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Endocrine and Enzymatic Changes in Flounder (*Platichthys flesus*) and in Mullet (*Mugil cephalus*) Chronically Exposed to Organic Contaminants in River Douro Estuary

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Endocrine and Enzymatic Changes in Flounder (*Platichthys flesus*) and in Mullet (*Mugil cephalus*) Chronically Exposed to Organic Contaminants in River Douro Estuary.

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Aos meus pais...

Por estarem sempre do meu lado...

Ao pai do Rui...

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ABSTRACT

In the last decades there was an increasing release of chemicals into the aquatic environment from industry, agriculture applications and domestic sewages, with consequences to aquatic populations, and ultimately to humans. Many of these contaminants interfere with endocrine functions, being responsible, even at sub-lethal doses, for changes in development and reproduction of wild populations; and for these reasons aquatic species are useful as bioindicator species for the presence of pollutants.

The persistency and the ability to bioaccumulate are recognised as criteria to establish priorities among chemical compounds. With these characteristics are the organochlorine compounds, such as Polychlorinated Biphenyls (PCBs) and Organochlorine Pesticides like dichlorodiphenyltrichloroethane (DDT), that due to their tendency to bioaccumulate and difficult elimination are considered to be toxic, even at low concentrations in the environment. Another group of contaminants frequent in the aquatic environment, the Polycyclic Aromatic Hydrocarbons (PAHs), are originated from a variety of anthropogenic and natural origins. The PAHs, originated from petroleum industry, maritime traffic and combustion, equally promote adverse effects in fish, such as genotoxicity, development alterations and endocrine disruption, among others.

This study was conducted with two resident species from River Douro estuary, flounder (*Platichthys flesus*) a benthonic species, and mullet (*Mugil cephalus*) a pelagic fish. We have evaluated the accumulation of Persistent Organic Pollutants (POPs), the PCBs, DDT and it stable metabolite the dichlorodiphenyldichloroethylene (DDE), in liver and muscle of both species. Eighteen PCB congeners were analysed, two of them considered to be "dioxin-like", the CB105 and the CB118. In all samples the CB138, 153 and 180 were the predominant congeners. The distribution of POPs in the tissues was associated with the fat depots, with the species showing a different pattern in their tissues accumulation. In mullet, we have also evaluated the presence of four PAH metabolites in bile, namely phenanthrene, naphthalene, pyrene and benzo(a)pyrene.

In environmental monitoring studies it has become frequent the use of several biomarkers in order to evaluate the quality of the systems. In this research we have evaluated the enzymes from phase I and phase II biotransformation, and oxidative stress, by means of antioxidant enzymes and oxidative damages. Both species were allowed to depurate for different periods (1, 4 and 8 months) in a cleaner environment with uncontaminated food, and the levels of pollutants and biomarkers were assessed after the depuration periods. Concerning the levels of POPs, no changes were observed in liver even after the 8 month period. However, in muscle a different pattern was seen with a decrease in these pollutants levels after 4 month of depuration. PAH metabolite levels,

because they are less lipophilic and more easily metabolised, have significantly decreased with depuration.

In flounder, biomarkers responses were conditioned by biological factors, mainly by reproduction, that have limited phase I responses (evaluated by the activity of ethoxyresorufin O-deethylase, EROD). In addition, the fasting condition during the depuration period, has limited oxidative stress biomarkers at the level of defences and also damages. For these reasons we have considered mullet, which had no food restriction and no influence on biomarkers responses during the depuration, to be a better sentinel species in this type of studies. In mullets, EROD activity has decreased significantly after one month depuration, maintaining the levels throughout the following depuration periods. Phase II biotransformation enzyme (evaluated as glutathione S-transferase, GST), in mullet revealed an increase in activity in some periods, however due to the physiological role of this enzyme it can be considered to be beneficial to the animal. Antioxidant enzymes in mullet have equally showed a decrease with depuration, however not so pronounced as phase I decreases. The decrease in oxidative damages in lipids, throughout the depuration, and in proteins after eight months, has confirmed that animals inhabiting Douro estuary are facing oxidative stress induced by the presence of pollutants in the environment.

Histological analysis of mullet's gonads has shown an intersex condition, testis-ova, in 21% of the males, characterised by the presence of oocytes in the testicular tissue. This condition was associated to the presence of compounds with xenoestrogen activity in the environment.

In conclusion, this study revealed the presence of several contaminants in River Douro estuary, and that these compounds induce changes at cellular and subcellular levels in the species inhabiting this aquatic environment. In addition, we can also conclude that mullet can be a useful sentinel species for the presence of several contaminants in European estuaries given their large geographic distribution.

RESUMO

Nas últimas décadas tem vindo a observar-se um aumento nos níveis de contaminantes de origem industrial, de aplicação na agricultura, e dos efluentes domésticos no meio aquático, com o consequente efeito nas populações que aí habitam, e, obviamente, na saúde humana. Muitos destes contaminantes interferem com as funções endócrinas, sendo os responsáveis, mesmo em doses sub letais, por anomalias no desenvolvimento e na reprodução das populações selvagens. Por essa razão muitas das espécies aquáticas são usadas como bioindicadores da presença de poluentes.

A persistência e a bioacumulação são reconhecidas como critérios para estabelecer prioridades entre os contaminantes químicos. Dentro deste grupo estão os compostos orgânicos clorados, como os Bifenilos Policlorados (PCBs) e os Pesticidas Organoclorados, como o Diclorodifeniltricloroetano (DDT), que por se acumularem e serem de difícil eliminação, são considerados de elevada toxicidade, mesmo em baixa concentração no meio ambiente. Outro grupo, o dos compostos policíclicos aromáticos, provenientes de uma variedade de fontes antropogénicas e naturais, são frequentes no meio aquático. Os hidrocarbonetos policíclicos aromáticos (PAHs), provenientes da indústria do petróleo, do tráfego marítimo e da combustão, promovem igualmente efeitos adversos nos peixes, como genotoxicidade, alterações no desenvolvimento e disrupção endócrina, entre outros.

Este estudo foi conduzido em duas espécies residentes no estuário do Rio Douro, a solha (*Platichthys flesus*), uma espécie bentónica, e a tainha (*Mugil cephalus*), uma espécie pelágica. Foi avaliada a acumulação dos poluentes orgânicos persistentes (POPs) - os Bifenilos policlorados (PCBs) e o Diclorodifenilotricloroetano (DDT) e o seu metabolito estável o Diclorodifenilodicloroetileno (DDE) -, no fígado e no músculo das duas espécies. Foram analisados 18 congéneres, dos quais dois considerados como "dioxin-like", o CB105 e o CB118. Em todas as amostras os CB138, 153 e 180 foram predominantes. A distribuição dos POPs nos tecidos verificou-se ser dependente da acumulação de gorduras, tendo-se observado uma diferente acumulação nos tecidos das duas espécies. Na bílis de tainha foi analisada a presença de metabolitos fluorescentes de quatro PAHs, nomeadamente o fenantreno, o naftaleno, o pireno e o benzo(a)pireno.

O recurso a vários biomarcadores para a avaliação da qualidade dos sistemas é frequente em estudos de monitorização ambiental. Neste trabalho foram utilizados como biomarcadores, as enzimas das duas fases de biotransformação de xenobióticos e o stresse oxidativo, nomeadamente as enzimas antioxidantes e os danos oxidativos. Após a captura, as duas espécies foram sujeitas a períodos de depuração (1,4 e 8 meses) em água e com alimento não poluído, sendo medidos os níveis dos poluentes e dos

biomarcadores após a depuração. Relativamente aos POPs não se verificou qualquer alteração nos níveis no fígado, mesmo ao fim dos 8 meses de depuração. Contudo, o músculo apresentou um comportamento diferente, com uma diminuição nos níveis destes poluentes ao fim de 4 meses. Este diferente comportamento deveu-se a uma redistribuição dos lípidos do músculo, devido a uma diferente alimentação. Quanto aos metabolitos dos PAHs, porque estes são compostos menos lipofílicos e mais facilmente metabolizados, diminuíram significativamente durante a depuração.

Na solha, as respostas dos biomarcadores analisados foram condicionadas por factores biológicos, principalmente pela reprodução, que limitaram a resposta da fase I (medida pela actividade da etoxiresorufina O-dietilase, EROD). Uma outra condicionante resultou do facto da solha não se alimentar durante o período de depuração, o que condicionou as respostas do stresse oxidativo, quer ao nível das defesas, quer dos danos oxidativos. Por este facto a tainha foi considerada a melhor espécie sentinela para este tipo de estudos, uma vez que se alimentou sem qualquer restrição durante toda a depuração, não existindo limitações no uso dos biomarcadores. Relativamente à actividade do EROD nas tainhas verificou-se um decréscimo significativo após um mês de depuração, mantendo-se os níveis de actividade desta enzima até aos oito meses. A fase II de biotransformação (avaliada pela actividade da glutationa S-transferase, GST) na tainha revelou um aumento, em alguns dos períodos de depuração, o que pode ser considerado como um factor positivo, dada a função desta enzima. As enzimas antioxidantes na tainha mostraram igualmente um decréscimo da sua actividade durante a depuração, não sendo no entanto tão evidente como a variação actividade do EROD. O stresse oxidativo foi também confirmado pela descida dos danos oxidativos em lípidos durante a depuração, e pela diminuição do teor em proteínas oxidadas, ao fim dos oito meses.

A análise histológica da gónada de tainha evidenciou uma condição de intersexo em 21% dos machos, caracterizada pela presença de oócitos no tecido testicular, indicando que existem no meio compostos com actividade xenoestrogénica.

Em conclusão, este estudo mostrou a presença de vários contaminantes no estuário do Rio Douro, e que estes induzem alterações a nível subcelular nas espécies aí residentes. Este trabalho mostra ainda que a tainha pode ser considerada como uma boa espécie sentinela para a presença de vários tipos de poluentes em estuários da Europa dada a sua larga distribuição geográfica.

RÉSUMÉ

Les dernières décennies ont vu l'augmentation du niveau de contamination d'origine industrielle, agricole et domestique dans le milieu aquatique, avec des conséquences sur les populations indigènes, et par conséquent sur la santé humaine. Nombre de ces contaminants perturbent les fonctions endocrines et sont responsables, même à des niveaux sub-létaux, d'anomalies du développement et de la reproduction des populations. Ainsi, de nombreuses espèces aquatiques sont utilisées comme bioindicateurs pour la présence de polluants.

La persistance et la bioaccumulation sont considérés comme les critères adéquats pour l'établissement de priorités entre les composés chimiques. Les composés organiques chlorés, tels que les biphényls polychlorés (PCBs) et les pesticides organochlorés comme le DDT possèdent ces caractéristiques. Leur tendance à s'accumuler et leur difficile élimination en font des composés hautement toxiques, même à basse concentration dans l'environnement. Un autre groupe, celui des composés polycycliques aromatiques, originaires de diverses sources anthropogéniques et naturelles, sont également fréquents dans le milieu aquatique. Les hydrocarbures polycycliques aromatiques (PAHs), provenant de l'industrie du pétrole, du trafic maritime et de la combustion, provoquent également des effets négatifs sur les poissons, tels que génotoxicité, des altérations du développement et des perturbations endocriniennes.

Cette étude a été conduite sur deux espèces indigènes de l'estuaire du Douro, la sole (*Platichthys flesus*), espèce benthonique, et le rouget (*Mugil cephalus*), espèce pélagique. L'accumulation de polluants organiques persistants (POPs), les biphényls polychlorés et le dichlorodiphényltrichloroéthane (DDT) et son métabolite stable le dichlorodiphényldichloroéthylène (DDE), a été mesurée dans le foie et les muscles des deux espèces. Dix-huit congénères ont été analysés, dont deux considérés comme étant "dioxin-like", le CB 105 et le CB 118. Dans tous les échantillons, les CB 138, 153 et 180 étaient prédominants. La distribution des POPs dans les tissus a été associée à l'accumulation des graisses, après l'observation de différences entre les tissus des deux espèces. Pour le rouget, la présence de métabolites fluorescents de 4 PAHs, le phénanthrène, le naphtalène, le pyrène et le benzo(a)pyrène, a été analysée dans la bile.

Le recours à divers biomarqueurs pour l'évaluation de la qualité des systèmes est désormais fréquent lors d'études de suivi environnemental. Dans ce cas, ont été utilisés les enzymes des deux phases de biotransformation, ainsi que le stress oxydatif, à travers les enzymes antioxydants et les dommages oxydants. Après leur capture, les deux espèces ont été soumises à une période de dépuration (1, 4 et 8 mois) dans un milieu propre avec de la nourriture non contaminée. Les niveaux de polluants et les

biomarqueurs ont été évalués après chaque période de dépuration. En ce qui concerne les POPs, aucun changement n'a été observé dans le foie, même après 8 mois. Cependant, dans le muscle, une diminution du niveau de polluants a été observée après 4 mois de dépuration. Les métabolites des PAHs, moins lipophiles et plus facilement métabolisés, ont quant à eux diminué de façon significative pendant le drainage.

Pour la sole, les réponses des biomarqueurs ont été conditionnées par des facteurs biologiques tels que la reproduction, qui présente une réponse de phase I limitée (mesurée par l'activité éthoxyresorufine O-déethylase, EROD). De plus, le jeûne observé par la sole durant le dépuration a conditionné les réponses au stress oxydatif, tant au niveau des défenses qu'au niveau des dommages oxydants. Ainsi, le rouget a été considéré comme la meilleure espèce sentinelle pour ce type d'étude, n'ayant pas limité sa nourriture durant le dépuration, ainsi n'influant pas les réponses aux biomarqueurs. L'activité EROD a également significativement diminué pour le rouget après un mois de dépuration, maintenant ces niveaux durant les huit mois de cette période. La phase II de la biotransformation a été déterminée à travers l'activité de la glutathione S-transférase (GST). Celle-ci a présenté une augmentation à certains moments de la période de dépuration, ce qui, étant donné le rôle physiologique de cet enzyme, peut être considéré comme positif pour l'animal. Les enzymes anti-oxydants du rouget ont également démontré une baisse de leur activité pendant le dépuration, cependant moins évidente que la variation d'activité de l'EROD. La diminution des dommages oxydants des lipides pendant toute la dépuration et la diminution de la teneur en protéines oxydées après huit mois, ont permis de confirmer que les animaux vivants dans l'estuaire du Douro sont soumis à un stress oxydatif dû à la présence de polluants dans leur environnement.

L'analyse histologique des gonades du rouget a mis évidence une situation d'intersexe chez 21% des mâles, caractérisée par la présence d'oocytes dans le tissu testiculaire. Ceci démontre la présence de composés présentant une activité xenoestrogène dans l'environnement.

En conclusion, cette étude a permis de démontrer la présence de divers polluants dans l'estuaire du Douro. Ces composés provoquent des changements au niveau cellulaire et sub-cellulaire chez les espèces vivant dans cet environnement. De plus, ce travail permet également d'avancer que le rouget peut être considéré comme une bonne espèce sentinelle pour la présence de ces polluants dans les estuaires d'Europe, étant donnée sa large distribution géographique.

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ABBREVIATIONS

WFD – Water Framework Directive
ETAR – Treatment plant for domestic sewage
PCB – Polychlorinated biphenyl
OCP – Organochlorine pesticide
PAH – Polycyclic Aromatic Hydrocarbon
PCDF – Polychlorinated dibenzofuran
PCDD – Polychlorinated dibenzo-p-dioxin
DDT – Dichlorodiphenyltrichloroethane
DDE – Dichlorodiphenyldichloroethylene
DDD - Dichlorodiphenyldichloroethane
EPA – US Environmental Protection Agency
BaP – Benzo(a)pyrene
MFO – Mixed Function Oxidase System
POP – Persistent Organic Pollutant
WHO – World Health Organization
MO – Microsomal Monooxygenase
AhR – Aryl Hydrocarbon Receptor
CYP1A1 – Cytochrome P450 1A1
EROD – Ethoxyresorufin O-deethylase
ERA – Environmental Risk Assessment
GSH –Glutathione Reduced
GA – Glucoronic Acid
GST – Glutathione S-transferase
GSSG –Glutathione Oxidised
VTg – Vitellogenin
CDNB – 1-chloro-2,4-dinitrobenzene
ROS – Reactive Oxygen Species
NADPH – Nicotinamide Adenine Dinucleotide
SOD – Superoxide Dismutase
CAT – Catalase
GPx – Glutathione Peroxidase
GR – Glutathione Reductase
CuZnSOD – Cupper/Zinc SOD
MnSOD – Manganese SOD
LP – Lipid Peroxidation
PUFA – Polyunsaturated Fatty Acids
MDA – Malondialdehyde
PO – Protein Oxidation
DNPH – 2,4-dinitophenylhydrazine
PHAH – Polyhalogenated Aromatic Hydrocarbon
HSI – Hepato-somatic Index
GSI – Gonado-somatic Index
CF – Condition Factor
BTI – Biotransformation Index
FAC – Fluorescent Aromatic Compound
FF – Fixed Wavelength Fluorescence
TBT – Tributyltin
EDCs – Endocrine Disrupting Chemicals
E2 - 17β-Estradiol
EE2 – Ethinyl estradiol

INTRODUCTION

INTRODUCTION

As a consequence of the Industrial Revolution started the release of large amounts of different chemical compounds into the environment. The increase of environmental pollution is certainly related with the demanding and development of new living facilities. However for many years citizens were not aware of the negative impact pollution was, and it was necessary occur several environmental disasters for building such awareness.

In 1978, 239 families were relocated from Love Canal, USA, that was declared a health hazard due to the high levels of polychlorinated biphenyls (PCBs). Later in 1989, the oil tanker Exxon Valdez spilled about 37,000 tonnes of crude oil in Arkansas coast line. This catastrophic event leads to one of the most thorough examinations of the effects of oil in the environment. More recently, in 2002 the Prestige oil spill where more than 80% of the tanker's 77,000 tonnes of fuel oil was spilled of Spain's North-East coast. The amount of oil spill was more than for the Exxon Valdez and the toxicity higher, because of the higher temperature.

Upon these disasters society became aware that to maintain the quality of the environment there was a need for actions, namely to establish some regulations by police makers to diminish the release of toxic chemicals to the environment.

The aquatic environment has the major contaminant input, from untreated sewage, rain off, industrial activities, maritime transport that makes this environment, and the animals, highly exposed to the effects of chemicals compounds.

Some chemicals due to their characteristics are persistent compounds that can biomagnify and bioaccumulate thus it is necessary to monitor the presence, and the amount of these compounds in organisms and environment.

Xenobiotics are chemicals present in the environment that can be uptake by organisms inducing changes in the physiological mechanisms. These chemicals have been proven to exert a profound effect on fish, and even at sublethal doses can influence the success of wildlife populations. During the last decades, several tools have been developed aiming at evaluating and monitoring the presence of xenobiotics. The use of fish species functioning as sentinel, or bioindicators, can give the alert if the systems are disturbed due to the presence of pollutants. However, before the effects are visible in the whole animal, several alterations may occur at the cellular and molecular level that can be used as biomarkers of environmental pollution.

This dissertation will present the results of some types of contaminants that were found in River Douro Estuary and their chemical characteristics. It is revealed that

different fish species chronically exposed to xenobiotics can adopt different strategies to adapt and survive in a polluted environment. In addition, the analysis of biomarkers can indicate different responses according to the species. In a second part, data is presented on the ability of one of the species to recover from the chronic effects induced by the presence of xenobiotics, after long-term depuration in an unpolluted environment.

1. Aquatic Environment

In the past, damage to the environment has largely been identified as a result to acute events such as major disasters. However, long term and chronic exposure to environmental stressors, including chemical pollutants or other anthropogenic factors, will have a negative effect not only in the environment but also in the food chain. The impact will be gradual, subtle and frequently difficult to distinguish from the process and effects of natural environmental changes (Moore et al., 2004).

The focus of ecotoxicological research is aimed on understanding the toxicological effects in a variety of biota of different complexity, population and the ecosystem as a whole (Fent, 2003). The knowledge of ecotoxicological effects in contaminated ecosystems needs an integrated approach involving environment, chemical, toxicological and ecological concepts. The multitude of chemicals in ecosystems, species diversity, biological and ecological functions and structures makes it difficult to estimate possible effects of contaminants in ecosystems.

There are several causes to environmental stress:

- Natural forces: sea levels rise, climate changes, soil erosion.
- Unplanned development: haphazard urbanisation and industrialisation.
- Depletion of resources: over-fishing, deforestation, bad use of agricultural land.
- Unregulated discharges: municipal sewage and industrial waste.
- Illegal practices: Disposal of dangerous toxic waste.

A major aim of environment science is to drive robust, practical and relatively low cost procedures for risk assessment to the health of the biosphere, and to use the capability to predict the likely consequences of exposure to potentially harmful toxic pollutants (Rice, 2003).

The European Union's Water Framework Directive (WFD) is one of the most important pieces of environmental legislation produced in recent years. The objectives of the WFD (2000/60/EC) are to improve, protect and prevent further deterioration of water

quality across Europe. The Directive aims to achieve and ensure good quality status for all water bodies throughout Europe by 2015 (Allan et al., 2006).

Monitoring is required to cover a number of water quality elements including, physico-chemical, hydro-morphological, biological and chemical parameters. In the Directive there are three modes of monitoring specified, that will form part of the management plans that must be introduced by December 2006:

1. *surveillance* monitoring aimed at assessing long-term water quality changes and providing baseline data on river basins allowing the design and implementation of other types of monitoring,
2. *operational* monitoring aimed at providing additional and essential data on water bodies at risk or failing environmental objectives of the WFD,
3. *investigative* monitoring aimed at assessing causes of such failure.

The complexity of the aquatic systems is associated with a number of issues related with monitoring their quality. It is clear that WFD will rely on the effective use of a combination of monitoring methods according to their suitability for the questions being asked and the characteristics of the given site of sampling.

2. River Douro Estuary: Study Area

By definition an estuary is an area where "sea water is measurably diluted with freshwater derived from land drainage" (Dyer, 1997), and can be seen as unique ecosystem structured by abiotic factors setting up a complexity of gradients (Chapman and Wang, 2001). Additionally, estuaries receive significant anthropogenic inputs from sources upstream and from metropolitan areas and industries located on, or near, those areas (Mucha et al., 2005).

The Douro estuary has a single, narrow channel approximately 20 km in length, and an average depth of 10 m (Ramalhosa et al., 2005). Since 1985, the Douro estuary has been confined to the last 21.6 km stretch of one of the longest rivers of the Iberian Peninsula (930 km) (figure 1), because of the construction of numerous dams, including the Crestuma-Lever dam that created a 106 km^3 reservoir for hydroelectric power production (Bordalo and Vieira, 2005).

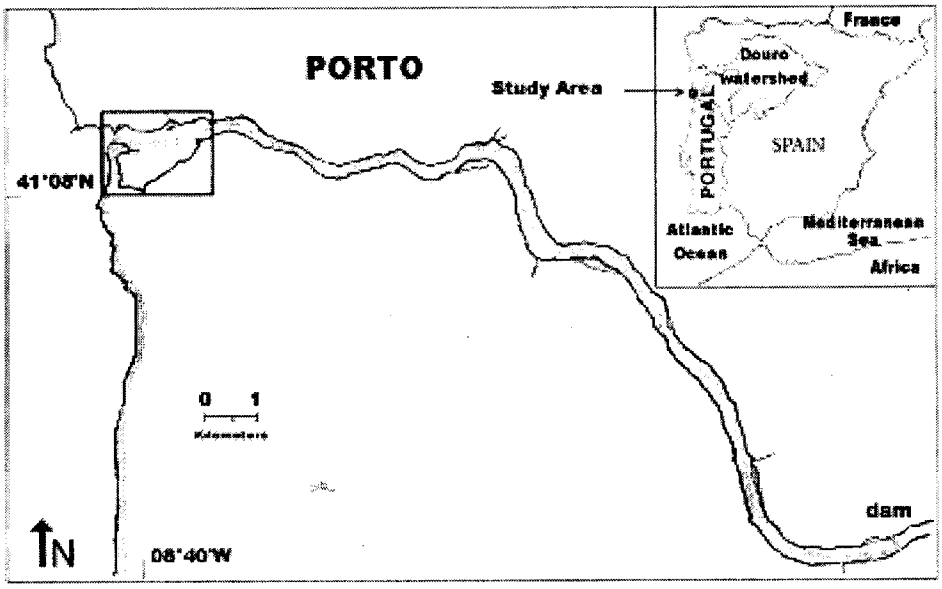


Figure 1: Map of the Douro Watershed, and the Douro estuary. (Picture kindly given by Professor Bordalo e Sá)

The Douro is an urban estuary from the river mouth to approximately 9 km upstream, with two major cities located within the estuarine region, Porto with 250,000 inhabitants on the north bank, and Vila Nova de Gaia with 300,000 inhabitants in the south bank (Bordalo and Vieira, 2005). Besides urban population, several industries like planting, soap, tannery and textiles are present along the Douro, that still discharge their effluents into the stream without treatment, despite the existence of urban sewage system (Mucha et al., 2004).

Recently, two new systems for the treatment of domestic sewage (ETAR) were installed in the city of Porto, presumably reducing the inputs of anthropogenic materials to the estuary: in 2000, the Freixo ETAR with the ability to the treatment of residual waters of about 40,000 m^3/day, and in 2003, the Sobreiras ETAR, with the ability to treat 154,000 m^3/day, of residual waters.

3. Sentinel species: mullet (*Mugil cephalus*) and flounder (*Platichthys flesus*)

The use of biological monitors for classifying water quality has a long history (Washington, 1984). The term "sentinel" was applied to species used in the first efforts to detect and map radioactivity in the 1950s and which later developed into the "Mussel Watch" programme (Goldeberg et al., 1983).

Sentinel species can be defined as biological indicators that accumulate a pollutant in their tissues without significant adverse effects (Beeby, 2001), offering a potentially simple solution to both the problem of measuring bioavailability and of summarising complex patterns of contamination. Given that their feeding and habitat are known, they could serve to quantify bioavailability for other species exposed to the same sources (Beeby, 2001).

Sentinel species in biomonitoring can have different uses:

1. As accumulators: to increase analytical sensitivity for a contaminant; to compare the scale of contaminantion between sites; to summarise a complex pollution signal.
2. As integrators: to provide a running mean over time and space.
3. As a measure of exposure: to quantify bioavailability of a pollutant from a particular source.

Practical limitations in ecotoxicology may lead to the extrapolation of laboratory data measured in non representative species, such as invertebrates, due to their ease culture and sampling, rather then for their ecological relevance. At some study sites, the species may not be available, or if they are, their responses may not be indicative of the sensitivity of other functional groups within the community (Galloway et al., 2004).

The characteristics of the ideal sentinel species have been described (Berger and Dallinger, 1993; Beeby, 2001; Galloway et al., 2004). Sentinel species should be widely distributed, easy to identify in the field, abundant and large enough to provide material for analysis. Nowadays, by using species available in ecosystems already impacted by human-induced stress, it is likely that a shifting baseline may have occurred. This problem, common to all ecosystem monitoring efforts, could be reduced by choosing species that exhibit different feeding strategies, and a diverse range of habitats in estuarine and coastal areas (Galloway et al., 2004).

Monitoring of aquatic pollution can be carried out by means of sentinel organisms, and bivalves have been used extensively for this purpose (Goldeberg et al., 1978), but fish have also been selected for monitoring (Pastor et al., 1996). Fish species can be good sentinels because:

- they concentrate pollutants in their tissues directly from water (low aqueous concentration levels are difficult to analyse), and also through diet, thus enabling the assessment of transfer of pollutants through the trophic web (Goerke and Weber, 2001);
- they generally exhibit a low metabolism for organochlorines and consequently should reflect the levels of pollution in the aquatic environment (Muir et al., 1988);

- they occupy different habitats in the same ecosystem and have different feeding behaviours, thus offering the potential to study the influence of environmental and biological factors on the bioaccumulation of pollutants (Porte and Albaigés, 1994).

Mullet (*Mugil cephalus*) and flounder (*Platichthys flesus*) are both eurihaline fish species, very common in southern European estuarine systems, and therefore may be considered a good tool for monitoring programs.

Mullets, members of the Mugilidae family, order Perciformes, are fish that inhabit all tropical and temperate waters (figure 2). They are found inshore, and enter lagoons, estuaries and some rivers, being characterised by an extreme salinity tolerance.

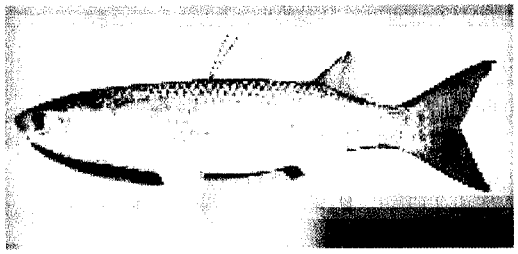


Figure 2: Picture of mullet (*Mugil cephalus*) specimen.

Mullets are characterised by a wide range of feeding adaptations to the estuarine environment, according to trophic availability (Boglione et al., 2006). This specie is mainly diurnal, feed on zooplankton, benthic organisms and detritus, with preference for sandy substrates and larger particles (Mariani et al., 1987). Reproduction takes place at open sea, from July to October. Females spawn 5 to 7 million eggs provided with a notable vitellus and are sexually mature at 7 to 8 years (www.fishbase.org). In Portugal, mullet is not a commercial species; however is a very important commercial fish species in many parts of the world being widely cultivated in freshwater ponds in Southeast Asia. A study in the Mediterranean Sea has shown that there has been a decline in fry availability of some mullet species in recent years due to pollution and overfishing of parent stocks, and including *M. cephalus* (Crosetti et al., 1998).

Flounder (*Platichthys flesus*), member of the Pleuronectidae family, order Pleuronectiformes, is an estuarine/coastal species that resides for the majority of the year in estuaries, only migrating to deeper water in the winter (Hylland et al., 1996) (figure 3).

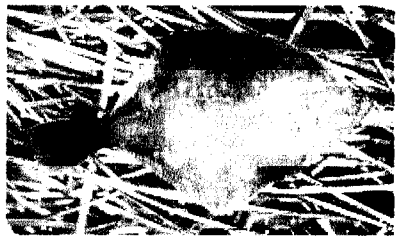


Figure 3: Picture of founder (*Platichthys flesus*) specimen.

Flatfish are particularly vulnerable to sediment contamination, through direct contact with the substrate, prey ingestion, as well as sediment particle ingestion (Moles et al., 1994). Flounder is also an abundant fish in the Douro estuary, but contrary to mullet, is a commercially important flatfish (Vinagre et al., 2004), and is an European flatfish that spends most of its life in low salinity of its home estuary, and migrates into the open sea to breed (Matthiessen et al., 1998). Flounder is distributed in coastal and brackish waters of Western Europe and from the White Sea to the Mediterranean and Black Sea. This species is nocturnal and burrowing, and juveniles and adults feed on benthic fauna, including small fishes and invertebrates (www.fishbase.org).

It is assumed that flounder would be more heavily exposed to sediment-associated lipophilic contaminants than the midwater fish, like mullet, both through direct exposure when they bury in sediment, and through their heavy consumption of benthic invertebrates. Moreover, their confinement to a home estuary for at least 8 months of the year ensures that any contaminant-related effects will probably be due to local inputs (Matthiessen et al., 1998).

For the stated reasons, fish species have attracted considerable interest in studies assessing biological and biochemical responses to environmental contaminants (Power, 1989). Fish play a major ecological role in the aquatic food-webs, and the understanding of toxicant uptake, behaviour and responses in fish may, therefore, have a high ecological relevance and distinct impact (Van der Oost et al., 2003). Indeed, besides the high ecological relevance the impact on the food chain is also meaningful. However, there are considerable variations between fish species, in both physiological features and the responses of certain biomarkers towards environmental pollution. Despite their limitations, such as high mobility, fish are generally considered to be the most feasible organisms for pollution monitoring in aquatic systems (Van der Oost et al., 2003).

4. Xenobiotics in the environment

The environment is continuously loaded with foreign organic chemicals (xenobiotics) released by urban communities and industries. These compounds will be transported and subsequently converted into other chemicals. These processes together with emission pattern, environment parameters and physicochemical properties of the substances, will govern their distribution and concentration in environmental compartments such as water, air, soil, sediment and biota (ECETOC, 1993).

