, Systat Software Inc.) was used for statistical analyses. Data were tested for goodness of fit to normal distribution and requirements of homogeneity of variances. Student's *t*-test (α=0.05) was used to detect the differences in fish populations and mercury concentrations between sediments and biota from both sampling sites. Significant differences between the correlations parameters were calculated using linear regression (α=0.05). All data suffered a *ln* transformation prior to analysis, except for the fish total length comparison.

Results

Analysis of the water column

The physicochemical parameters, including water temperature, salinity, conductivity, dissolved oxygen and pH for each sampling site are presented in Table 5. Physicochemical parameters are similar in both sites, except for the dissolved oxygen concentration which was higher at Barra.

Table 5 Physicochemical parameters on the both sampling sites at Ria de Aveiro (Cais do Bico and Barra)

	Cais do Bico	Barra
Temperature (ºC)	21.7	21.1
Salinity (ppt)	31.9	32.9
Conductivity (mS/cm)	49.1	50.5
Dissolved oxygen (mg/L)	9.0	14.3
pH	7.8	8.0

The two sites present similar values of dissolved Hg (Table 6). Cais do Bico has higher particulate Hg concentration. The partition coefficient (K_d) is higher in Cais do Bico than Barra.

Table 6 Mercury concentration and respective *SD*, in the water column of the two sampling sites (Cais do Bico and Barra): dissolved Hg, particulate Hg and partition coefficient (K_d).

Sampling site	Dissolved Hg (ng/L)	Particulate Hg (µg/g)	K_d
Cais do Bico	6.8 ± 0.1	0.757 ± 0.082	4.4
Barra	5.5 ± 0.6	0.125 ± 0.057	1.3

Mercury in sediments

In the fraction <63µm of sediments, although Cais do Bico shows a high value for the concentration of THg (0.188 µg/g) than Barra. The concentration of OHg and subsequently the %[OHg]/[THg] is higher for Barra (Table 7).

Table 7 Total mercury concentration ([THg]), organic mercury concentration ([OHg]) and percentage of organic mercury in the total burden of mercury (%[OHg]/[THg]) and respective standard deviation (*SD*) in the fraction <63 µm of the sediments from the two sampling sites (Cais do Bico and Barra).

Sampling site	[THg] (µg/g)	[OHg] (µg/g)	% [OHg]/[THg]
Cais do Bico	0.188 ± 0.003	0.009 ± 0.001	4.8 ± 0.5
Barra	0.071 ± 0.003	0.018 ± 0.015	24.4 ± 10.1

Mercury in fish

No statistical differences were found between size (total length) of fish captured in the two study sites (*t*-test, t_{15}=-0.873; p=0.397). However, the comparison between both sampling sites show significant differences for THg content of fish muscle (*t*-test, t_{13}=-27.687; $p \leq 0.001$) and for THg content of liver tissue (*t*-test, t_{15}=-16.870; $p \leq 0.001$ – Table 8).

Organic mercury accumulation (Table 8) also showed significant differences between sampling sites (OHg muscle (t_8=-8.887; $p \leq 0.001$)), being Cais do Bico once again the sampling site with high values. The ratio [THg] liver / [THg] muscle was higher than 1 in both sampling sites and Cais do Bico showed a statistic significant high value than Barra (t_{13}= -4.240, $p \leq 0.001$). The % [OHg] / [THg] showed no significant differences between sampling sites (t_8=1.542, p=0.162 – Table 8).

The condition factor (Fulton's K) of fish sampled in Barra is significant different from the ones sampled in Cais do Bico (t_{15}= 6.814, p≤0.001) and are respectively 0.86 ± 0.04 and 0.70 ± 0.05.

Table 8 Means and *SD* of total mercury concentrations in the liver and muscle tissue, organic mercury in muscle tissue, ratio of liver-to-muscle total mercury and percentage of organic mercury in fish from both sampling sites (Cais do Bico and Barra). Significant differences are designated by different letters (*t*-test, p<0.05).

