UNIVERSIDADE DO ALGARVE

FACULDADE DE CIÊNCIAS DO MAR E DO AMBIENTE

CHEMICAL AND BIOCHEMICAL TOOLS TO ASSESS POLLUTION EXPOSURE IN AQUATIC ECOSYSTEMS

(Dissertation presented at the University of Algarve to obtain the degree of Doctor in Philosophy in Environmental Sciences and technologies, Area of Aquatic Environment)

Denise Robina Teixeira Fernandes

Orientador: Doutora Maria João Bebianno

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Constituição do júri:

Presidente: Reitor da Universidade do Algarve

Vogais: Doutor Christophe Minier, Doutor William J. Langston, Doutora Maria Armanda Reis Henriques, Doutora Maria Helena F. R. da Costa, Doutora Maria João Bebianno, Doutora Alexandra Cravo, Doutora Cinta Porte Visa, Doutora Ângela Serafim

FARO

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General Abstract

Aquatic environments offer and sustain important habitats for many fish species, but also attract an ever-increasing level of human activities. These environments are the ultimate sink for many of the chemical contaminants released from anthropogenic activities, which can represent a great threat to organisms living in these ecosystems, as to human health. In recent years, there has been a growing awareness of the need to detect and assess the adverse effects of contaminants in organisms exposed to different levels of pollutants. Among the available techniques, the integrated use of chemical analysis and biochemical responses to pollutants is a sound procedure for detecting impact of anthropogenic contaminants in aquatic systems. Besides wild fish consumption, the aquaculture industry is an expanding activity, due to the world population demand of fish however, the intensive production and the extensive use of chemicals in current practises, has raised concerns over the quality of cultured fish in comparison to wild fish. Although, both wild and cultured fish are important components of the Atlantic and Mediterranean diet, there is little information regarding pollutant levels in farmed fish and the risks associated to consumption. In the present thesis the impact of pollutants were investigated in both wild and cultured fish from different aquatic ecosystems, by the combined analysis of chemical residues in fish tissues together with biochemical responses. Specific biological responses (MT, phase I and II enzymes, CAT, AChE, Vtg) to a range of environmental stressors (metals, OCs, PAHs, APEs) were assessed in wild sea bass (*Dicentrarchus labrax* L.) from the Arade Estuary (S Portugal) and in cultured sea bass from different aquaculture facilities located in Southern Europe. Furthermore, the impact of pollution along the Northern

Iberian coast was assessed using two commercial fish species, the four-spotted-megrim (*Lepidorhombus boscii*) and the pouting (*Trisopterus luscus*) as sentinel organisms. Finally, since the interference of xenobiotics with steroid synthesis and steroid metabolism in fish may alter the bioavailable amounts of active hormones within the organism, the metabolism of 17α-hydroxyprogesterone ($17P_4$) in male sea bass gonads was investigated, as a potential tool for the detection of endocrine alterations in male fish, by evaluating the *in vitro* effect of model endocrine disrupters (NP, BaA, TBT, *p,p*'-DDE, KCZ)

Keywords: aquatic ecosystems, fish, biomarkers, pollution, aquaculture, endocrine disruptors, 17α-hydroxyprogesterone

Resumo Geral

Como consequência da actividade humana, o ambiente aquático recebe múltiplos aportes de contaminantes, que representam não só uma grande ameaça aos organismos que nele habitam mas também á saúde humana. Nos últimos anos, tem havido uma crescente consciencialização na necessidade de detectar e avaliar os efeitos adversos desses contaminantes em organismos expostos a diferentes níveis de poluentes. Entre as técnicas disponíveis, o uso integrado de análises químicas e de respostas bioquímicas a poluentes é considerado um procedimento adequado para detectar o impacto de contaminantes antropogénicos em sistemas aquáticos. Para além do consumo de peixe selvagem, a indústria da aquacultura é uma actividade em grande expansão, devido á necessidade da população mundial em pescado, contudo a produção intensiva e o uso de compostos químicos nesta actividade, tem levantado grandes preocupações quanto á qualidade dos peixes de cultivo comparativamente com os selvagens. Apesar, de tanto o peixe selvagem como o de cultivo serem componentes importantes da dieta Mediterrânea e Atlântica, existe pouca informação sobre os níveis de contaminantes em peixes de cultivo, e os riscos associados ao seu consumo. Nesta tese, o impacto de contaminantes tanto em peixes selvagens como de cultivo, provenientes de diferentes ecossistemas aquáticos foi investigado, através da análise integrada de resíduos químicos em tecidos com respostas bioquímicas. Respostas bioquímicas específicas (MT, enzimas da fase I e II, CAT, AChE, Vtg) a uma série de contaminantes ambientais (metais, OCs, PAHs, APEs) foram avaliadas em robalo (*Dicentrarchus labrax*) selvagem proveniente de diferentes zonas do Estuário do Arade (S Portugal) e de cultivo proveniente de diferentes aquaculturas localizadas no Sul da Europa.

Adicionalmente, foi avaliado o impacto da poluição ao longo da costa Norte da Península Ibérica utilizando duas espécies de peixe comercial, o areeiro-de-quatro-manchas (*Lepidorhombus boscii*) e a faneca (*Trisopterus luscus*), como organismos sentinela. Finalmente, devido ao facto de certos compostos xenobioticos poderem interferir na síntese e/ou o metabolismo de hormonas sexuais em peixes e alterar a quantidade biodisponível de hormonas activas no organismo, foi investigado, nas gónadas masculinas de robalo, o metabolismo de 17α-hidroxiprogesterona ($17P_4$), como uma potencial sonda para detectar alterações endócrinas em machos, tendo-se avaliado o efeito *in vitro* de compostos químicos considerados modelo de disrupção endócrina (NP, BaA, TBT, *p,p*'-DDE, KCZ).

Palavras-chave: ecossistemas aquáticos, peixes, biomarcadores, poluição, aquacultura, disruptores endócrinos, 17α-hidroxiprogesterona

This thesis has been accomplished in the Ecotoxicology and Environmental Chemistry group from the Faculty of Marine and Environmental Sciences (F.C.M.A.) of the Algarve University, and in the Environmental Chemistry Department from the Superior Counsel of Scientific Investigations (CSIC) of Barcelona, thanks to a PhD fellowship (SFRH/BD/6123/2001) from the Portuguese Fundação para a Ciência e Tecnologia (FCT) of the Ministry of Science and Technology of Portugal

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2.1.

Acronym List

ACRONYM LIST

11-KT: 11-Ketotestosterone

17P$_4$: 17α-Hydroxyprogesterone

AChE: Acetylcholinesterase

AD: Androstenedione

AhR: Arylhydrocarbon Receptor

APE: Alkylphenol ethoxylate

βA: 11β-Hydroxyandrostenedione

BaA: Benzo[a]anthracene

BSTFA: Bis(trimethylsilyl) trifluoroacetamide

CAT: Catalase

Cd: Cadmium

CF: Condition factor

Cr: Cromium

Cu: Copper

CuSO$_4$: Copper sulphate

CYP: Cytochrome P450

CYP17: Cythochrome P450 17α-hydroxylase and C17,20-lyase

CYP19: P450 aromatase

CYP11B: 11β-Hydroxylase

DDD: Dichlorodiphenyldichloroethane

DDE: Dichlorodiphenyldichloroethylene

DDT: Dichlorodiphenyltrichloroethane

EDs: Endocrine Disruptors

EPA: Environmental Protection Agency

EROD: 7-Ethoxyresorufin *O*-Deethylase

ERs: Estrogen Receptors

FAO: Food and Agriculture Organization (United Nations)

Fe: Iron

FF: Fixed Wavelength Fluorescence

GC-MS: Gas Chromatography-Mass Spectrometry

GC-ECD: Gas Chromatography-Electron Capture Detector

GST: Glutathione *S*-Transferase

HCB: Hexaclorobenzene

γ-HCH: Hexaclorocyclohexane (lindane)

Hg: Mercury

HPLC: High Performance Liquid Chromatography

HSD: Hydroxysteroid Dehydrogenase

HSI: Hepatosomatic Index

KCZ: Ketoconazole

MT: Metallothionein

NADPH: β-Nicotinamide Adenine Dinucleotide Phosphate

NP: Nonylphenol

OCs: Organochlorinated Compounds

OP: Octylphenol

P450scc: Cytochrome P450 side chain cleavage

PAH: Polycyclic Aromatic Hydrocarbon

Pb: Lead

PCB: Polychlorinated Biphenyls

POPs: Persistent Organic Pollutants

SULT: Sulfotransferase

T: Testosterone

TBT: Tributyltin

UGT: UDP-glucuronosyltransferase

UNEP: United Nations Environmental Programme

Vtg: Vitellogenin

WHO: World Health Organization

Zn: Zinc

Chapter 1. General Introduction

1. Contamination of the aquatic environment

The massive increase of human population has lead to an exponential enhancement in technological and industrial advancements all around the world. The outburst of these advancements has greatly increased the consideration and concern for the quality of the environment in the last decades, mainly due to the awareness that pollution can be found everywhere. Pollution can be defined as any human-caused change in the physical, chemical, or biological properties of the environment, be that air, water or soil (Bardach, 1997). The production and emission of pollutants is usually derived from human settlements, resource uses and interventions such as infrastructural development and construction, agricultural activities, industrial development, urbanization, tourism, etc.

Aquatic environments offer and sustain important habitats for many fish species, but also attract an ever-increasing level of human activity, of both commercial and recreational nature. These environments are the ultimate sink for many of the chemicals released from anthropogenic activities, either due to direct (e.g. municipal, industrial and domestic effluents) or diffuse discharges (e.g. agriculture and urban runoff) or to atmospheric processes (Stegeman and Hahn, 1994). Many thousands of persistent organic pollutants (POPs) have been produced and in part released into the environment. POPs are usually divided into three categories: pesticides, industrial chemicals and unintentionally produced by-products, and they persist in the environment for long periods of time, since they resist photolytic, chemical and biological degradation. Due to their low water solubility they bond strongly to particulate matter in water column, and as a result, sediments can serve as a reservoir or

"sink" (Edlund, 2001). Moreover, POPs can travel through the atmosphere and oceans, thousands of miles from their source of origin, existing evidence of these compounds in remote regions where they have never been used nor produced (Muir *et al*., 1988; Simonich and Hites, 1995; Vilanova *et al*., 2001).

In the last decades of the 20th century, mankind became aware of the potential long-term adverse effects of synthesized chemicals and by-products, and their risk for aquatic and terrestrial ecosystems, since these compounds are rapidly bioaccumulated in the tissues of resident organisms and biomagnify through the food chain. To address this global concern, the United States joined forces with 90 other countries and the European Community to sign the treaty known as the Stockholm Convention (Sweden, May 2001), where countries initiated measures to protect human health and the environment by reducing and/or eliminating emissions and discharges of an initial list of twelve key POPs. The chemical substances in this list were aldrin, chlordane, dichlorodiphenyl trichloroethane (DDT), dieldrin, endrin, heptachlor, hexachlorobenzene (HCB), mirex, toxaphene, polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). Organochlorines, such as PCBs and DDTs, are among the most widespread and persistent contaminants in coastal environments (Varanasi *et al*., 1992). Even though a number of POPs are no longer produced (i.e. PCBs, banned in the 70s), they have remained in the environment for many decades, due to their persistency, and may continue to be unintentionally produced during some industrial processes. Nowadays, there is a rising interest on other organic compounds such as: polycyclic aromatic hydrocarbons (PAHs), nonylphenols (NPs), octylphenols (OPs), phthalates,

pentachlorophenol, hexabromobiphenyl, organomercury, organotin and organolead compounds, among others (UNEP, 2003).

PAHs are not commonly considered as persistent pollutants because of their extensive metabolism and excretion by fish and mammals. Nonetheless, depending on the chemical structure and the level of exposure, PAHs and their metabolites have the potential to produce toxic, mutagenic and/or carcinogenic effects in fish and other vertebrates, including humans (Albers, 2003), and for this reason, the increased levels of PAH in the last decades is a cause of concern. Increasing urbanization of coastal areas leads to high concentrations of PAHs in coastal environments. Nonetheless, most of the PAHs associated with coastal sediments originate from combustion (e.g. combustion of fossil fuel), with industrial and municipal waste-water, surface water runoff from urban areas and atmospheric deposition being an important source of PAHs to coastal waters (Varanasi *et al*., 1992; Albers, 2003). As a consequence of their hydrophobic characteristics, PAHs tend to accumulate in sediments (Liu *et al*., 2000) where they can reach concentrations 3 to 4 orders of magnitude higher than in the water column (Notar *et al*., 2001).

Alkylphenols (APs) are mainly used in the synthesis of alkylphenol polyethoxylates (APEs) surfactants (detergents) which are used in a variety of industrial activities, though alkylphenols themselves can be used as plasticizers, and its derivates (alkylphenol phosphates) can be used as UV stabilizers in plastics. It is estimated that 60% of the APEs find their way into the aquatic environment as nonylphenol (NP) and octylphenol (OP), the major degradation products (Groshart *et al*., 2001). The major routes of entrance are through waste water discharges into rivers and the sea and from sewage sludge. Several studies have identified NP as the most critical metabolite of

APEs because of its enhanced resistance towards biodegradation, toxicity, and ability to bioaccumulate in aquatic organisms (Ahel *et al*., 1994; White *et al*., 1994; Tyler *et al*., 1998). Moreover, the estrogenic properties of NP have been documented in a number of *in vivo* and *in vitro* studies (Jobling and Sumpter 1993; Arukwe *et al*., 1998; Celius and Walther, 1998). Additionally, the potential for human exposure to alkylphenols is quite high since these compounds have wide environmental distribution as a result of their manufacture, use and disposal. Hence, APE degradation products have been detected in drinking water (Clark *et al*., 1992); NP leaches from PVC tubing during milk processing (Junk *et al*., 1974) and from plastics used in food packaging (Gilbert *et al*., 1982).

Another important source of aquatic pollution are agricultural activities which are reported to contribute about 50% of the total pollution of surface water, by means of the higher nutrient enrichment (Islam and Tanaka, 2004). Moreover, pesticides from agricultural and forest sprays have been highlighted as potential estuarine contaminants (Thomas *et al*., 1999; Solé *et al*., 2000), since they enter the system through water runoff. In most European countries, the restriction on the use of organochlorine pesticides has led to a great increase in the use of organophosphate (OP) and carbamate (C) compounds. Although less persistent than organochlorine compounds, OP and C are generally more toxic and have been responsible for major ecological accidents and various fish killings (Capel *et al*., 1988; Horsberg *et al*., 1989).

Metals, on the other hand, differ from organic pollutants in that they are neither synthesized nor destroyed by humans, therefore they cannot be banned in the same way as synthetic organic chemicals (Hill, 2004). Metals occur naturally in marine environments and are classified as pollutants only when added by human activities in sufficient amounts to produce deleterious effects on the ecological system. They are

among the major contaminants reaching aquatic environments, and their levels have been increasing over the last decades as a result of technological development (Cajaraville *et al*., 2000). Their utilization by humans influences the environmental transport, by anthropogenic contributions into air, water, soil and food, and alters the speciation or biochemical form of the element, which might lead to potential health threats (Goyer and Clarkson, 2001). Metals (e.g. methylmercury) have been shown to bioaccumulate – *viz*. accumulation of a chemical from the surrounding medium into an organism by virtue of its lipophilicity – and biomagnify through the food chain – *viz*. increasing concentration of a chemical as food energy is transformed within the food chain – (Neff, 2002).

The need for effective antifoulants, which prevent the settlement and growth of marine organisms on submerged structures, such as ship's hulls and fish cages, among others, is recognised universally. For many years, tributyltin (TBT) was the most widely used active ingredient in paint formulations. However, since 1990, the use of TBT in some antifouling paints has been banned internationally due to its severe impact on the aquatic ecosystem (Fent, 1996). This ban has lead to an increase in copper-based antifouling paints (Katranitsas *et al*., 2003; Konstantinou and Albanis, 2004). As a result, important coastal concentrations of copper have been found in water and sediments in areas of high traffic activities, particularly in harbors and marinas (Schiff *et al*., 2004; Warnken *et al*., 2004).

Persistent hydrophobic chemicals may accumulate in aquatic organisms through different mechanisms: (a) direct uptake from water by gills or skin (bioconcentration), (b) ingestion of suspended particles, and (c) consumption of contaminated food (biomagnification) (Van der Oost *et al*., 2003). Pollutants interact with environmental

and biological systems according to their intrinsic physicochemical properties and reactivity, yielding a characteristic pattern of environmental and internal exposure concentrations for each pollutant. Final exposure and effect assessment according to this concept will always be subject to uncertainty due to inherent variability and complexity of both environmental and biological systems (Fig. 1).

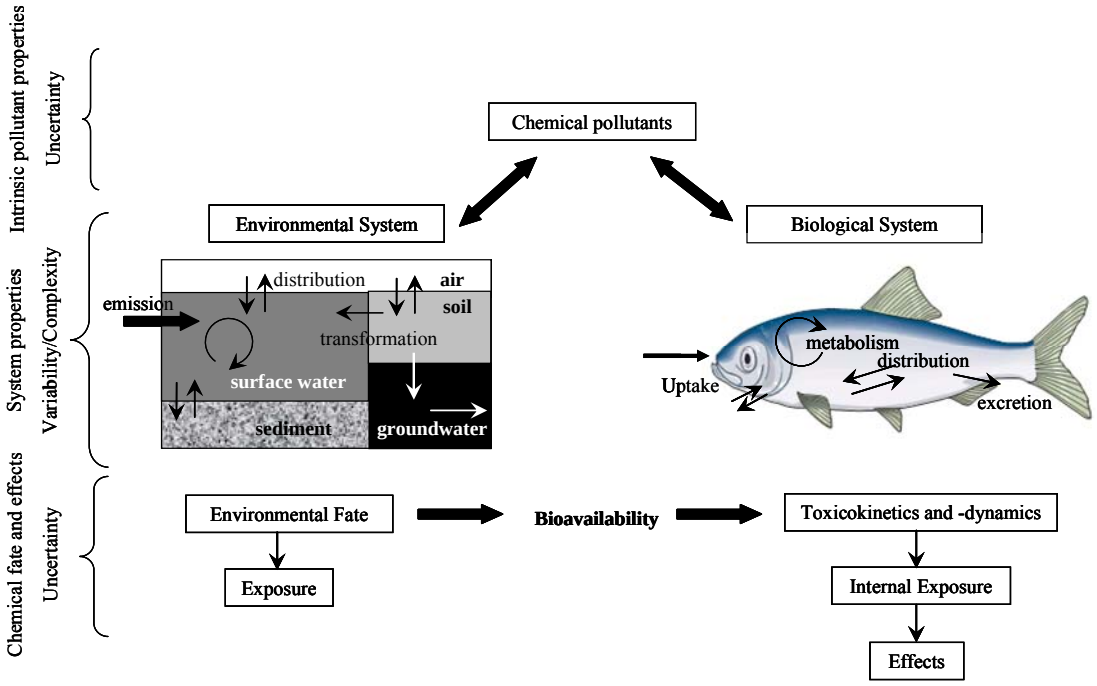


Figure 1. Pollutant input, distribution and fate in an environmental and biological system. Illustration of the key features and commonalities between exposure and effect assessment (adapted from Schwarzenbach *et al*., 2006).

1.1. Aquaculture industry

Aquaculture has been developed in the past decades as a consequence of the increase of fish consumption by the world population, since fisheries have possibly reached their maximum yield due to overexploitation (FAO, 2003) (Fig. 2a). Half of all monitored stocks are now fully exploited; another quarter are overexploited, depleted, or slowly recovering; the remaining quarter are under- or moderately exploited.

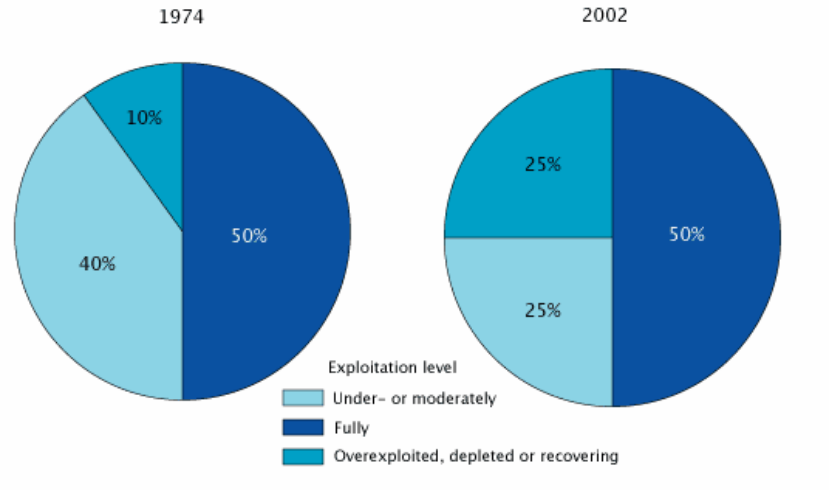


Figure 2a. State of the world marine fish stocks (FAO, 2003).

This industry is growing more than 10% per year (FAO 2003, Fig. 2b), and a production of 47 million tons of aquaculture products, mainly fish, is estimated for the year 2010 (Dar, 1999). Fish may be cultured in raceways, ponds or net cages in open and coastal waters. However, current aquaculture practises raise environmental concerns, such as (a) organic enrichment of surrounding waters by nutrients and solid wastes, (b) extensive use of chemicals (e.g. therapeutants, antibiotics, antifoulings) with their subsequent release into the aquatic environment, (c) introduction of pathogens, and

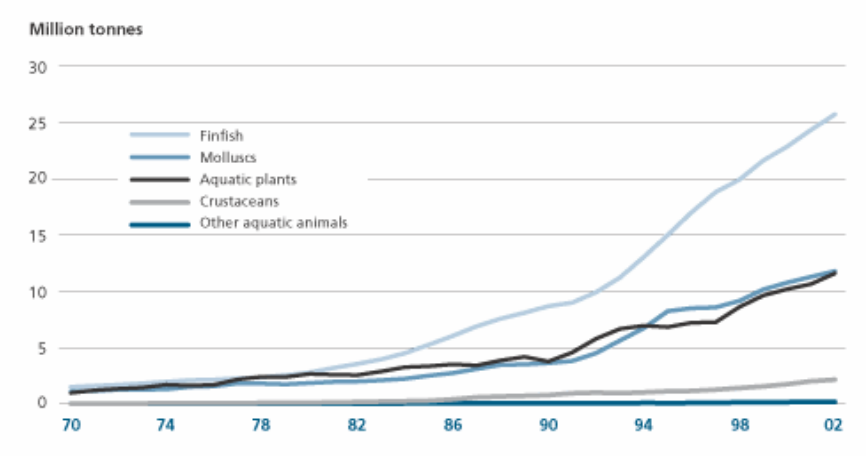


Figure 2b. Trends in world aquaculture production: major species groups (FAO, 2003).

(d) introduction of new genetic strains of cultured fish (Seymour and Bergheim, 1991; Wu, 1995; Katranitsas *et al*., 2003; Matos *et al*., 2006). Furthermore, the intensive production has raised concerns over the quality of cultured fish in comparison to wild fish (Alasalvar *et al*., 2002). But, although, aquaculture practises have been criticized for their ecological effects, the potential human health risks of cultured fish consumption have not been rigorously examined. The use of therapeutants as well as the contaminants present in the surrounding waters, can to some extent affect the well being and quality of cultured fish as well as those of non-target organisms. Moreover, as the aquaculture industry develops further, greater effects both on the environment and even in its own operations, may occur. Even though, clean water is one of the prerequisites of aquaculture, polluted conditions often arise gradually; e.g. when new industries are established in the coastal zone. Thus, evaluating pollutant loads and biological responses in cultured fish is an urgent need from the environmental, nutritional and toxicological point of view.

Marine fish culture is an increasing activity in the Atlantic and Mediterranean regions. It is mainly focused on two species: the sea bass (*Dicentrarchus labrax*) and the gilthead sea bream (*Sparus aurata*), working in parallel with traditional fisheries. Both cultured and wild fish are important components of the Atlantic and Mediterranean diet.

Eating fish provides nutritional benefits to humans; apart from being a good source of protein, fish is known to contain long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA), also known as omega-3 fatty acids, that help to reduce the risk of cardiovascular disease (Ulbricht and Southgate, 1991; La Vecchia *et al*., 2001) and of certain types of cancer (Terrry *et al*., 2001, 2002). Moreover, omega-3 fatty acids

cannot be synthesized by humans and therefore must be obtained from the diet (Alasalvar *et al*., 2002). However, fish consumption is considered a major route of chemical exposure for humans (Dougherty, 2000) and most importantly, children are more at risk because of their great intestinal absorption (Chance and Harmsen, 1998). Several pollutants are often detected in fish and seafood; e.g. metals such as mercury, cadmium, arsenic and lead, persistent organic compounds such as dioxins and PCBs, or residues of antibiotics and hormones used in aquaculture.

Organochlorine compounds (OCs) have been detected in tissues of wild fish (Lewis *et al*., 2002; Porte *et al*., 2002), smoked fish (Zabik *et al*., 1996), fish oils (Jacobs *et al*., 2002), fish feed and cultured fish (Easton *et al*., 2002; Antunes and Gil, 2004; Hites *et al*., 2004; Carlson and Hites, 2005). Elevated levels of PCBs in humans via fish consumption, as well as development deficits, neurodevelopmental problems, increased cancer risks and endocrine-disrupting effects have also been identified (Johnson *et al*., 1999; Carpenter *et al*., 2002; Borja *et al*., 2005). Furthermore, fish often contain significant amounts of methylmercury and lead, which have significant neurobehavioral effects and lead to development deficits in humans at high levels of exposure (Johnson *et al*., 1999; Carpenter *et al*., 2002). These findings suggest that the benefits of eating fish, primarily the possible prevention of sudden cardiac diseases, may be outweighed by the risks of consuming contaminated fish.

1.2. Environmental biomonitoring

As recognised in the last decades by international organisations and environmental agencies, risk assessment cannot be solely based on chemical analysis of

environmental samples (e.g. water and sediments), as no indication of the deleterious effects of contaminants on the biota is provided. In recent years, there has been a growing awareness of the need to detect and assess the adverse effects of contaminants in organisms exposed to different levels of pollutants. Among the available techniques, the integrated use of chemical analyses and biochemical and cellular responses to pollutants is a sound procedure for detecting impact of anthropogenic contaminants in aquatic systems.

The systematic use of living organisms to evaluate changes in water quality is defined as biomonitoring. Fish are often used to monitor urban and industrial effluents and to assess contaminant levels of coastal, brackish and freshwater environments. Fish species play an increasingly important role in the worldwide assessment of the quality of the aquatic ecosystems because they respond with great sensitivity to changes in the aquatic environment (Pastor *et al*., 1996). They can be found virtually everywhere in the aquatic environment and they play a major ecological role in the aquatic food webs because of their function as a carrier of energy from lower to higher trophic levels (Beyer, 1996). Their diversity of habitats makes them also useful for ecotoxicology studies, since it allows the examination of a wide range of chemical compounds and its potential effects. Despite their limitations, such as relatively high mobility, fish are generally considered one of the most feasible organisms for pollution monitoring in the aquatic systems.

1.3. Biomarkers

The need for early detection and assessment of the impacts of contamination in the aquatic environment has led to the development of biomarkers (biological indicators) (Peakall, 1994). A biomarker is generally used in a broad sense to include almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological (WHO, 1993). A biomarker is defined as a "change in a biological response, ranging from molecular through cellular and physiological responses to behavioural changes, which can be related to exposure to or toxic effects of environmental chemicals (Peakall, 1994). Generally, when deleterious effects on populations or ecosystems became clear, there is already considerable environmental degradation (Hylland, 2006). Therefore, the sequential order of responses to pollutants stress within a biological system, from the molecular to the ecosystem level, have triggered the research to establish "early-warning signals" or biomarkers reflecting adverse biological responses towards anthropogenic environmental pollutants (Bucheli and Fent, 1995) (Fig. 3). Thus, these "early biomarker signals" can be used in a predictive way, allowing the initiation of bioremediation strategies before irreversible environmental damage occurs.

In order to assess the bioavailability and toxicity of pollutants in fish, biomarkers on all levels of biological organization have become valuable ecotoxicological tools. The great advantage of a biomarker is providing evidence of the state of pollution in a comprehensive way based on the synergistic and antagonistic effects of all contaminants involved (Collier *et al*., 1995). Nonetheless, the physiological value of many parameters

may vary greatly in relation to the fish species age, sex, reproductive stage, as well as seasonality (Hylland, 2006).

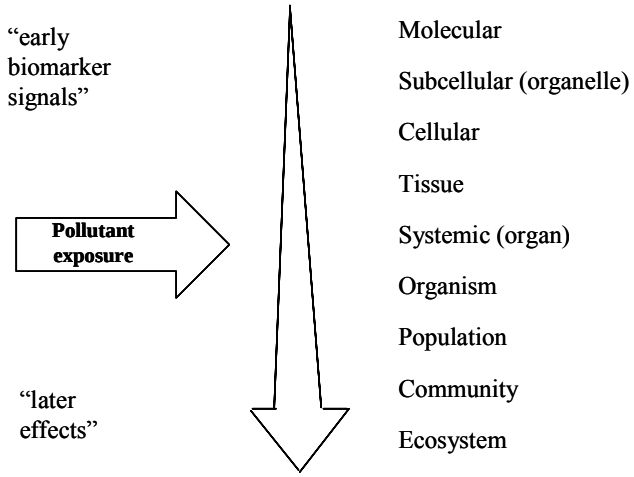


Figure 3. Schematic representation of the sequential order of responses to pollutants stress within a biological system (adapted from Van der Oost *et al*., 2003).

