Organic Contaminants in the Mondego Estuary Fish Assemblage

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Abstract

The main objective of this thesis was to study PCBs and HCB accumulation in an estuarine fish assemblage. PCBs and HCB are contaminants very persistent in the environment. Their high stability can lead to a relatively long half-live, low metabolic transformation and hydrophobicity. These contaminants are very lipophilic, therefore they tend to accumulate in organisms, including fish. PCBs are constituted by 209 congeners. In the present thesis the most abundant congeners were CB 138, 153 and 180. These congeners are high chlorinated congeners, therefore very persistent in the environment and in biological samples.

Estuaries are very important ecosystems, since they provide several habitats for many comercial species, particularly fish. Fishes can use estuaries as migration routes, refuge against predators and nursery grounds. However, estuaries are exposed to high anthropogenic activities, which can lead to a high uptake of contaminants. The Mondego estuary is a small estuary located in the western coast of Portugal, with limited and diffuse contamination sources. The main contamination sources are wastewaters, from domestic and industrial sewage, fertilizers and pesticides, from agricultural runoff, and the Figueira da Foz harbour.

In chapter 1 an introduction to the topic of HCB and PCBs is presented highlighting their importance in the natural environment. Chapter 2 addresses a study performed in the Mondego estuary fish community. No

studies regarding the concentration of PCBs in the whole fish community had been made so far, therefore this study fills a gap in knowledge in how PCBs are distributed in fish assemblages. Species that spent more than three years in the estuary presented higher concentration, than species that spent less than three years in the estuary. Furthermore, a positive correlation was found between lipid content and concentration. So the main factors influencing PCBs accumulation in the Mondego estuary were the time spent in the estuary, lipid content and dietary intake.

Chapter 3 addresses two studies performed in mullet species, *Liza ramada*, *Liza aurata* and *Chelon labrosus*. Both studies were performed along the species lifespan and in PCBs. In the first study *L. ramada* and *L. aurata* increased their concentration with age. Among the three mullet species *L. ramada* presented the highest concentration, which could be attributed to the fact that this species spent more time inside the estuary then the other two species. In the second study *C. labrosus* PCBs concentrations' were analyzed in both genders, in liver and muscle. No significant differences were found in both sexes and tissues concentration, although males increased their concentration with age. Liver presented higher concentration than muscle.

Chapter 4 addresses a study regarding PCBs and HCB concentration in *Platichthys flesus* from the Mondego estuary and adjacent coastal water. *P. flesus* was analyzed along its lifespan, in three tissues, muscle, liver and gills. PCBs concentration decreased along its lifespan, in all tissues. HCB concentration was only detected in older fishes from the adjacent coastal water, and its concentration inceased with age. Liver and gills presented the highest concentration followed by the muscle, for both contaminants.

Most fish species analyzed in this thesis were commercial species, therefore consumed by humans. Regarding PCBs all studied species presented low concentration and below the regulation limit establish by the European Union, 75 ng g^{-1} (wet wt). *P. flesus* daily intake was also below the regulation limit of US Department of Health and Human Services, regarding HCB, and WHO tolerance limit, regarding PCBs. Therefore, fishes from the Mondego estuary are considered safe for human intake.

Resumo

O principal objectivo da presente tese foi estudar a acumulação de PCBs e HCB numa população estuarina de peixes. PCBs e HCB são contaminantes muito persistentes no ambiente. Como são muito estáveis possuem um tempo de meia-vida relativamente longo, uma transformação metabólica baixa e são hidrofobicos. Estes contaminantes são muito lipofílicos, levando a uma acumulação em organismos, incluindo peixes. Os PCBs são constituídos por 209 congéneres. Nesta tese os congéneres mais abundantes são o CB 138, 153 e 180. Estes congéneres possuem um grau de clorinação elevado, sendo deste modo mais persistentes no ambiente e em amostras biológicas.

Os estuários são ecossistemas muito importantes, uma vez que fornecem inúmeros habitats para muitas espécies de interesse económico. Os peixes utilizam o estuário como rotas de passagem durante a migração, refugio contra predadores e locais de viveiro. Contudo, os estuários estão expostos a muitas actividades antropogénicas, levando a uma elevada absorção de contaminantes. O estuário do Mondego é um estuário de pequenas dimensões localizado na costa oeste de Portugal. Este estuário possui fontes de contaminação limitadas e difusas. As principais fontes de contaminação são efluentes, provenientes de esgotos domésticos e industriais, fertilizantes e pesticidas, provenientes da agricultura, e o porto da Figueira da Foz.

O capítulo 1 introduz o tópico HCB e PCBs, na qual é apresentada a sua importância no ambiente. O Capítulo 2 apresenta um estudo realizado em toda a comunidade de peixes do estuário do Mondego. Como não existem estudos referentes à análise de PCBs em toda a comunidade de peixes, este estudo preenche, deste modo, uma falha no conhecimento existente. As espécies que permaneciam no estuário por mais do que três anos apresentavam concentrações superiores em relação às espécies que permaneciam no estuário menos de três anos. Foi ainda encontrada uma correlação positiva entre o conteúdo lipídico e a concentração de PCBs. Deste modo, os principais factores que influenciam a acumulação de PCBs no estuário do Mondego são o tempo de permanência no estuário, o conteúdo lipídico e a dieta.

O Capítulo 3 aponta para dois estudos realizados em três espécies de taínhas, *Liza ramada*, *Liza aurata* e *Chelon labrosus*. Ambos os estudos são realizados para PCBs e ao longo da vida das espécies. No primeiro estudo a *L. ramada* e a *L. aurata* apresentam um aumento de concentração com a idade. De entre as três espécies de taínhas a *L. ramada* é a espécie que apresenta uma concentração mais elevada, o que poderá ser atribuído ao facto de esta espécie permanecer mais tempo no estuário. No segundo estudo a concentração de PCBs foi analisada em fêmeas e machos, e em fígado e músculo de *C. labrosus*. Não foram encontradas diferenças significativas entre a concentração nos dois sexos e nos dois tecidos, apesar de os machos aumentarem a sua concentração com a idade. O fígado apresenta uma concentração superior ao músculo.

O Capítulo 4 descreve um estudo realizado com PCBs e HCB em *Platichthys flesus* do estuário do Mondego e da zona costeira adjacente. A concentração em *P. flesus* foi analisada ao longo da vida e em três tecidos, músculo, fígado e guelras. A concentração de PCBs diminui ao longo da vida, em todos os tecidos. A concentração de HCB foi apenas detectada em indivíduos adultos e da zona costeira adjacente. O fígado e as guelras apresentam uma concentração superior ao músculo, para os dois contaminantes.

A maioria dos peixes analisados nesta tese são espécies comerciais, sendo deste modo consumidos por humanos. Relativamente aos PCBs todos os estudos apresentam baixa concentração e abaixo do limite de tolerância estabelecido pela União Europeia, de 75 ng g^{-1} (peso húmido). O consumo diário de *P. flesus* apresenta-se também abaixo do valor estabelecido pelo US Department of Health and Human Services, relativamente ao HCB, e do limite de tolerância estabelecido pela WHO, relativamente aos PCBs. Deste modo, os peixes presentes no estuário do Mondego são considerados seguros para consumo humano.

Nota: A presente tese não foi escrita segundo o acordo ortográfico.

Chapter 1

General introduction

Polychlorinated biphenyls in the environment

Polychlorinated biphenyls (PCBs) are synthetic organic chemicals, represented by a mixture of 209 chlorinated hydrocarbons congeners (Bazzanti et al., 1997; Mills et al., 2007; Pocar et al., 2003). PCBs are highly stable and very persistent in the environment, mainly due to their relatively long half-life, low metabolic transformation and hydrophobicity (Bazzanti et al., 1997; Eichinger et al., 2010; Stapleton et al., 2001). PCBs are characterized by different physical-chemical properties, such as the coefficient octanol-water (K_{OW}), which informed on the lipophicity of the compound. PCBs K_{OW} range from 5.9 to 7.0, with high chlorinated congeners presenting higher K_{OW} (Bodin et al., 2008). Generally, water solubility, vapor pressure and biodegradability decrease with a high degree of chlorine substitution. On the contrary, hydrophobicity and sorption tend to increase with a high degree of chlorination (Vallack et al., 1998).

PCBs production started in early 20s until they were banned in most industrialized countries in the 1970s (Bodiguel et al., 2009). Due to their nonflammable and heat resistant properties, they were used in dielectric fluids in transformers and large capacitors, plasticizers in sealants, heat exchange fluids, condensers, hydraulic lubricants, cutting oils, flame retardants, dedusting agents, plastics, paints, manufacture adhesives and textiles (Bazzanti et al., 1997; Pocar et al., 2003, Vallack et al., 1998). Thousands of tons of PCBs were produced being some released into the environment, either by diffuse sources or by direct discharges (Antunes et al., 2007). PCBs innumerous sources can be from historical or current inputs, although the most important sources are industrial points, urban runoff and combined sewages (Ashley et al., 2003), leakage from old equipment, building materials, stockpiles and landfill sites (Vallack et al., 1998; Xing et al., 2005). PCBs were produced for commercial mixtures under various trade names, such as Aroclor®, Clophen® or Phenoclor® (Bazzanti et al., 1997). Nowadays, commercial mixtures (Aroclor®, Clophens®) are not truly representative of human exposure, since PCBs have already been filtered by the ecosystem (Ulbrich and Stahlmann, 2004).

The presence of PCBs in the environment and related hazards were probably first identified in biological samples in 1966 (Jensen, 1966). PCB analysis in environmental samples and the identification of the different congeners are fundamental for the study of bioaccumulation, transformation and transport processes. The aquatic environment is the ultimate sink for these contaminants (Bocquené and Abarnou, 2012; Stegeman and Hahn, 1994), due to atmospheric precipitation or direct discharges in aquatic systems (Zhou et al., 2001).

PCBs toxicity can pose a risk for the environment and human health (Vallack et al., 1998). Human exposure occurs mainly through environmental contamination, especially by contaminated food products. Fish and fishery products are the main source for human contamination (Fattore et al., 2008). Measurements in human plasma and tissues have indicated that the exposure to PCBs have declined since they were banned (Ulbrich and Stahlmann, 2004). The three more persistent congeners in environmental samples are CB 138, 153 and 180, are also the most persistent in humans, as well (Ulbrich and Stahlmann, 2004).

PCBs toxicological data have indicated a number of physiological processes important during development, specifically in nervous and endocrine system (inducing estrogenic and antiestrogenic effects) (Fattore et al., 2008). Moreover can also have adverse effects in thyroid, immunological function, reproduction function, behavior and can have carcinogenic effects (Sirot et al., 2012).

Hexachlorobenzene in the environment

Hexachlorobenzene (HCB) was introduced in the environment in 1933 as a fungicide, for seed treatment (Barber et al., 2005; Vallack et al., 1998). Nevertheless, HCB was also used for a variety of applications such as wood preservation, aluminum fluxing and degassing or intermediate in organic synthesis (Bailey, 2001).

HCB has been observed worldwide in air, water and biota (Bailey, 2001). Its vapor pressure, water solubility and persistence in the environment are combined to facilitate the long-range transport and accumulation (Bailey, 2001). This contaminant is highly insoluble in water, with high partition coefficient (log K_{OW}= 3.03 – 6.42), as a result it accumulates in fat tissues. HCB is in many lists of regulation and management, due its bioaccumulation, persistence and potential toxicity (Bailey, 2001). Generally, rivers presented HCB higher concentration, followed by estuaries, lakes and sea (Barber et al., 2005).

HCB has a wide range of toxic effects on experimental animals including hepatoxicity, immune suppression, neurotoxicity, thyroid dysfunction, reproductive toxicity and carcinogenicity (Song et al., 2006; Roy et al., 1995). At its peak, thousands Tons of HCB were used each year. In 1970s the estimated production ranged from 1000 to 2000 tons per year? (Barber et al., 2005). In Europe emission was estimated to be 192 tons/year (Pacyna et al., 1999). Due to its chemical stability, persistence and long-range transport, HCB can be found throughout the environment (Song et al., 2006). HCB as a pesticide was banned globally in many countries and was severely restricted or voluntarily withdrawn in several others (Vallack et al., 1998). In the European Union HCB production was banned in the 1978, in Canada was banned in 1976 and in the United Stated was banned in 1984 (Barber et al., 2005). Although HCB production has ceased in most countries it is still produced as a by-product or impurity in several chemical processes, such as the manufacture of chlorine solvents, chlorine aromatics and pesticides, it can also be released by incomplete combustion or from old dumpsites (Falcó et al., 2004). In many countries, such as China or Russia, it is still used as an intermediate compound (Barber et al., 2005).

Estuarine environment and open coastal waters

Estuaries are transitional areas with strong environmental gradients, mainly due to salinity gradients (Elliot and McLusky, 2002). These transitional areas are very important for the fish fauna, as they provide important nursery habitats, reproduction grounds, refuge against predators and migratory routes (Cabral et al., 2007; Martinho et al., 2008). Estuaries are among the most productive ecosystems (Dolbeth et al., 2007; McLusky and Elliot, 2004). Their high primary productivity added to their habitat richness provides optimal settlement conditions for a wide variety of species (Beck et al., 2001). Many marine juvenile fishes that occur in the estuary are commercially important, thus estuaries are important ecosystems for the renewal of fisheries resources (Nicolas et al., 2007).

Estuaries are located in human-populated areas, hence exposed to high anthropogenic stress (Cabral et al., 2012). Aquatic ecosystems act as sinks for many organic and inorganic pollutants, resulting from anthropogenic activities. Human activities, such as agricultural activities, industrial outflow and urban runoff, overfishing, bank reclamation and general environmental degradation, have significant impact on the estuarine fish communities (Cabral et al., 2001). Estuaries can be directly and indirectly exposed to these activities. Industrial development and intensive agriculture are the main cause for estuarine up-take of pollutants (Vasconcelos et al., 2007). These activities may have negative impact in the estuarine system by reducing its nursery function (Courrat et al., 2009).

Estuaries are more contaminated than open coastal waters. Estuaries accumulate higher quantities of organic nutrients and organic materials (Elliot and Whitfield, 2011), therefore accumulating higher quantities of organic contaminants (Parnell et al., 2008). Moreover, open coastal waters are less contaminated due to their high energy climate and more remote settings (Parnell et al., 2008). Coastal environmental main sources of disturbances are urban and industrial effluent discharges, agricultural runoff and contaminant transport by rivers and estuaries (Silva et al., 2004).

Fish assemblage and organic contamination

In order to understand the effects of human activities in estuaries, it is important to classify and categorize estuarine fish fauna. Guilds have been used to provide information on functional, hierarchical structure and to simplify ecosystems, assessing resemblances and dissimilarities between areas, according to the different biological characteristics (Martinho et al., 2010).

Ecological guilds have been used to indicate the importance of the estuarine system on fish (Elliot and Dewailly, 1995). In this thesis estuarine fish community was classified into 5 ecological guilds: Estuarine residents (ER): estuarine species capable of spending their entire life cycle within the estuarine environment; Catadromous (CA): species that spend all their trophic life in freshwater and migrate to the sea to spawn; Marine stragglers (MS): species that spawn and live at sea, entering the estuary in low numbers; Marine estuarine-opportunists (MMO): marine species that regularly enter estuaries in substantial numbers, particularly as juveniles; Marine estuarine-dependent (MMD): marine species that require sheltered estuarine habitats as juveniles, but live along coasts where there are no such habitats, thus these species depend on estuaries (Elliot et al., 2007).

The trophic guild system was designed to allow the aggregation of fish species that use similar food resources (Elliot et al., 2007). In the present thesis the estuarine fish community was classified into 5 trophic guilds: Detritivore (DE): species that feed mainly on detritus and/microphytobenthos; Omnivore (OV): species that feed mainly on filamentous algae, macrophytes, periphyton, epifauna and infauna; Piscivore (PV): species that feed mainly on fish, but can include large nektonic invertebrates; Zoobenthivore (ZB): species that feed mainly on invertebrates associated with the substratum; Zooplanktivore (ZP): species that feed mainly on zooplankton (Elliot et al., 2007).

Fishes have an extreme economic importance, since they are considered one of the most important food resources for the human population. Many fishes have high economic value such as *Sardina pilchardus* (Amenzoui et al., 2006) or *Chelidonichthys lucerna* (Cicek et al., 2008), other fishes are important in the functioning of an ecosystem, such as *Pomatoschistus microps* (Leitão et al., 2006).

Fishes are often used as indicators for environmental contamination, since they are moderately large and easy to identify (Blasco et al., 1998), also they have a widespread distribution and sometimes a high trophic position.

Despite being very mobile, they are considered one the most viable organisms for pollution monitoring in aquatic systems (van der Oost et al., 2003).

Bioaccumulation of PCBs, HCBs and other lipophilic contaminants are partly controlled by exposure. Fishes can accumulate contaminants by direct uptake from water by gills or skin (bioconcentration) or by intake of contaminated food (biomagnification) (Mackay and Fraser, 2000; van der Oost, 2003). Biomagnification processes are highly dependent on exposure, mostly related to their diet and therefore trophic position and the target species physiology (biotransformation capacity) (Ashley et al., 2003; Bodin et al., 2008). Numerous biotic and abiotic factors can control the exposure to a given source. For instance, the proximity to the source has been suggested to be main factor determining the fish body burden. Fish ecology and physiology is also expected to affect bioaccumulation, since factors related to diet, feeding and assimilation rate, growth rate and lipid loss through metabolism (Ashley et al., 2003) are important. PCBs and HCBs concentrations increase with the trophic position in aquatic food webs (Borga et al., 2001). Hence top predators are expected to have higher levels, leading to a higher risk for humans.

Mondego estuary

The study presented in this thesis was carried out in the Mondego estuary. The Mondego estuary is a small estuary with about 8.6 Km^2, located in a warm temperate region in the western coast of Portugal (40º08'N; 8º50'W) (Fig. 1). It comprises two arms: the north and the south, separated by the Murraceira Island, joining again near the mouth, about 7Km from the shore. The two arms present different hydrologic characteristics. The north arm is deeper, with 5-10m in high tide and 2-3m in tidal range. It is dredged frequently to maintain its depth, constituting the main navigation channel and the location of the Figueira da Foz harbour.

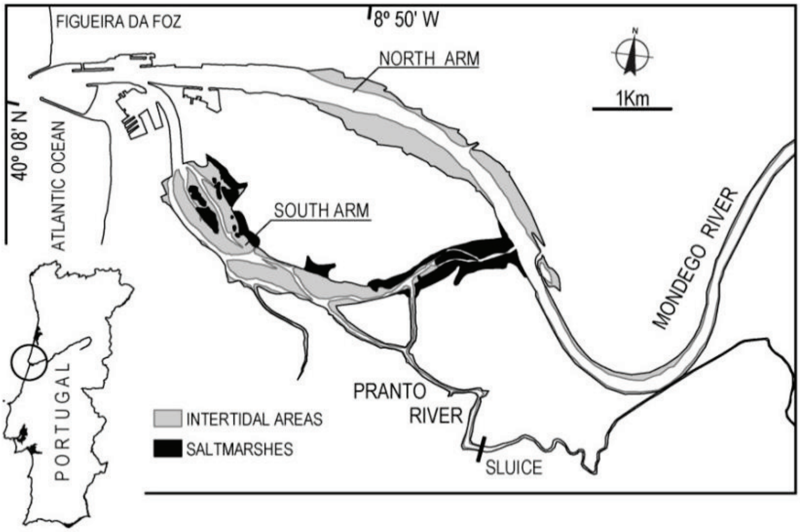

Figure 1. Detailed scheme of the Mondego estuary, showing the two arms, intertidal and saltmarsh areas

The south arm is shallower with 2 – 4m deep in high tide and 1 – 3m in tidal range, and it is characterized by large areas of intertidal flats during low tide (about 75% of the total area). It is characterized by several seagrass meadows (*Zostera noltii*) and saltmarshes (*Scirpus maritimus*, *Spartina maritima*) at the downstream areas (Lillebø et al., 2004).

The main freshwater circulation is carried out through the north arm, whereas the water circulation in the south arm is mainly dependent on tides and freshwater input from the Pranto River, a small tributary river, which is controlled by sluice, according to water needs of the surrounding rice fields.

The Mondego estuary has been classified has highly productive, especially in the intertidal macrobenthonic assemblages (Dolbeth et al., 2003). Regarding special protection legislation the estuary has been classified as an Important Bird Area (IBA; PT039) and as RAMSAR site (site no. 1617).

The main anthropogenic pressures that occur in the Mondego estuary are eutrophication, bank reclamation and shipping activities. During 1990–1998 the ecological quality of the south arm declined due to anthropogenic activities mainly harbour facilities, aquaculture farms and intensive agriculture (with intensive use of fertilizers in the rice fields). All these activities contributed to an eutrophication process, which affected *Z. noltii* meadows (Cardoso et al., 2010). Nowadays, since the implementation of a restoration programme, in 1998, the major aim is to restore the system original condition. After the application of the restoration measures (including public education, highlight of the ecological importance of the intertidal vegetation for the health and related socio-economic activities of the estuary), the results were significant: nutrient loading reduction, improvement of water dynamics and transparency and seagrass bed protection from human disturbance (Cardoso et al., 2008; Grilo et al., 2012).

Figueira da Foz city has over 62.000 habitants (Instituto Nacional de Estatística, statistics Portugal), whose wastewater (partially untreated) is still discharged in the north arm of the estuary. In addition, the traditional salt-farms in the Murraceira Island have been converted into semi-intensive aquacultures, whose wastewaters are discharged in both arms. Figueira da Foz harbour is responsible for the main industrial pressure in the estuarine area. Overfishing of some target seasonal species, such as lamprey (*Petromizon marinus*), coupled with illegal catches of European eel (*Anguilla anguilla*), sea bass (*Dicentrarchus labrax*) and sole (*Solea solea*) (Leitão et al., 2007) increase the anthropogenic pressure that the estuary is exposed to.

General aims and Thesis outline

The general aim of this thesis was to increase the knowledge and understanding of PCBs and HCB in the Mondego estuary fish assemblage. In order to understand differences in accumulation in the different species of the Mondego estuary, the whole fish community was analyzed.

Another major aim of this thesis was to investigate the fate of PCBs and HCB along the species lifespan. To accomplish this four species were used (*Liza aurata*, *Liza ramada*, *Chelon labrosus* and *Platichthys flesus*), and were involved 4 different tissues muscle, liver, gills and gonads.