Sampling site	[THg] liver (µg/g)	[THg] muscle (µg/g)	[OHg] muscle (µg/g)	[THg] liver/ [THg] muscle	% [OHg]/[THg]
Cais do Bico	0.284 ± 0.100[a]	0.066 ± 0.021[c]	0.046 ± 0.011[e]	4.2 ± 0.8[g]	86.9 ± 13.6[i]
Barra	0.044 ± 0.005[b]	0.015 ± 0.001[d]	0.015 ± 0.001[f]	2.8 ± 0.4[h]	96.2 ± 1.5[i]

Mercury accumulation on muscle and its relationship with the length of fish are showed in Fig. 8. No significant correlations were found for both sampling sites: Barra (r = 0.563; *df*=7; p=0.146) and Cais do Bico (r = 0.731; *df*=5; p=0.099).

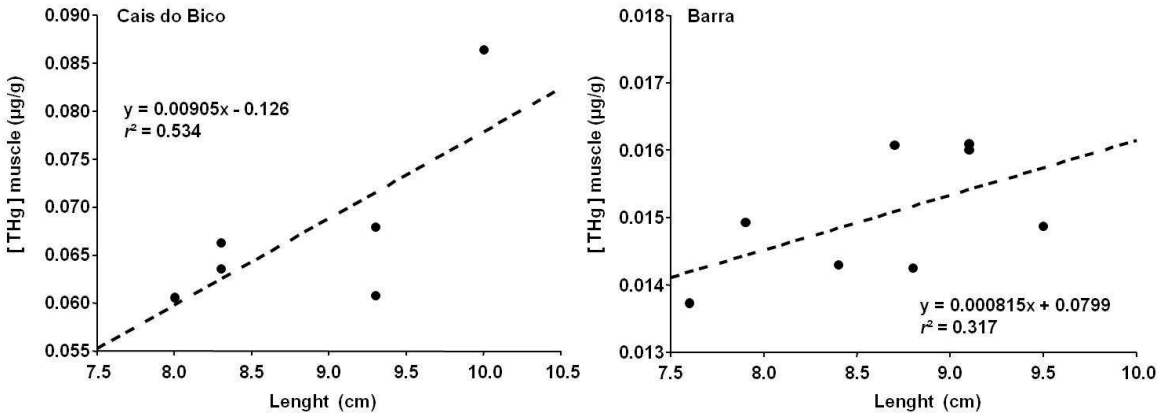

Fig. 8 Mercury concentrations (µg/g, wet weight) in muscle versus specimen length (cm) of *L. aurata* captured in sampling sites (Cais do Bico and Barra).

As for the correlation between organic mercury and total mercury concentrations in the muscle of *L. aurata* it was significant for Barra (r = 0.984; df=4; p=0.002) but not for Cais do Bico (r = 0.816; df=4; p=0.092 – Fig. 9).

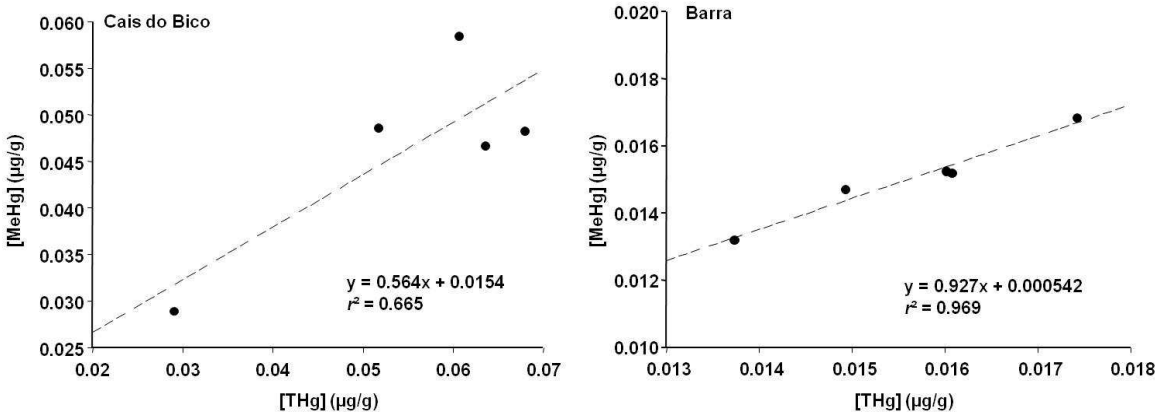

Fig. 9 Linear relationship between organic mercury (OHg µg/g, wet weight) and total mercury (THg µg/g, wet weight) in muscle of *L. aurata* from Cais do Bico and Barra.