The biomarker approach has been incorporated into several pollution monitoring programmes in Europe and the USA (e.g. the North Sea Task Force Monitoring Master Plan and the NOAA's National Status and Trends Program). Likewise, different methods for biological effect measurement have been evaluated in a series of practical workshops organised by the International Council for the Exploration of the Sea (ICES) and the Intergovernmental Oceanographic Commission (IOC). The United Nations Environment Programme has funded a biomonitoring programme in the Mediterranean Sea including a variety of biomarkers (UNEP, 1997). Recently, biomarkers have also been included in the Joint Monitoring Programme of the OSPAR convention where Portugal and Spain are members (Cajaraville *et al*., 2000).

Nonetheless, the use of biomarkers in marine biomonitoring programmes is still a developing research field. In general, it would appear that no individual biomarker can

provide a complete diagnosis of pollution effects in coastal environments. Rather, a set of biomarkers should be used, in conjunction with other chemical and biological measurements. Selected biomarkers should indicate that the organism has been exposed to pollutants (exposure biomakers) and/or the magnitude of the organism's response to the pollutant (effect biomakers or biomarkers of stress) (Cajaraville *et al*., 2000). Furthermore, the criteria for candidate biomarkers are that they should be sensitive, reliable and easy to measure and that they should be related with the "health" and "fitness" of the organisms (Stegeman *et al*., 1992).

1.4. Xenobiotic metabolism: Enzymes involved in biotransformation

Most toxicants that are absorbed and penetrate into the body are lipophilic substances. This characteristic enables them to cross cellular membranes more easily and to be distributed throughout the body. Biotransformation of lipophilic compounds into more hidro-soluble metabolites is generally required before excretion from living organisms. Some exogenous chemicals may be excreted largely unchanged (original form), but most persistent environmental contaminants are highly lipophilic and require metabolic conversion before elimination from the organism (Ahokas *et al*., 1994). Without biotransformation, lipophilic xenobiotics would be excreted from the body so slowly that they would eventually overwhelm and kill the organism (Parkinson, 2001). Nonetheless, this change in chemical properties may also result in changes in their biological activity; in some cases, biotransformation not only leads to inactivation of toxic compounds, but also can result in more toxic carcinogenic products (Stegeman and Lech, 1991). Moreover, since some xenobiotics share common metabolic pathways

with endogenous substrates such as hormones (Fig. 4), it is likely that they may potentially interfere with normal endogenous metabolism and trigger endocrine alterations (Waxman, 1988).

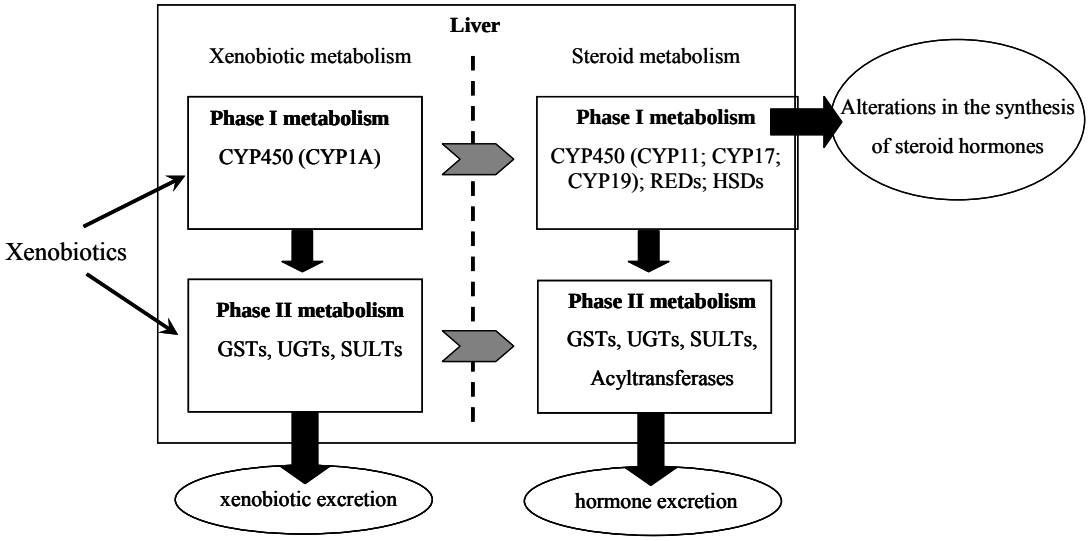


Figure 4. Scheme illustrating the common metabolic pathways for xenobiotic and endogenous steroid metabolism.

Biotransformation usually involves two types of enzymatic reactions: phase I and phase II reactions. Phase I is a non-synthetic alteration of the original foreign molecule (oxidation, reduction or hydrolysis), which can then be conjugated in phase II. The majority of the phase II type enzymes (e.g. GSTs, UGTs) catalyze synthetic conjugation reactions, which facilitate the excretion of the phase I reaction products by the addition of an endogenous polar group to the molecule (Commandeur *et al*., 1995).

1.4.1. Phase I enzymes

The first step in the metabolism of most lipophilic chemicals is oxidation by the cytochrome P450-dependent monooxygenase system localized in the endoplasmatic reticulum of the liver and other tissues. Its terminal component, cytochrome P450 (CYP) exists as a superfamily of heme-thiolate isoenzymes, all of which possess an iron protoporphyrin IX (Fe^{3+}) as the prosthetic group (Stegeman and Hahn, 1994). The monomer of the enzyme has a molecular weight of 45-55 KDa. It is present in a wide variety of species, from bacterial to mammals (Lewis *et al*., 2006). Another component of the monooxygenase system is the flavoprotein NADPH cytochrome P450 reductase that transfers electrons to the P450 cytochrome (CYP) (Stegeman and Hahn, 1994). Fish, along with many other vertebrates, have a well-developed xenobiotic metabolizing system (Ahokas and Pelkonen, 1984). In general, cytochromes are present at high levels in the liver, accounting for 1 to 2% mass of hepatocytes (Lester *et al*., 1993). However, they are also expressed in extrahepatic tissues, including intestine, kidney, lungs, brain, skin, etc (Husøy *et al*., 1994; Stegeman and Hahn, 1994; Ortiz-Delgado *et al*., 2002). Cytochrome P450 present a great functional versatility which is reflected both in the variety of processes which they catalyse and in the increased number of substrates that they can metabolize (Table 1). CYP1, CYP2 and CYP3 families are primarily associated with the metabolism of xenobiotics, but are also involved in endogenous metabolic processes. CYP11, CYP17, CYP19 and CYP21 families are mainly responsible for the biosynthesis of steroid hormones (Lewis *et al*., 2006).

Table 1. Nomenclature of cytochrome P450 isoenzymes based on species-specificity or function (Lewis, 2001)

Family designation	Main characteristics
CYP 1	Metabolism of foreign compounds
CYP 2	Metabolism of foreign compounds
CYP 3	Metabolism of foreign compounds
CYP 4	Metabolism of foreign compounds
CYP 5	Thromboxane biosynthesis
CYP 6	Insect isoforms
CYP 7	Bile acid biosynthesis and metabolism of steroids
CYP 8	Prostacycline biosynthesis
CYP 9	Insect isoforms
CYP 10	Mollusc isoforms
CYP 11	Biosynthesis of steroids
CYP 17	Biosynthesis of steroids
CYP 19	Biosynthesis of steroids
CYP 21	Biosynthesis of steroids
CYP 24	Vitamin D degradation
CYP 26	Metabolism of retinoids
CYP 27	Biosynthesis of bile acids
CYP 51-70	Fungal isoforms
CYP 71-100	Plant isoforms
CYP 101-140	Bacterial isoforms

Cytochrome P4501A (CYP1A). Among the different forms of cyt P450s, the CYP1A family has gained particular attention because of its role in biotransformation of many xenobiotic compounds including PAHs, PCBs, dioxins, furans, pesticides, petroleum products and an array of drugs (Stegeman and Lech, 1991; Van der Oost *et al*., 2003). Induction of CYP1A is mediated through the binding of xenobiotics to a cytosolic aryl hydrocarbon receptor protein (AhR) (Fig. 5, Route II). The xenobiotic binds as ligand to the AhR, releasing the heat shock protein (HSP90). The Ah receptor complex then binds to the Ah receptor nuclear translocator protein (ARNT) and migrates to the cell nucleus, where it interacts with the DNA with specific xenobiotic response elements (XREs) near the promoter region of the CYP1A gene transcription,

followed by increased levels of CYP1A messenger RNA, new synthesis of CYP1A protein and, finally enhanced CYP1A catalytic activity (Sarasquete and Segner, 2000). Thus, pollutants with a high binding affinity to AhR also have a high capacity to induce CYP1A (Billiard *et al*., 2002). The prototype ligand for the Ah receptor is 2,3,7,8-tetra-chloro-dibenzo-*p*-dioxin (TCDD) which is one of the most potent CYP1A inducers. All steps of the CYP1A induction cascade i.e. mRNA, protein and catalytic activity can be used to measure CYP1A in fish (Goksøyr and Husøy, 1992; Stegeman and Hahn, 1994; Bucheli and Fent, 1995).

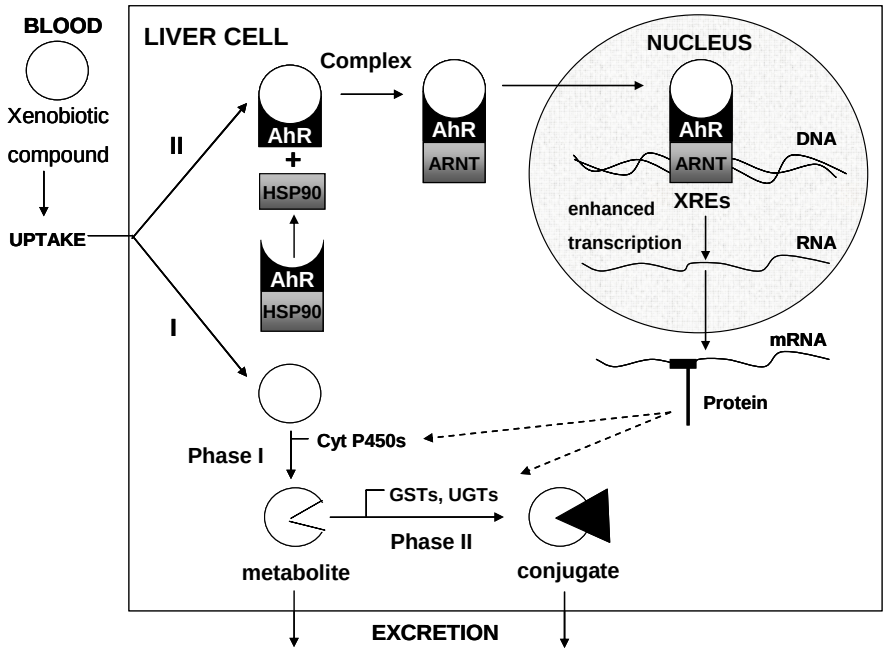


Figure 5. Schematic diagram presenting the fate of certain xenobiotic compounds in the liver cell. Route I: detoxification or toxication pathways; Route II: Enzyme induction through binding to the AhR: aryl hydrocarbon receptor; HSP90: 90 KDa heat shock protein; ARNT: Ah receptor nuclear translocator; XREs: xenobiotic response elements; Cyt P450s: cytochrome P450 isozymes; GSTs: glutathione *S*-transferases; UGTs: UDP-glucuronosyltransferases (adapted from Van der Oost *et al*., 2003).

7-Ethoxyresorufin *O*-deethylase (EROD) activity appears to be the most sensitive catalytic probe for determining the inductive response of the CYP1A in fish (Goksøyr and Förlin, 1992). This activity is most often measured in liver microsomes

by following the increase in fluorescence of the reaction product 7-hydroxyresorufin (Burke and Mayer, 1974). CYP1A induction in fish is considered as a sensitive "early warning" signal of pollution exposure, and EROD activity has been widely used as a biomarker of contamination in the aquatic environment (Van der Oost *et al*., 1996; Fernandes *et al*., 2002). Increased EROD activity has been related to, or positively correlated with, exposure to organic pollutants such as PAHs, PCBs and petroleum hydrocarbons in numerous field studies (Bucheli and Fent, 1995; Goksøyr, 1995; Kirby *et al*., 1999; Whyte *et al*., 2000; Porte *et al*., 2002). However, a direct straightforward relation between the concentration of an inducing chemical and CYP1A response is not always to be expected in the natural environment. Thus, certain types of chemicals or complex mixtures of xenobiotics normally present in the environment can repress EROD induction or inhibit the catalytic activity in fish, these include organotin (e.g. TBT) and metallic compounds (e.g. Cd , Hg and Cu), among others (Viarengo *et al*., 1997; Fent *et al*., 1998; Rice and Roszell, 1998; Bozcaarmutlu and Arinc, 2004). Likewise, EROD activity can additionally be influenced by abiotic and biotic factors such as water temperature, seasonality, nutrition, age, sex and reproductive status (Andersson and Förlin, 1992; Lemaire *et al*., 1992; Arukwe and Goksøyr, 1997). Nevertheless, EROD activity in fish liver can be considered as a sensitive biomarker of great value in assessing exposure of potentially harmful effects of many organic trace pollutants. Research on mechanisms of CYP1A-induced toxicity suggests that EROD activity may not only indicate chemical exposure, but may also precede effects at various levels of biological organization (Whyte *et al*., 2000). The confounding variables, which may affect the enzyme activity, however, have to be considered when interpreting the responses of this parameter.

1.4.2. Phase II enzymes

Phase II type enzymes catalyze conjugation reactions, facilitating the excretion of chemicals by the covalent addition of more polar groups (e.g. glutathione (GSH) and glucuronic acid (GA)) to the molecule (Commandeur *et al*., 1995; Van der Oost *et al*., 2003), thereby transforming a xenobiotic or endogenous compound into a more hydro-soluble product which can be excreted from the organism through bile, urine or through the gills (Clarke *et al*., 1991; Goksøyr and Förlin, 1992) (Fig. 5, Route I). Phase II enzymes play an important role in homeostasis as well as in the detoxification and clearance of xenobiotics and products of oxidative stress (Hayes and Pulford, 1995). The major pathway for electrophilic compounds and metabolites is conjugation with GSH, while for nucleophilic compounds conjugation with GA is the major route. Other pathways, i.e. conjugation with sulphate, play a minor role in fish and are the preferred route for only a few compounds (George, 1994). While some xenobiotics posses functional groups (e.g. –COOH, -OH or $–NH_2$) for direct metabolism by conjugative phase II enzyme systems, others have to be metabolized by phase I enzymes (George, 1994). In addition to CYP1A genes, the Ah gene battery also comprises phase II genes like NADPH menadione oxidoreductase, aldehyde dehydrogenase, UGTs and GSTs (Nebert *et al*., 1990; Celander, 1993). The mechanism of induction for most forms of phase II enzymes is, therefore, probably regulated via the Ah-receptor as well (Sutter and Greenlee, 1992).

UDP-glucuronosyltransferase (UGTs). The transfer of glucuronic acid (GA) in an activated form as uridine diphosphate glucuronic acid (UDPGA) to a wide variety of endogenous (e.g. bilirubin, steroid hormones and bile acids) and exogenous aglycone

compounds (e.g. phenolic xenobiotics) is performed by UDP-glucuronosyltransferases and constitutes a major pathway for their metabolic inactivation and subsequent excretion (George, 1994). UGTs are a family of enzymes present in the endoplasmatic reticulum differing from other phase II enzymes which are located in the cytosol. Mutiple UGT isoforms with differing substrate specificities are present in fish, and are generally named after their acceptor substrates, e.g. phenol, steroid, bilirubin (Van der Oost *et al*., 2003). In addition to its capacity to metabolize contaminants, UGTs also play an important role in the balance of endogenous compounds. Glucuronidation of endogenous compounds is important in fish species for biliary elimination of bilirubin (Clarke *et al*., 1992), cessation of hormone action via excretion (Förlin and Haux, 1985), and chemical signalling i.e. dihydroprogesterone glucuronides are the sex pheromones (Van der Kraak *et al*., 1989). The liver is quantitatively the most important site for glucuronidation of xenobiotics in fish, nevertheless significant activities have also been detected in extrahepatic tissues, including kidney, gills and intestine (George, 1994).

In fish, the UGT isoform, which preferentially conjugates planar phenols, is induced by PAHs, inferring the involvement of the Ah receptor (Nebert *et al*., 1990; George, 1994). Several studies have reported increases in hepatic UGT activities in both marine and freshwater fish from polluted sites (Van der Oost *et al*., 1996; Vigano *et al*., 1998; Porte *et al*., 2002; Gaworecki *et al*., 2004). Although not as sensitive as phase I enzymes, UGT appears to be the phase II parameter which is most responsive to pollutant exposure (Van der Oost *et al*., 2003), and has been used as a valid biomarker.

Glutathione S-transferases (GSTs). Glutathione is one of the most important molecules in the cellular defence against toxic compounds, due in part to its

involvement in conjugation reactions. The reduced form of glutathione (GSH) is a tripeptide thiol, composed of glutamic acid, cysteine and glycine (γ-glu-cys-gly). The presence of cysteine provides a sulphydryl group, which is nucleophilic, and so glutathione will react with electrophiles (Hayes and Pulford, 1995). These electrophiles may be chemically reactive (*viz*. metabolic products of phase I reactions) or they may be more stable foreign compounds which have been ingested. The conjugation reaction is catalyzed by glutathione *S*-transferases (GSTs), a multigene superfamily of dimeric, multifunctional, primarily soluble enzymes located in the liver cytosolic fraction (Van der Oost *et al*., 2003). Apart from their essential functions in intracellular transport (heme, bilirubin and bile acids) and the biosynthesis of leukotrienes and prostaglandins, GSTs play a critical role in the detoxification of xenobiotics and carcinogens (which are mostly electrophilic chemicals), as well as in the defence against oxidative damage and peroxidative products of DNA and lipids (Geroge, 1994; Hayes and Pulford, 1995). Induction of these enzymes must be considered beneficial, although metabolic activation of halogenated xenobiotics by GST is also well recognized (Commandeur *et al*., 1995).

Most studies determine the total GST activity using the artificial substrate 1-chloro-2,4-dinitrobenzene (CDNB), which is conjugated by most of the GST isoforms (George, 1994). Increases in hepatic GST activity have been reported in several studies after exposure of fish to PAHs, PCBs, organochlorine pesticides and dioxins, but most field studies did not demonstrate any significant differences between fish from control and polluted sites. Furthermore, the exposure to pollutants like dioxins and PAHs may cause both induction and inhibition of the enzyme activity (studies reviewed by Van der Oost *et al*., 2003). Thus, field studies on the effects of organic pollutants on GST

activity have been somewhat inconclusive, showing induction, no change, or inhibition of the enzyme activity. Moreover, information regarding sexual, seasonal and developmental differences in GST activity in fish is limited (Stegeman *et al*., 1992). Therefore, hepatic total GST activity in fish does not seem to be a feasible biomarker in field studies (Van der Oost *et al*., 2003).

1.5. Antioxidant enzymes

Many environmental pollutants or their metabolites may exert toxicity related to oxidative stress (Winston and Di Giulio, 1991). Oxygen toxicity is defined as injurious effects due to cytotoxic reactive oxygen species (ROS), also referred to as oxyradicals. The reduction products of molecular oxygen (O_2) such as the superoxide anion radical (O^{2-}), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH) are extremely potent oxidants capable of reacting with critical cellular macromolecules, possibly leading to enzyme inactivation, lipid peroxidation, DNA damage and, ultimately, cell death (Winston and Di Giulio, 1991). The defence systems that tend to inhibit the formation of oxyradicals include antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). These enzymes are critically important in the detoxification of radicals to non-reactive molecules, and their catalytic activity is often measured as a biomarker of oxidative stress (Van der Oost *et al*., 2003). The xenobiotics capable of causing oxidative stress are mostly metals, quinines, nitroaromatics and aromatic diols (Winston and Di Giulio, 1991). PAH metabolism can lead to the formation of oxyradicals through phenolic or quinone products (Ingelman-Sundberg and Hagbjörk, 1982).

Superoxide dismutase (SOD). SODs are a group of metalloenzymes that catalyse the conversion of reactive superoxide anions (O^{2-}) to yield hydrogen peroxide (H_2O_2), which is subsequently detoxified by CAT or glutathione peroxidase (GPx). SOD isoenzymes are found in the cytosol, mitochondria and in chloroplasts; SOD appears to be the most important antioxidant enzyme as it dismutates O^{2-} (Stegeman *et al*., 1992). Most techniques for the measurement of SOD activity are indirect assays in which an indicating scavenger of O^{2-} (e.g. Cyt c) competes with endogenous SOD for O^{2-}. A unit of SOD activity is defined as the amount that causes 50% inhibition of the reduction of the scavenger under specified conditions (McCord and Fridovich, 1969).

Catalase (CAT). CATs are hematin-containing enzymes that facilitate the removal of hydrogen peroxide (H_2O_2), which is metabolized to molecular oxygen (O_2) and water. Unlike some peroxidases that can reduce various lipid peroxides as well as H_2O_2, CATs can only reduce H_2O_2. This enzyme is primarily located in the peroxisomes and is involved in fatty acid metabolism (Stegeman *et al*., 1992). A commonly employed assay for the measurement of CAT activity follows the disappearance of exogenous H_2O_2 spectrophotometrically (Aebi, 1974).

Glutathione peroxidase (GPx). Peroxidases are enzymes that reduce a variety of peroxides (ROOH) to their corresponding alcohols (ROH). 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. The principal peroxidase in fish is a selenium-dependent tetrameric cytosolic enzyme that employs GSH as a cofactor (Van der Oost *et al*., 2003). GPx catalyses the metabolism of H_2O_2 to water, involving a concomitant oxidation of reduced GSH to its oxidized form (GSSG), which can be measured spectrophotometrically (Günzler and Flohé, 1985).

1.6. Acetylcholinesterase (AChE)

Acetylcholinesterase (AChE) enzymes are responsible for the removal of the neurotransmitter acetylcholine (ACh) from the synaptic cleft through hydrolysis (Habig and DiGiulio, 1991). The activity of this system is vital to the normal behaviour and muscular function and represents a prime target on which some contaminants can exert a detrimental effect (Kirby *et al*., 2000). Thus, inhibition of the AChE enzymes, result in a build up of acetylcholine, causing a continuous and excessive stimulation of the nerve/muscle fibres, producing acute toxic effects (Bruinink *et al*., 2002). Generally, the inhibition of AChE has been accepted as an easy-to-measure biomarker, indicative of exposure to organophosphate (OP) and carbamate (C) pesticides, and other contaminants with neurotoxic action, including hydrocarbons and metals (Payne *et al*., 1996; Forget *et al*., 1999). This activity is determined in different tissues (e.g. muscle and brain) and fluids (e.g. blood), and within the same species, enzyme sensitivity can vary considering the compartments where the activity is measured. The measurement of AChE activity in fish has become a biomonitoring tool in marine (Bocquené *et al*., 1990; Minier *et al*., 2000) and continental waters (Payne *et al*., 1996; Kirby *et al*., 2000; de la Torre *et al*., 2002).

1.7. Metallothionein (MT)

The exposure of marine organisms to metals leads to the alteration of several biochemical processes that have the potential to be used as biomarkers of exposure. First reported in mammals in the late 1950s (Margoshes and Vallee, 1957) and

subsequently in the oyster *Crassostrea virginica* and the mussel *Mytilus edulis* (Casterline and Yip, 1975; Noel-Lambot, 1976), metallothioneins (MTs) have been identified in various aquatic organisms including fish species (Hogstrand and Haux, 1991; Roesijadi, 1992). Metallothioneins (MTs) (i.e. a metallo-derivate of the sulphur-rich proteins, thioneins) are inducible metal-binding cytosolic proteins, characterized by having a low molecular weight ranging from 6 to 10 KDa, heat-stability, high content of cysteine (about 30 %), a lack of aromatic amino acid residues and a strong affinity for binding 6-12 atoms of class B metals such as Ag, Cd, Cu, Hg and Zn (George and Olsson, 1994; Langston *et al*., 1998). MTs take part in multiple biological processes (Table 2), they play an important role in the transport and storage of essential metals (e.g. Cu and Zn), and they provide a protective role against the toxic effect of metals, whether essential or non-essential (e.g. Cd and Hg), by sequestering and reducing the amount of free metal ions and making them less available for interaction with sensitive biomolecules. Metals such as Cu, Cd and Hg can be highly toxic since they have high reactivity with sulphydryl groups (– SH) of functionally important cellular molecules including amino acids, peptides and proteins (George and Young, 1986).

Table 2. Function of metallothioneins (Nordberg, 1998)

1.	Transport of metals
2.	Detoxification of non-essential metals (e.g. Cd; Hg)
3.	Protection from metal toxicity
4.	Free radical scavenger
5.	Storage of metals
6.	Metabolism of essential metals (e.g. Cu; Zn)
7.	Immune response

The mechanism of metal detoxification by MT occurs via metal-initiated transcriptional activation of MT genes, resulting in an increase in MT synthesis, and subsequent binding of free metals to the MT proteins (Hogstrand and Haux, 1991). So, the protective role of MTs is related to the induction of the protein in a stress situation or by the presence of a toxic agent, and is the first detectable sign of metal exposure at the cellular level. Therefore, MTs are often used as biomarkers in marine (Hamza-Chaffai *et al*., 1995; Filipović and Raspor, 2003) and brackish/freshwater (Linde *et al*., 1999; Rotchell *et al*., 2001) biomonitoring programs. Results from field studies support the hypothesis that MT is a significant factor for the accumulation of trace metals in fish and that MT levels reflect the degree of exposure (Hogstrand and Haux, 1991). Tissue distribution of MTs in fish primarily includes liver, gills, kidney and intestine, but their value as biomarkers of metal exposure varies with species, reproductive condition, sex, age, diet and water temperature (Langston and Bebianno, 1998; Langston *et al*., 2002), so these factors must be taken into consideration when using MTs as biomarkers.

1.8. Biotransformation products

Elevated levels of biotransformation products, such as metabolite levels of non-persistent compounds in body fluids, may also be considered as biomarkers. In general, exposure to xenobiotic compounds that are easily metabolized in fish, such as PAH, phenols and aromatic amines cannot be assessed by simply measuring their tissue levels (Melancon *et al*., 1992). The rapid metabolism and elimination of these xenobiotics by fish result in low residual concentrations in liver and muscle tissues. PAH biotransformation occurs in many aquatic organisms, but it is most effective in the

liver of fish (Van der Oost *et al*., 2003). Some PAHs are excreted as hydroxylated metabolites via the gallbladder (in bile), but most PAHs are excreted after conjugation by phase II enzymes (Vermeulen *et al*., 1992). Laboratory and field studies have both demonstrated that the presence of PAH metabolites in bile is well correlated with levels of exposure (Yu *et al*., 1995; Aas *et al*., 2001). Thus, biliary levels of conjugated metabolites of xenobiotics, such as PAHs have been accepted as fish biomarkers. Analysis of bile metabolites is a relatively rapid method for monitoring PAH contamination in fish, that can be determined either by analyzing the total level of PAH metabolites as fluorescent aromatic compounds (FAC) or by selecting single metabolites as markers for total PAH metabolism; using fixed wavelength fluorescence (FF), high-performance liquid chromatographic/fluorescence detection (HPLC/F) and/or chromatography-mass spectrometric analysis (GC-MS) (ICES, 2005a).

1.9. Endocrine disruption

An environmental endocrine disruptor (ED) has been defined by the U.S. Environmental Protection Agency (EPA) as an "exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body, that are responsible for the maintenance of homeostasis, reproduction, development and/or behaviour". A number of both natural and/or man-made chemicals with widespread distribution in the environment may act as EDs, which may affect reproduction and development of wildlife and therefore might threaten the existence of susceptible species. These include natural hormones (human, myco- and phytoestrogens), synthetic hormones (i.e. diethylbestrol, ethynylestradiol), and many

synthesised chemicals, that were designed for a specific use, but that are now suspected of unintentionally disrupting the endocrine system of humans and wildlife (organochlorine pesticides, PCBs, bisphenol A, phthalate plasticizers, alklyphenols) (Colborn *et al*., 1993). Table 3 summarizes examples of substances that are known to act as EDs, their use and their sources of entry into the environment.

Table 3. Source, category (type) and examples of substances that have been reported as potential endocrine disrupters (Labelle, 2000).