Organic contaminants, such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzofurans (PCDFs) and dibenzo-*p*-dioxins (PCDDs) are ubiquitously present in the aquatic environment. The behaviour of these hydrophobic chemicals in sediment, water and biota is mainly determined by lipid and organic matter (Meador et al., 1995). The most widespread organochlorines in the environment and in animal tissues are PCBs and dichlorodiphenyltrichloroethane (DDT), and specially the DDT degradation product, dichlorodiphenyldichloroethylene (DDE) (Toft, 2003). The elimination of PAHs is generally very efficient, by chemical oxidation or biological transformation, and no bioaccumulation of these compounds has been demonstrated (Van der Oost et al., 2003).

4.1 Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls, or PCBs, are a group of 209 structurally related chemical compounds (congeners), consisting of two connected benzene rings and 1-10 chlorine atoms (figure 4) and with a general formula $C_{12}H_{10-n}Cl_n$. PCBs were discovered over 100 years ago and their production and commercial use began in 1929, because of their remarkable electrical insulating properties and their flame resistance, they soon gained widespread use as insulators and coolants in transformers and other electrical equipments (Ross, 2004). PCBs were also routinely used in the manufacture of a wide variety of common products such as plastics, adhesives, paints and varnishes, carbonless copying paper, newsprint, fluorescent light ballasts, and caulking compounds.

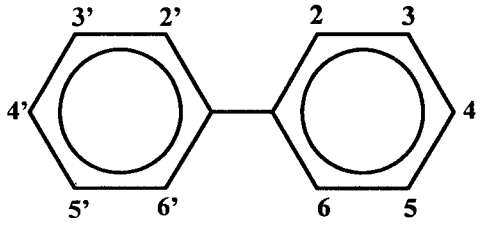


Figure 4: Basic chemical structure of PCBs.

It was only the late 1960s and afterwards that concern over PCBs in the environment began to rise. Furthermore, it was demonstrated that the rate of biodegradation (natural breakdown) was very slow for the more highly chlorinated PCBs congeners (Jensen, 1972), which resulted in environmental persistence of certain types of PCBs, a characteristic that added concerns over their potential health effects. The production of PCBs finished world-wide around the late 1970s to early 1980s after authorities became aware of the adverse effects of PCBs on the environment, due to their persistency, bioaccumulative properties and toxicity (de Boer, 2001). Even so, the last PCB production facility, located in Russia, was shut down in 1993 (Breivik et al., 2002). PCB mixtures were produced under the names of Aroclor (USA), Chlophen (Germany), Kanechlor (Japan), Fenclor (Italy), and other (Bolgar et al., 1995; Lang, 1992).

Organochlorine compounds, like PCBs, are still being released into the environment by (1) use, disposal or accidental release from previously produced material, (2) volatilization of previously released material, and (3) creation of PCBs and dioxins during combustion processes (Breivik et al., 2002; Katami et al., 2002).

Unfortunately, the same properties that made PCBs attractive for industrial use caused them to persist and bioaccumulate in the environment. They constitute a particular problem in aquatic ecosystems such as the Great Lakes (Kamrin and Fischer, 1999) and the Baltic Sea (Falandysz et al., 1994), and consumption of PCB-contaminated fish from polluted waters is a major source of PCB exposure for humans (Easton et al., 2002) with negative effects on human health. The most common PCB congener, the CB153 (figure 5) has been suggested as an indicator of overall exposure to persistent organochlorines (Richthoff et al., 2003).

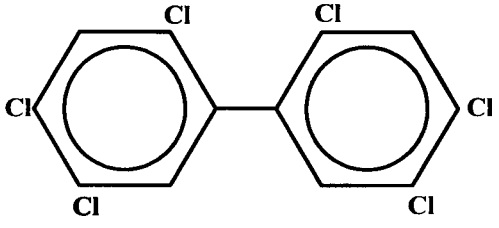


Figure 5: Basic chemical structure of CB153 (2,2',4,4',5,5'-hexachlorobiphenyl).

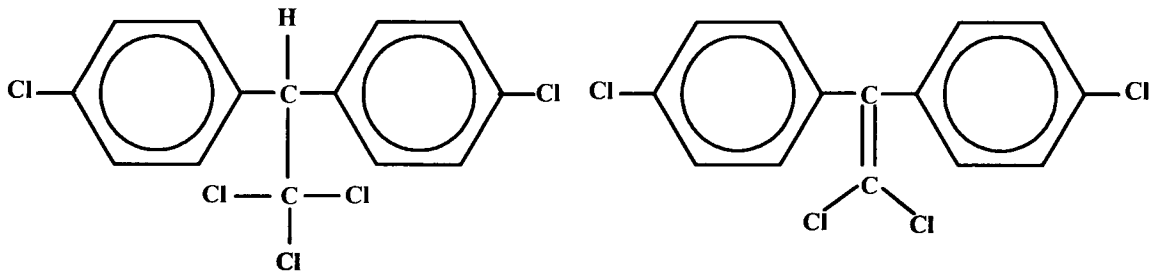
Due to their chemical stability, high lipophicity, and resistance to biotic and abiotic degradation they tend to bioaccumulate and biomagnify in the food chain. The highest PCBs concentrations are found in organisms with a long life (including humans) at the top of the food chain. Because of the cessation of PCBs production, regulation of their use, and cleanup of heavily contaminated sites general exposure to PCBs in fish and other foods has been significantly reduced, and PCBs in humans are now at lower levels (Ross, 2004).

These compounds have been associated with health effects in laboratory animals, but typically at very high dose levels relative to realistic human environmental exposures; and, for purpose of regulation, several governmental agencies, including the US Environmental Protection Agency (EPA), have concluded that there is sufficient evidence to consider PCBs to be animal carcinogens (Ross, 2004).

Organochlorine compounds may accumulate in aquatic organisms throught different mechanisms: via the direct uptake from water by gills or skin (bioconcentration), via uptake of suspended particles (ingestion), and via the consumption of contaminated food (biomagnification) (Van der Oost et al., 2003). It is most likely that biomagnification through trophic transfer is the primary mechanism governing the accumulation of PCBs, as was confirmed by numerous field studies (van der Oost et al., 1996b; Metcalfe and Metcalfe, 1997). Although PCBs are very persistent chemicals, there are indications for the biotransformation of lower chlorinated PCBs congeners in fish (de Boer et al., 1993; Elskus et al., 1994). de Boer et al. (1994) demonstrated, with eel, that the elimination half-lives of tetra- and penta-CBs ranged from 340 to 1450 days, while for hexa-, hepta- and octa-CBs no measurable elimination was observed. Moreover, the highly chlorinated congeners are less efficiently transferred in the food web due to restricted membrane permeability (Kannan et al., 1998).

4.2 Dichlorodiphenyltrichloroethane (DDT)

Another important group of organochlorine contaminants are the dichlorodiphenyltrichloroethane (DDT), and specially the DDT degradation product dichlorodiphenyldichloroethylene (DDE) (figure 6).



p,p'-DDT p,p'-DDE

Figure 6: Structural formula of dichlorodiphenyltrichloroethane (*p,p'*-DDT) and dichlorodiphenyldichloroethylene (*p,p'*-DDE).

As for PCBs, the use of DDT was banned or strongly restricted in the 1970s in many countries due to its environmental toxicity and persistence (Pereira et al., 1994). The DDT is a pesticide that was widely used in the past to control the spread of arthropod borne diseases and agricultural pests (You, 2000).

Because of their high chemical stability, lipophilicity, and persistence, it tends to bioconcentrate and biomagnify in the food chains and persists in the environment for many years (Naso et al., 2005). Long term chronic exposure to organochlorine pesticides (OCPs) and PCBs has been correlated with severe injury to the nervous, endocrine, reproductive, and immune systems in birds, fish and mammals (Kelce et al., 1995; Skaare et al., 2000; Toft et al., 2003).

Biotransformation has been reported to influence the accumulation of several OCPs compounds in fish (Sijm and Opperhuizen, 1989). The DDT can be partially metabolised by fish to the metabolite DDE (Streit, 1992). Indications of biomagnification of DDT metabolites (DDE and dichlorodiphenyldichloroethane, DDD) were also found in various fish species from different trophic levels (Ford and Hill, 1991).

4.3 Polycyclic Aromatic Hydrocarbons (PAHs)

The polycyclic aromatic hydrocarbons, PAHs, are a large group of organic compounds with two or more fused aromatic rings (figure 7). PAHs are formed mainly as a result of pyrolytic processes, especially the incomplete combustion of organic materials during industrial and other human activities, such as processing of coal and crude oil, combustion of natural gas, including for heating, combustion of refuse, vehicle traffic, cooking and tobacco smoking, as well as in natural processes like carbonization. There are several hundred PAHs; the best known is the benzo[*a*]pyrene (BaP).

PAH inputs to the marine environment are mainly: (1) produced by organisms (biogenic), (2) derived from incineration processes (pyrogenic), (3) derived from fossil fuels (petrogenic), and (4) derived from transformation processes in soil and sediments (diagenic) (Hylland, 2006). The four processes produce different PAHs, and it is generally possible to identify the contribution from each one to PAHs in environmental samples, and only pyrogenic and petrogenic PAHs have quantitative importance in marine ecosystems.

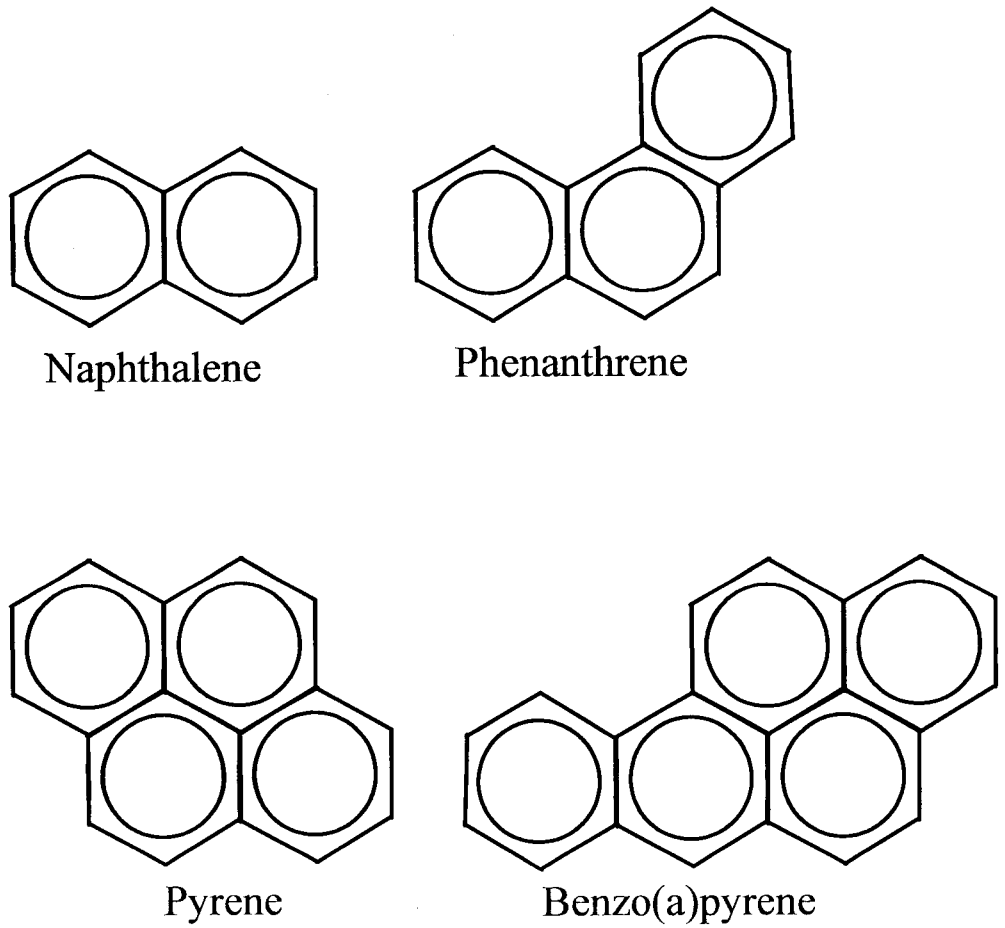


Figure 7: Structures of selected Polycyclic Aromatic Hydrocarbons (PAHs).

PAHs with few benzene rings are more water-soluble than heavier PAHs and therefore generally more bioavailable. There is also a general difference in bioavailability between petrogenic and pyrogenic PAHs. Pyrogenic PAHs are to a large extent associated with particles, even incorporated in their structure, which significantly decreases their bioavailability (Axelman et al., 1999; Rust et al., 2004). Petrogenic PAHs are generally though to be largely available for uptake by marine organisms (Neff and Burns, 1996).

In contrast to the very slow environmental breakdown of most organohalogen contaminants, PAHs are degraded through both chemical and biological processes in atmosphere and in water. PAH compounds in the aquatic environment can be

transformed by chemical (photo) oxidation or biological transformations (Valerio et al., 1984), that occurs, in many aquatic organisms, more effectively in the liver (Van der Oost et al., 2003). PAHs are easily metabolised by the phase I enzymes of the mixed function oxygenase system (MFO) to more hydrophilic products like phenols, dihydrodiols, quinones and epoxides (Lech and Vodicnik, 1985; Sijm and Opperhuizen, 1989). The carcinogenic potential of different PAHs is associated with the propensity of phase I to generate reactive epoxides which can bind to cellular components, such as DNA (Collier et al., 1992). Some PAHs can be excreted directly as unconjugated polar metabolites in bile (via the gallbladder), but most PAH will be excreted after conjugation by phase II enzymes (Van der Oost et al., 2003).

PAHs are taken up by marine organisms directly from water, sediments, or the diet. There have been some suggestions that the diet is less relevant than water exposure, and dietary uptake in fish is generally not very efficient (Neff and Burns, 1996). Dietary exposure to PAHs may however be high in species that preferentially feed on organisms that have low ability to metabolize these compounds, such as bivalves (Peterson et al., 2003).

Since the elimination of PAHs is generally very efficient in fish, low bioaccumulation of these compounds has been found. In rainbow trout, (Meador et al., 1995) reported half-lifes of parental PAHs that ranged from 1 day for acenaphtylene to 9 days for phenanthrene. In contrast to most persistent organic pollutants, PAH are not biomagnified in marine food chains, therefore, PAH fish tissue levels are not indicative of the levels to which the animals were exposed and should not be used as bioaccumulation markers of exposure assessment. In order to assess the exposure of fish to PAHs it is more appropriate to determine PAH metabolite levels in bile or tissue DNA adduct levels (Meador et al., 1995; Vigano et al., 2002), and other genotoxicity markers like erythrocytic micronuclei (Vigano et al., 2002; Gravato and Santos, 2003).

5. Biomarkers

Physicochemical analyses to water and/or sediments shed no light on the biological status of the ecosystems and a biological approach is needed to evaluate environmental health. The ecotoxicological approach based on biomarkers measured in individuals relies on the fact that changes occur at low levels of biological organization before the community is affected. The most compelling reason for using biomarkers is the fact that the information obtained is on the biological effects of pollutants, rather than a mere quantification of their environmental levels.

The term biomarker is generally used in a broad sense to include almost any measurement indicating an interaction between a biological system and a potential hazard that may be chemical, physical or biological (WHO, 1993). They are indicators of either a normal status, or changes in the members of the population analysed (Vasseur and Cossu-Leguille, 2003). According to the World Health Organisation, biomarkers can be sub-divided into three classes:

1. Biomarkers of exposure: covering the detection and measurement of an exogenous substance, and its metabolite, or the product of an interaction between a xenobiotic agent and some target cells or molecules;

2. Biomarkers of effect: including measurable biochemical, physiological or other alterations within tissues or body fluids of an organism, that can be recognised as associated with an established or possible health impairment or disease;

3. Biomarkers of susceptibility: indicating the inherent or acquired ability of an organism to respond to an exposure to a specific xenobiotic substance, including genetic factors and changes in receptors which alter the susceptibility of an organism to the exposure.

In general, the effects are more visible at a cellular level than at higher levels of biological organization, so biochemical responses may be similar in a large variety of organisms. Biomarkers may be used after exposure to dietary, environmental or occupational sources, to elucidate cause-effect or dose-effect relationships in health risk assessment, in clinical diagnoses and for monitoring purposes.

Biomarkers may provide insight into the potential mechanisms of contaminant effects. By screening multiple biomarker responses, information will be obtained regarding the mechanisms of toxicity of chemicals. A pollutant stress situation normally triggers a cascade of biological responses, each of which may, in theory, serve as a biomarker (Van der Oost et al., 2003).

Since both overestimation and underestimation of effects may occur, laboratory observations on biomarkers must always be validated with field research. Biomarkers applied in both laboratory and field, can provide an important linkage between laboratory toxicity and field assessment. In the field, biomarkers data may provide an important index of the total external load that is biologically available.

Various biochemical parameters in fish have been tested for their responses to toxic substances and their potential use as biomarkers of exposure or effect. The most extensively investigated are the enzymes involved in the detoxification process of xenobiotics and their metabolites, like biotransformation enzymes and oxidative stress parameters.

5.1 Biotransformation Enzymes

Biotransformation or metabolism can be defined as an enzyme-catalysed conversion of a xenobiotic compound into a more water-soluble form, which can be more easily excreted from the body than the parent compound (Lech and Vodicnik, 1985). The toxicity of a foreign compound may be affected by metabolism, which can either be beneficial (detoxification) or harmful (bioactivation) to an organism (figure 8). Metabolism is an important determinant of the activity of a compound, the duration of its activity and the half-life of the compound in the body (Timbrell, 1991).

Xenobiotic compounds may be biotransformed in liver by enzymes from phase I and phase II. Phase I is a non-synthetic alteration (oxidation, reduction or hydrolysis) of the original foreign molecule, which can then be conjugated in phase II (Commandeur et al, 1995). Generally, the most sensitive effect biomarkers are alterations in levels and activities of biotransformations enzymes (Van der Oost et al., 2003).

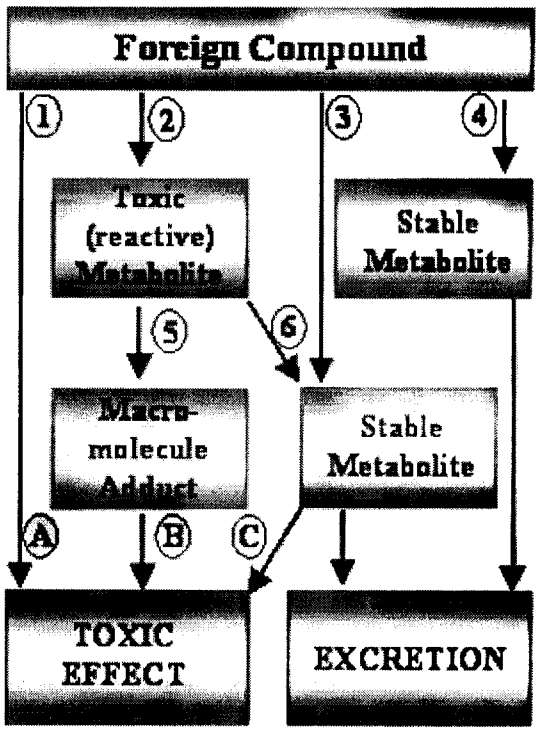


Figure 8: Possible toxication and detoxification pathways of xenobiotic compounds: (1) direct toxic effect (A); (2) metabolic activation; (3) formation of a stable metabolite which may cause a toxic effect (C); (4) detoxification. The reactive metabolite formed by bioactivation (2) may cause a toxic effect (B) through reaction with critical targets (5) or be detoxified through reaction with a protective agent (6). Adapted from Van der Oost et al. (2003).

5.1.1 Phase I Enzymes

For the majority of xenobiotic compounds the phase I reactions are catalyzed by microsomal monooxygenase (MO) enzymes, also known as the mixed-function oxidase (MFO) system (i.e. cytochrome P450, cytochrome b_5, and NADPH cytochrome reductase). Cytochromes P450, comprising a large and still expanding family of membrane proteins, predominantly located in the endoplasmatic reticulum of the liver (Stegeman et al., 1992; Bucheli and Fent, 1995). The most important feature of the MFO system is its ability to facilitate the excretion of certain compounds by phase I metabolism, as it transforms lipophilic xenobiotics to more water-soluble compounds (Bucheli and Fent, 1995).

Several toxic and biochemical effects of xenobiotics are mediated through the aryl hydrocarbon receptor (AhR), which is highly conserved in numerous taxa (Hahn, 1998). The ligands for AhR are hydrophobic aromatic compounds with planar structure of particular size, which fit the binding site; after binding of an agonist, AhR is translocated to the nucleus, where it forms a heterodimer with the AhR nuclear translocator protein and binds to dioxin-responsive elements in the promotor regions of certain genes, which are then upregulated (figure 9) (Fent, 2003). It has been shown that the binding strength of a ligand to the AhR is roughly directly proportional to the enhanced genes transcription and associated toxicity (Stegeman and Hahn, 1994).

Since the MFO system is sensitive to certain environmental pollutants, its activity may be used as a biological monitor for exposure to some classes of xenobiotic chemicals (Bucheli and Fent, 1995). The induction of cytochrome P450 1A1 (CYP1A1), in response to organochlorinated pollutants, such as PCBs, dioxins, DDT/DDE, and PAHs has long been considered as an adaptative process (Vasseur and Cossu-Leguille, 2003). An increased activity of the isoform CYP1A1 in vertebrates is used to identify these contaminants and demonstrate their availability (Stegeman and Hahn, 1994). In general, the structural features associated with CYP1A1 induction in fish are similar to those in mammals. However, even for the best known groups of inducers our understanding is not sufficient to identify the most important contributors to environmental induction (Van der Oost et al., 2003).

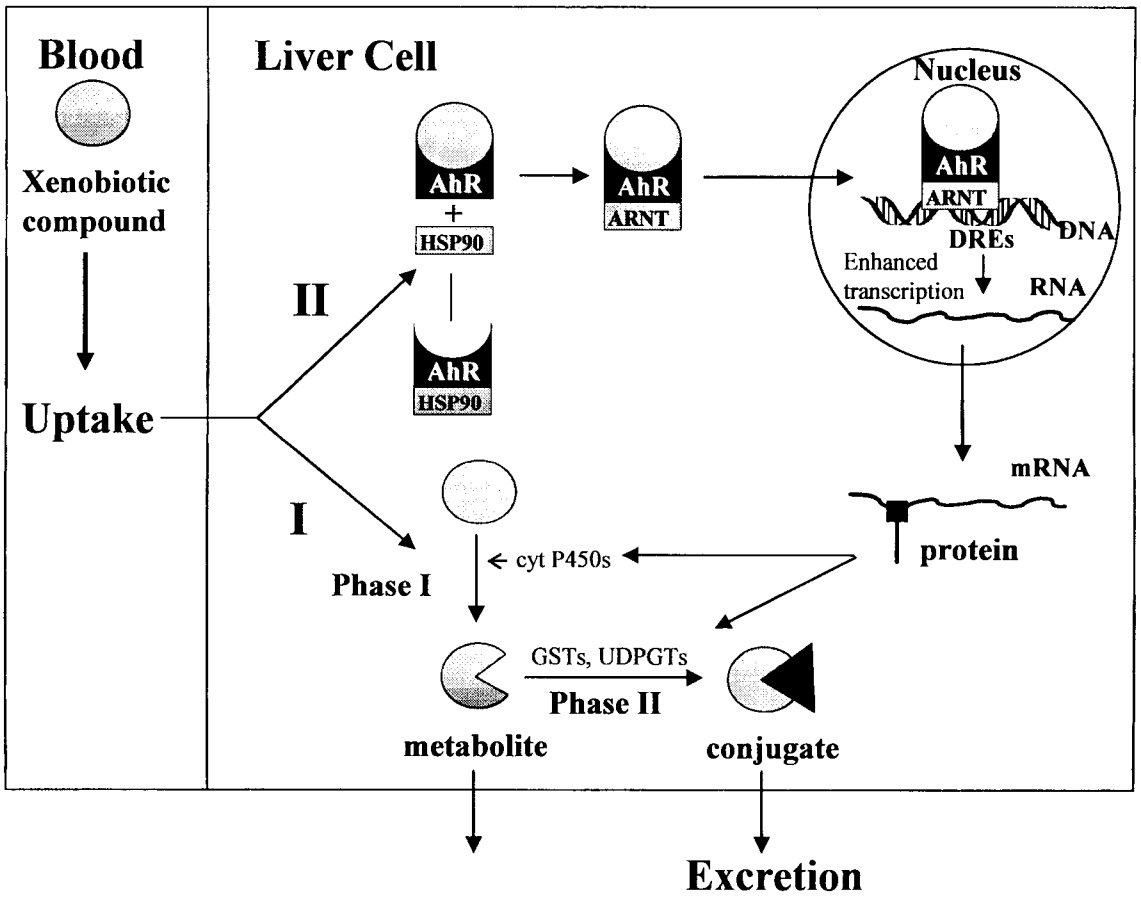


Figure 9: Simplified presentation of the fate of xenobiotic compounds in the liver cell. Route I, a possible mechanism for detoxification or toxication, and Route II, a possible mechanism for enzyme induction. AhR, aryl hydrocarbon receptor; HSP90, 90 kDa heat shock protein; ARNT, Ah receptor nuclear translocator; DREs, dioxin responsive elements; cyt P450s, cytochrome isoenzymes; GSTs, glutathione S-transferases; UDPGTs, UDP-glucuronyl transferases. Adapted from Van der Oost et al. (2003).

There are different approaches to evaluate phase I induction, like CYP1A and mRNA levels, and in addition to examine the responses of the CYP1A isoenzyme catalytic activity. The activity of ethoxyresorufin O-deethylase (EROD) appears to be the most sensitive catalytic probe for determining the inductive response of the cyt P450 system in fish (Goksoyr and Forlin, 1992). The EROD activity is measured by following the increase in fluorescence of the reaction product resorufin (Burke and Mayer, 1974). Generally, a good correlation is observed between CYP1A1 protein levels and EROD activity (Van der Oost et al., 1996a). Increases in EROD activity have been observed in many fish species after exposure to organic trace pollutants, like PAHs, PCBs, PCDDs and PCDFs that caused very strong increases (>500% of control) in CYP1A1 catalytic activities (Van der

Oost et al., 2003). Several field studies demonstrated a strong and significant increase in CYP1A protein levels and activity in many fish species from polluted environments (Van der Oost et al., 2003). But, the contrary was also true, strong and significant decreases were also observed in EROD activity in species of fish from polluted environments.

Although certain chemicals may inhibit EROD induction or activity, this interference is generally not a drawback to the use of EROD as a biomarker (Whyte et al., 2000). CYP1A catalytic activity may be used both for the assessment of exposure and as an early warning sign for potential harmful effects of many organic trace pollutants. EROD activity may not only indicate chemical exposure, but may also precede effects at various levels of biological organization (Whyte et al., 2000). Even so, EROD activity in fish liver is a very sensitive biomarker, and may thus be of great value in Environmental Risk Assessment (ERA) processes.

5.1.2 Phase II Enzymes

The second phase of metabolism involves the conjugation of the xenobiotic parent compound or its metabolites with an endogenous ligand (figure 9). Conjugations are addition reactions in which large and often polar chemical groups or compounds, such as sugars and amino acids, are covalently added to xenobiotic chemical (Lech and Vodicnik, 1985). These synthetic conjugation reactions facilitate the excretion of chemicals by the addition of more polar groups (e.g. glutathione (GSH) and glucuronic acid (GA)) to the molecule (Commandeur et al., 1995).

Some xenobiotics compounds contain the requisite functional groups for direct metabolism by conjugative phase II enzyme systems, while others are metabolised by integrated steps involving prior action of the phase I enzymes (George, 1994). Phase II enzymes can play an important role in homeostasis as well as detoxification and clearance of many xenobiotic and the major pathway is the conjugation with GSH (George, 1994). In addition to phase I CYP1A genes, the Ah gene battery also comprises phase II genes like glutathione S-transferase (GST) (figure 9) (George, 1994).

The conjugation of electrophilic compounds (or phase I metabolites) with GSH is catalysed by the GSTs. The GSTs are a multigene superfamily of dimeric, multifunctional, soluble enzymes, which occurs ubiquitously, having been identified in virtually every living species studied (Stenersen et al., 1987; Hayes and Pulford, 1995). All eukaryotic species have multiple cytosolic and membrane-bound GST isoenzymes, with distinct catalytic as well as noncatalytic binding properties. The cytosolic enzymes are encoded by at least five distantly related gene families (designated class alpha, mu, pi, sigma and theta GST)

(Hayes and Pulford, 1995). Most studies determine the total GST activity using the artificial substrate 1-chloro-2,4-dinitrobenzene (CDNB), which is conjugated by all GST isoforms with the exception of theta class (George, 1994; Van der Oost et al., 2003).

The toxicity of many exogenous compounds can be modulated by induction of GSTs. The effects of inducing agents on total GST activity in fish livers, measured by CDNB conjugation have been reported, mostly in conjugation with studies of CYP1A induction (George, 1994). Due to the role that GSTs play in conjugating reactive epoxide species and other electrophiles, induction of these enzymes must be considered to be beneficial, and metabolic activation of halogenated xenobiotics by GSTs is also well recognised (Armstrong, 1986; Commandeur et al., 1995). An increase in hepatic GST activity has been reported after exposure of fish to PAHs, PCBs, OCPs and PCDDs, but most studies did not reveal any significant alterations (Van der Oost et al., 2003).

5.2. Oxidative Stress

The normal fate of most of the molecular oxygen consumed by animals is the tetravalent reduction to water, coupled to the oxidation of substrates or biomolecules and the production of energy. Partial reduction results in the formation of Reactive Oxygen Species (ROS), it has been estimated that about 1-3 % of O_2 consumed in animal systems is converted to ROS (Halliwell and Gutteridge, 1999), such as (a) the superoxide anion radical ($O_2^{\cdot-}$), (b) hydrogen peroxide (H_2O_2), and (c) the hydroxyl radical ($HO^{\cdot}$). This later radical is an extremely potent oxidant capable of reacting with cellular macromolecules (Winston and Di Giulio, 1991).

ROS are produced in animals as unwanted by-products, from various endogenous sources and processes, including certain enzymes, auto-oxidation, haem proteins, and mitochondrial, endoplasmatic reticulum and nuclear membrane electron transport (Halliwell and Gutteridge, 1999). Of more immediate interest with respect to environmental biomarkers is the ability of a number of structurally diverse compounds to enhance intracellular oxyradical production through the process of redox cycling. Rates or amounts of ROS production can be increased by the presence of a wide range of natural and man-made xenobiotics. Redox active compounds include aromatic diols and quinones, nitroaromatics, transition metals, PAH (benzene, PAH oxidation products), halogenated hydrocarbons, dioxins, and metal contaminants (Halliwell and Gutteridge, 1999). In the redox cycle, the parent compound is typically first enzymatically reduced by a NAPDH dependent reductase to yield a xenobiotic radical. This radical donates its unshared electron to molecular O_2, yielding $O_2^{\cdot-}$ and the parent compound. Thus at each turn of the

cycle, two potentially deleterious events have occurred: a reductant has been oxidised and an oxyradical has been produced (Winston and Di Giulio, 1991). Such a diverse array of chemicals can have different mechanisms of toxicity, and several mechanisms may exist for a single contaminant, each contributing to various degrees to the final overall deleterious effect (Livingstone, 1991).

ROS produced in biological systems are detoxified by antioxidant defences, which are generally ubiquitous in animal species and different tissue-type (Livingstone, 2001). Defence systems that tend to destroy or to scanvage the radicals, to stop propagation and to prevent oxidative damage, include the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione-dependent peroxidade (GPx) and glutathione reductase (GR) (figure 10), and some molecules such as glutathione and uric acid. The mitochondria consume over 90 % of the cellular oxygen in unstressed cells and are considered the major sites of aerobic cellular ROS production (Lenaz, 1998; Han et al., 2001). Moreover, ROS are also produced by the microsomal systems of the endoplasmatic reticulum (Winston et al., 1996), and by various enzymatic reactions.