Fish from both sampling sites presented significant differences between THg BSAF (t_{14}=-7.927, p≤0.001) with a mean value of 0.348 and 0.216 for Cais do Bico and Barra respectively. The OHg BSAF mean values for Barra (0.852) and Cais do Bico (5.141) also presented significant differences (t_8=-14.345, p≤0.001).

Discussion

Environmental mercury values were as expected higher in sediments and water column in Cais do Bico due to its proximity to the Hg industrial source. As expected the lower values of Hg obtained in Barra for all compartments reflect the distance to the contamination source and the proximity of the marine water entrance (Turner et al. 2001). The Hg concentrations in the water are highly dynamic, being influenced by abiotic factors among which the water flow, so the

dissolved Hg concentrations were, in general, low in Ria de Aveiro and all over the world (Mieiro et al. 2010).

Mercury has high affinity for SPM, so SPM plays an important role on mercury bioavailability, since the Hg will associate with it and will become more available for fish to consume. Particulate mercury content is usually higher in environments with elevated SPM, especially in shallow regions of estuaries where resuspension process is higher (Mieiro et al. 2010). The high particulate mercury associated with SPM in Cais do Bico when compared to Barra can be explained by the proximity to the Hg source and consequently the high Hg resuspension in this area. Cais do Bico shows higher K_d compared to Barra station not only due to the higher values of particulate mercury in the most contaminated area but also because of the comparatively higher fraction of dissolved Hg in Barra (similar to the dissolved fraction in Cais do Bico) induced by complexation processes caused by tidal dynamics and sea water mixture.

Total mercury concentrations in sediments revealed high human-induced environmental mercury gradient in Ria de Aveiro, being the highest concentrations found in Cais do Bico, the nearest sampling site to the contamination source. So, the results obtained for mercury concentrations in the sediments are mostly related with distance to the industrial source of this metal, with concentrations as high as 0.188 µg/g in Cais do Bico and 0.071 µg/g in Barra.

Ria de Aveiro estuary is characterized by muddy, organic-rich sediments, resultants from reducing conditions, so favorable for methylation. As a result, the higher percentage of OHg found in sediments from Barra can be explained by the Hg transported by flooding events, that could also transfer Hg to downstream sites and other biotic/abiotic parameters that can also induce a high bacterial flora responsible for this methylation. The transfer of mercury downstream may also increase the quantity of Hg available for methylation. In fact, a number of recent studies have reported that newly deposited or freshly added Hg is more readily methylated than environmental Hg (Carrasco et al. 2011). When we take in consideration together the total mercury and organic mercury results from both sampling sites, they are in accordance with previous studies (Tavares et al. 2011;

Coelho et al. 2005). The same authors found that the concentration of mercury in sediments would give a better response of the sites contaminations than other parameters like dissolved Hg and in some cases the SPM. This is not the case of this study since almost all the measured concentration of mercury in the different parameters gave significant differences, but it should be taken in consideration the proximity of Cais do Bico to the contamination source. If another place between the two sites would be chosen, the same conclusion maybe be obtained.

All values were lower than 1 µg Hg/L, the value permitted by Council Directive 76/464/EEC, for mercury concentrations in estuary coastal water, and lower than 0.36 µg/g of the Water Framework Directive environmental quality standard for sediments.

The fish condition factor is commonly used as indicator of the overall nutritional status of fish. A K bellow 1 give the information that the organisms are undernourished or thin, and is the case of this study. The recent study by Vasconcelos et al. (2009), found also Ks bellow 1 for several other juvenile species in Ria de Aveiro.