Source	Category (type)	Substances
Incineration, landfill	Polychlorinated compounds (from industrial production or by-products of mostly banned substances)	Polychlorinated dioxins (PCDD), polychlorinated biphenyls (PCBs)
Agricultural runoff / Atmospheric transport	Organochlorine pesticides (found in insecticides, many now not in use)	DDT, dieldrin, lindane
Agricultural runoff	Pesticides currently in use	Atrazine, trifluralin, Permethrin
Harbours	Organotins (found in antifouling paint)	Tributyltin (TBT)
Industrial and municipal effluents	Alkylphenols (surfactants – certain kinds of detergents used to remove oil – and their metabolites)	Nonylphenol (NP)
Industrial effluent	Phthalates (found in placticisers)	Dibutyl phthalate, butylbenzyl, phthalate
Municipal effluent and agricultural runoff	Natural Hormones (produced naturally by animals); synthetic steroids (found in contraceptives)	17β-Estradiol, estrone, testosterone, ethynyl estradiol
Pulp mill effluents	Phytoestrogens (found in plants)	Isoflavones, lignans, Coumestans

Xenobiotics that are capable of causing endocrine modulation *in vivo* have one of the three following characteristics: they are present in the environment at high concentrations, they are persistent and bioaccumulative, or they are constantly entering the environment (Tyler *et al*., 1998). Among EDs, primary concerns in the last decades have been for substances with estrogenic activity (xenoestrogens).

1.9.1. Vitellogenin (Vtg)

One of the most sensitive responses to estrogens in fish and other oviparous vertebrates is induction of vitelogenin (Vtg), the serum phospholipoglycoprotein precursor of egg yolk proteins (Nilsen *et al*., 1998). Vitellogenin, is normally synthesized in the liver of oviparous females as a response to estrogens circulating in the plasma. It is secreted into the blood and transported to the ovaries where it is incorporated into the developing oocytes and subsequently processed to form the yolk proteins, lipovitellin and phosvitin, which are stored in the yolk, and serve as food reserve for the developing embryo (Hyllner *et al*., 1991). In normal conditions, males do not synthesize this protein because of the very low, or even undetectable, levels of estradiol (E_2) in plasma. However, they do have the potential to synthesize it to the same degree as females as a consequence of exposure to xenoestrogens (Hyllner *et al*., 1991; Purdom *et al*., 1994). Vtg expression in male fish has, therefore, become an accepted assay for measuring exposure to estrogenic chemicals (Sumpter and Jobling, 1995; Solé *et al*., 2001; Canapa *et al*., 2002; Van den Belt *et al*., 2003). Additionally, very low levels of Vtg in females have also been proposed as a biomarker of

xenoestrogen exposure (Kime *et al*., 1999). Hence, the VTG response in fish may be used as a biomarker of exposure to estrogenic compounds.

Controlled laboratory studies with male rainbow trout, have demonstrated that nonylphenol (NP) at environmentally relevant concentrations (20 µg/L) was able to induce Vtg production and retard testicular growth (Jobling *et al*., 1996). Vtg induction in male fish was reported in flounder caught near sewage treatment works (STWs) in the UK, and later work suggested that alkylphenols (APEs) might have been partly responsible for this response (Lye *et al*., 1997; 1999). In fact, APEs are among the most likely estrogenic substances in domestic sewage (Purdom *et al*., 1994). Several cases of Vtg production in wild male fish, have been documented (Allen *et al*., 1999; Hashimoto *et al*., 2000; Fossi *et al*., 2002; Solé *et al*., 2003; Lavado *et al*., 2004), and partly related to exposure to alkylphenolic or organochlorinated compounds.

1.9.2. Potential pathways of endocrine disruption

Steroid hormones are synthesized in the gonadal cells, being then secreted into the blood were they may become available to target cells through passive diffusion or may be transported bound to sex-hormone binding globulins (SHBGs). Once in the cell, the free steroid hormone diffuses into the perinuclear region where it will bind to unoccupied receptors, which will undergo a conformational change, exposing key protein binding sites and forming homodimers. The homodimers accumulate transcriptional factors, forming a transcriptional complex, which binds to specific sequences on the DNA of hormone-dependent genes, known as hormone response elements (HRE). The transcriptional complex then initiates mRNA synthesis (mRNA),

which is transported out of the cell into the cytoplasm and translated into protein molecules. Since this pathway involves action on the genome it is known as "genomic steroid action" (Falkenstein *et al*., 2000).

Recently, it has been shown that EDs can also act by non-genomic mechanisms, altering steroid synthesis or steroid metabolism; e.g. alkylphenol inhibits the inactivation of estrogens by glucuronidation, so causing a rise in levels of the free active endogenous estrogens (Thibaut and Porte, 2004). In contrast to genomic steroid actions, the non-genomic steroid actions act through binding to membrane receptors and initiating intracellular signalling cascades (Olsson *et al*., 1998). Figure 6 illustrates a schematic diagram of both the genomic and non-genomic action of steroids.

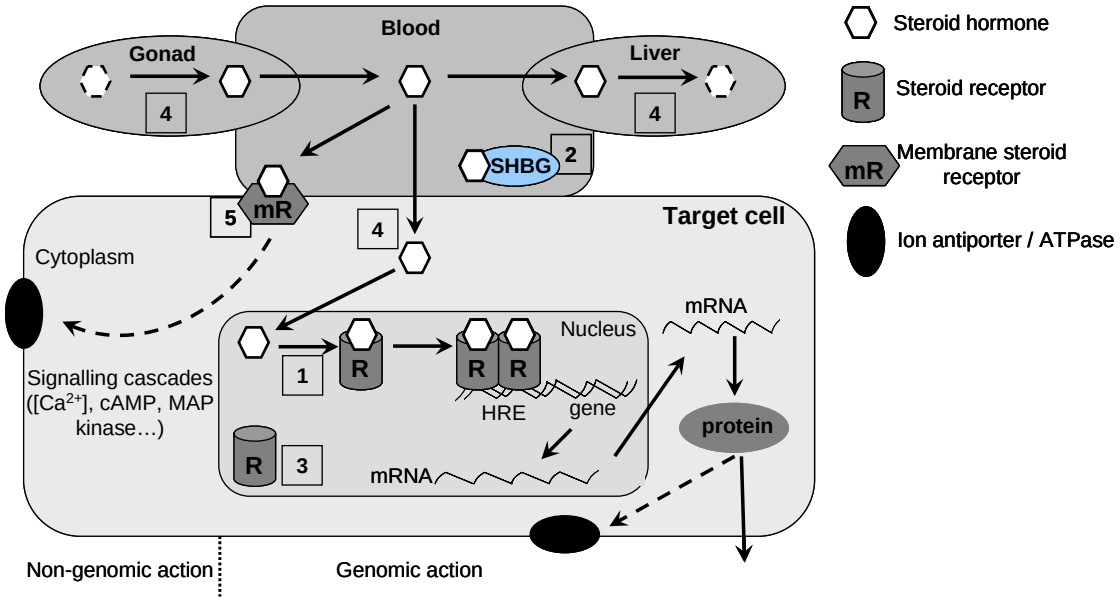


Figure 6. Schematic diagram depicting several key steps in steroid hormone action that may be sensitive to disruption by environmental chemicals. 1) Binding to steroid receptor; 2) Binding to steroid hormone binding globulin (SHBG) or altering levels of SHBG; 3) Alteration of steroid receptor levels; 4) Alterations of biosynthesis and metabolism pathways, either in the target cell, liver or gonad; 5) Interaction with the non-genomic action of steroid hormones. Adapted from WHO (2002) and Falkenstein *et al*. (2000).

The complexity of both pathways enables several possible targets and mechanisms of action of environmental endocrine disrupters. EDs can exert their effect by mimicking (i.e. estrogenic or androgenic compounds) or antagonizing (i.e. antiestrogenic or antiandrogenic compounds) the action of naturally produced hormones, altering the natural pattern of hormone synthesis or metabolism (Sonnenschein and Soto, 1998).

1.9.3. Steroidogenic enzymes in fish

The biosynthesis of steroid hormones is the result of sequential hydroxylation and reduction reactions which convert cholesterol to physiological active androgens and estrogens (Payne and Hales, 2004). Specific genes involved in steroid biosynthesis are differentially expressed in the somatic cells of testis and ovary (Omura and Morohashi, 1995), which results in the production of an array of steroids. Species and maturational stage largely influences the type of reproductive hormones (i.e. androgens, estrogens and progesterone) synthesized in the gonads. Nonetheless, the major androgens in fish include androstenedione, testosterone, 11-ketoandrostenedione and 11-ketotestosterone, whereas the predominant estrogens are 17β-estradiol and estrone. Sex steroids in immature fish influence gonadal differentiation, whereas these same hormones play an important role in gametogenesis, ovulation and spermiation in mature fish (Devlin and Nagahama, 2002).

As in other vertebrates, a complex series of enzymes are responsible for the biosynthesis of sex steroids in fish. These enzymes are members of either the cytochrome P450 superfamily or members of the steroid dehydrogenase family, and associated with the mitochondrial membrane or the endoplasmatic reticulum (Omura

and Morohashi, 1995; Payne and Hales, 2004). Among the steroid synthesis pathways, C17,20-lyase also called cleavage P450c17 or CYP17 (P450 17α-hydroxylase), is a key enzymatic pathway that occurs in fish gonads and plays a key role on the conversion of 17α-hydroxyprogesterone ($17P_4$) to androstenedione (AD), which is a precursor of testosterone (T). In female gonads, the androgen hormones are converted to estrogens i.e. estrone and 17β-estradiol (E_2) by the CYP19 enzyme (P450 aromatase). Meanwhile, in male gonads both, T and AD can be transformed into its respective 11-hydroxylated metabolites by 11β-hydroxylases (Fig. 7), and further metabolized to 11-ketoandrostenedione (11-KAD) and 11-ketotestosterone (11-KT) by the 11β-hydroxysteroid dehydrogenase (11β-HSD). 11-KT is considered to be the main

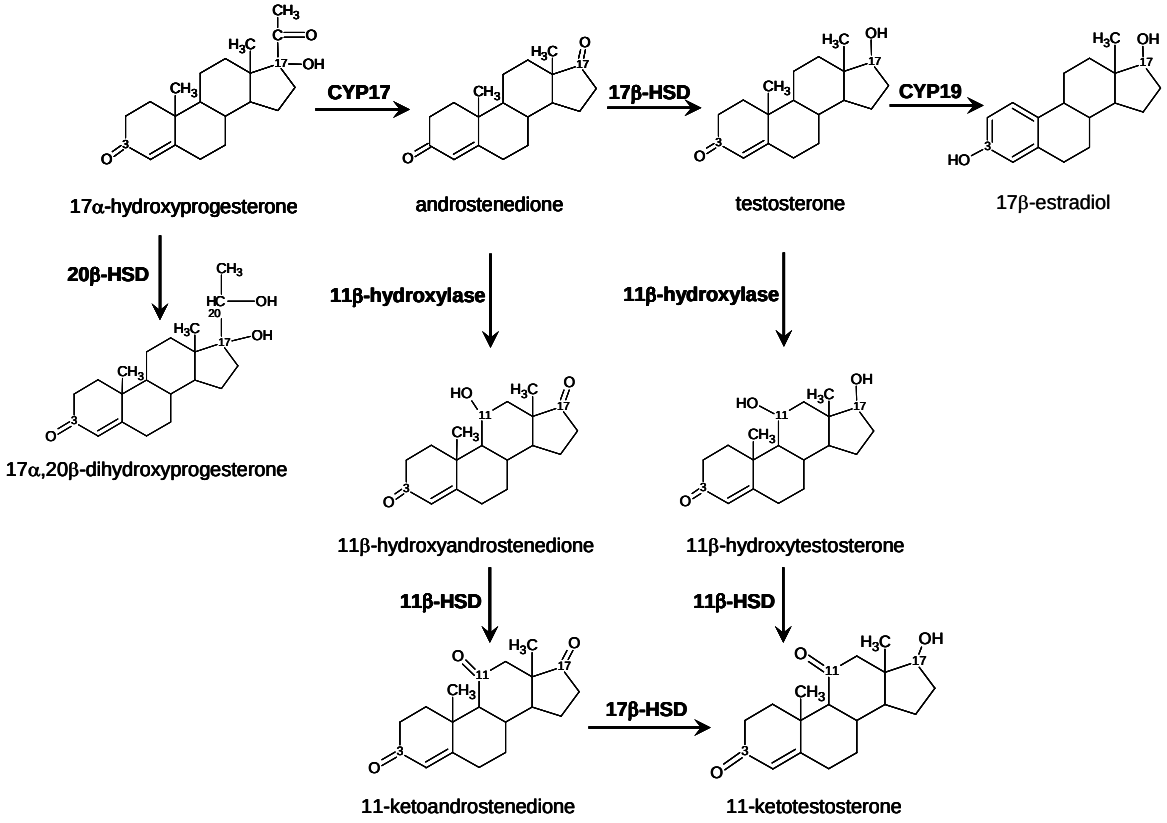


Figure 7. Sex steroid biosynthesis pathway in teleost fish. CYP17: C17,20-lyase (cleavage P450c17); HSD: hydroxysteroid-dehydrogenases; CYP19: P450 aromatase; 11β-hydroxylase (CYP11B).

androgen in male teleosts, being more effective than T in stimulating secondary sexual characters, influencing spermatogenesis and stimulating reproductive behaviour (Borg, 1994).

One of the major differences between the P450 enzymes and the hydroxysteroid dehydrogenases (HSDs) is that each of the P450 enzymes is a product of a single gene, whereas there are several isoforms of HSDs, each a product of a distinct gene (Payne and Hales, 2004). Both, P450 and HSDs enzymes play a key role in steroid biosynthesis pathways and therefore, can be potential targets for EDs.

CYP17. The rate limiting step in biosynthesis of steroid hormones is the transport of free cholesterol from the cytoplasm into the mitochondria. This transport is facilitated by the binding of gonadotropins (GtHs) (follicle stimulating hormone and luteinising hormone) to their receptors on the membrane of the steroidogenic cells. The pathway initiates with the synthesis of the steroid precursor pregnenolone via side-chain-cleavage of cholesterol by P450scc. Pregnenolone can be hydroxylated at its C_{17} position by the action of cytochrome P450 17α-hydroxylase (CYP17), originating 17α-hydroxypregnenolone. CYP17 can also act in the conversion of progesterone (P_4) into 17α-hydroxyprogesterone ($17P_4$) and subsequently into androstenedione (AD) (C17,20-lyase activity) (Devlin and Nagahama, 2002). Having both 17α-hydroxylase and C17,20-lyase activity, CYP17 is positioned at a key branch in steroid hormone synthesis. Pregnenolone is converted into mineralcorticoids (e.g. aldosterone) when CYP17 remains inactive, into glucocorticoids (e.g. cortisol) when CYP17 only displays 17α-hydroxylase activity, or into sex steroids when CYP17 displays both activities, 17α-hydroxylation and C17,20-lyase (Miller, 2002).

11β-hydroxylase or CYP11B. An 11β-hydroxylase enzyme catalyzes the hydroxylation of testicular steroids (i.e. T and AD) at their C_{11} position, in the presence of molecular oxygen and consists of an NADPH-specific flavoprotein, a non-heme iron protein, and cytochrome P450. The products of this catalysis are 11β-hydroxytestosterone (*β*T) and 11β-hydroxyandrostenedione (*β*AD).

Hydroxysteroid dehydrogenases. The hydroxysteroid dehydrogenases belong to the same phylogenetic protein family, namely the short-chain alcohol dehydrogenase reductase superfamily. They are involved in the reduction and oxidation of steroid hormones requiring NAD^+/$NADP^+$ as acceptors and their reduced forms as donors of reducing equivalents. Their number of isoforms varies in tissue distribution, catalytic activity (whether they function predominantly as dehydrogenases or reductases), substrate and cofactor specificity, and subcellular localization (Payne and Hales, 2004).

17β-HSD. The 17β-hydroxysteroid dehydrogenases (17β-HSDs) are enzymes involved in the formation of active sex steroids such as estradiol (E_2) and testosterone (T). They catalyze the last and key step in the formation of all estrogens and androgens since sex steroids require the presence of a hydroxyl group at position 17β on the steroid nucleus (Luu-The, 2001). This enzymatic pathway is present in all vertebrates (Mindnich *et al*., 2004). The most widely known is the testicular enzyme, which catalyzes the transformation of 4-androstene-3,17-dione (AD) into testosterone (Inano and Tamaoki, 1986). The 17β-HSDs are found as either membrane-bound (i.e. mitochondrial and microsomal fraction) or soluble enzymes (i.e. cytosolic fraction), and to date 11 different isoforms have been identified, mainly based on mammal studies (Payne and Hales, 2004). Some 17β-HSDs seem to show a preference for reduction of androgens (type 3); others for reduction of estrogens (type 1 and 7); and yet others for

oxidation of androgens and estrogens (type 2) (Luu-The, 2001). In fish, only recently the first 17β-HSD, the type 1 homolog has been cloned (Kazeto *et al*., 2000).

11β-HSD. 11β-hydroxysteroid dehydrogenase (11β-HSD) is an enzyme with both oxidative and reductive activity. However, the ratios of oxidative and reductive activity change with development, reduction predominating in prepubertal testes, and oxidation in the adult (Gomez-Sanchez and Gomez-Sanchez, 1997). Thus, 11β-HSD catalyses the oxidation of 11β-hydroxylated androgens (i.e. *β*AD and *β*T) giving rise to 11-KAD and 11-KT, the main androgens in male fish.

20β-HSD. In many species of teleost, 20β-hydroxysteroid dehydrogenase (20β-HSD) is a key steroidogenic enzyme in the formation of the principal progestin – 17α,20β-dihydroxy-4-pregnen-3-one (17,20*β*P) –, related to sperm release and final oocyte maturation. 20β-HSD activity is located mainly in the membrane-bound fractions of the mitochondria and microsomes (Vizziano *et al*., 1996; Kazeto *et al*., 2001).

In fish, early studies of steroid biosynthesis focused mainly on salmonids, where E_2, T, and 11KT or 17,20*β*P predominate in the reproductive cycle. Nowadays, it is clearly established that 11-oxygenated androgens predominate during the testicular recrudescence of many teleost fish, but as spawning approaches there is a switch to the production of progestins, of which 17,20*β*P is one of the most commonly measured. This switch is analogous to that which occurs in females in which production of testosterone and 17β-estradiol decreases as 17,20*β*P appears, coincident with the gonadotropin (GtH) surge associated with final oocyte maturation (Kime and Abdullah, 1994; Nagahama, 1997; Devlin and Nagahama, 2002). The interference of xenobiotics with the steroid synthesis and steroid metabolism in fish may alter the bioavailable

amounts of active hormones within the organism, and be a potential mechanism of endocrine disruption *viz*. ovarian tissue of flounder (*Platichthys flesus*) exposed to high doses of PAHs showed an inhibition of the steroidogenic enzyme CYP17 (C17,20-lyase) and CYP19, leading to reduced secretion of AD and 17β-estradiol (E_2) (Monteiro *et al*., 2000). Others for instance have shown that exposure to certain pesticides and nonylphenol can either induce 20β-HSD and/or inhibit 17β-HSD activities in carp (*Cyprinus carpio*), these being key enzymes involved in the formation of 17,20βP and T, respectively (Thibaut and Porte, 2004). However, much more information is needed regarding the interference of pollutants with the synthesis of 11-oxygenated androgens in fish gonads (CYP17, CYP11B, 17β-HSD, 11β-HSD), and the final effect on fish reproduction and sexual maturation. The characterization and better knowledge of these enzymatic pathways can offer new complementary tools for the assessment of reproductive disturbance in fish exposed to polluted environments.

1.10. Selected species

Fish are used as bioindicator organisms of water quality, due to its size/quantity of tissue and its important commercial value. Furthermore, they provide an essential protein source and, hence, are of crucial significance for the nutrition of mankind. Therefore, the health and protection of these organisms is of vital importance.

The selected species for this thesis were as follows:

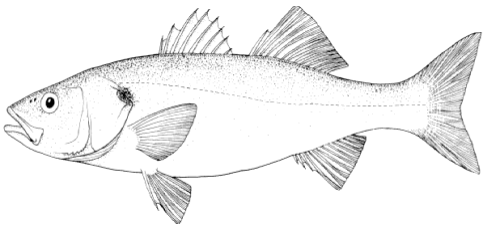
Sea bass (*Dicentrarchus labrax*) belongs to the family Moronidae, Order Perciformes (Fig. 8A). A demersal species distributed along the Easter Atlantic, also known from the Mediterranean and the Black Sea. It inhabits the littoral zone on various kinds of bottoms on estuaries, lagoons and occasionally rivers. Feeds mainly on shrimps, molluscs and on fishes. Its reproduction period is between January and March (Muzavor *et al*., 1993). Sea bass is a species of great commercial interest and rose in aquaculture, reaching in the last year's high production and high economic value in European countries (FAO, 2003).

Four-spotted megrim (*Lepidorhombus boscii*) belongs to the Scopthalmidae family, Order Pleuronectiforms (Fig. 8B). It is a demersal flatfish of commercial interest distributed along the Northeast Atlantic and Mediterranean Sea. Feeds mainly on detritivorous crustaceans, it has low migratory capacity and the reproduction period is between December and March (ICES, 2005b). It dwells on muddy bottoms on the middle and outer shelf at maximum depths of 800 m. Studies carried out in the NW Mediterranean Sea have demonstrated that four-spotted megrim is a sensitive species to PAH exposure (Pietrapiana *et al*., 2002). It has been selected for monitoring the impact of anthropogenic activities in the Northern Iberian coast.

Pouting (*Trisopterus luscus*) belongs to the family Gadidae, Order Gadiformes (Fig. 8C). It is a benthopelagic species distributed along the Northeast and Eastern Atlantic and Western Mediterranean. They have gregarious habits and live mostly on the inner shelf, moving inshore to depths of 50 m or less for spawning between

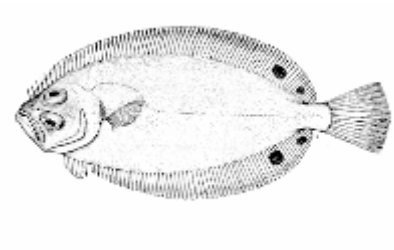
December and March (França *et al*., 2004). Feeds on benthic crustaceans but also on small fish, molluscs and polychaetes. It has been selected for monitoring the impact of anthropogenic activities in the Northern Iberian coast, because of its commercial interest.

(A)



Dicentrarchus labrax

(B)



Lepidorhombus boscii

(C)

Trisopterus luscus

Figure 8. Selected species (pictures obtained from *FishBase*)

1.11. Objectives and structure of the thesis

The aim of the present thesis was to investigate the impact of pollutants in both wild and cultured fish from different ecosystems (i.e. ponds, marine and estuary systems) by the combined analysis of chemical residues in fish tissues together with biochemical responses, and to further develop new *in vitro* tools to assess the effect of endocrine disruptors. In order to accomplish these main objectives, the thesis was structured into several chapters as follows:

In the first chapter of this dissertation (**Chapter 1**), a general introduction is presented. This chapter reviews the widespread distribution of persistent pollutants in aquatic ecosystems, the actual development and environmental concerns of the aquaculture industry, and the available techniques for detecting impact of anthropogenic contaminants in aquatic systems. Furthermore, the role of a suite of biochemical responses (Phase I and II enzymes, antioxidant enzymes, AChE, MT, Vtg) as biomarkers of pollutant exposure/effect in fish is considered, as well as the potential pathways of environmental endocrine disruptors (EDs). Additionally, a description of the selected species was included.

In **chapter 2** specific biological responses to a range of environmental stressors (metals, organic pollutants) in wild and cultured sea bass (*Dicentrarchus labrax* L.) from South Europe were investigated, and those that offer a greater potential for future biomonitoring programmes selected. This work was first designed to assess exposure to metals and PAHs in wild and cultured sea bass from the South coast of Portugal, particularly from the Arade Estuary. Sea bass was selected for the study because it is a common wild species in the Arade estuary and its culture has increased considerably in

the last years reaching high levels of production as well as high economic interest in the region. The Arade Estuary is an area of ecological and economic importance in the South Portuguese coast that is subjected to industrial and domestic effluents, which, over time, can have serious consequences for estuarine biota. However, to our knowledge, there is no information regarding the impact of pollutants in both wild and cultured fish living in the area. Thus, the existence of seasonal trends and the responsiveness of several biochemical responses to pollutant exposure (MT, phase I and II enzymes, CAT, AChE), in both wild and cultured specimens, were investigated. Due to the rather limited information regarding pollutant levels in farmed fish and the risks associated to consumption, particular emphasis was placed to assess pollutant exposure to a broad range of contaminants (metals, OCs, PAHs, APEs) in sea bass from different aquaculture facilities located in Southern Europe. The usefulness of several biochemical responses (MT, Cyt P450 system, Vtg) as screening tools in aquaculture practises is discussed.

In **Chapter 3** another field study was conducted in order to assess pollution exposure in commercial wild fish along the Northern Iberian shelf. The Northern Iberian coast is an area of great socioeconomic importance and highly vulnerable to anthropogenic pressure due to the co-existence of several human activities. Moreover, the accident of the *Prestige* tanker taking place one year before this study caused massive oil pollution along the Northern Iberian shelf (Sánchez *et al*., 2006). However, information on the fate and toxicity of chemicals that are currently released into the area is scarce, and very few monitoring programs have addressed the integrated use of chemical analyses with biochemical responses to pollutants along the Northern Iberian shelf. Therefore, considering the high productivity of Galician waters, the importance of

fisheries along the North Iberian coast, and the existence of potential land-ocean inputs of pollutants which bioavailability and impact have not been previously characterized, this chapter reports on levels of chemical residues (metals, OCs, PAHs, APEs) in several tissues together with a suite of biochemical responses (MT, phase I and II enzymes, CAT) in order to assess the impact of pollution along the Northern Iberian shelf using two commercial fish species, *Lepidorhombus boscii* and *Trisopterus luscus*, as sentinel organisms.

In recent years, special attention has been given to the alteration of fish reproduction by chemical pollutants and the possible consequences on wildlife populations. Several studies have demonstrated the potential of a large number of chemicals, like pesticides, PCBs, APEs, PAHs, among others, to impair the reproductive function of fish (e.g. Choudhury *et al*., 1993; Monosson *et al*., 1994; Jobling *et al*., 1995, 1996; Monteiro *et al*., 2000). Endocrine disruptors (EDs) can exert their effects at a variety of sites on the hypothalamus-pituitary-gonadal axis and alter several different signalling pathways, including binding globulins, growth factors, different receptor systems, and/or steroidogenic enzymes, and as a consequence impair the reproductive development and/or behaviour of susceptible organisms. Thus, in **Chapter 4**, the metabolism of 17α-hydroxyprogesterone ($17P_4$) in male sea bass gonads was investigated, as a potential tool for the detection of endocrine alterations; being $17P_4$ (C21 steroid) the starting precursor for the synthesis of androgens and 11-oxysteroids (C19 steroids) that are characteristic of male teleost fish, with great importance in male fish reproduction. This study was first designed to characterize the gonadal metabolism of $17P_4$ in male sea bass by different subcellular fractions and at different sexual maturation stages (SMS). Once characterized the enzymatic pathways,

the *in vitro* effect of model endocrine disruptors (i.e. 4-nonylphenol (NP), benzo[a]anthracene (BaA), trybutiltin (TBT), *p,p*'-DDE and ketoconazole (KCZ)) was evaluated.

Finally, a general discussion (**Chapter 5**) is presented, giving an overview of pollutant concentrations and possible sources, their relationship with the studied biomarkers, and their potential to impair testicular steroid biosynthesis. Furthermore, the final conclusions and future perspectives are summarised in the last chapter (**Chapter 6**).

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Chapter 2. Chemical and Biochemical Tools to Assess Pollution Exposure in Wild and Cultured Sea Bass (*Dicentrarchus labrax*)

2.1.

CHEMICAL RESIDUES AND BIOCHEMICAL RESPONSES IN WILD AND CULTURED EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX* L.)

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Resumo

Robalos (*Dicentrarchus labrax*) de aquacultura e selvagens provenientes de diferentes zonas do Estuário do Rio Arade foram amostrados no Verão e no Inverno e o grau de exposição a metais e a hidrocarbonetos aromáticos policíclicos (PAHs) avaliados, conjuntamente com algumas respostas bioquímicas i.e. metalotioneína, enzimas da fase I e da fase II, catalase e acetilcolinesterase. Os níveis mais elevados de cobre (até 977 $\mu g.g^{-1}$ peso seco) e de cádmio (até 4.22 $\mu g.g^{-1}$ peso seco) foram detectados no fígado e no rim de espécimes de cultivo, enquanto que a exposição mais elevada de PAHs foi observada em organismos selvagens. Além disso, foram detectadas alterações significativas nalguns marcadores bioquímicos associados á exposição aos contaminantes analisados. Assim sendo, as concentrações de metalotioneína foram mais elevadas nos tecidos dos peixes de cultivo e correlacionados positivamente com os resíduos de metais. A actividade de 7-etoxiresorufina *O*-deetilase variou de 28 pmol/min/mg proteína nos peixes de cultivo a 83 pmol/min/mg proteína nos peixes selvagens recolhidos no Estuário na área da marina. Tanto os peixes de cultivo como os selvagens da área da marina mostraram uma inibição na actividade da acetilcolinesterase em tecido muscular e uma infecção nas gónadas provocada por parasitas. Os resultados obtidos suportam a utilidade do uso combinado de marcadores químicos e bioquímicos na avaliação do impacto de contaminantes antropogénicos tanto em peixes selvagens como de cultivo.