The general aims were accomplished by addressing the following specific objectives:

- To assess the most prevalent congeners on the Mondego estuary;

- To evaluate differences among the fish community concentration in the Mondego estuary;
- To detect the main factors that influence interspecies trends in PCBs accumulation;
- To detect differences in accumulation patterns in *L. ramada*, *L. aurata* and *C. labrosus*, along their lifespan;
- To assess differences between *C. labrosus* genders concerning PCB concentration;
- To evaluate if HCB and PCBs accumulate in different tissues of *P. flesus* along its lifespan;
- To asses if fish species from the Mondego and adjacent coastal waters are safe for human intake.

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Chapter 2

PCBs in the fish assemblage of a southern European estuary

Abstract

The Mondego estuary fish assemblage was studied for PCBs accumulation. The fishing took place in three sampling stations along an estuarine salinity gradient, and 15 species were collected. PCBs analysis revealed no significant differences among the sampling stations, although differences were observed among the fish assemblages. Fish assemblage could be divided into three groups. The first group with higher concentration (more than 10 ng g^{-1}, dry wt.), included the species *G. niger*, *S. pilchardus*, *A. anguilla*, *P. microps*, *C. lucerna* and *L. ramada*; the second group with medium concentration (5 – 10 ng g^{-1}, dry wt.), included the species *P. minutus*, *D. labrax*, *A. presbyter*, *C. labrosus*, *D. vulgaris*, *P. flesus* and *C. mustela*; and a third group with low concentration (less than 5 ng g^{-1}, dry wt.), included the species *S. solea* and *C. lyra*. A positive correlation was found between lipid content and PCBs concentrations. To evaluate the influence of the residence time of species on the accumulation of PCBs, species were divided into two groups: species that spend more than 3 years in the estuary, and species that spend less than 3 years in the estuary. Species that spend more than 3 years in the estuary presented higher concentration than species that spend less than 3 years in the estuary. CBs

138 and 153 had higher concentration, and tended to increase with time spend in the estuary.

Additional keywords: PCBs, Mondego estuary, fish assemblage, accumulation

Introduction

Estuaries are transitional areas that have strong environmental gradients, mainly due to salinity (Elliot and McLusky, 2002). Transitional areas have a very important role for fish fauna, since they provide nursery habitats, reproduction grounds, refuge against predators, and migratory routes (Cabral et al., 2007; Martinho et al., 2008). Many marine species with commercial value use transitional areas in part of their life-cycle, being estuaries important for the renewal of fish resources (Nicolas et al., 2007). In general, estuaries are exposed to high degrees of anthropogenic stress (Elliot and Quintino, 2007), such as agricultural activities, industrial outflow and urban runoff, overfishing, bank reclamation, and general environmental degradation (Nicolas et al., 2007).

Polychlorinated biphenyls (PCBs) are a class of environmental contaminants, which tend to accumulate in fish. Their persistence in marine ecosystems can be due to their relatively long environmental half-live, low metabolic transformation and hydrophobicity (Stapleton et al., 2001).

The study of organic pollutants is an important feature for the estuarine environment. Estuaries with persistent organic pollutants present low quality habitats and habitat losses, which can have consequences for fish growth, survival and population renewal (Courrat et al., 2009). Fishes can be important for ecosystems monitoring, although the bioaccumulation pattern varies among species. Fishes can concentrate pollutants directly from water (bioconcentration) (Costa et al., 2008) and through their diet (biomagnification) (Borga et al., 2001), allowing the transfer of pollutants through the trophic web. Furthermore, they accumulate according to their trophic level, different assimilation efficiencies, depuration rates, and lipid content (Stapleton et al., 2001). Fishes are considered to be useful tools to assess anthropogenic impact, because they have low congener metabolism rates (Muir et al., 1988) reflecting the levels of pollution in the aquatic environment. Fishes occupy different habitats in the same ecosystem and have different feeding behaviour. As a result, they are used as a good proxy to assess the influence of the environment and biological factors on the bioaccumulation of pollutants (Pastor et al., 1996).

Both commercial and non-commercial species are important for the function of the estuarine environment. So it becomes important to determine and compare PCBs concentrations in commercial and non-commercial species. Many studies performed in estuaries only compared the contamination among different estuaries (Courrat at al., 2009; Harvey et al., 2008) and in commercial species, like *Anguilla anguilla* (Ashley et al., 2003), *Solea senegalensis* (Costa et al., 2008), or *Dicentrarchus labrax* (Pastor et al., 1996). Only a few studies have been made regarding the entire estuarine fish assemblage (Veltman et al., 2005) including the non-commercial estuarine species. As a result, the present study aims to detect PCBs in the estuarine fish assemblage of the Mondego estuary, including many species with commercial and non-commercial value. In accordance, some questions can be addressed: a) Are there differences among the fishes PCBs concentration from different areas of the estuarine system?; b) Are there differences among different species of the estuarine fish community, regarding PCBs accumulation?; c) What are the main factors influencing interspecies trends in PCBs accumulation?

Material and methods

Study site and fish community

The Mondego estuary (Fig.1) is a small estuary, with 8.6 km^2 located in the western coast of Portugal (40º08'N, 8º50'W). It comprises two arms, the north and the south arm, with distinct hydrologic characteristics. The north and the south arm are separated about 7 km from the shore joining again near the mouth of the estuary.

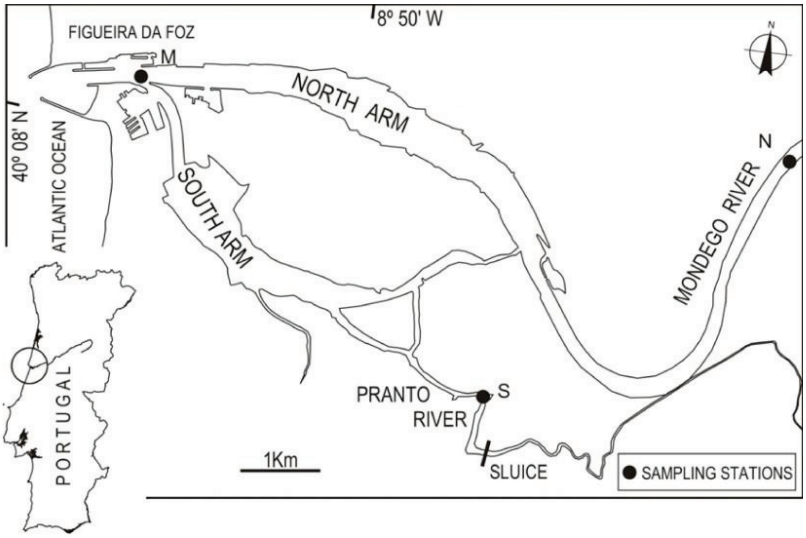

Figure 1. Mondego estuary and location of the sampling stations

The north arm is deeper, 5-10 m in high tide with 2-3 m of tidal range. It is dredged frequently to maintain its depth, because it is the main navigation channel of the Figueira da Foz harbour. The south arm is shallower, 2-4 m in high tide, with 1-3 m of tidal range, presenting about 75% of intertidal mudflats. The south arm is largely silted up in the upstream areas causing the water to flow mainly through the north arm. The water circulation in the south arm is mainly depending on the tides and on small freshwater inputs from the Pranto River, which is controlled by a sluice, according to the needs in the rice fields from the Mondego agricultural valley.

Sampling and laboratory work

Fishing was carried out at three sampling stations (M, N and S in Fig. 1) following a typical salinity gradient; M station is the more downstream station presenting higher salinity values, whereas N and S stations are located more upstream, and with lower salinity values. N and S station have distinct hydrologic characteristics. N station is located in the north arm, where the runoff is higher, while S station is located in the south arm, where the runoff depends on inputs from the Pranto River. Since the three sampling stations have different environments the fish population within these stations is also different. The fishing took place in these three stations, in order to obtain a greater number of fish species of the Mondego estuary.

The fishes were collected between November and December 2009. Fishing was carried out during the night, using a 2 m beam trawl, with 5 mm stretched mesh size in the cod end. Each survey consisted of three hauls of five minutes at each sampling station, covering at least an area of 500 m^2. The fishes were taken to the laboratory where they were identified to the species level and muscle tissues were removed. For each species and each sampling station, 3 samples were analysed for PCBs, given a total of 81 samples analysed. Due to the lack of mass small fishes were analysed as a composite sample, using 2 to 8 individuals in each sample. After laboratory processing, samples were freeze-dried (Snijders scientific), homogenised and stored at -20ºC, until analytical procedure. Sediments were collected between November and December 2009, at two sampling stations (M and S in Fig. 1).

PCBs analysis

Sediment samples were freeze-dried, sieved to <1 mm, homogenized and frozen (−20 °C) wrapped in aluminium foil. Representative aliquots were Soxhlet-extracted with a hexane/acetone mixture (2:1) for 6 h at a rate of 4–6 cycles/h, in a pre-washed glass fibre thimble (Folch et al., 1996). Activated copper granules were added to remove elemental sulphur. The resultant extract was concentrated using a rotavapor and submitted to an alumina cleanup (Supelclean® neutral-alumina and anhydrous sodium sulphate at the top) using a solid phase extraction (SPE) system. The column was eluted with hexane:DCM (9:1) and hexane:DCM (2:1). The eluate was then concentrated down to 1 mL using a rotavapor, dried under a gentle stream of nitrogen and solvent changed to hexane. The extract was further submitted to an acid silica gel (Supelclean® silica gel with 44% w/w concentrated sulphuric acid) cleanup and PCBs were eluted with hexane. The eluate was concentrated down to 1 mL using a rotavapor, dried under a gentle stream of nitrogen and solvent changed to iso-octane for further processing by gas chromatography coupled to mass spectrometry (GC–MS).

For biota analysis 3 g of muscles was accurately weighted and extracted by sonication (Branson 3510) with a *n*-hexane:acetone (1:1) mixture. The extract was decanted and the process repeated three times. The extract volume was reduced by solvent evaporation using a rotavapor.

The content in lipids was gravimetrically determined using 10% of the extract. The remaining (90%) volume of the extract was reduced by solvent evaporation under a gentle stream of nitrogen and lipids were removed with sulphuric acid (97%). Afterwards, the lipid-free extract passed through a multilayered column packed with florisil (Supelclean® Florisil) and anhydrous sodium sulphate and PCBs eluted with hexane. Samples were dried, under a gentle stream of nitrogen. A known mixture of PCBs congeners (CB 34, 62, 119, 131 and 173) were used as internal standards (Ayris et al., 1997), added to the dried extracts, and diluted with iso-octane to a final volume 200 μL.

Samples were analysed on a gas chromatograph equipped with a MDN-12 silica capillary column (30 m; 0.25 mm i.d.; 0.25 μm film thickness) coupled to a mass spectrometry detector (GCMS-QP5050A, Shimadzu) using electron impact ionization and selected ion monitoring (SIM) acquisition. Helium was employed as the carrier gas, and samples were injected (1 μL) in splitless mode with column temperature at 40ºC and held for 2 minutes, then programmed to ramp 15ºC/min to 180ºC, and held for 1 minute, 8ºC/min to

300ºC and held for 15 minutes (injector temperature = 280ºC; interface temperature = 300ºC). Total PCBs content dry weight (ng g^{-1}, dw) was based on the summed concentrations of the 12 congeners detected ($\sum_{12}$ PCB) (IUPAC nos. 18, 28 and 31-tri; 44 and 52-tetra; 101 and 118-penta; 138, 149 and 153-hexa; 170 and 180-hepta). Within these congeners, 7 of them are considered ecological indicators to assess marine pollution, by the International Council for the Exploitation of the Sea (ICES) ($\sum_{7}$ ICES) (28, 52, 101, 118, 138, 153, and 180).

For quality assurance and quality control of the PCBs quantification method, contamination was evaluated by blank controls and results were always below detection limit. The accuracy of the analytical method was evaluated by analysis of spiked samples and recoveries for the analysed congeners ranged between 71-106%. The precision was calculated on replicate analysis giving an overall variability of 4-20%. The detection limit for individual PCBs ranged between 0.1 and 1.0 ng g^{-1}.

Data analysis

To determine differences among the fish species concentration in the different stations a one-way ANOVA (SigmaPlot 11.0) was performed, using the PCBs concentrations of each species from each sampling station. No transformation was applied to the data, since all values had a normal distribution.

The species from the Mondego estuary presented different concentrations. So, several ANOSIM (Primer 6β) analyses were performed to detect differences in their concentration, habitat, ecological guild and trophic guild. To detect differences in the fish community concentration the samples were group *a prior*, and three groups could be distinguished: low concentration group (less than 5 ng g^{-1}), medium concentration group (5 – 10 ng g^{-1}) and high concentration group (more than 10 ng g^{-1}). To test differences in their habitat the species were grouped *a priori* and according to their position in the water column: demersal, pelagic-neritic and benthopelagic. To test differences in the ecological guilds the species were grouped *a priori* according to five ecological guilds: catadromous (CA), estuarine residents (ER), marine stragglers (MS), marine estuarine opportunist (MMO) and marine estuarine dependent (MMD). To test differences in their trophic guild the species were grouped *a priori* according to their trophic guild: zoobenthonic+omnivore (ZB+OV), piscivorous (PV), detritivore+omnivore (DE+OV), zoobenthonic (ZB) and zooplanktivore (ZP).

Estuarine fish species were divided into two groups according to the time spent inside the estuary: a group that spend more than 3 years in the estuary, and a group that spend less than 3 years in the estuary. To detect differences among the groups a t-test was performed. *S. pilchardus* was not included in the analysis, since this species is not typically estuarine, only entering the estuary sporadically. No transformation was applied to the data, since all values had a normal distribution.

Regarding lipids, a Spearman correlation (SigmaPlot 11.0) was developed correlating lipid content and PCBs concentrations.

A Principal Component Analysis (PCA) was performed to compare PCB congeners profile among the estuarine fish community. The PCA was performed using the data of the 12 analysed congeners. In order to remove the confounding effect of congener concentration, the concentration data was rescaled with values from 0-1, using the formula:

$$Normalized\ data = \frac{Ccongener - Cmin}{Cmax - Cmin}$$

Results

Mondego estuary fish assemblage

In order to obtain a higher number of individuals along the estuarine salinity gradient, the Mondego estuary fish assemblage was studied in three sampling stations. A one-way ANOVA was performed in order to detect differences in the PCBs concentrations among the species of the three sampling stations. No significant differences were found among the three sampling stations ($p=0.405$; $p\leq0.05$), therefore the fish assemblage analysis was performed using the estuary as a whole. Sediments were analysed for station M and S, due to the lack of sediments samples station N was not analysed. Station M presented a concentration of 2.36 ng g^{-1} (dry wt.), whereas station S presented a concentration of 0.81 ng g^{-1} (dry wt.).

The estuarine fish assemblage (Table 1) consisted of 15 species, belonging to 13 families, 5 ecological guilds and 4 trophic guilds. The lipid percentage varied between 0.6 to 8.8% (Fig. 2), and Σ_{12} PCBs concentrations varied between 3.0 to 17.7 ng g^{-1} (dry wt.) (Table 1). Species, such as *Gobius niger*, *Sardina pilchardus*, *Anguilla anguilla*, *Pomatoschistus microps*, *Chelidonichthys lucerna* and *Liza ramada*, presented the highest concentrations, over 10 ng g^{-1} (dry wt.) (Table 1).

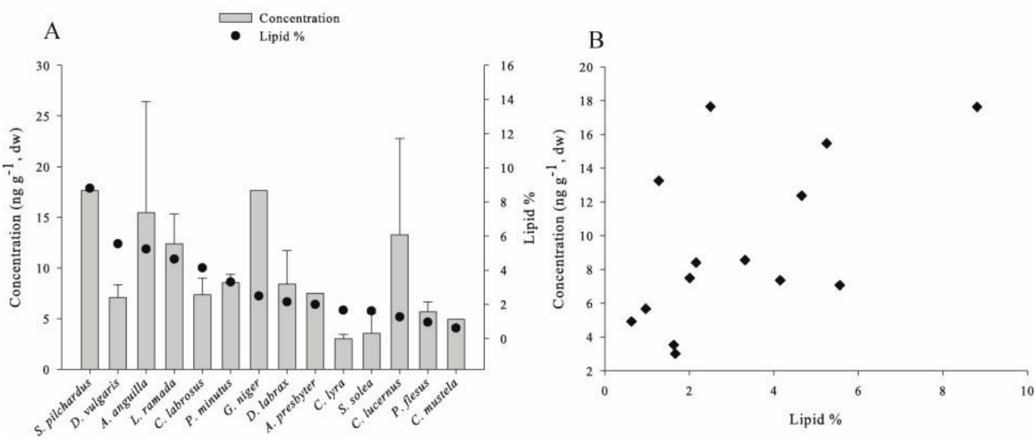

Figure 2. ΣPCBs concentrations (ng g^{-1}, dry wt.) in the fish assemblage according to their lipid content (%) (A). Relation between mean lipid content (%) and ΣPCBs concentration (ng g^{-1}, dry wt.) of the entire fish population (B)

On the other hand, species, such as *Solea solea* and *Callionymus lyra*, had the lowest concentrations, with less than 5 ng g^{-1} (dry wt.) (Table 1).

Ecological indicators concentrations ($\sum_7$ ICES) varied from 2.44 to 14.3 ng g^{-1} (dry wt.) in the estuarine population (Table 1). The species with higher concentration were *S. pilchardus* and *A. anguilla*, with 14.3 and 14.0 ng g^{-1} (dry wt.), respectively. The species with lower concentration were *C. lyra* and *S. solea*, with 2.44 and 2.95 ng g^{-1} (dry wt.), respectively.

Table 1. The Mondego estuary fish assemblage distribution by species, family, ecological guilds (EG), trophic guilds (TG), habitat, lipids (%), age, $\sum_{12}$ PCB concentration (ng g^{-1}, dry wt.), and $\sum_{7}$ ICES concentration (ng g^{-1}, dry wt.). (CA – catadromous; ER – estuarine resident; MS – marine stragglers; MMO – marine estuarine opportunist; MMD – marine estuarine dependent; ZB – zoobenthonic; OV – omnivorous; PV – piscivore; DE – detritivorous)

Species	Family	Common name	EG	TG	Habitat	Age	**$\sum_{12}$ PCB**	**$\sum_{7}$ ICES**
Anguilla anguilla	Anguillidae	European eel	CA	ZB+OV	Demersal		15.45	14.00
Atherina presbyter	Atherinidae	Sand smelt	ER	ZB+OV	Pelagic-neritic	2+	7.50	6.36
Callionymus lyra	Callionymidae	Dragonet	MS	ZB+OV	Demersal		3.01	2.43
Chelidonichthys lucerna	Triglidae	Tub gurnard	MMO	PV	Demersal		13.26	10.87
Chelon labrosus	Mugilidae	Thicklip grey mullet	MMO	DE+OV	Demersal	2+	7.34	5.77
Ciliata mustela	Gadidae	Fivebeard rockling	MMO	ZB	Demersal		4.94	4.27
Dicentrarchus labrax	Moronidae	Sea bass	MMD	PV	Demersal	1+	8.92	7.78
Diplodus vulgaris	Sparidae	Seabream	MMO	ZB+OV	Benthopelagic		7.09	5.85
Gobius niger	Gobiidae	Black goby	ER	ZB	Demersal	2+	17.65	13.31
Liza ramada	Mugilidae	Thinlip grey mullet	CA	DE+OV	Pelagic-neritic	3+	12.24	10.11
Platichthys flesus	Pleuronactidae	European flounder	MMD	ZB	Benthopelagic	1+	5.59	4.71
Pomatoschistus microps	Gobiidae	Common goby	ER	ZB	Denersal	2+	14.94	11.81
Pomatoschistus minutus	Gobiidae	Sand goby	ER	ZB	Demersal	2+	8.56	6.95
Sardina pilchardus	Clupeidae	Sardine	MMO	ZP	Pelagic-neritic		17.64	14.33
Solea solea	Soleidae	Common sole	MMD	ZB+OV	Benthopelagic	0+	3.68	3.03

Estuarine species analysis

The ANOSIM analysis was used to test differences among the concentration of the estuarine fish community (Table 2), trophic guilds, ecological guilds and fish habitat. The ANOSIM related to the fish concentration revealed significant differences (Table 2), the Pairwise test revealed significant differences between all groups ($R=0.928$, $p=0.001$; $p \leq 0.05$) (Table 2). *C. mustela* presented a concentration of 4.93 ng g^{-1} (dry wt.) which was considered 5 ng g^{-1}, therefore was included in the group with medium concentration. Only the ANOSIM related to the fish concentration revealed significant differences among our groups. No significant differences were found within the trophic guild ($p=0.659$; $p \leq 0.05$), ecological guild ($p=0.247$; $p \leq 0.05$) or fish habitat ($p=0.475$; $p \leq 0.05$).

Table 2. Results of ANOSIM and Pairwise test analysis

Groups	R	p
High, Medium	0.925	0.003
High, Low	1	0.036
Medium, Low	0.877	0.028

Global R – 0.928 Significance level of sample statistic – p=0.001

Regarding lipids, *S. pilchardus* was the species with higher lipid content (Fig. 2A). *A. anguilla*, *D. vulgaris*, *L. ramada* and *C. labrosus*, also, had high lipid content (Fig. 2A). The species with lower lipid content were *P. flesus*, *C. mustela*, *S. solea* and *C. lyra* (Fig. 2A). Since PCBs are very lipophilic, they tend to accumulate in species with more lipids. A positive correlation was found between lipids and PCBs concentrations (p=0.037; p≤0.05); consequently species with more lipids tend to accumulate more PCBs (Fig. 2B).

Species spending more than 3 years in the estuary are *A. anguilla*, *L. ramada*, *G. niger*, *P. microps*, *P. minutus* and *A. presbyter* (Fig. 3A), whereas *S. pilchardus*, *C. lyra*, *S. solea*, *C. labrosus*, *C. lucerna*, *D. labrax*, *P. flesus*, *C. mustela* and *D. vulgaris* spend less than 3 years in the estuary (Fig. 3A). Fish species that spent more than 3 years inside the estuary tend to exhibit higher body burdens (Fig. 3B). According to the t-test differences were detected among the two groups (p=0.009; p≤0.05).