As mercury is a non-essential element, is not probable to have its uptake/elimination actively regulated and subsequently its tissue concentrations can vary in a wide range, reflecting exposure to environmental levels and feeding behavior (Mieiro et al. 2009). Therefore, mercury body burdens in bioindicator species provide realistic indications of aquatic pollution as well as of possible impact on organism health. Significant differences between fish from Cais do Bico and Barra were detected not only in muscle, but also in liver tissues. In fact, the higher accumulation of total Hg in liver may be considered the primarily signal of metal exposure. It was possible to infer a buffering action of liver, protecting the other tissues, namely muscle, against mercury accumulation and the subsequent toxicity. Liver always demonstrated higher levels than muscle (4x in Cais do Bico and 3x in Barra), which is in agreement with previous studies of fish exposure to Hg in Ria de Aveiro, with liver tissues presenting around 3x higher concentration of mercury than muscle tissues (Guilherme et al. 2008a; Abreu et al. 2000). This differential tissue accumulation may be explained by its physiological role, with

liver being a main target and also a detoxifying organ (greater likelihood of Hg to associate with metallothioneins (Tavares et al. 2011)), and muscle remaining as a secondary target and as a reservoir (Guilherme et al. 2008a; Mason et al. 2000). Along with the previous results the liver/muscle ratio of mercury concentrations is once again higher in Cais do Bico than in Barra presenting different intensities of stress (Abreu et al. 2000).

To better understand Hg species distribution in tissues, different Hg species ratios were estimated. The mean percentage value of OHg in THg was for Cais do Bico and Barra respectively 86% and 96%, which indicates high toxicity since the OHg is several times more toxic than the inorganic Hg. A recent study on mercury speciation in a near place of Cais do Bico (Laranjo) also found a percentage higher than 90% of OHg in the muscle of the same specie (*L. aurata*) (Tavares et al. 2011). It is noteworthy that although Barra presents lower concentrations of mercury, the percentage of OHg is higher, due to nonlinearities between total Hg input, OHg formation, and OHg bioaccumulation; and between OHg concentrations in the water and sediments and OHg levels in the biota (Carrasco et al. 2011). In other hand, accumulation from food is the dominant uptake mechanism for OHg in fish and a further study on *L. aurata* prey may be useful to better understand these results (Mason et al. 2000; Coelho et al. 2008).

The lowest percentage of OHg (86%) was found in the most contaminated area (Cais do Bico). Analogous results were previously reported and described as the “mercury accumulation paradox”, which consists in the induction of mer-encoded enzymes responsible for the degradation of organic mercury. This enzymes induction is proportional to the environmental mercury concentration; high levels induced the mer-encoded system, promoting the demethylation of mercury, and, as consequence, low organic mercury accumulation rates in biota (Mieiro et al. 2009).

Previous studies have found positive relationships between mercury concentration and fish length, mainly due to the fact that methylmercury is very slowly eliminated once incorporated into fish muscle (Al-Majed and Preston 2000; McClain et al. 2006). In this study and for the specie *L. aurata*, a correlation was

not obtained between total mercury in muscle and fish length for both sampling sites, although statistically the probabilities were very close to $p \leq 0.05$ and $r > 0.5$. These results may be explained by the low number of sampled organisms, and the similar length between organisms, so a new study that takes in consideration these two facts may be in accordance with the previous studies stated above.

The correlation between total mercury and organic mercury in the muscle showed differences between sampling sites. Although a positive correlation between the two variables was expected in both sampling sites, the lower concentration of mercury in the sampling site Barra may help explain with a near ratio between the organic mercury and the total mercury, with a slope equal to 0.927. As for the Cais do Bico this ratio tends to the total mercury (slope equal to 0.564), since it as a higher mercury input.

As expected a high liver/muscle ratio was found in the most contaminated sampling site (Cais do Bico). An explanation to this fact can be the successive binding and immobilization of inorganic Hg to metalothioneins, highly produced in the liver that will result in increased liver concentrations to allow this detoxification process to occur. Also, a prior demethylation process occurs in the same organ that transforms the organic mercury into inorganic mercury leading to the above detoxification process by the metallothioneins (Mieiro et al. 2009).