Palavras-chave: Robalo; metalotioneína; catalase; 7-etoxiresorufina *O*-deetilase; acetilcolinesterase; poluição; *Sphaerospora testicularis*

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Chemical residues and biochemical responses in wild and cultured European sea bass (*Dicentrarchus labrax* L.)

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Abstract

Cultured and wild sea bass (*Dicentrarchus labrax*) from the Arade Estuary were sampled in summer and winter and the degree of exposure to metals and polycyclic aromatic hydrocarbons (PAHs) assessed, together with some biochemical responses against those and other pollutants. The highest levels of copper (up to 997 $\mu g\,g^{-1}$ dry weight) and cadmium (up to 4.22 $\mu g\,g^{-1}$ dry weight) were detected in the liver and kidney of cultured specimens, whereas the highest exposure to PAHs was observed in wild fish. Significant alterations in some biochemical markers were detected and associated to pollutant exposure. Thus, metallothionein concentrations were higher in the tissues of cultured fish and positively correlated with metal residues. The activity 7-ethoxyresorufin *O*-deethylase ranged from 28 pmol/min/mg protein in cultured fish to 83 pmol/min/mg protein in wild fish collected near a marina area. Cultured fish and wild fish from the marina area had depressed acetylcholinesterase in muscle tissue and a parasitic infection in the gonads. The obtained results support the usefulness of the combined use of chemical and biochemical markers to assess the impact of anthropogenic pollutants in both wild and cultured fish.

Keywords: Sea bass; Metallothionein; Catalase; 7-ethoxyresorufin *O*-deethylase; Acetylcholinesterase; Pollution; *Sphaerospora testicularis*

1. Introduction

In recent years, there has been a growing awareness of the need to detect and assess the adverse effects of contaminants in aquatic organisms. Among the available techniques, the integrated use of chemical analyses and biochemical and cellular responses to pollutants is a sound procedure for detecting the impact of anthropogenic contaminants in aquatic systems (Fernandes et al., 2002). The measurement of biochemical and physiological responses in fish tissues may serve to assess the degree of exposure to environmental pollutants as well as the risks for the health and survival of contaminant-exposed populations (Stien et al., 1998).

Estuaries and their adjacent coastal waters are often vulnerable to the presence of xenobiotic compounds due to the impact of river effluents and the proximity of point and diffuse sources of contamination exposing fauna inhabiting these areas (Forget et al., 2003). The Arade estuary (Algarve, Southern Portugal) is a system of ecological and economic importance that is under increasing anthropogenic pressure due to the existence of a large fishery harbor and a marina. The estuary receives treated wastewater of the adjacent city of Portimão (45 500 inhabitants), and in recent years, there has been an increase in fish farm practices that release nutrients and chemical compounds into the area. However, data on pollutant residues in biota or information on the fate and toxicity of the chemicals released into this aquatic environment are unavailable at this time.

The present study was designed to assess exposure to metals and polycyclic aromatic hydrocarbons (determined as hydroxylated PAHs in fish bile, Escartín and Porte, 1999), along with several biochemical responses in both wild and cultured European sea bass (*Dicentrarchus labrax* L.) from the Algarve region. Sea bass was selected as a bioindicator species because it is a common wild

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species in the estuary and is of high economic interest in the region. Sea bass culture has increased considerably in the last few years, reaching a high production and a high commercial value in Europe (FAO, 2003). This intensive production has raised concerns over the quality of cultured fish in comparison to wild fish (Alasalvar et al., 2002). Thus, evaluating pollutant loads and biological responses in cultured and wild fish is a major need.

The biochemical markers measured in this study were: (A) liver and kidney metallothioneins (MTs), as a tool to monitor environmental impact of metals (George and Olsson, 1994; Viarengo et al., 1999); (B) liver 7-ethoxyresorufin *O*-deethylase (EROD) activity, commonly used as a biomarker of exposure to polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and petroleum hydrocarbons in wild fish (Au and Wu, 2001; Whyte et al., 2000); (C) liver catalase (CAT) activity, a defense mechanism against oxidative damage, since many pollutants in aquatic ecosystems exert their toxic effects through oxidative stress (di Giulio et al., 1989); (D) brain and muscle acetylcholinesterase (AChE) activity, intended to diagnose exposure to organophosphorus pesticides along with other chemicals which have the potential to decrease AChE activity in exposed organisms (de la Torre et al., 2002); (E) liver glutathione *S*-transferase (GST) and UDP-glucuronosyltransferase (UGT), which are induced by a variety of natural and synthetic compounds, and play a key role catalyzing the conjugation and potential excretion of different xenobiotics (Clarke et al., 1992). In addition, histological examination of gonads was performed to determine the state of maturation of individuals and to investigate the occurrence of abnormalities within this organ.

2. Materials and methods

2.1. Sample collection and preparation

Sea bass (*D. labrax*) were collected from the Arade Estuary (S Portugal), and supplied by a fish farm (FF) (S Portugal) in June 2003 and February 2004. Three sites were selected in the estuary, according to different environmental characteristics (Fig. 1). Site A1, an area influenced by the marina of Portimão city, is located 12 km from the estuary mouth; site A2, an area influenced by the outlet of the city sewage treatment plant (STP) is located 24 km from the estuary mouth; and site A3, located upstream the STP, is about 45 km from the estuary mouth. It was not possible to find fish in the upper stream site (A3) during the summer sampling. The name and exact location of the fish farm will not be identified due to confidential policy.

Immediately after collection, individuals were killed by a blow to the head, total length and weight recorded and target tissues (liver, kidney, bile, brain, muscle) immediately dissected, frozen in liquid nitrogen and stored at −80 °C upon arrival at the laboratory. A subsample from the central part of the gonad was fixed for histological examination.

2.2. Chemical analysis

2.2.1. Analysis of metals

Liver and kidney samples (0.5–2.0 g wet weight) were homogenized in ice-cold 0.02 M Tris–HCl pH 8.6 containing 0.9% NaCl, and dried at 80 °C for 24 h, weighted and wet digested with concentrated HNO_3. Digested samples were evaporated to dryness before dissolution in 1 M HCl. Analysis of copper (Cu) and zinc (Zn) were carried out by flame atomic absorption spectrophotometry. Concentrations of cadmium (Cd) were determined by graphite furnace atomic absorption spectrophotometry, using standard addition methods. The analytical procedure was checked using a standard reference material (cod muscle CRM 422) provided by the Community Bureau of Reference-BCR, the values obtained were not statistically different from the certified ones and the results are shown in Table 1. Metal concentrations are expressed on a dry weight basis.

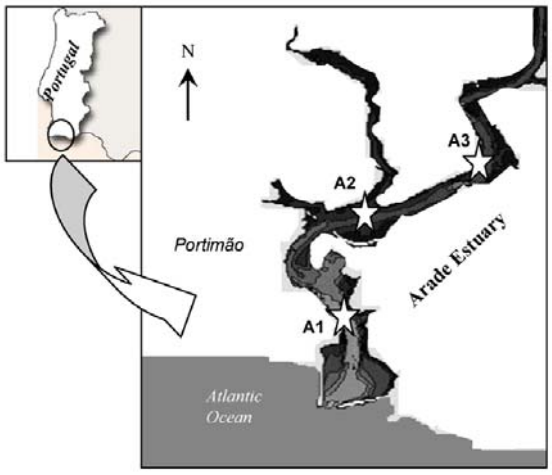

Fig. 1. Schematic diagram of the Arade estuary (S Portugal), indicating the sampling sites where sea bass (*Dicentrarchus labrax*) were collected: A1: area influenced by the marina; A2: area influenced by the STP; A3: upperstream site.

2.2.2. Analysis of hydroxylated-PAHs

Bile samples were hydrolyzed using a modification of the method described in Escartín and Porte (1999). Briefly, 100 μL of bile were incubated for 1 h at 40 °C in the presence of 1 ml 0.4 M acetic acid/sodium acetate buffer pH 5.0, containing 2000 units of β-glucuronidase and 50 U of sulphatase. Hydrolyzed metabolites where extracted with 1 ml ethyl acetate (× 3), the extracts recombined and reduced to near dryness under a nitrogen stream. This final extract was analyzed by fixed wavelength fluorescence (Kontron Instruments SFM-25 spectrofluorometer), at the excitation/emission wavelength pairs of 1-naphthol (290/335 nm) and 1-pyrenol (345/395 nm). To estimate the concentration of naphthalene-like and pyrene-like metabolites, calibration curves were performed using 1-naphthol and 1-pyrenol as standards, and the results expressed as equivalents.

2.3. Biochemical determinations

MTs were determined on individual liver and kidney samples. Subsamples (0.5–2.0 g wet weight) were homogenized in ice-cold 0.02 M Tris–HCl pH 8.6 containing 0.9% NaCl. An aliquot of each homogenate (3 mL) was centrifuged at 30000*g* for 45 min. To partially purify MT for electrochemical quantification, the supernatant was decanted and heated in a water bath at 80 °C for 10 min in order to denature high molecular weight proteins. The resulting preparation was further centrifuged at 30000*g* for 45 min, and the heat-treated supernatant, containing thermally stable MT, separated from precipitated proteins. MT was measured using

Table 1
Analysis of the reference material cod muscle CRM 422 (Community Bureau of Reference)

Metal	Certified values	n	Recorded values
Cd	0.02±0.002	5	0.02±0.002
Cu	1.05±0.07	5	1.12±0.09
Zn	19.60±0.50	5	19.76±0.53

Mean values (μg/g dry weight) ±SEM.

differential pulse polarography (DPP), as described by Bebianno and Langston (1989). An aliquot of heat-treated supernatant (10–25 μL), together with 250 μL Triton-X were added to 20 mL hexaminocobalt chloride buffer and the polarographic response of the MT measured during a potential scan between −1.4 and −1.7 V (646VA Processor autolab type II and an ECO Chemie IME663 mercury drop electrode). Comparisons of peak heights with those of standard additions of purified rabbit MT (Sigma) enabled the quantification of MT. Concentrations are expressed as μg/g dry weight of tissue.

Microsomal and cytosolic fractions were prepared as described in Lavado et al. (2004). Briefly, after weighing, livers were flushed with ice-cold 1.15% KCl, and homogenized in 1:5 w/v of cold 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, containing 150 mM KCl and supplemented with 1 mM dithiothreitol (DDT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM ethylenediaminetetra acetic acid (EDTA). Homogenates were centrifuged at 1500*g* for 15 min and the fatty layer removed. The obtained supernatant was then centrifuged at 12000*g* for 20 min. The resulting supernatant was further centrifuged at 100 000*g* for 60 min to obtain the cytosolic and microsomal fractions. Microsomal pellets were resuspended in a ratio of 0.5 ml buffer/g liver tissue in 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, containing 150 mM KCl, 20% w/v glycerol and supplemented with 1 mM DDT, 0.1 mM PMSF and 1 mM EDTA. Microsomal and cytosolic proteins were measured by the method of Lowry et al. (1951), using Folin's reagent and bovine serum albumin (BSA) as standard.

EROD activity was determined in the microsomal fraction of the liver as described in Fernandes et al. (2002). Hepatic microsomes (200 μg) were incubated at 30 °C for 10 min in a final volume of 1 ml containing 100 mM K_2HPO_4/KH_2PO_4 pH 7.4, 0.25 mM NADPH and 4.15 μM 7-ethoxyresorufin. The reaction was stopped by adding 2 ml of ice-cold acetone. Samples were centrifuged at 3000 rpm for 10 min and 7-hydroxyresorufin fluorescence was determined using a Kontron Instruments SFM-25 spectrofluorometer at 537/583 excitation/emission wavelengths. Activity was calculated as the amount of resorufin (pmol) generated per milligram of protein per minute of reaction time.

CAT activity was measured in liver cytosol according to Aebi (1974). This technique is based in the direct determination of hydrogen peroxide consumption. The assay was performed at room temperature in a final volume of 3 ml, containing 50 mM KH_2PO_4/K_2HPO_4 pH 6.5 and 50 mM 30% H_2O_2 as substrate. After addition of the sample, the decrease in absorbance was measured at 240 nm and the enzyme activity calculated in terms of mmol H_2O_2 consumed/min/mg protein using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

AChE activity was assayed in individual brain and dorsal muscle tissue according to the colorimetric method of Ellman et al. (1961). Samples were homogenized in ice-cold 100 mM Tris–HCl pH 8.0, at a ratio 1:5 (w/v) using a Polytron homogeniser. The homogenate obtained was centrifuged at 12000*g* for 30 min and the resulting supernatant immediately used for AChE activity, in the presence of 10 mM acetylthiocholine iodide (ATC) as a substrate and 1 mM 5,5′-dithiobis-2-dinitrobenzoic acid (DTNB) as the thiol indicator. Spectrophotometric measurements were done in duplicate and average change in absorbance per minute determined at 405 nm. Activity was calculated in terms of nmol ATC hydrolysed/min/mg protein using a molar extinction coefficient of 13.6 mM^{-1} cm^{-1}.

Phase II enzymes were assayed as described in Fernandes et al. (2002). Glutathione *S*-transferase (GST) activity was measured in the cytosolic fraction of liver, using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The final reaction mixture containing 80 mM K_2HPO_4/KH_2PO_4 pH 7.4, 1 mM CDNB and 1 mM reduced glutathione in a total volume of 1 ml. The change in absorbance, at room temperature, was recorded at 340 nm and the enzyme activity was calculated as μmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}. Hepatic UDP-glucuronosyltransferase (UGT) assay, which contained 0.25 mg of microsomal protein (pretreated for 15 min with 0.2% TritonX-100 on ice), was initiated by the addition of 81 μM *p*-nitrophenol (*p*NP) and run for 30 min at 30 °C in a shaking water bath. The reaction was stopped by the addition of 0.2 M ice-cold trichloracetic acid, centrifuged and alkalinized with 0.1 ml of 10 N KOH and the remaining *p*NP was measured spectrophotometrically at 405 nm. Activity was calculated as the amount of *p*NP (nmol) generated per milligram of protein per minute of reaction time.

2.4. Histological analysis

Gonads were fixed in 10% buffered formalin for 24 h, dehydrated through a graded ethanol series and embedded in paraffin. Tissue sections (6 μm thick) were stained with hematoxylin-eosin. Sexual maturity stages (SMS) were classified as described in Goodbred et al. (1996).

2.5. Statistical analysis

Biochemical activities and chemical analyses were conducted individually in four to six organisms per station and run in duplicate. Values are presented as means ±SEM. Statistical significance was assessed using one-way ANOVA with Tukey's test, using the software package SPSS/PC^{TM}, version 12.0 (SPSS Inc., Chicago, IL). P values lower than 0.05 were considered statistically significant. Pearson's correlation coefficients were calculated and only $P<0.01$ was accepted as significant.

3. Results

3.1. Biological data of samples

The biological parameters of the sampled fish are listed in Table 2. Individuals collected near the estuary mouth (A1) were bigger in size and weight than those sampled upstream (A2, A3). In both seasons, the hepatosomatic index (HSI) was significantly higher in cultured sea bass (FF) when compared to wild specimens (about 4-fold higher in winter). During the summer, the condition factor (CF) was similar for all individuals (1.0 1.1) but, in winter, cultured fish showed significantly higher CF (1.23) than wild sea bass (0.94 1.0).

3.2. Chemical analysis

Metal concentrations in the liver and kidney of sea bass are shown in Table 3. Concentrations of Cu in wild specimens increased from upstream (A3, A2) to downstream areas (A1). This tendency was observed both in summer and winter. Levels of Cu in the liver of fish from FF were up to 997 μg/g dry weight during the summer sampling, which was about 4 40-fold higher than those detected in wild fish at stations A1 and A2, respectively. During the winter, Cu concentrations in the liver of fish from FF were reduced to 100 μg/g. Levels of Cu in the kidney from FF were also higher by approximately

Table 2
Biological data of sea bass (*Dicentrarchus labrax*) collected from the Arade estuary (A1, A2, A3) and in a fish farm (FF) (S Portugal)

Site	*n*	Weight (g)	Length (cm)	HSI (%)	CF (g cm^{-3})
Summer					
A1	4	285±50^{a}	30.5±2.06^{a}	0.45±0.05^{a}	0.99±0.03^{a}
A2	6	120±9^{b}	22.8±0.49^{b}	0.56±0.10^{a}	1.00±0.03^{a}
FF	6	464±30^{c}	35.2±0.81^{a}	0.97±0.08^{b}	1.06±0.03^{a}
Winter					
A1	4	382±85^{a}	33.1±2.57^{a}	0.66±0.05^{a}	1.01±0.04^{a}
A2	6	50±5^{b}	17.1±0.49^{b}	0.56±0.05^{a}	0.99±0.03^{a}
A3	4	44±9^{b}	16.5±1.00^{b}	0.75±0.09^{a}	0.94±0.05^{a}
FF	6	458±17^{a}	33.5±0.41^{a}	2.43±0.16^{b}	1.22±0.03^{b}

Fish were sampled in summer and winter. HSI: hepatosomatic index, calculated as (liver weight/body weight) × 100. CF: condition factor, calculated as [body weight/(length)^{3}] × 100. Values are mean ±SEM. Distinct letters indicate significant differences between sites, according to Tukey's test ($P<0.05$). *n*: number of organisms analysed.

Table 3
Metal concentrations (μg/g dry weight) in sea bass (*Dicentrarchus labrax*) collected from the Arade estuary (A1, A2, A3) and in a fish farm (FF)

Sites		Cu	Cd	Zn
Summer				
A1	Liver	258±129^{a}	0.22±0.05^{a}	620±180^{a}
	Kidney	1.83±0.22^{a}	0.03±0.001^{a}	100±30
A2	Liver	26±5.0^{b}	0.11±0.01^{a}	170±10^{b}
	Kidney	1.56±0.09^{a}	0.02±0.01^{a}	80±10
FF	Liver	997±75^{c}	1.18±0.03^{b}	220±3.0^{ab}
	Kidney	3.55±0.22^{b}	0.09±0.002^{b}	90±5.0
Winter				
A1	Liver	189±95^{a}	0.20±0.04^{ab}	360±190^{a}
	Kidney	2.33±0.07^{b}	0.12±0.003	60±10^{a}
A2	Liver	44±26^{bc}	0.40±0.05^{b}	450±110^{a}
	Kidney	1.07±0.09^{a}	0.17±0.01	10±1.0^{b}
A3	Liver	6.0±1.0^{b}	0.07±0.01^{a}	80±10^{b}
	Kidney	0.87±0.13^{a}	0.19±0.01	10±3.0^{b}
FF	Liver	104±25^{ab}	0.11±0.04^{a}	150±60^{b}
	Kidney	4.22±1.01^{b}	0.19±0.02	60±3.0^{a}

Fish were sampled in summer and winter. Values are expressed as mean ±SEM ($n=4$). Distinct letters indicate significant differences between sites, according to Tukey's test ($P<0.05$).

2 4-fold than those detected in wild fish in both summer (3.55 μg/g dry weight) and winter (4.22 μg/g dry weight).

Fish from FF had the highest residues of Cd in the liver (1.18 μg/g dry weight) and kidney (0.09 μg/g dry weight) during the summer sampling. However, in winter, the highest concentration of Cd was detected in the liver of wild sea bass from A2 (0.40 μg/g dry weight), about 6- and 4-fold higher than the levels detected in fish from A3 and FF, respectively. No differences between sampling sites were observed regarding Cd levels in kidney (0.12 0.19 μg/g dry weight) in winter, however, those levels were about 2 8-fold higher than in the summer.

Table 4
Biliary levels of hydroxylated-PAHs (μg/g of bile) in sea bass (*Dicentrarchus labrax*) collected from the Arade estuary (A1, A2, A3) and in a fish farm (FF)

	Arade estuary			Fish farm
	A1	A2	A3	FF
Summer				
1-Naphthol	39.9±5.4^{a}	37.1±7.9^{a}	—	22.3±3.2^{a}
1-Pyrenol	0.67±0.10^{a}	0.37±0.20^{b}	—	0.62±0.20^{a}
Σ OH–PAHs	40.6±5.5	37.5±8.1	—	22.9±3.4
Winter				
1-Naphthol	34.7±0.1^{ab}	51.4±6.5^{b}	24.7±7.9^{ab}	16.4±3.5^{a}
1-Pyrenol	0.21±0.10^{a}	0.24±0.10^{a}	0.08±0.03^{b}	0.57±0.20^{a}
Σ OH–PAHs	34.9±0.2	51.6±6.6	24.8±7.9	17.0±3.7

Fish were sampled in summer and winter. Results are expressed as naphthol and pyrenol equivalents. Values are mean ±SEM ($n=4$). Distinct letters indicate significant differences between sites, according to Tukey's test ($P<0.05$).

The highest levels of Zn were observed in the summer in the liver of wild fish from A1 (620±180 μg/g dry weight), 4- and 3-fold higher than those observed in A2 and FF, respectively. No such differences among sampling sites were observed when looking at Zn residues in kidney (80 100 μg/g dry weight). During the winter, fish from A2 showed rather high Zn levels in the liver (450 μg/g dry weight), but in the kidney, the highest residues were detected in fish from A1 (60 μg/g dry weight) and FF (60 μg/g dry weight).

Concentrations of hydroxylated PAHs in fish bile are presented in Table 4. The highest concentrations of the sum of 1-naphthol and 1-pyrenol equivalents were found in the bile of wild fish from sites A1 and A2. Differences among sites were observed in terms of 1-naphthol equivalents, the highest values detected in the bile of wild sea bass, particularly in those from A2 in the winter. The highest levels of 1-pyrenol equivalents were detected in the bile of fish from FF in both samplings, and in fish from A1 (marina area) during the summer sampling.

3.3. Biochemical markers

During the summer, cultured fish showed higher hepatic MT levels (15590±280 μg/g dry weight) than wild specimens, however no such difference was observed in the kidney (Fig. 2). Hepatic MTs were positively correlated with Cu ($r=0.971$, $p<0.01$) and Cd ($r=0.991$, $p<0.01$) residues in the liver. In the winter, MT concentrations in the liver of cultured fish (FF) were significantly reduced (4780±1170 μg/g dry weight) compared to the summer MT levels of 15590±280 μg/g dry weight. Expression of MT in the liver and kidney of wild sea bass increased from upstream (A3) to downstream (A1) areas; renal concentrations of MT showed a significant induction in sea bass from site A1 (9860±160 μg/g dry weight) and FF

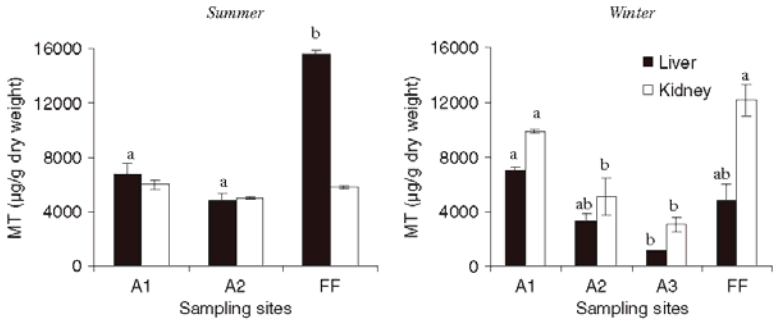

Fig. 2. Metallothionein (MT) levels in liver and kidney of sea bass (*Dicentrarchus labrax*), sampled in summer and winter. Values are expressed as mean ± SEM ($n = 4$). Distinct letters indicate significant differences ($P < 0.05$) between sites, according to Tukey's test.

(12140 ± 1130 µg/g dry weight), when compared to A3 (Fig. 2).

EROD activity (Fig. 3) showed a pattern similar to that of the sum of hydroxylated PAHs in fish bile (Table 4). During the summer, EROD was significantly elevated in wild sea bass from site A1 (83 ± 11 pmol/min/mg protein), about 2- and 4-fold higher than in fish from site A2 and FF, respectively. In both seasons, cultured specimens (FF), showed lower EROD activity (28 ± 2.4 and 38 ± 6.5 pmol/min/mg protein) than wild sea bass.

For catalase activity determined in liver cytosolic fractions, significant differences among sampling sites were only recorded in the summer. Interestingly, the high Cu and Cd concentrations detected in cultured fish in the summer, coincided with a significant depletion of CAT activity. This depletion was also evident in winter, but differences among sites were not statistically significant (Fig. 3).

Significant differences among sampling sites were not observed for GST activity -both seasons-, and for UGT -winter sampling-. During the summer, fish from site A2 had a 2-fold higher UGT activity than those from the other sites (Fig. 3).

Brain AChE activity did not show significant differences among sites. When the activity was determined in muscle tissue, decreased AChE activity was detected in sea bass from A1 (61 ± 5.6; 67 ± 7.8 nmol/min/mg protein) and FF (57 ± 4.0; 59 ± 6.6 nmol/min/mg protein), in both samplings when compared to the other sites (A2, A3) (Fig. 4). This depletion of AChE activity corresponded to a 50% inhibition.

3.4. Histology

During the summer sampling, sea bass from site A2 were classified as SMS-0 (undeveloped). SMS of the specimens collected in sites A1 and FF was difficult to determine due to the invasion of the gonadal tissue by myxosporean endoparasites. Infected gonads were significantly smaller and harder than non-infected ones and showed many orange-yellowish spots. The histological study revealed that most of the testicular tissue was occupied by large necrotic granulomata, which corresponded to seminiferous tubules harboring a myxosporean parasite and/or their debris. The cysts were encapsulated by fibrotic layers (Fig. 5). The myxosporidian was identified as *Sphaerospora testicularis* (personal communication, Dr. Sitjà-Bobadilla, IATS-CSIC). The mean prevalence of the infection was of 100%, with a very high intensity. Interestingly, sites (A1 and FF) where sea bass was infected were the most contaminated in terms of metals and PAHs.

In winter, all the individuals were at the early stages of sexual development; being classified as SMS-1 (early spermatogenic and previtellogenic), and no evidences of parasite infection were observed.

4. Discussion

This study indicates that both wild (Arade Estuary) and cultured sea bass (*D. labrax*) from Southern Portugal had significant alterations in some of the biochemical markers tested (MT, EROD, CAT, UGT, AChE), and that those alterations are at least partially associated to pollutant exposure.

Cu residues in the liver and kidney of cultured fish (FF) were very high, particularly during the summer sampling, and they were associated to a Cu treatment that occurred 3 weeks before sampling. During the summer, increased water temperature favors the development of an ectoparasitic algae, which lounges in the gills of the fish killing them by asphyxiation; $CuSO_4$ is commonly added in the water to treat this and other ectoparasitic infections (Schlenk et al., 1999; Straus, 1993). In winter, hepatic Cu levels in cultured fish were in the same range of those detected in wild sea bass from A1. Nonetheless, Cu levels in kidney were still elevated. Despite of the efficacy of Cu in treating these infections, it is unknown how exposure to therapeutic concentrations can affect the general well being of fish (Schlenk et al., 1999). Moreover, the increasing anthropogenic use of Cu, Zn, and other metals may

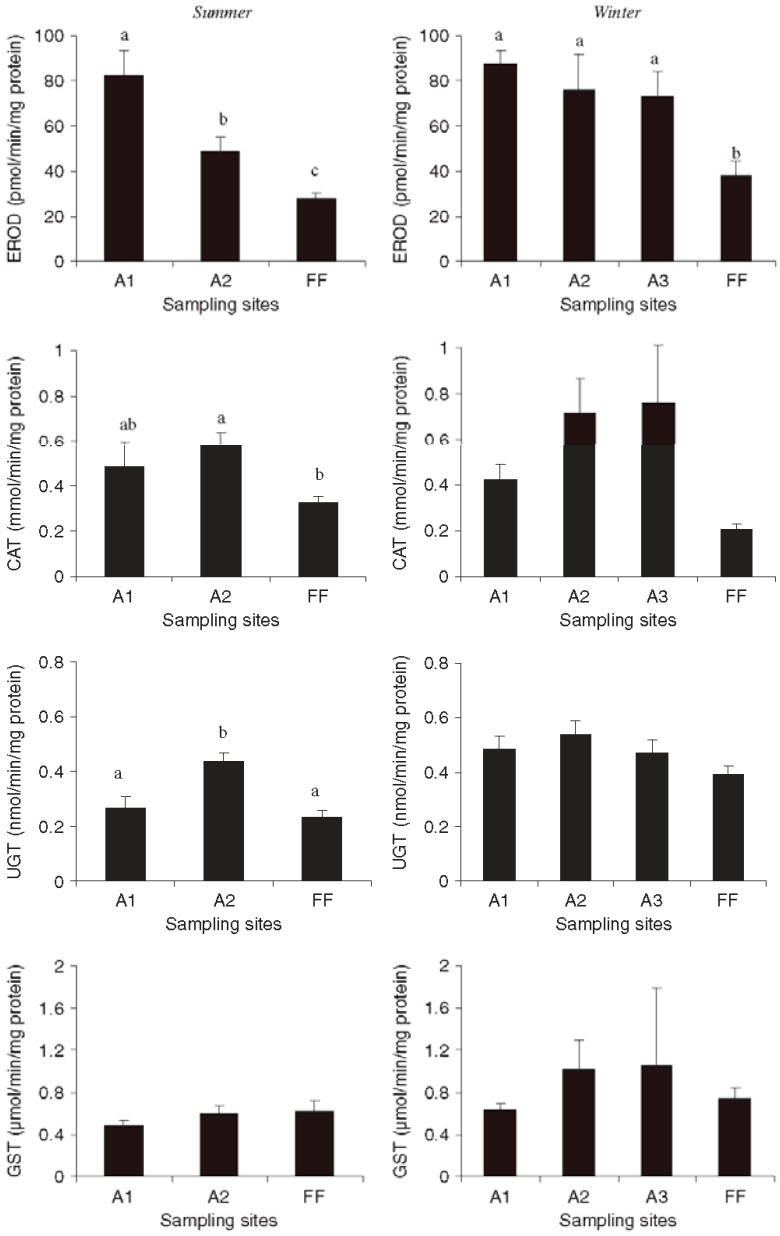

Fig. 3. 7-Ethoxyresorufin *O*-deetylase (EROD); catalase (CAT); UDP-glucuronosyltransferase (UGT) and glutathione *S*-transferase (GST) activities in liver of sea bass (*Dicentrarchus labrax*), sampled in summer and winter. Values are expressed as mean ± SEM ($n = 4-6$). Distinct letters indicate significant differences ($P < 0.05$) between sites, according to Tukey's test.

certainly contribute to an increase in its environmental concentrations (Sánchez-Bayo and Goka, 2005; Schiff et al., 2004).