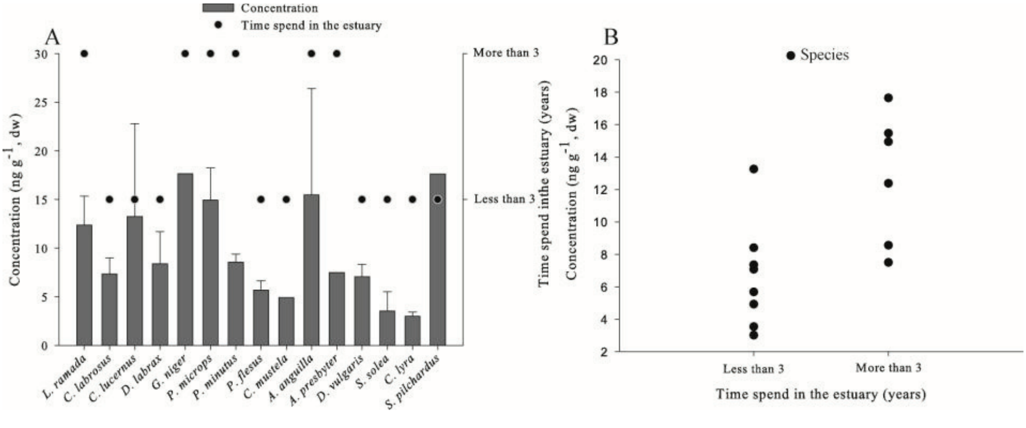

Figure 3. ΣPCBs concentrations (ng g^{-1}, dw) in the fish assemblage according to their trophic guild and time spent in the estuary (years) (A). Relation between the time spent in the estuary (years) and concentration (ng g^{-1}, dw) oh the entire fish population (B). (DE – detritivorous; OV – omnivorous; PV – piscivorous; ZB – zoobenthonic; ZP – zooplanktivorpus)

Considering each trophic guild (Fig. 3A), species spending more than three years in the estuary had higher body burdens. In the DE+OV guild, *L. ramada* spent more time in the estuary and consequently its concentration is higher than *C. labrosus*. In the ZB guild, the species with higher concentration were *G. niger* and *P. microps*, while the species with lower concentration were *C. mustela* and *P. flesus*. The species *C. mustela* and *P. flesus* spent less time inside the estuary (Fig. 3A). In the ZB+OV guild, *A. presbyter* and *A. anguilla* were the species spending more time inside the estuary, but *A. anguilla* had higher PCBs concentrations than *A. presbyter*. On the other hand, *C. lyra* and *S. solea* were the species with lower concentration, spending less than 3 years inside the estuary (Fig. 3A). Concentration tends to increase with time spent in the estuary.

PCB congeners analysis

Species that spend more than 3 years inside the estuary have higher individual congener concentration than the other species (Fig. 4). In this group of species concentrations of less chlorinated congeners were detected, while in the other group these congeners were not detected (Fig. 4). For the two groups the congeners with higher concentration were CBs 138 and 153.

CB 118 is the most toxic congener, within the detected congeners, and its concentration increases with the time spent inside the estuary. The mean concentration of this congener for the species that spend more than 3 years in the estuary was 0.65 ng g^{-1} (dry wt.), while in species that spend less than 3 years in the estuary the mean was 0.22 ng g^{-1} (dry wt.) (Fig. 4). CBs 118, 138 and 153 tend to increase their concentration with the time spent inside the estuary by the different estuarine species (Fig. 4).

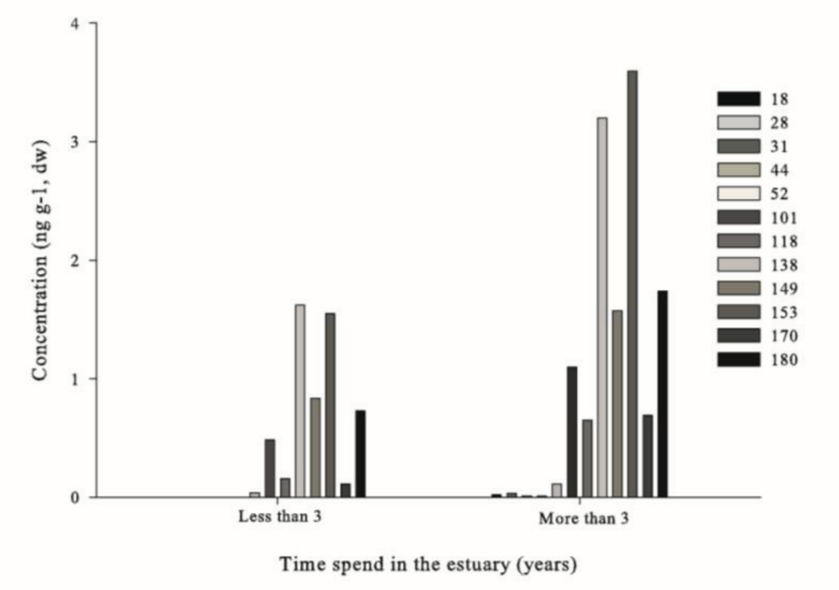

Figure 4. PCBs congeners pattern (mean concentrations (ng g^{-1}, dry wt.) according to the time spend in the estuary

A PCA analysis (Fig. 5) was used to evaluate the congeners' distribution within the estuarine fish community. PC1 explained 82% of the variation, and PC2 explained 9% of the variation.

All congeners are positively associated with PC1. Congeners 101, 138, 149 and 180 are negatively associated with PC2, whereas congeners 18, 28, 31, 44, 52, 118, 153 and 170 are positively associated with PC2.

S. pilchardus and *L. ramada* are highly associated with low chlorinated congeners, such as CBs 18, 28, 31, 44 and 52. Species are distributed along a concentration gradient. Species with medium and lower concentration are negatively associated with PC1, while species with higher concentration are positively loaded with PC1. High chlorinated congeners are highly associated with species higher concentration. Congeners 101, 138, 149, 153, 170 and 180 are extremely influenced by PC1.

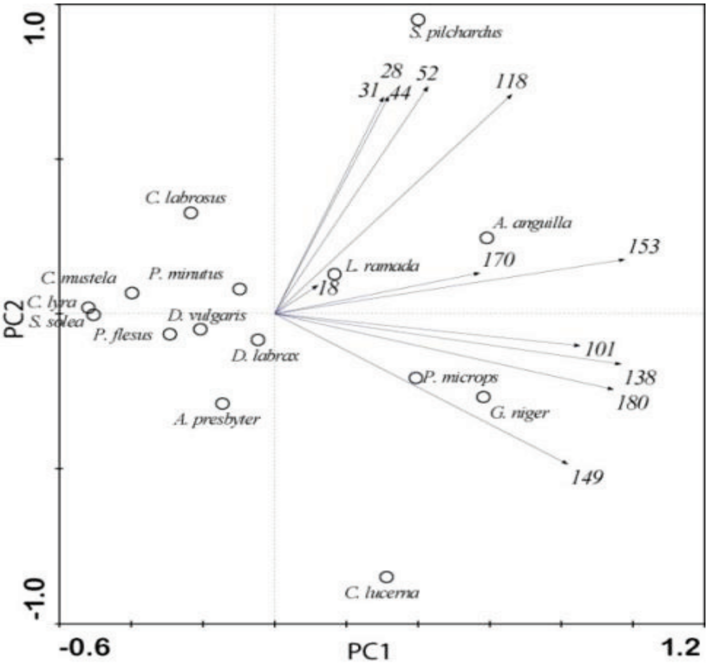

Figure 5. Principal component analysis (PCA) of the PCB congener of the Mondego fish assemblage

Discussion

The Mondego estuary and the fish assemblage

Estuaries are important sites for fishes and fisheries (Martinho et al., 2007), since they are very productive (Dolbeth et al., 2008a), and provide several habitats. Many species use estuarine waters as migration routes (e.g. *A.*

anguilla) (Zompola et al., 2008), refuge against predators and nursery grounds, like the common sole (*S. solea*), sea bass (*D. labrax*) and plaice (*P. flesus*) (Martinho et al., 2010). In estuaries, environmental gradients, especially the salinity gradient, are very strong, consequently species can be restricted to a particular section of the estuary (Jaureguizar et al., 2003). The Mondego estuary fish assemblage follows a typical spatial distribution: freshwater species are found in the upstream areas, marine species are found in the downstream areas and estuarine residents and marine estuarine dependent species are found in the middle section (Baptista et al., 2010). The life strategies of the different estuarine organisms create this structure in the estuarine ecosystem, thus reflecting the functioning of estuaries (Franco et al., 2008). The Mondego estuary fish assemblage studied had 15 species, from 13 families. Studies from Whitfield (1983) emphasized that estuarine size may influence species richness, since bigger estuaries frequently present greater exchanges with the ocean and can provide a wider range of potential habitats for juveniles.

The Mondego estuary is a small estuary with limited and diffuse contamination sources. The most common are wastewaters, resulting from domestic and industrial sewage, agricultural runoff, which requires the use of large amounts of fertilizers and pesticides, and the Figueira da Foz harbour (Nunes et al., 2011). In addition, these specific anthropogenic emissions in the Mondego estuary are not typical sources of PCBs. In fact, studies performed by Pereira et al. (2005) revealed that sediments from the Mondego estuary have low concentration, which is in accordance with the present results. Despite presenting distinct characteristics, the three sampling stations did not show significant differences in PCBs concentrations. This can be due to the small size of the estuary and to fish mobility. Since fishes are very mobile species they could have been feeding in different locations, besides the capture site. Another explanation is that species and sampling locations can be confounded.

Lipid content is among the most important factors that determine species body burden. A positive correlation was found between lipid content and PCBs concentrations, which is in accordance with other studies (e.g. Pastor et al., 1996). This feature explains why *S. pilchardus* and *C. lucerna* presented high concentrations of PCBs despite the lower time spent inside the estuarine system.

Many species present in the Mondego estuary are consumed by humans presenting a high socio-economic importance. Among these species are included *A. anguilla*, *C. lucerna*, *C. labrosus*, *C. mustela*, *D. labrax*, *D. vulgaris*, *L. ramada*, *P. flesus*, *S. pilchardus* and *S. solea*. Although, *A. presbyter* is a non-commercial species, it is consumed by humans through subsistence fishing. Other species, such as *C. lyra*, *G. niger*, *P. microps* and *P. minutus*, despite being some of the most abundant species in the estuary, are considered non-commercial and are not consumed by humans. Though, the study of organic compounds in these species is very important, because they are essential for the structure and function of the ecosystem.

Some species with high commercial value had the highest PCBs concentrations. Within these species are included *A. anguilla*, *C. lucerna*, *L.*

ramada and *S. pilchardus*. *P. microps* is prey to many piscivorous species, and its PCB concentration is high. Since PCBs tend to move through the trophic web, these species may be a possible source of contamination for top predators feeding on them. On the other hand, some commercial species presented low PCBs concentrations, such as *C. mustela* and the flatfishes, *P. flesus* and *S. solea*.

It is difficult to compare concentrations between studies due to variation in the congeners that are analyzed (specific congeners and the number of congeners). Another difficulty in the intercomparison of results from different studies is the expression of results, which can be expressed in dry weight, wet weight and fat weight. Studies performed by Nunes et al. (2011) in the Mondego estuary revealed that the concentration of ∑dl-PCB increases from the primary producers, to the benthic feeders and ultimately to fish. Nunes et al. (2011) detected that *A. anguilla* presented the highest concentration, followed by *D. labrax* and *S. solea*, which presented the lowest concentration. These results are in accordance with the present results since *A. anguilla* presented higher concentration than *D. labrax*, that presented higher concentration than *S. solea*.

Some species from the Mondego estuary have higher concentration then other studies. The data from *C. lucerna* and *G. niger* was transformed into fat weight and only the 7 ecological indicators were used, for a better comparison of the results. So, *C. lucerna* and *G. niger* from the current study have higher concentration then the same species found in Turkey (Table 3). For these species the congeners 138 and 153 had the highest concentrations, among the 7 analyzed congeners, which is in accordance with the present results. On the other hand, *S. pilchardus* from the current study has lower concentration than *S. pilchardus* from Turkey, with 89.0 and 199 ng g^{-1} (fat weight), respectively (Table 3). For *S. pilchardus* were only used the 7 ecological indicators and the concentration was expressed in fat weight. *S. solea* from the current study has much lower concentration then specimens analyzed in France, with 2.95 and 58 – 121 ng g^{-1} (dry wt.), respectively (Table 3).

Table 3. Comparison of PCBs concentration (∑PCBs) in different species of the Mondego estuary and other regions

Species	Concentration (ng g^{-1})	Number of congeners	Location	Reference
Chelidonichthys lucerna	84.52 (lipid wt.)	7	Turkey	Coelhan et al., 2006
	699 (lipid wt.)	7	Portugal	Current study
Dicentrarchus labrax	13 (±2.5) (dry wt).	18	Portugal	Antunes and Gil, 2004
	10.3–19.1 (dry wt.)	13	Portugal	Fernandes et al., 2008
	8.41 (dry wt.)	12	Portugal	Current study
Gobius niger	298 (lipid wt.)	7	Turkey	Coelhan et al., 2006
	1210 (lipid wt.)	7	Portugal	Current study
Sardina pilchardus	199 (lipid wt.)	7	Turkey	Coelhan et al., 2006
	3–51 (dry wt.)	30	Portugal	Antunes et al., 2007
	89.01 (lipid wt.) 17.63 (dry wt.)	7 12	Portugal	Current study
Platichthys flesus	23–52 (dry wt.)	18	Portugal	Ferreira et al., 2004
	5.68 (dry wt.)	12	Portugal	Current study
Solea solea	58.3–121.3 (dry wt.)	7	France	Dierking et al., 2009
	2.95 (dry wt.)	7	Portugal	Current study

According to Dierking et al. (2009) the contaminant levels in the Mediterranean Sea are relatively high, thus the differences in the Mondego (Portugal) and France. Regarding studies performed in Portugal, *D. labrax* concentration, from the current study, was slightly lower than *D. labrax* analyzed by Antunes and Gil (2004) and Fernandes et al. (2008) (Table 3). *D. labrax* analyzed by Antunes and Gil (2004) was capture in Ria de Aveiro (Portugal), a more contaminated site than the Mondego estuary and this study included more congeners than the current study (18 congeners). *D. labrax* from Fernandes et al. (2008) study was performed with fish from aquaculture. Generally, cultured fish has higher concentration than wild fish (Ferreira et al., 2008).

Fish assemblage congeners

CBs 138 and 153 had the highest concentrations in all estuarine species analyzed, which is in accordance with former studies developed in Italy, Turkey and Portugal (Bazzanti et al., 1997; Coelhan et al., 2006; Fernandes et al., 2008). Antunes et al. (2007) stated that CB 138 and 153 are the prevailing compounds reported in biological samples. CBs 138 and 153 are hexachlorinated congeners, and being high chlorinated congeners they tend to bioaccumulate. These congeners are more persistent in the environment, more lipophilic, less volatile, more resistant to microbial degradation (Bazzanti et al., 1997; Zhou et al., 2001) and have slow metabolic elimination rates (Stapleton et al., 2001; Wu et al., 2008).

Among the detected congeners CB 118 is the most toxic, being the only analyzed congener having an assigned TEF-value (Toxic Equivalent Factor, indicating a relative dioxin-like activity) (Van den Berg et al., 1998). The concentrations of CB 118 for the species that spend less than 3 years in the estuary were very low, however the concentration generally tended to increase with the time spent in the estuary. Most species that spend more than three years in the estuary presented higher concentration of this congener. Yang et al. (2008) stated that this congener is a good indicator of environmental contamination.

It is useful to examine the similarity of the congener patterns with the Aroclor mixtures. The high proportion of congeners such as 101, 138, 149, 153 and 180 in the Mondego estuary fish assemblage resembles Aroclor 1260 where these congeners were predominant. Although these results have to be interpret with caution. Aroclor mixtures production was banned in the 1980’s so the pattern of the congeners may have suffered some alterations, mostly in their proportion/inicial ratio. Furthermore, biotransformation can occur in biological samples (Stapleton et al., 2001) which can alter the congener environmental composition. Aroclor 1260 was used in transformers, hydraulic fluids and dedusting agents (Rodenburg et al., 2011).

Estuarine fish assemblage life strategies

The species in the Mondego estuary presented different organochlorines concentrations, with the highest concentration found in species that spent more time in the estuary. According to Bodin et al. (2008) PCBs concentrations is area dependent, a fact also observed by Melwani et al. (2009). Therefore species that spend most of their life time near the contamination source (inside the estuary) are more likely to increase their body burden, and species that spend most of their lifespan in less contaminated environments (eg. coastal waters) are more susceptible to have lower body burden.

S. pilchardus and *C. lucerna* are among the species with higher PCBs concentrations. Despite the different life strategies, these species have similar concentrations. *S. pilchardus* concentration can be due to its high lipid content. *C. lucerna* is a piscivorous species that feeds mainly on fish, but can feed on crustaceans and mollusks, as well (Dolbeth et al., 2008b). Since is a top predator this species has a higher probability to have higher body burden through biomagnification. On the other hand, *C. lucerna* may accumulate contaminants more easily and be more susceptible to its effects (Cabral et al., 2001). *D. labrax* presented lower concentration than *C. lucerna*. Both species are piscivorous but present differences in their diet, reflecting differences in their PCBs concentrations. *D. labrax* as a juvenile is not exclusively piscivorous, also feeding on invertebrates and plankton, increasing its fish consumption with age. On the other hand, *C. lucerna* is a more exclusive piscivorous, starting to feed on fish at a younger age (Dolbeth et al., 2008b).

S. solea and *C. lyra* have the lowest PCBs concentrations in the estuarine fish assemblage. *C. lyra* is a marine straggler, so lives at sea its entire life cycle, entering the estuary in low numbers in order to feed (Elliot et al., 2007), remaining in the estuary for only a few months. It was the only marine species found in the Mondego estuary within the study period. Since the contamination in the marine environment is expected to be low, the results found were expected. *S. solea* use the estuary as a nursery ground (Jorge et al., 2002), and the individuals caught in the estuary were less than one year old. This species exhibited low PCBs concentrations, which can be associated to its low lipid content and the short amount of time spent in the estuary. In other studies performed by Bocio et al. (2007), *S. pilchardus* is one of the fish species with higher concentration, whereas, *S. solea* is one with lower concentration, which is in accordance with the present results.

P. flesus use the estuary as a nursery ground and presented low body burden. This can be explained by its low lipid content and the short period of time spent in the estuary. Although, *P. flesus* and *S. solea* had low concentration, they are susceptible to accumulate organic contaminants. Eichinger et al. (2010) stated that the benthonic life style and the fact that estuaries are considered nursery grounds for flatfishes make them especially vulnerable to chronic or accidental pollution.

Estuarine residents' *P. microps* and *G. niger* are among the species with higher concentration. Both species are capable of spending their entire life cycle inside the estuary. *P. minutus* and *A. presbyter* have lower concentrations than the other estuarine residents, since these species do occasional migrations to the sea (Leitão et al., 2006; Pombo et al., 2005). The marine environment is expected to be less contaminated than the estuary, therefore lowering these species body burden. Moreover, species from the same genus, like *P. microps* and *P. minutus* have different body burden. These species are opportunistic carnivorous species, feeding according to its availability; but tend to occupy different areas reducing the potential for competition among them (Leitão et al., 2006).

A. anguilla and *L. ramada* are among the species with higher concentration. These species spend their adult life in brackish or less saline water, only migrating to sea to spawn. *A. anguilla* high concentration can be due to their high lipid content. This species has strong site fidelity during their adult life, so they can be good indicators for a local contamination (Ashley et al., 2003). *L. ramada* is often found in polluted waters, increasing this species body burden.

Conclusions

Multiple factors influence the PCBs body burden in the fish assemblage of Mondego estuary, such as lipid content, time spend in the estuary and trophic strategies. A positive correlation was found between species concentration and lipid content.

Species that spend more than three years in the estuary presented high PCBs concentrations. The most abundant congeners were CB 138 and 153 and their concentration tended to increase with the time spent in the estuary. Furthermore, the concentration of CB 118, the most toxic congener analyzed, increase with the time spent in the estuary. The PCB congener pattern of the fish assemblage of the Mondego estuary is similar with the congener pattern of Aroclor 1260. The study of organochlorine contaminants in non-commercial species is as important as the study in commercial species, since these species are very essential for the structure and function of the ecosystem.

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Chapter 3

Polychlorinated biphenyls (PCBs) bioaccumulation along three grey mullet species lifespan – A comparison study

Abstract

Polychlorinated biphenyls (PCBs) are lipophilic contaminants that tend to accumulate in organisms. PCBs were detected in *C. labrosus*, *L. aurata* and *L. ramada* from the Mondego estuary, along the species lifespan. *L. ramada* presented the highest concentration, and it increased with age, whereas *C. labrosus* and *L. aurata* concentration remained stable. *L. ramada* high concentration can be attributed to its lifespan location, since this species is able to accumulate PCBs along its lifespan even in low environmental contamination conditions. CBs 101, 118, 138, 149, 153, 170 and 180 were the congeners that more contributed to these species contamination, being CB 138 and 153 the congeners with higher concentration. Mullets are edible in many countries, being important in fisheries and aquaculture. *L. ramada* is the most common mullet for capture and human consumption. All species presented concentrations below the regulation limit establish by the European Union.

Additional keywords: PCBs, *Chelon labrosus*, *Liza aurata*, *Liza ramada*, Mondego estuary, Bioaccumulation, Public health

Introduction

Polychlorinated biphenyls (PCBs) have been an environmental concern for many years, due to their high toxicity and bioaccumulative properties (Bodiguel *et al.*, 2009). PCBs are widely spread in the environment, and can accumulate in organisms (Masmoudi *et al.*, 2007). PCBs are considered dangerous pollutants, given a broad spectrum of toxicological responses, including immunotoxicity, endocrine disruption, reproductive deficits and tumor and carcinoma development (Bodiguel *et al.*, 2009). Due to this it is important to determine PCBs values, in order to determine the quality status of the environment.

Fishes are often used as indicators of contamination in coastal systems, because they are relatively large and easy to identify (Coelhan *et al.*, 2006; Tavares *et al.*, 2011). Many authors pointed out that the consumption of fish contaminated by PCBs increases the risk for human contamination (Fu and Wu, 2005; Matthiessen and Law, 2002; Ulbrich and Stahlmann, 2004). So, obtaining information on organochlorine concentration and bioaccumulation in fish is essential given the importance for public health.

The Mugillidae family can be found in estuaries, coastal waters and rivers (Almeida, 1996), this success can be associated to its food plasticity. They are omnivorous and detritivorous consuming a wide selection of food items (Boglione *et al.*, 2006; Zouiten *et al.*, 2008). Mugillidae have the advantage of using food resources provided by the primary producers, and therefore contribute decisively to the energy and organic matter flux in the estuarine ecosystem (Almeida, 2003).

Mullet species are very appreciated as fish food in many tropical and subtropical countries, and play an important role in the world fisheries and aquaculture (Boglione *et al.*, 2006; Cardona *et al.*, 2008), like in Italy (www.fao.org), Tunisia (Masmoudi *et al.*, 2007) or Egypt (El-Halfway *et al.*, 2007; Mousa, 2010). *Liza ramada* has great nutritious value and it is an important alternative to other food resources (El-Halfawy *et al.*, 2007), achieving high market prices (Mousa, 2010).