As well as tissue-types, antioxidant enzyme activities also vary with other endogenous factor such as age, and with endogenous/exogenous factors such as seasonality, reproductive cycle, and elevated temperature (Livingstone, 2001).

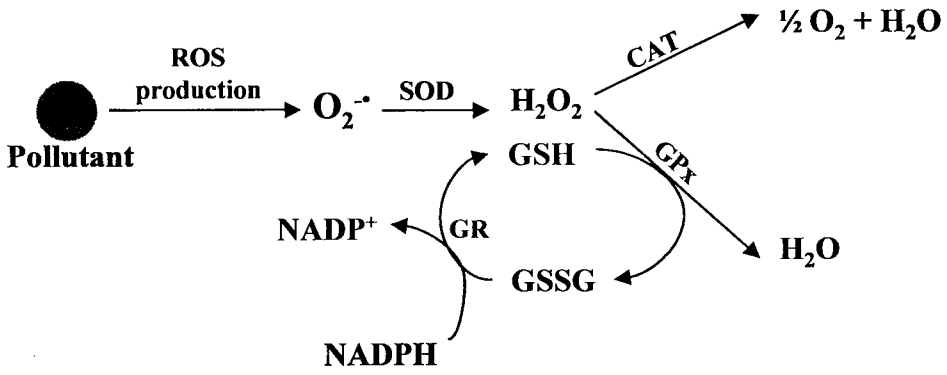


Figure 10: Antioxidant defences against ROS production due to the presence of pollutants. SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; GR: glutathione reductase.

In aquatic ecosystems, dissolved oxygen and temperature are environmental variables that are likely to influence oxidative processes (Parihar et al., 1997). These variables must be carefully controlled in laboratory experiments examining oxidative stress, and also considered in field studies including this phenomenon in aquatic species (Winston and Di Giulio, 1991).

In the normal situation, the production of ROS and other reactive species is balanced by antioxidant defence systems. However, if the equilibrium is not perfect, will lead to oxidative damage to molecules like DNA, proteins and lipids (Halliwell and Gutteridge, 1999). Subsequently, the oxidative damaged molecules are either repaired or degraded by enzymes. In aquatic organisms increased levels of oxidative damage will occur when exposed to contaminants that stimulate ROS and other pro-oxidant production (Livingstone, 2001). Increases in oxidative damage are detected in fish, for all three types of major macromolecules, by single and mixed contaminants, including redox cycling (e.g. Cu, Fe, paraquat) and non redox cycling (e.g. Cd, PAHs) contaminants. The observed oxidative damage includes lipid peroxidation, protein oxidation (non-peptide carbonyl groups) and DNA damage (8-hydroxydeoxyguanosine and other oxidised nucleotides) (Livingstone, 2001).

Oxidant-mediated effects with potential suitability as biomarkers include either adaptative responses, such as increased activities of antioxidant enzymes, or evaluation of oxidant-mediated toxicity by assessing oxidation of proteins, lipids and nucleic acids (Winston and Di Giulio, 1991; Filho, 1996). Although a significant amount of data has been collected in laboratory and field studies, so far a detailed knowledge on the regulation of antioxidant systems in aquatic organisms in relation to either endogenous sources of ROS and other pro-oxidants requires new data, and new experimental approaches (Livingstone, 2001).

5.2.1 Antioxidant Enzymes

5.2.1.1 Superoxide Dismutase (SOD)

The SODs are a group of metalloenzymes that catalyse the conversion of reactive super anions ($O_2^{\cdot-}$) to yield hydrogen peroxide (H_2O_2), an important ROS as well (eq. 1).

Equation 1: $$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$$

SODs are considered to play a key role within the primary antioxidant defences, and their importance is indicated by their presence in all aerobic organisms so far examined (Halliwell and Gutteridge, 1999). There are two forms of SOD with different metals centers, the copper/zinc SOD (CuZnSOD) and the manganese SOD (MnSOD). The CuZnSOD is mostly located in cytosol, while the MnSOD is largely (if not entirely)

located in the mitochondria, and the relative activities depend on the tissue and the species (Halliwell and Gutteridge, 1999).

Most techniques for the measurement of SOD activity are indirect assays in which an indirect scavenger competes with the endogenous SOD for $O_2^{\cdot-}$. A unit of SOD activity is defined as the amount that causes 50 % inhibition of the reduction of the scavenger under specified conditions (Stegeman et al., 1992).

Studies on the exposure of fish to contaminants have reported an increase in SOD activities, and in the field the percentage of reports showing an increase was even higher (Van der Oost et al., 2003). However, the induction of SOD activity was not a strong induction (>500% of control) in any of the studies reported.

5.2.1.2. Catalase (CAT)

Dismutation of $O_2^{\cdot-}$ generates H_2O_2, a species also generated by several oxidase enzymes *in vivo*, including xanthine and urate (Halliwell and Gutteridge, 1999). Hydrogen peroxide is usually removed in aerobic organisms by two types of enzymes (CAT and GPx). The catalases directly catalyse the decomposition of H_2O_2 to ground-state O_2, and water (eq. 2).

Equation 2: $$2H_2O_2 \rightarrow 2H_2O + O_2$$

CATs are heme-containing enzymes localised in the peroxisomes of most cells and are involved in fatty acid metabolism, and changes in activities are often difficult to interpret (Stegeman et al., 1992; Halliwell and Gutteridge, 1999). Peroxisomes contain many of the cellular enzymes that generate H_2O_2, such as glycolate oxidase, urate oxidase and flavoprotein dehydrogenases involved in the β-oxidation of fatty acids (Halliwell and Gutteridge, 1999). Although much of CAT activity detected in homogenates of animals was found not to be organelle-associated, this was probably due to the rupture of peroxisomes during the homogenization (Halliwell and Gutteridge, 1999).

Hepatic CAT activities have showed increases and decreases in studies with fish exposed to several contaminants like PCBs and PAHs. Most significant increases were seen in field studies, similar as for SOD activities, and also there were no strong increases reported (Van der Oost et al., 2003).

5.2.1.3 Glutathione Peroxidase (GPx)

Peroxidases are enzymes that reduce a variety of peroxides to their correspondent alcohols. While CAT employs one molecule of H_2O_2 as a donor in the reduction of another H_2O_2 molecule, peroxidases employ other reductants. Glutathione peroxidase removes H_2O_2 by coupling its reduction to H_2O with oxidation of reduced glutathione (GSH) (eq. 3).

Equation 3: $$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

GPx enzymes are widely distributed in animal tissues and specific for GSH as a hydrogen donor, however they can act on peroxides other the H_2O_2 (eq. 4), and in all cases the peroxide group is reduced to an alcohol.

Equation 4: $$LOOH + 2GSH \rightarrow GSSG + H_2O + LOH$$

GPxs consist of four protein subunit, each of which contains one atom of selenium (Se). GSH, the substrate for GPx is a low molecular weight thiol containing tripeptide (Stegeman et al., 1992; Halliwell and Gutteridge, 1999). The ratios of reduced to oxidised glutathione (GSH/GSSG) in normal cells are high so there must be a mechanism for reducing GSSG back to GSH (eq. 5) (Halliwell and Gutteridge, 1999).

Equation 5: $$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

GPx is considered to play an important role in protecting membranes from damage due to lipid peroxidation (LP). The major detoxification function of GPx is the termination of radical chain propagation by quick reduction (Van der Oost et al., 2003).

In comparison with SOD and CAT, GPx has also showed increases and decreases in activity, in several fish species exposed to a variety of contaminants. Similar to SOD and CAT the hepatic GPx has not shown strong increases due to exposures (Van der Oost et al., 2003). The use of this enzyme as a potential biomarker is still less explored, and the available information suggests that GPx activities in animals may be less responsive to pro-oxidants.

5.2.2. Oxidative Damages

5.2.2.1. Lipid Peroxidation (LP)

Lipid peroxidation (LP) has been defined as the "oxidative deterioration of polyunsaturated fatty acids (PUFA)". Damage to PUFAs tends to decrease membrane fluidity, which is known to be essential for the proper functioning of biological membranes (Halliwell and Gutteridge, 1999). The process of LP proceeds by a chain reaction and, as in the case of redox cycling, demonstrates the ability of a single radical species to propagate in a number of deleterious biochemical reactions.

Lipids, in fish and in other organisms, are in close juxtaposition to electron transport chains and heme iron proteins, which can act as sources of radical oxygen species under normal conditions, the lipids may sustain high degrees of damage (Almroth et al., 2005).

Lipid peroxidation products are formed with the abstraction of a hydrogen atom from an unsaturated fatty acid, and double bounds are rearranged to form dienes. Attack by molecular oxygen produces a lipid peroxyradical that can abstract a hydrogen atom from an adjacent lipid to form a lipid hydroperoxide (Almroth et al., 2005). Malondialdehyde (MDA) is a well characterised oxidation product of PUFA. Oxidatively damaged molecules may themselves be removed either through elimination/repair, or through reactions with other biological molecules, e.g. MDA is a highly reactive pro-oxidant (Livingstone, 2001).

Several studies have demonstrated enhancement of LP in various tissues from fish species exposed to a variety of chemicals (Ploch et al., 1999; Romeo et al., 2000). The increase in oxidative damage with exposure to non-directly redox cycling contaminants possibly indicates that other contaminant pro-oxidant mechanisms may be occurring, such as biotransformation of PAHs to redox cycling quinones, induction of CYPs by PAHs and PCBs, auto-oxidation of CYPs in the presence of poorly metabolised PCBs, and disruption of membrane systems by lipophilic contaminants (Livingstone et al., 2000; Livingstone, 2001).

5.2.2.2. Protein Oxidation (PO)

Membrane fluidity is modified upon lipid peroxidation as well as the structure of biomolecules associated with the membrane, such as membrane proteins. A by product of LP is 4-hydroxynonenal (4-HNE) a highly oxidizable lipid that attacks proteins leading to the formation of oxidised proteins (Almroth et al., 2005). Exposure to reactive oxygen compounds leads to modification and interconversion of amino acid side chain and consequently, to alterations in the protein structure and amino acid sequence with possible scission or cross-linking (Davies, 1987).

Among the various oxidative modifications of amino acids in proteins, carbonyl formation may be an early marker for protein oxidation (Davies, 1987). This alteration was characterised as metal-catalysed oxidation of proteins, redox cycling cations such as Fe^{2+} and Cu^{2+} can bind to cation binding locations on proteins and with the aid of further attack by H_2O_2 or O_2 can transform side chains amine groups and several amino acids into carbonyls (Stadtman, 1990). The most likely amino acids residues to form carbonyl derivates are lysine, arginine, proline and histidine (Levine et al., 1994; Halliwell and Gutteridge, 1999). Protein carbonyls can also be formed via secondary mechanisms resulting from reactions of free radicals with other cellular constituents, such as lipids, carbohydrates and nucleic acids (Grune et al., 2003; Requena et al., 2003).

The formation of carbonyl derivates is non-reversible, causing conformational changes, decreased catalytic activity in enzymes and ultimately resulting in preferential target for proteolysis (Almroth et al., 2005). Protein oxidation can be increased by xenobiotic exposure (Gibson et al., 1996) and their detection can be used as a potential non specific biomarker. The carbonyls can be measured by derivatisation with 2,4-dinitrophenylhydrazine (DNPH) (Fessard and Livingstone, 1998). When coupled with imunodetection analysis, the method can be used to measure individual or groups of oxidised proteins (Keller et al., 1993), and be useful as a potential biomarker of contaminant-mediated oxidative damage in fish liver (Fessard and Livingstone, 1998).

Biotransformation enzymes, like the CYP1A, and oxidative damages can be correlated in the presence of polyhalogenated aromatic hydrocarbons (PHAH). Non-ortho-PCBs when bound to CYP1A establish conditions in the active site that favour radical formation (Schlezinger et al., 2006). PCB congeners like CB126 and CB169 stimulated the release of ROS from induced liver microsomes, and caused the inactivation of CYP1A (Schlezinger et al., 1999). The non-ortho congeners that are more toxic also are the most potent AhR agonist, and more strongly stimulates ROS release and inhibit and inactivate

CYP1A (Schlezinger et al., 2000; Schlezinger and Stegeman, 2001). Although PHAH bound to CYP1A can release ROS, other sources of free radicals may also contribute to oxidative stress caused by PHAH (Nebert et al., 2000).

5.3 Limitations of Biomarkers

Biological monitoring may be performed at a number of levels, from the organism to a subcellular level. Biomarkers sensitive to early detection of degradation of water quality can be used (Allan et al., 2006). A successful implementation of biomarkers in environmental monitoring programmes requires a good understanding of the mechanisms underlying the responses (Van der Oost et al., 2003).

Biomarkers responses are powerful because they integrate a wide array of environmental, toxicological and ecological factors that control and modulate exposure to, as well as effects of, environmental contaminants. However these same factors may also complicate interpretation of the significance of the biomarker responses in ways that may not always be anticipated (Power and McCarty, 1997). Many non pollution related variables may have an additional impact on the various enzyme systems, and may thus interfere with biomarker responses when experimental conditions are not thoroughly analysed or controlled.

Confounding factors are, for example, the organism health, condition, sex, age, nutritional status, metabolic activity, migratory behaviour, reproductive and developmental status, and population density. There are some other non biological factors that can also affect the biomarker responses like season, ambient temperature, heterogeneity of the environmental pollution, etc (Van der Oost et al., 2003; Allan et al., 2006).

In addition, since various substances may affect the same biomarkers, most biomarkers responses are not specific for individual compounds. The use of specific biomarkers, which respond to a single class of compounds, may be an advantage in environmental monitoring. Some examples of more specific biomarkers are the measurement of the vitelloprotein, vitellogenin (Vtg), as an indicator of exposure to Endocrine Disrupting Chemicals (EDCs) (Hinck et al., 2006), and the imposex in gastropods as an indicator of exposure to tributyltin (TBT) (Santos et al., 2002; Santos et al., 2006).

The feasibility of a decrease in the levels of chemical pollutants in the environment will certainly lead to improve the status of the ecosystem. This improvement will produce

effects in the food chain and thus in human health. The decrease of pollutants release into the aquatic system, and its associated hazards, will conduct to a reduction of pollutants inputs in animals. The evaluation of the aquatic ecosystems quality, using biological and biochemical tools, will permit to monitor the health of the environment. This monitoring will provide relevant information to protect the populations from exposure, for example trough fish consumption.

OBJECTIVES

OBJECTIVES

The degradation of the aquatic environment, due to the increase of foreign chemicals has become a problem in the last decades. The presence of contaminants, even at sublethal concentrations, can induce changes at subcellular levels, as an adaptative response, by the species inhabiting those contaminated environments.

This work intended to study the accumulation of Persistent Organic Pollutants (POPs), using as models two fish species from the Douro Estuary with different life strategies that would reflect different types of exposure. As models we have selected flounder (*Platichthys flesus*), a benthonic fish, and mullet (*Mugil cephalus*) a pelagic species. Fish can be useful as sentinel to alert for the degradation of the aquatic systems before it reaches the populations levels.

In muscle and liver of both species we will measure the content in Polychlorinated Biphenyls (PCBs) and the insecticide DDT, and their metabolites, DDE and DDD. In order to evaluate exposure to Polycyclic Aromatic Hydrocarbons, we will assess the presence of Fluorescent Aromatic Compounds (FACs) in bile.

The presence of xenobiotics in the aquatic environment will induce changes and adaptations in the species. The phase I and phase II biotransformation enzymes will be assessed as biomarkers of the exposure to xenobiotics. Another feature of chemical compounds in the aquatic environment is their ability to induce oxidative stress, traduced in changes in the primary antioxidant defences and oxidative damages in macromolecules that will be evaluated as biomarkers.

In addition, this research intended to evaluate the ability of these species to recover from the damages induced by chronic exposure to sublethal concentration of contaminants. To achieve this, mullet and flounder will be allowed to depurate, during three different periods (one, four and eight months), in controlled exposure to a cleaner environment and feed with non-contaminated food. Biomarkers, biotransformation enzymes of xenobiotics and oxidative stress, will be assessed in both species before and after depuration.

Finally, this work will show River Douro estuary quality and the different patterns in POPs accumulation in both species. In addition we will be able to distinguish the differences between species and in biomarkers responses, to the exposure to contaminants. Ultimately, we can distinguish the differences in the ability to recover for the damages induced by sublethal exposure to the chemicals present in the Douro estuary.

CHAPTER 1

Organochlorine contaminants in flounder (*Platichthys flesus*) and mullet (*Mugil cephalus*) from Douro estuary, and their use as sentinel species for environmental monitoring.

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Organochlorine contaminants in flounder (*Platichthys flesus*) and mullet (*Mugil cephalus*) from Douro estuary, and their use as sentinel species for environmental monitoring

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Abstract

In order to monitor the presence of organic pollutants in Douro estuary (NW Portugal), two sentinel fish species, grey mullet (*Mugil cephalus*) and flounder (*Platichthys flesus*), were periodically sampled from May 2001 to April 2002. At all sampling periods, several specimens of both species were allowed to depurate in clean sea water. Levels of PCBs and DDTs were quantified in liver and muscle of both species. The accumulation of PCBs was higher in muscle of mullet, with a maximum of 345 ng/g dw, than in flounder, with a maximum 52 ng/g dw. In the liver, flounder showed the highest levels (811 ng/g dw). Of the 18 congeners analysed, CBs 180 (hepta), 153 and 138 (hexachlorobiphenyls) were predominant in the tissue of both species. The maximum concentration of tDDT was measured in flounder liver (301 ng/g dw). In contrast, a 10-fold higher tDDT was recorded in mullet muscle (63–69 ng/g dw and 8–16 ng/g dw for mullet and flounder, respectively). The hepatic ethoxyresorufin *O*-deethylase (EROD) activity was periodically determined. Consistently, a 10-fold higher enzymatic activity was present in mullet in comparison with flounder (1536 pmol/min/mg protein in mullet and 156 pmol/min/mg protein in flounder). In mullet, no correlation could be found between EROD activity and gonado-somatic index (GSI) or hepato-somatic index (HSI). On the contrary, during the reproductive season, female flounder showed a negative correlation between EROD activity and GSI. Despite being in clean sea water for 1 month period, no significant decrease in the tissue content of PCBs and tDDT was found. However, mullet's EROD activity followed a clear pattern, with a decrease enzymatic activity after being in captivity. Female flounder displayed a similar trend during the resting season. Yet, during the reproductive season, an increased EROD activity was recorded after being in captivity for 1 month, which may be associated with a modulation effect of steroids on CYP 1A1. Histological analyses of gonad revealed that 21% of male mullet displayed testis-ova, while no male flounder was found to show gonadal abnormalities. Overall,

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the study reports the accumulation of significant levels of PCBs and DDTs in the tissues of the studied species. It also provides important evidences supporting the use of grey mullet as a sentinel species for monitoring the presence of organic contaminants and xeno-estrogenic pollution in southern European estuaries.

Keywords: Organochlorine; Cytochrome P450; Flounder; Mullet; Environmental monitoring

1. Introduction

A large number of studies have demonstrated that many organic compounds are present in the marine environment, the highest concentrations being often detected in estuaries and coastal areas. As some of these chemicals are not readily degraded by physical, chemical or biological processes, they persist in the environment for long periods adsorbed to the sediments, in the water surface microlayer and in the tissue of marine organisms. Among a large number of man-made chemicals, organochlorines such as polychlorinated biphenyls (PCBs) have been widely used by a large variety of industries over the past 50 years (McManus et al., 1983). However, studies have demonstrated that they are toxic to a variety of marine organisms (Safe, 1984, 1990). Additionally, PCBs biomagnify through the food chain and residues are prevalent in humans, fish and wild life (Bergman et al., 1994; Jacobson et al., 1989). As detoxification of endogenous waste products and xenobiotics in fish, as in all other vertebrates, is carried out primarily in the liver, pathological liver alterations have been reported in fish exposed to these organic compounds (Grinwis et al., 2000). Disturbance on reproduction and thyroid metabolism from PCB exposure have been demonstrated (Tryphonas, 1995; Gray et al., 1993), their toxicity and modes of action being associated with the degree of chlorination and molecular structure (Safe, 1984, 1990).

The 1,1,1-trichloro-2 (*p*-chlorophenyl) 2-(*o*-chlorophenyl) ethane (DDT) is a chlorinated pesticide widely used in the past to control the spread of arthropod borne diseases and agricultural pests (You, 2000). DDT is metabolised slowly in the body and the metabolite DDE the 1-1 dichloro-2,2-bis (*p*-chlorophenyl) ethylene is particularly persistent. DDE has the ability to alter sexual development because it acts as an androgen receptor antagonist, interfering with the binding of testosterone to the androgen receptor (You, 2000; Kelce et al., 1995). Recent data indicate that DDE is still detectable in a large proportion of the population, even in developed countries where the ban has been in effect for a long time (Harris et al., 1999). In fish, DDT and metabolites interfere not only with steroid receptors, but also with cytochrome P450 system leading to disturbance of steroid metabolism (Goksøyr and Förlin, 1992; McKinney and Waller, 1994).

The induction of certain CYP 450 isoforms has become an important tool for monitoring environmental exposures of fish to organic compounds (Hodson et al., 1991). Among these, cytochrome P450 1A1 (CYP 1A1) a isoenzyme involved in the metabolism of endogenous lipophilic compounds as well as different xenobiotics including PCBs, has been largely used as a biomarker of exposure to organic compounds (Bergman et al., 1994). Ethoxyresorufin *O*-deethylase (EROD) activity is CYP1A1 dependent, and therefore was measured in this study as a biomarker of CYP1A1 induction. As PCBs and organochlorinated pesticides are efficiently sequestered in lipid-rich tissues of aquatic organisms, the percentage of lipids in the body play an important role in PCBs and DDT/DDE bioaccumulation (Addison, 1982; Kawai et al., 1988). Hence, the consumption of fish from contaminated areas may be a significant human dietary source for these chemicals (Safe, 1998; Schlummer et al., 1998).

The purpose of this study was to determine the induction of the hepatic microsomal CYP 1A1 in two representative fish species of Douro estuary, and to evaluate whether 1 month depuration in clean seawater would have any measurable effects in EROD activity. The analytical determination of chlorinated compounds (PCBs and DDTs) was performed in the liver and muscle to ascertain whether the two fish species showed a different accumulation pattern. Flounder (*Platichthys flesus*) and mullet (*Mugil cephalus*), both eurihyaline fish species, were selected as the sentinel species, because flounder is an estuarine/coastal species that resides for the majority of the year in

estuaries, only migrating to deeper water in the winter (Hylland et al., 1996) and it feeding preferences make it particularly vulnerable to sediment associated pollution. Mullet was chosen because it possesses several characteristics required in an estuarine sentinel species, such as the extreme salinity tolerance, but also because it is very common in southern European estuarine systems, and therefore may be a good tool for monitoring programs.

2. Materials and methods

2.1. Study area

The present work was carried out in the lower Douro estuary. The Douro is one of the longest rivers in the Iberian Peninsula (930 km), sharing its 98.000 km^2 of watershed with Spain and Portugal. It drains into the Atlantic Ocean at 41°08′N and 8°42′W, near Porto. Domestic sewage as well as industrial effluents are still discharged, mostly without treatment, directly into the estuary and its tributaries.

2.2. Sampling

Mullet and flounder were caught in Douro estuary from May 2001 to May 2002. A total of 96 mullets (43 females and 53 males) and 109 flounders (73 females and 36 males) were sampled. In each sampling campaign, six animals from both species (group I) were sacrificed within 24 h after capture. Body, liver and gonads were dissected, weighted and the hepato-somatic (HSI), and gonado-somatic indices (GSI) calculated. Gonads were directly placed in Bouin's fixative prior to histological analysis. The remaining liver and small pieces of muscle were frozen in liquid nitrogen and stored at −80 °C until they were assayed for EROD activity and the concentrations of polychlorinated compounds determined.

At each sampling, specimens of both species (Group II) were allowed to depurate separately for 1 month in 3000 L tanks at a salinity of 20‰ with a flow rate of 5 L/min. Water was continuously filtered through an extensive biological filter, and a charcoal filter before being recycled. Aeration was provided in the tanks to maintain 100% oxygen saturation in the water. Fish were maintained in natural photoperiod and the temperature the animals experienced in captivity was similar to the observed in the Douro Estuary. The animals were feed with fresh fish (hake) which was also analysed, as for muscle and liver, and did not revealed any detectable levels of these compounds.

2.3. PCBs and DDTs measurements

PCBs and DDTs were analyzed in composite samples of muscle and liver of fish captured in Douro estuary in May and in individual fish in June and September 2001. Samples were Soxhlet extracted with hexane for 6 h. Fat content was determined gravimetrically from aliquots of the extracts and the remaining extracts were cleaned-up with Florisil and sulphuric acid. After concentration, each sample was injected into a Hewlett-Packard 5890 series II gas chromatograph equipped with an electron capture detector. A DB-5 (J&W Scientific, Folsom, CA) capillary column (60 m × 0.25 mm i.d., 0.25 μm film thickness) was used. The column was held at 60 °C for 1 min, then programmed in three levels: at a rate of 20 °C min^{-1} to 210 °C (8 min); 2 °C min^{-1} to 250 °C (17 min) and 4 °C min^{-1} to a final temperature of 260 °C (15 min). The injector temperature was kept at 270 °C and the detector was maintained at 320 °C. Helium and argon:methane were used as the carrier and the make up gases, respectively. A mixture of 18 individual CBs (IUPAC Nos. 18, 26, 52, 49, 44, 101, 118, 105, 151, 149, 153, 138, 128, 187, 183, 180, 170, 194), *p,p′*-DDE, *p,p′*-DDD and *p,p′*-DDT was used as external standard for quantification.

The recovery of the Florisil column was evaluated with a standard solution and more than 85% of each compound was obtained. Procedural blanks were also analyzed in each batch of 10–16 samples.

2.4. EROD activity

Livers were homogenized in ice-cold buffer (50 mM Tris–HCl, pH 7.4, containing 0.15 M KCl). Microsomes were prepared in resuspension buffer (50 mM Tris–HCl, 1 mM NaEDTA, pH 7.4, 1 mM dithiothreitol, 20% (v/v) glycerol), and were obtained by centrifugation of the 10,000 rpm supernatant at 20,000 rpm for 90 min in a SIGMA 3K30 centrifuge. The obtained pellet was resuspended, washed in resuspension buffer, and spun down at 20,000 rpm for 120 min (Fent and Bucheli, 1994). Microsomes resuspended in EDTA-

free resuspension buffer were stored at −80 °C until use. The ethoxyresorufin *O*-deethylase (EROD) activity was evaluated by the fluorimetric method described by Pacheco and Santos (1998).

2.5. Gonad histology

Once fixed in Bouin's fixative and embedded in paraffin, 5 μm-thick histological sections of gonad tissue were made and stained hematoxilin-eosin.

2.6. Statistical analysis

Differences between groups were tested using a one-Way ANOVA with a multiple comparison test (LSD) at a 5% significant level. Some data had to be logarithmized in order to fit ANOVA assumptions. All testes were performed using the software Statistica 5.0 (Statsoft, Inc., 1995).

3. Results

3.1. Biological parameters

Table 1 shows the gonado-somatic index (GSI) and hepato-somatic index (HSI) for mullet at capture day (Group I) and after 1 month in clean seawater (Group II). Consistently, both males and females showed a low GSI during the sampling periods, the lowest values being observed in May (0.55 ± 0.04 and 0.14 ± 0.04 for females and males, respectively). Additionally, no significant variation after 1 month in captivity was observed for GSI. For female flounder, a seasonal difference was recorded (Table 2) with higher GSI values being found in January and February (11.1 ± 4.6 and 21.0 ± 6.4, respectively), corresponding to a maturation phase of the ovary. The male's GSI (maximum 2.28 ± 0.47), also increased in the same annual period (December to February). HSI showed a similar trend, with higher values observed in January and February, the period of the maximum development of ovaries and testis.

3.2. PCBs and tDDT levels

The 18 analysed PCB congeners (Fig. 1), tPCBs and DDT compounds (Tables 3 and 4) were detected in all samples of muscle and liver of mullet and flounder. The PCB and DDT patterns remained highly similar in the two species irrespective of tissues type or sampling period, thus concentrations could be evaluated on the basis of tPCB, calculated as the sum of individual CBs levels and tDDT, as the sum of concentrations of *p,p'*-DDE, *p,p'*-DDD and *p,p'*-DDT. In mullet CBs 180 (hepta), 153 and 138 (hexachlorobiphenyls) were

Table 1
Female and male GSI, and HSI in mullet at capture day (Group I) and after 1 month in captivity (Group II)

Month of capture	Group	*n*	GSI female (%)	GSI male (%)	HSI female (%)	HSI male (%)
May 2001	I	6	0.55 ± 0.04 (a)	0.14 ± 0.04 (a)	1.71 ± 0.01	1.91 ± 0.09
	II	6	1.33	1.31 ± 0.15	1.33	1.31 ± 0.15
June 2001	I	5	1.70 ± 0.11 (b)	2.07 ± 0.14 (b)	1.70 ± 0.11	2.07 ± 0.14
	II	6	1.74	1.20 ± 0.16	1.73	1.20 ± 0.16
September 2001	I	6	1.62 (b)	1.84 ± 0.16 (b)	1.62	1.84 ± 0.16
	II	7	1.20 ± 0.18	1.29 ± 0.18	1.20 ± 0.18	1.29 ± 0.18
November 2001	I	6	1.54 ± 0.32 (b)	1.28 ± 0.17 (b)	1.54 ± 0.32	1.28 ± 0.17
	II	6	1.53 ± 0.45	1.70 ± 0.23	1.53 ± 0.45	1.70 ± 0.23
January 2002	I	6	2.00 (b)	1.48 ± 0.10 (b)	2.00	1.48 ± 0.10
	II	6	1.20	1.30 ± 0.10	1.20	1.29 ± 0.10
February 2002	I	6	1.41 ± 0.20 (b)	1.43 ± 0.13 (b)	1.41 ± 0.20	1.43 ± 0.13
	II	6	1.06 ± 0.13	1.35 ± 0.11	1.06 ± 0.13	1.33 ± 0.11
May 2002	I	6	1.75 ± 0.08 (b)	1.70 (b)	1.75 ± 0.08	1.70
	II	6	1.20 ± 0.21	1.21 ± 0.20	1.20	1.21 ± 0.20

Dissimilar letters denote significant differences ($P < 0.05$) between months, while, *, denote significant differences ($P < 0.05$) between groups.

Table 2
Female and male GSI, and HSI in flounder at capture day (Group I) and after 1 month in captivity (Group II)

Month of capture	Group	n	GSI female (%)	GSI male (%)	HSI female (%)	HSI male (%)
May 2001	I	4	1.87 ± 0.84 (a)	0.08	1.52 ± 0.21 (a.c)	1.25 ± 0.23
	II	6	2.41 ± 0.90	0.35	0.83 ± 0.07	0.76
June 2001	I	5	1.25 ± 0.17 (a)	0.18	1.43 ± 0.13 (a, c)	0.81
	II	5	0.74 ± 0.32	0.05 ± 0.02	1.06 ± 0.02	0.86 ± 0.14
September 2001	I	6	1.03 ± 0.05 (a)	0.07 ± 0.01	1.19 ± 0.30 (a, c)	1.51 ± 0.10
	II	7	1.27 ± 0.07	0.08 ± 0.01	1.22 ± 0.16	0.86 ± 0.18
November 2001	I	6	1.42 ± 0.09 (a)		1.20 ± 0.11 (a, c)	
	II	6	2.44 ± 0.37	0.21 ± 0.01	1.30 ± 0.28	0.72 ± 0.06
December 2002	I	6	3.76 ± 2.11 (a)	2.28 ± 0.47	1.63 (a)	0.87 ± 0.06
	II	6	2.80 ± 0.34	1.82 ± 0.61	0.91 ± 0.06	0.75 ± 0.04
January 2002	I	6	8.48 ± 1.97 (b)		2.13 ± 0.24 (b, c)	
	II	6	11.1 ± 4.60	1.47 ± 0.15	1.18 ± 0.01	0.93 ± 0.12
February 2002	I	6	13.44 ± 1.74 (c)	0.93	2.01 ± 0.09 (c)	1.19
	II	6	21.03 ± 6.40	1.74 ± 0.15	0.98 ± 0.14	0.88 ± 0.09
April 2002	I	6	3.95 ± 1.17 (a)		1.45 ± 0.13 (a)	
	II	6	2.16 ± 0.27	0.21	0.92 ± 0.20	0.67

Dissimilar letters denote significant differences ($P< 0.05$) between months, while, *, denote significant differences ($P< 0.05$) between groups.

predominant and accounted for 55–57% of tPCBs; in flounder CBs 153 and 138 were the most abundant representing 41–44% of the tPCB.