Metals such as Cu and Zn are essential for fish metabolism (Dugo et al., 2006), however when their intake is excessively high, toxic effects in the organisms can occur

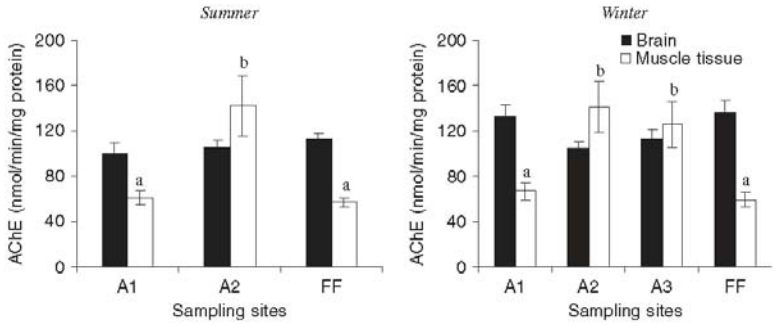

Fig. 4. Acetylcholinesterase (AChE) activity in brain and muscle tissue of sea bass (*Dicentrarchus labrax*), sampled in summer and winter. Values are expressed as mean ± SEM ($n = 4-6$). Distinct letters indicate significant differences ($P < 0.05$) between sites, according to Tukey's test.

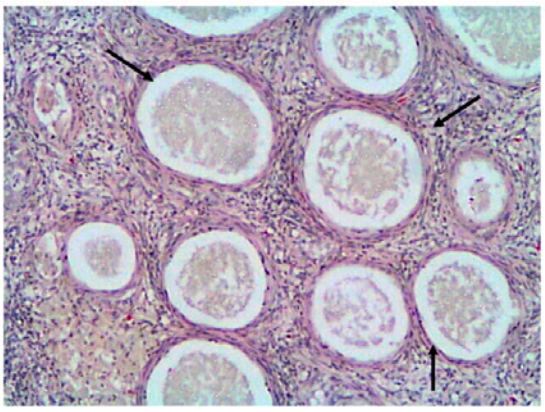

Fig. 5. Testicular tissue of sea bass (*Dicentrarchus labrax*) infected with *Sphaerospora testicularis*. Arrows indicate big necrotic granulomatous areas around infected seminiferous tubules with intensive fibrotic reaction produced after the infection (magnification 100 ×).

(Pipe et al., 1999; Suresh et al., 1992). Considering wild sea bass, the highest residues of Cu and Zn were detected in specimens collected in the marina area (A1), probably associated to their use in antifouling paints on vessel hulls and in harbor infrastructures. Actually, high levels of these trace metals have been reported in water and sediments from harbors and marinas (Baruthio, 1991; Osman et al., 1995; Schiff et al., 2004). Cu and Zn residues in specimens of A1 were significantly higher in summer than in winter, probably due to the increased presence of tourist boats in the area during the summer.

While Cu and Zn are essential metals, others such as Cd are potentially toxic and pose a serious risk for human health when entering into the food web (Rojas et al., 1999). Curiously, significantly high levels of Cd were detected in the liver (1.18 μg/g dry weight) of cultured fish (FF), but only in the summer. No such levels were detected in winter. The fish farm facility drains water from a lagoon system that has a high anthropogenic input, particularly during the summer months. High levels of Cd were found in hepatic tissue of wild sea bass collected near the STP area (A2) in winter. Cd has been often associated to discharges of treated wastewater effluents (Brown et al., 1984).

In agreement with metal residues, MT levels were significantly high in the liver and kidney of cultured fish (FF), and correlated with Cu and Cd residues in the liver, as previously indicated by other authors (Buton et al., 1987). In the summer sampling, cultured fish had higher levels of hepatic MT (2 4-fold) than wild fish, probably linked to Cu storage in the liver. No significant differences were detected regarding MT levels in kidney. However, several months later, during the second sampling, MT levels in the liver of cultured fish decreased significantly, but increased in the kidney. This increase coincided with the high levels of Cd detected in kidney, and it is in agreement with other studies that indicated an increase in renal MT levels in Cd-exposed rainbow trout and sea bass (Olsson et al., 1996; Roméo et al., 2000).

High EROD activity was recorded in the liver of sea bass from the estuary, mainly in the area influenced by the marina (A1), which confirms the presence of CYP1A inducing agents in the area. In fact, high levels of 1-pyrenol equivalents, which is regarded as the best general indicator of PAH exposure in fish (Ruddock et al., 2002), were detected in the bile of fish from site A1 (marina area) and FF (fish farm). These high levels were detected mainly in the summer sampling, and are possibly associated to an increase of tourist recreational activities. Despite the similar levels of 1-pyrenol equivalents in the bile of fish from A1 and FF, cultured sea bass (FF) showed significantly lower EROD activity than wild fish. A potential inhibition of EROD activity due to co-exposure to high levels of metals (Cu & Cd) cannot be discarded. Several authors have demonstrated the inhibitory effect of Cu and Cd in EROD activity in sea bass (Oliveira et al., 2004; Stien et al., 1997; Viarengo et al., 1997); and low EROD activity was reported in sea bass from hydrocarbon polluted sites that had high tissue metal residues (Roméo

et al., 1994; Stien et al., 1997). Moreover, the fact that fish from FF were starved before sampling may be another factor lowering EROD activity (Lemaire, 1990).

Generally, EROD activities measured in the present work were in the higher range of those obtained in sea bass from other field studies (8–42 pmol/min/mg protein) or laboratory kept animals (8–13 pmol/min/mg protein) (Deviller et al., 2005; Stien et al., 1998). Taking into account the two sampling periods, and regardless of sampling sites, hepatic EROD activity was significantly lower in summer than in winter. Differences in the water concentration of CYP1A inducers may exist between the two sampling periods, but water temperature as well as the physiological status of the sea bass may also interfere (Stien et al., 1998). Temperature is probably one of the most dominant of the abiotic factors that exert influence on cytochrome P450 dependent enzymes (Bucheli and Fent, 1995). Mean temperatures of the water in the sampled areas were 22.6 °C in the summer and 14.8 °C in the winter. Sleiderink et al. (1995) has suggested that a negative compensation takes place, and fish increase their hepatic EROD activity at low temperatures. Also, Stien et al. (1998) reported that hepatic EROD in sea bass, caged in the Bay of Cannes, showed lower activity in autumn (19.9 °C) than in spring (13.4 °C).

Phase II enzymes were less responsive to pollutant exposure than EROD, and this is in agreement with several studies that reported no changes on GST activities of different fish species exposed to complex mixtures of pollutants (Fenet et al., 1998; Lavado et al., 2006; Teles et al., 2004). Seasonal variation on GST activity was rather low: mean GST activity was 0.57 ± 0.07 ($n = 16$) in June and of 0.86 ± 0.26 ($n = 20$) in February, and this is in agreement with other studies in this species (Stien et al., 1998). If the induction of liver monooxygenases was a physiological adaptive process in fish, one would expect that conjugation enzymes would be induced for subsequent metabolic steps of detoxification (Soimasuo et al., 1995). However, low hepatic UGT activity was observed in sea bass specimens exposed to high PAHs and metal concentrations.

Considering the other biochemical markers analysed, it is worth mentioning the significant decrease of CAT activity in cultured fish (FF), possibly related to high metal exposure. CAT has been shown to be either induced or inhibited by metals, depending on the dose, the species and/or the route of exposure (Sanchez et al., 2005). Zebrafish (*Brachydanio rerio*) exposed to 40 and 140 μg Cu L^{-1} as $CuSO_4$ presented an induction of hepatic CAT within 2 weeks of waterborne exposure (Paris-Palacios et al., 2000). Conversely, in carp (*Cyprinus carpio*), hepatic CAT was inhibited after 96 h exposure to 100 and 250 μg Cu L^{-1} as $CuSO_4$ (Dautremepuits et al., 2002). An inhibition of CAT activity in the liver of rainbow trout (*Oncorhyncus mykiss*) and killifish (*Fundulus heteroclitus*) exposed to Cd has also been reported (Pruell and Engelhardt, 1980; Palace et al., 1992).

The sensitivity of brain and muscle AChE appears to be species specific (Fulton and Key, 2001). In the present work, a significant inhibition of AChE activity was reported in muscle tissue of sea bass from sites A1 and FF, in both seasons, whereas no differences were observed in the brain. Thus, our results suggest that AChE in sea bass is more sensitive in muscle tissue than in the brain, and that not only pesticides (organophosphates and carbamates), but metals may have an inhibitory effect on AChE activity. Other studies have reported depressed AChE activity in the muscle of sea bass (*D. labrax*) caged in a harbor area (Stien et al., 1998), and in the muscle of wild carp (*C. carpio*) exposed to high levels of Cd (Lavado et al., 2006), or experimentally exposed to $CuSO_4$ (Szabó et al., 1992).

The histological study of gonads revealed no alterations except those due to the presence of parasites in specimens from A1 and FF in the summer, which corresponded to individuals with significantly high metal concentrations (e.g. Cu). Some parasites have been reported to proliferate in fish living in polluted areas due to fish immunodepression. There is, however, a very poor knowledge on the alterations of the fish immune system due to a parasitic infection and on the bioenergetic cost to combat a parasite or a disease (Hecker and Karbe, 2005; Muñoz et al., 1998). The identified parasite *Sphaerospora testicularis*— is a systemic Myxosporean which naturally infects European sea bass in cultured stocks (Sitjá-Bobadilla and Álvarez-Pellitero, 1993a); and this is the first time the infection is reported in wild fish. Only male fish were found to be parasitized by *S. testicularis* which is a parasite specific to the testes, and acts destroying testicular tissue and significantly reducing the fecundity of males (Sitjá-Bobadilla and Álvarez-Pellitero, 1993b). Unexpectedly, the parasite was not found in winter, the season when sea bass gonads mature and *S. testicularis* is more prevalent (Sitjá-Bobadilla and Álvarez-Pellitero, 1993a).

Overall, metal tissue residues and levels of hydroxylated PAHs in bile, in conjunction with altered biochemical responses (MT levels and EROD, CAT, AChE activities) and the histopathological findings are indicative that both wild and cultured sea bass populations are under different types of stress. Elevated EROD activity and depressed AChE were observed in wild sea bass from the marina area, suggesting the presence of CYP1A inducing agents in the water apart from rather high levels of metals (Cu and Zn). Cultured fish (FF) showed a clear response to $CuSO_4$ treatment that was evident even 7 months after exposure, they also had depressed CAT and AChE activity. Pollutant exposure can lead to immunodepression, which would result in increased susceptibility of polluted fish to parasitic infections. Although no markers of the immune system were measured in this study, the correspondence between Cu contamination and the infestation with *S. testicularis* in cultured and wild sea bass may support this hypothesis. However, the present study was not designed to answer this specific question and future research will be necessary to

test the hypothesis. The obtained results further support the usefulness of the combined use of chemical and biochemical markers to assess the impact of anthropogenic pollutants in both wild and cultured fish.

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2.2.

CHEMICAL AND BIOCHEMICAL TOOLS TO ASSESS POLLUTION EXPOSURE IN CULTURED FISH

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Resumo

Existe pouca informação sobre os níveis de contaminantes em peixes de cultivo, e os riscos associados ao seu consumo. Este estudo foi projectado com a finalidade de avaliar os níveis de exposição a metais, compostos organoclorados, hidrocarbonetos aromáticos policíclicos (PAHs) e alquilfenois (APEs) no Robalo (*Dicentrarchus labrax*) cultivado em cinco aquaculturas localizadas no Sul da Europa. Adicionalmente, diversas respostas bioquímicas (metalotioneína, 7-etoxiresorufina *O*-deetilase, vitelogenina) foram determinadas como ferramentas complementares. Os dados obtidos indicam que a exposição a contaminantes em peixe de cultivo é similar aos níveis observados em espécimes selvagens da área. No entanto, algumas respostas bioquímicas foram observadas nos organismos analisados, *viz*. a indução da metalotioneína em indivíduos expostos a cobre (Cu), a indução de 7-etoxiresorufina *O*-deetilase (EROD) e da vitelogenina nos indivíduos expostos a PAHs e APEs. O estudo apoia ainda a utilidade dos biomarcadores como um primeiro método de selecção para descriminar entre níveis de exposição basal e elevado em peixes de cultivo.

Palavras-chave: Robalo; peixes de cultivo; poluição; marcadores bioquímicos

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Chemical and biochemical tools to assess pollution exposure in cultured fish

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Pollution assessment in cultured fish: chemical and biochemical tools.

Abstract

There is little information regarding pollutant levels in farmed fish, and the risks associated to consumption. This study was designed to assess levels of exposure to metals, organochlorinated compounds, polycyclic aromatic hydrocarbons (PAHs) and alkylphenols (APEs) in farmed sea bass *Dicentrarchus labrax* from five aquacultures located in Southern Europe. Additionally, several biochemical responses (metallothionein, 7-ethoxyresorufin *O*-deethylase, vitellogenin) were determined as complementary tools. The obtained data indicate that pollutants exposure in farmed fish is similar to the levels reported in wild specimens from the area. Nonetheless, some biochemical responses were observed in the studied organisms, viz. metallothionein induction in Cu exposed organisms, and 7-ethoxyresorufin *O*-deethylase (EROD) and vitellogenin induction in PAHs and APEs exposed ones. The study further supports the usefulness of the biomarker approach as a first screening method to discriminate between basal and high levels of exposure in cultured fish.

Keywords: Sea bass; Farmed fish; Pollution; Biochemical markers

1. Introduction

Aquaculture has been developed in the past decades as a consequence of the increase of fish consumption by the world population, since fisheries have possibly reached their maximum yield due to overexploitation (FAO, 2003). Marine fish may be cultured in raceways, ponds or net cages in open or coastal waters. However, current aquaculture practises raise environmental concerns, such as: (a) organic enrichment of surrounding waters by nutrients and solid wastes; (b) extensive use of chemicals (e.g. therapeutants, antibiotics, anti-foulants) with their subsequent release into the aquatic environment; (c) introduction of pathogens; and (d) introduction of new genetic strains of cultured fish (Seymour and Bergheim, 1991; Wu, 1995; Katranitsas et al., 2003; Matos et al., 2006). Despite of these problems, aquaculture is growing more than 10% per year (FAO, 2003), and a production of 47 million tons of aquaculture products, mainly fish, is estimated for the year 2010 (Dar, 1999).

Regarding the health and quality of cultured fish, it is worth asking whether the chemicals used in aquaculture practises, as well as the contaminants present in the surrounding waters, can have a negative impact on fish health and/or in the consumers. Hites et al. (2004) reported higher contaminant burdens (e.g. PCBs and DDTs) in European cultured salmon than in wild salmon, and suggested that consumption of farmed salmon could result in exposure to a variety of persistent bioaccumulative contaminants. Also, Antunes and Gil (2004) found that sea bass from natural environments showed

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lower PCB levels than cultured fish. However, despite of these reports, there is still rather limited information on the levels of pollutants to which cultured fish are exposed to, their bioaccumulation and the toxicological consequences.

Within this context, the present study was designed to integrate chemical analysis of selected contaminants -metals, organochlorinated compounds, polycyclic aromatic hydrocarbons (PAHs), alkylphenolic compounds (APEs)- with several biochemical markers (metallothioncins, cytochrome P450 system, vitellogenin), with the aim of investigating pollutant exposure of cultured fish collected from different aquacultures. Sea bass (*Dicentrarchus labrax* L.) was selected for the study because its culture has increased considerably in the last years, reaching high production and commercial value in European countries (FAO, 2003). Sea bass were collected from five aquaculture facilities located in Southern Portugal and Northeast of Spain. Metals were determined in liver and muscle tissue, while organochlorinated compounds were measured in muscle tissue and in the food -commercial pellets-. PAHs and APEs were determined in bile as a measure of recent exposure to these compounds (Escartín and Porte, 1999). The biochemical markers considered were: hepatic metallothionein content (MT), as a tool to monitor environmental exposure to metals (Viarengo et al., 1999); liver 7-ethoxyresorufin *O*-deetylase (EROD) activity, which has commonly been employed as a biomarker of exposure to dioxin-like compounds (Au and Wu, 2001; Porte et al., 2002); and plasma vitellogenin (Vtg) as a biomarker of exposure to estrogenic compounds (Hemmer et al., 2001). Histological examination of the gonads was performed in order to investigate the occurrence of abnormalities within this tissue.

2. Materials and methods

2.1. Sample collection and preparation

Sea bass (*Dicentrarchus labrax*) of marketable size were supplied by five aquaculture facilities between January and March 2005. Four of these facilities were located in Southern Portugal (SN, VM, AP, CM) and one in the Northeast of Spain (RC). SN and RC were respectively, semi-intensive and intensive production systems based on net cages in coastal Atlantic and Mediterranean waters. VM, AP and CM were semi-intensive production systems based on ponds. The names and the exact localization of the facilities are not given because of confidential policy. Furthermore, sea bass kept under controlled conditions in a laboratory facility (BC) were sampled for comparative purposes. Those fish were kept in 2500 L round aquaria under natural conditions of photoperiod (12:12), temperature (17 °C) and salinity (37‰) in aerated (90% dissolved O_2) and filtered sea water (pH 8.0) from the coast of Barcelona (NE Spain), set at a flow rate of 3 L/min. All fish were treated in agreement with the regulation of animal welfare (European convention for the protection of vertebrate animals used for experimental and other scientific purposes; ETS N° 123, 01/01/91).

Immediately after collection, total length and weight of the individuals was recorded, and blood (≈3 ml) was taken from the caudal vein with a heparinized syringe. The blood was transferred to heparinized tubes and, after centrifugation, plasma was frozen in liquid nitrogen. Individuals were then killed by a blow to the head and target tissues (liver, bile, muscle) immediately dissected, frozen in liquid nitrogen and stored at −80 °C upon arrival to the laboratory. A subsample from the central part of the gonad was fixed in 10% buffered formalin for histological examination.

2.2. Chemical analysis

2.2.1. Analysis of metals

Samples of liver and muscle (1 g) were homogenized in 1:5 w/v of cold 20 mM Tris–HCl buffer pH 8.6, containing 0.9% NaCl, 1 mM DTT and 0.2 mM PMSF in an ice bath using a polytron homogenizer. An aliquot of each homogenate (2 ml) was dried at 80 °C for 24 h, weighted and wet digested with concentrated HNO_3. Digested samples were evaporated to dryness before dissolution in 1 M HCl. Analysis of copper (Cu) and zinc (Zn) were carried out by flame atomic absorption spectrophotometry. Concentrations of cadmium (Cd) were determined by graphite furnace atomic absorption spectrophotometry, using standard addition methods. Standard reference material (cod muscle CRM 422) provided by the Community Bureau of Reference-BCR was analyzed to check the validity of the analyses. Results (1.10 ± 0.08 μg Cu/g; 19.80 ± 0.51 μg Zn/g; 0.02 ± 0.002 μg Cd/g; $n = 5$) were in good agreement with the certified values (1.05 ± 0.07 μg Cu/g, 19.60 ± 0.50 μg Zn/g and 0.02 ± 0.002 μg Cd/g). Metal concentrations are expressed on a dry weight basis.

2.2.2. Analysis of organochlorine compounds

Fish samples were pooled (5–6 individuals per site), homogenized, lyophilized and a subsample of 5 g of muscle tissue was Soxhlet-extracted with *n*-hexane:dichloromethane (4:1) for 18 h. Procedural blanks were also included. Commercial food pellets (subsample of 5 g) were extracted similarly. The solvent extract was evaporated to near dryness, the residue dissolved in 3 ml of *n*-hexane and cleaned up by vigorous shaking with 1–2 ml of conc. H_2SO_4, in order to remove lipids and other interfering substances. Further cleanup was based on solid-liquid adsorption chromatography: glass columns containing 5 g of alumina were eluted with *n*-hexane:dichloromethane (1:2). The recovery of the column was evaluated with a standard solution, being more than 80% for the analyzed compounds. The cleaned extract was concentrated by vacuum rotary evaporation, transferred to vials, evaporated to near dryness under a gentle stream of nitrogen, and analyzed by gas chromatography-electron capture detector (GC-ECD). The instrument was a Hewlett Packard 5890 GC. The column, a 50 m × 0.25 mm i.d. CP-Sil 5 CB fused silica (Chrompack, Middelburg, NL), was programmed from 80 to 180 °C at 15 °C/min and from 180 to 280 °C at 3 °C/min, keeping the final temperature for 15 min. The carrier gas was helium at a linear flow-rate of 50 cm/s. The injector and detector temperature was set at 280 and 300 °C, respectively. Quantitation was performed using an external standard calibration mixture of selected congeners (I.U.P.A.C. Nos.: 18, 31, 28, 52, 44, 149, 101, 118, 153, 138, 180, 170, 194, 209) supplied by Promochem (Wesel, Germany) and recommended by the International Council for the Exploration of the sea (ICES) for assessing marine pollution (Duinker et al., 1988). These congeners were quantified separately and the PCB concentration defined as its sum. Total DDTs, hexachlorobenzene (HCB) and lindane were determined and quantified using an external calibration curve. Concentrations are expressed as ng/g dry weight.

2.2.3. Analysis of hydroxylated-PAHs and alkylphenols (APEs) in bile

Bile samples were hydrolyzed by a modification of the method described in Escartín and Porte (1999). Briefly, 100 μl of bile were incubated for 1 h at 40 °C in the presence of 1 ml 0.4 M acetic acid/sodium acetate buffer pH 5.0, containing 2000 units of β-glucuronidase and 50 U of sulphatase. Hydrolyzed metabolites were extracted with 1 ml of ethyl acetate (×3), the extracts recombined and reduced under a nitrogen stream. Dry residues of bile metabolites were derivatized by the addition of 100 μl of bis-(trimethylsilyl)trifluoroacetamide (BSTFA), heated for 1 h at 70 °C, reduced under a nitrogen stream and analysed by gas chromatography-mass spectrometry electron impact mode (GC-MS-EI). The equipment was a Fisons GC 8000 Series chromatograph interfaced to a Fisons MD800 mass spectrometer. The column, a 30 m × 0.25 mm i.d. HP-5MS crosslinked 5% PH ME siloxane (Hewlett-Packard, USA) was programmed from 90 to 140 at 10 °C/min and from 140 to 300 at 4 °C/min. The carrier gas was Helium at 80 Kpa. The injector temperature was 250 °C and the ion source and the analyser were maintained at 200 °C and 250 °C, respectively. Just prior to sample analysis, calibration curves were performed with reference compounds and operating in selected ion monitoring mode (SIM). Hydroxylated-PAHs and alkylphenols were identified by

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comparison of retention times and spectra of reference compounds. The silylation derivative ions used for monitoring and quantification of OH-PAHs were: m/z 216 and 201 for 1-naphthol; m/z 254 and 165 for 9-fluorenol; m/z 266 and 251 for 9-phenanthrol; and m/z 290 for 1-pyrenol. The silylation derivative ions used for monitoring APEs were: m/z 207 and 193 for 4-nonylphenol (NP) and m/z 207 for 4-*tert*-octylphenol (OP). Concentrations are expressed as ng/g of bile.

2.3. Biochemical determinations

2.3.1. Metallothionein (MT)

Individual livers (1 g) were homogenized in 1:5 w/v of cold 20 mM Tris–HCl buffer pH 8.6, containing 0.9% NaCl, 1 mM DTT and 0.2 mM PMSF in an ice bath. An aliquot of each homogenate (3 ml) was centrifuged at 30,000 × *g* for 45 min. To partially purify MT for electrochemical quantification, the supernatant was decanted and heated in a water bath at 80 °C for 10 min in order to denature high molecular weight proteins. The resulting preparation was further centrifuged at 30,000 × *g* for 45 min, and the heat-treated supernatant, containing thermally stable MT, separated from precipitated proteins. MT was measured by differential pulse polarography (DPP), as described by Bebianno and Langston (1989), using a 646VA Processor autolab type II and an ECO Chemie IME663 mercury drop electrode. Comparisons of peak heights with those of standard additions of purified rabbit MT (Sigma) enabled the quantification of MT. Concentrations are expressed as μg/g dry weight of tissue.

2.3.2. Microsomal isolation

Hepatic microsomal fractions were prepared as described in Lavado et al. (2004). Briefly, after weighing, livers were flushed with ice-cold 1.15% KCl, and homogenized in 1:5 w/v of cold 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, containing 150 mM KCl and supplemented with 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM ethylenediaminetetraacetic acid (EDTA). Homogenates were centrifuged at 500 × *g* for 15 min, the fatty layer removed and the obtained supernatant centrifuged at 12,000 × *g* for 20 min. The resulting supernatant was further centrifuged at 100,000 × *g* for 60 min to obtain the microsomal fraction. Microsomal pellets were resuspended in a ratio of 0.5 ml buffer/g liver tissue in 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, containing 150 mM KCl, 20% w/v glycerol and supplemented with 1 mM DTT, 0.1 mM PMSF and 1 mM EDTA. Proteins were measured using Folin's reagent and bovine serum albumin (BSA) as standard (Lowry et al., 1951).

2.3.3. 7-Ethoxyresorufin O-deetylase (EROD)

The activity was assayed by incubating 100 μg of liver microsomal protein with 3.7 μM of 7-ethoxyresorufin and 225 μM of NADPH in 100 mM K_2HPO_4/KH_2PO_4 pH 7.4 (final volume 250 μl) at 30 °C for 10 min. The reaction was stopped by adding 400 μl of ice-cold acetonitrile (ACN) and after centrifugation (3000 rpm/10 min) an aliquot of the supernatant was transferred to a 96-multiwell plate. Fluorescence was read at the excitation/emission wavelengths pairs of 537/583 using a Gemini XPS SepctraMax Plus microplate reader (Molecular Devices Corporation). Quantification was performed by using a 7-hydroxyresorufin calibration curve and the activity calculated as the amount of 7-hydroxyresorufin (pmol) generated per milligram of protein per minute of reaction time.

2.3.4. Vitellogenin (Vtg)

Plasma levels of Vtg were quantified using a homologous enzyme-linked immunosorbent assay (ELISA) following the procedure described by Mañanós et al. (1994). Plasma samples were diluted 1:20 and analyzed by duplicate. The range of the standard curve was 0.78–400 ng/ml, corresponding to 94–20% of binding, respectively. The intra- and inter-assay coefficients of variation at 50% of binding were 5.3% ($n = 5$) and 9.8% ($n = 9$), respectively.

2.4. Histological analysis

Gonads were fixed in 10% buffered formalin for 24 h, dehydrated through a graded ethanol series and embedded in paraffin. Tissue sections (6 μm thick) were stained with hematoxylin-eosin.

2.5. Statistical analysis

Biochemical activities were determined individually in 5–6 male organisms per station and run per duplicate. Chemical analyses were conducted individually in 5–6 organisms (metals, PAHs, APEs) or in pools of 5–6 individuals (PCBs and organochlorinated pesticides) per station. Values are presented as means ± SEM. Transformation of the data was performed when the assumption of normality of residuals was not met. Statistical significance was assessed using one-way ANOVA with Tukey's test using the software package SPSS/PC™ version 12.0 (SPSS Inc., Chicago, IL); p values lower than 0.05 were considered statistically significant. Pearson's correlation coefficients were calculated and $p < 0.01$ was accepted as significant.

3. Results

3.1. Biological data of samples

Biological parameters of sampled fish are listed in Table 1. Individuals were all males and rather homogenous in size and weight, except for laboratory kept specimens (BC), which were smaller. The highest hepatosomatic index (HSI) was observed in specimens from CM (2.61 ± 0.06) and the lowest in individuals from RC (1.19 ± 0.16). When, looking at the condition factor (CF), individuals from CM and RC showed higher CF (1.24–1.28) than those form AP, SN and VM (1.00–1.06). No direct relationship between production system and CF was observed.