The biota-sediment accumulation factor is useful to determine the ability of the organism to accumulate mercury from the associated sediment. The BSAF for THg and OHg are in accordance with the differences between the Hg content of the two sites, with Cais do Bico showing higher Hg concentrations bioavailable to biota. The OHg BSAF values are higher than the THg BSAF values, and to our knowledge no other work developed in this area as ever reported this fact. A possible explanation to these results may be the OHg assimilated from preys and/or SPM than the OHg from sediments. In fact, since the sampled organisms were 1+ age, compared to adults their behavior is based in a more predatory habit. For the fish results in our study, the BSAF values are always below one, except for the OHg from Cais do Bico (5.1). These BSAF values show us that a small bioamplification occurs from the sediment to the organism. As for the OHg BSAF

found for Cais do Bico can be explained by a high assimilation efficiency, and a very slow elimination compared to the uptake (McGeer et al. 2003).

Conclusion

Liza aurata was selected in the present work because it is one of the most dominant species in the surveyed lagoon Ria de Aveiro, being easy to identify and capture. As a benthopelagic species, its feeding behavior and its life history makes it particularly appropriate to the current goals. In fact, the juveniles of *L. aurata* showed in this study the ability to detect inter-sites differences, and contrary to other studies, they did not present the mobility usually described for other fish species, that could live between the two sampling sites. Furthermore, the adoption of juvenile specimens provides information on short-term variations of mercury concentrations in the environment. As *L. aurata* is a migratory species, the mercury accumulated by its juveniles can be transported to the marine environment.

Mercury concentrations in fish tissue correlate to Hg levels in their aquatic environment, and sediments did not seem to be the major source for Hg accumulated in fish. On the other hand, SPM and trophic chain structure may be an important factor influencing *Liza aurata* mercury burden. It is reinforced the importance of monitoring mercury burden, particularly in the edible tissue of fish species included in the human diet, in order to safeguard public health. Total mercury concentration in muscle from fish captured in this study were not higher than the legal limit of 0.5 µg/g established by European Union, however, human risk associated to the regular ingestion of fish inhabiting in the Cais do Bico and Barra areas cannot be excluded. As well as the consumption of seafood from the two sampling sites, since the organic mercury concentrations in sediments are high in both sites.

References

Abreu SN, Pereira E, Vale C, Duarte AC (2000) Accumulation of Mercury in Sea Bass from a Contaminated Lagoon (Ria de Aveiro, Portugal). Mar Pollut Bull 40 (4):293-297

Al-Majed NB, Preston MR (2000) An Assessment of the Total and Methyl Mercury Content of Zooplankton and Fish Tissue Collected from Kuwait Territorial Waters. Mar Pollut Bull 40 (4):298-307

Baeyens W, Leermakers M, Papina T, Saprykin A, Brion N, Noyen J, De Gieter M, Elskens M, Goeyens L (2003) Bioconcentration and Biomagnification of Mercury and Methylmercury in North Sea and Scheldt Estuary Fish. Archives of Environmental Contamination and Toxicology 45 (4):498-508. doi:10.1007/s00244-003-2136-4

Carrasco L, Barata C, García-Berthou E, Tobias A, Bayona JM, Díez S (2011) Patterns of mercury and methylmercury bioaccumulation in fish species downstream of a long-term mercury-contaminated site in the lower Ebro River (NE Spain). Chemosphere

Ciesielski T, Pastukhov MV, Szefer P, Jenssen BM (2010) Bioaccumulation of mercury in the pelagic food chain of the Lake Baikal. Chemosphere 78 (11):1378-1384

Coelho JP, Pereira ME, Duarte A, Pardal MA (2005) Macroalgae response to a mercury contamination gradient in a temperate coastal lagoon (Ria de Aveiro, Portugal). Estuarine, Coastal and Shelf Science 65 (3):492-500

Coelho JP, Reis AT, Ventura S, Pereira ME, Duarte AC, Pardal MA (2008) Pattern and pathways for mercury lifespan bioaccumulation in *Carcinus maenas*. Mar Pollut Bull 56 (6):1104-1110

Cossa D, Coquery M (2005) The Mediterranean Mercury Anomaly, a Geochemical or a Biologocal Issue. In: The Mediterranean Sea, vol 5K. The Handbook of Environmental Chemistry. Springer Berlin / Heidelberg, pp 121-130. doi:10.1007/b107147