The mean concentrations in dry weight and lipids basis of tPCB for both species in the three sampling periods are represented in Table 3. The maxima accumulation of PCBs was higher in muscle of mullet (344.4 ng/g dry wt and 4564.1 ng/g lipids), than in flounder (51.7 ng/g dry wt and 1126.0 ng/g lipids). However, high standard deviation variability was observed, particularly in mullet, suggesting a high mobility of this species in the estuary and different feeding strategies. In the liver, when the concentration was represented in the basis of dry weight, flounder presented higher levels in all samples (415.1–810.9 ng/g in flounder; 190.3–686.4 ng/g in mullet), although

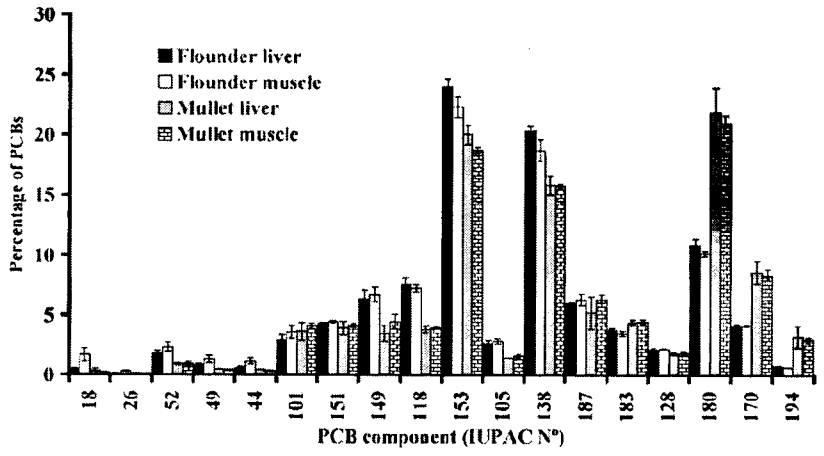

Fig. 1. Percentage of polychlorinated biphenyls (PCBs) in flounder and mullet, liver and muscle. Values are given as mean ± S.E.

Table 3
Concentrations of tPCB and lipids percentage in mullet and flounder, liver and muscle (ng/g), in a dry weight basis (dw)

Month	Species	n	Lipids (%)		tPCB (ng/g DW)	
			Liver	Muscle	Liver	Muscle
May 2001	Flounder	5	44.9	4.5 ± 0.6	749.6. (1669.6)	51.7 ± 8.7 (1126.0±92.1)
	Mullet	5	22.7 ± 2.3	8.0 ± 3.0	686.4 ± 77.7 (3212.5±530.1)	310.7 ± 57.5 (4564.1±512.7 [a])
June 2001	Flounder	5	53.6 ± 5.9	4.6 ± 0.8	810.9 ± 84.0 a (1533.2±116.7)	40.8 ± 3.7 (969.1±84.0)
	Mullet	4	24.5 ± 5.4	11.3 ± 1.8	577.1 ± 99.4 (2421.5±397.6)	324.4 ± 52.6 (2913.7±523.1 [a])
September 2001	Flounder	5	46.7 ± 4.5	4.0 ± 0.6	415.1 ± 27.1 b (946.9±97.8)	23.3 ± 1.7 (615.9±54.0)
	Mullet	4	12.7 ± 2.4	17.7 ± 5.7	190.3 ± 21.0 (1452.0±60.6)	344.4 ± 13. 5 (1372.2±306.8 [b])

Values in parentheses represent ng of tPCB/g of lipid. Values given as mean ± S.E.; dissimilar letters denote significant differences ($P < 0.05$) between months.

Table 4
Concentrations of tDDT and lipids percentage in mullet and flounder, liver and muscle (ng/g), in a dry weight basis (dw)

Month	Species	n	Lipids (%)		tDDT (ng/g DW)	
			Liver	Muscle	Liver	Muscle
May 2001	Flounder	5	44.9	4.5 ± 0.6	300.3 (668.9)	14.4 ± 1.7 (316.6±8.0 [a])
	Mullet	5	22.7 ± 2.3	8.0 ± 3.0	114.6 ± 3.3 a (518.8±38.4 [a])	64.7 ± 15.6 (798.3±66.9 [a])
June 2001	Flounder	5	53.6 ± 5.9	4.6 ± 0.8	301.0 ± 24.2 (568.8±46.6)	15.9 ± 1.4 (377.6±32.1 [a])
	Mullet	4	24.5 ± 5.4	11.3 ± 1.8	137.8 ± 15.7 a (562.0±29.2 [a])	63.1 ± 4.1 (607.0±43.4 [a])
September 2001	Flounder	5	46.7 ± 4.5	4.0 ± 0.6	159.4 ± 6.9 (351.3±19.6)	8.3 ± 0.6 (214.4±12.0 [b])
	Mullet	4	12.7 ± 2.4	17.7 ± 5.7	39.2 ± 2.6 b (321.2±16.5 [b])	69.4 ± 21.2 (291.7±55.3 [b])

Values in parentheses represent ng of tDDT/g of lipid. Values given as mean ± S.E.; dissimilar letters denote significant differences ($P < 0.05$) between months.

statistical differences were only observed in September. However, when related to lipids, higher mean levels were observed in mullet (1452.0–3212.5 ng/g lipids), although differences were not statistically significant. The major metabolic product of DDT, *p,p'*-DDE represented more than 69% of the tDDT, in the liver and muscle of both species. The highest mean concentration of tDDT was measured in flounder liver

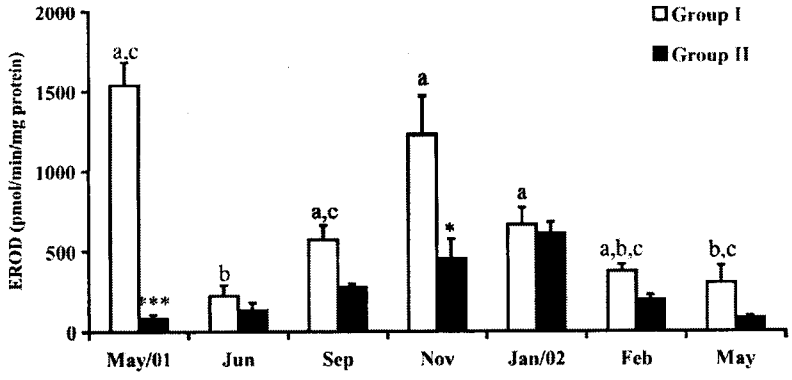

Fig. 2. Ethoxiresorufin *O*-deethylase (EROD) activities of liver microsomes of mullets at capture day (Group I) and after 1 month in captivity (Group II). Enzyme activities are given as mean ± S.E. (*, ***) Significant differences between Group I and Group II. Different letters represent significant differences ($P < 0.05$) between Group I.

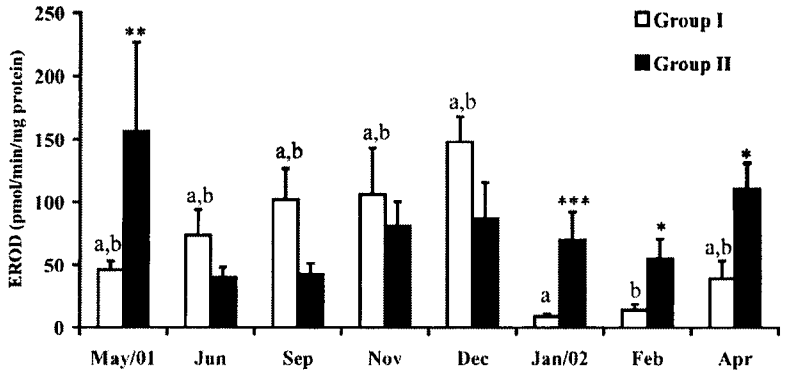

Fig. 3. Ethoxyresorufin *O*-deethylase (EROD) activities in liver microsomes of flounder at capture day (Group I) and after 1 month in captivity (Group II). Enzyme activities given as mean ± S.E. (*,**,***) Significant differences between Group I and Group II. Different letters represent significant differences ($P < 0.05$) between Group I.

(301.0 ng/g dw), nevertheless this difference is less evident when calculated in a lipid basis, indicating that these results are due to an increase in mullet lipid content (24.5% of lipids and 53.6% of lipids in flounder and mullet, respectively). After 1 month in clean seawater the levels of tPCBs and tDDT in muscle and liver remained at the same levels for both species.

3.3. EROD activity

EROD activity for both species is displayed in Figs. 2 and 3; at all sampling periods, mullet showed a mean EROD activity ten fold higher in comparison with flounder. Considering the seasonal EROD activity, mullets collected in June showed the lowest enzymatic activity (220.4 ± 69.7 pmol/min/mg) protein) which was significantly lower than those recorded in May, September, November and January (Fig. 2). No correlation between EROD and GSI was found. Mullets kept for 1 month in captivity presented lower EROD activity than at the capture day, but significant differences were found only in May and November (Fig. 2). For flounders, the lower EROD activities were observed in January and February (8.9 and 14.1 pmol/min/mg protein, respectively) (Fig. 3). Unlike mullets, flounder's EROD activity from Group I was negatively correlated with GSI ($R^2 = 0.68$). Additionally, animals kept in captivity did not show a consistent pattern: from June to December they showed a decrease EROD activity after 1 month in clean sea water. On the contrary, from January to May the EROD activity increased in comparison to the sampling day, probably due to differences in the maturation stages, which may modulate EROD activity.

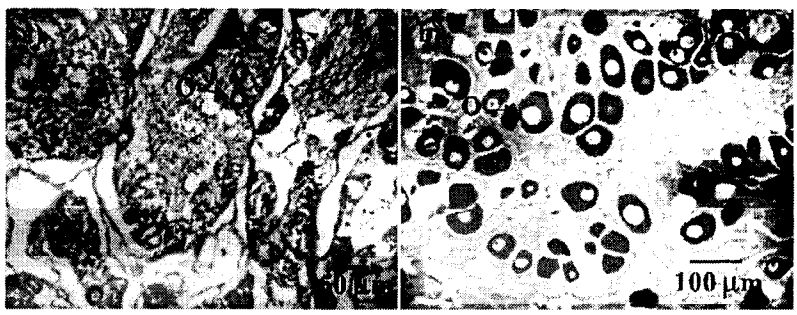

Fig. 4. Testicular sections showing testis-ova. (a) Oocytes in a pre-vitellogenic phase, and (b) oocytes in an early vitellogenic phase, within the testicular tissue.

3.4. Gonad histology

Flounder females and males presented gonads in early development stages in November and December. Mature fish were captured in January and February. Mature mullets were not found during this study. Male mullet (21%) showed an intersex condition, the testis-ova (Fig. 4a), characterised by the presence of oocytes in the testis. In May and June the histology showed follicular cells, spermatogonia and a few oocytes in a previtellogenic stage. In November a large number of spermatocytes, spermatydes, spermarozoa and a high number of vitellogenic oocytes were observed (Fig. 4b).

4. Discussion

Considering the levels of organic compounds in animal tissues, the eighteen analysed PCB congeners and DDT compounds were detected in most samples of muscle and liver of grey mullet and flounder. For each sampling period, the PCB and DDT patterns remained highly similar in the two species irrespective of tissue type. The difference in muscular concentration of tPCB between both species may be associated with a different lipid distribution in the body: over the studied period, mullet showed a lipid content approximately three times higher when compared with flounder ($17.7 \pm 5.7\%$ and $4.6 \pm 0.8\%$ for mullet and flounder, respectively), which may contribute for a higher accumulation of contaminants in mullets. PCBs concentration in muscle (Table 3) was two to four times higher in mullet than in flounder (310–344 ng/g dw versus 23–52 ng/g dw). On the lipids basis the differences are less evident. On the contrary, the levels of PCBs in the liver showed a different pattern when reported to dry weight, the highest concentrations being found in flounders, although when calculated on the basis of lipids the higher concentrations were again found in mullet. This may be associated with the fact that flounder, besides showing a high liver lipid content, is a lean fish, while mullet have their main fat reservoirs peritoneally and subcutaneously. Similar species-differences in tissue distribution have been reported for the CB-105 orally administrated to cod and rainbow trout (Bernhoff et al., 1994), the results revealing high concentrations of this congener in the liver and brain of cod, while in rainbow trout the distribution was mainly confined to extrahepatic fat depots. Additionally, the lower potential for induction of the hepatic xenobiotic metabolising enzyme systems in cod, was explained by differences in distribution of lipids in the body. In an environmental study using three estuarine animals from Weser estuary, Goerke and Weber (2001) found that in *P. flesus* the concentration pattern of CBs 44, 52, 87, 95, 101, 153 was very similar to that of Clophen A60. Likewise, our study shows that both studied species have similar percentages of congeners 44, 52 and 101, 153, with flounders displaying the highest concentrations of hexachloro congeners 153 and 138. Porte and Albaigés (1993) also reported the penta-, hexa-, and hepta-chlorobiphenyls as the dominant components in marine samples from the Mediterranean coast, with the CB 138 as the major component in the different organisms, followed by CB153 in mussel and red mullet. The congeners 118 (2,3′,4,4′5-pentachlorobiphenyl) and 105 (2,3,3′,4,4′-pentachlorobiphenyl) considered by EPA (US Environmental Protection Agency) as 2 of the 12 "Dioxin-like" congeners have also been detected in both species, the higher levels being found in flounder tissues (CB105: 2.7–2.8%; CB118: 7.3–7.6% of tPCBs for flounders and CB105: 1.4–1.6%; CB118: 3.8–3.9% for mullets); for the studied species, no differences in tissue concentration of CB 105 and CB 118 were observed (Fig. 1). Since the two species were collected in the same area, the higher levels of PCBs in mullet tissues can be imputed to the distinct feeding strategies or life style, which leads to a high uptake of contaminants in comparison with flounder. Alternatively, it could also be related with difference efficiency in the metabolism/excretion processes. Other studies using several edible marine species from the Adriatic sea (Bayarri et al., 2001) demonstrated that the highest accumulation of PCBs were found in makerel (17.7–32.4 ng/g fw), followed by red mullet (8.1–9.8 ng/g fw) and anchovy (6.4–11 ng/g fw). In the present study only mullet showed comparable values both in muscle and liver, when calculated in the basis of fresh weight. Over all, high levels of hexa, hepta and penta congeners were found in the different marine species studied, with a clear predominance of the hexachlorinated isomers. The individual PCB congener's distribution observed in mullet and flounder from Douro estuary is in accordance with earlier

published results obtained in marine invertebrates and vertebrates colleted in polluted areas, with the congeners 101, 118, 138,153, 170, 180, 187 as the major components of the mixture (Porte and Albaigés, 1993; Goerke and Weber, 2001; Řekula, 2001; Bayarri et al., 2001).

The higher levels of tDDT were detected in flounder liver, but when concentrations were expressed on a lipid basis, the species differences were less evident, indicating that much of the trend of increasing dry weight results from the coincident increased lipid content. In flounder, lipid content explains 71% of the variability in tPCB concentrations and 80% of the tDDT and in mullet 71% of the tDDT and only 46% of the tPCB, probably due to the high mobility and influence of contrasting weight sources. On the contrary, DDT concentrations in muscle show a different pattern: mullet show approximately a two fold higher DDT levels than flounder (63–69 ng/g dw; 291–798 ng/g lip and 8–14 ng/g dw; 214–377 ng/g lip, respectively).The levels found in this study (15.5–17.1 ng/g fw in mullet) are similar to DDE concentrations in red mullet (8.1–9.8 ng/g fw) from the Adriatic sea (Bayarri et al., 2001), but lower than those measured in the same species from the Spanish Mediterranean coast (ranging from 12.6 to 31.6 ng/g fw) (Sanchez et al., 1993). The maximum concentrations of both tPCB (1135 ng/g dw) and tDDT (397 ng/g dw) detected in our study were one to two orders of magnitude higher than values observed in golden mullet from Ria de Aveiro (80 and 35 ng/g, respectively), a coastal lagoon in the NW Portugal, surrounded by industries (Antunes et al., 2001).

Although DDT has been banned in European countries since 1970s, its degradation products are still present in all ecosystems (Wiktelius and Eduards, 1997), and although the main metabolite *p,p'*-DDE, has lost the insecticide effect, it is able to impair the reproduction of many animals (Blus, 1995). Maximum residue limits for tDDT and their metabolites in some foods of animal origin (meat, milk and eggs) were established by the European Union, but not yet for these organic compounds in fish. The established limit in those animal products is 1000 ng/g on a lipid basis (Directive 86/363/EC and later modifications). Our results for both marine species are well below this level.

Liver microsomal EROD activity was in all sampling periods higher in mullet than in flounder. In our study, the highest enzymatic activity measured in a mullet sample (1536 pmol/min/mg protein), is at the same order of magnitude of that reported by Arinç and Sen (1999) for mullet *Lysa saliens* (1293 pmol/min/mg protein) and for common sole *Solea vulgaris* (2000 pmol/min/mg protein) collected along the Izmir Bay in Turkey, and may reflect the exposure to organic compounds such as PAHs, PCBs (Elksus et al., 1989; Arinç and Sen, 1994; Livingstone et al., 1997; Van der Oost et al., 2003). For flounder, EROD activity correlated negatively with GSI (R^2 = 0.68). This negative correlation could be imputed to the modulating effect that estrogens appear to have on the expression of cytochrome P4501A1 in fish. In an in vitro study using rainbow trout liver culture cells, the lowest EROD activity was found in the presence of higher levels of 17β-estradiol (16 ngE2/ml plasma) (Navas and Segner, 2000). In our study, the observed increase in EROD activity when flounder were maintained in clean seawater during 1 month (January, February and March), was followed by an increase on the GSI and an increase maturation of gonad. These results further demonstrate that the reproductive status of female flounder may interfere with EROD activity, which limits the use of this enzymatic activity in female flounder as a biomarker of exposure to organic compounds.

In spite of the observed high EROD activity in male and female mullet, the GSI was always low (2.0 and 2.1), both females and males showing only early stages of gonad development. Consequently, the high EROD activity in mullet was most likely not affected by an active steroidogenesis, but only attributed to the exposure to a variety of contaminants including the polychlorinated compounds. This is further supported by the observed decrease in EROD activity of wild specimens that were kept in clean seawater for 1 month. In a reproductive behaviour study, Chubb et al. (1981) described that mullet spawn at the sea during the cold months, and juveniles after entering the estuary remain there before returning to spawn at the sea at age 1+ (length 200–300 mm) or 2+ length >400 mm). Based on this we assumed that in our study the specimens were caught before the migration to the sea, and therefore the final development of the gonads will be attained far from the coast.

Despite the decrease on EROD activity after 1 month in captivity, tissue levels of the polychlorinated compounds did not change over this period. Therefore, this

decrease in the enzymatic activity may be due to the presence of other EROD inducers, which are easily metabolised, such as PAHs (Billiard et al., 2002).

Our previous study, aimed at evaluating the presence of endocrine disruption in male fish from the Douro estuary. Ferreira et al. (2002) showed that mullet males displayed testis-ova, while no male flounder was found to show gonadal abnormalities typically associated to xenoestrogens exposure. Of the 53 mullet males examined 21% contained in the testis oocytes. In May and June, the sections of the testis showed follicular cells and spermatogonia, and a few oocytes in a previtellogenic phase. In September, a large number of oocytes in an early vitellogenic phase were present, although mature oocytes were never found (Ferreira et al., 2002). These observations let us to confirm that in Douro estuary mullet can be used as a suitable sentinel species for the presence of estrogenic compounds. In a field study conducted in Swan-Canning estuary in Western Australia, Webb and Gagnon (2002) used the EROD induction as a biomarker of exposure to xenoestrogenic compounds and sea mullet as one of the selected species.

In conclusion, this study demonstrates the presence of high levels of persistent organic compounds (PCBs and tDDT) in the tissues of mullets and flounders collected in the Douro Estuary, indicating that these populations are under sub-lethal contaminant exposure. Since grey mullet does not seem to maturate while living in the estuary, EROD activity can be used as a biomarker of organic contaminant exposure without any interference of the animal steroidogenesis. On the contrary, the evidences suggest that flounder's EROD activity is dependent on the animal's maturation stage, which limits the use of this biomarker during certain phases of the reproductive cycle. Clearly, further studies are needed to improve the understanding how steroids modulate cytochrome P450 system. In addition, the study reports testis-ova development in male mullets. Although the presence of testis-ova in males of several aquatic fish species have already been reported in many studies, almost no data is available for the Portuguese aquatic ecosystems; our results show that estrogenic compounds are present in Douro estuary and points grey mullet as a promising candidate species for monitoring the presence of xenoestrogens in southern European estuarine systems.

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CHAPTER 2

Oxidative stress biomarkers in two resident species, mullet (*Mugil cephalus*) and flounder (*Platichthys flesus*) from a polluted site in River Douro Estuary, Portugal.

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Oxidative stress biomarkers in two resident species, mullet (*Mugil cephalus*) and flounder (*Platichthys flesus*), from a polluted site in River Douro Estuary, Portugal

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Abstract

Exposure of marine animals to certain pollutants can enhance reactive oxygen species (ROS) production with subsequent damage to macromolecules and alterations in oxidant defences levels. Aimed at correlating the tissue concentration of certain contaminants (PCBs, DDT) with antioxidant defence levels and oxidative damages, two fish species with different life strategies (mullet, *Mugil cephalus*, and flounder, *Platichthys flesus*) were collected in the Douro Estuary (NW Portugal). After capture, the fish were left to depurate for 1 month in clean seawater. The levels of the two antioxidant enzyme activities revealed that they are species-dependent with mullet's livers showing higher superoxide dismutase (SOD) (13.2 ± 0.5 U/mg protein) and catalase (CAT) (15.5 ± 1.0 mmol/min/mg protein) activities than flounder (SOD: 7.9 ± 0.9 U/mg protein; CAT: 11.1 ± 0.8 mmol/min/mg protein). After 1 month in captivity the antioxidant enzymes activities in liver decreased in mullets, while for flounders the responses were not consistent because during the experimental period flounders did not ate and responses of antioxidant enzymes and oxidative damages were dependent on the fasting condition. The liver oxidative damages were evaluated by estimating oxidised lipids and proteins. Both species showed similar levels for these two parameters. The hepatic lipid peroxidation in flounder increased after 1 month in captivity, while in mullet an increase was observed only in summer and autumn. The oxidised protein content increased for both species after the depuration period.

This study reveals differences between species under oxidative stress when exposed to pollutants. In a clean environment, the mullet's primary antioxidant defences decreased indicating that the animals living in Douro estuary were facing an oxidative stress. The data indicate that, namely in mullet, the presence of pollutants induce oxidative stress responses.

Keywords: Oxidative stress; Flounder; Mullet; Contaminants

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1. Introduction

The development of anthropogenic activities is the main factor leading to the increasing levels of contaminants in the marine environment. These contaminants usually are present as very complex mixtures, and the interactive effects on the biota renders difficult to evaluate their specific effects namely by means of chemical analysis. To overcome this difficulty, the use of biomarkers can offer an integrated evaluation of the effects of pollutants in wildlife. Indeed, integrated approaches involving both chemical analyses and biomarker determinations have been recommended for biomonitoring programs (Cajaraville et al., 2000; Solé, 2000). In this work, two biomarkers have been selected, namely antioxidant enzymes and oxidative damage alterations.

Recent data indicate that the pollution toxicity in aquatic organisms may be associated to an increased production of 'reactive oxygen species' (ROS) leading to oxidative damage (Di Giulio et al., 1989; Livingstone, 1991; Livingstone et al., 1990). Under physiological conditions, cells have antioxidant defences that scavenge or prevent the generation of ROS, and repair or degrade oxidatively modified molecules (Halliwell and Gutteridge, 1999).

Changes in the levels of antioxidants have been proposed as biomarkers of contaminant-mediated prooxidant challenge in a variety of marine organisms, including invertebrates, as mussels (Regoli and Principato, 1995; Livingstone, 2001; Regoli et al., 2004), and in fish (Orbea et al., 2002; Regoli et al., 2002). Potential suitable biomarkers include either components of the oxidative adaptative responses, such as activities of enzymes or detection of oxidant-mediated damages on proteins, lipids and nucleic acids (Winston and Di Giulio, 1991; Filho, 1996). Defence systems that prevent the formation of ROS, include the antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT). Antioxidant enzymes are generally less responsive to pollutants than phase I and II enzymes, and the relationships between response and contaminant exposure are still less well established, however a significant SOD and CAT induction was observed in most of the field surveys, while most of the laboratory studies did not report any significant responses (Van der Oost et al., 2003).

In the aquatic environment, despite the presence of constitutive or enhanced antioxidant defence systems, increased levels of oxidative damage will occur in organisms exposed to contaminants which stimulate the production of ROS (Livingstone, 2001). This increased ROS production and subsequent oxidative damage has been associated with pollutant-mediated mechanism of toxicity in fish liver (Lemaire and Livingstone, 1993). Lipid peroxidation or the oxidation of polyunsaturated fatty acids are observed and has been used to analyse the effect of pollutants (Stegeman et al., 1992; Hageman et al., 1992). Indeed, it was revealed an increase in lipid peroxidation in the liver of organisms exposed to contaminants such as PAHs, PCBs and other (Livingstone et al., 1993; Di Giulio et al., 1993). Proteins constitute also a target for oxidative damage with subsequent alteration of their functions (Levine et al., 1981). In flounders, living in contaminated waters with xenobiotics, increased levels of oxidised proteins were reported (Fessard and Livingstone, 1998).

Aiming at correlating the alterations in antioxidant defences and oxidative damages with the presence of pollutants in the estuary of river Douro, we analysed these changes in two fish species, mullet and flounder. This two species were selected due to different life strategies. Flounder (*Platichthys flesus*) is a pleuronectiform species living in the sediment of coastal and estuarine areas, and as a bottom-feeding fish it is particularly vulnerable to sediment-associated chemical pollution. Mullet (*Mugil cephalus*) is a perciform species, that feeds mainly on zooplankton, benthic organisms and detritus, and was chosen because it possesses several characteristics required in an estuarine sentinel species, such as the extreme salinity tolerance, and also because it is very common in southern European estuarine systems. The levels in oxidative defences and damages were monitored after transfer of the fishes to a cleaner environment in captivity and the following parameters were analysed: antioxidant enzymes (CAT, SOD and MnSOD) and oxidative damages (lipid peroxidation and oxidised proteins) at capture and after 1 month. The purpose of this study was to evaluate oxidative stress responses in both species, due to the presence of pollutants in Douro estuary. Subsequently, the changes in the activities of key antioxidant defences and the levels of oxidative damages were analysed when the fishes were transferred to a cleaner environment.

2. Materials and methods

2.1. Study area

The present work was carried out in the lower Douro estuary. The Douro is one of the longest rivers in the Iberian Peninsula (930 km), sharing its 98 000 km^2 of watershed with Spain and Portugal. It drains into the Atlantic Ocean at 41°08′N and 8°42′W, near Porto. Domestic sewage as well as industrial effluents are still discharged, mostly without treatment, directly into the estuary and its tributaries.

2.2. Sampling

Adult mullets and flounders were captured always in the same site in Douro estuary from May 2001 to May 2002. A total of 96 mullets (43 females and 53 males) and 109 flounders (73 females and 36 males) were sampled. In each sampling campaign, six animals from both species (Group I) were sacrificed within 24 h after capture. Livers were frozen in liquid nitrogen and stored at −80 °C until they were assayed.

At each sampling, six specimens of both species (Group II) were allowed to depurate separately for 1 month in 3000 L tanks at a salinity of 20‰ with a flow rate of 5 L/min. Tanks with flounders had a 10–20 cm thick bottom sand layer. Water was continuously filtered through an extensive biological filter, and a charcoal filter before being recycled. Aeration was provided in the tanks to maintain 100% oxygen saturation in the water. Fish were maintained under natural photoperiod and temperature. Mullets were fed with uncontaminated frozen fish (hake), while flounders did not eat any kind of food (shrimps, clams, commercial pellets or fish) during the depuration period.

2.3. Biochemical analysis

Livers were homogenised in ice-cold sodium phosphate buffer 50 mM, Na_2EDTA 0.1 mM, pH 7.8. Mitochondrial fractions were obtained after centrifugation at 10 000 × *g* for 20 min.

Catalase activity was determined by measuring the consumption of H_2O_2 at 240 nm (ext. coef. 40 mM^{-1} cm^{-1}) according to Aebi (1974). The reaction volume was 1 mL and contained 67.5 mM potassium phosphate buffer, pH 7.5, and 12.5 mM H_2O_2. The reaction was started by the addition of the sample. SOD activity was determined by an indirect method involving the inhibition of cytochrome *c* reduction. In this method, SOD competes with cytochrome *c* for the superoxide anion generated by the hypoxanthine and xanthine oxidase reaction. SOD activity was determined in the mitochondrial fraction as the degree of inhibition of cytochrome *c* reduction at 550 nm (McCord and Fridovich, 1969). The concentration of the reactives was: potassium phosphate buffer 50 mM, pH 7.8, hypoxanthine 50 μM, xanthine oxidase 1.98 mU/mL and cytochrome *c* 10 μM (Orbea et al., 2002). The activity of MnSOD was evaluated adding to the reaction KCN 2 mM. The activity is given in SOD units (1 SOD unit = 50% inhibition of the xanthine oxidase reaction).

2.4. Oxidative damages

The peroxidative damage to lipids that occurs with free radical generation, and results in the production of malondialdehyde (MDA) was assessed by the determination of TBARS. MDA was determined by the thiobarbituric acid method, the liver homogenate was incubated with TCA 100%, after centrifugation the supernatant was incubated at 100 °C for 30 min, with TBA 1%, NaOH 0.05 M and BHT 0.025% (Niki, 2000). The absorbance was measured at 532 nm. Lipidic peroxidation (LP) is expressed as MDA equivalents per mg of protein.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using minigels consisting of a 4% stacking gel and 12.5% running gel. Fish liver samples were prepared to a final protein concentration of 4 μg μL^{-1} and derivatised with DNPH by incubation at dark for 30 min, a control was incubated with trifluoroacetic acid (TFA) 10% (Levine et al., 1994), without preheating the sample, 5 μL per lane were loaded in the gels. After electrophoresis, separated proteins were transferred onto nitrocellulose membranes using a HOEFER TE 22. Membranes were blocked overnight at 4 °C in phosphate-buffered saline (PBS) plus 0.05% Tween-20 (TPBS) containing 5% non-fatty dried milk. Incubations with the diluted antibody rabbit anti-DNP (1:5000) (DAKO) were performed for 1 h at room temperature. Membranes were washed and incubated with the diluted secondary antibody, anti-rabbit IgG-peroxidase (1:5000) (SIGMA) for 1 h at 4 °C. Activity

was visualised with an enhanced chemiluminescence (ECL) kit (Amersham Life Sciences).

In all assays, total protein was measured by Lowry method adapted to microplates.

2.5. Statistical analysis

Differences between groups were tested using a one-way ANOVA with a multiple comparison test (LSD) at a 5% significant level. Some data had to be log-transformed in order to fit ANOVA assumptions. All tests were performed using the software Statistica 5.0 (Statsoft, Inc., 1995).