3.2. Chemical analysis

Metal concentrations in the liver and muscle tissue of cultured sea bass are shown in Fig. 1. The accumulation pattern of metals in the liver followed the order Cu > Zn > Cd; whereas in the muscle was Zn > Cu > Cd. In general, aquaculture specimens had significantly higher levels of hepatic Cu (4–10 fold) than those kept in the laboratory (BC); the highest residues of Cu detected in RC (828 ± 74 μg/g dry weight) (Fig. 1A). Also, hepatic Cd was high in sea bass from RC (1.03 ± 0.12 μg/g dry weight), SN (1.00 ± 0.24 μg/g dry weight) and AP (0.89 ± 0.11 μg/g dry weight) (Fig. 1B). In contrast, Zn residues in the liver were similar in fish from all sites (33–63 μg/g dry weight), with the exception of AP that had the highest values (111 ± 9 μg/g dry weight) (Fig. 1C).

Table 1
Biological data of male sea bass (*Dicentrarchus labrax*) collected from different aquaculture facilities: AP, SN, VM, CM (S Portugal); RC (NE Spain)

Sites	n	Weight (g)	Length (cm)	HSI (%)	CF (g/cm^3)
AP	6	421 ± 28^{c}	34.7 ± 0.5^{ab}	1.85 ± 0.17^{bc}	1.00 ± 0.03^{c}
SN	6	479 ± 32^{bc}	35.5 ± 0.4^{a}	1.73 ± 0.15^{bc}	1.06 ± 0.04^{bc}
VM	6	406 ± 27^{c}	33.6 ± 0.5^{b}	1.97 ± 0.20^{ab}	1.06 ± 0.03^{bc}
CM	6	609 ± 36^{a}	36.6 ± 1.1^{a}	2.61 ± 0.06^{a}	1.24 ± 0.06^{a}
RC	6	549 ± 26^{ab}	35.0 ± 0.7^{ab}	1.19 ± 0.16^{c}	1.28 ± 0.04^{a}
BC	5	195 ± 6^{d}	25.4 ± 0.2^{c}	1.42 ± 0.20^{bc}	1.19 ± 0.02^{ab}

BC: laboratory kept specimens. HSI: hepatosomatic index, calculated as (liver weight/body weight) × 100. CF: condition factor, calculated as [body weight/(length)^3] × 100. Values are mean ± SEM. Distinct letters indicate significant differences between sites according to Tukey's test ($p < 0.05$). n: number of organisms analyzed.

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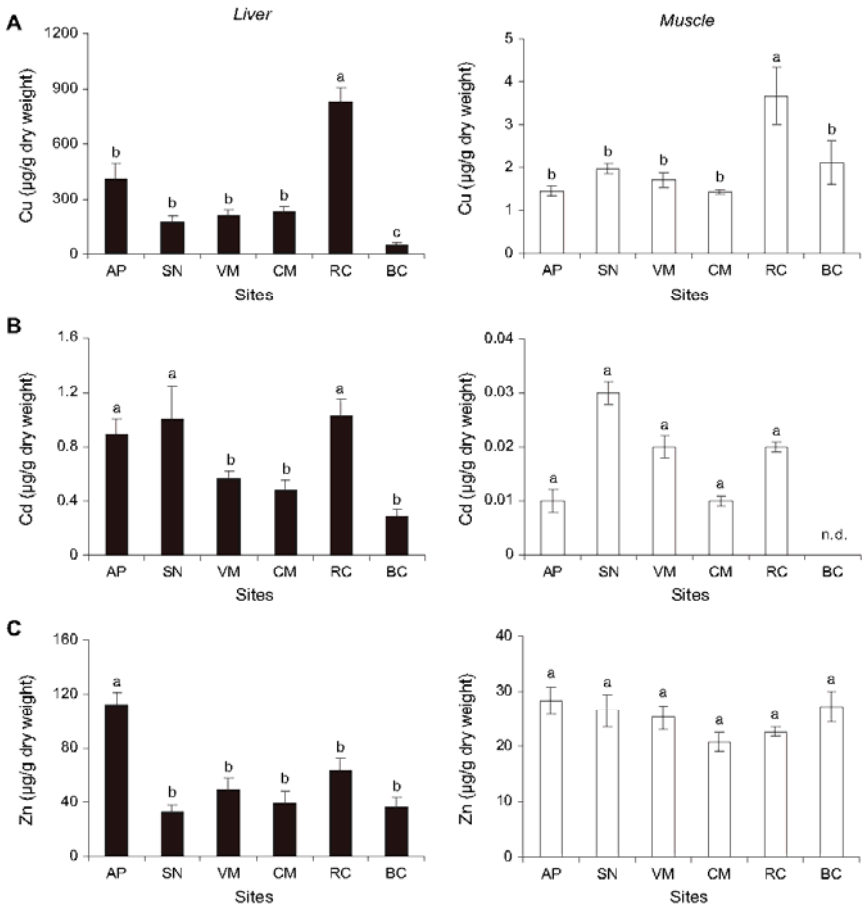

Fig. 1. Metal concentrations in the liver and muscle of male sea bass (*Dicentrarchus labrax*) from different aquaculture facilities: AP, SN, VM, CM (S Portugal); RC (NE Spain). BC: laboratory kept specimens. (A) Cu; (B) Cd; and (C) Zn. Values are expressed as mean ± SEM ($n = 5–6$). Distinct letters indicate significant differences between sites according to Tukey's test ($p < 0.05$). n.d.: below detection limit.

The concentration of metals in muscle tissue was significantly lower than in the liver, particularly Cu (1–4 µg/g in muscle *vs.* 100–900 µg/g in the liver) and Cd (n.d.–0.03 µg/g in muscle *vs.* 0.4–1.2 µg/g in the liver). No significant differences between aquacultures were observed regarding Zn (21–28 µg/g dry weight) and Cd (0.01–0.03 µg/g dry weight) residues in muscle. In agreement with liver data, significantly high concentrations of Cu were observed in the muscle of specimens from RC (3.67 ± 0.52 µg/g dry weight) (Fig. 1A).

The PCB load of sea bass was estimated on the basis of 14 PCB congeners selected from the GC-ECD profile. These congeners were quantified separately, and results are given as its sum (Table 2). The highest PCB residues in muscle were observed in sea bass from CM and SN, almost 2-fold higher than those from RC (7.0 ng/g dry weight). For DDTs, although the spatial pattern was similar to that exhibited by PCBs, significantly high residues were observed in laboratory kept specimens (BC: 8.6 ng/g dry weight), being 4–6-fold higher than the concentrations of DDTs recorded in sea bass from

Table 2
Organochlorine residues (ng/g dry weight) in the muscle tissue of sea bass (*Dicentrarchus labrax*) from different aquaculture facilities: AP, SN, VM, CM (S Portugal); RC (NE Spain)

Sites	Σ PCBs	Σ DDTs	*p,p'*-DDE/ΣDDTs	HCB	γ-HCH
AP	10.3	1.5	0.83	0.29	n.d.
SN	17.3	2.6	0.85	0.39	0.21
VM	13.1	2.0	0.88	0.59	0.25
CM	19.1	3.4	0.86	0.73	0.26
RC	7.0	1.4	0.80	n.d.	n.d.
BC	10.6	8.6	0.93	0.27	n.d.

BC: laboratory kept specimens. Data correspond to pools of 5–6 organisms analyzed in duplicate. n.d.: below detection limit.

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the different aquacultures, with exception of CM (3.4 ng/g dry weight) (Table 2). A higher contribution of p,p'-DDE to total DDTs was detected in BC. Measurable levels of HCB and lindane were not found in all the samples. The highest HCB residues were detected in fish from CM (0.73 ng/g dry weight) and VM (0.59 ng/g dry weight), whereas no differences among sites were observed for lindane residues (Table 2).

When all the identified PCB congeners (from tri- to octachlorobiphenyls) were considered, it was possible to observe further differences among sampling sites. Hexachlorobiphenyls represented 50–60% of the whole PCB mixture, followed by heptachlorobiphenyls (13–15%). The relative abundance of lower molecular weight isomers (tri- and tetrachlorinated) was higher in the Mediterranean (RC and BC) (14–16%) than in the Atlantic sites (5–9%), while heptachlorinated isomers were relatively less abundant at BC (8%) than in aquaculture specimens (13–15%). No significant differences between net cages (coastal) and ponds (inland) based facilities were observed in terms of organochlorine contamination.

As for the PCB load of commercial food pellets, results are given in Table 3. No significant differences between the different brands and types of food analyzed were observed in terms of concentration of PCBs and other organochlorinated compounds. Residues of PCBs in pellets were lower (8–10 fold) than those reported in muscle on a dry weight basis (Table 2). Nevertheless, the PCB congener distribution resembled that of the muscle tissue, with the most predominant congeners being the hexachlorobiphenyls (60–70%). Regarding DDTs, residues were in the same range as in fish muscle, with the exception of individuals from BC, which had higher residues. Some differences in the contribution of p,p'-DDE to total DDTs were observed between the analyzed pellets (p,p'-DDE/ΣDDTs: 0.40–0.81). Measurable levels of HCB were found in most food pellet samples, but lindane was only detected in 5 out of 10 analyzed samples (Table 3).

The concentrations of hydroxylated PAHs in fish bile are shown in Table 4. When looking at the sum of OH-PAHs, the highest concentration of PAHs was observed in the bile of laboratory kept specimens (BC: 567 ± 57 ng/g of bile), followed by aquaculture VM (419 ± 21 ng/g of bile). The lowest concentration of OH-PAHs was detected in specimens from RC (108 ± 22 ng/g of bile). Further differences among sampling sites were observed in terms of individual PAHs. Hence, 9-fluorenol was particularly abundant in specimens from VM (31% of total detected metabolites), whereas individuals from SN and BC were mostly enriched in 1-pyrenol (50–55% of total PAH metabolites). Fish from cage based aquacultures (SN and RC) indicated very low levels of 9-phenanthrol (2–4%), when compared to those cultured in ponds (19–27% of total PAH metabolites).

Table 3
Organochlorine residues (ng/g dry weight) in commercial food pellets

Diet pellet	Σ PCBs	Σ DDTs	p,p'-DDE/ΣDDTs	HCB	γ-HCH
Brand 1					
A3	1.4	1.5	0.43	0.18	n.d.
M2	1.9	2.5	0.40	0.19	0.09
Brand 2					
IM3	2.0	2.9	0.43	0.35	0.09
M3	1.9	1.9	0.52	0.17	n.d.
M5	2.1	1.8	0.70	0.22	0.07
M7	1.8	1.9	0.81	0.18	n.d.
A2	1.8	2.4	0.47	0.25	n.d.
O2	2.2	n.d.	–	n.d.	n.d.
Brand 3					
A22	2.3	2.4	0.56	0.26	0.07
S	1.5	2.0	0.40	0.47	0.16

Brand 1, 2 (IM3, A2) and 3 provided by Southern Portugal aquacultures. Brand 2 (M3, M5, M7, O2) used to feed the laboratory kept specimens and RC (NE Spain). n.d.: below detection limit.

Additionally, the analyses of bile samples by GC-MS allowed the identification of peaks corresponding to NP and OP (Table 4). NP concentrations are reported as the sum of 11 isomers, and their identification was shown unequivocally by the complete match of profiles between samples and the NP technical mixture. NP was above detection limit in all the samples analysed and showed significantly higher concentrations than OP. In general, the highest average concentration of NP (1554 ± 246 ng/g of bile) and OP (84 ± 26 ng/g of bile) was detected in the bile of laboratory kept specimens (BC). When considering the aquacultures, although NP (360–898 ng/g of bile) and OP (6–10 ng/g of bile) were present in the bile of all cultured specimens, no significant differences between sites were observed in terms of bile residues.

3.3. Biochemical responses

Cultured sea bass had higher concentrations of hepatic MT than those BC specimens (Fig. 2A); individuals from RC had the highest MT levels (3802 ± 598 μg/g dry weight). In fact, hepatic MTs were positively correlated with Cu residues in the liver ($r = 0.9870$, $p < 0.01$).

In contrast, higher hepatic EROD activity was detected in laboratory kept sea bass (BC: 105 ± 21 pmol/min per mg protein) in comparison with cultured fish which had EROD activities in the range of 30–50 pmol/min per mg protein, with the exception of fish from CM, where EROD was significantly lower (10 ± 2 pmol/min per mg protein) (Fig. 2B).

As for Vtg levels in plasma of male sea bass, laboratory kept specimens (BC: 100 μg/ml) had significantly higher concentrations (about 500-fold higher) than the aquaculture ones (Fig. 3). Vtg levels (0.2 μg/ml) exhibited by the cultured specimens, may correspond to low background levels of Vtg in male sea bass.

3.4. Histology

No abnormalities within gonadal tissue were observed with the exception of fish from RC (Mediterranean). The histological examination of the gonads of individuals from RC revealed a disorganization of the connective tissue caused by an endoparasitic infection. The infected gonads showed a great reduction in size compared with the non-infected ones, a hardening and several whitish or orange-yellowish spots. Histological analyses revealed an extremely thickened and in some

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Table 4
Biliary levels of hydroxylated PAHs and alkylphenols (ng/g of bile) detected in sea bass (*Dicentrarchus labrax*) from different aquaculture facilities: AP, SN, VM, CM (S Portugal); RC (NE Spain)

Sites	1-naphthol	9-fluorenol	9-phenanthrol	1-pyrenol	ΣOH-PAHs	NP	OP
AP	109 ± 14	72 ± 5	54 ± 9	79 ± 9	287 ± 24^{b}	360 ± 146^{b}	6 ± 2^{b}
SN	102 ± 14	74 ± 6	5 ± 1	155 ± 26	281 ± 41^{b}	675 ± 112^{b}	6 ± 0.3^{b}
VM	103 ± 14	128 ± 5	102 ± 20	119 ± 22	419 ± 21^{ab}	898 ± 94^{b}	9 ± 1^{b}
CM	97 ± 11	99 ± 13	95 ± 16	114 ± 9	355 ± 42^{b}	614 ± 103^{b}	8 ± 2^{b}
RC	78 ± 11	37 ± 14	5 ± 1	5 ± 1	108 ± 22^{c}	446 ± 136^{b}	10 ± 3^{b}
BC	129 ± 19	96 ± 9	117 ± 10	283 ± 47	567 ± 57^{a}	1554 ± 246^{a}	84 ± 26^{a}

BC: laboratory kept specimens. NP: nonylphenol; OP: octylphenol. Values are mean ± SEM ($n = 5–6$). Distinct letters indicate significant differences between sites according to Tukey's test ($p < 0.05$).

cases hyalinised connective tissue, with abundance of macrophage aggregates and necrotic granulomatous areas around the infected seminiferous tubules (Fig. 4). Histopathological analyses (conducted by Dr. Sitjà-Bobadilla, IATS-CSIC) allowed the identification of the parasite as *Sphaerospora testicularis* (Myxosporea: Sphaerosporidae). The mean prevalence of the infection was of 50%.

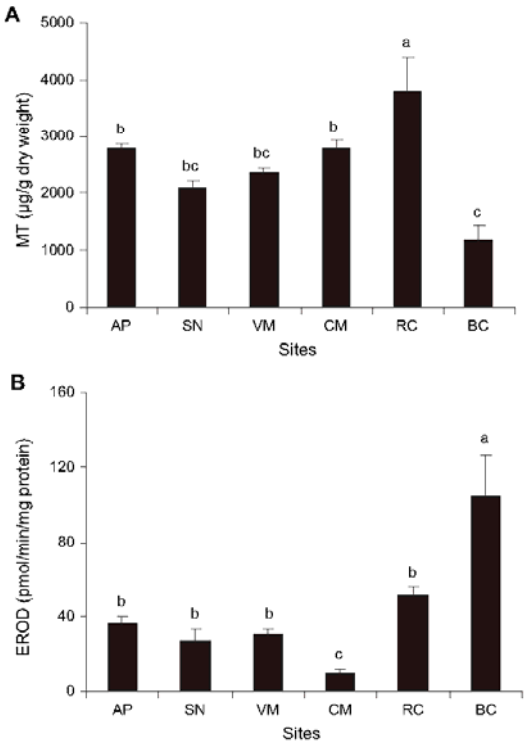

Fig. 2. (A) MT concentrations; and (B) EROD activity in the liver of male sea bass (*Dicentrarchus labrax*) from different aquaculture facilities: AP, SN, VM, CM (S Portugal); RC (NE Spain). BC: laboratory kept specimens. Values are expressed as mean ± SEM ($n = 5–6$). Distinct letters indicate significant differences between sites according to Tukey's test ($p < 0.05$).

4. Discussion

Assessing pollutant levels in cultured sea bass tissues (mainly muscle) is an urgent need from the environmental, nutritional and toxicological point of view. In this study, some contaminants (metals, organochlorinated compounds) were measured in the muscle tissue to define food quality for human consumption, but also in the liver (metals), as the main accumulating organ (Mai et al., 2006).

The observed metal concentrations in the muscle of cultured sea bass were all below the recommended FAO maximum limits for commercial fish species (Cd: 0.5 mg/kg; Cu: 30 mg/kg, and Zn: 30 mg/kg) (FAO, 1983). Nonetheless, cultured sea bass caged in the Mediterranean coast (RC) indicated high concentrations of Cu, both in the liver (828 ± 74 µg/g d.w.) and in the muscle tissue (3.67 ± 0.52 µg/g d.w.) when compared to other sites. Although, essential metals (Cu & Zn) are present in pellet feed, the high concentrations of Cu detected in specimens from RC suggest an additional exposure to Cu in the aquaculture facility; the main source of this contamination being unknown.

Cu contamination in the aquatic environment has been of increasing concern in the last ten years (Katranitsas et al., 2003; Konstantinou and Albanis, 2004; Kwok and Leung, 2005). In the aquaculture industry, antifouling paints are used to treat cage netting in order to prevent the attachment and growth of fouling organisms (Katranitsas et al., 2003); copper-based antifouling paints have regained increased attention in replacement of tributyltin-based paints that were found to have severe impact on the aquatic ecosystem (Fent, 2003). Alternatively, Cu is used as a therapeutant (Schlenk et al., 1999). In a previous study, high concentrations of hepatic Cu (up to 1000 µg/g d.w.) were detected in cultured sea bass exposed to $CuSO_4$ to treat an ectoparasitic infection of *Amyloodinium ocellatum* (Fernandes et al., 2007). Despite of the use of Cu as a therapeutant, several studies have shown Cu to be immunosuppressive in fish (Zelikoff, 1993; Schlenk et al., 1999), and to act through a general stress response increasing the production of cortisol (Carballo et al., 1995). Interestingly, individuals from RC showed an infection by the myxosporean parasite *Sphaerospora testicularis*, which destroys testicular tissue and reduces the fecundity of males (Sitjà-Bobadilla and Alvarez-Pellitero, 1990). The same infection was previously detected in gonads of both cultured and

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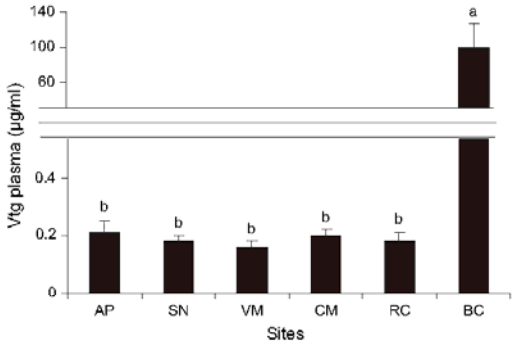

Fig. 3. Plasma vitellogenin levels in male sea bass (*Dicentrarchus labrax*) from different aquaculture facilities: AP, SN, VM, CM (S Portugal); RC (NE Spain). BC: laboratory kept specimens. Values are expressed as mean ± SEM ($n = 5–6$). Distinct letters indicate significant differences between sites according to Tukey's test ($p < 0.05$).

wild sea bass showing high Cu residues in their livers (Fernandes et al., 2007). Indeed, further studies are needed in order to determine whether therapeutic treatment of cultured fish with $CuSO_4$ and/or exposure to antifouling paints may impede resistance to parasites or diseases.

Regarding the use of MT as a biomarker to assess metal exposure in cultured fish, hepatic MT were significantly high in specimens from RC that showed the highest Cu residues in the liver. The obtained results support the use of MT as a biomarker of exposure to relatively high concentrations of Cu.

Considering levels of organochlorine compounds in the muscle tissue of cultured sea bass, the highest concentrations (PCBs, DDTs, HCB) were reported in specimens from CM (Table 2). This aquaculture drains water from an important river located in the South of Portugal that is highly impacted by anthropogenic activities (e.g. urban and industrial effluents). The highest concentration of DDTs was reported in sea bass from BC (8.6 ng/g d.w.) and this was mainly due to the presence of *p,p′*-DDE. Nonetheless, DDT residues in fish from BC were far from exceeding the threshold of 14 ng/g wet weight for the protection of wildlife consumers of aquatic biota (Canadian Environmental Quality Guidelines, 2003). The most predominant PCB congeners in muscle tissue corresponded to PCB 138 and 153, which agrees with most of the authors' findings in aquatic organisms (Fernandes et al., 2002; Serrano et al., 2003; Lavado et al., 2006). Nevertheless, significant differences were found among samples, with cultured sea bass from Portugal exhibiting higher levels of hexa- and heptachlorobiphenyls than those from Spain.

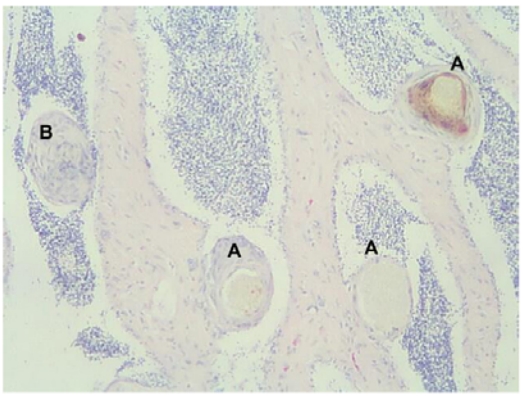

Fig. 4. Testicular tissue of cultured sea bass (*Dicentrarchus labrax*) from site RC, showing an infection of the seminiferous tubules caused by a parasitic infestation. (A) granulomata areas around the infected seminiferous tubules; and (B) aggregate of macrophages (magnification 100×).

Antunes and Gil (2004) reported higher levels of PCBs (26–36 ng/g d.w.) and DDTs (22–39 ng/g d.w.) in cultured sea bass than in wild specimens from Northern Portugal. However, in the present study levels of PCBs (7.0–19 ng/g d.w.) and DDTs (1.4–8.6 ng/g d.w.) were in the same range as those detected in wild sea bass from Northern Portugal (Antunes and Gil, 2004). Commercial pellets have been suggested to be a major source of PCBs and other organochlorine compounds in cultured specimens, this together with the higher lipid content of cultured in comparison to wild specimens can lead to a rather high bioaccumulation of lipophilic persistent pollutants in the former (Easton et al., 2002; Antunes and Gil, 2004; Hites et al., 2004). However, no significant differences in OCs residues between pellet feed from different aquaculture facilities was observed in the present study, suggesting that water (aquaculture location) does account as a source of contaminants for the analyzed specimens. Thus, the high *p,p′*-DDE, OH-PAHs and APEs residues in BC (laboratory kept specimens) might be attributed to specific inputs of those compounds in the coastal water of Barcelona (Escartín and Porte, 1999; Porte et al., 2002); similarly, the relatively high PCBs residues in fish from CM might mirror the water quality of this specific aquaculture rather than a specific input of PCBs through contaminated food.

Aromatic hydrocarbon exposure in marine organisms can be assessed by measuring the concentrations of PAHs in their tissues (Baumard et al., 1998; Vives and Grimalt, 2002). However, fish often show only trace levels of PAHs due to their ability to metabolize these compounds (Meador et al., 1995). An alternative technique for assessing PAH exposure is the determination of PAHs excreted through the bile as conjugated metabolites (Fernandes et al., 2002). Laboratory and field studies have both demonstrated that the presence of PAH metabolites in bile is well related with levels of exposure (Yu et al., 1995; Escartín and Porte, 1999). The observed levels of hydroxylated PAHs (567 ± 57 ng/g of bile) in bile of sea bass from site BC indicate higher exposure of these individuals to PAHs than in aquaculture fish. Further differences among sampling sites were observed in terms of individual hydroxylated-PAH (Table 4). Hence, 1-pyrenol was particularly abundant in the bile of sea bass from sites BC and SN (Atlantic coast); pyrene is produced by many pyrolitic and petrogenic processes and it is regarded as the best general indicator of PAH exposure in fish (Van der Oost et al., 1994; Ruddock et al., 2002). Interestingly, considerable levels of 9-phenanthrol were detected in the bile of fish from inland

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facilities (AP, CM, VM), whereas 9-phenanthrol was almost not detectable in fish from coastal aquacultures (SN and RC). Factors such as the feeding status of the organisms can certainly affect levels of bile metabolites. Bile accumulation in non-feeding fish may lead to relatively high metabolites as compared to feeding fish (Brumley et al., 1998; Aas et al., 2000), thus in this study all individuals were starved before sampling, being therefore all in the same conditions.

In agreement with PAHs exposure data, laboratory kept specimens (BC) reported significantly high EROD activity (105 pmol/min per mg protein) when compared to cultured specimens, indicating higher exposure to CYP1A-inducing agents. Interestingly, individuals from CM had significantly low EROD activity despite of the high PCBs residues in their tissue. A large number of field studies have shown a positive correlation between piscine EROD activity and environmental levels of CYP1A inducing chemicals such as PAHs or PCBs. (Whyte et al., 2000; Van der Oost et al., 2003). However, such positive correlation can not always be expected since in the natural environment, fish are exposed to complex mixtures of contaminants that can act both as inducers and inhibitors of CYP1A (Burton et al., 2002).

Additionally, concentrations of NP and OP were recorded in the bile of cultured specimens indicating recent exposure to alkylphenolic compounds (APEs). APEs are present in paints, herbicides, pesticides and other formulated products, but mainly in detergents. APEs are the most likely estrogenic substances in domestic sewage and have been recognized as endocrine disrupter chemicals (EDCs) in fish (Purdom et al., 1994; Jobling et al., 1996). In fact, APEs have been show to bind to the estrogen receptor and to induce the synthesis of Vtg in fish (Jobling and Sumpter, 1993). In this study, the highest levels of NP and OP were detected in sea bass from site BC, 5–10-fold higher than in the other sites (Table 4). Nonetheless, these levels were in the low range of those reported for freshwater fish from the Ebro River (Lavado et al., 2006). The relatively lower abundance of OP compared to NP has already been reported in other studies, and attributed to its lower commercial use (Lye et al., 1999; Lavado et al., 2006).

The detection of high concentrations of Vtg circulating in the plasma of male individuals from BC strongly suggests the presence of estrogenic compounds in the water of the laboratory facility. Low background levels of Vtg are likely to be normal in males (Gross et al., 2003) and only significantly increases above background levels, as observed in specimens from BC, are indicative of estrogenic exposure. Despite the high levels of Vtg circulating in the plasma of BC individuals, no gonadal alteration or case of intersex was observed.

Overall, results indicate an influence of aquaculture location on pollutant exposure and tissue residues in cultured fish. This was particularly evident when considering the laboratory kept specimens (BC), which were fed with the same commercial pellet as the others but had significantly high concentrations of DDTs in their tissues, and high levels of OH-PAHs and APEs in bile, which is possibly a reflection of the poor water quality of the city of Barcelona, highly impacted by industrial and anthropogenic activities.

Regarding pollutant residues in cultured sea bass, it should be mentioned that the detected concentrations of organochlorine compounds and metals in muscle were lower than international standards and FAO/WHO recommended values for commercial species. Thus, consumption of cultured sea bass from the studied areas does not pose potential risks for human health. Nonetheless, some biochemical responses were observed; namely induction of MT in RC specimens exposed to Cu; induction of EROD activity and Vtg in BC individuals exposed to PAHs and APEs. Thus, in view of the increasing availability of cultured sea bass in the market and the increasing consumption rates among European residents, this study points the need for developing methodologies that allow the continuous monitoring of cultured fish quality and the development of consistent consumption advice guidelines for the protection of public health. In this regard, the use of biochemical responses as a first screening tool to assess significant exposure to certain chemicals offers a great potential and has proved to be useful in the present study.

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Chapter 3. The Use of Wild Fish for the Assessment of Pollution along the Northern Iberian Shelf

3.1.

HEPATIC LEVELS OF METAL AND METALLOTHIONEINS IN TWO COMMERCIAL FISH SPECIES OF THE NORTHERN IBERIAN SHELF

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Resumo

Concentrações de metais (Cd, Pb, Hg, Cr, Fe, Cu, Zn) em conjunto com metalotioneínas (MTs) foram determinadas no fígado de duas espécies de peixe comercial, recolhidos ao longo da costa Norte da Península Ibérica. O areiro-de-quatro-manchas (*Lepidorhumbos boscii*) e a faneca (*Trisopterus luscus*) foram seleccionados como espécies representativas da plataforma media/exterior (200-500m) e interior (70-120m), respectivamente. A contaminação metálica na plataforma media/exterior foi detectada na maioria das áreas das Asturias e do País Basco, enquanto que na plataforma interior, esta se mostrou disseminada ao longo da costa. Concentrações significativamente elevadas de Pb, Cd, Cr, e Hg foram detectados em *T. luscus* amostrados em diferentes locais e associados à pressão antropogénica. Os peixes com maiores níveis hepáticos de Zn, Cd, Cr e Hg mostraram indução de MT, apoiando o uso de MT como biomarcador de exposição a metais. Este estudo confirma a utilidade das espécies de peixe seleccionadas como organismos sentinela para futuros estudos de biomonitorização.