The presence and distribution of PCBs in edible fish is important not only for public health, but also from an ecological perspective. Many studies regarding contaminants have been made in mullet species (Baker *et al.*, 1998; Bilbao *et al.*, 2010; Tavares *et al.*, 2011), but only a few studies have been made for PCBs, for *Liza aurata* (Masmoudi *et al.*, 2007; Licata et al., 2003), *L. ramada* (Bocquené and Abarnou, 2012) or *C. labrosus* (Narbonne, 1979). Moreover there are no studies regarding PCBs along the three species lifespan. So, the purpose of this work was to assess PCBs contamination in three Mugilidae species: thinlip grey mullet (*Liza ramada*), golden grey mullet (*Liza aurata*) and thicklip grey mullet (*Chelon labrosus*). Accordingly, some questions were addressed: a) Do these species bioaccumulate PCBs along their lifespan?; b) Are there differences between species in the accumulation patterns?; c) Is the size/age of the fish important, concerning public health?; d) Which congeners have higher contribution for PCBs contamination?

Material and Methods

Sampling location

The Mondego estuary is a small estuary located in the western coast of Portugal (40º08’N, 8º50’W), with an area of 8.6 km^2 (Fig. 1). The estuary comprises two arms, the north and the south arm, separated at about 7 km from the shore joining again near the mouth of the estuary. The north and the south arm have distinct hydrologic characteristics.

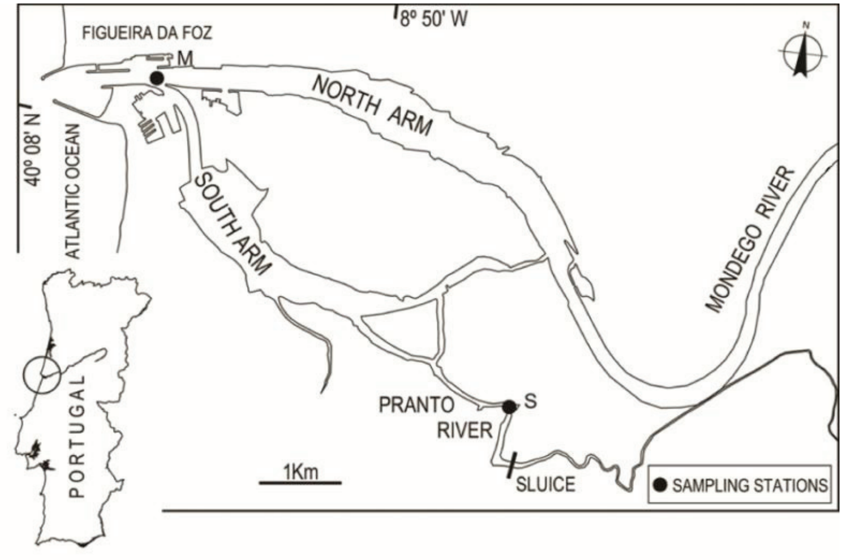

Figure 1. Mondego estuary sampling sites location

The north arm is deeper with 5-10 m of water column in high tide and 2-3 m tidal range. It is dredged frequently to maintain its depth, due to harbor activities. The south arm is shallower with 2-4 m deep in high tide and 1-3 m tidal range. The water circulating in the south arm is mainly depending on the tides and on small fresh water input from the Pranto River. The Mondego estuary is a well described system and poorly contaminated (Vale et al., 2002). In this scenario of low environmental contamination it is important to understand how fish species accumulate PCBs along their lifespan and specifically the ones that are consumed by humans reaching considerable prices in some markets.

Sampling and laboratory work

Three species of mullets were caught in the Mondego estuary: *Chelon labrosus*, *Liza aurata and Liza ramada*. Fishes and surface sediments were collected from September 2007 to April 2008 at the south arm (mainly juveniles, and adults from *L. aurata* and *L. ramada*) and at the mouth (mainly *C. labrosus* adults) (Fig.1). A traditional beach-seine net was used to catch the younger fish, and a trammel net was used to catch the older fishes. Fishes were taken into the laboratory where they were weighted (g) and measured (cm) before collecting muscles samples. Fishes were aged using scales analysis. For each species and age 3 individuals were stored and analysed for PCBs. Due to the lack of mass some samples were analysed as composite samples with 3 individuals. For *C. labrosus* were used individuals from 2+ to 8+ years, for *L. aurata* from 0+ to 4+ years and for *L. ramada* from 3+ to 11+ years. Due to the lack of mass for PCB analysis, age 10+ of *L. ramada* and age 6+ of *C. labrosus* were not determined. After laboratory processing, samples were freeze-dried (Snijders scientific), homogenised and stored in freezer (-20^{o}C), until the analytical procedure.

PCBs analysis

Freeze-dried sediment samples were sieved to <1 mm, homogenized and frozen (−20°C) wrapped in aluminum foil. Representative aliquots were Soxhlet-extracted with a hexane/acetone mixture (2:1) for 6 h at a rate of 4–6 cycles/h, in a pre-washed glass fiber thimble (Cachada et al., 2009). Activated copper granules were added to remove elemental sulphur (US EPA method 3660B – Sulfur cleanup). The resultant extract was concentrated using a rotavapor and submitted to an alumina cleanup (Supelclean® neutral-alumina and anhydrous sodium sulphate at the top) using a solid phase extraction (SPE) system. The column was eluted with hexane:DCM (9:1) and hexane:DCM (2:1). The eluate was then concentrated down to 1 mL using a rotavapor, dried under a gentle stream of nitrogen and solvent changed to hexane. The extract was further submitted to an acid silica gel (Supelclean® silica gel with 44% w/w concentrated sulphuric acid) cleanup and PCBs were eluted with hexane. The eluate was concentrated down to 1 mL using a rotavapor, dried under a gentle stream of nitrogen and solvent changed to iso-octane for further processing by gas chromatography coupled to mass spectrometry (GC–MS) as described below for tissue samples.

For fish analysis 3 g of muscles was accurately weighted and extracted by sonication (Branson 3510) with a *n*-hexane:acetone (1:1) mixture. The extract was decanted and the process repeated three times. The extract volume was reduced by solvent evaporation using a rotavapor.

The content in lipids was gravimetrically determined using 10% of the extract. The remaining (90%) volume of the extract was reduced by solvent evaporation under a gentle stream of nitrogen and lipids were removed with 97% sulphuric acid (US EPA methods 3665A – Sulfuric acid cleanup). Afterwards, the lipid-free extract passed through a multilayered column packed with florisil (Supelclean® Florisil) and anhydrous sodium sulphate and PCBs eluted with hexane. Samples were dried, under a gentle stream of nitrogen.

The internal standards CB 34, 62, 119, 131 and 173 (Ayris et al., 1997), were added to the dried extracts, and diluted with iso-octane to a final volume 200 μL. Samples were analysed on a gas chromatograph equipped with a MDN-12 silica capillary column (30 m; 0.25 mm i.d.; 0.25 μm film thickness) coupled to a mass spectrometry detector (GCMS-QP5050A, Shimadzu) using electron impact ionization and selected ion monitoring (SIM) acquisition. Helium (0.7 mL/min) was employed as the carrier gas, and samples were injected (1 μL) in splitless mode with column temperature at 40ºC and held for 2 minutes, then programmed to ramp 15ºC/min to 180ºC, and held for 1 minute, 8ºC/min to 300ºC and held for 15 minutes (injector temperature = 280ºC; interface temperature = 300ºC). Total PCBs content dry weight (ng g^{-1}, dry wt.) was based on the summed concentrations of the 12 congeners analysed ($\sum_{12PCBs}$) (IUPAC nos. 18, 28 and 31-tri; 44 and 52-tetra; 101 and 118-penta; 138, 149 and 153-hexa; 170 and 180-hepta). Within these congeners, 6 of them are considered ecological indicators to assess marine pollution, by the European Union ($\sum_{6ICES}$ 28, 52, 101, 138, 153, and 180) (Commission Regulation (EU) No 1259/2011).

For quality assurance and quality control of the PCBs quantification method, contamination was evaluated by blank controls and results were always below detection limit. The recoveries of the analytical method for the analysed congeners were tested by analysis of spike samples and the recoveries mean was 98% (standard deviation of 11%). Reproducibility was calculated on replicate analysis giving an overall variability lower than 20%. The detection limits for individual PCBs ranged between 0.1 and 1.0 ng g^{-1} (dry wt.).

Statistical analysis

To detect differences in PCB concentration and in the lipid content of the different age classes a linear regression was performed (SigmaPlot 11.0). To detect differences in the concentration of all species a Kruskal-Wallis one-way analysis of variance on ranks was performed. Afterwards, an All Pairwise multiple comparison, Dunn's test, was applied in order to detect differences between each species. To detect differences between lipids of all species a one-way ANOVA was developed using the average lipid percentage for the three species (SigmaPlot 11.0). A data square-root transformation was applied to the data. A MDS analysis (Primer 6β) was performed to detect differences between the mullet species. The MDS analysis was performed using the congeners' concentration of each species. A SIMPER analysis (Primer 6β) was used to detect differences on the congeners' distribution of each species. This analysis was performed using the Bray-Curtis similarity, with a cut-off of 90%, and the congeners' concentration of each species' age. A 5% significance level was used for all the analysis.

Results

PCBs analysis in C. labrosus, L. aurata and L. ramada

Sediments from the Mondego estuary presented low PCBs concentrations. M station presented the highest concentration ($\sum_{12PCBs}$) with 2.63 ng g^{-1} (dry wt.), whereas the south arm sediment concentration was 1.35 ng g^{-1} (dry wt.).

Three mullet species were captured in the Mondego estuary: *Chelon labrosus*, *Liza aurata* and *Liza ramada*. *C. labrosus* age varied between 2+ and 8+ years, its size varied between 19.3 and 51.4 cm and its weight between 77 and 1477 g. *L. aurata* age ranged between 0+ and 4+ years, its size varied between 14.3 and 34.3 cm and its weight between 27 and 282 g. *L. ramada* age varied between 3+ and 11+ years, its size varied from 15 and 50 cm and its weight varied from 29.7 and 1096 g (Fig. 2).

At the age of 3+ *C. labrosus* is bigger than the other mullet species with approximately 30 cm, while *L. ramada* and *L. aurata* have approximately 15 and 22 cm (respectively) (Fig. 2). At age 4+ *L. aurata* and *C. labrosus* have similar concentration with 5 ng g^{-1} (dw), presenting also similar size, with 34 – 35 cm. *L. ramada* at the same age is smaller (18 cm) (Fig. 2), but its concentration is much higher, with 15 ng g^{-1} (dry wt.) (Fig. 3).

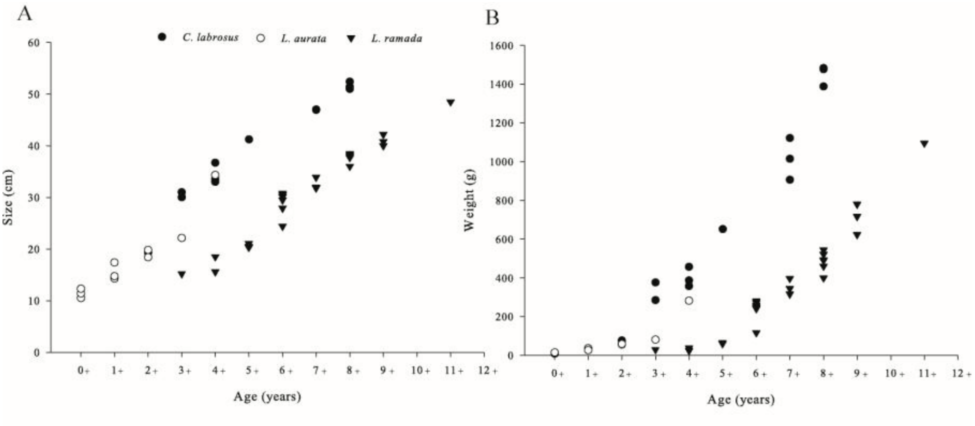

Figure 2. Age (years), size (cm) (A) and weight (g) (B) variation in *C. labrosus*, *L. aurata* and *L. ramada*

C. labrosus PCBs concentrations varied between 1.6 and 8.4 ng g^{-1} (dry wt.). No significant differences were found in PCBS concentration of different size/age ($R^2 = 0.0093$, $p = 0.856$). *L. aurata* concentration varied between 3.3 and 6.4 ng g^{-1} (dry wt.), and significant differences were found along the species lifespan ($R^2 = 0.976$, $p = 0.012$). Although *L. aurata* PCBs concentrations tend to increase, the data are not clear to ensure that this species increases its bioaccumulation with the lifespan since only individuals until age 4+ were analyzed. *L. aurata* age 0+ PCBs concentrations were below the detection limit, and therefore not able to be quantified. *L. ramada* PCBs concentrations varied between 8.8 and 52.3 ng g^{-1} (dry wt.) (Fig. 3).

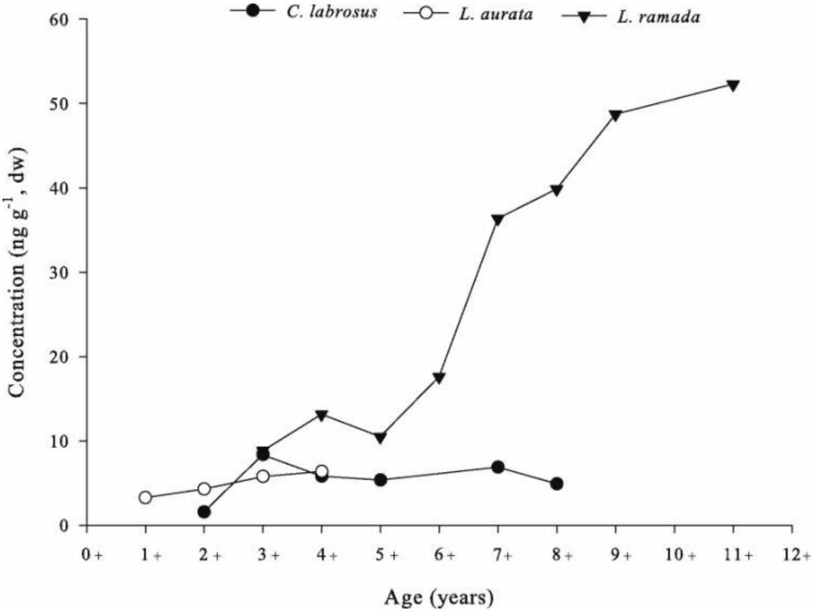

Figure 3. PCBs concentration (ng g^{-1}, dry wt.) in *C. labrosus*, *L. aurata* and *L. ramada* along their lifespan

Significant differences were found in PCBs concentrations of different size/age (R^2 = 0.869, p = 0.002). *L. ramada* had the highest concentrations among the three mullet species and greatly increased with the species lifespan. No relation was found between lipid and age (R^2 = 0.456, p = 0.066; R^2 = 0.018, p = 0.867; R^2 = 0.456, p = 0.141, for *L. ramada*, *L. aurata* and *C. labrosus*, respectively), therefore lipid content tends to remain stable with age, for the three mullet species. According to the Kruskal-Wallis test significant differences were found between each species concentration (p = 0.002). An All Pairwise test reveled differences between *L. ramada* vs *L. aurata*and *C. labrosus* (post hoc test, p < 0.05). On the other hand, *L. aurata* vs *C. labrosus* did not present significant differences (post hoc test, p > 0.05).

According to the one-way ANOVA no significant differences were found between lipid content of the three species (Fig. 4) (p = 0.523).

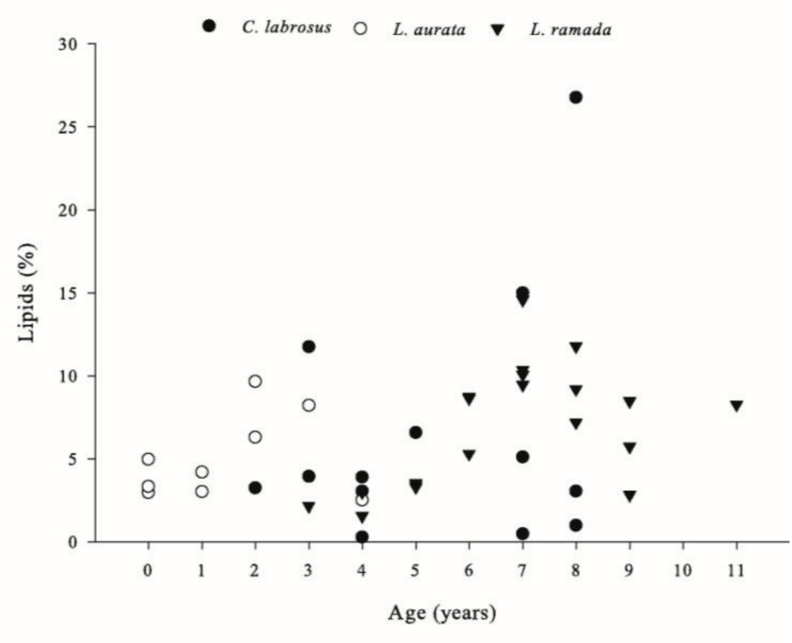

Figure 4. Lipid (%) variation in *C. labrosus* (A), *L. aurata* (B) and *L. ramada* (C)

A MDS analysis was performed to detect differences in the mullet species concentrations. *L. ramada* older individuals are separated from the other species and from their younger individuals (Fig. 5), due to its high PCBs concentrations.

Until age 6+ *L. ramada* is included in the same group as *L. aurata* and *C. labrosus*, since they present similar concentrations (Fig. 5). Due to the low concentration of *C. labrosus* at age 2+, this age was separated from the other groups (Fig. 5).

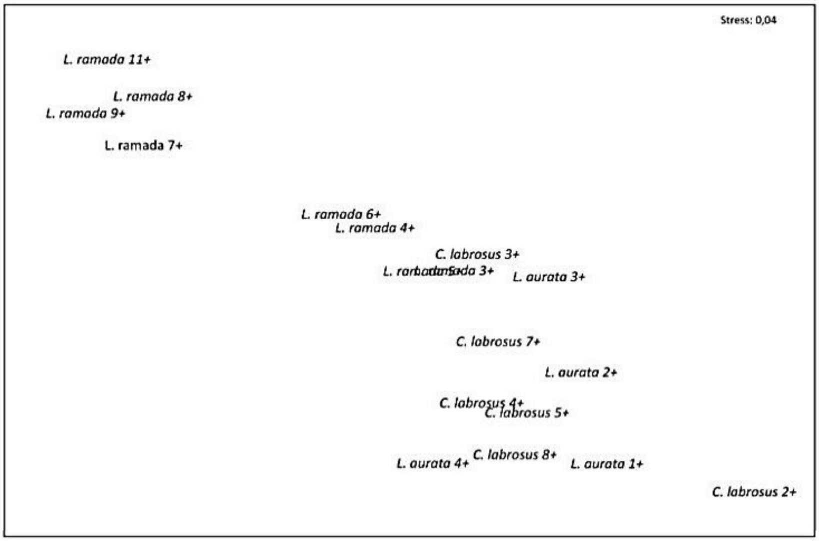

Figure 5. MDS analysis with the *C. labrosus*, *L. aurata* and *L. ramada* at all ages

According to the SIMPER analysis only higher chlorinated congeners were found to be important for the mullet species contamination (Table 1). In *L. aurata*, only CBs 138, 149, 153 and 180 were found to be important for this species contamination, with 28%, 16%, 38% and 16%, respectively (Table 1). In *C. labrosus* six congeners contributed for the contamination, being CB 153 the congener with higher contribution, (29%) (Table 1). The congeners with lower contributions were CB 101 and 118, with 6%.

In *L. ramada* all seven chlorinated congeners contributed for the contamination. CB 153 had the highest contribution with 27%, on the other hand CBs 118 and 170 had the contributions, with 8% and 6%, respectively (Table 1).

Table 1. SIMPER analysis with the congeners that more contribute (%) for the species contamination

	Contribution (%)						
	101	**118**	**138**	**149**	**153**	**170**	**180**
Chelon labrosus	6.31	5.68	25.00	15.41	28.97	—	10.12
Liza aurata	—	—	27.85	15.89	37.52	—	15.69
Liza ramada	10.37	7.71	21.35	9.71	26.76	5.83	11.91

CB 118 is the only congener analyzed considered as a dioxin-like PCB. Both *L. aurata* and *C. labrosus* presented values lower than 0.5 ng g^{-1} (dry wt.), while *L. ramada* presented higher concentration varying between 0.5 and 6.5 ng g^{-1} (dw) (Fig. 6A). Until age 5+ *L. ramada* and *C. labrosus* presented similar concentrations of CB 118. Afterwards, *L. ramada* CB 118 concentration increases while *C. labrosus* concentration remains stable.

L. ramada CB 138 concentration varied between 2.0 and 10 ng g^{-1} (dry wt.), increasing with age (Fig. 6B). On the other hand, *L. aurata* and *C. labrosus* concentrations are similar and remained stable with age, varying between 1.0 – 2.0 ng g^{-1} (dry wt.) (Fig. 6B).

CB 153 has the higher concentration within the three mullet species. *L. ramada* concentration increases with age, ranging from 2.0 to 15 ng g^{-1} (dry wt.). *L. aurata* and *C. labrosus* had similar concentration varying between 0.3 and 2.6 ng g^{-1} (dry wt.) (Fig. 6C). CB 153 concentration for these two species remains stable with age.