Green-Ruiz C, Ruelas-Inzunza J, Páez-Osuna F (2005) Mercury in surface sediments and benthic organisms from Guaymas Bay, east coast of the Gulf of California. Environmental Geochemistry and Health 27 (4):321-329. doi:10.1007/s10653-004-5741-x

Guilherme S, Válega M, Pereira ME, Santos MA, Pacheco M (2008a) Antioxidant and biotransformation responses in *Liza aurata* under environmental mercury exposure

- Relationship with mercury accumulation and implications for public health. Mar Pollut Bull 56 (5):845-859

Guilherme S, Válega M, Pereira ME, Santos MA, Pacheco M (2008b) Erythrocytic nuclear abnormalities in wild and caged fish (*Liza aurata*) along an environmental mercury contamination gradient. Ecotoxicology and Environmental Safety 70 (3):411-421

Hope B (2003) A basin-specific aquatic food web biomagnification model for estimation of mercury target levels. Environmental Toxicology and Chemistry 22 (10):2525-2537. doi:10.1897/02-395

Mason RP, Laporte JM, Andres S (2000) Factors Controlling the Bioaccumulation of Mercury, Methylmercury, Arsenic, Selenium, and Cadmium by Freshwater Invertebrates and Fish. Archives of Environmental Contamination and Toxicology 38 (3):283-297. doi:10.1007/s002449910038

McClain W, Chumchal M, Drenner R, Newland L (2006) Mercury Concentrations in Fish from Lake Meredith, Texas: Implications for the Issuance of Fish Consumption Advisories. Environmental Monitoring and Assessment 123 (1):249-258. doi:10.1007/s10661-006-9194-9

McGeer JC, Brix KV, Skeaff JM, DeForest DK, Brigham SI, Adams WJ, Green A (2003) Inverse relationship between bioconcentration factor and exposure concentration for metals: Implications for hazard assessment of metals in the aquatic environment. Environmental Toxicology and Chemistry 22 (5):1017-1037. doi:10.1002/etc.5620220509

Mieiro C, Ahmad I, Pereira M, Duarte A, Pacheco M (2010) Antioxidant system breakdown in brain of feral golden grey mullet (*Liza aurata*) as an effect of mercury exposure. Ecotoxicology 19 (6):1034-1045. doi:10.1007/s10646-010-0485-0

Mieiro CL, Bervoets L, Joosen S, Blust R, Duarte AC, Pereira ME, Pacheco M (2011a) Metallothioneins failed to reflect mercury external levels of exposure and bioaccumulation in marine fish - Considerations on tissue and species specific responses. Chemosphere 85 (1):114-121

Mieiro CL, Pacheco M, Pereira ME, Duarte AC (2009) Mercury distribution in key tissues of fish (*Liza aurata*) inhabiting a contaminated estuary-implications for human and ecosystem health risk assessment. Journal of Environmental Monitoring 11 (5):1004-1012

Mieiro CL, Pereira ME, Duarte AC, Pacheco M (2011b) Brain as a critical target of mercury in environmentally exposed fish (Dicentrarchus labrax)--Bioaccumulation and oxidative stress profiles. Aquatic Toxicology 103 (3-4):233-240

Monterroso P, Abreu SN, Pereira E, Vale C, Duarte AC (2003) Estimation of Cu, Cd and Hg transported by plankton from a contaminated area (Ria de Aveiro). Acta Oecologica 24 (Supplement 1):S351-S357

Nevado J, Martín-Doimeadios R, Bernardo F, Moreno M, Ropero M, de Marcos Serrano A (2011) Mercury Speciation in Fish Tissues from a Mediterranean River Basin: The Tagus River (Central Spain) as a Case Study. Archives of Environmental Contamination and Toxicology:1-11. doi:10.1007/s00244-011-9666-y

Oliveira M, Ahmad I, Maria VL, Serafim A, Bebianno MJ, Pacheco M, Santos MA (2010) Hepatic metallothionein concentrations in the golden grey mullet (*Liza aurata*) - Relationship with environmental metal concentrations in a metal-contaminated coastal system in Portugal. Marine Environmental Research 69 (4):227-233