Palavras-chave: metalotioneína, metais, fígado, *Lepidorhumbos boscii*, *Trisopterus luscus*, plataforma Nórdica da Península Ibérica

Abstract

Metal levels (Cd, Pb, Hg, Cr, Fe, Cu, Zn) together with metallothioneins (MTs) were determined in the liver of two commercial fish species collected along the Northern Iberian coast. The four-spotted megrim (*Lepidorhumbos boscii*) and the pouting (*Trisopterus luscus*) were selected as representative species of the middle/outer (200-500m) and inner shelf (70-120m), respectively. Metal pollution in the middle/outer shelf was mostly detected in the Asturias and Basque Country areas, whereas in the inner shelf, pollution was widely spread along the coast. Significantly high levels of Pb, Cd, Cr and Hg were detected in *T. luscus* from different sampling sites and associated to anthropogenic pressure. MTs were induced in fish that had higher amounts of Zn, Cd, Cr and Hg in their livers, supporting the use of MT as a biomarker of metal exposure. The study supports the usefulness of the selected fish species as sentinel organisms for future biomonitoring studies.

Keywords: Metallothionein; metals; liver; *Lepidorhombus boscii*; *Trisopterus luscus*; Northern Iberian shelf

1. Introduction

The contamination of water resources, biota, sediments and soils by metals is of major concern especially in many industrialized countries because of their toxicity, persistence and bioaccumulative nature (Ikem *et al*., 2003). Many metals occur naturally in marine environments and are classified as pollutants only when added by anthropogenic sources (e.g. industries, agriculture, mining, harbour activities, dumping, oil spills) in sufficient amounts to produce deleterious effects on the ecological system (Prego and Cobelo-García, 2003; Dural *et al*., 2006).

Different fish species are used as biomonitors to assess the bioavailability of metals in the marine environment. Among them, marine benthic fish have been shown to reflect environmental concentration of metals (Roméo *et al*., 1999; Çoğun *et al*., 2006). Fish require essential metals (e.g. Cu, Zn) as micronutrients for endogenous metabolism, which are taken up from the surrounding medium (e.g. water and sediment) or from the diet (Clearwater *et al*., 2002; Filipović and Raspor, 2003). Non-essential metals are similarly taken up and accumulated in fish tissues (Filipović and Raspor, 2003) including mercury (Hg), lead (Pb) and cadmium (Cd) which are considered the most dangerous metals in the aquatic environment (Neff, 2002). However, essential metals, such as copper (Cu) and zinc (Zn), have also shown toxicity when their intake was excessively high (Suresh *et al*., 1992; Pipe *et al*., 1999).

The bioaccumulation of metals depends on their bioavailability and on biotic and abiotic factors such as water temperature, age, size, and feeding habits of the organisms (Al-Yousuf *et al*., 2000; Çoğun *et al*., 2006). Being indestructible in biological tissues, metals when incorporated into the organism have their toxicological effects regulated by

two general mechanisms: the binding to specific ligands and excretion (Oliveira *et al*., 2004). Generally, the liver is a good indicator of chronic exposure to metals as it plays a key role in the storage and inactivation of metals (Miller *et al*., 1992).

Metallothioneins (MTs) are an inducible family of metal binding proteins; they are low molecular weight cysteine-rich proteins that play an important role in the transport and storage of essential metals, but also provide protection against the toxic effect of metals, whether essential or non-essential, by binding free metal ions and making them less available for interaction with sensitive biomolecules (Langston *et al*., 2002; Van der Oost *et al*., 2003). So, induced MTs reduce the toxic effects caused by metals and are the first detectable sign of metal exposure at the cellular level (Filipović and Raspor, 2003). Therefore, MTs are often used in biomonitoring programs as biomarkers. Tissue distribution of MTs in fish primarily includes liver, gills, kidney and intestine (Hogstrand and Haux, 1991; Hamza-Chaffai *et al*., 1995), but their value as biomarkers of metal exposure varies with species, reproductive condition, sex, age and diet, so these factors must be taken into consideration when using MTs as biomarkers (Langston *et al*., 2002).

The Northern Iberian coast is an area with a great socioeconomic importance, where the co-existence of industrial activities, agriculture, mining, aquaculture, boat traffic, harbours, tourism, and fisheries makes the area vulnerable to anthropogenic pollutants (Franco *et al*., 2002; Prego and Cobelo-Garcia, 2003). Additionally, the *Prestige* oil spill occured in the area in November 2002 (Sánchez *et al*., 2006), the fuel contained among other components several trace metals (e.g. Cu, Pb, Cd and Zn) at a concentration of 0.21 to 3.39 $\mu g.g^{-1}$ (Prego and Cobelo-Garcia, 2004; Morales-Casellas *et al*., 2006). Therefore, considering the high productivity of Galician waters, the

importance of fisheries along the North Iberian coast, and the existence of potential land-ocean inputs of metals which bioavailability and impact have not been previously characterized, this work aimed at assessing metal levels along the Northern Iberian shelf by measuring liver residues and liver metallothioneins (MTs), as a biomarker of metal exposure, in two commercial fish species: the four-spotted megrim (*Lepidorhombus boscii*) and the pouting (*Trisopterus luscus*). The selection of these species was based on their wide distribution, territorial behaviour and relative abundance in catches. *L. boscii* dwells on muddy bottoms on the middle and outer shelf at maximum depths of 800 m (Pietrapiana *et al*., 2002), while *T. luscus* is a benthopelagic fish which lives mostly on the inner shelf and moves inshore to depths of less than 50 m for spawning (França *et al*., 2004). The simultaneous use of both species will allow obtaining information on metal bioavailability/exposure in different shelf environments: 70-120 m depth (*T. luscus*) and 200-500 m depth (*L. boscii*).

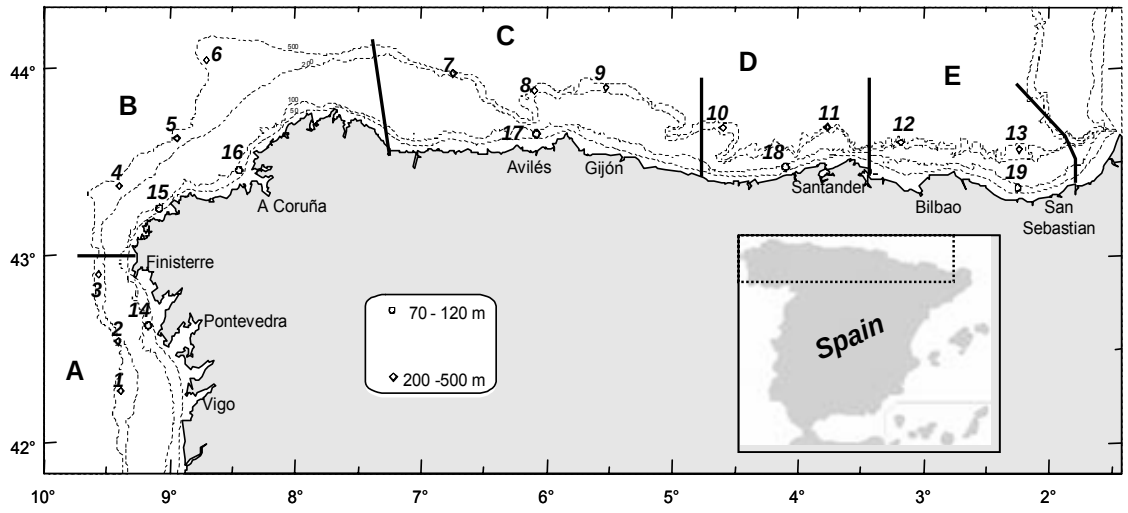


Figure 1. Map of the sampling sites. (A) S Galicia; (B) N Galicia; (C) Asturias; (D) Cantabria; (E) Basque Country. Depth ranges of sampled fish: 70-120 m (*Trisopterus luscus*); 200-500 m (*Lepidorhombus boscii*).

2. Material and Methods

2.1. Sampling procedure

Lepidorhombus boscii and *Trisopterus luscus* were collected from 19 stations along the Northern Iberian shelf by 30 minute bottom trawls (baca 44/60 otter trawl gear) at depth ranges of 70-120 m for *T. luscus* and 200-500 m for *L. boscii*. Sampling was carried out in October 2003, coinciding with the prespawning period of both species. Stations were fixed along 15 transects drawn from coast to open sea. For the scope of this study, the continental shelf was divided into five geographical areas: (A) S Galicia; (B) N Galicia; (C) Asturias; (D) Cantabria; (E) Basque Country (Figure 1). Immediately after collection, fish were sacrificed by severing their spinal cord, sexed and weighed; their length was measured, the liver dissected, immediately frozen in liquid nitrogen, and stored at – 80ºC upon arrival to the laboratory.

2.2. Analysis of metals

Liver samples were weighed and homogenized in 1:5 w/v of cold 20 mM Tris-HCl buffer pH 8.6, containing 0.9% NaCl, 1 mM DTT and 0.2 mM PMSF in an ice bath using a polytron homogenizer. An aliquot of each homogenate (2 ml) was dried at 80ºC for 24 h, weighted and wet digested with concentrated HNO_3. Digested samples were evaporated to dryness before dissolution in 1 M HCl. Quantitative determinations of Cd, Cu, Zn, Cr, Pb, Fe were carried out by flame or graphite furnace atomic absorption spectrometer (Analyst 800 P.E.), while total Hg was determined by cold-vapour atomic

absorption spectrometry (CVAAS) after reduction with $NaBH_4$ (FIAS-Furnace P.E.). The standard addition method was used to correct for matrix effects. The instrument was calibrated with standard solutions prepared from commercial materials. Analytical blanks (n = 3) were run in the same way as the samples and determined using standard solutions prepared in the same acid matrix. Precision was checked against standard reference material (cod muscle CRM 422) provided by the Community Bureau of Reference-BCR, and was within the range of certified values. Recovery of all metals was over 90%.

2.3. Metallothionein (MT) analysis

Approximately 1 g of individual livers were homogenized in 1:5 w/v of cold 20 mM Tris-HCl buffer pH 8.6, containing 0.9% NaCl, 1 mM DTT and 0.2 mM PMSF in a ice bath using a polytron homogenizer. An aliquot of each homogenate (3 ml) was centrifuged at 30,000 *g*, 4ºC for 45 min and the obtained supernatant was heat denatured at 80ºC for 10 min in order to denature high molecular weight proteins. The resulting preparation was further centrifuged at 30,000 *g* for 45 min at 4ºC, and the heat-treated cytosol, containing thermally stable MT, separated from precipitated proteins. MT was measured in the partially purified (heat-treated) supernatant, using differential pulse polarography (DPP), as described by Bebianno and Langston (1989). This method takes advantage of two features of MT: firstly, the stability of MT at high temperatures enables other potentially interfering proteins to be removed by heat denaturation; secondly, the characteristically high cysteine (– SH) content, typical of MT, and its

special metal complexing configuration is used as the basis of determining concentrations of the metal-binding protein by DPP.

An aliquot of the heat-treated supernatant (10 µl), together with 250 µl Triton-X (0.025% v/v) were added to 20 ml hexaminocobalt chloride buffer (the supporting electrolyte), in a polarographic cell. The cell was purged for 2 min with purified N_2 prior to analysis. The polarographic response of the target species MT was measured during a potential scan between -1.4 and -1.7 V (646VA Processor autolab type II and an ECO Chemie IME663 mercury drop electrode). Comparisons of peak heights with those of standard additions of purified rabbit liver metallothionein (Sigma) enabled the quantification of MT. Results are expressed as $\mu g.g^{-1}$ dry weight of tissue.

2.4. Statistical procedure

Analyses were conducted individually in three to six organisms of each species per station. Results are presented as means ± SEM. Statistical significance was assessed using one-way ANOVA with Tukey's test using the software package SPSS/PC^{TM} version 12.0 (SPSS Inc., Chicago, IL). *P*-values of less than 0.05 were considered as statistically significant. Pearson's correlation coefficients were calculated among selected parameters in order to measure the strength of association between variables. Data was log-transformed prior to statistical analysis when the assumption of normality of residuals was not met.

3. Results

3.1. Biological data of samples

The number of analyzed individuals and main morphometric parameters are presented in Table 1. Fish were rather homogeneous in body weight and length and no significant differences were observed among sampling sites. However, there were

Table 1. Morphometric data of *Lepidorhombus boscii* and *Trisopterus luscus* from the collection sites. Values are mean ± SEM. HSI: hepatosomatic index, calculated as (liver weight / body weight) x 100. CF: condition factor, calculated as [body weight / (length)^3] x 100. n: number of individuals analyzed. Distinct letters indicate significant differences between sites according to Tukey's test (p<0.05).

Area	Site	n	Weight (g)	Length (cm)	HSI (%)	CF (g.cm^{-3})
L. boscii						
A	1	6	104 ± 18^a	25 ± 2.0^a	1.53 ± 0.23^b	0.66 ± 0.03^a
	2	6	131 ± 4.0^a	25 ± 0.3^a	2.47 ± 0.27^{ab}	0.81 ± 0.03^a
	3	6	124 ± 10^a	25 ± 1.0^a	2.29 ± 0.19^{ab}	0.76 ± 0.03^a
B	4	4	128 ± 24^a	26 ± 1.0^a	2.04 ± 0.13^{ab}	0.75 ± 0.02^a
	5	5	114 ± 19^a	24 ± 2.0^a	2.80 ± 0.30^a	0.87 ± 0.14^a
	6	6	167 ± 21^a	28 ± 1.0^a	2.30 ± 0.15^{ab}	0.76 ± 0.03^a
C	7	6	128 ± 5.0^a	26 ± 0.4^a	1.90 ± 0.25^{ab}	0.77 ± 0.03^a
	8	6	194 ± 37^a	30 ± 2.0^a	2.44 ± 0.19^{ab}	0.71 ± 0.05^a
	9	6	105 ± 6.0^a	24 ± 0.5^a	1.74 ± 0.21^{ab}	0.72 ± 0.01^a
D	10	6	120 ± 16^a	25 ± 1.0^a	1.85 ± 0.17^{ab}	0.78 ± 0.01^a
	11	3	128 ± 14^a	26 ± 1.0^a	2.06 ± 0.23^{ab}	0.74 ± 0.01^a
E	12	6	163 ± 31^a	26 ± 1.0^a	2.35 ± 0.18^{ab}	0.83 ± 0.03^a
	13	6	157 ± 24^a	27 ± 1.0^a	1.71 ± 0.16^b	0.76 ± 0.02^a
T. luscus						
A	14	6	131 ± 9^a	23 ± 1.0^a	4.35 ± 0.50^a	1.05 ± 0.03^b
B	15	6	188 ± 12^a	26 ± 0.4^a	3.05 ± 0.32^{ab}	1.07 ± 0.03^b
	16	6	128 ± 17^a	21 ± 0.5^a	1.69 ± 0.19^b	1.33 ± 0.09^a
C	17	6	146 ± 11^a	24 ± 1.0^a	2.88 ± 0.57^{ab}	1.01 ± 0.02^b
D	18	3	121 ± 42^a	23 ± 2.0^a	2.07 ± 0.28^b	0.95 ± 0.10^b
E	19	6	174 ± 13^a	26 ± 1.0^a	3.35 ± 0.19^{ab}	1.03 ± 0.03^b

significant differences in the hepatosomatic index (HSI) among sampling sites and these differences were species-specific. For *L. boscii*, the lowest HSI was detected in sites A1 (1.53 ± 0.23) and E13 (1.71 ± 0.16), and the highest in B5 (2.80 ± 0.30) ($P<0.05$). Differences among sampling sites were more evident in *T. luscus*; HSI ranged from 1.69-2.07% in sites B16 and D18 to 4.35% in A1 ($P<0.05$). As for the condition factor (CF), no differences between sites were observed for *L. boscii* ($P>0.05$), while, *T. luscus* from site B16 (1.33 ± 0.09) had significantly higher CF than specimens from the other sites ($P<0.05$).

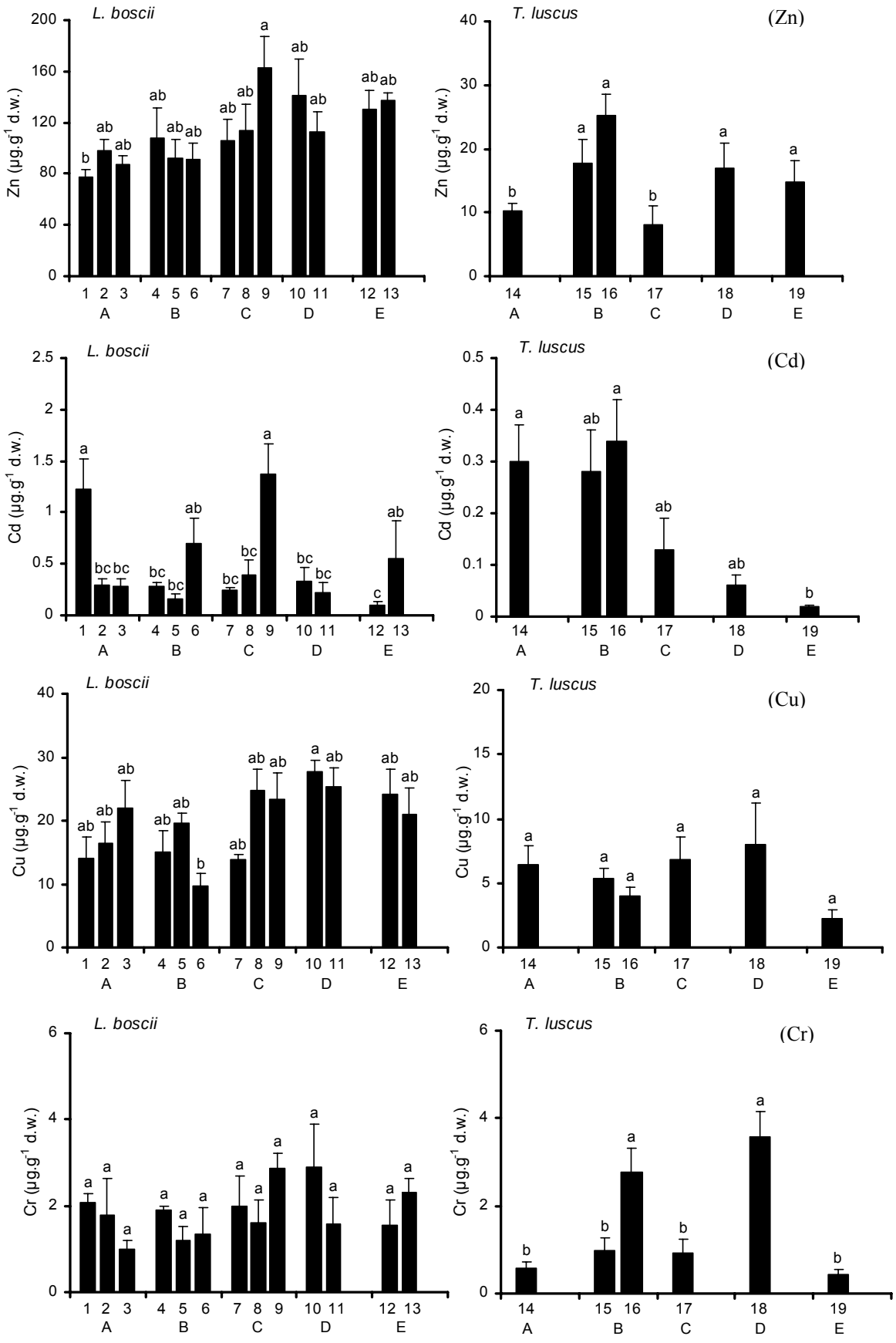
3.2. Metal bioaccumulation in the liver

Measured metal concentrations in the liver are given in Figure 2. Metal content was significantly higher in the liver of *L. boscii* (up to 10-fold), with the exception of the high levels of Cr and Pb exhibited by *T. luscus* in some of the sampled sites. Both species indicated a similar pattern in the accumulation of essential metals (Fe>Zn>Cu>Cr). For the non-essential metals different patterns of bioaccumulation were observed for *L. boscii* (Cd>Hg>Pb) and *T. luscus* (Cd>Pb>Hg).

Regarding *L. boscii*, hepatic levels of Zn and Cu were lower in the Galician area, especially in sites A1 for Zn (77.8 ± 5.6 $\mu g.g^{-1}$ d.w.), and B6 for Cu (9.8 ± 1.8 $\mu g.g^{-1}$ d.w.) (Fig. 2). The highest levels of Zn and Cu were shown in sites C9 (162.8 ± 24.5 $\mu g.g^{-1}$ d.w.) and D10 (27.7 ± 1.91 $\mu g.g^{-1}$ d.w.), respectively. As for Cd residues, values were uneven among the sampling sites, with specimens from sites A1 and C9 showing significantly higher levels than those from the others sites (1.22 ± 0.3 $\mu g.g^{-1}$ d.w. and 1.37 ± 0.3 $\mu g.g^{-1}$ d.w., respectively; Fig. 2). High residues of Hg were observed in the

liver of individuals from sites C9 (0.399 ± 0.11 μg.g^{-1} d.w.), D11 (0.422 ± 0.15 μg.g^{-1} d.w.) and E13 (0.403 ± 0.09 μg.g^{-1} d.w.), while those from South Galicia (0.03 μg.g^{-1} d.w.) and site C8 (0.011 ± 0.004 μg.g^{-1} d.w.) reported significantly lower concentrations. Meanwhile, no significant differences between sites were observed for Cr (1.0–2.9 μg.g^{-1} d.w.), Pb (0.002–0.011 μg.g^{-1} d.w.) and Fe (101.4–228.5 μg.g^{-1} d.w.) (Fig. 2).

Metal residues in liver of *T. luscus* collected from the inner shelf differ from those observed for *L. boscii* (Fig. 2). Significantly lower levels of hepatic Zn were observed in *T. luscus* from sites A14 (10.2 ± 1.2 μg.g^{-1} d.w.) and C17 (8.0 ± 2.0 μg.g^{-1} d.w.) compared to the other sites. As for Cd, the highest residues were observed in specimens from the Galician coast (A14: 0.30 ± 0.07 μg.g^{-1} d.w.; B15: 0.28 ± 0.08 μg.g^{-1} d.w.; B16: 0.34 ± 0.08 μg.g^{-1} d.w), whereas the lowest concentration was found in specimens from the Basque Country (E19: 0.02 ± 0.003 μg.g^{-1} d.w.). Furthermore, individuals from E19 showed low concentrations of Cr (0.48 ± 0.11 μg.g^{-1} d.w.) in comparison with those from D18 (3.59 ± 1.0 μg.g^{-1} d.w.) and B16 (2.77 ± 0.56 μg.g^{-1} d.w.). Significant negative correlations between the concentration of Cr in the liver of *T. luscus* and biometric parameters such as HSI (R^2 = -0.754; P<0.01) and length (R^2 = -0.650; P<0.01) were found. Specimens collected from North Galicia (sites B15 and B16) showed significantly lower levels of Hg (0.001 μg.g^{-1} d.w.) compared to the other areas, while fish from B15 reported the highest residues of Pb in liver (0.05 ± 0.002 μg.g^{-1} d.w.). As for residues of Cu (2.26–8.01 μg.g^{-1} d.w.) and Fe (68.5–117.8 μg.g^{-1} d.w.) no significant differences between sites were observed (Fig. 2).


L. boscii
T. luscus
(Zn)
Zn (µg.g-1 d.w.)
(Cd)
Cd (µg.g-1 d.w.)
(Cu)
Cu (µg.g-1 d.w.)
(Cr)
Cr (µg.g-1 d.w.)
A
B
C
D
E

(*Continued*)

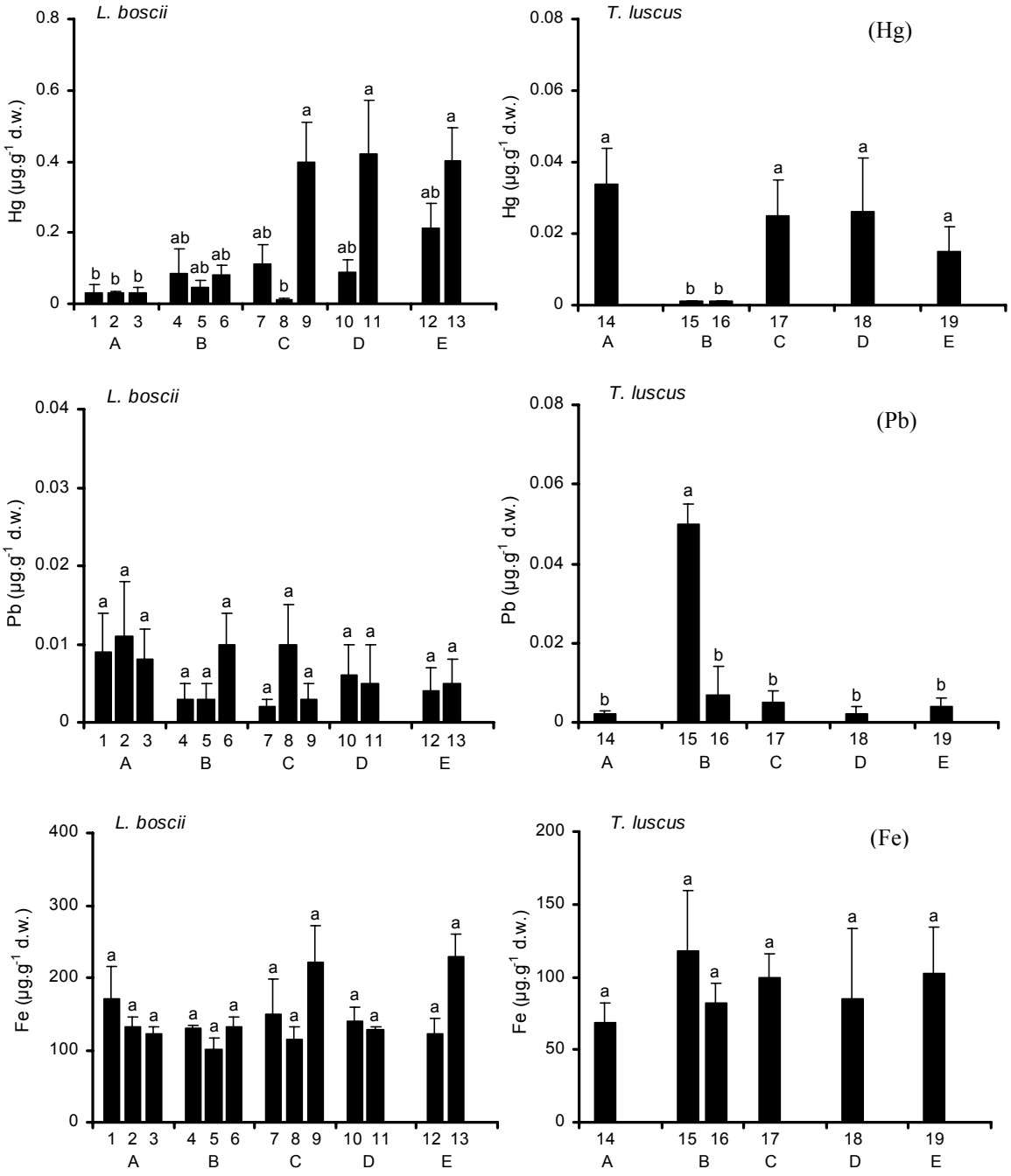


Figure 2. Metal concentrations (µg.g^{-1} d.w.) in the liver of *Lepidorhombus boscii* and *Trisopterus luscus* collected along the Northern Iberian shelf. Values are expressed as mean ± SEM (n=3-6). Distinct letters indicate significant differences between sites according to Tukey's test (p<0.05).

3.3 MT levels in the liver

The cytosolic MTs measured in the liver of *L. boscii* and *T. luscus* are presented in Figure 3. *L. boscii* showed higher levels of MT (about 2-fold) than *T. luscus*, and this is in accordance with the high levels of metals accumulated in the liver of *L. boscii*. The highest concentrations of MTs in *L. boscii* were observed in sites C9 (7920 ± 692 $\mu g.g^{-1}$ d.w.) and E13 (6773 ± 283 $\mu g.g^{-1}$ d.w.), where organisms showed high levels of Zn, Cd and Hg in the liver (Figs. 2 and 3). Furthermore, positive correlations between MT and Cd levels (R^2 = 0.573; P<0.01) and MT and Zn levels (R^2 = 0.537; P<0.01) were found. As for *T. luscus*, the lowest concentrations of MTs were observed in sites E19 (1479 ± 129 $\mu g.g^{-1}$ d.w.) and A14 (1694 ± 139 $\mu g.g^{-1}$ d.w.), while the highest were noted in sites D18 (3100 ± 350 $\mu g.g^{-1}$ d.w.) and B16 (2669 ± 261 $\mu g.g^{-1}$ d.w.), in agreement with the high metal (Zn, Cd, Cr and Hg) residues reported in the liver (Fig. 3). However, significant positive correlations were only observed between MT and Cr (R^2 = 0.784; P<0.01).