3. Results

Weight and length from flounders after capture were, 287.2 ± 18.5 g and 28.5 ± 0.5 cm, respectively, with a condition factor (CF) of 1.19 ± 0.02. A slight decrease occurred in CF (1.09 ± 0.02) after the depuration period reflecting a weight loss due to the fact that animals did not ate. Mullets from Group I weighted 509.7 ± 25.4 g and measured 39.9 ± 0.7 cm, and in Group II the average weight was 441.0 ± 23.8 g and length was 37.9 ± 0.8 cm. Contrary to flounders in mullets, a slight increase in CF occurred after the depuration period (Group I: 0.78 ± 0.01 and Group II: 0.80 ± 0.01).

3.1. Antioxidant enzyme activities

From both species, the livers were isolated to estimate antioxidant enzymes activities (CAT, SOD and MnSOD) (Table 1). No sex-dependent differences were observed and therefore the activities from males and females were grouped for both species. At the time of capture, the enzymes activities were higher in mullets than in flounders, and mullets collected in spring revealed a CAT activity significantly higher (17.93 ± 1.86 nmol/min/mg protein), while flounders had the highest CAT activity in summer (12.46 ± 1.56 nmol/min/mg protein). In mullets a significant decrease ($P < 0.05$) in liver CAT activity in Group II was found when captured in winter. In contrast, the liver CAT activity in flounders colleted in spring and winter increased after the depuration period (in autumn CAT activity was not evaluated in Group

Table 1
Activities of antioxidant enzymes in liver of mullets and flounders at capture (Group I) and after 1 month in recovery (Group II), in spring, summer, autumn and winter

	Group I					Group II					
	Spring	*Summer*	*Autumn*	*Winter*	*Average*	*Spring*	*Summer*	*Autumn*	*Winter*	*Average*
Mullet										
CAT	17.9 ± 1.8	13.1 ± 1.4	14.1 ± 1.6	13.4 ± 0.7	15.5 ± 1.0	16.3 ± 0.4 (12.8 ↓)	12.6 ± 1.8 (4.5 ↓)	13.7 ± 1.5 (2.8 ↓)	9.8 ± 0.9* (35.1 ↓)	14.7 ± 0.9 (5.2 ↓)
SOD	13.7 ± 0.9	13.6 ± 1.4	14.2 ± 1.0	10.3 ± 0.9	13.2 ± 0.5	10.9 ± 1.0* (20.5 ↓)	10.8 ± 1.8 (20.8 ↓)	12.3 ± 0.6 (12.8 ↓)	9.0 ± 0.4 (12.7 ↓)	10.8 ± 0.6* (18.2 ↓)
MnSOD	11.1 ± 0.4	10.1 ± 0.3	11.3 ± 0.8	7.1 ± 0.2	10.1 ± 0.4	9.4 ± 0.9 (15.3 ↓)	9.8 ± 1.4 (2.6 ↓)	8.5 ± 0.8* (24.7 ↓)	6.9 ± 0.2 (2.0 ↓)	8.6 ± 0.6 (14.8 ↓)
Flounder										
CAT	11.9 ± 1.8	12.5 ± 1.6	9.5 ± 1.7	11.0 ± 1.2	11.1 ± 0.8	12.2 ± 1.5 (1.7 ↑)	10.3 ± 1.6 (17.5 ↓)	–	12.1 ± 0.5 (10.9 ↑)	11.4 ± 0.7 (2.7 ↑)
SOD	9.0 ± 1.9	7.6 ± 1.3	9.9 ± 1.0	5.2 ± 1.5	7.9 ± 0.9	9.5 ± 0.1 (5.3 ↑)	10.9 ± 0.6 (43.6 ↑)	–	6.9 ± 2.2 (33.7 ↑)	8.9 ± 1.0 (12.7 ↑)
MnSOD	3.5 ± 0.7	5.8 ± 0.1	6.2 ± 1.6	1.0 ± 0.3	4.1 ± 0.9	4.7 ± 0.1 (32.4 ↑)	7.6 ± 0.9 (29.6 ↑)	–	3.2 ± 1.5 (211.7 ↑)	5.2 ± 0.9 (26.8 ↑)

Values in parentheses represent percentage of variation. Values are presented in mean ± S.E. CAT – catalase (nmol/min/mg protein); SOD – superoxide dismutase (U/mg protein).
* $P < 0.05$: Significant statistical differences to Group I.

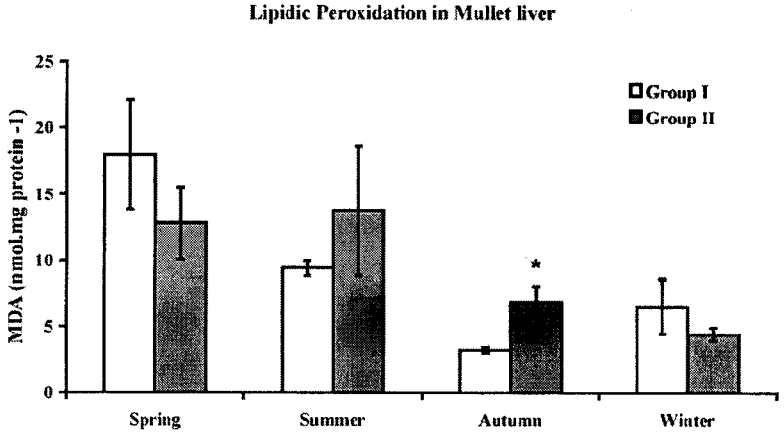

Fig. 1. Hepatic LPOX in mullets at capture (Group I) and after 1 month (Group II). Data presented as mean ± S.E. $^{*}P < 0.05$: Significant differences between Group I and Group II.

II due to limited amount of sample). Differences between species were more evident in SOD activities and namely for MnSOD with mullet presenting the higher activity levels (Table 1).

Regarding the SOD activities, the results showed that mullets sampled in autumn had higher activities (14.16 ± 1.01 U/mg protein). After 1 month, the values decreased in mullets from Group II. In mullets captured in spring the SOD activity was significantly low ($P < 0.05$). Flounders captured in autumn presented higher levels of SOD activity (9.94 ± 1.01 U/mg protein). In contrast with mullets, flounders revealed an increase in SOD activity after the depuration period (in autumn SOD activity was not evaluated due to limited amount of sample). The activity of MnSOD was also evaluated in liver of both species, and as occurred in total SOD activity the higher values were registered in autumn, for both mullets (11.35 ± 0.76 U/mg protein) and flounders (6.23 ± 1.62 U/mg protein). Although not always statistically significant, a decrease in MnSOD activity was observed in mullets from Group II; in contrast with flounder that showed an in-

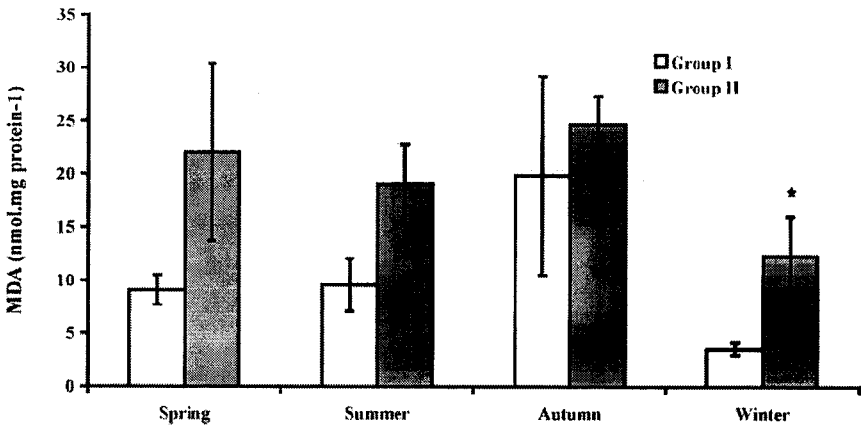

Fig. 2. Hepatic LPOX in flounders at capture (Group I) and after 1 month (Group II). Data presented as mean ± S.E. $^{*}P < 0.05$: Significant differences between Group I and Group II.

Fig. 3. Example of Western blot of carbonyl groups (derivatised with DNPH) from oxidised mullet liver proteins. In lanes 1 and 3 liver proteins from mullets captured in Douro estuary, in lanes 2 and 4, from mullets kept in captivity for 1 month.

crease in this enzymatic activity after the depuration period.

3.2. Oxidative damage

Oxidative damages in liver lipids (measured as TBARS) and proteins were evaluated in both species. No sex-dependent differences were observed in TBARS levels or oxidised proteins in liver with similar levels for both species. Mullets showed higher TBARS levels in spring (17.96 ± 4.13 nmol/mg protein) (Fig. 1), and the higher levels in flounder were registered in autumn (Fig. 2) (19.85 ± 9.33 nmol/mg protein). Flounders displayed a consistent pattern during the different sampling periods with an increase in hepatic TBARS after depuration. In summer and autumn, hepatic TBARS levels increased in mullet after the depuration period, while a decrease was observed during spring and winter.

In both species, hepatic oxidised proteins were analysed at capture and after the depuration period. The pattern of proteins is shown in Figs. 3 and 4 (mullets and flounders, respectively). An increase in proteins carbonyls content for both species was observed after the depuration in clean water.

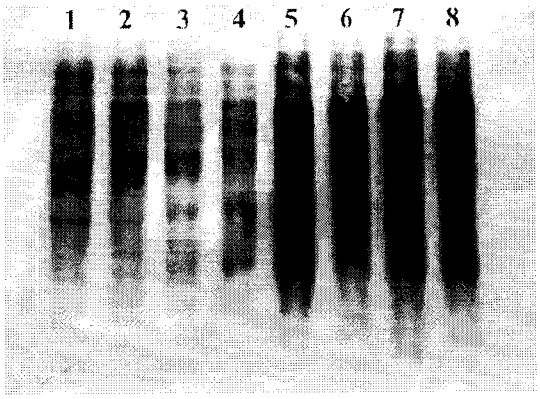

Fig. 4. Example of Western blot of carbonyl groups (derivatised with DNPH) from oxidised flounder liver proteins. In lanes 1–4 liver proteins from Group I flounders captured in Douro estuary, in lanes 5–8, from Group II flounders.

4. Discussion

The indiscriminate dumping and release of waste containing hazardous substances into rivers might lead to environmental disturbance which could be considered as potential source of stress to biotic community. Many classes of environmental pollutants are known to enhance the intracellular formation of ROS causing oxidative damage to biological systems. These pollutants may promote the production of superoxide anion radicals by redox cycling, while transition metals such as iron catalyse the reaction of superoxide anion radicals and hydrogen peroxide to produce hydroxyl radicals by Fenton reactions (Aust et al., 1985; Sies, 1988; Winston and Di Giulio, 1991). The fish uptake of these pollutants can occur from sediments, suspended particulate material, water-column and food sources; and the major routes of input will depend on the particular dietary and ecological lifestyles of the organisms (Van Veld, 1990; Livingstone, 1991, 1998).

This work aimed at evaluating the induction of oxidative stress in two fish species – mullet and flounder – living in a polluted environment as they display different lifestyles. The oxidative stress was monitored by evaluating the levels of SOD and CAT activities and lipid and protein oxidation at capture and after a period of 1 month living in a cleaner environment. Moreover, contaminants usually appear as very complex mixtures that can cause interactive effects on the biota. In this particular area, Mucha et al. (2003) detected a clear signature of anthropogenic contamination in Douro estuary in terms of zinc (Zn), copper (Cu), lead (Pb)

and chromium (Cr), rising the possibility of those sediments being toxic for living organisms, it is well known that metals are oxidative stress inducers. In addition to metal contamination, organic components are also present in this estuary, like was shown in our previous work measuring PCBs and DDT in mullet and flounder tissues (Ferreira et al., 2004). The accumulation of PCBs was higher in muscle of mullet with a maximum of 345 ng/g dry weight (dw), than in flounder, with a maximum of 52 ng/g dw. In the liver, flounder showed the highest levels (811 ng/g dw) while in mullets the levels were 687 ng/g dw. The maximum concentration of tDDT was measured in flounder liver, 301 ng/g dw. Mullet presented higher levels in muscle than flounder (69 and 16 ng/g dw, respectively). According to Albaigés et al. (1987), these two tissues reflect different contaminants inputs, muscle reflects chronic pollutants inputs while liver would reflect acute inputs. The higher levels in mullet for the measured antioxidant enzymatic activities are in accordance with the levels for EROD activity, a 10-fold higher EROD activity, 1536 pmol/min/mg protein, in mullet in comparison to flounder (156 pmol/min/mg protein) (Ferreira et al., 2004) that could be related to possible damages to the tissues.

The activities of the two antioxidant enzymes (SOD and CAT) revealed that the levels are species-dependent. The major difference was found for Mn-SOD activity in mullet, suggesting that mullets liver mitochondria can deal with the increase in superoxide anion more efficiently than flounders. In regard to the oxidative damages in liver lipids, the values for TBA in flounder were slightly higher than the ones estimated for mullets. This difference may be related with less efficient defences which is consistent with the lower levels of the antioxidant primary defences assessed. In addition to that, the difference in fatty acid composition of the liver membranes may also be involved (Halliwell and Gutteridge, 1999). It has to be referred that the food uptake can have an effect on the antioxidant enzymes activities and oxidative damages, as the fishes did not ate during the depuration period, as Pascual et al. (2003) showed in *Sparus aurata*, in conditions of fasting or limited food availability after 3 weeks increased MDA levels. As the lipid storage is mobilised to cope with metabolic needs, lipids become more exposed target to oxidation. Indeed, an increase in SOD activity in fasting fish, in agreement with ours results, was reported by the same authors. The results obtained with flounder showed that it is important to maintain as close as possible the natural conditions as biomarkers determinations can be masked by the nutritional status of the animal in the captivity. So far, we cannot conclude about the effects of the pollutants present in the estuary using flounders as model system.

In contrast, with mullets feeding conditions were achieved, and the results obtained could be related to the presence of pollutants in the environment; oxidative stress and adaptative responses were observed, revealing higher enzyme activities of SOD and CAT.

After the period in captivity, it was observed a decrease in SOD and CAT activities. This indicating that the higher enzyme activities contribute to avoid oxidative damages. The higher values for SOD and CAT activities in spring can be accounted to the concentration increase of pro-oxidants during the period before capture, providing further evidence that the pollutants present in the River Douro can induce oxidative stress. These higher levels are in agreement with the levels of EROD activity observed in mullets in spring (Ferreira et al., 2004).

Rodriguez-Ariza et al. (1993), in mullet, found increased antioxidant activities in animals from a contaminated site with PCBs, PAHs and pesticides, and proposed that antioxidant enzymes might be useful tools for biomonitoring of environmental pollution.

Previous data from Rudneva-Titova and Zherko (1994) in a laboratory study with red mullet (*Mullus barbatus*) exposed to PCB (Aroclor 1254) showed induction of CAT activity but no responses for SOD activity, while an induction in both antioxidant enzyme activities was reported in a field study aimed at evaluating the effects of PAHs in antioxidant defences (Burgeot et al., 1996). In addition, increases in antioxidant enzyme activities with laboratory exposure to a variety of organic and metal contaminants have been observed. The responses are transient and dependent on the species, enzymes and single or mixed contaminants, and even greater variability has been registered in field situations (Livingstone, 1991; Livingstone et al., 1993). Indeed, it is not possible to evaluate the levels of pollutants, or what kind of pollutants, that cause the oxidative stress in the marine environment and the data have to be analysed taking into account these limitations. In fact, these animals live in an environment with a mixture of pollutants, some of them with estrogen-like activity as it

were evidenced by the presence of testis–ova in male mullet's gonads (Ferreira et al., 2002, 2004), the xenoestrogens includes a variety of estrogen-mimicking chemicals such as 4-nonylphenol, bisphenol-A, various plasticisers, herbicides and pesticides (Colborn et al., 1993) and it is generally accepted that endocrine-disrupting chemicals are at least partially responsible for disruption of reproduction and development in some wildlife populations (Tyler et al., 1998; Vos et al., 2000). In field conditions, a complex interaction among pollutants can occur, and these interactions could be synergistic or antagonist. Antioxidant systems can be considerer as non-specific biomarkers of exposure to pollutants, and also as an indicator of toxicity. An induction can be regarded as an adaptative response to an altered environment; an inhibition could mean cell damage and toxicity of bioavailable pollutant in a dose-dependent manner (Vassuer and Cossu-Leguille, 2003).

Oxidative damage can occur when antioxidant and detoxifying systems are deficient and not able to neutralise the active intermediates produced by xenobiotics and their metabolites. The oxidation of polyunsaturated fatty acids leads to lipid peroxidation (LPOX) (Stegeman et al., 1992; Hageman et al., 1992). In mullets, the effect of the transfer to non-polluted waters led to a decrease of the values of LPOX except in summer and autumn. The increased levels in TBARS observed in summer/autumn could be related to an increase in water temperature, in the laboratory conditions, as was reported by Pellerin-Massicotte (1997).

Rodríguez-Ariza et al. (1993) showed an inhibition for LPOX in the same fish species in the presence of PCBs, PAHs and pesticides, but in other mullet species an increase was observed after exposure to PCB (Aroclor 1254) (Rudneva-Titova and Zherko, 1994). Likewise for antioxidant enzyme activities, the results showed great variability for this biomarker, although results for laboratory exposures to contaminated sediments (PAHs and PCBs) showed increases in oxidative damages in liver of dad, *Limanda limanda* (Livingstone et al., 1993), and liver of catfish, *Ictalurus punctatus* (Di Giulio et al., 1993). Increases in oxidative damage (lipid peroxidation, oxidised proteins and DNA damages) are seen for both fish and invertebrates, for single and mixed contaminants, including Cu, Fe, Cd, PAHs and PCBs (Livingstone, 2001).

Protein oxidation can be increased by xenobiotic exposure (Gibson et al., 1996; Fessard and Livingstone, 1998) and the assessment of individual or groups of oxidised proteins can be used as potential biomarkers of contaminant-mediated oxidative damage in fish liver. Our results showed that for flounders the lack of food can contribute to the increase in protein carbonyls content, while for mullets the decrease observed in antioxidant defences in Group II have to be considered to justify the increase in oxidised proteins. Indeed, the decrease in activities of both SOD and CAT can lead to promote the levels of ROS and subsequently the effects on the oxidation of proteins. In a preliminary work it was observed that when mullets were maintained for longer periods (4 and 8 months) in unpolluted water it is observed a tendency to a decrease in the content in oxidised proteins possibly indicating that more time is needed for repair or replacement of the damaged proteins. Additional studies should be performed to validate the usefulness of this parameter for biomonitoring purposes.

5. Conclusions

In conclusion, this study revealed that mullets and flounders display different levels of antioxidant primary defences when living in polluted waters. These differences could be related to PCBs and DDT accumulation and reflect damages to the tissues. The lower levels of antioxidant enzymes measured in flounder can justify the higher levels in liver lipid peroxidation. The increased levels of SOD and CAT activities and also oxidative damages after 1 month in captivity can be related to the fasting condition experienced by flounders. Indeed, the biomarkers responses can be masked by the nutritional status of the animals and in future works in captivity all potential confounding factors should be minimised. It has been proposed that animals inhabiting in chronically polluted environments can develop some adaptation or compensatory mechanisms (Regoli and Principato, 1995). The decrease in antioxidant enzyme activities observed in mullets after captivity confirms that the animals are being exposed to oxidative stress due to pollutants and can reprogramme the cell response when transferred to an unpolluted environment. The experimental data obtained with mullets can be considered as a useful reference for comparisons

with biomarkers response of organisms living in polluted environments. This can be extended to detect future spillages of toxic compounds in the study area and their influence on the biota.

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CHAPTER 3

Depuration of PCBs and DDTs in mullet under captivity clean conditions.

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Chemosphere (In Press)

Abstract

Contaminated mullet (*Mugil cephalus*) from Douro estuary was allowed to depurate in clean water and fed with uncontaminated food. Levels of PCBs and DDTs in muscle and liver, and ethoxyresorufin O-deethylase (EROD) activity were measured at day 0, 21, 120 and 270. In specimens captured in the estuary total PCB and total DDT concentrations were 311 and 65 $ng.g^{-1}$ in muscle and 686 and 115 $ng.g^{-1}$ in liver, respectively. At day 21, after an initial 10-15 days period of starvation, organochlorines levels increased in both analyzed tissues. Thereafter levels of all PCB congeners and DDT compounds decreased in muscle, and at the end of the 270 days were 49 $ng.g^{-1}$ and 13 $ng.g^{-1}$, respectively. These decreases were correlated to the lipids consumption. In liver no relationship between those variables was observed, suggesting different elimination processes and eventual exchange of contaminants between muscle and liver. EROD activities decreased in the first days of depuration experiment, but showed no relations with analysed organochlorines.

Introduction

Thousands of tons of polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT) were produced in the 20th century and partially released to the environment. Either due to diffuse sources or direct discharges to the terrestrial and atmospheric compartments, the aquatic environment is the ultimate sink for these contaminants (Stegeman and Hahn, 1994). Fish captured in the coastal zone often contains enhanced residues of these compounds in their tissues, as result of environmental contamination. Accumulation in the tissues does not necessarily imply injurious effects to the organisms. The biological response is usually evaluated through the presence of chemical biomarkers (van der Oost et al., 2003), namely ethoxyresorufin O-deethylase (EROD) one of the hepatic cytochrome P-450 dependent monooxidase (Ferreira et al., 2004).

The elimination of organochlorines in fish is barely known despite the number of studies related to the uptake kinetics by fish. Organochlorines can be eliminated by excretion (Moermond et al., 2004) and lower chlorinated PCB congeners biotransformed (van der Oost et al., 2003). However, most of the understanding comes from laboratory experiments with species exposed to contaminants (Goerke and Weber, 2001), and few works report results from naturally contaminated fish. In a long-term elimination study, PCB-contaminated eels captured in a natural environment were transferred to a relatively clean lake (de Boer et al., 1994), and half-lives of tetra- and penta-CBs ranged from 340 to 1450 days, but most of hexa-, hepta- and octa-CBs showed no measurable elimination.

This work presents the levels of PCBs and DDTs in muscle and liver of contaminated mullet (*Mugil cephalus*) from the Douro estuary when exposed to clean sea water and uncontaminated food, and examines whether elimination of organochlorines is a realist mechanism during the life-time of species that migrate from estuarine contaminated systems to coastal waters.

Materials and Methods

Sampling

Twenty two mullets (*Mugil cephalus*) were captured in the Douro estuary in May 2001. Five individuals were sacrificed within 24 hours after capture, body liver and gonads were dissected, weighted and the hepato-somatic (HSI: ratio between the weights of the liver

and the fish), and gonado-somatic indices (GSI: ratio between the weights of the gonads and the fish) calculated. The remaining liver and small pieces of muscle were frozen in liquid nitrogen and stored at -80ºC until they were assayed for EROD activity and the concentration of PCB congeners and DDT compounds. The other specimens were allowed to depurate in a 3000 L tank with a flow rate of 5 L/minute of brackish water (salinity 20‰). Water was continuously filtered through an extensive biological filter coupled to a charcoal filter before being recycled. The tank was aerated to maintain 100% oxygen saturation in the water. Fishes were maintained in natural photoperiod and temperature, and fed with uncontaminated hake fillet. During the first 10 days the added food was not consumed, probably due to the stress of captivity. At the day 15 it was observed that all individuals were eating normally. Five individuals were sampled at days 21, 120 and 270 following the same procedure.

Analytical procedure

Samples for analysis of PCBs and DDTs were prepared individually. The method has been described previously in Antunes and Gil (2002, 2004) and is summarized below. Freeze dried tissues were extracted with hexane using Soxhlet apparatus. Fat content was determined gravimetrically from aliquots of the extracts and the remaining extracts were cleaned with Florisil before the analysis in a HP 5890 series II gas chromatography, equipped with an electron capture detector, and a DB-5 (J&W Scientific) capillary column (60m x 0.25 mm i.d. x 0.25 μm film thickness). PCBs and DDTs were quantified using a six point calibration curve. A standard solution containing 18 PCB congeners (IUPAC Nos. 18, 26, 52, 49, 44, 101, 151, 149, 118, 153, 105, 138, 187, 183, 128, 180, 170, 194), p,p'-DDT and metabolites (p,p'-DDD and p,p'-DDE) was used as external standard. Procedural blanks were analyzed each 10 to 16 samples to monitor possible laboratory contamination and blank subtractions were made before quantification. Recovery of the Florisil column was evaluated with a standard solution and more than 85% of each compound was obtained. The ethoxyresorufin O-deethylase (EROD) activity was evaluated by the fluorimetric method described by Pacheco and Santos (1998). One way analysis of variance or student's t-test was used to compare concentrations. A 5% significance level was used for the statistical tests.

Results and Discussion

PCBs and DDTs in mullets from the Douro estuary

Mullets captured in the Douro estuary contained relatively high values of PCBs and DDTs in their muscle and liver tissues. The mean concentrations and standard error (n=5) of tPCB (calculated as the sum of individual analysed CB levels) in muscle and liver were 311±58 and 686±135 $ng.g^{-1}$, respectively, and of tDDT (sum of concentrations of p,p'-DDT, p,p'-DDE and p,p'-DDD) were 65±35 and 115±5.7 $ng.g^{-1}$. In spite of the differences of residue levels among the five analysed individuals, values are one order of magnitude higher than the concentrations reported by Antunes et al. (2001) for golden mullet (*Liza aurata*) captured in Ria de Aveiro (80 $ng.g^{-1}$ of tPCB and 22 $ng.g^{-1}$ of tDDT), a coastal lagoon with permanent connection to the sea, located about 70 Km south of the Douro estuary. 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 (Chubb et al., 1981). Levels of organochlorines in the specimens captured in the Douro indicate local contamination. Values are one to two orders of magnitude higher than concentrations of PCB (2.5 $ng.g^{-1}$ fresh weight, sum of the IUPAC congeners Nos 28, 52, 101, 118, 138, 153 and 180) and DDTs (7.2 $ng.g^{-1}$) in sea mullet from the Ebro Delta, a zone influenced by agro-industrial activities, and comparable to levels of PCBs in samples of mackerel and anchovy from an area exposed to pollution (Bayarri et al., 2001).

The contribution of each analysed CBs to the total PCB was the same in muscle and liver in mullets from Douro, and the CB180 (hepta-), CB153 and CB138 (hexa-chlorinated) were the predominant congeners (Figure 1).

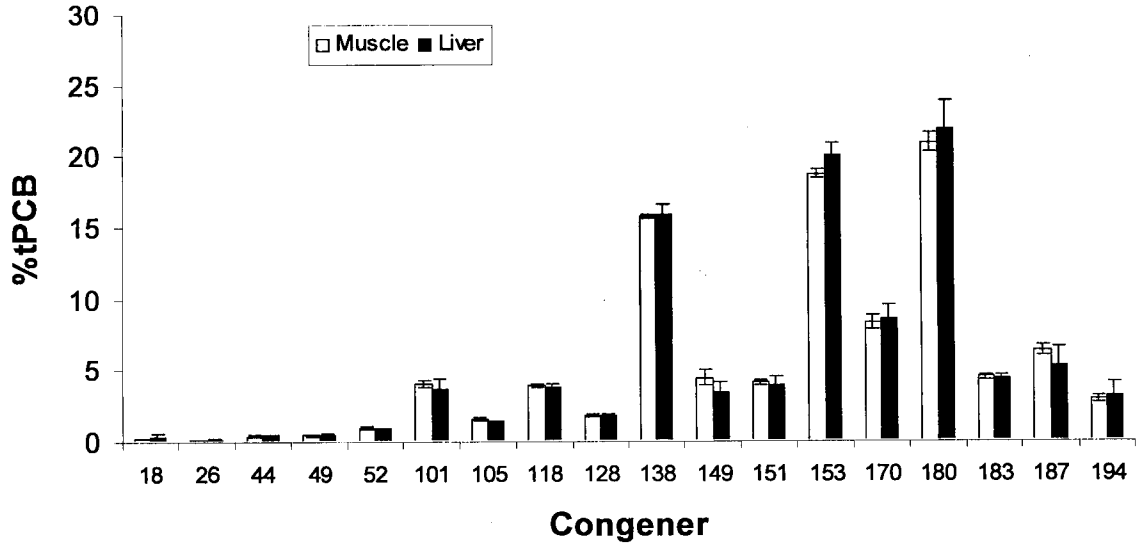

Figure 1: PCB pattern in muscle and liver of mullet from Douro estuary (mean ± SE).

These compounds contain chlorines at *para* positions in both biphenyl rings, and usually are the prevailing compounds reported in biological samples (Bayarri et al., 2001). The CB138 and CB153 are dominant components in *Platichthys flesus* from Douro (Ferreira et al., 2004), in several species from Ria de Aveiro (Antunes et al., 2001) and in sea bass from Seine estuary (Loizeau et al., 2001). The metabolite *p,p'*-DDE accounted to more than 69% of the total DDT in muscle and liver tissues of mullet from Douro.

Laboratory experiment

Mullet conditions

The physiological conditions of mullet during the experiment are presented in Table 1. The specimens kept in captivity lost progressively weight. Weight variations between the sampling dates were significant ($p<0.05$) and the largest differences occurred between day 21 and day 120. The hepato-somatic index (HSI) decreased slightly and reached the lowest value at day 120. The lipid content presented pronounced decreases in liver (67%) and muscle (22%) between day 21 and day 120. Gonad weight was almost constant indicating that individuals did not reached sexual maturation.

Table 1: Physiological conditions of mullet (*Mugil cephalus*) during the experiment. Mean values are given ± SE. Different letters denotes significant differences between sampling days.

Sampling day	Whole fish		Liver		Gonad	Muscle
	Length (cm)	Weight (g)	HSI	Lipids (%)	GSI	Lipids (%)
0	36.1±0.3	459^{c} ±20.3	1.85^{b} ±0.04	23^{b} ±1.3	0.28±0.05	$8.0^{a,b}$ ±1.6
21	35.7±0.8	$412^{b,c}$ ±16.5	1.31^{a} ±0.05	23^{b} ±2.3	0.13±0.01	11^{b} ±0.6
120	33.9±0.4	$325^{a,b}$ ±14.6	0.94^{a} ±0.05	$18^{a,b}$ ±0.8	0.28±0.07	3.6^{a} ±0.4
270	35.6±0.3	316^{a} ±6.0	1.26^{b} ±0.05	12^{a} ±1.0	0.12±0.01	3.0^{a} ±0.2

HSI, hepato-somatic index; GSI, gonado-somatic index.

Mobilization of PCB and DDT in mullet during starvation

The time-course evolutions of PCB and DDT concentrations in muscle and liver of mullet during the experiment are presented in Table 2. At day 21 concentrations of all analysed CBs and DDTs in muscle were higher than values recorded at wild individuals captured in Douro (day 0). Due to the variability observed in levels of organochlorines in mullet from Douro estuary, the increase is not statistically different (t-test). Similar situation during the adaptation period has been observed in a preliminary experiment (Antunes, unpublished data). A plausible explanation for this apparent discrepancy of increasing contaminant concentrations at cleaner conditions is the non-consumption of added food during the first 10 to 15 days coupled with mobilization of organochlorines in tissues induced by the lipid consumption. The enrichment of these compounds was less clear in liver than in muscle, as illustrated by the ratio between concentrations at day 21 and at day 0, presented as bars in Figure 2. During starvation fish weight started to decrease, in special liver weight, simultaneously with increases of PCBs and DDTs in muscle, which may indicates exchange between muscle and liver due to the metabolic role of this organ (van der Oost et al., 2003).

Table 2: Concentrations of organochlorines (ng g^{-1} dw) in muscle and liver of mullet over the 270-d study. Each value represents the mean ± SE of five fishes.