Oliveira M, Maria VL, Ahmad I, Serafim A, Bebianno MJ, Pacheco M, Santos MA (2009) Contamination assessment of a coastal lagoon (Ria de Aveiro, Portugal) using defence and damage biochemical indicators in gill of *Liza aurata* - An integrated biomarker approach. Environmental Pollution 157 (3):959-967

Pacheco M, Santos MA, Teles M, Oliveira M, Rebelo JE, Pombo L (2005) Biotransformation and Genotoxic Biomarkers in Mullet Species (*Liza* Sp.) From a Contaminated Coastal Lagoon (Ria De Aveiro, Portugal). Environmental Monitoring and Assessment 107 (1):133-153. doi:10.1007/s10661-005-5308-z

Pereira M, Lillebø A, Pato P, Válega M, Coelho J, Lopes C, Rodrigues S, Cachada A, Otero M, Pardal M, Duarte A (2009) Mercury pollution in Ria de Aveiro (Portugal): a review of the system assessment. Environmental Monitoring and Assessment 155 (1):39-49. doi:10.1007/s10661-008-0416-1

Pereira ME, Duarte AC, Millward GE (1995) Seasonal variability in mercury inputs into the Ria de Aveiro, Portugal. Aquatic Ecology 29 (3):291-296. doi:10.1007/bf02084227

Pérez Cid B, Boia C, Pombo L, Rebelo E (2001) Determination of trace metals in fish species of the Ria de Aveiro (Portugal) by electrothermal atomic absorption spectrometry. Food Chemistry 75 (1):93-100

Pickhardt PC, Folt CL, Chen CY, Klaue B, Blum JD (2005) Impacts of zooplankton composition and algal enrichment on the accumulation of mercury in an experimental freshwater food web. Science of The Total Environment 339 (1-3):89-101. doi:10.1016/j.scitotenv.2004.07.025

Ramalhosa E, Monterroso P, Abreu S, Pereira E, Vale C, Duarte A (2001) Storage and export of mercury from a contaminated bay (Ria de Aveiro, Portugal). Wetlands Ecology and Management 9 (4):311-316. doi:10.1023/a:1011864702531

Ramalhosa E, Pereira E, Vale C, Válega M, Duarte AC (2005) Distribution of mercury in the upper sediments from a polluted area (Ria de aveiro, Portugal). Pollut Bull 50 (6):682-686. doi:10.1016/j.marpolbul.2005.02.043

Tavares S, Oliveira H, Coelho JP, Pereira ME, Duarte AC, Pardal MA (2011) Lifespan mercury accumulation pattern in Liza aurata: Evidence from two southern European estuaries. Estuarine, Coastal and Shelf Science 94 (4):315-321

Turner A, Millward GE, Le Roux SM (2001) Sediment−Water Partitioning of Inorganic Mercury in Estuaries. Environ Sci Technol 35 (23):4648-4654. doi:10.1021/es010933a

Ullrich SM, Tanton TW, Abdrashitova SA (2001) Mercury in the Aquatic Environment: A Review of Factors Affecting Methylation. Critical Reviews in Environmental Science and Technology 31 (3):241-293. doi:10.1080/20016491089226

Válega M, Abreu S, Pato P, Rocha L, Gomes A, Pereira M, Duarte A (2006) Determination of Organic Mercury in Biota, Plants and Contaminated Sediments Using a Thermal Atomic Absorption Spectrometry Technique. Water, Air, & Soil Pollution 174 (1):223-234. doi:10.1007/s11270-006-9100-7

Vasconcelos RP, Reis-Santos P, Fonseca V, Ruano M, Tanner S, Costa MJ, Cabral HN (2009) Juvenile fish condition in estuarine nurseries along the Portuguese coast. Estuarine, Coastal and Shelf Science 82 (1):128-138. doi:10.1016/j.ecss.2009.01.002

Zhang L, Zang X, Xu J, Xie P, Zhu Z, Su J (2007) Mercury Bioaccumulation in Fishes of Three Gorges Reservoir After Impoundment. B Environ Contam Tox 78 (3):262-264. doi:10.1007/s00128-007-9117-7

Chapter V: Final remarks and Conclusions

Final remarks and Conclusions

The work developed in this Thesis intended to be a contribution to the knowledge of mercury behavior and toxicity within aquatic environments. To better understand these main endpoints, a sequential work based in three steps was made. In the initial step of this work a study based in the toxicity of mercury to several key organisms was determined (producers, primary consumers and detritivorous). The following step was to determine the mercury transfer within the trophic chain and the final step was a field work developed in Ria de Aveiro to study a natural estuarine environment polluted by a mercury source and to understand how it affects the environment and its possible harmfull effects to Humans.