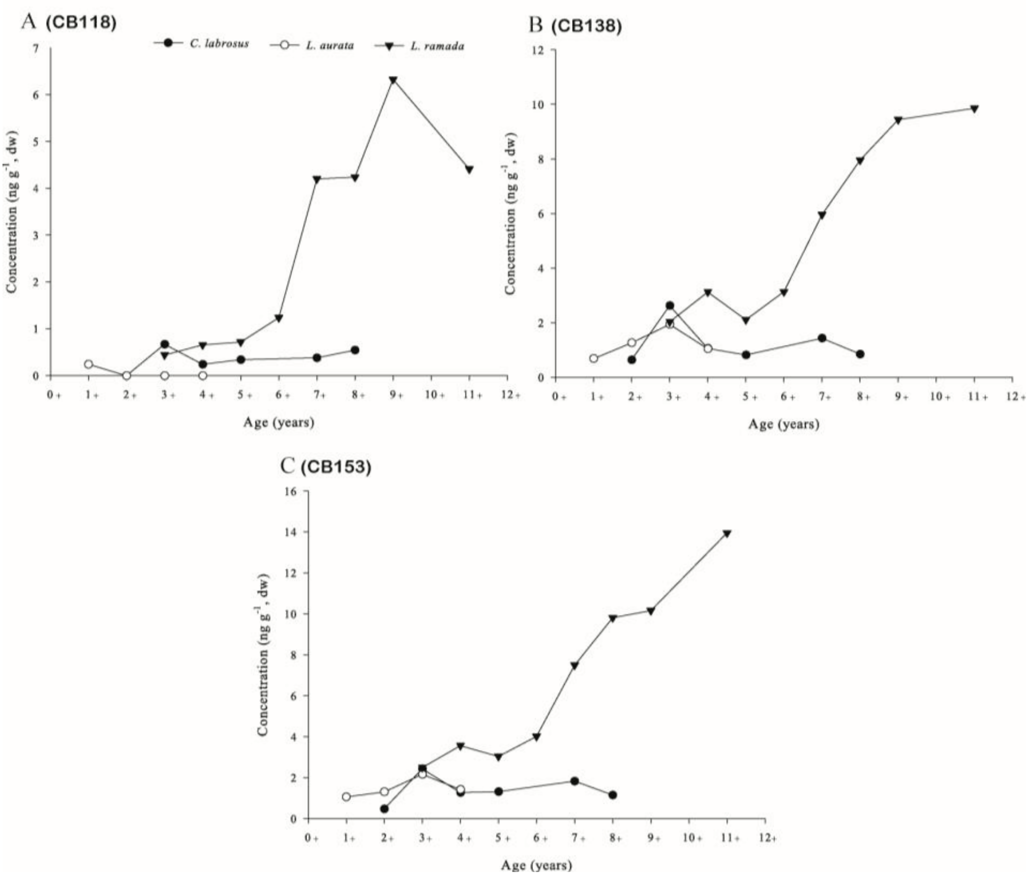

Figure 6. Congeners 118 (A), 138 (B) and 153 (C) concentrations (ng g^{-1}, dry wt.) along the lifespan of *C. labrosus*, *L. aurata* and *L. ramada*

Discussion

Mugilidae PCBs contamination

The Mondego estuary can be considered a poorly contaminated. Pereira et al. (2005) stated that sediments from the Mondego estuary have low concentration, which is in accordance with the present results. Moreover, PCBs concentrations present in sediments from the Mondego estuary are lower than those found by Hong et al. (2003), in Masan Bay, Korea, and Howell et al. (2008), in the Houston Ship channel, USA. The Mondego estuary is a small estuary with and its few contamination sources, domestic and industrial sewage, agricultural runoff (which requires the use of fertilizers and pesticides) and Figueira da Foz harbor (Nunes et al., 2011). Although, they do not discharge large quantities of PCBs.

PCBs are very lipophilic and in many studies higher lipid content is associated with higher PCBs concentrations (eg. Coelhan et al., 2006; Trocino et al., 2009). However, in the current study lipid levels are not associated with PCBs concentration, since all species presented similar lipid content and different PCBs concentrations. *L. ramada* PCBs concentrations increases with age but its lipid content remains stable. So, other factors must also play a key role in the metabolic pathways involved in the bioaccumulation of PCBs by the fish species.

Mullets can run long distances in short periods of time eventually moving out of the estuarine systems, or remain inside particularly around urban sewages discharge (Antunes et al., 2007), leading to a high uptake of contaminants (Masmoudi et al., 2007). Juveniles' mullet species presented similar PCBs concentrations, which can be explained by the fact that the three species juveniles spent a part of their life in the same environment, inside the Mondego estuary (Fig. 7). *L. ramada* concentration increased with age, because this species spends its entire lifespan inside or near the estuarine system, only migrating to the sea to spawn (Fig. 7). As a catadromous species, *L. ramada* has the capacity to osmoregulate even in freshwater (Cardona et al., 2008). Furthermore, this species is often found in more contaminated areas (Kottelat and Freyhof, 2007), and is more tolerant to coastal organic pollution and eutrophication (Boglione et al., 2006).

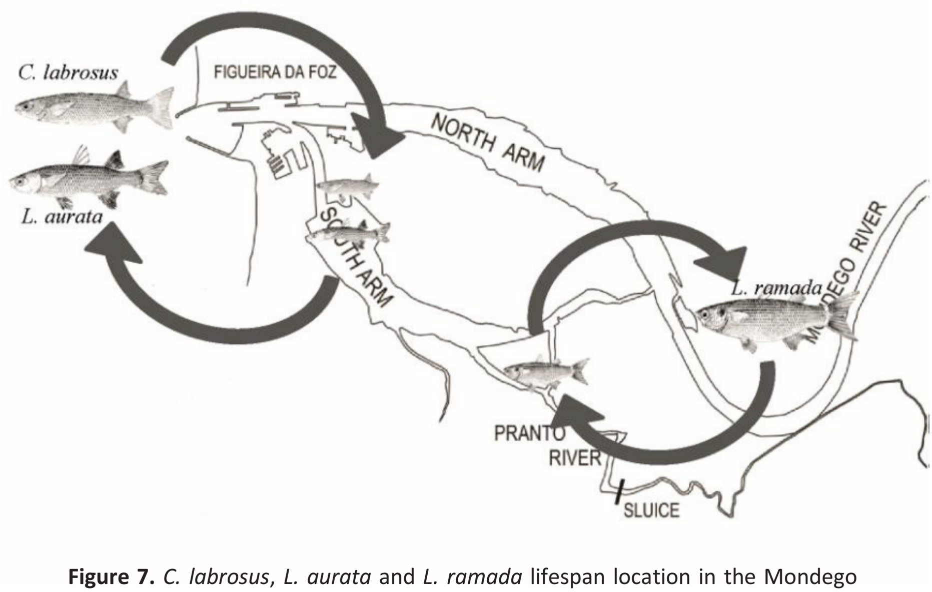

Figure 7. *C. labrosus*, *L. aurata* and *L. ramada* lifespan location in the Mondego estuary

L. aurata and *C. labrosus* presented lower PCBs concentrations than *L. ramada*. These two species are marine estuarine opportunists, entering the estuary regularly in substantial numbers, particularly as juveniles, but use near shore marine waters as a preferential habitat. According to Tavares et al. (2011), *L. aurata* mercury contamination, in the Mondego estuary, decreases with fish age. The decrease of this bioaccumulative pollutant can be explained by the ecology of the species that tend to spent most of the time in the coastal area and not inside the estuarine system, which can lead to a low uptake of contaminants. This can also explain the fact that PCBs increased very slowly in *L. aurata* individuals.

In the Mondego estuary, the three mullet species have similar feeding behavior, being detritivorous and omnivorous. Mugilidae are characterized by a wide range of feeding adaptations to the estuarine environment according to the trophic availability (Zouiten et al., 2008). Each species is able to utilize the food distributed from the thin water surface film to the bottom mud, either by direct grazing or using plant-detritus food chain (Boglione et al., 2006). PCBs contaminations in mullet species can also be attributed to their feeding strategies (Ferreira *et al.*, 2004), since different species have different assimilation rates originating different contamination patterns along the lifespan.

The relation between species concentration and age is not well established in literature. This relation can occur due to extended exposure to contaminants in older fish. Nevertheless, a wide range of variables can affect bioaccumulation, such as growth rate, diet and lifespan location. The relation between concentration/age is only observed in *L. ramada* corresponding to the only species that spent the entire life cycle inside the estuarine system.

PCBs congeners

CBs 101, 118, 138, 149, 153, 170 and 180 are high congeners, and had higher contributed for PCBs contaminations in the three mullet species from the Mondego estuary. High chlorinated congeners are slowly eliminated metabolically (Stapleton *et al.*, 2001; Wu *et al.*, 2008), less volatile and more resistant to microbial degradation (Bazzanti *et al.*, 1997; Zhou *et al.*, 2001), therefore more persistent in the environment. So, once in the organism they tend to bioaccumulate, as they are not readily metabolized and excreted (Borga *et al.*, 2001).

The predominant congeners for the three mullet species were CB 138 and 153, which are, usually, the prevailing in biological samples (Antunes et al., 2007). Furthermore, these congeners were among the congeners that more contributed for the contamination of the three mullet species analyzed. According to Ulbrich and Stahlmann (2004) these congeners have great impact in humans. CBs 101, 118, 138 and 153 are toxic for humans, by increasing tumor promoting activity, oxidative stress, and also leading to DNA damage (Martabini et al., 2011).

CB 118 is the only congener analyzed that is considered a dioxin-like PCB, therefore having an assigned TEF-value (Toxic Equivalent Factor, indicating a relative dioxin-like activity) (Van den Berg *et al.*, 1998). CBs 118, 138 and 153 increased their concentration with age in *L. ramada*, due to the species ecology, lifespan and diet. On the other hand, these congeners in *L. aurata* and *C. labrosus* remain stable with age, also due to these species ecology.

Fishing, aquaculture and human impact

Mugilidae family has a great economic importance in many countries, such as Egypt (El-Halfawy et al., 2007), Italy or Greece (Hotos et al., 2002). Mullets suitable fishes for aquaculture purposes in brackish and freshwater ponds (Arruda et al., 1991). In Italy the aquaculture practice of these three species is very common, being used the cultured-based fisheries (valliculture) (www.fao.org).

L. ramada is the most common species captured for human consumption, due to its great nutritious value and as an alternative food resource (El-Halfawy et al., 2007), and can achieve high market prices (Hotos et al., 2002; Mousa et al., 2010). It is a very abundant species despite the massive fishing, though this species tends to accumulate PCBs during its lifespan, which can lead to possible health risks in high contaminated estuaries, due to its high PCBs concentration specifically in older/bigger individuals.

Length-frequency distributions are the first step in determining the numbers and sizes of different ages or year classes in the catch, and they are needed for an analytical assessment of a stock. They can also be used to establish the age structure of the population, fish growth, the age at which the fish become liable to capture, and how quickly the population is reduced as a result of fishing and natural mortality (www.fao.org). In Portugal the minimum size for mullet fishing is 20 cm (Diário da República nº 162 17-7-1987, anexo IV). With 20 cm *L. ramada* is older with 5 years and presented a concentration of around 10 ng g^{-1} (dry wt.), whereas *C. labrosus* and *L. aurata* are 2 years old, and presented concentration around 3 and 4 ng g^{-1} (dry wt.), respectively.

PCBs levels in food have been gradually decreasing, since environmental legislation on use and disposal of PCBs was introduce by the European Union (www.efsa.europa.eu). Though, human exposure by PCBs is mostly through food products (Marabini et al., 2011; Ulbrich and Stahmann, 2004). European Union has recommended a tolerance limit of 75 ng g^{-1} (wet wt.), for the sum of the 6 ecological indicators (IUPAC $\sum_{6ICES}$ 28, 52, 101, 138, 153 and 180), for fish muscle and fishery products (Commission Regulation (EU) No 1259/2011). In the Mondego estuary, *L. ramada* has the higher concentration of the three mullet species (Fig. 8). *L. ramada* concentration varied between 1 – 9 ng g^{-1} (wet wt.), whereas *L. aurata* is around 1 ng g^{-1} (wet wt.) and *C. labrosus* varied between 0.25 – 1 ng g^{-1} (wet wt.) (Fig. 8).

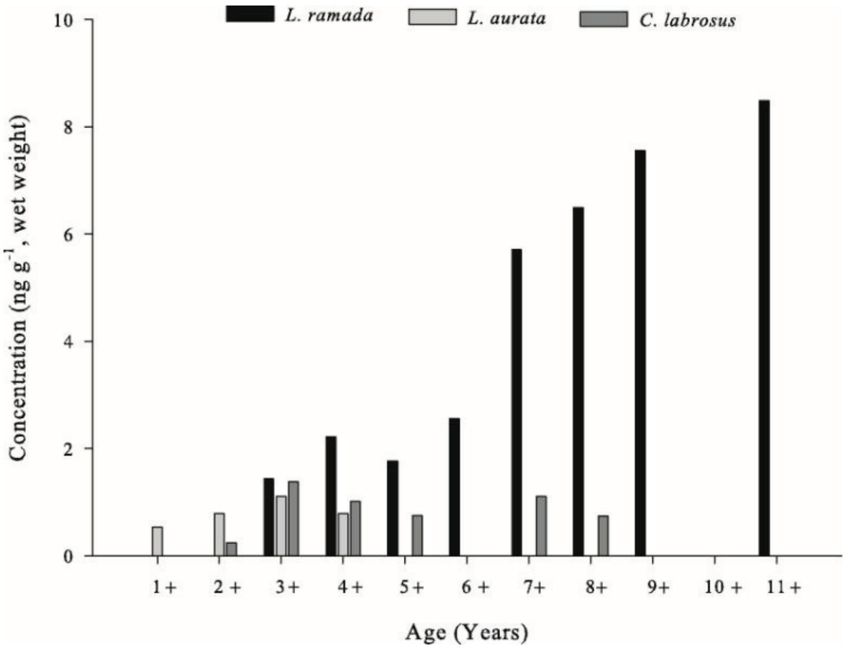

Figure 8. PCBs ecological indicators concentration in wet weight (ng g^{-1}, wet wt.) in *C. labrosus*, *L. aurata* and *L. ramada* along their lifespan

The human consumption of *L. ramada*, *L. aurata* and *C. labrosus*, and the implementation of aquaculture units in medium contaminated estuaries can be made safely, since these species presented values far below the concentration limit.

Conclusions

PCBs concentration was measured in three mullet species (*C. labrosus*, *L. aurata* and *L. ramada*) from the Mondego estuary. *L. ramada* presented higher concentration and its concentration increased along the lifespan. *C. labrosus* and *L. aurata* concentration remained stable with the lifespan. This dissimilarity in the PCBs accumulation in the different species emphasizes the importance of studying specific accumulation behavior in different species. The main factors that influenced PCB accumulation in the mullet species was the time spent in the estuary and probably their diet, rather than lipids. Higher chlorinated congeners contributed more for the accumulation in the mullet species, and the congeners with higher concentration were CB 138 ad 153.

Mullet species are often used in aquaculture, and can achieve high market prices. *L. ramada* is commonly used for human intake, due to its great nutritious value. European Union has recommended a tolerance limit for the ecological indicators of 75 ng g^{-1} (wet wt.). All the studied mullet species presented concentrations were below this tolerance limit.

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Influence of sex and age on PCBs accumulation in the commercial fish C*helon labrosus*

Abstract

Chelon labrosus is an important commercial species and has been studied worldwide. However, no recent studies have been made regarding polychlorinated biphenyls (PCBs) in wild *C. labrosus*. Due to that, the concentration of 13 PCBs congeners were measured in muscles and livers, of males and females, of *C. labrosus* of different ages, allowing the estimation of PCBs bioaccumulation along the lifespan, in a southern European temperate estuary. Male muscle samples concentrations ranged from 32 to 96 ng g^{-1} (lipid wt.) and in females from 32 to 62 ng g^{-1} (lipid wt.). In male liver samples concentration ranged from 106 to 158 ng g^{-1} (lipid wt.), while females concentration ranged from 88 to 129 ng g^{-1} (lipid wt.). The most abundant congeners presenting higher percentages in all samples were CB 138, 153 and 180. No significant differences were found between the concentrations in both sexes, but muscle and liver PCBs concentration in males tended to increase with age whereas in females remains stable along the lifespan. Significant differences were found between muscle and liver.

Additional keywords: PCBs, fish, bioaccumulation, *Chelon labrosus*, sex, age

Introduction

Polychlorinated biphenyls (PCBs) are environmental pollutants that tend to persist in the environment for a long period of time. Due to their lipophilic character they tend to accumulate in fishes (Howell et al., 2008; Nfon and Cousis, 2006). Fishes can accumulate PCBs from the surrounding environment (bioconcentration) (Antunes and Gil, 2004) or by prey intake (biomagnification) (Mackay and Fraser, 2000). The differences in the PCB congeners' pattern can demonstrate their distribution in the aquatic environment. High chlorinated congeners are the most persistent ones and with higher accumulative properties (Bazzanti et al., 1997; Bodin et al., 2008).

The Mondego estuary has been highly studied for many years, regarding its estuarine fish (Martinho et al., 2007), benthic (Cardoso et al., 2010) and plankton community (Primo et al., 2011). Concerning contaminants some studies have been made in mercury (Tavares et al., 2011), and only a few studies have been made in PCBs in fish and sediments (Nunes et al., 2011; Pereira et al., 2005). The main contamination sources of the Mondego estuary are wastewaters (most resulting from high population density, by domestic sewage) industrial activities (industrial sewage), agriculture runoff (which requires great amounts of fertilizers and pesticides) and Figueira da Foz harbour (responsible for industrial pressure in the estuarine area) (Nunes et al., 2011).

Thicklip grey mullet (*Chelon labrosus*) is a marine-estuarine opportunist species, since it enters the estuaries mainly as juveniles, as an alternative habitat (Baptista et al., 2010). This species is characterized by an omnivorous feeding (feeds mainly on benthic diatoms, epiphytic algae, small invertebrates and detritus) (Zouiten et al., 2008). More recently, the research in *C. labrosus* has increased due to its economic relevance and to its potential to be cultured in fish farms (Boglione et al., 2006; Khemis et al., 2006; Zouiten et al., 2008), moreover *C. labrosus* usually supports artisanal fisheries (Cardona et al., 2008). Since they are consumers of low trophic layers, they can be used in an economic and efficient way of extensive culture (Khemis et al., 2006). PCBs studies on this species are scarce, and only laboratory studies have been made (Narbonne, 1979). In fact, only the accumulation of a mixture of PCBs was considered and no field studies regarding bioaccumulation of specific congeners along the species lifespan have been developed so far. For that reason, the aim of this work was to study PCBs bioaccumulation according to age and sex in *C. labrosus*, in two different tissues (muscle and liver). Furthermore, it was expected to find higher concentration in liver than in muscle, differences in male and female bioaccumulation, and concentrations below the European Union tolerance limit for human intake. Accordingly, several hypothesis were raised: A) Does these contaminants bioaccumulate during *C. labrosus* life cycle?; B) Are there any differences among sexes concerning bioaccumulation?; C) Are there any differences in PCB congeners bioaccumulation on both sexes?; D) Is this species safe for human intake in low contaminated estuaries?

Material and Methods

Study site and laboratory work

The Mondego estuary is a small estuary (with 8.6 km^2), located in the western coast of Portugal (40º08’ N, 8º50’ W) (Fig.1). The estuary comprises two arms, the north and the south, separated 7 km from the shore and joining again near the mouth of the estuary.

Figure 1. The Mondego estuary and sampling stations location

The two arms present different hydrologic characteristics. The north arm is deeper with 5-10 m in high tide and 2-3 m tidal range being frequently dredged to maintain its depth, due to harbour activities. The south arm is shallower with 2-4 m depth in high tide and 1-3 m tidal range, and 75% are intertidal mudflats. The south arm is largely silted up in the upstream areas causing the freshwater to flow mainly through the north arm, while the water circulation in the south arm is mainly depending on the tides and on a small freshwater input from a tributary, the Pranto River, which is controlled by a sluice, according to the water needs of the rice fields from the Mondego agricultural valley.

Due to the fishes' life-cycle, juveniles are preferentially located in the inner areas of the estuarine system, and adults are located close to the mouth of the estuary. Fishes were collected from September 2007 to April 2008, in the south arm (mainly juveniles, age 2+) and at the Mouth of the Mondego estuary (adults, age ≥3+) (Fig.1). 3 individuals, from each age group and sex, were analysed for PCBs, given a total of 35 individuals were analysed. Fishing took place during low tide, using a traditional beach-seine net, to catch the younger fishes, and a trammel net to catch the older fishes. All fishes were weighted (g) and measured (cm) before collecting samples of muscles and livers. Sex was determined by examination of the gonads. Fishes were aged using scales analysis being all individuals <3+ considered immature (Table 1).

Table 1. C. labrosus mean size according to age

Age	Size (cm)
2+	19.30
3+	29.68 (±1.47)
4+	34.52 (±1.98)
5+	38.92 (±1.35)
6+	44.28 (±1.38)
7+	47.36 (±0.57)
8+	53.13 (±2.02)

For both sexes ages were estimated between 3+ and 8+. Immature muscle samples (2+) were also included in the analysis. From each age and sex, 3 individuals were stored and analysed for PCBs individually. Due to the lack of mass for the PCBs analysis, female liver age 5+ and 6+ were not determined. After laboratory processing fish samples were freeze-dried, homogenised and stored in the freezer (-20ºC), until the analytical procedure.

Sediments (0-5 cm depth) are composed mainly of mud and silt. They were collected in August of 2008, in the south arm and at the mouth of the estuary. After sampling sediments were taken into the laboratory, where they were freeze-dried, homogenised and stored at -20ºC until analytical procedure. For comparison with the European Union limits *C. labrosus* wet weight concentration was calculated using the formula: $\sum_6$ PCB concentration (dry wt.)]*(100 – water (%)).

PCBs analysis

The procedures for extraction and cleanup of fish tissues were adapted from US Environmental Protection Agency methods. A detailed description can be found as Electronic Supplementary Material. Briefly, 3 grams of muscle and 1g of liver of each individual were extracted by sonication with *n*-hexane:acetone (1:1) followed by a cleanup with sulphuric acid and solid phase extraction with florisil. Liver extracts were further cleaned with acid silica gel. Sediment (10 g) samples were Soxhlet-extracted, submitted to an alumina cleanup as described by Cachada et al. (2009), and an additional cleanup was performed with acid silica gel (as performed for liver samples).

Instrumental analyses of the extracts were performed by GC-MS with selected ion monitoring (details can be found in Supplementary Material). Thirteen congeners (IUPAC nos. 18, 28, 31, 44, 52, 101, 118, 138, 149, 153, 170, 180 and 194) according to EN 12766/CEN and EN 61619 were analysed in muscle, liver and sediments samples. Total PCB ($\sum_{13}$PCB) content (ng g^{-1}, lipid wt.) was based on the sum of the concentrations of the detected congeners.

For quality assurance and quality control of PCBs quantification methods, contamination was evaluated by blank controls and the recovery checked by analysis of spiked samples. Blanks levels were below detection limit and for the analyzed congeners mean recoveries in spiked samples ranged between 71-106%. Reproducibility was calculated on replicate analysis giving an overall error of 4-20%.

Statistical analysis

A linear regression (SigmaPlot 11.0 software) analysis was performed to determine the tendency of males and females PCBs concentration for $\sum_{13PCB}$. To evaluate differences within males and females, for both muscle and liver, was performed a one-way ANOVA. No transformation was applied to the data, since all values had a normal distribution. A 5% significance level was used for all the analysis.

Results and Discussion

Sediment samples from the Mouth station (M station) presented higher $\sum_{13}$ PCB concentration, with 2.0 ng g^{-1} (dw), than the south arm, with 0.28 ng g^{-1} (dw). Moreover, at M station were detected nine congeners, whereas in the south arm were only detected two congeners (Fig. 2A).