	Muscle				Liver			
	Depuration time (days)				Depuration time (days)			
Compound	0	21	120	270	0	21	120	270
Trichlorobiphenyl								
CB18	0.6±0.1	1.0±0.3	0.6±0.04	0.3* ±0.01	2.7±0.5	2.0±0.4	1.8±0.2	3.1±0.3
CB26	0.5±0.1	0.5±0.1	0.2±0.02	nd	1.2±0.2	0.9±0.1	0.8±0.1	0.5±0.1
Tetrachlorobiphenyl								
CB44	1.1±0.2	1.8±0.3	0.7±0.06	0.4* ±0.02	3.3±0.3	2.7±0.4	2.7±0.1	2.0±0.2
CB49	1.3±0.2	2.9±0.6	1.1±0.1	0.3* ±0.01	3.6±0.3	4.2±0.9	4.6±0.3	3.5±0.2
CB52	3.0±0.6	5.1±0.8	1.7* ±0.2	0.6±0.02	6.4±0.6	7.5±1.7	7.3±0.4	7.5±0.4
Pentachlorobiphenyl								
CB101	12.7±2.3	31±7.5	3.7* ±0.5	2.1±0.1	24±3.3	37±11	11±0.9	29* ±1.3
CB105	4.7±0.8	9.7±1.5	2.5* ±0.3	0.8* ±0.02	9.8±1.1	9.1±1.7	7.8±0.7	9.8±0.4
CB118	12.7±2.5	32±6.4	6.8* ±0.9	2.1* ±0.1	26±2.2	36±8.1	21±1.8	26±1.1
Hexachlorobiphenyl								
CB128	5.9±1.1	18±3.3	2.3* ±0.3	1.0±0.05	13±1.6	16±3.3	6.6±0.6	10±0.4
CB138	50±9.4	133±32.5	16* ±1.9	8.4±0.5	107±11	153±35	45±3.2	84* ±5.4
CB149	14±2.3	22±4.	2.1* ±0.2	1.8±0.2	24±3.8	22±4.8	6.5* ±0.2	19* ±1.3
CB151	13±2.2	31±8.8	2.5* ±0.3	1.9±0.2	27±3.3	40±10	8.4±0.2	27* ±1.7
CB153	57±10.2	172±42.4	18* ±2.0	9.9±0.6	135±12	205±51	57±4.4	101* ±5.7
Heptachlorobiphenyl								
CB170	27±5.7	78±18.0	6.8* ±0.7	3.8±0.2	60±8.3	78±16	16* ±1.3	33* ±2.5
CB180	66±12.9	185±41.2	16* ±1.7	9.3±0.6	152±20	166±39	40* ±3.4	82* ±6.2
CB183	14±2.4	40±8.5	3.1* ±0.3	2.2±0.1	31±3.9	40±9.0	9.4* ±0.7	21* ±1.2
CB187	19±3.0	35±9.7	2.5* ±0.2	3.0±0.2	37±8.7	35±8.6	7.1* ±0.4	36* ±2.0
Octachlorobiphenyl								
CB194	9±1.8	24±7.0	1.7±0.1	1.2±0.1	23±4.5	15±4.7	5.0±0.5	9.8* ±0.6
∑PCB	311±58	821±270	88* ±21	49±6.7	686±135	870±350	259±39	503±64
p,p'-DDE	40±7.8	61±3.0	29* ±3.6	11±0.7	81±2.6	79±14.2	79±8.4	123±8.5
p,p'-DDD	8.3±2.3	17±0.9	5.0* ±0.6	1.2* ±0.1	30±1.4	25±1.6	12* ±0.9	18±1.1
p,p'-DDT	17±5.6	29±1.8	7.4* ±1.0	0.5* ±0.08	3.4±0.9	4.0±1.1	20* ±1.5	14±1.2
∑DDT	65±35	106±27	41* ±12	13±1.8	115±5.7	108±27	111±23	153±22

* denotes significant differences ($p<0.05$) to previous sampling day.

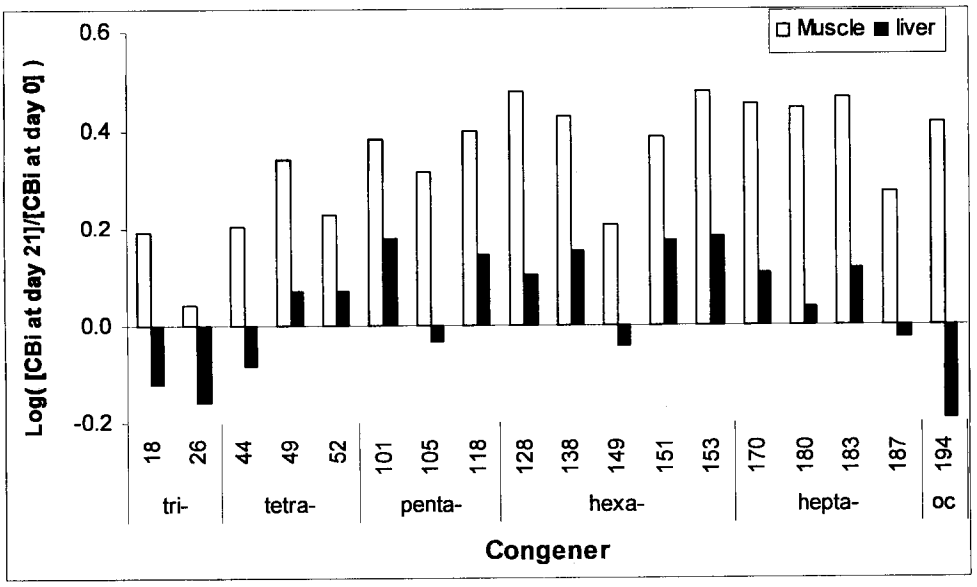

Figure 2: Logarithm of the ratio between concentration of each congener after 21 days, and the concentration at day 0.

Elimination of PCBs and DDTs in mullet

At day 120, after a long period in which mullets have ingested uncontaminated food, tPCB concentrations in muscle decreased from 821 to 88 ng.g^{-1} and tDDT from 106 to 41 ng.g^{-1} (Table 2). These values were lower than concentrations of wild individuals (day 0). At day 270 tPCB and tDDT residues were much lower being 49 and 13 ng.g^{-1}, respectively. The decrease of these compounds in muscle parallels with the consumption of lipids. Significant correlations between all the analysed CBs and lipid content (r2 ranged from 0.61 to 0.94) and DDT compounds and lipids (r2 from 0.81 to 0.93) suggest that lipid consumption is a major factor driving the elimination kinetics of PCBs and DDTs in muscle tissues of mullets. The decrease of PCBs in liver was much smaller (from 686 to 503 ng.g^{-1}) and tDDT showed even a slight increase between day 0 and day 270 (115 to 153 ng.g^{-1}). In muscle p,p'-DDD and p,p'-DDT were eliminated faster than p,p'-DDE: more than 60% of p,p'-DDD and p,p'-DDT were eliminated after 120 days, and 90% at day 270, while concentrations of p,p'-DDE decreased only 48% and 80%, respectively. This slower decrease may result from conversion of p,p'-DDT to p,p'-DDE (Pastor et al., 1996). In liver an erratic variability of DDT compounds was recorded (Table 2).

Contrary to muscle, organochlorines concentrations in liver did not follow the decrease of lipid content during the experiment. The contrast is illustrated in Figure 3, showing the relationships of CB44 (tetrachlorobiphenyl) and lipid content in muscle and liver, as an example. The lack of relationship in liver may be explained by the involvement of liver in

metabolization and mobilization of these compounds from muscle and other organs (van der Oost et al., 2003).

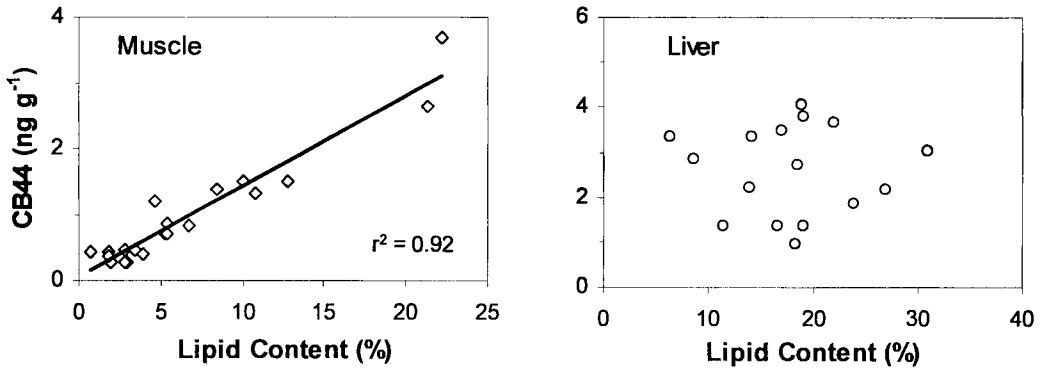

Figure 3: Relationship between concentration of tetrachlorobiphenyl CB44, in muscle and liver of mullet, and lipid content.

Behaviour of individual CBs during elimination

Assuming that elimination started only at day 21, the elimination efficiency of each congener can be calculated dividing the concentration at day 120 and day 270 by that at day 21 (Figure 4).

After 120 days less than 50% of tri- and tetrachlorobiphenyls were eliminated in the muscle, while the higher chlorinated CBs were eliminated more than 66%. At day 270 decreases were higher than 70% for all quantified congeners, and up to 95% for the more chlorinated compounds. In liver, only the CB101, hexa- and heptachlorobiphenyls showed a significant decrease at day 120. These congeners, and some others, increased at day 270. Due to the absence of external sources, this can only be attributed to a mobilization to the liver. In a bioconcentration/elimination laboratory experiment Goerke and Weber (2001) observed that the lower level of chlorination and free *m,p* position of CBs favoured elimination in *Platichthys flesus*, and calculated half-lives between 9 days (CB44) and 70 days (CB153). Niimi and Olivier (1983) had already calculated half-lives of PCBs in rainbow trout, achieving values from 29 to 127 days in muscle, smaller than in whole fish.

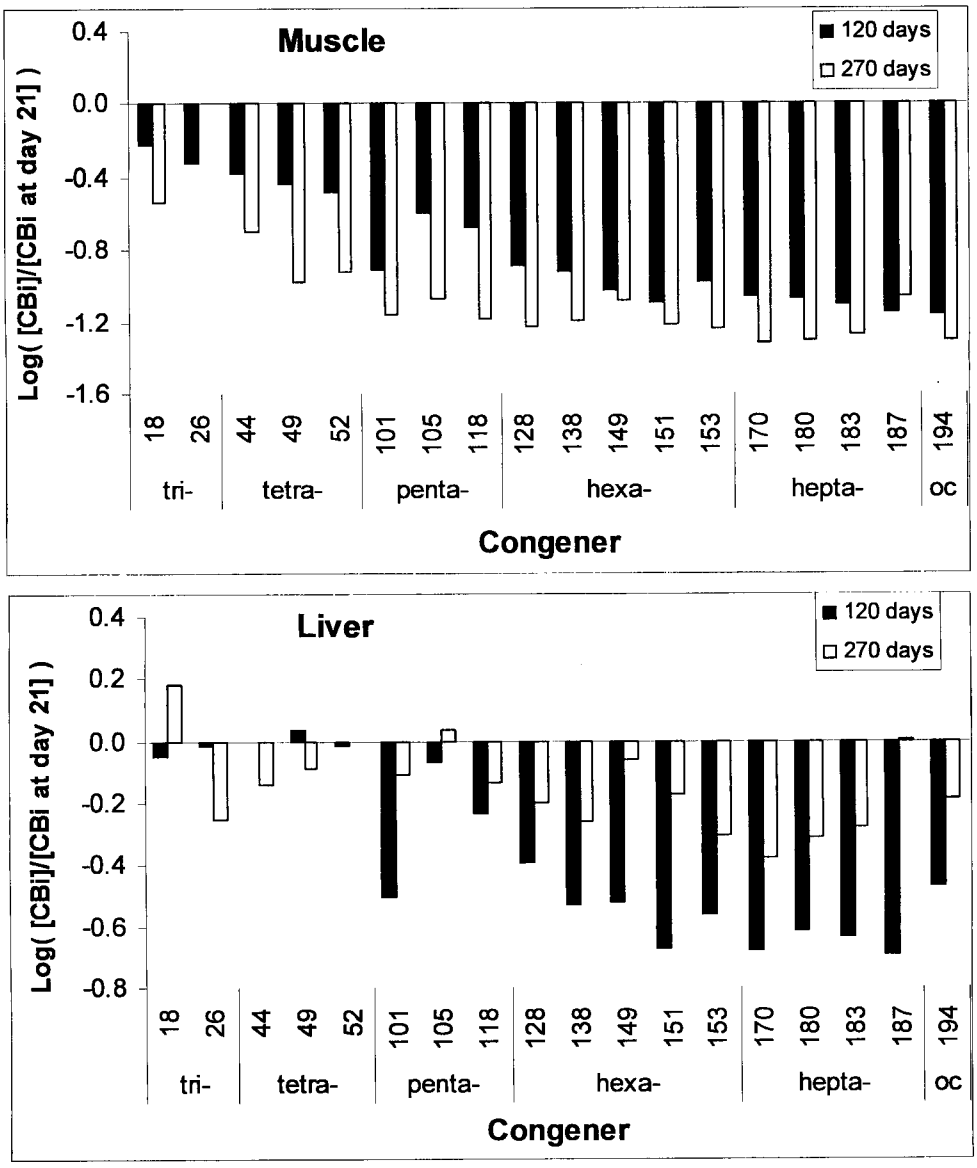

Figure 4: Logarithm of the ratio between concentration of each congener after 120 and 270 days, and the concentration at day 21.

In muscle the mobilization of congeners, tri- to decachlorobiphenyls, from muscle to the whole fish was not influenced by chlorine content. In our study the reductions of tri- and tetra-CBs in muscle were slow, but all the other analysed congeners were eliminated faster and there were no substantial dependence on the component. In spite of the variability among individuals and the small number of observations, half-times in mullets would be less than 100 days. These results differ from those obtained in a field experiment with ells (de Boer et al., 1994), where elimination of higher chlorinated congeners was not measured during the 8-year study, in a clean lake. The good correlations obtained with lipid content in our experiment show that the lipids had an important role in PCB mobilization, but do not explain the differences in the elimination of

less chlorinated congeners. Assuming multi compartments in a fish, pharmacokinetic models predict initial increments of POP concentrations in tissues of deep peripheral compartments, followed by a slow decrease (Kulkarni and Karara, 1990; Cahill et al., 2003). The observed increase at day 21 in mullet tissues agrees with those findings, and suggests that muscle may be considered as a peripheral compartment in bioaccumulation models of organochlorines.

Levels of EROD in liver of mullet

Several authors have been used EROD induction as a biomarker for assessment PCB and PAH pollution (Livingstone et al., 1997). Mullet livers of the individuals collected in Douro estuary, presented high induction of EROD (1536±145 pmol.min^{-1}.mg protein^{-1}, Ferreira et al., 2004) which reflects the exposure to pollution. This is in accordance with the relatively high concentrations of PCBs and DDTs in muscle and liver of the wild fishes. After 21 days of depuration, EROD activity decreased more than one order of magnitude (mean value was 154±20 pmol.min^{-1}.mg protein^{-1}, Ferreira et al., 2004). However, this decrease did not correspond to a decrease on concentrations of PCBs and DDTs in liver (Table 2). This lack of correspondence was observed along all the experiment. These results indicate that the high values of EROD activity of mullet from Douro estuary cannot be attributed solely to the accumulation of these organochlorine compounds, but presumably to a mixture of non-persistent contaminants which were eliminated after 21 days under captivity conditions.

Conclusions

The transfer of contaminated wild mullets to clean laboratory conditions showed that lipid consumption leads to redistribution of PCBs and DDTs during the adaptation starvation period, and to elimination from muscle when individuals consumed low-fat uncontaminated food. Approximately 90% of higher chlorinated CBs were efficiently eliminated from muscle, whereas 29% of lower chlorinated CBs remained at residual concentrations during the 270-day of experiment. Residues accumulated in liver remained at relatively high levels. Consequently, it is expected that PCB pattern changed to higher contribution of the tri- and tetra- CBs, when fishes during their life time move from contaminated estuarine systems to shelter or open coastal waters.

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CHAPTER 4

The effect of long-term depuration on phase I and phase II biotransformation in mullets (*Mugil cephalus*) chronically exposed to pollutants in River Douro Estuary, Portugal.

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The effect of long-term depuration on phase I and phase II biotransformation in mullets (*Mugil cephalus*) chronically exposed to pollutants in River Douro Estuary, Portugal

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Abstract

Pollutants such polycyclic aromatic hydrocarbons (PAHs) are released into the environment by urban communities and industries and the enzymes that catalyse the biotransformation of pollutants play a key role regarding the accumulation of these compounds in fish species inhabiting these areas. In this study the relationship between phase I (EROD activity) and phase II (GST activity) and PAH metabolites was measured in grey mullet (*Mugil cephalus*) after capture in the Douro estuary, and after long-term depuration in an unpolluted laboratory environment. The results showed a significant decrease in EROD activity after 1 month and in bile metabolites after 4 months in captivity, with both maintaining reduced levels at 4 and 8 months depuration. Liver GST activity did not showed significant changes. This study provides evidence that Douro estuary waters contain bioavailable PAHs that can be associated with the induction of cytochrome P450, and that mullets have the ability to metabolise and eliminate PAHs.

Keywords: Biomarkers; Mullet (*Mugil cephalus*); FACs; Pollutants

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1. Introduction

In the 20th century, many thousands of organic pollutants, such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzofurans (PCDFs) and dibenzo-*p*-dioxins (PCDDs) were produced and released into the environment (Van der Oost, Beyer, & Vermeulen, 2003). These pollutants have a net effect on life in the aquatic environment and the toxicity is dependent on the rate of their biotransformation. It is not economically feasible to monitor all contaminants of anthropogenic (predominantly halogenated hydrocarbons) and natural origin (heavy metals and most PAHs). The enzymes catalysing the biotransformation of the pollutants play a key role regarding the accumulation of the compounds in the organism (Matthews & Dedrick, 1984). The liver is the main site of PAH metabolism (Au, Wu, Zhou, & Lam, 1999) and the metabolites are secreted into the bile and stored in the gall bladder before being excreted into the alimentary tract. As many fish species are highly mobile, measurement of bile fluorescent aromatic compounds (FACs) is often a more reliable method for assessing exposure to PAHs, than measuring PAHs in the sediment where the fish were captured (Arcand-Hoy & Metcalfe, 1999; Collier & Varanasi, 1991), and bioavailability is taken into account. Measuring PAH concentrations in fish tissues, to estimate exposure, is often not reliable due to the fast metabolism of PAH in fish (Livingstone, 1998), together with the difficulties associated with the analytical measurement of volatile PAH compounds. Therefore, the analysis of bile metabolites has become a convenient and a relatively rapid method for monitoring PAH contamination in fish (Lin, Cormier, & Racine, 1994). The biotransformation systems generally consist of two subsystems: phase I and phase II systems. In phase I, usually an oxidative step, the cytochrome P450 (CYP) system holds a central role as catalyst, resulting in the hydroxylation, leading to a suitable substrate for the phase II reaction. Within the different members of the cytochrome P450 family, the CYP1A plays a particular role in the metabolism of a large number of compounds, such as dioxins, furans, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) (Ferreira, Antunes, Gil, Vale, & Reis-Henriques, 2004; Stegeman & Hahn, 1994). The phase II metabolism involves the conjugation of the xenobiotic parent compound or its metabolites with an endogenous substrate, thus facilitating the excretion of chemicals by the addition of more polar groups to the molecule (Van der Oost et al., 2003). The conjugation of electrophilic compounds (or phase I metabolites) with reduced glutathione (GSH) is catalyzed by the glutathione S-transferases (GSTs). Glutathionation is a step within the detoxification of pollutants catalysed by GSTs. However, the data available are not consistent, with some reports indicating an increase in the enzyme activity in contrast with others that do not observe changes or even reported significant decreases (Otto, Buttner, Arquette, & Moon, 1996; Tuvikene et al., 1999; Van der Oost et al., 2003). Mullet was chosen because it possesses several characteristics required in an estuarine bioindicator species, such as the extreme salinity tolerance, and also because it is very common in southern European estuarine systems, and therefore may be a good tool for monitoring programs (Ferreira et al., 2004, Ferreira, Moradas-Ferreira, & Reis-Henriques, 2005).

This study aims to analyse the induction of phase I and II biotransformation enzymes systems in mullets (*Mugil cephalus*) chronically exposed to contaminants, and to evaluate whether long-term depuration leads to significant alterations. The experimental approach

involved the measurement of EROD and GST activities in liver of mullets captured in an assumed polluted site in River Douro estuary, and after 3 periods of depuration of 1, 4 and 8 months. The ratio between phase I and II enzymes activities, the 'biotransformation index' (BTI) introduced as a biomarker by Van der Oost et al. (1998), was assessed. FACs were also analysed in bile, in order to assess PAH exposure, and to evaluate which types of PAHs the animals were exposed to.

2. Materials and methods

2.1. Study area

The present work was carried out in the lower Douro estuary. The Douro is one of the longest rivers in the Iberian Peninsula (930 km), sharing its 98,000 km^2 of watershed with Spain and Portugal. It drains into the Atlantic Ocean at 41°08'N and 8°42'W, near Porto. Domestic sewage as well as industrial effluents are still discharged, mostly without treatment, directly into the estuary and its tributaries.

2.2. Sampling

Mullets were netted in an assumed polluted site in Douro estuary in May and June of 2001, January and May 2002, and April 2003 with a total of 83 mullets sampled. In each sampling campaign, six animals (group I) were sacrificed within 24 h after capture. Mullets were measured and weighed to determine condition factor (CF) (body weight (g) × 100/(length (cm))^3). After dissection livers were weighed to determine hepato-somatic index (HSI) (liver weight (g)/body weight (g) × 100). Livers were frozen in liquid nitrogen and stored at −80 °C until they were assayed. Bile samples were collected with a syringe from the gall bladder and stored at −80 °C until analysed (only collected after January 2002). At each sampling occasion, mullets were allowed to depurate separately for 1 month (group II), 4 months (group IV) (except in January 2002 and April 2003) and 8 months (group VIII) (except in May 2002) in 3000 L tanks at a salinity of 20‰ (similar to the salinity in the estuary where the mullets were captured) with a flow rate of 5 L per minute. Water was continuously filtered through an extensive biological filter, and a charcoal filter before being recycled. Aeration was provided in the tanks to maintain 100% oxygen saturation in the water. Fish were maintained in natural photoperiod and temperature and fed with uncontaminated frozen fish (hake). During the first 10 days the added food was not consumed, probably due to the stress of captivity. At day 15 it was observed that all fishes were eating normally.

2.3. FAC analysis

Fluorescent aromatic compounds (FACs) in the bile were determined through fixed wavelength fluorescence (FF). Five microliters of bile diluted in 5 ml of ethanol 48% were used for FF determining at the excitation/emission wavelength pairs 260/380, 290/335, 341/383 and 380/430 nm, denoted $FF_{260/380}$, $FF_{290/335}$, $FF_{341/383}$ and $FF_{380/430}$ respectively. Phenanthrene type metabolites are detected by $FF_{260/380}$ (Krahn et al., 1993). By $FF_{290/335}$, mainly naphthalene type of metabolites, typically associated with petroleum products are detected, and benzo[*a*]pyrene type of metabolites are more efficiently

detected by $FF_{380/430}$ (Krahn, Burrows, MacLeod, & Malins, 1987; Lin, Cormier, & Torsella, 1996). By $FF_{341/383}$, mainly pyrene-derived metabolites are detected (Aas, Baussant, Balk, Liewenborg, & Andersen, 2000). Measurements were performed on a BIOTEK SFM25 fluorimeter. The FF values were expressed as arbitrary fluorescence units (a.f.u.) after deducting the signal levels of the solvent. Total FACs were calculated as the sum of the specific fluorescence for the four types of metabolites measured. The bile pigment biliverdin was measured at 380 nm in all samples to estimate bile density, and the concentration (mM) was calculated based on the extinction coefficient of 40,738 $M^{-1} cm^{-1}$. PAH metabolites fluorescence was normalised to biliverdin. Biliverdin was used as control, namely to check if differences in fluorescence intensity could result from differences in bile densities.

2.4. Biochemical analysis

To assess the activity of ethoxyresorufin *O*-deethylase (EROD) the liver was homogenized in ice-cold buffer (50 mM Tris–HCl, pH 7.4, 0.15 M KCl). Microsomes were prepared according to the method described by Fent and Bucheli (1994). The ethoxyresorufin *O*-deethylase (EROD) activity was evaluated by the fluorimetric method described by Pacheco and Santos (1998). Liver EROD activity is given in pmol/min/mg protein.

Glutathione S-transferase (GST) in the liver was determined according to the method of Habig, Pabst, and Jakoby (1974) adapted to microplate (Frasco & Guilhermino, 2002), using glutathione (GSH) 10 mM in phosphate buffer 0.1 M, pH 6.5, and 1-chloro-2,4-dinitrobenzene (CDNB) 60 mM in ethanol prepared just before the assay. The reaction mixture consisted of phosphate buffer, GSH solution and CDNB solution in a proportion of 4.95 ml (phosphate buffer):0.9 ml (GSH):0.15 ml (CDNB). In the microplate, 0.2 ml of the reaction mixture was added to 0.1 ml of the sample, with final concentration 1 mM GSH and 1 mM CDNB in the assay. The GST activity was measured immediately every 20 s, at 340 nm, during the first 5 min, and calculated in the period of linear change in absorbance. Liver GSH activity is expressed in nmol/min/mg protein.

2.5. Statistical analysis

Differences between groups were tested using a one-way ANOVA with a multiple comparison test (LSD) at a 5% significance level. Some data had to be log transformed in order to fit ANOVA assumptions. All tests were performed using the software Statistica 5.0 (Statsoft, Inc., 1995).

3. Results

The results in Table 1 indicate that HSI values showed significant decreases in every periods analysed after 1, 4 or 8 months in depuration. The values for CF decreased significantly after 4 and 8 months in May/01 and after 1 and 8 months in Jun/01, in contrast with the pattern occurring in Jan/02, May/02 and Apr/03 where a net increase in CF was observed after 4 and 8 months in unpolluted conditions.

Table 1
Mullets mean weight (g) and length (cm), hepato-somatic index (HSI) (%) and condition factor (CF) at capture day (Group I), after 1 month (group II), 4 months (Group IV) and after 8 months (Group VIII) in captivity in the 5 periods analysed

	Group	Weight (g)	Length (cm)	HSI (%)	CF
May/01	I ($n=6$)	469 ± 26	38 ± 1	1.8 ± 0.1	0.88 ± 0.02
	II ($n=6$)	407 ± 32	36 ± 1	1.3 ± 0.1**	0.87 ± 0.03
	IV ($n=5$)	362 ± 46	36 ± 2	1.0 ± 0.1***	0.78 ± 0.04*
	VIII ($n=4$)	337 ± 23*	36 ± 1	1.3 ± 0.1***	0.70 ± 0.04***
Jun/01	I ($n=6$)	589 ± 86	41 ± 2	1.8 ± 0.1	0.85 ± 0.03
	II ($n=6$)	443 ± 33*	40 ± 1	1.3 ± 0.2**	0.71 ± 0.02**
	IV ($n=5$)	362 ± 46**	36 ± 2*	1.0 ± 0.1***	0.78 ± 0.04
	VIII ($n=4$)	337 ± 23***	36 ± 1*	1.3 ± 0.1***	0.70 ± 0.04**
Jan/02	I ($n=5$)	548 ± 77	43 ± 2	1.4 ± 0.1	0.69 ± 0.01
	II ($n=5$)	417 ± 45*	38 ± 2*	1.2 ± 0.1	0.76 ± 0.03
	VIII ($n=5$)	571 ± 22	41 ± 1	1.0 ± 0.0**	0.83 ± 0.03**
May/02	I ($n=5$)	577 ± 70	42 ± 2	1.7 ± 0.1	0.78 ± 0.03
	II ($n=4$)	610 ± 55	43 ± 1	1.2 ± 0.1**	0.77 ± 0.03
	IV ($n=4$)	277 ± 33***	31 ± 1*	1.0 ± 0.1***	0.95 ± 0.03**
Apr/03	I ($n=5$)	462 ± 35	40 ± 1	1.6 ± 0.1	0.70 ± 0.02
	II ($n=4$)	410 ± 4	39 ± 1	1.3 ± 0.1*	0.69 ± 0.04
	VIII ($n=4$)	508 ± 52	39 ± 1	1.2 ± 0.1*	0.86 ± 0.07**

Values presented as mean ± SE. Statistical differences to Group I : $*p < 0.05$; $**p < 0.01$ and $***p < 0.001$.

Total PAHs metabolites are presented in Fig. 1 as arbitrary fluorescence units normalised to the biliverdin content. In Jan/02 FACs in bile from fish after capture were lower when compared to bile values from mullets captured in May/02 and Apr/03. The decrease

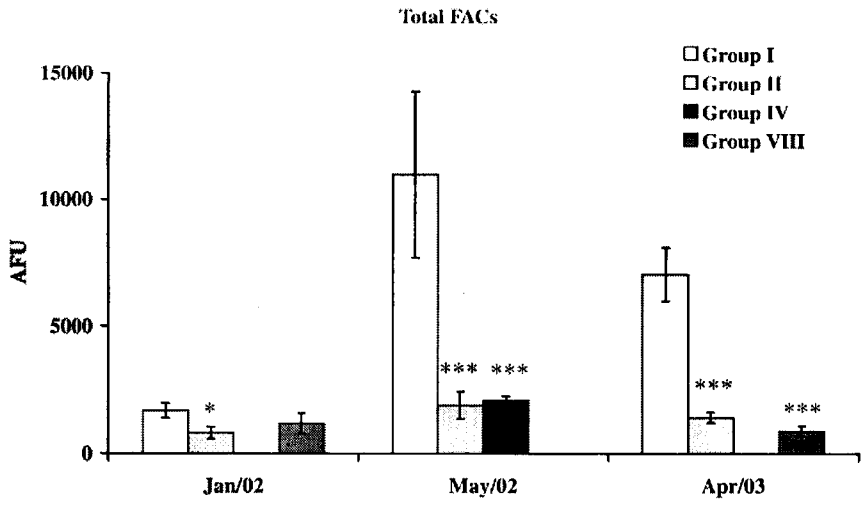

Fig. 1. Total fluorescent aromatic compounds, in mullet's bile at capture day (Group I), after 1 month (Group II), 4 months (Group IV) and 8 months (Group VIII) in captivity in the 3 periods analysed. Levels expressed as arbitrary fluorescence units, normalized to biliverdin. Values are given as mean ± SE. Significant differences to Group I: $*p < 0.05$; $***p < 0.001$.

observed in the first month of depuration could be in fact a result of the biliverdin increase due to different feeding, and the period of non consumption of the added food. Independently of the values at capture, the levels become similar after a longer period in depuration indicating metabolization of PAHs compounds. No differences were observed for the four types of PAHs metabolites analysed after depuration (Table 2) presenting similar decrease rates.

EROD activity in mullet liver, for each sampling and subsequent depuration periods, is displayed in Fig. 2. EROD activities in mullet's liver at capture ranged from 220 ± 70 to 1536 ± 145 pmol/min/mg protein. This biomarker appears to attain basal levels of activity after 4 months of depuration, with values of activity at 4 and 8 months between 60 and 190 pmol/min/mg protein in every sampling period. An overall decrease in EROD activity was observed after the depuration in unpolluted water in captivity. The most significant decreases in liver EROD activity after depuration occurred in May/01. A decrease, although not statistically different, was seen in Jun/01. In Jan/02 a significant decrease in EROD activity was recorded (from 662 ± 107 to 59 ± 8 pmol/min/mg protein after 8 months in captivity). Mullets captured in May/02 showed a significant decrease (72%) in EROD activity after 1 month in depuration (from 300 ± 105 to 82 ± 14 pmol/min/mg protein), similar to the percentage decrease in total FACs in the same period. The response in Apr/03 was similar to the one observed in Jan/02, with a decrease in liver EROD activity from 432 ± 40 to 89 ± 29 pmol/min/mg protein after 8 months in depuration. Compared to variations in liver EROD activity in mullets from Group I, bile metabolites showed a different trend, with higher activities corresponding to lower PAH metabolites.