Mercury toxicity to aquatic biota was assessed using as primary producers the microalgae *Pseudokirchneriella subcapitata*, *Chlorella vulgaris* and the vascular plant *Lemna minor*, as primary consumers the microcrustaceans *Daphnia longispina* and *Daphnia magna*, and as a detritivorous the *Chironomus riparius* larvae. For the microalgae, there are a few studies that report the toxicity of mercury in terms of inhibition of some photosynthetic enzymes; here we assessed the effect of mercury for growth inhibition, so the possible impact in diminution of the population size. The primary producers' microalgae could be affected by low values of mercury in the environment, potentially affecting the equilibrium of all the subsequent trophic chain. For our knowledge, this is the first work describing EC_{50} value for *Daphnia longispina* and larvae of I instar of *Chironomus riparius*. Species that have two life stages, like insects with larvae phase in the aquatic medium, can be potential vectors of mercury transfer from the aquatic to the terrestrial environment and to areas away from the point sources.

The increasing mercury tolerance along the several organisms belonging to higher trophic levels suggests a natural selection or adaptability to higher levels of mercury exposure as trophic position and the complexity of the organism increase. Nevertheless, the variability of mercury tolerance between organisms of the same

trophic level reinforces the idea that a toxicity characterization must be performed for several aquatic organisms, including autochthonous species, in order have to legislation that better protect the structure and functionality of the aquatic ecosystems.

The bioconcentration of mercury for primary producers is a fact, and, despite the effect of growth dilution can occur, mercury still is magnified to the primary consumers, and upper trophic levels. We also expect that the data obtained with this work can contribute for bioremediation studies too and to the development of new studies that can use as base mercury concentration within the different tissues and its increment with time exposure.

When analyzing the Ria de Aveiro estuary, mercury burden still is a real concern with the transfer of mercury to the sediments to particulate matter and consequently bioavailable to the biota.

The use of juveniles' specimens to evaluate the presence of mercury seems to be appropriated and have some advantages. In this study, the juveniles of *Liza aurata* showed the ability to detect inter-sites differences, and contrary to other studies, they did not present the mobility usually described for other fish species, that could live between the two sampling sites. Furthermore, the adoption of juvenile specimens provides information on short-term variations of mercury concentrations in the environment. As *L. aurata* is a migratory species, the mercury accumulated by its juveniles can be transported to the marine environment.

Mercury concentrations in fish tissue correlate to mercury levels in their aquatic environment, but sediments did not seem to be the major source for mercury accumulated. Instead the mercury associated with suspended particulate matter and trophic chain structure may be important factors. Further studies can be performed in order to evaluate the mercury burden (total and organic mercury) and biomagnification factors in prey of *L. aurata* and complement this work.

Regarding the impact of mercury that is being continually inputted into the Ria de Aveiro estuary more studies and environmental risk assessments (ERA)

should be made, since this estuary is an important source of seafood and fish for the local population. Although, the samples analyzed in this study did not pass the legal limit of 0.5 µg Hg/g established by European Union, the human risk associated to the regular ingestion of seafood and fish inhabiting in the two sampling sites cannot be excluded, since the concentration of organic mercury continuous exposure in the two sampling sites is considerable. Also we demonstrate that the continues exposure to mercury leads to higher accumulation of this metal, so analysis made to other adult species instead of juveniles may show mercury values higher than the legal limit. Furthermore, the knowledge and understanding of mercury mobility in sediments and the subsequent bioavailability to living organisms are crucial for a successful remediation of contaminated areas.

In a general conclusion, the main objectives of this work showed that mercury is a pollutant that produces great harmful effects not only to the environment but also potentially to Humans, and that the studies of estuarine environments are an important and still incomplete task, much necessary to protect all the living organisms that cohabit this amazing living place.