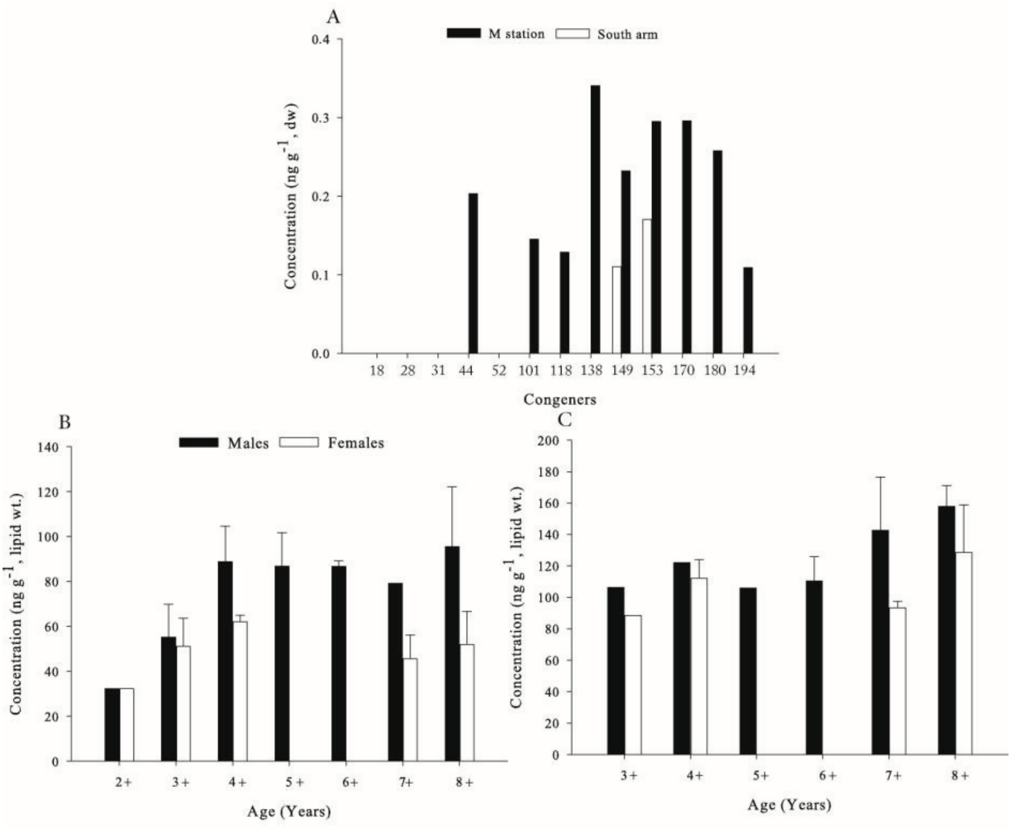

Figure 2. PCB congeners concentration (ng g^{-1}, dry wt.) in sediments (A); and mean $\sum_{13}$ PCB (ng g^{-1}, lipid wt.) concentration according to age and gender in muscle (B) and liver (C). Error bars represent the standard error.

This pattern results from the higher urban pressure in this station, including the proximity of a boat touristic marina only 100m away. Studies performed in the Mondego estuary by Pereira et al. (2005) and Vale et al. (2002) are in accordance with the present results. Sediment concentration from the Mondego estuary is low when compared with others estuaries, eg. Sado Estuary (Portugal), with 87 to 1100 ng g^{-1}, dw (Costa et al., 2008), or Er-Jen Estuary (Taiwan), with 5 to 65 ng g^{-1}, dw (Fu and Wu, 2006).

In the Mondego estuary *C. labrosus* size varied between 19 and 53 cm (Table 1). Male muscle $\sum_{13}$ PCB concentrations varied between 32–96 ng g^{-1} (lipid wt.), while in females muscle varied between 32–62 ng g^{-1} (lipid wt.) (Fig. 2B). According to the one-way ANOVA male and female muscle concentration did not present significant differences ($p=0.268$). Nevertheless, male muscle increased its concentration with age, and significant differences were found along the species lifespan ($r^2=0.634$; $p=0.032$). On the other hand, female muscle concentration remained constant ($r^2=0.094$; $p=0.617$). Differences in female and male accumulation can be explained by differences in their gross growth efficiency. If one sex requires more energy (food intake) than the other sex to attain the same size, then the sex requiring more energy would have higher PCBs concentration (Madenjian, 2011). Another explanation, and the most common, is that females can lose a portion of their PCBs body burden by releasing their eggs. Depuration systems can be observed in other species, such as in largemouth bass (*Micropterus salmoides*) (Rypel et al. 2007), hake

(*Merluccius merluccius*) (Bodiguel et al., 2009) or sea bass (*Dicentrarchus labrax*) (Loizeau et al., 2001).

Regarding liver samples, male $\sum_{13}$ PCB concentration varied between 106–158 ng g^{-1} (lipid wt.), while female $\sum_{13}$ PCB concentration varied between 88–129 ng g^{-1} (lipid wt.) (Fig. 2C). According to the one-way ANOVA no differences were found between both sexes (p=0.707). Both male and female concentration remained constant along the species lifespan (r^2=0.646, p=0.054; r^2=0.300, p=0.452, respectively), although males' concentration had a tendency to increase with age (Fig 2C).

Muscle and liver concentrations presented significant differences (p=0.011, for males; p=0.021 for females). Liver presented higher concentration since is the main organ for PCBs storage and metabolism (Bodiguel et al., 2009; Fernandes et al., 2008), also this organ presented higher lipid content than the muscle (Table 2). In males muscle lipids varied between 2–17%, while in liver varied between 21–35% (Table 1). In females muscle lipids varied between 2–10%, while liver varied between 17–31% (Table 2).

For all samples the most abundant congeners were CB 138, 153 and 180 (Table 2). High chlorinated congeners are the most prevalent and significant in environmental samples (Bazzanti et al., 1997; Fernandes et al., 2008). Similar results were found in sea bass (Antunes and Gil, 2004), sardines (Antunes et al., 2007; Coelhan et al., 2006) and hake (Bodiguel et al., 2009).

Human exposure occurs mostly through contaminated food. *C. labrosus* is a suitable species for aquaculture, therefore is part of the human diet (Boglione et al., 2006; Zouiten et al., 2008). Recently the European Union has recommended a tolerance limit of 75 ng g^{-1} (wet wt.) for fish muscle and fishery products, and 200 ng g^{-1} (wet wt.) for fish liver, for the sum of the 6 ecological indicators ($\sum_6$ PCB 28, 52, 101, 138, 153 and 180) (Commission Regulation (EU) No 1259/2011). *C. labrosus* muscle and liver $\sum_{6PCB}$ concentration are far below the respective tolerance limit. Male muscle concentration varied between 1.7–3.3 ng g^{-1} (wet wt.) and female muscle was around 1.1–2.4 ng g^{-1} (wet wt.). Male liver values varied between 7.2–13.4 ng g^{-1} (wet wt.), while values in female liver varied between 5.8–9.0 ng g^{-1}(wet wt.). Therefore, the intake of *C. labrosus* from the Mondego estuary can be considered safe for human intake, regarding PCBs.

The Mondego estuary is a poorly contaminated system, with few contamination sources. Contrarily to expectations, no statistical differences were found between PCBs concentration of each sex for each tissue. Nevertheless, male *C. labrosus* increased its PCBs concentration with age, whereas female concentration tended to remain stable. Despite the fact that male liver did not present significant differences along the species lifespan, PCBs concentrations tended to increase with age. As expected, liver presented higher concentration than muscle due to its high lipid content. High chlorinated congeners (CB 138, 153 and 180) presented higher concentration. The Mondego estuary can be a useful site for the study and calibration of PCBs uptake. Moreover, *C. labrosus* concentration is below the tolerance limit, therefore safe for human intake.

Table 2. *C. labrosus* congeners (%), $\sum_{13}$ PCB concentration (ng g^{-1}, lipid wt.) and lipid content (%), for male and female, muscle and liver according to age.

		18	28	31	44	52	101	118	138	149	153	170	180	194	$\sum_{13}$PCB (	Lipid (%)
Age										**Muscle**						
2+	**Male**								40.3	29.2	30.5				32.3	3.25
	Female								40.3	29.2	30.5				32.3	3.25
3+	**Male**					4.5	5.1	7.7	20.6	9.4	28.5	11.9	12.3		55.2	4.15
	Female						6.4	8.0	31.4	13.3	29.1	3.7	8.1		51.1	7.83
4+	**Male**	3.0	4.5	3.8	2.7	7.3	6.8	7.4	18.4	7.6	21.7	8.7	8.1		88.8	2.44
	Female	1.2			1.6	5.7	18.4	4.2	18.3	10.4	21.9		14.6	3.7	62.0	2.49
5+	**Male**			1.2	1.4	3.6	8.2	6.7	22.8	10.4	26.8	8.3	10.6		86.7	4.28
	Female															
6+	**Male**	3.6	1.6	1.8	0.8	2.4	5.3	1.8	25.2	10.4	28.4	4.4	13.7	0.6	86.7	9.95
	Female															
7+	**Male**	0.3		0.8	2.6	7.1	8.5	7.0	19.9	9.4	26.0	7.1	10.9	0.4	79.1	6.80
	Female	3.5	3.5		1.2	5.0	6.6	5.6	20.9	11.7	26.8	2.7	10.6	1.9	45.6	6.86
8+	**Male**		0.8		1.6	4.5	11.0	9.5	22.2	10.7	25.4	4.4	9.9		95.5	5.59
	Female	1.4			5.3	9.2	8.0	11.2	17.5	6.8	23.7	6.3	10.6		51.9	10.26
										Liver						
3+	**Male**	1.0	4.9	3.4	0.2	4.5	7.5	4.7	20.0	12.8	24.9	2.8	13.1	0.2	106.3	28.33
	Female	3.7	1.2	4.2	9.2	6.9	13.7	2.7	7.9	5.5	34.8		10.2		88.4	20.89
4+	**Male**	4.6	5.5	4.4	1.0	5.5	8.4	7.7	12.3	10.9	17.3	12.3	8.7	1.4	122.2	33.45
	Female	1.8	6.5	4.3	6.5	8.2	2.3	4.1	16.7	14.0	27.0	5.9	2.7		112.2	16.52
5+	**Male**		4.7	3.2	2.1	3.5	7.7	5.5	16.7	10.3	27.2	8.4	9.2	1.3	106.1	24.75
	Female															

6+	**Male**	2.1	3.5	2.9	3.7	3.2	2.8	3.2	19.3	11.0	30.5	3.3	10.7	3.8	110.4	35.36
	Female															
7+	**Male**	2.4	5.5	3.7	3.5	5.3	8.2	3.5	11.5	9.3	26.9	9.2	8.8	2.2	142.6	24.19
	Female	1.1	3.1	1.1	1.7	5.6	8.7	9.3	18.8	15.1	25.4	3.6	6.5		93.4	34.95
8+	**Male**		0.6			1.0	5.9	7.0	28.1	12.1	35.7	2.7	6.9		157.9	20.90
	Female	0.1	9.3	12.0	1.3	5.1	5.4	8.0	11.0	9.9	22.6	3.0	8.5	3.8	128.5	29.84

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Chapter 4

Organochlorine contaminants in different tissues from *Platichthys flesus* (Pleuronectidea, Pisces)

Abstract

Polychlorinated biphenyls (PCBs) and hexachlorobenzene (HCB) are organic contaminants that tend to accumulate in fish. Estuaries are exposed to high anthropogenic activities, therefore tending to accumulate more contaminants than the adjacent coastal waters. *P. flesus*, a fish with high economic value, was analysed for PCBs and HCB along its lifespan, in liver, gills, gonads and muscle. Younger fishes were caught in the estuary, whereas older fishes were caught in the adjacent coastal waters and acquired at Figueira da Foz Regional Office of Docapesca-Portos e Lotas, SA. Both contaminants concentrations follow the pattern: liver > gills > muscle. Hepatosomatic index had the lowest values in younger fishes and the highest values in older fishes. Condition factor values remain stable with age. Younger fishes had higher PCBs concentrations than the older fishes. In opposition HCB was only detected in fish from 3+ to 5+ and its concentration tended to increase with age. Overall *P. flesus* is considered safe for human intake.

Additional keywords: *P. flesus*, PCBs, HCB, estuary, fish market, Condition factor

Introduction

Organochlorine contaminants are very persistent in the environment, and tend to accumulate in organisms, due to their lipophilic characteristics, resistance to chemical and metabolic degradation. Organic contaminants toxic characteristics can pose risks for environment and human health (Vallack et al., 1998). Human exposure occurs mainly from environmental contamination of food products (Ulbrich and Stahlmann, 2004). Hexachlorobenzene (HCB) was widely used as a fungicide. Due to its persistence in the environment, it was banned globally. In the European Union HCB was banned in 1978, in Australia in 1972 and in USA in 1984 (Barber et al., 2005). Nevertheless, HCB is still present in the environment as an unintended by-product in chemical processes, incomplete combustion and an impurity in pesticides (Barber et al., 2005). Polychlorinated biphenyls (PCBs) are synthetic organic chemicals, highly stable, hydrophobic and persistent in the environment (Eichinger et al., 2010). PCBs tend to accumulate in organisms, due to their lipophilic properties (Eichinger et al., 2010). PCBs were introduced in the environment in the 1920s, and due to its high persistence in the environment were banned in many countries in 1970s (Bodiguel et al., 2009).

Aquatic ecosystems act as sinks for many organic and inorganic pollutants, resulting from anthropogenic activities. Estuaries are exposed to these activities through urban and industrial development and intensive agriculture (Vasconcelos et al., 2007). Due to the increasing quantities of nutrients and organic material, estuaries accumulate higher quantities of organic contaminants than the adjacent coastal waters. In general, open coastal habitats are less contaminated, due to their high energy climate and more remote settings (Parnell et al., 2008).

Fish species are very important for the aquatic food webs and human intake. For this reason, fishes are widely used to evaluate the aquatic ecosystems health. The European flounder (*Platichthys flesus*; Pleuronectidea, Pisces) is a demersal fish, with a wide distribution (from Norway to Portugal, Mediterranean, Baltic, North White and Black seas) (Morais et al., 2011), being present in both coastal waters and estuarine ecosystems (Vasconcelos et al., 2008). This species is among the most important components of estuarine fish assemblages in temperate latitudes (Franco et al., 2008; Martinho et al., 2010). They reproduce in offshore waters and use estuaries as nursery areas or temporary habitats (alternative to coastal areas), because it increases their chances of survival (Martinho et al., 2010). *P. flesus* is a highly exploited commercial species, but can be particularly vulnerable to chronic and accidental pollution, due their benthic lifestyle and to the fact that nurseries are located in estuarine areas (Eichinger et al., 2010). *P. flesus* was chosen for this study due to its easy sampling and high ecological and economic relevance. Furthermore, PCBs and HCB exposure to humans occurs mainly by environmental contamination of food products (Ulbrich and Stahlmann, 2004), highlighting the importance to know if the consumed fish is appropriated for human intake and if fish size is relevant for risks associated with consumption. *P. flesus* is a widely studied species regarding PCBs (Ferreira et al., 2004; Goerke and Weber, 2001), but HCB was rarely addressed (Kleinkauf et al., 2004). Regarding PCBs some studies were performed in wild animals (Ferreira et al., 2004), but many studies were performed in laboratory, in order to provide information on bioaccumulation and elimination (Goerke and Weber, 2001) or disease induction (Grinwis et al., 2000). No studies of accumulation of these organochlorine contaminants along the species lifespan were performed so far. The aim of this work was to study the bioaccumulation along the lifespan of PCBs and HCB in *P. flesus* from the Mondego estuary and adjacent coastal area. *P. flesus* contamination was measure in four different organs, muscle, liver, gonads and gills, along the species lifespan. So, some questions can be posed: Do HCB and PCBs bioaccumulate along the species lifespan?; Are these patterns similar?; Are there differences in the concentration of the different tissues?; Does the contamination present in the Mondego estuary and adjacent coastal waters pose a risk for *P. flesus* general health? Does the intake of *P. flesus* represent a risk for human health?

Material and Methods

Study site

The Mondego estuary is a small estuary (8.6 Km^2), located in the western coast of Portugal (40º08’N, 8º50’W) (Fig.1).

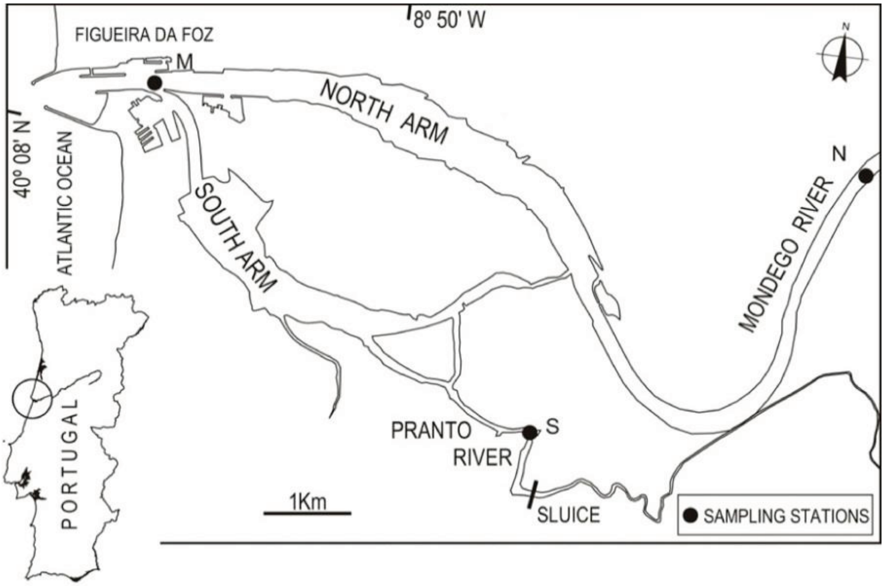

Figure 1. Mondego estuary sampling sites location.

It comprises two arms, the north and the south arm, separated about 7 Km from shore, joining again near the mouth of the estuary. The two arms have distinct hydrological characteristics. The north arm is deeper with 5-10 m at high tide and 1-3 m tidal range, being dredged frequently to maintain its depth, since is the main navigation channel. The south arm is shallower with 2-4 m depth during high tide and 1-3 m tidal range. The south arm is largely silted up in the upstream areas causing the water to flow mainly through the north arm. The water circulation in the south arm is mainly dependent on tides and on a small freshwater input from the Pranto River, which is controlled by a sluice, according to the water needs of the rice fields of the Mondego agricultural valley.

Fish sampling and laboratory work

P. flesus studied analysed was aged from 0+ to 5+ (Table 1). Younger fishes (0+ to 2+) were caught in the Mondego estuary, in November and December 2009. Fishing took place during the night and during high tide at 3 sampling stations (M, N and S) (Fig. 1). During the fishing was used a 2m beam trawl, with 5mm stretched mesh size in the cod end. Each survey consisted of three five-minute hauls at each sampling station. Older fishes (3+ to 5+) were acquired at Figueira da Foz Regional Office of Docapesca-Portos e Lotas, SA, the institution in charge of the first fish sales along the Portuguese mainland.

Table 1. Medium size (cm) and medium weight (g) variation according to age (years), standard deviation between brackets.

Age (years)	Size (cm)	Weight (g)
0+	12.85 (1.50)	20.17 (6.27)
1+	17.38 (1.68)	51.63 (5,86)
2+	24.01 (2.24)	150.35 (41.46)
3+	27.33 (0.55)	226.65 (32.05)
4+	29.85 (1.90)	336.76 (103.81)
5+	33.53 (0.96)	427.63 (54.54)

All fishes were taken into the laboratory, where they were measured (cm), and weighted (g), before collecting the muscles, livers, gonads and gills. Sex was determined by visualization of the gonads, which were only removed from females. Three individual samples from each tissue and age were stored and analysed for PCBs and HCB, given a total of 74 samples analysed.

The samples were freeze-dried, homogenized and stored in freezer (-20ºC), until analytical procedure. Due to the lack of mass, in juvenile samples (0+) were used composite samples for analysis, ranging from 3 to 23 individuals depending on the necessary mass.

PCBs and HCB analysis

For the analysis was used 3 g of muscles, 1 g of liver, 1.5 g of gonads and 2 g of gills. The samples were extracted by sonication (Branson 3510) with a *n*-hexane:acetone (1:1) mixture. The extract was decanted and the process repeated three times. The extract volume was reduced by solvent evaporation using a rotavapor.

The content in lipids was gravimetrically determined using 10% of the extract. The remaining (90%) volume of the extract was reduced by solvent evaporation under a gentle stream of nitrogen and lipids were removed with sulphuric acid (97%). Afterwards, the lipid-free extract passed through a multilayered column packed with florisil (Supelclean® Florisil) and anhydrous sodium sulphate, for muscle and gills, and acid silic and florisil (Supelclean® Florisil), for liver, and PCBs eluted with hexane. Samples were dried, under a gentle stream of nitrogen. A known mixture of PCBs congeners (CB 34, 62, 119, 131 and 173) were used as internal standards (Ayris et al., 1997), added to the dried extracts, and diluted with iso-octane to a final volume 200µL. Samples were analysed on a gas chromatograph equipped with a MDN-12 silica capillary column (30 m; 0.25 mm i.d.; 0.25 µm film thickness) coupled to a mass spectrometry detector (GCMS-QP5050A, Shimadzu) using electron impact ionization and selected ion monitoring (SIM) acquisition. Helium was employed as the carrier gas, and samples were injected (1 μL) in splitless mode with column temperature at 40ºC and held for 2 minutes, then programmed to ramp 15ºC/min to 180ºC, and held for 1 minute, 8ºC/min to 300ºC and held for 15 minutes (injector temperature = 280ºC; interface temperature = 300ºC). Total PCBs content (ng g^{-1}, lipid weight) was based on the summed concentrations of 6 ecological indicators, considered by the European Union to assess marine pollution ($\sum_{6PCB}$ 28, 52, 101, 138, 153, and 180), and CB 118 ($\sum_{tPCBs}$).

For quality assurance and quality control of the PCBs quantification method, contamination was evaluated by blank controls and results were always below detection limit. The recoveries of the analytical method for the analysed congeners were tested by analysis of spike samples and results ranged between 71-106%. Reproducibility was calculated on replicate analysis giving an overall variability of 4-20%. The detection limits for individual PCBs ranged between 0.1 and 1.0 ng g^{-1}.

Data analysis

To detect differences in $\sum_{tPCBs}$ concentrations a potential tendency line was performed for each tissue along the species lifespan, obtaining the R^2 values.

In order to detect differences among the lipid percentage for each tissue a Tukey test (SigmaPlot 11.0) was also performed.