GST activities in mullet's liver, for each sampling and forthcoming depuration period are shown in Fig. 3. After 1 month of depuration no significant changes were detected. In fact a small decrease in activity was reported in May/01 and Apr/03, or an increase in the other analysed periods. After 4 months in captivity a significant increase in GST activity was estimated in May/01 (from 203 ± 31 to 260 ± 26 nmol/min/mg protein) and

Table 2
Biliverdin concentration in mullet's bile, and arbitrary fluorescence units (a.f.u.), normalized to biliverdin, for phenanthrene, naphthalene, pyrene and benzo[*a*]pyrene types of metabolites in mullet's bile in Jan/02, May/02 and Apr/03, in Group I, II, IV and VII

Month	Group	Biliverdin (mM)	Phenanthrene type (a.f.u.)	Naphthalene type (a.f.u.)	Pyrene type (a.f.u.)	Benzo[*a*]pyrene type (a.f.u.)	Total FACs (a.f.u.)
Jan/02	I	0.48 ± 0.04	181 ± 37	952 ± 169	422 ± 83	113 ± 13	1667 ± 290
	II	2.26 ± 0.93**	59 ± 16**	451 ± 125*	210 ± 71*	71 ± 25	791 ± 234*
	VIII	0.30 ± 0.14	161 ± 80	636 ± 177	278 ± 114	91 ± 44	1166 ± 390
May/02	I	0.14 ± 0.04	1279 ± 427	5845 ± 1679	3182 ± 1004	663 ± 191	10969 ± 3270
	II	0.55 ± 0.15**	131 ± 34***	729 ± 212***	848 ± 254***	176 ± 52***	1883 ± 542***
	IV	0.15 ± 0.10	225 ± 25***	1240 ± 290***	455 ± 95***	150 ± 10***	2070 ± 170***
Apr/03	I	0.30 ± 0.09	745 ± 118	3944 ± 527	1927 ± 337	418 ± 92	7035 ± 1051
	II	0.82 ± 0.22	115 ± 15***	849 ± 173***	342 ± 21***	86 ± 9***	1391 ± 204***
	VIII	0.34 ± 0.10	76 ± 21***	499 ± 125***	198 ± 51***	86 ± 26***	859 ± 203***

Total FACs represent the sum of the four types of metabolites analysed in each sample.
Significant differences to group I: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

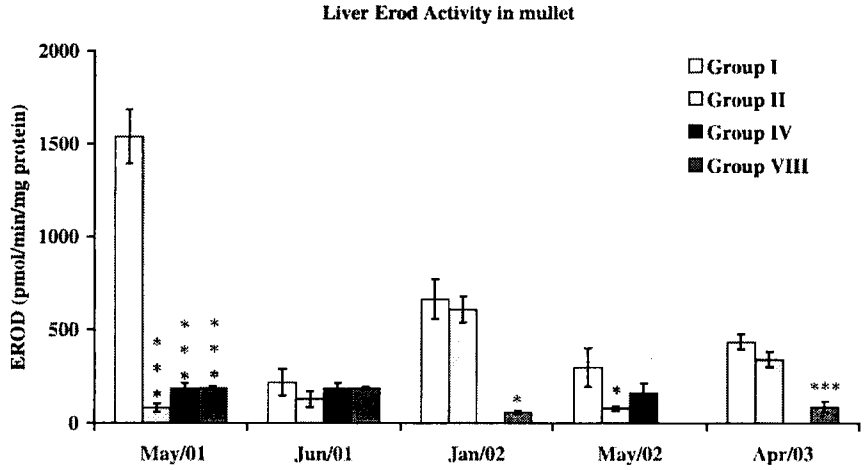

Fig. 2. Ethoxyresorufin *O*-deethylase (EROD) activities in mullet's liver at capture day (Group I), after 1 month (Group II), 4 months (Group IV) and 8 months (Group VIII) in captivity in the 5 periods analysed. Enzyme activities are given as mean ± SE. Significant differences to Group I: $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

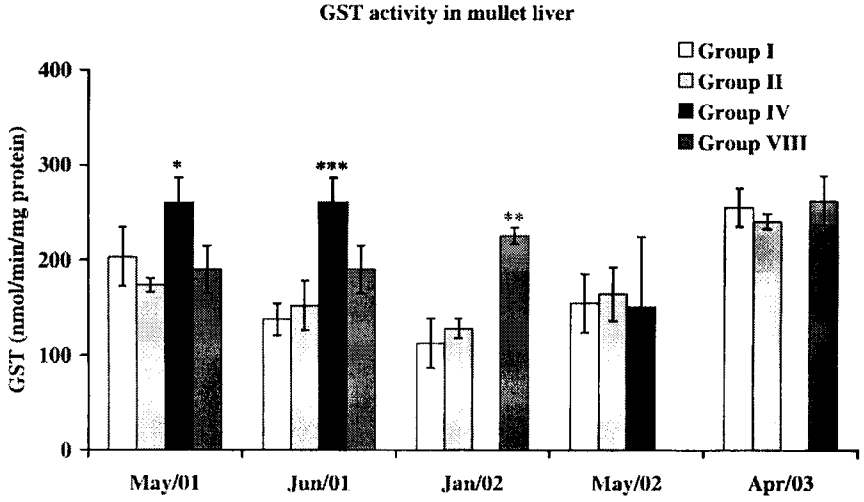

Fig. 3. Glutathione S-transferase (GST) activities in mullet's liver at capture day (Group I), after 1 month (Group II), 4 months (Group IV) and 8 months (Group VIII) in captivity in the 5 periods analysed. Enzyme activities are given as mean ± SE. Significant differences to Group I: $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

in Jun/01 (138 ± 17 to 260 ± 26 nmol/min/mg protein), whereas in May/02 no significant changes occurred. GST activity in mullet after 8 months of depuration increased significantly, by 100%, in Jan/02, while for the other analysed periods no changes were observed.

The biotransformation index (BTI), introduced by Van der Oost et al. (1998), as a new biomarker shows the balance between bioactivation and detoxification, by the ratio between phase I and phase II enzyme activities. In this experiment the BTI (Fig. 4) decreased significantly after 1 and 4 months in captivity only in mullets captured in May/01. Mullets captured in May/01, Jan/02 and Apr/03 have shown a significant decrease in BTI after 8 months in an unpolluted environment.

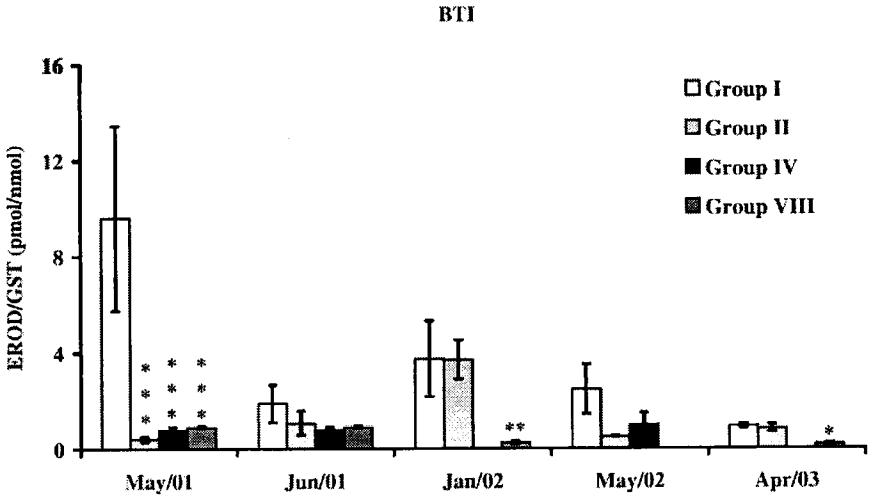

Fig. 4. Biotransformation indices, i.e. phase I:phase II activity ratios EROD:GST in mullet's liver at capture day (Group I), after 1 month (Group II), 4 months (Group IV) and 8 months (Group VIII) in captivity in the 5 periods analysed. Biotransformation indices are given as mean ± SE. Significant differences to Group I: $*p < 0.05$, $** p < 0.01$ and $***p < 0.001$.

4. Discussion

Previous studies described the presence of contaminants in Douro estuary, such as organic contaminants like PCBs and DDTs, and xenoestrogens (Ferreira, Antunes, Gil, Vale, & Reis-Henriques, 2002, 2004) and metals from anthropogenic origin (Mucha, Vasconcelos, & Bordalo, 2003). The present study aims at analysing the presence of an important group of contaminants, the polycyclic aromatic hydrocarbons (PAHs), and the effects of a long term depuration on the phase I and II biotransformation enzymes. In addition, to assess PAH metabolites in bile as a biomarker for PAH exposure of mullets.

Organo-somatic indices, like hepato-somatic index (HSI), provided the status of the organ system, which may change in size due to environmental factors more rapidly than organism weight and lengths increase or decrease. The condition factor (CF) is an organism-level response with factors such as nutritional status, pathogens effects, and toxic chemicals exposure causing increased or decreased weights. In this study the HSI has decreased after the period in captivity. As nutritional quality and diets can affect liver size (Daniels & Robinson, 1986; Foster, Houlihan, & Hall, 1993), the decrease in HSI values can be explained by the different diet the animals had during the captivity period in comparison to the feeding in the natural habitat, that consist mainly of zooplankton, benthic organisms and detritus. HSI from group I was similar on every sampling occasion, except in Jan/02, when the lower levels were probably due to less available food in this period. In addition, the decrease in HSI values, after depuration period, also indicate a recovery from liver enlargement due to chemical exposure, as was reported in rainbow trout (*Oncornhynchus mykiss*) (Poels, van der Graag, & van de Kerkhff, 1980), Atlantic cod (Kiceniuk & Khan, 1987), and winter flounder (*Pleuronectes americanus*) (Fletcher, King, Kiceniuk, & Addison, 1982) exposed to waters containing a mixture of PAHs and other pollutants, and in redbreast sunfish exposed to industrial discharge containing PAHs and PCBs (Adams et al., 1989). In general, CF varies directly with nutrition but can also vary in either direc-

tion outside normal range in response to chemical exposure. The decreases observed in CF after the first month in captivity, in every sampling period, can be directly related to the nutritional status of mullets that experienced a short period of non-consumption of the added food. In the experiments carried out in May/01 and Jun/01 the CF has decreased significantly after the 4th and 8th months in captivity. In the other periods, a significant increase in CF was observed. Reproductive effects were not taken into account, because the mullets used in this study were not developed, and did not attain a vitellogenic or mature state during the captivity.

PAHs are pollutants rapidly metabolized in the liver and secreted into the bile (Brown & Steiner, 2003), and a specific biomarker that has been used to quantify the exposure to PAHs in fish is the level of fluorescent aromatic compounds (FACs) in bile. Fixed wavelength fluorescence (FF) has proven to be a simple and sensitive method for screening PAH contamination in fish, although the FF technique is not for quantification purposes of specific PAH compounds but rather to discern between sites of varying PAH exposure (Aas et al., 2000). In this particular study, the FF technique was used to compare wild and depurated mullets, regarding the PAHs, and the obtained data showed a significant decrease in bile metabolites after depuration. Indeed, metabolites levels in an organism or in its excreta are the result of a clear interaction of a chemical with the biological matrix and often reflect the induction of enzymatic reactions (Van der Oost et al., 2003). In this study a number of important PAH metabolites were detected that are present in the bile of mullets, respectively, phenanthrene, naphthalene, pyrene and benzo[*a*]pyrene. Previous data (unpublished data) have detected PAHs in the coastal area near Douro estuary, mainly low molecular weight PAHs and from petrogenic origins. The dietary status of the animals can affect bile metabolite concentration (Aas et al., 2000; Brumley, Haritos, Ahokas, & Holdway, 1998) and after 1 month in depuration we observed an increase in biliverdin in bile that accounted for the decrease in PAH metabolite in the first month of depuration. After a longer period the biliverdin levels were similar to those observed in Group I and an effective decrease in PAH metabolite occurred indicating PAH metabolization by mullets. Indeed, care should be taken in analysing bile metabolites due to the different bile densities as a result of the feeding status. This study also revealed that independently of the levels of PAHs at capture, after depuration the levels become similar in every sampling period, which means that mullets have the ability to metabolize these compounds despite the levels present at capture. The metabolites in fish bile can provide additional information about the possible sources of PAH contamination. Phenanthrenes are released to the atmosphere during the combustion of fossil fuels, particularly coal, oil and its refined products (O'Malley, Abrajano, & Hellou, 1996; Varanasi, Stein, & Nishimoto, 1989), naphthalene and its derivates are the main contamination due to petroleum fuel (Krahn, Kittle, & MacLeod, 1986), pyrene a widespread and common PAH is generated by many pyrolytic and petrogenic industrial processes (Ariese et al., 1993; Varanasi et al., 1989; Zhou et al., 1998), and benzo[*a*]pyrene is produced by many industrial processes including oil refineries (Lin et al., 1994) often located near estuaries. Depending on the chemical structure and level of exposure, PAHs and their metabolites are putative toxic products that lead to mutagenic and/or carcinogenic effects in fish and other vertebrates, including humans (MacRae & Hall, 1998; Monteiro, Reis-Henriques, & Coimbra, 2000).

So far, in all the periods analysed, a decrease in EROD activity occurred. This is in agreement with our previous study (Ferreira et al., 2004) that showed a decrease in EROD

activity in mullets after a period of 1 month of depuration in unpolluted seawater. The different levels of liver EROD activity at capture can be season related or as a result of recent discharges to the estuary. The higher values observed in May/01 could be related to a recent discharge in the estuary. In Jan/02 we observed higher values that reflect the lower temperatures experienced in this period, leading to higher levels of activity as stated for *Limanda limanda* from the North sea (Sleiderink et al., 1995). Increases in EROD activity have been observed in many fish species after exposure to organic trace pollutants, such as PAHs, PCBs, PCDDs and PCDFs causing very strong increases in CYP1A catalytic activities (Van der Oost et al., 2003). In mullets from Douro estuary, polluted with polychlorinated compounds (Ferreira et al., 2004), xenoestrogens (Ferreira et al., 2002), and metals (Mucha et al., 2003), EROD activity in liver was induced, and a decrease was observed after transfer to a cleaner environment. In addition, we have observed a negative correlation between liver EROD activity and bile metabolites at capture, finding higher levels of bile metabolites in mullets with lower levels of EROD activity. The same tendency was also found in eels (*Anguilla anguilla*) with results showing that induction of cytochrome P450 and accumulation of PAHs metabolites in bile were not necessary correlated in the field (Gorbi & Regoli, 2004). After longer periods of depuration, EROD activity levels may be considered to have achieved a constant activity (from 60 to 190 pmol/min/mg protein), as for the FACs that have similar levels of fluorescence intensity after the same period in depuration. Hepatic EROD activity, in many fish species, seems to be a sensitive biomarker for aquatic pollution. We have observed a significant reduction in PAH metabolites in bile after 4 and 8 months, accompanied by a significant decrease in hepatic EROD activity after 1, 4 and 8 months depuration. In our previous study we observed a clear decrease in liver EROD activity after 1 month of depuration, but the levels of organochlorine contaminants (PCBs and DDTs) did not decrease in the same period (Ferreira et al., 2004). This indicated that not all contaminants induce CYP1A in the same manner, and that care should be taken in analysing biomarker responses at polluted sites using this biomarker.

In this study, while for EROD activities, the phase I biomarker, an overall decrease was observed, for the phase II biotransformation biomarker, the GST activity, an increase occurred with no differences observed in GST activity after 1 month in uncontaminated seawater. Significant decreases in GST activity were reported in rainbow trout exposed to TCDD (Hektoen, Bernloft, Ingebrigtsen, Skaare, & Goksøyr, 1994), sea bass and sunfish exposed to PAH (Lemaire, Förlin, & Livingstone, 1996; Oikari & Jimenez, 1992); and also in fish species from polluted environments (Tuvikene et al., 1999; Otto et al., 1996). The GST liver activity did not show a clear correlation with levels of PAHs metabolites in bile. Similar results were obtained with PCBs and DDTs previously measured in this species (Ferreira et al., 2004) and therefore this enzyme does not seem to be very useful as an exposure biomarker.

The balance between bioactivation and detoxification can be crucial for the assessment of possible hazards due to the exposure to toxic substances. The biotransformation index (BTI) which expresses the ratio between phase I and II enzymes activities has decreased during depuration. This change can be due to the decrease in EROD activities, with the increase of GST activity having no effect on the BTI, in contrast with results obtained with caged carp exposed to pollutants (Van der Oost et al., 1998). The decrease in BTI and EROD can be accounted for by the disappearance of pollutants in the fish as they are metabolised.

5. Conclusion

In conclusion, the results of the present study showed that, the high levels of the phase I biotransformation system, assessed as hepatic EROD activity, may be attributed to the presence of pollutants, including PAHs. However, after 1 month of depuration the enzyme activity attained a lower, basal level. In addition, the biliary fixed fluorescence values, reflecting the PAH and their metabolites, showed a similar pattern with a significant decrease ($p < 0.001$) at the end of the same period of depuration, indicating that these metabolites can provide a useful biomarker for monitoring specific exposure to PAH. This is the first time that the presence of bioavailable PAHs in the Douro estuary has been demonstrated and also that this species has the ability to metabolise and eliminate this type of contaminant.

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CHAPTER 5

The effect of long-term depuration on levels of oxidative stress biomarkers in mullets (*Mugil cephalus*) chronically exposed to contaminants.

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Abstract

The present study aimed to obtain additional data on the effect of long-term depuration in the levels of oxidative stress biomarkers, and to clarify the role of mullets for monitoring pollution in River Douro estuary. Mullets chronically exposed to a mixture of contaminants in Douro estuary were captured in Spring of 2001, 2002 and 2003. The activities of antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx); and oxidative damages in lipids (lipid peroxidation) and in proteins (protein carbonyl content) were assessed at capture day and after transfer to unpolluted seawater for one, four and eight months. An overall decrease in the activities of the antioxidant enzymes was detected, except for the GPx after 4 months depuration. CAT activity exhibited the more significant decrease after the long-term depuration, and the decrease in SOD activity after one month in depuration was maintaining during the other depuration periods. Regarding oxidative damages, a decrease in lipid peroxidation was estimated after depuration as well as the content on oxidised proteins. Indeed, at capture the activities of antioxidant defences were higher as a result of the formation of ROS from the metabolism of pollutants. The oxidative damaged molecules were repaired or degraded during the depuration period, supporting that the damages can be used as indicators of pollution.

Introduction

Environmental contaminants such as metals, polychlorinated compounds (PCBs), polycyclic aromatic hydrocarbons (PAHs) are known to enhance intracellular formation of reactive oxygen species (ROS) with subsequent oxidative damage to macromolecules. The response of organisms to the increase in levels of ROS production involves up-regulating antioxidant defences, such as the activities of antioxidant enzymes, and of scavenger's compounds. The level of antioxidants against ROS overproduction associated with tissue damage can be considerer as a toxicity measure in aquatic organisms exposed to pollution (Ahmad et al., 2000; Di Giulio et al, 1989; Ferreira et al., 2005). The importance of oxidative stress response as potential biomarkers of environmental pollution has been addressed by different experimental approaches (Ferreira et al., 2005; Orbea et al., 2002; Rodriguez-Ariza et al., 2003). The biomarkers utilized include components of oxidative adaptative responses, such as antioxidant enzyme activities (catalase (CAT), superoxide dismutases (SODs) or glutathione peroxidase (GPx)), or the estimation of oxidative damages in lipids, proteins and DNA (Filho, 1996; Winston and Di Giulio, 1991). When defence mechanisms are unbalanced regarding the increased presence of ROS generated compounds, e.g. by the presence of pollutants, oxidative damage will occur, indicating a mechanism of toxicity in aquatic organisms. It has been reported that despite an increase in antioxidant enzymes, oxidative damage occurs in animals living in polluted sites (Van der Oost et al., 2003).

It has been reported previously, several contaminants namely polychlorinated biphenyls (PCBs) and DDT (Ferreira et al., 2004), xenoestrogens (Ferreira et al., 2002), metals from anthropogenic origin (Mucha et al., 2003), and polycyclic aromatic hydrocarbons (PAH) (Ferreira et al., 2006) that are present in River Douro Estuary. An earlier report has shown that mullets (*Mugil cephalus*) contain elevated levels of hepatic SODs and CAT activity that decrease after one month depuration in captivity conditions, and also showed lipid peroxidation in liver (Ferreira et al., 2005). When comparing these results, after depuration, with phase I biotrasformation enzyme, namely ethoxyresorufin O-deethylase (EROD) (Ferreira et al., 2004, 2006), the responses were not so evident. Lipid peroxidation (LP) in mullet liver, after one month depuration, have decreased in some occasions; and regarding oxidised proteins there was an increase after the depuration (Ferreira et al., 2005).

The present work was aiming at evaluating changes in antioxidant defences, measured as hepatic CAT, SOD and GPx activity, associated with longer time in depuration, when animals are transferred from a polluted environment to a laboratory setting. In addition, we

assessed the oxidative damages after long-term depuration, to check if mullets are able to repair mainly the damages observed in liver proteins.

Material and Methods

Study area

The present work was carried out in the lower Douro estuary. The Douro is one of the longest rivers in the Iberian Peninsula (930 Km), sharing its 98,000 Km^2 of watershed with Spain and Portugal. It drains into the Atlantic Ocean at 41°08' N and 8°42' W, near Porto. Domestic sewage as well as industrial effluents are still discharged, mostly without treatment, directly into the estuary and its tributaries.

Sampling

Mullets (N=66, mean weight, 406.4±23.0 g, and mean length, 33.7±1.6 cm) were netted in an assumed polluted site in Douro estuary in the spring of 2001, 2002 and 2003. In each sampling campaign, six mullets (Group I) were sacrificed within 24 hours after capture. Mullets were measured and weighed to determine condition factor (CF) (body weight (g) x 100/ (length (cm))^3). After dissection livers were weighed to determine hepato-somatic index (HSI) (liver weight (g)/body weight (g)x100). Livers were frozen in liquid nitrogen and stored at -80°C until further use. At each sampling occasion, mullets were allowed to depurate separately for one month (Group II), four months (Group IV) and eight months (Group VIII) in 3000 L tanks at a salinity of 20 ‰ (similar to the salinity in the estuary where the mullets were captured) with a flow rate of 5 L/min. Water was continuously filtered through an extensive biological filter, and a charcoal filter before being recycled. Aeration was provided in the tanks to maintain 100% oxygen saturation. Fish were kept under natural photoperiod and temperature. Mullets were fed with uncontaminated frozen fish (hake), during the first 10 days the added food was not consumed probably due to the stress of captivity. At day 15 it was observed that all fish were eating normally.

Antioxidant enzyme activities

Livers were homogenized in ice-cold sodium phosphate buffer 50 mM, Na_2EDTA 0.1 mM, pH 7.8. Mitochondrial fractions were obtained after centrifugation at 15 000 g for 20 min. Catalase activity was determined by measuring the consumption of H_2O_2 at 240 nm (ext. coef. 40 $mM^{-1}.cm^{-1}$) according to Aebi (1974). The reaction volume was 1 ml and contained 67.5 mM potassium phosphate buffer, pH 7.5, and 12.5 mM H_2O_2. The reaction

was started by the addition of the sample. CAT activity is expressed as mmol/min/mg protein. SOD activity was determined by an indirect method involving the inhibition of cytochrome *c* reduction. In this method SOD competes with cytochrome *c* for the superoxide anion generated by the hypoxanthine and xanthine oxidase reaction. SOD activity was determined in the mitochondrial fraction as the degree of inhibition of cytochrome *c* reduction at 550 nm (McCord and Fridovich, 1969). The concentration of the reactives was: potassium phosphate buffer 50 mM, pH 7.8, hypoxanthine 50 μM, xanthine oxidase 1.98 mU/ml and cytochrome *c* 10 μM (Orbea et al., 2002). The activity of MnSOD was evaluated adding to the reaction KCN 2 mM. The activity is given in SOD units (1 SOD unit = 50 % inhibition of the xanthine oxidase reaction). GPx activity was measured with H_2O_2 as substrate, i.e. the activity of the selenium (Se)-containing GPx (Halliwell and Gutteridge, 1999). The activity was monitored by following the decrease in NADPH concentration at 340 nm (ext. coef. 6.22 $mM^{-1}.cm^{-1}$), which is consumed during the generation of GSH from oxidized glutathione, according to Günzler and Flohe (1985). The concentration of the reactives was: potassium phosphate buffer 50 mM, pH 7.8, 2.5 mM GSH, 0.6 mM sodium azide, 1.25 U glutathione reductase, 0.15 mM NADPH, 0.2 mM H_2O_2. GPx activity is expressed as nmol/min/mg protein.

Oxidative damages

The peroxidative damage to lipids that occurs with free radical generation, and results in the production of malondialdehyde (MDA) was assessed by the determination of TBARS. MDA was determined by the thiobarbituric acid method, the liver homogenate was incubated with TCA 100%, after centrifugation the supernatant was incubated at 100°C, for 30 min, with TBA 1%, NaOH 0.05 M and BHT 0.025 % (Niki, 2000). The absorbance was measured at 532 nm. Lipid peroxidation (LP) is expressed as MDA equivalents per mg of protein.

Sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE) was performed using minigels consisting of a 4 % stacking gel and 12.5 % running gel. Fish liver samples were prepared to a final protein concentration of 4 $\mu g.\mu l^{-1}$ and derivatizated with DNPH by incubation at dark for 30 min, a control was incubated with trifluoroacetic acid (TFA) 10% (Levine et al., 1994), without preheating the sample, 5 μl per lane were loaded in the gels. After electrophoresis, separated proteins were transferred onto nitrocellulose membranes using a HOEFER TE 22. Membranes were blocked overnight at 4°C in phosphate buffered saline (PBS) plus 0.05 % Tween 20 (TPBS) containing 5 % non-fatty dried milk. Incubations with the diluted antibody rabbit anti-DNP (1:5000) (DAKO) were preformed for

1h at room temperature. Membranes were washed and incubated with the diluted secondary antibody, anti-rabbit IgG-peroxidase (1:5000) (SIGMA) for 1h at 4ºC. Activity was visualized with an enhanced chemiluminescence (ECL) kit (Amershan Life Science). In all assays total protein was measured by Lowry method adapted to microplates.

Statistical analysis

Differences between groups were tested using a one-Way ANOVA with a multiple comparison test (LSD) at a 5% significance level. Some data had to be log transformed in order to fit ANOVA assumptions. All tests were performed using the software Statistica 6.0 (Statsoft, Inc., 2001). No differences between years were observed and therefore the data were grouped. Differences between genders were also assed, and no statistical differences were observed and therefore the data are presented with both sexes grouped.

Results

Biological parameters such as HSI and CF can be used to assess the status of the organ and the organism. The values for HSI and CF in mullets after capture and after the three periods of depuration revealed significant decreases after one, four and eight months of depuration for HSI, whereas no differences were observed for CF (Table 1).

Table 1: Mullets mean weight (g) and length (cm), hepato-somatic index (HSI) (%) and condition factor (CF) at capture day (Group I), after 1 month (group II), 4 months (Group IV) and after 8 months (Group VIII) in captivity.

	Weight (g)	Length (cm)	HSI (%)	CF
Group I (n=18)	521.5 (29.2)	40.0 (0.8)	1.75 (0.05)	0.80 (0.02)
Group II (n=18)	470.0 (25.1)	39.2 (0.7)	1.27 (0.07)***	0.76 (0.02)
Group IV (n=15)	333.8 (34.4) ***	34.1 (1.6) ***	1.09 (0.06)***	0.84 (0.04)
Group VIII (n=15)	441.7 (37.0) *	37.6 (0.8)	1.23 (0.09)***	0.86 (0.04)

Values presented as mean (Standard Error). Statistical differences to Group I : * $p<0.05$; ** $p<0.01$ and *** $p<0.001$.

Hepatic CAT, totalSOD, MnSOD and GPx activities are displayed in fig.1. CAT activity has shown a slightly increase after one month of depuration, however, after four months the activity was lower (18.4 %; $p<0.05$), and was maintained during the following months of depuration. TotalSOD activity decreased after the first month of depuration (15.2 %; $p<0.05$) maintaining similar values in the forthcoming periods of depuration. Hepatic

MnSOD activity presented the same pattern as liver totalSOD activity, although more pronounced with significant decreases in group II (16.2 %; $p<0.05$), in group IV (22.5 %; $p<0.05$) and in Group VIII (16.2 %; $p<0.05$). Liver GPx activity decreased after one month of depuration (18.8 %; $p<0.05$), and in contrast with the assessed antioxidant enzymes, an increase was observed at the forth month of depuration, and decreasing after eight months of depuration to values similar to the ones measured in group II.

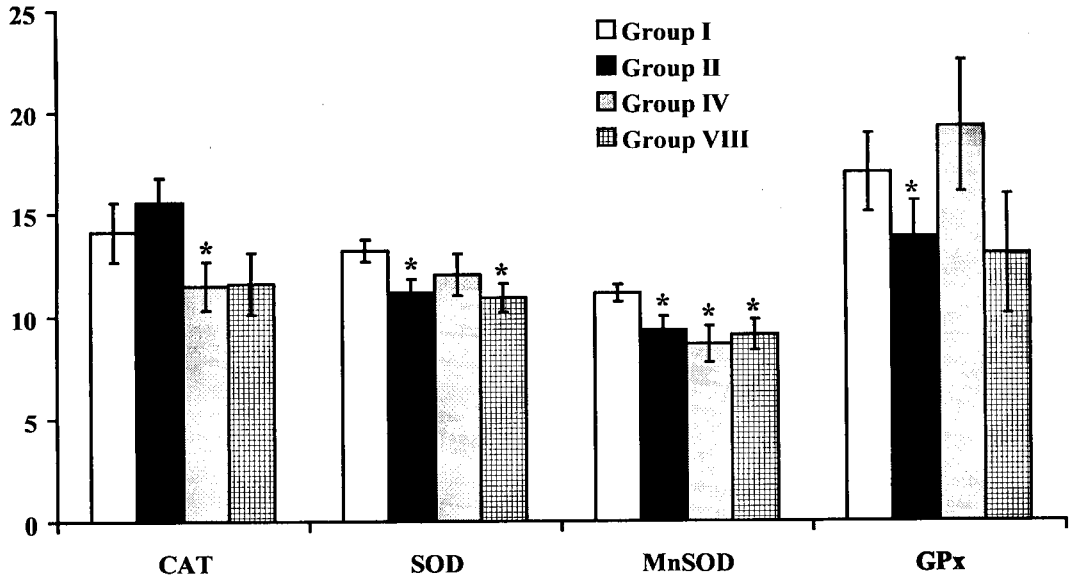

Figure 1: Hepatic antioxidant enzymes activities in mullets after capture in Douro estuary (Group I), and after one month (Group II), 4 months (Group IV) and 8 months (Group VIII) of depuration. Values presented as mean±SE. Statistical differences to Group I: * $p<0.05$. CAT in mmol/min/mg protein, Total SOD and MnSOD in U/mg protein and GPx in nmol/min/mg protein.

Oxidative damages in lipids evaluated as lipid peroxidation levels (LP), using the TBA test, showed that after one month of depuration LP decreased significantly (33.5 %; $p<0.05$), and continued to decrease in group IV (48.8 %;$p<0.05$) (fig. 2). In group VIII however, an increase was observed although bellow the values measured in mullets after capture. Oxidative damages in proteins were evaluated by imunoblot of carbonyl groups (fig.3). One month of depuration led to an increase in oxidised protein content, in agreement with our previous results and only after eight months of depuration was observed a reduction of carbonyl groups.