In order to detect differences in HCB concentration in the different tissues a Kruskal-Wallis one-way analysis of variance on ranks was performed (SigmaPlot 11.0 software). An All Pairwise Multiple Comparison, Dunn's method, was performed to detect differences between each tissue. Log transformation was applied to the data. A linear regression was performed on each tissue to detect differences in HCB concentration along the lifespan.

A one-way ANOVA (SigmaPlot 11.0) was performed in the PCBs congeners for each age in order to detect differences between the different tissues for each congener. No transformation was applied to the data, since all value had a normal distribution.

A decrease in fish general health can affect the normal physiology. Therefore, the Hepatosomatic Index (HSI) was calculated from the liver weight of the fish in relation to its body weight, using the equation:

$$\mathrm{HSI} = \frac{Liver\ weight}{Total\ body\ weight} * 100$$

A linear regression (SigmaPlot 11.0) was performed with HSI values in order to detect differences among them. The condition factor was calculated from the weight of the fish in relation to its length, and serves as an indicator of growth, nutritional state and energy content of the fish. The condition factor was calculated using the equation:

$$CF = \frac{Total\ body\ weight}{(body\ length)^3} * 100$$

A 5% significance level was used for all the analysis.

Results

PCBs concentrations along P. flesus lifespan

PCBs concentrations were measured along *P. flesus* lifespan. Muscle samples had the lowest concentrations, ranging from 28 to 553 ng g^{-1}, lipid wt. (Fig. 2), whereas liver had the highest concentrations ranging from 75 to 5548 ng g^{-1}, lipid wt. (Fig. 2). Gills concentrations ranged from 79 to 587 ng g^{-1}, lipid wt. (Fig. 2). For all tissues PCBs concentrations decreases with age (R^2 = 0.95, for muscle; R^2 = 0.98, for liver; R^2 = 0.99, for gills) (Fig. 2).

Table 2. Hepatosomatic index, condition factor and lipid (%), in muscle, liver, gills and gonads according to age.

Age	**Hepatosomatic Index**	**Condition Factor**	**Lipids (%)**			
			Muscle	**Liver**	**Gills**	**Gonads**
0+	0.48	1.18	0.87	18.16		
1+	0.26	1.03	1.01	15.97	3.26	
2+	0.98	1.03		20.69	8.46	
3+	1.07	1.11	2.21	19.28	9.87	
4+	1.66	1.25	2.70	19.97	11.92	1.41
5+	1.54	1.13	2.79	24.11	13.56	4.15

Gonads concentration was measured in ages 4+ and 5+, being 70 ng g^{-1} and 23 ng g^{-1}, lipid wt., respectively.Liver had the highest lipid content, with 16 to 24%, muscle had low lipid content, with 0.87 to 2.8% (Table 2). Gills tended to increase their lipid content with age, varying from 3.3 to 14% (Table 2). Statistical differences were found in the lipid content amongst the 3 tissues ($p < 0.001$).

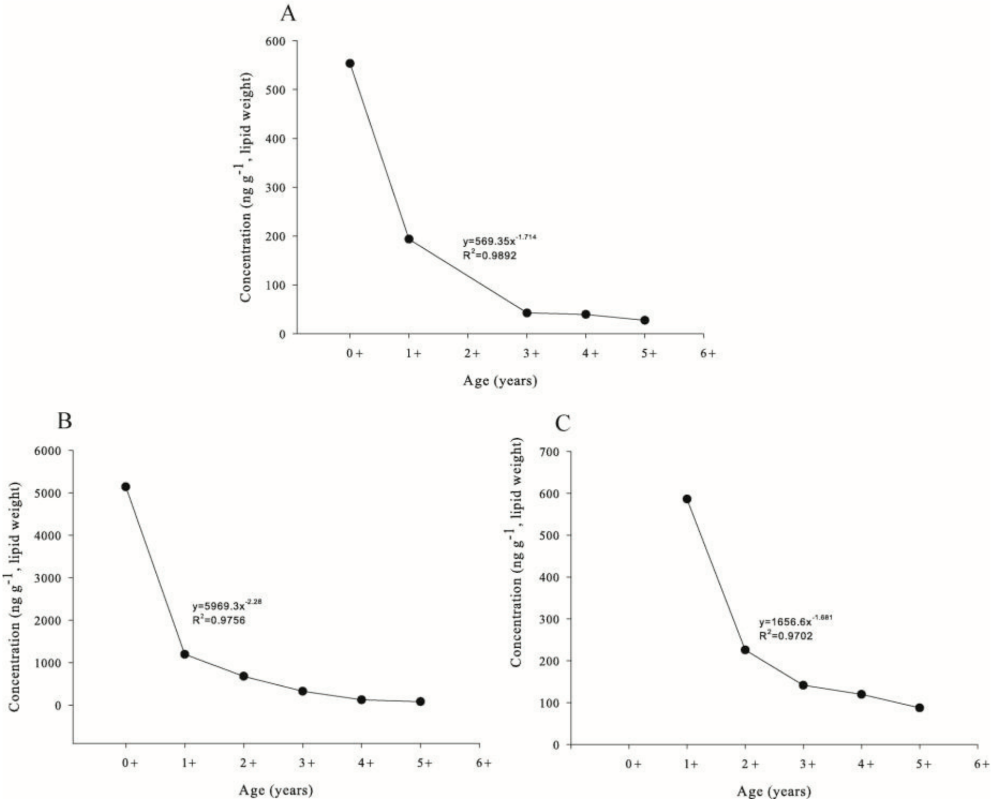

Figure 2. $\sum_{tPCB}$ muscle concentration (ng g^{-1}, lipid wt.) in the estuarine and marine environment, for muscle (A), liver (B) and gills (C).

HCB concentration along P. flesus lifespan

HCB concentration was only detected in individuals older than 3+ (Table 3). The liver had the highest concentration, whereas the muscle had the lowest

(Table 3). HCB concentration tended to increase with age for muscle, liver and gills ($R^2 = 0.998$; $R^2 = 0.9753$; $R^2 = 0.829$, respectively).

A Kruskal-Wallis one-way analysis of variance on ranks was performed to detect differences among the three tissues. Dunn's method detected significant differences were found between liver and muscle, and between gills and muscle (post hoc test, $p < 0.05$). No significant differences were found between liver and gills (post hoc test, $p > 0.05$).

Table 3. HCB concentration (ng g^{-1}, lipid wt.) according to age.

	3+	**4+**	**5+**
Muscle	2.16	2.75	3.43
Gills	6.22	6.45	8.42
Liver	5.90	7.96	9.13

PCB congeners' pattern

All PCB congeners tend to decrease their concentration with age (Fig. 3). Less chlorinated congeners have lower concentration than higher chlorinated congeners, being CB 138 and 153 the most abundant (Fig. 3).

Although, CB 138 and 153 are high chlorinated congeners their concentration decreases faster, in the first two years, than the other congeners.

Liver has higher individual congeners' concentration among the three tissues. The congeners' concentrations in the tissues follow the pattern: liver > gills > muscle.

A one-way ANOVA was performed to detect differences in each tissue for each congener. High chlorinated congeners, CB 118, 138, 153 and 180, presented significant differences between the three tissues, for each age.

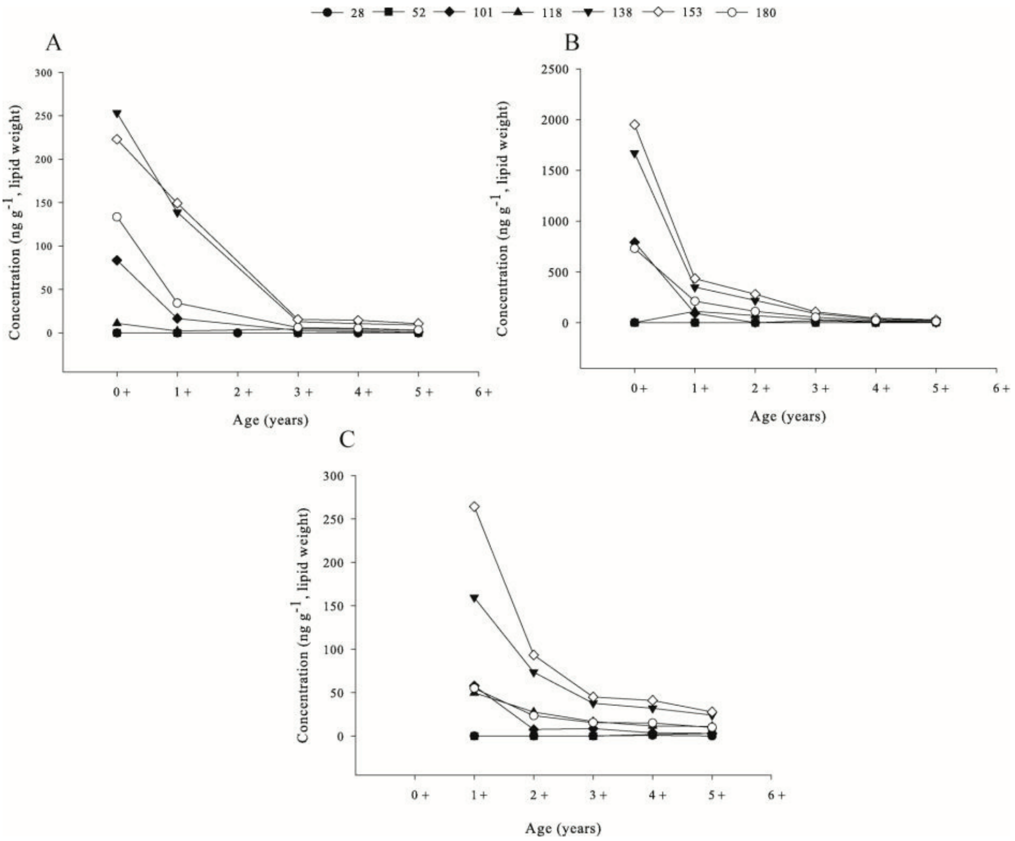

Figure 3. PCB congener analysis in the muscle (A), liver (B) and gills (C).

Physiological conditions of P. flesus along its lifespan

The Hepatosomatic index (HSI) and the condition factor can give valuable information about the fish general health, and can be influenced by environmental contaminants. HSI values were measured for each age. Lower values were associated with younger individuals and higher PCBs concentrations. Estuarine individuals, ages 0+ and 1+, had lower HSI values than marine individuals, ages 2+ to 5+ (Table 2). Significant differences were found in the HSI values with age ($p = 0.009$). The condition factor was measured for each age, and the values did not vary along the species lifespan, ranging from 1.03 to 1.25 (table 2), and no significant differences were found in the condition factor along the species lifespan ($p = 0.564$).

Discussion

PCBs contaminations along P. flesus lifespan

Estuaries are sinks for many chemical contaminants due to their proximity to urbanized areas (Gravato et al., 2010). *P. flesus* is a marine estuarine-dependent species, using estuaries as a nursery ground. Younger individuals, age 0+, tend to move to the upstream areas, where the salinity values are lower. As they mature (age 1+), they move towards the downstream areas of the estuary. At age 2+, mature individuals move to the adjacent coastal waters, where they will continue their lifecycle. After spawning, *P. flesus* larvae move again to the upstream areas of the estuary, completing the lifecycle (Fig. 4).

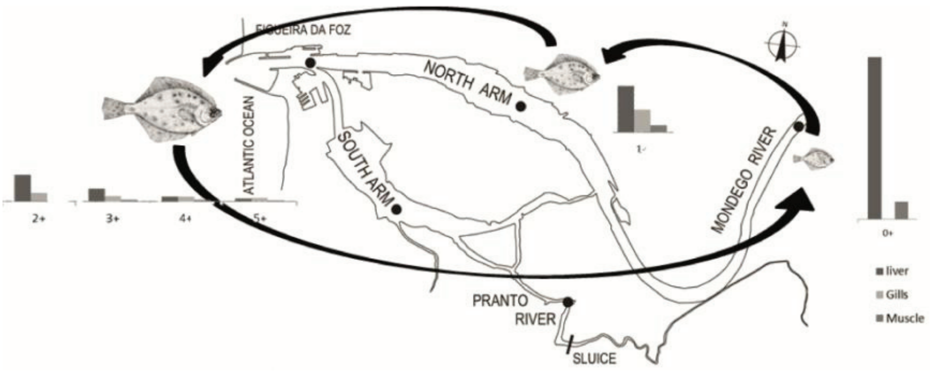

Figure 4. *P. flesus* lifecycle in the Mondego estuary and the $\sum_{tPCB}$ concentration, for liver, gills and muscle

The juvenile stages (from the estuarine system) had higher $\sum_{tPCBs}$ concentrations, than the mature stages (from the adjacent coastal waters). Class 0+ presented the highest concentration, being this class located in the most upstream areas of the estuary near the Mondego agricultural valley. Mature fishes have lower concentration, since the marine environment is less contaminated, due to their remote setting (Parnell et al., 2008) (Fig. 4). $\sum_{tPCBs}$ concentrations decreased with *P. flesus* lifespan. This can also be associated to an increase in metabolic activity and growth dilution (Pastor et al., 1996). Growth dilution can be defined as biomass-specific concentration of pollutants diminishes by accumulation of new biomass, causing lower concentrations in faster growing individuals (Pickhardt et al., 2002). Growth dilution was observed in other species, such as in the Northern pike (*Esox lucius*), for mercury contamination (Verta, 1990). The migration pattern of *P. flesus* from a more contaminated to a less contaminated environment and growth dilution may not be the only factors associated to its decontamination. *P. flesus* can, also, eliminate PCBs by metabolic conversion (Mackay and Fraser, 2000), by excretion of parent compounds or by biotransformation, it is also capable of oxidative transformation and excretion (Eichinger et al., 2010), therefore decreasing its body burden.

The high concentration from class 0+ can be attributed to many factors, such as gonadal pre-contamination, contaminating the recently hatched *P. flesus*. As observed in other species, such as largemouth bass (*Micropterus salmoides*) (Rypel et al., 2007) or sea bass (*Dicentrarchus labrax*) (Loizeau et al., 2001), reproduction can be used as a depuration system. Furthermore, class 0+ inhabits at the more upstream areas of the estuary near the Mondego rice fields, which can be a possible contamination source of the estuarine system.

Liver presented higher $\sum_{tPCBs}$ concentrations than gills or muscles. The liver is the main organ for PCB storage (Bodiguel et al., 2009), and has higher lipid content. PCBs are very lipophilic and are more prone to accumulate in liver. Nevertheless, gills are associated with higher concentration, as well. Gills can accumulate PCBs by bioconcentration, since they are in direct contact with the surrounding environment (Björk and Gilek, 1997) presenting a large surface area per gram. Moreover, gills are continually transferring organic pollutants from both water and suspended particles into its surface (Yang et al., 2007).

Muscle low contamination can be attributed to its low lipid content. Gonads contamination can be attributed to its lipid content and a dislocation of the contamination from the muscle to the gonads. According to Bodiguel et al. (2009), muscle is the major contributor for gonadal contamination.

PCB congeners in P. flesus

PCB congeners' concentrations in *P. flesus* decrease with age in all tissues. Congeners can be eliminated by excretion and/or by biotransformation (Eichinger et al., 2010), and are only excreted after they are metabolized (Goerke and Weber, 2001).

The higher rate of decontamination is observed in the first years, 0+ and 1+. In these first years CB 138 and 153 concentrations decrease at a higher rate than the other congeners. CB 153 can decrease exponentially in function of elimination time, as demonstrated by Goerke and Weber (2001), which is in accordance with our results. Furthermore, *P. flesus* is able to eliminate this congener by oxidative transformation and excretion (Goerke and Weber, 2001). Antunes et al. (2007) has demonstrated a decrease in CB 153 in *Mugil Cephalus*, after providing unspiked food. The decrease in the congeners' concentration can also be attributed to the dislocation of this species into a less contaminated environment.

The congeners that more contributed to the differences in concentration among the three tissues were the high chlorinated congeners, CB 118, 138, 153 and 180. These differences can be explained by the fact that high chlorinated congeners are more slowly degraded metabolically (Wu et al., 2008), have higher accumulative properties, and therefore are more persistent in the environment and in the biological tissues (Bodin et al., 2008).

HCB in P. flesus lifespan

HCB has a long-half life in water and sediment and therefore is extremely persistent in the environment (Barber et al., 2005). HCB can be transported for great distances in the atmosphere before removal, deposition or degradation (Barber et al., 2005).

The muscle is the organ with lower HCB concentration, whereas the liver and gills have higher concentration. The liver is the main organ for contaminants storage and has higher lipid content, followed by the gills. Gills presented higher concentration, due to its larger surface and by the continuously transfer of organic pollutants from both water and suspended particles onto its surface (bioconcentration) (Björk and Gilek, 1997; Yang et al., 2007).

During *P. flesus* lifespan is observed an increase in HCB concentration. HCB residues were only detected at age class 3+, 4+ and 5+. *P. flesus* from the present study presented lower concentration than *P. flesus* found in the Baltic Sea (Szlinder-Richert et al., 2008). Estuaries are known to have higher HCB concentration than the adjacent open waters nearby (Barber et al., 2005). So it is possible to assume that generally, *P. flesus* contamination is derived from the estuaries rather than the sea. According to Vasconcelos et al (2008) the Douro estuary (located 80 Km north) has higher contribution (53.3%) for the fish stock caught in the coastal area of Figueira da Foz, while the Mondego estuary only contributed with 23%. HCB contamination detected in older *P. flesus* could be due to a contamination already present in fishes from the Douro estuary (Ferreira et al., 2004).

HCB contamination in *P. flesus* increased along the species lifespan, whereas PCBs concentrations decreased. PCBs are metabolized primarily in the liver by the enzyme cytochrome P450 1A (Ferreira et al., 2004). In fishes this enzyme increases substantially after an exposure to organic contaminants (Collier et al., 1998). Although, this enzyme is important for PCBs detoxification, HCB is not an inductor of cytochrome P450 1A (Mundy et al., 2012). So, the differences in the contaminants accumulation along *P. flesus* lifespan can be due to differences in detoxification processes. HCB detoxification may not be as efficient as PCB detoxification.

P. flesus physiological conditions

HSI can give valuable information about the fish general health, and can be influenced by chemical contamination (Güngördü et al., 2012). There is some discrepancy in the literature regarding the interpretation of the decrease or increase in HSI values in fish (Güngördü et al., 2012). Some authors pointed out that exposure to pollutants may cause liver dysfunction capable of interfering with normal development, increasing HSI values (Kleinkauf et al., 2004). Though, exposure to PAHs or heavy metals results in a decrease of HSI values (Yang and Baumann 2006). Accordingly, our results show lower values of HSI with higher concentration of PCBs. But, these results may be interpreted with caution, because multiple factors, other than contaminants, can cause changes in the liver size (Yang and Baumann, 2006). Among these factors can be included sampling season, nutrition or disease (Yang and Baumann, 2006). HCB was only detected in mature individuals, therefore did not interfere in HSI of younger fish. Moreover, despite the opposite tendencies observed in PCBs and HCB the overall concentrations found were low. Accordingly HCB and PCBs contamination at these levels may not be the main influence in HSI values.

A decrease in the general health of *P. flesus* can affect their normal physiology and induce adverse changes. The condition factor can give important information on the physiological state of the fish. *P. flesus* general health can be indicative of their reproductive potential and the fitness of their offspring (Kleinkauf et al., 2004). Multiple factors can influence the condition factor, such as food availability, parasites and chemical contamination (Güngördü et al., 2012). In the Mondego estuary and adjacent coastal water the condition factor is similar at all ages, and it is, also, similar to those found by Kleinkauf et al. (2004), also for *P. flesus*. The relationship of weight and length remain constant throughout *P. flesus* lifespan, and the condition factor also remains constant along the species lifespan. Therefore, PCBs and HCB contamination, at these levels, does not seem to affect the condition factor.

P. flesus economical value and human intake

P. flesus is a commercial species, with great economic importance being consumed in many countries such as United Kingdom, France, Spain, Greece or Portugal. According to FAO fishery statistics, in 2010 around 20000 tons of fish were captured. A higher number than the captured fish in the year 2000, where only 16000 tons (www.fao.org). In Portugal, between the years 2009 and 2010 the estimated captured was 110 and 122.7 tons respectively (www.dgrm.min-agricultura.pt). *P. flesus* is a high commercial species, so the average prices for *P. flesus* in 2009 and 2010 ranged between 2.96 and 3.10 €/kg, for the first fish sale. The average price for *P. flesus* for the public sale was around 10€/kg, a much higher value than the first fish sale. Figueira da Foz Regional office of Docapescas-Portos e Lotas, SA provided 10.6 tons of captured fish to the mainland, with an average commercial value of 2.91 euro/kg, for the first fish sale. The Mondego estuary contributed with 2.47 tons (www.dgrm.min-agricultura.pt).

Recently, the European Union has recommended a tolerance limit of 75 ng g^{-1} (wet wt.), for fish muscle and fish products, for the sum of the 6 ecological indicators (28, 52, 101, 138, 153 and 180), (Commission Regulation (EU) No 1259/2011). *P. flesus* concentration from the fish market is around 1.46 ng g^{-1} (wet wt.). A much lower value than the one established by the European Union. Therefore, fish from the Figueira da Foz Regional office of Docapescas-Portos e Lotas, SA, representing the adult fish, can be considered safe for human intake, regarding PCBs.

Also regarding PCBs, the limit of the daily intake was set in 10 ng/kg bw/day (WHO, 2003). The weekly consumed fish for the Portuguese population is around 1250g (Lourenço et al., 2006) representing one of the highest consumption rates of fish per capita in the world (www.fao.org). Since the

Portuguese average body weight is around 65 kg and the used concentration was 1.46 ng g^{-1} (wet wt.) the Portuguese daily intake is around 4 ng/kg bw/day, which is lower than the limit set by WHO (1993). The values estimated in the present work are lower to the ones estimated in Germany (11.2 ng/Kg bw/day) (Fromme et al., 2009) and in Italy (11.2 ng/kg bw/day) (Fattore et al., 2008), and closer the ones estimated in France (5.42 ng/kg bw/day) (Sirot et al., 2012)

HCB values estimated in the present work (2.2 – 3.4 ng g^{-1}, lipid wt.) are lower than those found in previous studies, such as Falandysz et al. (2004), with 6 – 27 ng g^{-1} (lipid wt.) in perch, or by Wang et al. (2010), with 11 – 37 ng g^{-1} (dry wt.) in carp. Although, no limit was set for HCB intake in fish, the fish daily intake can be considered. The daily intake of HCB by the Portuguese population, with a concentration of 0.09 ng g^{-1} (wet wt.) is around 0.25 ng/kg bw/day. According to the US Department of Health and Human Services for non-cancer effects the daily intake limit is 170 ng/kg bw/day and for neoplastic effects the limit is 160 ng/kg bw/day (WHO, 1997). The daily intake levels regarding HCB of the Portuguese population are lower, than values found in Spain (2.4 ng/kg bw/day) (Falcó et al., 2004), Finland (24.2 ng/kg bw/day) (Moilanen et al., 1986) or Netherlands (14.3 ng/kg bw/day) (Greve, 1986).

Conclusions

PCBs and HCB were measure in *P. flesus* from the Mondego estuary and adjacent coastal waters, along the species lifespan, in liver, gills and muscle. PCBs concentrations decrease along the species lifespan, for all tissues. Liver and gills presented a higher concentration than muscle. HCB residues were not detected in juvenile fishes (estuarine environment), but was detected in the mature fishes (marine environment), with a tendency to increase its concentration along the lifespan. Liver and gills presented higher concentration than muscle.

Human exposure to such contaminants occurs mainly by environmental contamination of food products. Regarding PCBs, the concentration is below the 75 ng g-1 (wet wt.), moreover PCBs daily intake is below the limit establish by WHO (1993). Regarding HCB the daily intake is also below the US Department of Health and Human Services tolerance limit. Therefore, *P. flesus* can be considered safe for human intake.

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Chapter 5

General discussion

General overview

This section presents an overview on the various topics of the previous chapters, by summarizing and discussing PCBs bioaccumulation in an estuarine fish assemblage, and along 4 estuarine fish species lifespan. The present studies are focused mainly on the 7 ecological indicators to assess marine pollution, chosen by the International Council for the Exploration of the Sea (ICES) ($\sum_{7ICES}$ 28, 52, 101, 118, 138, 153, and 180), and more recently, the 6 ecological indicators ($\sum_{6PCBs}$ 28, 52, 101, 138, 153 and 180) chosen by the European Union. On the overall, 13 congeners were analyzed due to their different chlorination degrees ($\sum_{12PCB}$, IUPAC nos. 18, 28 and 31-tri; 44 and 52-tetra; 101 and 118-penta; 138, 149 and 153-hexa; 170 and 180-hepta; 194-octa).

The present study represents one step forward on the detection of PCBs in estuarine fish communities since there is a lack in literature regarding the contamination of the whole fish community. It is also important to highlight that most species present in estuaries have high economic importance. Nevertheless it is always important to keep in mind that species with no economic interest are as important for the function of the ecosystem as commercial species. Many studies have been made in fish species, although studies regarding PCBs in the fishes from the Mondego estuary are scarce, with most studies only assessing the contamination in sediments or in dioxin-like PCBs (Nunes et al., 2011, Pereira et al., 2005). Furthermore, this study has filled the gaps related to PCBs bioaccumulation along 4 estuarine species lifespan (*Liza aurata*, *Liza ramada*, *Chelon labrosus* and *Platichthys flesus*).

The Mondego estuary fish community

The Mondego estuary fish community consists of 42 species, belonging to 23 families, 5 ecological guilds (Baptista et al., 2010; Martinho et al., 2007; Nyitrai et al., 2012) and 5 trophic guilds (Table 1). In the Mondego estuary the most representative guilds are marine-estuarine dependent (MMD) and estuarine residents (ER) (Nyitrai et al., 2012), which is similar to the typical European Atlantic estuarine community (Elliot and Dewailly, 1995). The marine-estuarine dependent species (MMD) *Dicentrarchus labrax*, *Platichthys flesus* and *Solea solea* represent a significant part of the Mondego estuary fish assemblage, although in terms of number the community is dominated by *Pomatoschistus microps*, a estuarine resident species (ER) (Martinho et al., 2007).

The Mondego estuary followed a typical spatial distribution, in which the marine species were located in the downstream areas of the estuary, freshwater species were found in the upstream areas, and the estuarine residents and marine-estuarine dependent were located in the middle areas. This pattern resulted from the salinity gradient (Baptista et al., 2010).

Estuarine organisms' life strategies can create the structure of the estuarine ecosystem, thus reflecting its function. Estuarine life strategies can also be used to determine spatial and temporal utilization of the available resources (Franco et al., 2008). Marine stragglers species entered the estuary in low densities, probably due to the small area and opening of the estuary, which may limit their entrance (Nyitrai et al., 2012; Martinho et al., 2007). Freshwater stragglers (FS) only appeared in the estuary until 2004, as a result of an upstream displacement of the salinity gradient (Baptista et al., 2010; Martinho et al., 2007; Nyitrai et al., 2012).

Feeding guilds can be used as indicators of the main sort of food exploited by fish in estuaries (Nyitrai et al., 2012). The most abundant trophic guild in the Mondego estuary was the zoobenthonic guild (ZB) (Table 1).

Table 1. The Mondego estuary fish community: distribution of species by family, common name, ecological guild and trophic guild

Species	**Family**	**Common name**	**Ecological guild**	**Trophic guild**
Ammodytes tobianus	Ammodytidae	Lesser sand eel	MMO	ZP
Anguilla anguilla	Anguillidae	European eel	CA	ZB+OV
Aphia minuta	Gobiidae	Transparent goby	MMO	ZP
Arnoglossus laterna	Scophthalmidae	Scaldfish	MMO	PV
Atherina boyeri	Atherinidae	Big-scale sand smelt	ER	ZB
Atherina presbyter	Atherinidae	Sand smelt	ER	ZB+OV
Barbus bocagei	Cyprinidae		FS	ZB+DE
Buglossidium luteum	Soleidae	Solenette	MS	ZB
Callionymus lyra	Callionymidae	Dragonet	MS	ZB+OV
Carassius auratus	Cyprinidae	Goldfish	FS	ZB+OV
Chelidonichthys lucerna	Triglidae	Tub gurnard	MMO	PV

Chelon labrosus	Mugilidae	Thicklip grey mullet	MMO	DE+OV
Ciliata mustela	Gadidae	Fivebeard rockling	MMO	ZB
Conger conger	Congridae	European conger	MS	PV
Dicentrarchus labrax	Moronodae	Sea bass	MMD	PV
Dicologlossa hexophthalma	Soleidae	Ocellated wedge sole	MMO	ZB
Diplodus vulgaris	Speridae	Seabream	MMO	ZB+OV
Echiichthys vipera	Trachinidae	Lesser weever	MS	PV
Engraulis encrasicolus	Engraulidae	European anchovy	MS	ZP
Gaidropsarus mediterraneus	Gadidae	Shore rockling	MS	ZP
Gambusia holbrooki	Poeciliidae	Eastern mosquitofish	FS	ZB
Gobius niger	Gobiidae	Black goby	ER	ZB
Liza aurata	Mugilidae	Golden grey mullet	ER	OV
Liza ramada	Mugilidae	Thinlip grey mullet	CA	DE+OV
Mugil cephalus	Mugilidae	Flathead grey mullet	MMO	OV
Mullus surmuletus	Mullidae	Surmullet	MMO	ZB+DE
Nerophis lumbriciformis	Syngnathidae	Worm pipefish	ER	ZB
Parablennius gattorugine	Blennidae	Tompot blenny	MS	ZB
Platichthys flesus	Pleuronactidae	European flounder	MMD	ZB
Pomatoschistus microps	Gobiidae	Common goby	ER	ZB
Pomatoschistus minutus	Gobiidae	Sand goby	ER	ZB
Sardina pilchardus	Clupeidae	Sardine	MMO	ZP
Scophthalmus rhombus	Scophthalmidae	Brill	MMO	PV
Solea lascaris	Soleidae	Sand sole	MS	ZB
Solea senegalensis	Soleidae	Senegalese sole	MMO	ZB
Solea solea	Soleidae	Common sole	MMD	ZB+OV
Sparus aurata	Sparidae	Gilthead seabream	MMO	OV
Spondyliosoma cantharus	Sparidae	Black seabream	MS	OV
Symphodus bailloni	Labridae	Baillon's wrasse	MS	ZB
Syngnathus abaster	Syngnathidae	Black-striped pipefish	ER	ZB
Syngnathus acus	Syngnathidae	Greater pipefish	ER	ZB

Trisopterus luscus	Gadidae	Bib	MS	ZB

In the present thesis the studied fish assemblage consisted of 15 species, belonging to 13 families, 5 ecological guilds and 5 feeding guilds. The Mondego estuary presented few and diffuses contamination sources, being the most common wastewaters, from domestic and industrial sewage, fertilizers and pesticides, from agricultural runoff, and the Figueira da Foz harbour (Nunes et al., 2011).

Regarding PCBs, the estuarine species presented different concentrations. Species that spend more than three years in the estuary presented higher PCBs concentration than the species spending three years or less. Ashley et al. (2003) stated that the proximity to the contamination source in eels is one of the main factors influencing their body burden. Species such as *L. ramada*, *Sardina pilchardus*, *Chelidonichthys lucerna*, *Anguilla anguilla*, *Gobius niger* and *Pomatoschistus microps* presented higher body burden, than the other species. Variability in concentration can be due to their feeding habitat and strategies, proximity to the contamination source, lipid content (Coelhan et al., 2006), contact with superficial sediment or by water through respiration (van Geest et al., 2011). For instance, *P. microps*, *G. niger*, *A. anguilla* and *L. ramada* spent their entire lifespan inside or near the estuary. Moreover, catadromous species *A. anguilla* and *L. ramada* also spent most of their lifespan near the estuary. Morgan and Lohman (2010) observed that most fishes that exceed safety limit were migratory fishes. Diet and lipid content could also have a crucial role in species body burden.

C. lucerna diet (feeds mainly on fish, crustaceans and mollusks) was the main factor influencing this species bioaccumulation. Finally, *S. pilchardus* high body burden was due to its high lipid content.

P. microps and *P. minutus* presented high concentration due to the fact that they spent their entire lifespan inside the estuarine environment (Leitão et al., 2006). These species are very well known to be a part of the many piscivorous species diet (Leitão et al., 2006). The study of non-commercial species has an extreme importance, since most non-commercial species are a part of commercial species diet, and by biomagnification they can increase the concentration of PCBs throughout the food chain, leading to the humans, as a final consumer.

Species from the same family might present differences in their concentration, as was observed in the three mullet species from the Mondego estuary. *L. ramada* presented higher concentration than *L. aurata* and *C. labrosus*. Furthermore, *L. ramada* concentration increased along the species lifespan. This species spent more time in the estuarine environment than the other two species. Moreover, *L. ramada* is often found in more contaminated areas, leading to a high uptake of contaminants (Kottelat and Freyhof, 2007). *L. aurata* and *C. labrosus* are marine-estuarine opportunists' species, and only entered the estuary as juveniles, migrating afterwards to the sea (Baptista et al., 2010).

Callionymus lyra presented the lowest concentration among the estuarine fish community. As a marine straggler, *C. lyra* spends its entire lifespan in marine waters, only entering the estuary in low numbers and in order to feed (Elliot et al., 2007).

In the studied fish assemblage was detected a positive correlation between lipid content and PCBs concentration. PCBs tend to accumulate in organs with higher lipid content (Coelhan et al., 2006; Trocino et al., 2009). This fact was observed in *P. flesus* and *C. labrosus* from the present study, and in *D. labrax* from aquaculture (Antunes and Gil, 2004). *L. ramada* lipid content did not vary along its lifespan, although its PCBs concentration increased with age. Vives et al (2005) stated that lipid content in fish muscle is not correlated with age, and the increasing concentration along the lifespan cannot be attributed to the increasing lipid accumulation.

Liver presented higher lipid content than muscle or gills', therefore presenting higher PCBs concentration. This fact was observed in *P. flesus* and *C. labrosus*, and was also observed in *S. pilchardus* by Antunes et al. (2007). Liver is the main organ for PCBs storage (Bodiguel et al., 2009) and metabolism (Ferreira et al., 2004). PCBs are metabolized in liver by the enzyme cytochrome P450 1A (Ferreira et al., 2004). In fishes this enzyme increase after organic contamination exposure as observed by Collier et al. (1998).

In addition to all factors mentioned above, age and sex can also be determinant when assessing fish body burden. Amongst all the species analyzed along the species lifespan *L. ramada*, *L. aurata* and male *C. labrosus* increased their body burden with age. On the other hand, *P. flesus* concentration decreases along the species lifespan, since this species only uses the estuary as a nursery ground, migrating to the sea afterwards. Since PCBs concentration depends directly on exposure, this confirms a relation between accumulation and time of exposure, also observed by Bocquené and Abarnou (2012). Sex can also be a factor to determine fish body burden. Male *C. labrosus* increased its concentration with age, whereas female's concentration remained stable. Many factors can explain differences in PCBs accumulation among sexes. The most common is the mobilization of lipids for the maturation of the gonads (Loizeau and Abarnou, 1994). Females are able to decrease PCBs body burden by the releasing of the eggs. Although, differences in accumulation pattern could be observed between male and females, no significant differences were found between both sexes concentration a fact, also observed in *L. ramada* from Vilaine estuary (France) (Boquené and Abarnou, 2012).

Regarding HCB, *P. flesus* from the estuary did not present detectable values, whereas *P. flesus* from the adjacent coastal waters increased its concentration with age. Estuaries are known to have higher contaminant concentration than the adjacent coastal waters (Barber et al., 2005), which is contradictory to the present results. Though, the majority of fishes caught in the adjacent coastal area derived from the Douro estuary, a more contaminated estuary than the Mondego (Ferreira et al., 2004). The Douro estuary is located about 80 km north of the Mondego estuary, and contributes around 53.3% for the fish stock caught in the coastal area of Figueira da Foz. The Mondego estuary only contributes with 23% of the fish stock (Vasconcelos et al., 2008).

Studies along fish species lifespan are very important, since it is possible to understand the fate of contaminants within each species. Size can be an indicator of the fish age, and can be one of the most important indicators of chemical bioaccumulation (Pérez-Fuentetaja et al., 2010). Different species present different strategies of accumulating pollutants.

PCBs congeners

In the present study 13 PCB congeners were analyzed, CB_{IUPAC} 18, 28, 31, 44, 52, 101, 118, 138, 149, 153, 170 , 180 and 194. These 13 congeners were chosen because they present different degrees of chlorination. The congeners 18, 28 and 31 are trichlorinated, CB 44 and 52 are tetrachlorinated, CB 101 and 118 are pentachlorinated, CB 138, 149 and 153 are hexachlorinated, CB 170 and 180 are heptachlorinated and finally CB 194 is octachlorinated. Among these congeners seven are considered ecological indicators to assess marine pollution by the International Council for the Exploration of the Sea (ICES) ($\sum_{7ICES}$ 28, 52, 101, 118, 138, 153 and 180). Recently, in December 2011, the European Union removed CB 118 from the 7 ecological indicators, due to the fact that this congener was a dioxin-like PCB. So, instead of the 7 ecological indicators only 6 (CB 28, 52, 101, 138, 153 and 180) are considered ecological indicators for marine pollution assessment (Commission Regulation (EU) No 1259/2011). These congeners were chosen because they represent 50% of all PCB congeners in food (Sirot et al., 2012), and because they are important for the prediction of the degree of contamination (Coelhan et al., 2006).

It is useful to examine the similarity of the congeners' pattern with the Aroclors mixtures, to better understand and identify each factor (Rodenburg et al., 2011). In the Mondego estuary the congeners found in higher proportion were CB 101, 138, 149, 153 and 180, which might resemble Aroclor 1260, since these congeners were also the predominant congeners in this mixture. On the other hand, PCB mixtures are no longer been produced, therefore congeners pattern may have suffered some alterations in their proportion/initial ratio. Moreover they may have been transformed by the environment altering the congener environmental composition (Stapleton et al., 2001; Ulbrich and Stahlmann, 2004).

In the present study, CB 138, 153 and 180 were the predominant congeners in the fish assemblage. These congeners also dominate PCB pattern in crustaceans (Bodin et al., 2008), and in humans (Ulbrich and Stahlmann, 2004). In some cases CB 153 predominates over the other congeners (Bocquené and Abarnou, 2012; Pérez-Funtetaja et al., 2010). Moreover, CB 138 and 153 increased with the time spent in the estuary, and species that spent more than three years in the estuary presented higher concentration. More specifically in *L. ramada* CB 138 and 153 increased their concentration along the species lifespan.

High chlorinated congeners dominate PCBs pattern since they were produced in higher proportions, and have higher bioaccumulation potential (Bodin et al., 2008) and are more persistent in the environemt than less chlorinated congeners (Vives et al., 2005). High bioaccumulative properties can be due to their slow metabolic rates (Wu et al., 2008), high lipophilicity, higher resistance to microbial degradation and are less volatile (Zhou et al., 2001).

Fish tissue accumulation

Contaminants that tend to pass from organism to organism in the food chain, which can result in progressively high concentration in high trophic levels (Mackay and Fraser, 2000). HCB and PCBs bioaccumulation in tissues can be a result of biomagnification and bioconcentration processes (Vives et al., 2005). Bioconcentration processes involve the uptake of chemicals by absorption from the water, it can occur via respiratory surface or by skin. Biomagnification is the uptake of chemicals by dietary intake Mackay et al., 2000). Both processes are dependent on the octanol-water partition coefficient (K_{ow}) of each compound (Vives et al., 2005). K_{OW} is used for the charactezition of the lipophilicity of each compound (Hansen et al., 1999). Fish biology is also very important for HCB and PCBs accumulation. In *C. labrosus* and *P. flesus* liver presented higher lipid content than muscle. Therefore in both species liver presented higher concentration than muscle. In *P. flesus* gills presented higher concentration than muscle, but lower concentration than liver. Associated to high lipid content, liver is responsible for the detoxification of endogenous products, such as PCBs, in fish (Ferreira et al., 2004).

Liver, muscle and gills can present different mechaminsms for PCBs accumulation. Liver and muscle accumulated PCBs by prey ingestion, whereas gills tended to accumulate mainly by bioconcentration. Gills are in direct contact with the surrounding environment (Björk and Gilek, 1997) and are constantly transferring organic pollutants from both water and suspended particles into its surface (Yang et al., 2007).

Human intake and contaminants fate

Even though HCB and PCBs production was banned, they are still present in the environment and, therefore, in food chains. These contaminants exposure in humans occurs mainly through contaminated food (Ulbrich and Stahlmann, 2004). Dietary intake is the most important source of HCB and PCBs for the general population (Baeyens et al., 2007; Ulbrich and Stahlmann, 2004). Fish and fishery products have the highest contribution in the contaminated dietary intake (Fattore et al., 2008).

Most species present in the Mondego estuary have high economic importance and are consumed by humans. Recently the European Union has recommended, for PCBs, a tolerance limit of 75 ng g^{-1} (wet wt.) for fish muscle and fish products (Commission Regulation (EU) No 1259/2011), or approximately 300-350 ng g^{-1} (dry wt.) (Bocquené and Abarnou, 2012). This tolerance limit only includes the six ecological indicators (CB 28, 52, 101, 138, 153 and 180), instead of the seven ecological indicators recommended by the International Council for the Exploration of the Sea (ICES). Fishes from the Mondego estuary and adjacent coastal waters are considered safe for human intake since their concentration is far below the 75 ng g^{-1} (wet wt.), or the 300 ng g^{-1} (dry wt.).

For the tolerable daily intake WHO (2003) has recommended 20 ng/kg bw/day, for all the PCBs congeners, and 10 ng/kg bw/day for the six ecological indicators. The Portuguese daily intake for *P. flesus* is around 4 ng/kg bw/day, which is far below the tolerable daily intake recommended. Regarding HCB the tolerable daily intake is 170 ng/kg bw/day, for non-cancer effects, or 160 ng/kg bw/day for neoplastic effects (WHO, 1997). The Portuguese daily intake for *P. flesus* daily is around 0.25 ng/kg bw/day, which is far below the tolerance limit.

In Portugal PCBs emissions are estimated to be 385 kg yr^{-1}, whereas HCB emissions are estimated to be 96 kg yr^{-1} (Van der Gon et al., 2007). Since HCB emissions in Portugal are lower than PCBs emissions, HCB concentration present in fish tissues is expected to be lower than PCBs concentration, which is in accordance with the present results.

Portuguese emissions of PCBs are higher than emissions from Greece (168 kg yr^{-1}), Ireland (49 $kgyr^{-1}$) or Netherlands (164 kg yr^{-1}). Nevertheless, Portuguese emissions are lower than emissions from the United Kingdom (1643 kg yr^{-1}), Italy (3648 kg yr^{-1}) or France (13380 kg yr^{-1}) (Van der Gon et al., 2007). Regarding HCB, Portuguese emissions are low, when compared with Italy (2863 kg yr-1), France (1800 kg yr-1) or United Kingdom (595 kg yr-1). On the other hand, Portuguese emissions are higher than Belgium emissions (28 kg yr^{-1}), Switzerland emissions (31 kg yr^{-1}) or Russia emissions

(8 kg yr^{-1}) (Van der Gon et al., 2007). It is predicted that PCBs and HCB concentration will tend decrease until 2020 (Van der Gon et al., 2007), since these contaminants are no longer being produced.

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Final remarks

One of the main goals of this work was to understand how PCBs are distributed in the fish community of the Mondego estuary. Some species from the Mondego estuary presented higher concentration, like *L. ramada* or *P. microps*, whereas other species presented lower concentration, such as *C. lyra* or *S. solea*. In the Mondego estuary species body burden increased with the time spent in the estuary, high lipid content and dietary intake. Furthermore, this work fills the gap in detecting PCBs in non-commercial species. The majority of the studies so far, were performed in commercial species, nevertheless the study of PCBs in non-commercial species present an extreme importance, since these are very important for the structure and functioning of the ecosystem.

This study, also, allowed understanding how organic contaminants accumulate along species lifespan. Some species increased their concentration along their lifespan, like *L. ramada*, whereas others decreased their concentration, like *P. flesus*. This can be due mostly to lifespan location (estuaries or adjacent coastal waters). Studies along the species lifespan are of extreme importance since it can be an indicator of chemical contamination. Sex can also be a factor determining fish body burden, as observed in *C. labrosus*. Moreover, different tissues present differences in contaminants concentration. In the present study liver presented higher concentration, followed by the gills. The muscle presented the lowest concentration among the studied organs.

Human consumption is one of the main issues approached in this thesis. There a lack in literature regarding fish contamination and their relation with human intake and tolerance limits. The European Union as recommended a tolerance limit of 75 ng g^{-1} (wet wt), for the sum of the 6 ecological indicators. In the present study the fish species analyzed are below the tolerance limit, including species from Figueira da Foz Regional Office of Docapesca-Portos e Lotas, SA, responsible for the first fish sales. Regarding HCBs the daily intake is far below the tolerance limit from 160 ng/kg bw/day. Therefore, species from the Mondego estuary and adjacent coastal waters are safe for human intake, regarding PCBs.

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