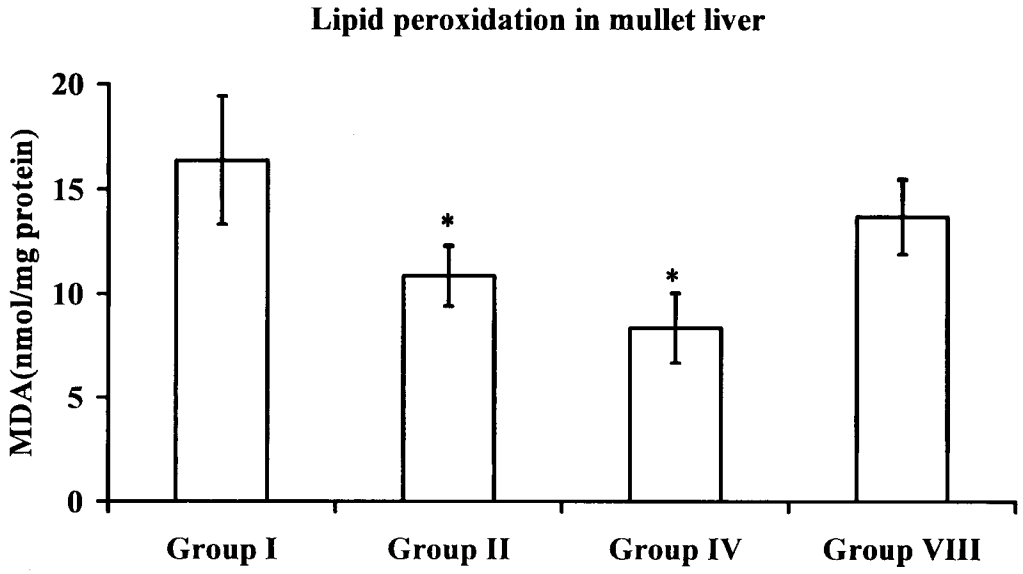

Figure 2: Hepatic lipid peroxidation in mullets after capture in Douro estuary (Group I), and after one month (Group II), 4 months (Group IV) and 8 months (Group VIII) of depuration. Values presented as mean±SE. Statistical differences to Group I: * $p<0.05$.

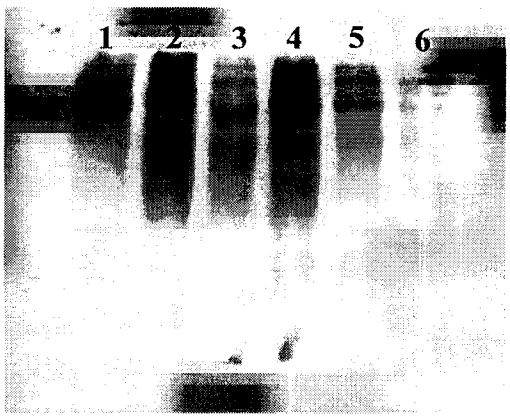

Figure 3: Example of Western blot of carbonyl groups (derivatizated with DNPH) from oxidised mullet liver proteins. In lanes 1 and 3 liver proteins from mullets captured in Douro estuary (Group I), in lanes 2 and 4, from mullets kept in captivity for 1 month (Group II) and in lanes 5 and 6 mullet depurated for 4 and 8 months respectively.

Discussion

Previous studies reported the presence of different types of contaminants in river Douro estuary (Mucha et al., 2003; Ferreira et al., 2004, 2006) inducing oxidative stress in fish inhabiting these waters (Ferreira et al., 2005). However, the changes on oxidative stress

biomarkers, and the ability to recover from damages after different periods of depuration has not been analysed.

Four types of PAH metabolites were found in mullet's bile captured in Douro estuary, and a significant decrease was observed after four and eight months of depuration (Ferreira et al., 2006). The presence of PCBs and DDT was also identified and it was observed that these pollutants are more persistent than PAHs as, one month in depuration, the levels of these contaminants in muscle and liver of mullets are maintained (Ferreira et al., 2004), and started to decrease only after four months depuration (Antunes et al., *in press*), in muscle and no differences for liver levels.

During the captivity period the mullets CF did not change, indicating that the captivity had no effect in the organism level. In addition, the decreased HSI values, after the depuration periods, indicate a recovery of liver enlargement due to chemical exposure (Ferreira et al., 2006).

Antioxidant systems can be considerer as non-specific biomarkers of exposure to pollutants, and also as an indicator of toxicity. The induction of levels of primary antioxidant defences preventing cell damage can be regarded as an adaptative response to an altered environment; in contrast an inhibition can lead to cell damage and toxicity of bioavailable pollutant in a dose-dependent manner (Vassuer and Cossu-Leguille, 2003). After one month of depuration it was observed a hepatic CAT activity increase, contrary to our previous study (Ferreira et al., 2005). This increase could be related to the period of non-consumption of food during the first days of captivity. Some authors reported the influence of starvation in oxidative stress responses with increases in some antioxidant enzyme activities including CAT (Morales et al., 2004). Nevertheless, after four and eight months of depuration CAT activity has significantly decreased, indicating that a longer period of time is needed to recover from oxidative stress induced by pollutants, directly or indirectly. Hepatic totalSOD and MnSOD activity, contrary to CAT activity, showed significant decreases after one month of depuration and the levels were maintained in the following periods analysed. The decrease observed for MnSOD activity was more evident, and so far there are few studies on this enzyme in aquatic organisms although it plays a key role in protecting mitochondria from oxidative stress (Reed, 2001). Interestingly, hepatic GPx activity, that revealed a significant decrease after one month of depuration, has increased in Group IV. This period was concomitant with summer months and the increase in GPx activity can be attributed, probably to the higher temperature experienced in this period, as was reported by Bacanskas et al. (2004) for killifish (*Fundulus heteroclitus*). In addition, the increase in GPx activity in this period may act as protective mechanism avoiding the increase in LP in summer that was reported in mullets in depuration (Ferreira et al., 2005).

Oxidative damage can occur when antioxidant and detoxifying systems are deficient and not able to neutralise the active intermediates produced by the metabolism of xenobiotics or their products. The presence of single and mixed contaminants, including metals, PAHs and PCBs led to an increase in oxidative damages in both fish and invertebrates (Livingstone, 2001). Previously we have reported a decrease in LP in mullet liver, in spring and winter, after one month of depuration, in contrast with the levels of oxidised proteins that were increased (Ferreira et al., 2005). In this study, in summer, LP levels continued to decrease, which could have been a result of the increased GPx activity observed in summer. Stephensen et al. (2002) speculated that GPx was not an important enzyme in destroying organic peroxides in fish, in mullet liver we have found a correlation between GPx activities and LP. Higher GPx activity in Group IV corresponded to lower levels of LP and after eight month of depuration the decrease in GPx activity was traduced to an increase in LPO.

Protein oxidation can be increased by xenobiotic exposure (Gibson et al., 1996; Fessard and Livingstone, 1998), and the assessment of individual or groups of proteins can be used as potential biomarkers of contaminant-mediated oxidative damage in fish liver. The formation of carbonyl derivates is non-reversible, causing conformational changes and decreased catalytic activity in enzymes (Almroth et al., 2005). Our previous study has shown an increase in oxidised protein content in mullet liver after one month of depuration (Ferreira et al., 2005). This increase could be a result of the decrease in antioxidant enzyme activities after the depuration, and because the formation of carbonyl derivates is a non-reversible process we should expect longer time to replace the damaged proteins. In fact, after eight months of depuration the content in oxidised proteins in liver has decreased.

In conclusion, the present study brings important information in the capacity of mullets, after long-term depuration, to recover oxidative damages induced due to the presence of pollutants. Antioxidant enzyme activities showed different responses, with CAT activity decrease after only four month of depuration, whereas for totalSOD and MnSOD activities the decrease was detected after one month of depuration. GPx activity has shown an increase in activity in summer period that inhibited the increase observed in LP. Oxidative damages in LP were back to normal values after only one month of depuration whereas the time for replacing the oxidative damaged proteins was longer.

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DISCUSSION

DISCUSSION

At present, a major task is to build a sustained environmental policy that will enable to maintain a cleaner environment. The main cause for pollution is the industrialization of the Northern Hemisphere with an impact on lakes, rivers, estuaries and ultimately the ocean, thus there is need to prevent further deterioration of water resources, protecting and enhancing ecosystems status. However, the multitude of chemicals in ecosystems, species diversity, biological and ecological functions and structures makes it difficult to estimate possible effects of contaminants in ecosystems (Fent, 2003). It has been recognised for the last years by international organisations and environmental agencies that risk assessment can not be solely based on chemical analysis of environmental samples, because this approach does not provide any indication of the deleterious effects of contaminants on the biota (Cajaraville et al., 2000). Thus, biological monitoring performed at cellular and intracellular levels, as specific biomarkers, is considered to be a more sensitive approach to evaluate water quality (Allan et al., 2006). Nevertheless, a causative correlation between effects and chemical residue analysis is often difficult to establish, and to understand the ecotoxicological effects in contaminated ecosystems we need an integrated approach considering environmental, chemical, toxicological and ecological concepts (Fent, 2003). One of the most important features of biomarkers is that they have the potential to anticipate changes at higher levels of biological organisation, i.e. population, community or ecosystem (Cajaraville et al., 2000), providing insight into the potential mechanisms of contaminant effects, mainly if applied by the screening of multiple biomarkers responses that will give important information about organism toxicant exposure and stress (Van der Oost et al., 2003).

This dissertation discusses the results regarding the accumulation of significant levels of organochlorine contaminants (PCBs and DDTs) in mullet and flounder tissues (muscle and liver) reflecting their presence in the estuary. The PCB and DDT patterns were very similar between species, irrespective of tissue type, indicating similar contamination sources. In mullet the most predominant congeners were the CB180, CB153 and CB138 that accounted for 55-57% of tPCB, whereas in flounder the CB153 and CB138 accounted for 41-44% of tPCB. In agreement with other studies the CB153 was the most predominant congener in all the samples analysed (Antunes and Gil, 2004; Naso et al., 2005). The PCBs patterns are always dominated by a large contribution from the hepta-, hexa-, and pentachlorobiphenyls CBs 180, 153, 138 and 118; being these congeners the most abundant in commercial PCB mixtures, such as Aroclor 1254 and

1260, which were the most common used in European countries (Naso et al., 2005). The individual PCB congener's distribution observed in mullet and flounder from Douro estuary was in accordance with earlier published results obtained in marine invertebrates and vertebrates colleted in polluted areas, with the congeners 101, 118, 138, 153, 170, 180, 187 as the major components of the mixtures (Porte and Albaigés, 1993; Goerke and Weber, 2001; Bayarri et al., 2001). From the 209 PCB congeners a dozen are considerer to be "dioxin-like" because of their toxicity and certain features of their structure which make them similar to the 2,3,7,8-tetrachlorodibenzo-p-dioxin (2378-TCDD). The congeners 118 (2,3',4,4'5-pentachlorobiphenyl) and 105 (2,3,3',4,4'-pentachlorobiphenyl), two of the twelve "dioxin-like" congeners, were detected in both species. In flounder tissues were found the higher percentages of CB105 and CB118, however tissue concentrations were found to be similar between species, and ranging from 0.02 to 0.9 ng/g FW (fresh weight). The one month depuration experience reflected the persistency of these types of compounds, with data showing no decrease in the levels of PCBs and DDTs in muscle and liver, of both species.

Mean tPCB levels were also calculated in a fresh weight basis in both species and tissues. Muscle of flounder presented 0.54 ng/g FW, whereas mullet showed considerably higher levels (4.6 ng/g FW); on the contrary, in liver, species showed a different trend with flounder presenting higher levels (9.4 ng/g FW) than mullet (6.9 ng/g FW). Concerning tDDT, in both species and tissues, the levels were considerable lower when comparing with tPCB levels. tDDT levels in flounder muscle (0.18 ng/g FW) where lower than in mullet muscle (0.93 ng/g FW), and the same trend, as for tPCB, was seen in liver with flounder presenting higher levels than mullet (3.6 ng/g FW in flounder and 1.4 ng/g FW in mullet). Mullet and flounder presented different lipid accumulation in liver and muscle, and the higher lipid content in mullet muscle and in flounder liver reflects the higher accumulation of POPs in these tissues.

The most studied tissue, concerning POPs accumulation, is muscle, the edible part of the fish, and a vehicle to human exposure through the diet. Due to their stable structure and lipophilic character they tend to concentrate and magnify in the food chain, particularly associated with fat. The ingestion of contaminated food contributes more than 90% to the total exposure and food of animal origin, such as fish and seafood, are recognised as one of the main contributors (Bordajandi et al., 2006). PCBs and DDTs are broadly distributed and high levels have been detected in flounders in estuaries and offshore in North America (Ray et al., 1998) and mullets in estuaries and fish farms in Asian countries (Fu and Wu, 2005; Fu and Wu, 2006). Data from studies performed in European countries had also showed higher levels in several fish species (de Boer et al., 2001; Roots, 2001). Mediterranean countries share eating habits with Portuguese, often

including fish and seafood in the diets. POPs accumulation, in mullet and flounder tissues from Douro estuary, were lower than the ones detected in mullets and flounders in estuaries in the north (Orbea et al., 2002) and in the south (Bordajandi et al., 2003; Bordajandi et al., 2006) of Spain, as well as in other fish species like sardine and sole (Bordajandi et al., 2006), carp and barbell (Lavado et al., 2006); the same picture occurs in Italy with several studies reporting data on PCBs and DDTs levels, in estuarine species and in edible tissues considerable higher than our data (Corsolini et al., 2005; Naso et al., 2005; Storelli et al., 2007). In contrast, mullet and seabass from Ria de Aveiro showed lower levels of PCBs and DDTs (Antunes et al., 2001; Antunes and Gil, 2004); than mullets and flounders from Douro estuary.

However, the fact that production of PCBs and DDTs has been limited since the 1970s in most developed countries seems to have some effect. It's a fact that of PCBs concentrations have dramatically declined in all environmental media (air, water, soil/sediment), in fish and other sea foods, and ultimately in humans (Ross, 2004). Even so, the hazard associated with PCBs and DDTs has not changed over the years. The DDE/DDT ratio is used to assess the chronology of DDT input into the ecosystem (Bordajandi et al., 2003; Naso et al., 2005). DDE/DDT ratio in flounder and mullet was found to be greater than 1 suggesting no recent input of DDT in the Douro estuary region. As mullet is not usually included in the Portuguese diet, and flounder's liver is not one of the edible parts, the input of these pollutants in humans through the diet is in some way reduced due to the different accumulation patterns in tissues.

The presence of chemicals with endocrine disrupting activity has been reported in Portuguese environments (Céspedes et al., 2004; Quiros et al., 2005; Rodrigues et al., 2006). The highest concentrations in endocrine disrupting chemicals (ECDs), in Portugal, were detected in industrial areas close to Porto and Lisbon (Céspedes et al., 2004), as well as the most potent estrogenic compounds (Quiros et al., 2005). In male mullet we have detected the presence of an intersex condition due to the exposure to xenoestrogens, characterised by the presence of oocytes in the testicular tissue, characteristic that was never observed in male flounders. We would expect to have observed this condition in flounder, a species more exposed to sediment-associated lipophilic contaminants, than midwater fish such as mullet, both through direct exposure when they bury themselves in sediment, and through their consumption of benthic invertebrates. A possible disadvantage is that the most estrogen-sensitive part of the cycle, the sexual differentiation in the larvae, probably occurs at sea under relatively uncontaminated conditions (Matthiessen et al., 1998).

Phase I biotransformation was evaluated by measuring hepatic EROD activity that showed to be species dependent, with mullet showing levels of activity ten fold higher than flounder. In mullet, one month depuration led to a decrease in EROD activity. In flounder, EROD activity had also decreased after depuration. However, from January to May, we have observed, besides lowered EROD activity levels, an inversion of the pattern, after the depuration period. These alterations in flounder's EROD activity may be due to differences in maturation stages which in turn may modulate EROD activity. A similar trend was observed by Eggens et al., (1996) in flounder, with lower EROD activity levels registered in January and February, and suggesting that monitoring programmes using flounder and EROD activity should be preformed outside the reproduction period. The mechanism for CYP1A suppression in spawning females is related to 17β-estradiol (E2) levels (Whyte et al., 2000) and in many fish species it has been reported that E2 or its synthetic homologue ethinylestradiol (EE2) suppresses EROD activity (Arukwe et al., 1997; Arukwe and Goksøyr, 1997; Solé et al., 2000). In fact, we have observed a negative correlation (R^2=0.68) between EROD activity and the gonadosomatic index (GSI) in flounder. Outside the reproduction period in flounders, and in mullets, hepatic EROD activities have decreased, after the one month period of depuration, however this decrease was not a result of lowered levels of organochlorine chemicals in species tissues after depuration. Most likely the decrease observed in EROD activity was associated with other EROD inducers present, such as PAHs more easily and rapidly metabolised by fish (Van der Oost et al., 2003).

Oxidative stress biomarkers were evaluated in both species as the primary antioxidant defences (CAT, tSOD) and oxidative damages in lipids and proteins. The activities of the two antioxidant enzymes (tSOD and CAT) revealed that they are species dependent, in agreement with phase I enzyme (EROD), flounders and mullets displayed different levels of primary antioxidant defences, with flounders showing lower levels of CAT and tSOD activities. The major difference was found for MnSOD activity in mullet, suggesting that mullets liver mitochondria can deal with the increase in superoxide anion more efficiently than flounders. In regard to the oxidative damages in liver lipids, the values for MDA in flounder were slightly higher then the ones estimated for mullets. This difference may be related with less efficient defences which is consistent with the lower levels of the antioxidant primary defences assessed. In addition to that, the difference in fatty acid composition of the liver membranes may also be involved (Halliwell and Gutteridge, 1999). However, the fasting conditions during the experimental period have conditioned the responses from antioxidant enzymes, and oxidative damages, in lipids and proteins, after the depuration period. The food availability can have an effect on the

antioxidant enzymes activities and oxidative damages, as Pascual et al. (2003) showed in *Sparus aurata*, in conditions of fasting or limited food availability after 3 weeks that increased MDA levels. As the lipid storage are mobilised to cope with metabolic needs, lipids become a more exposed target to oxidation. The results obtained with flounder showed that is important to maintain as close as possible the natural conditions; biomarkers determinations can be masked by the nutritional status of the animal in the captivity. In contrast, in mullets it was observed a decrease in CAT and SOD activity after one month depuration, confirming that the animals are being exposed to oxidative stress due to pollutants and can reprogramme the cell response when transferred to a cleaner environment.

In summary, we found that flounder was not an appropriate model sentinel to monitor the effects of pollutants present in the estuary. Thus, in this experimental design, mullet proved to be more suitable species for monitoring purposes. In mullet, the most widely used biomarker, EROD activity, was not influenced by reproductive factors that alter the levels of EROD activity. In addition, the fasting condition flounders experienced during the captivity conditions have influenced oxidative stress biomarkers responses, which were not correlated with the presence of pollutants in the field. Several studies, with environmental monitoring purposes were carried out using flounder as a sentinel species (Elskus et al., 1994; Eggens et al., 1996; Matthiessen et al., 1998; Roots, 2001; Vinagre et al., 2004). More recently several authors have also chosen mullet to be included in monitoring studies (Pastor et al., 1996; Orbea et al., 2002; Licata et al., 2003; Perugini et al., 2004; Fu and Wu, 2005).

The persistence of organochlorine contaminants is an environmental problem because they tend to accumulate in animals fat depots and bioaccumulate through the food chain; and the one month depuration experiment has confirmed PCBs and DDTs persistency in fish tissues. The long-term depuration (120 and 270 days) experiment showed a slightly decrease in tPCBs levels in mullet liver, while in muscle a significant decrease has occurred. DDT compounds in muscle followed the same trend, however in liver a slightly increase was observed between day 0 and day 270. Ninety percent of the higher chlorinated CBs were efficiently eliminated from muscle, whereas 29% of lower chlorinated CBs remained at residual concentrations during the 8 months depuration. The same trend was observed for tDDT, presenting decreases of 60% for DDD and 90% for DDT, while DDE showed slower decreases. The decrease in these compounds in muscle parallels with the decrease in lipids, suggesting that lipids consumption is the major factor driving the elimination kinetics of PCBs and DDTs in mullet muscle. Data obtained with

artic charr also showed an increase in PCB concentration in liver due to lipid mobilization (Jorgensen et al., 1999).

Until this point we have described the presence of POPs (PCBs and DDTs) and xenoestrogen compounds in the Douro estuary. In addition, previous data (unpublished data) have detected PAHs in the coastal area near Douro estuary, mainly low molecular weight PAHs and assumed from petrogenic origins. In order to evaluate mullet exposure to PAHs in Douro estuary, and their ability to metabolise them, we have analysed PAH metabolite in bile in a long-term depuration experiment. The liver is the main site of PAH metabolism (Au et al., 1999) and the metabolites are secreted into the bile and stored in the gall bladder before being excreted into the alimentary tract (Brown and Steinert, 2004). As many fish species are highly mobile, measurement of bile fluorescent aromatic compounds (FACs) is often a more reliable method for assessing exposure to PAHs, than measuring PAHs where the fish were captured (Collier and Varanasi, 1991; Arcand-Hoy and Metcalfe, 1999), and bioavailability is taken into account. Measuring PAH concentrations in fish tissues, to estimate exposure, is often not reliable due to the fast metabolism of PAH in fish (Livingstone, 1998), together with the difficulties associated with the analytical measurement of volatile PAH compounds. Therefore, the analysis of bile metabolites has become a convenient and a relatively rapid method for monitoring PAH contamination in fish (Lin et al., 1994), and fluorescent aromatic compounds (FACs) in bile have been used as a specific biomarker to quantify the exposure to PAHs in fish (King et al., 2005; Lee and Anderson, 2005; Vuorinen et al., 2006). Indeed, metabolites levels in an organism or in its excreta are the result of a clear interaction of a chemical with the biological matrix and often reflect the induction of enzymatic reactions (Van der Oost et al., 2003). In this study a number of important PAH metabolites were detected in the bile of mullets, namely phenanthrene, naphthalene, pyrene and benzo[*a*]pyrene.

The different types of PAH can give additional information about the possible sources of contamination. Phenanthrenes are released to the atmosphere during the combustion of fossil fuels, particularly coal, oil and its refined products (Varanasi et al., 1989; O'Malley et al., 1996), naphthalene and its derivates are the main contamination due to petroleum fuel (Krahn et al., 1986), pyrene a widespread and common PAH is generated by many pyrolytic and petrogenic industrial processes (Varanasi et al., 1989; Ariese et al., 1993; Zhou et al., 1998) and benzo[*a*]pyrene is produced by many industrial processes including oil refineries (Lin et al., 1994) often located near estuaries.

The dietary status of the animals can affect bile metabolite concentration (Brumley et al., 1998; Aas et al., 2000) and after one month of depuration we observed an increase in biliverdin levels in bile that accounted for the decrease in PAH metabolite. After a longer

period, biliverdin levels were similar to those observed in mullet after capture and an effective decrease in PAH metabolite occurred, indicating PAH metabolization by mullets. Indeed, care should be taken in analysing bile metabolites due to the different bile densities as a result of the feeding status. This study also revealed that independently of the levels of PAHs at capture, after depuration the levels become similar in every sampling period, which means that mullets have the ability to metabolize these compounds despite the levels present at capture. While for PCBs and DDTs no clear reduction in their levels, in liver, was observed after the depuration periods, FAC analysis revealed that total PAH metabolites, in bile, decrease 72% after one months depuration (although these values are dependent on the fasting period mullet had in captivity). However, after four and eight months a significant reduction has occurred in total PAH metabolites, 81% and 59% respectively.

A first level screen to identify potential pollutant exposure and effect can be accomplished on the basis of simple measurements of condition like the morphological parameters. The condition factor (CF), to assess the general condition of the fish, may be affected if the food availability is limited or food consumption is impaired due to stress factors. Mullets, after longer depuration periods, have increased their CF that could indicate a better health status in cleaner conditions. The contrary has occurred with flounders due to inhibition of food consumption in stress captivity that have also affected the hepatosomatic index (HSI). The HSI identify possible liver diseases, the organ may change in size due to environmental factors more rapidly than organism weight and length increase or decrease. Several studies have also reported higher HSI values in fish from more contaminated sites (Huuskonen and Lindstrom-Seppa, 1995; Kirby et al., 1999).

Biochemical changes in fish tissues such as enzymatic activities, mainly in liver, are the most used parameters as biomarkers, and we have evaluated different enzymatic activities in mullets after three periods of depuration in cleaner conditions, one and four and eight months, EROD activity to evaluate the phase I biotransformation, and GST activity to phase II. Mullet's hepatic EROD activity after one month depuration showed a 47.5% decrease, whereas GST presented an increase of 2.1%. The same trend was seen after longer period of depuration, four months depuration leads to an average decrease of 49.3% in hepatic EROD activity, after 8 months the average decrease was even higher (69.0%). The decrease observed in liver EROD activity clearly indicates that the chemicals present in the estuary have the ability to induce phase I enzymes. Due to their persistency, PCBs and DDT levels in liver were not decreased after the depurations periods, and can not be related to EROD activities recorded in mullet. However, the

significant decreases observed for PAH metabolites after depuration can be correlated to the decrease observed for EROD activity.

The phase II biotransformation, evaluated as hepatic GST activity, showed an overall increase after the depuration in cleaner conditions; one month depuration lead to an average 2% increase, and 38% and 34% average increase after four and eight months depuration, respectively. Data available on GST variation is controversial. Some studies have demonstrated increased GST activity, in mullet species with pollutants exposure (Sen and Kirikbakan, 2004), and other species like *Prochilodus lineatus*, (Camargo and Martinez, 2006), others reported no variations in mullet (Porte et al., 2002) and in three-spined stickleback (Sanchez et al., 2005) and even decreased activity in mullet after exposure to PCBs (Otto and Moon, 1996). Some authors have reported the induction of GST in rainbow trout due to PCB (Perez-Lopez et al., 1998; Perez-Lopez et al., 2002) and DDE exposure (Machala et al., 1998). Depuration, in this work, was not followed by a reduction in PCB and DDT levels and GST activity has not decreased as well, during the depuration. In comparison, these two biomarkers from phase I and phase II have given contrary results, and the responses obtained for EROD can be easily correlated to the presence of PAHs, the same does not occur with GST. We would expect GST activity to follow EROD activity, however due to the role that GSTs play in conjugating reactive epoxide species and other electrophiles, induction of these enzymes must be considered to be beneficial (Commandeur et al., 1995; Van der Oost et al., 2003). The use of this phase II enzyme in fish does not seem very useful as an exposure biomarker for monitoring purposes.

Antioxidant enzymes are generally less responsive to pollutants than phase I and phase II, and the relationships between response and contaminant exposure are still less well established (Van der Oost et al., 2003). In our study, antioxidant enzyme activities showed less marked decreases in their activities after the one month depuration period in comparison, particularly, to EROD activity. The long-term depuration experience have confirmed these features of the primary antioxidant defences showing less marked decreases after four and eight months in depuration. After one month depuration CAT activity maintained the same levels, SOD and MnSOD have decreased about 15% in activity, while GPx have decreased 24%, decreases significantly lower than the ones observed for EROD activity after the same period in depuration. SOD and MnSOD have maintained the same levels of activity during the forthcoming periods of depuration, whereas CAT activity has decreased only in the fourth month depuration, maintaining the levels at the eighth month. Hepatic GPx activity, on the contrary, has increased after 4 months in depuration, due to an increase in temperature during that period. On the other

hand, this increase in GPx during that period accounted for the decrease in LP levels. Some authors speculated that in rainbow trout GPx was not an important enzyme in destroying organic peroxides in fish (Stephensen et al., 2002), in mullet we have found a correlation between GPx activity and the levels of LP.

Although primarily a pathway for detoxification, there are various ways whereby cyt P450 activity can elicit toxic effects (Stegeman and Hahn, 1994) and some authors have correlated CYP1A1 induction with oxidative damage (Park et al., 1996; Schlezinger et al., 2006). Lipid peroxidation showed decreases after depuration that correlates well with the decreases registered for EROD activity. There is a possible correlation between the induction of cyt P450 being in part responsible for the oxidative stress that animals were facing in the estuary, as a consequence of cyt P450 activation. The decrease observed in EROD activity could have been at some degree responsible for the decrease in oxidative stress parameters.

Protein oxidation (PO) by the formation of carbonyl derivates is non-reversible, even causing conformational changes and decreased catalytic activity in enzymes (Almroth et al., 2005). The increase observed in PO, in mullet after one month depuration, could be due to deficient defence against ROS, indeed the activity of antioxidant enzymes decreased after the depuration. However, after eight months of depuration the content in oxidised proteins in liver decreased, mainly proteins with lower molecular mass. This decrease in PO content after a longer period in an unpolluted environment indicates that the presence of contaminants can induce oxidative damages in proteins by promoting the formation of ROS. Increased levels of PO due to exposure to contaminants have also been reported for flounder, in a laboratory study (Fessard and Livingstone, 1998), and in eelpout from the field (Almroth et al., 2005).

In the Douro estuary there is a complex mixture of pollutants such as of PCBs, DDTs and PAHs (measured in this study) but also xenoestrogens (Céspedes et al., 2004; Quiros et al., 2005), as well as metals from anthropogenic origins (Mucha et al., 2003), that are inducing changes in the species inhabiting this environment. However, care should be taken in analysing the responses because there are many other factors, than contaminant exposure, that could also make the interpretation of responses difficult to evaluate, and integrate. During the course of this study we encountered some factors that altered the biomarkers responses, not directed related with pollutants present in the environment. Mainly in flounders, the responses were conditioned by biological factors such as reproduction, and the experimental conditions (the fish did not eat the added food during the depuration period). During the experimental period no mature mullets were captured and they have eaten the added food (hake fillet) after a short period of

non-consumption (10 to 15 days), fact that overcame the problems that were encountered with flounders. Biomarkers responses in mullet were related with the presence of chemical stressors present in the Douro estuary. Another important factor in field studies is the multitude of chemicals present in the aquatic environment that makes it difficult to correlate/integrate biomarkers responses with specific pollutants, in a dose-response manner.

At present we have indications for the presence of several types of contaminants, in River Douro estuary, such as PCBs, DDTs, PAHs and xenoestrogens. Considering the xenoestrogens it would be useful to know the levels of these compounds. The multitude of chemicals, present in the estuary, has induced changes in the biomarkers assessed in this research. However, to know which chemicals have induced these changes in biomarkers we could perform exposures, in laboratory controlled conditions, with single and mixtures of contaminants. These exposures would give insights to the chemicals that are more potent to the formation of ROS and that induced oxidative stress. The use of oxidative stress biomarkers needs further research before being included in monitoring programmes.

The presence of chemicals in the environment, even at sublethal concentrations, induces changes, at cellular and subcellular levels, in mullet and flounder. These changes can have long term effects in the wild life, that can be translated in the decline in fish populations. The effects of exposure to pollutants in fish can be extrapolated to human life, given indications about the levels of exposure and ultimately the possible effects that these chemicals have in human health as well. Our goal is to maintain an unpolluted ecosystem in order to improve animal and human health and well being.

CONCLUSIONS

Conclusions

To finalise this research we summarize the following main conclusions:

- In both species, and tissues, we have reported the accumulation of significant levels of PCBs and DDT. The congeners showed a similar pattern in both species; however flounder showed a higher percentage of the "dioxin-like" congeners, CB105 and CB118.
- The depuration period was reflected in a decrease of PCBs and DDT in muscle, but not in liver, thus lipid consumption leads to a redistribution of PCBs and DDTs during the captivity.
- PAH are also present in the estuary and being less lipophilic chemicals, are easier metabolised in liver of fish species, reflected by a significant decrease of these metabolites in mullet's bile after depuration.
- In environmental monitoring is important the use of different species that could reflect different types of pollution inputs, like was shown in mullets, but not in flounders, presenting an intersex condition confirming the presence of chemicals with xenoestrogen activity.
- Biomarkers responses in flounder were masked by biological factors, like reproduction, and the experimental conditions. Reproduction in flounder has decreased the EROD activity, and starvation during depuration had effects at the level of antioxidant defences and oxidative damages. For the stated reasons, we can not conclude about biomarkers responses in flounder and mullet was considered to be a more suitable sentinel species for the presence of pollutants in River Douro estuary.
- In the field, monitoring should be preformed using different types of biomarkers due to the presence of several types of chemical pollutants. This study has shown that phase I biotransformation enzyme, assessed as EROD activity, presented a significant decrease with depuration, contrary to phase II that has showed an increase in some periods. Even so, in the field is not an easy task to correlate biomarkers responses with the respective contaminants.
- Antioxidant defences had also shown a decrease with depuration, but not as noticeable as for phase I activity. Depuration had also reflected a decrease in oxidative damages. For those reasons we can conclude that animals inhabiting Douro estuary are facing oxidative stress due to the presence of contaminants, and that the transfer to a cleaner environment leads to a recovery of damaged macromolecules.

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