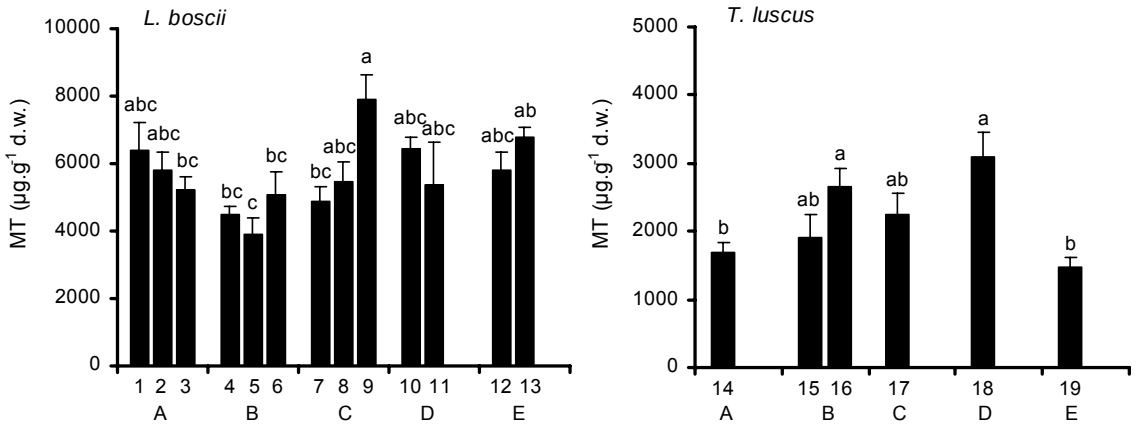


Figure 3. Hepatic MT concentrations ($\mu g.g^{-1}$ d.w.) in *Lepidorhombus boscii* and *Trisopterus luscus* collected along the Northern Iberian shelf. Values are expressed as mean ± SEM (n=3-6). Distinct letters indicate significant differences between sites according to Tukey's test (p<0.05).

4. Discussion

The analysis of metals in two different fish species provided data on metal bioavailability/exposure in different shelf environments (70-120 m and 200-500 m depth) along the Northern Iberian coast. Factors such as age, size and sex have been reported to affect metal bioaccumulation in fish (Al-Yousuf *et al*., 2000). In an attempt to minimize those effects, the analyzed individuals were adult females, with no significant differences in size between sampling sites. Under these constraints, both species showed higher levels of essential than non-essential metals, and indicated a similar pattern in the accumulation of essential metals (Fe>Zn>Cu>Cr), possibly related to their role in the regulation of key enzymatic and respiratory processes in both organisms (Çoğun *et al*., 2006). In contrast, for non-essential metals, a different bioaccumulation pattern was detected in *L. boscii* (Cd>Hg>Pb) and *T. luscus* (Cd>Pb>Hg); differences in metal speciation/bioavailability in the two shelf environments may account for the observed differences.

Thus, *L. boscii* (middle/outer shelf) showed elevated residues of Cd in some stations of areas A, B, C and E, while in *T. luscus* (inner shelf), the highest levels were mostly found in areas A and B (Galicia), up to 15-fold higher than in area E (Basque Country). This is in agreement with the high levels of Cd previously reported in sediments from some Galician Rias and associated to industrial and mining sewage inputs (Prego and Cobelo-Garcia, 2003). In contrast, elevated levels of total Hg were detected in the liver of both *L. boscii* and *T. luscus* from Asturias (site C9, C17), Cantabria (site D11, D18) and Basque Country (site E13, E19) suggesting a chronic

exposure to Hg in those areas. In general, anthropogenic inputs of Hg to the marine environment are due to direct releases from industrial and domestic activities, the contamination of marine organisms occurring mainly through ingestion of mercury-contaminated sediments and food (Neff, 2002). Curiously, *T. luscus* from South Galicia also reported high Hg residues, whereas those from North Galicia showed significantly low residues in their livers. These findings are consistent with data on sediment contamination and Hg enrichment in mussels from the Pontevedra Ria (South Galicia, near station A14) (Prego and Cobelo-Garcia, 2003).

High concentrations of Pb (up to 25–fold) were detected in *T. luscus* from site B15 (North Galicia). Interestingly, levels of Pb, 3 to 15 times higher than "pristine" values, where reported in the upper layers of the water column in the Galician coast, 12 days after the *Prestige* wrecking (Prego and Cobelo-Garcia, 2004). Additionally, previous studies have reported Pb enrichment in water and sediments of some Galician Rias, reflecting inputs from anthropogenic origins (e.g. industries and harbours) (Prego and Cobelo-Garcia, 2003). Nevertheless, those elevated levels of Pb were not observed in *L. boscii* (middle/outer shelf), which suggests a decrease in the concentrations of Pb with depth and evidences the anthropogenic origin of Pb from coastal urban settlements.

Regarding Cr, *L. boscii* showed no differences among sampling sites while *T. luscus* from North Galicia (site B16) and Cantabria (site D18) had higher levels than elsewhere, suggesting a higher availability of Cr in the surrounding medium. Those individuals had low hepatosomatic index (HSI) and a negative relationship between HSI and Cr residues in the liver was observed. Negative correlations between metals and HSI have often been interpreted as a negative impact of metals on fish health (Maes *et al*., 2005); in fact, a decrease of liver size as a consequence of the loss of

hepatic glycogen and/or lipid is a common morphologic response of fish liver to toxicity (Wolf and Wolfe, 2005). Furthermore, a negative correlation between Cr residues in the liver of *T. luscus* and fish size was observed.

Additionally, it is important to indicate the higher levels of metals (up to 10-fold) detected in the liver of *L. boscii* in comparison to *T. luscus* are possibly associated to its benthic habitat. In fact, it is often stated that sediments constitute the most important reservoir or sink of metals and other pollutants in the aquatic environment, and *L. boscii* lives in close contact with the sediment. In accordance with metal residues, levels of hepatic MT were higher in *L. boscii* than in *T. luscus*. MTs were elevated in *L. boscii* from site C9 (Asturias) and E13 (Basque Country) showing high residues of Zn, Cd and Hg in their livers (Fig. 2). It is well established that MT have a role in Zn homeostasis and that Zn is a constituent element of MT (Hamza-Chaffai *et al*., 1995). On the other hand, Cd and Hg are known to be toxic pollutants, whose deleterious effects are minimized when they are bound to MT. Nonetheless, it has been demonstrated that toxic metal binding by hepatic MT is very efficient with Cd and less efficient with Hg (Cosson, 1994), which can explain the observed correlations with Zn and Cd in the liver of *L. boscii*.

For *T. luscus*, significant high levels of MT were reported in specimens from sites B16 (North Galicia) and D18 (Cantabria), which are in agreement with high residues of Zn, Cd, Cr (B16) and Zn, Cr, Hg (D18) in their livers (Fig 2). The elevation of hepatic MT is a measure of the biologically available metal in the environment (Roch et al., 1986). However, binding of metals on MT sites is not only dependent on their respective affinity for cysteinic residues, but also related to their relative amount in the cell (Cosson, 1994). A positive correlation was observed between MT and Cr, whereas

none or very poor correlations were observed for the other analyzed metals. Several authors have interpreted good correlations between metal and MT content as an indication of metal sequestration by MTs, while poor correlations have been attributed to the metals have exceeded the binding capacity of MTs or the involvement of low molecular weight non-MT proteins in metal sequestration (Roméo *et al*., 1999; Rotchell *et al*., 2001; Filipović and Raspor, 2003).

Overall, metal concentrations in the liver of *L. boscii* and *T. luscus* from the Northern Iberian coast indicates that metal pollution in the middle/outer shelf is mostly concentrated in the Asturias (C) and Basque Country (E) area, whereas in the inner shelf it is widespread along the Iberian shelf (e.g. high levels of Zn in North Galicia (B), Cantabria (D) and Basque Country (E), Cd in Galicia (A &B), Cr in North Galicia (B) and Cantabria (D), Hg all along the coast apart from North Galicia). In fact, the anthropogenic pressure tends be higher near the coastline, due to the proximity of urban settlements. Metal concentrations in the liver of the studied fish are in the range or lower than those previously reported in wild fish from coastal areas, with the exception of the levels of Cr in both species, and Zn, Cd and Hg in *L. boscii*, which were higher than some of the values reported in the literature (Table 2). Additionally, MT seems to be induced in fish that had higher amounts of Zn, Cd, Cr and Hg in their livers, supporting its use as a biomarker of metal exposure in these species. Furthermore, the study shows the suitability of *L. boscii* and *T. luscus* as sentinel species for future biomonitoring programmes and the importance of selecting ecologically different sentinel species (different habitats) for the assessment of pollution impact in the marine environment.

Table 2. Hepatic metal concentrations (μg.g^{-1} dry weight) in wild commercial fish species from different coastal areas. Values are given as ranges of concentrations.

Area	Specie	Cu	Zn	Cr	Fe	Cd	Pb	Hg	Reference
Northern Iberian shelf	*Lepidorhombus boscii*	9.8 - 27.7	77.8 - 162.8	1.0 - 2.89	101.4 - 221.6	0.10 - 1.37	0.002 - 0.011	0.011 - 0.422	This work
	Trisopterus luscus	2.3 - 8.0	8.0 - 25.3	0.43 - 3.59	68.5 - 117.8	0.02 - 0.34	0.002 - 0.050	0.001 - 0.034	
Southern Atlantic Coast	*Solea vulgaris*	50.7 - 129	15.0 - 25.6	0.015 - 0.031	236 - 326	0.08 - 0.43	0.20 - 0.42	0.010 - 0.027	Usero et al. (2003)
	Liza aurata	13.7 - 164	30.6 - 81.8	0.011 - 0.029	185 - 397	0.14 - 0.51	0.25 - 0.48	0.012 - 0.042	
	Anguilla anguilla	16.4 - 32.5	31.9 - 44.6	0.029 - 0.065	365 - 560	0.12 - 0.48	0.40 - 0.60	0.011 - 0.023	
African Atlantic Coast	*Cephalopholis nigri*	10.8 - 19.0	85 - 115			13.2 - 22.3		0.53 - 0.97	Roméo et al. (1999)
	Pseudupeneus prayensis	10.7 - 16.9	90 - 116			7.0 - 13.6		0.11 - 0.19	
Eastern Mediterranean coast	*Sparus aurata*	2.32 - 2.88	60.2 - 61.5		57.5 - 224.3	0.58 - 1.33	1.87 - 3.92		Dural et al. (2006, 2007)
	Dicentrarchus labrax	0.35 - 0.69	26.3 - 94.9		51.4 - 212.7	0.98 -1.49	1.41 - 1.72		
	Mugil cephalus	4.77 - 12.0	70.3 - 125.7		96.1 - 143.6	0.94 - 1.64	1.85 - 3.12		
Northeast Mediterranean Sea	*Sparus aurata*	17.1 - 49.6	59.1 - 93.9	1.33 - 1.99	147.7 - 365.3	0.72 - 1.20	6.74 - 11.0		Canli and Atli (2003)
	Atherina hepsetus	18.8 - 89.6	45.3 - 95.1	0.92 - 6.46	221.8 - 564.6	0.39 - 1.95	35.0 - 47.5		Çoğun et al. (2006)
	Sardina pilchardus	16.1 - 42.5	53.4 - 93.1	7.59 - 26.7	174.0 - 277.0	1.68 - 4.30	21.5 - 57.3		Kalay et al. (1999)
	Mugil cephalus	79.6 - 98.6	69.8 - 86.5	1.29 - 2.54	224.3 - 284.5	5.90 - 8.10	11.2 - 16.8		
	Mullus barbatus	121.9 - 175.2	101.4 - 130.2	1.22 - 2.38	298.6 - 385.2	10.8 - 14.5	16.8 - 23.6		
Western Arabian Gulf coast	*Lethrinus lentjan*	3.64 - 7.58	33.5 - 106.7			0.45 - 1.11			Al-Yousuf et al. (2000)
Estern Arabian Gulf coast	*Epinephelus coioides*	18.6 - 276	143 - 1714	<0.01 - 0.062	478 - 2866	0.11 - 11.40	<0.001 - 0.108	0.287 - 4.65	de Mora et al. (2004)
	Lethrinus nebulosus	9.2 - 98.8	228 - 2400	<0.05 - 0.08	1207 - 1900	1.46 - 9.94	0.175 - 0.426	0.333 - 1.02	
Western Indian Ocean	*Xiphias gladius*	54.7 - 65.4	213 - 239		558 - 617	163 - 169	0.09 - 0.18	5.33 - 9.44	Kojadinovic et al. (2007)
	Thunnus albacares	121 - 240	439 - 516		690 - 734	126 - 138	0.05 - 0.13	0.65 - 3.27	

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3.2.

ASSESSMENT OF POLLUTION ALONG THE NORTHERN IBERIAN SHELF BY THE COMBINED USE OF CHEMICAL AND BIOCHEMICAL MARKERS IN TWO REPRESENTATIVE FISH SPECIES

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Resumo

Concentrações de compostos organoclorados no músculo assim como hidrocarbonetos aromáticos policíclicos (PAHs) e alquilfenois (APEs) na bílis foram determinados em duas espécies de peixe, o areeiro-de-quatro-manchas (*Lepidorhumbos boscii*) e a faneca (*Trisopterus luscus*) recolhidos ao longo da costa Norte da Península Ibérica. Adicionalmente, um conjunto de marcadores bioquímicos nomeadamente, 7-etoxiresorufina *O*-deetilase (EROD), UDP-glucuronosiltransferase (UGT) e catalase (CAT) foram medidos em fracções subcelulares do fígado. As análises químicas indicaram diferenças na exposição a contaminantes orgânicos nas distintas áreas geográficas, que foram ainda reforçadas pelas respostas dos biomarcadores. Deste modo, a actividade de EROD mostrou uma forte correlação com a quantidade de PCBs bioacumulado no músculo de ambas as espécies. Foi observado uma actividade elevada de UGT nos indivíduos expostos a maiores níveis de APEs e 1-naftol. Este estudo vem a renforçar o uso de espécies representativas de distintos habitats como organismos de sentinela apropriados em programas de biomonitorização, para além de apoiar o uso de biomarcadores na avaliação da contaminação em áreas costeiras.

Palavras-chave: poluição, biomarcadores, peixes bentónicos, costa Norte da Península Ibérica, 7-etoxiresorufina *O*-deetilase, UDP-glucuronosiltransferase, *Prestige*.

Abstract

Muscle concentrations of organochlorinated compounds as well as biliary levels of polycyclic aromatic hydrocarbons (PAHs) and alkylphenols (APEs) were determined in two different fish species, the four-spotted megrim (*Lepidorhombus boscii*) and the pouting (*Trisopterus luscus*) collected along the Northern Iberian coast. Additionally, a set of biochemical markers namely, 7-ethoxyresorufin *O*-deethylase (EROD), UDP-glucuronosyltransferase (UGT) and catalase (CAT) were measured in liver subcellular fractions. Chemical analysis indicated geographical differences in pollutant loads that were further reinforced by biomarker responses. Thus, EROD activity showed a good correlation with the amount of PCBs bioaccumulated in the muscle tissue of both fish species. Elevated UGT activity was observed in those individuals highly exposed to APEs and 1-naphthol. The study reinforces the need of slecting representative sentinel species from different habitats for biomonitoring purposes and provides further support on the use of biomarkers for assessing the health of coastal areas.

Keywords: Pollution, biomarkers, benthic fish, Northern Iberian coast, 7-ethoxyresorufin *O*-deethylase, UDP-glucuronosyltransferase, *Prestige* oil-spill

1. Introduction

Fish inhabiting coastal areas have been proposed as sentinel species for biomonitoring land-based pollution because they may concentrate indicative hydrophobic organic compounds in their tissues, directly from water, sediments and/or through the diet. Once in the organism, xenobiotic compounds undergo a series of biotransformation reactions catalyzed by different enzymatic systems, the activation of which may be an additional evidence of pollution exposure (Porte *et al*., 2002). Therefore, the comprehensive determination of chemical and biochemical markers in coastal fish is a sound procedure for assessing pollution exposure in coastal areas.

The cytochrome P450 (CYP) system consists of a family of enzymes that play a key role in the metabolism of xenobiotics and endogenous compounds. Cytochrome P450 activate, inactivate and facilitate the excretion of lipophilic chemicals, thus, modulating the duration and intensity of their toxicity (Navas and Segner, 2000). In fish, induction of cytochrome P4501A (CYP1A) by exposure to organic contaminants, namely polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), among others, has been considered as a sound biomarker in environmental monitoring (Collier *et al*., 1995; Van der Oost *et al*., 1996; Cajaraville *et al*., 2000). The metabolism of organic chemicals by different CYP isoenzymes may yield oxidized products, either inactive, or in some cases, reactive metabolites, which are more toxic than the parent compound, resulting in deleterious physiological effects (Stegeman and Lech, 1991). Some of these intermediate products can be conjugated with endogenous cellular molecules (e.g. glucuronic acid) and excreted through the bile, or may generate

oxidative stress. UDP-glucuronosyltransferases (UGTs) are one of the phase II enzymes that conjugate those intermediate products; UGTs are induced by a variety of natural and synthetic compounds and play a key role catalyzing the conjugation and potential excretion of different xenobiotics in fish (Clarke *at al*., 1992a). UGT responses have been observed in fish from polluted sites, although the magnitude of the response is not as sensitive as that of phase I enzymes (Van der Oost *et al*., 2003).

Additionally, antioxidant enzymes, such as catalase (CAT), belong to the cellular antioxidant system that counteracts the toxicity of reactive oxygen species. CATs are hematin-containing enzymes that facilitate the removal of hydrogen peroxide (H_2O_2), which is metabolized to molecular oxygen (O_2) and water (Van der Oost *et al*., 2003). Several classes of pollutants, including trace metals and organic compounds, are known to enhance the formation of reactive oxygen species. Variations in CAT activity along a pollutant gradient have been demonstrated in several studies and proposed as a biomarker of pollutant mediated oxidative stress (Porte *et al*., 2002; Lionetto *et al*., 2003; Pandey *et al*., 2003).

The Northern Iberian coast is an area of great socioeconomic importance and highly vulnerable to anthropogenic pressure due to the co-existence of urban, industrial, agriculture, mining, aquaculture, fisheries and leisure activities. Additionally, the *Prestige* tanker disaster happened in this region in November 2002 causing massive oil pollution and spilling a heavy fuel that drifted all along the Northern Iberian shelf (Sánchez *et al*, 2006). Regarding pollutant levels, most of the data available so far refers to water and sediment levels of metals and polycyclic aromatic hydrocarbons (PAHs) (Cobelo-García and Prego, 2003; Prego and Cobelo-Garcia, 2004; Franco *et al*., 2006). However, information on the fate and toxicity of chemicals that are currently released

into the area is scarce, and very few monitoring programs have addressed the integrated use of chemical analyses with biochemical and cellular responses to pollutants along the Northern Iberian shelf (Martínez-Gómez *et al*., 2006). Therefore, considering the high productivity of the Galician waters, the importance of fisheries and the existence of unknown land-ocean inputs of pollutants, this work aimed at assessing pollutant levels along the Northern Iberian shelf by measuring chemical residues – organochlorinated compounds (OCs), polycyclic aromatic hydrocarbons (PAHs) and alkylphenolic compounds (APEs)– in fish together with biochemical responses –7-ethoxyresorufin *O*-deethylase (EROD), UDP-glucuronosyltransferase (UGT) and catalase (CAT)–. The validation of the biomarker approach under field conditions in undoubtedly an important issue in marine environmental research and crucial in improving monitoring and assessment strategies.

The selected species for the study were the four-spotted megrim (*Lepidorhombus boscii*) and the pouting (*Trisopterus luscus*). Fish were collected from 20 stations along the Northern Iberian coast to provide a wide coverage of the area. *L. boscii* is a flatfish of high commercial value in Spain widely distributed in the area of study (Sánchez *et al*., 2006); it dwells on muddy bottoms on the middle and outer shelf at maximum depths of 800 m. Studies carried out in the NW Mediterranean Sea have demonstrated that *L. boscii* is a sensitive species to PAH exposure (Pietrapiana *et al*., 2002). *T. luscus* is a benthopelagic fish which lives mostly on the inner shelf and moves inshore to depths of less than 50 m for spawning (França *et al*., 2004). The simultaneous use of both species will allow obtaining information on pollution exposure in different shelf environments: 70-120 m depth (*T. luscus*) and 200-500 m depth (*L. boscii*).

2. Material and Methods

2.1. Sampling procedure

Lepidorhombus boscii and *Trisopterus luscus* were collected from 20 stations along the Northern Iberian shelf by 30 minute bottom trawls (baca 44/60 otter trawl gear) at depth ranges of 70-120 m for *T. luscus* and 200-500 m for *L. boscii*. Sampling was carried out in October 2003, coinciding with the prespawning period of both species. Stations were fixed along 15 transects drawn from coast to open sea. For the scope of this study, the continental shelf was divided into five geographical areas: (A) S Galicia; (B) N Galicia; (C) Asturias; (D) Cantabria; (E) Basque Country (Figure 1). Immediately after collection, fish were sacrificed by severing their spinal cord, sexed and weighed; their length was measured, the target tissues (liver, bile, muscle) dissected and immediately frozen in liquid nitrogen, and stored at – 80ºC upon arrival to the laboratory.

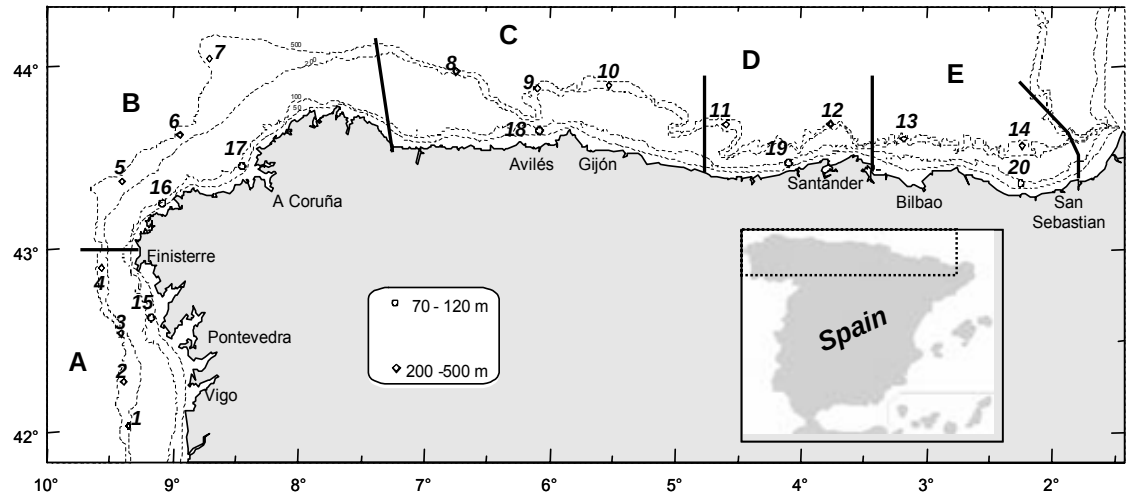

Figure 1. Map of sampling sites. (A) S Galicia; (B) N Galicia; (C) Asturias; (D) Cantabria; (E) Basque Country. Depth ranges of sampled fish: 70-120 m (*Trisopterus luscus*); 200-500 m (*Lepidorhombus boscii*).

2.2. Chemical analysis

2.2.1. Analysis of organochlorine compounds.

Fish samples were pooled (4-6 individuals per site), homogenized, lyophilized and a subsample of 5 g of muscle tissue was Soxhlet-extracted with *n*-hexane:dichloromethane (4:1) for 18 h. Procedural blanks were also included. The solvent extract was evaporated to near dryness; the residue dissolved in 3 ml of *n*-hexane and cleaned up by vigorous shaking with 1-2 ml of conc. H_2SO_4, in order to remove lipids and other interfering substances. Further cleanup was based on solid-liquid adsorption chromatography: glass columns containing 5 g of alumina were eluted with *n*-hexane:dichloromethane (1:2). The recovery of the column was evaluated with a standard solution, being more than 85 % for the analyzed compounds. The cleaned extract was concentrated by vacuum rotary evaporation, transferred to vials, evaporated to near dryness under a gentle stream of nitrogen, and analyzed by gas chromatography-electron capture detector (GC-ECD). The instrument was a Hewlett Packard 5890 GC. The column, a 50 m X 0.25 mm i.d. CP-Sil 5 CB fused silica (Chrompack, Middelburg, NL), was programmed from 80 to 180 ºC at 15 ºC/min and from 180 to 280 ºC at 3 ºC/min, keeping the final temperature for 15 min. The carrier gas was helium at a linear flow-rate of 50 cm/s. The injector and detector temperature was set at 280 and 300 ºC, respectively. Quantification was performed using an external standard calibration mixture of selected congeners (I.U.P.A.C. Nos.: 18, 31, 28, 52, 44, 149, 101, 118, 153, 138, 180, 170, 194, 209) supplied by Promochem (Wesel, Germany) and recommended by the International Council for the Exploration of the Sea (ICES) for

assessing marine pollution (Duinker *et al*., 1988). These congeners were quantified separately and the PCB concentration defined as its sum. Total DDTs were determined and quantified using an external calibration curve. Concentrations are expressed as ng/g dry weight.

2.2.2. Analysis of hydroxylated-PAHs and alkylphenols (APEs) in bile.

Bile samples were pooled (3-4 individuals per site) and hydrolyzed by a modification of the method described in Escartín and Porte (1999a). Briefly, 100 mg of bile were incubated for 1 h at 40ºC in the presence of 1 ml 0.4 M acetic acid/sodium acetate buffer pH 5.0, containing 2000 units of β-glucuronidase and 50 U of sulphatase. Hydrolyzed metabolites were extracted with 1 ml of ethyl acetate (× 3), the extracts recombined and concentrated under a nitrogen stream. For the analysis of OH-PAHs, dry residues of bile metabolites were derivatized by the addition of 100 µl of bis-(trimethylsilyl)trifluoroacetamide (BSTFA), heated for 1 hour at 70 ºC, and further reduced under a nitrogen stream. Analyses were carried by gas chromatography-mass spectrometry electron impact mode (GC-MS-EI). The equipment was a Fisons GC 8000 Series chromatograph interfaced to a Fisons MD800 mass spectrometer. The column, a 30 m x 0.25 mm i.d. HP-5MS crosslinked 5 % PH ME siloxane (Hewlett-Packard, USA) was programmed from 90 to 140 at 10ºC/min and from 140 to 300 at 4ºC/min. The carrier gas was Helium at 80 Kpa. The injector temperature was 250 ºC and the ion source and the analyser were maintained at 200ºC and 250ºC, respectively. Just prior to sample analysis, calibration curves were performed with reference compounds and operating in selected ion monitoring mode (SIM). Hydroxylated-PAHs and

alkylphenols were identified by comparison of retention times and spectra of reference compounds. The silylation derivative ions used for monitoring and quantification of OH-PAHs were: m/z 216 and 201 for 1-naphthol; m/z 254 and 165 for 9-fluorenol; m/z 266 and 251 for 9-phenanthrol; and m/z 290 for 1-pyrenol. The ions used for monitoring and quantification of APEs were: m/z 149 and 135 for 4-nonylphenol (NP) and m/z 206 for 4-*tert*-octylphenol (OP). Concentrations are expressed as ng/g of bile.

2.3. Biochemical determinations

Hepatic microsomal fractions were prepared as described in Fernandes *et al*. (2007). Briefly, after weighing, livers were flushed with ice-cold 1.15 % KCl, and homogenized in 1:5 w/v of cold 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, containing 150 mM KCl and supplemented with 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM ethylenediaminetetra acetic acid (EDTA). Homogenates were centrifuged at 500 *g* for 15 min, the fatty layer removed and the obtained supernatant centrifuged at 12,000 *g* for 20 min. The resulting supernatant was further centrifuged at 100,000 *g* for 60 min to obtain the cytosolic and microsomal fractions. Microsomal pellets were resuspended in a ratio of 0.5 ml buffer/g liver tissue in 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, containing 150 mM KCl, 20 % w/v glycerol and supplemented with 1 mM DTT, 0.1 mM PMSF and 1 mM EDTA. Microsomal and cytosolic proteins were measured by the method of Bradford (1976), using serum albumin (BSA) as standard.

2.3.1. 7-Ethoxyresorufin O-deethylase (EROD)

EROD activity was determined in the microsomal fraction of the liver and was assayed by incubating 25 µl of microsomes with 3.7 µM of 7-ethoxyresorufin and 225 µM of NADPH in 100 mM K_2HPO_4/KH_2PO_4 pH 7.4 (final volume 250 µl) at 30 ºC for 10 min. The reaction was stopped by adding 400 µl of ice-cold acetonitrile (ACN) and after centrifugation (3000 rpm/10 min) an aliquot of the supernatant was transferred to a 96-multiwell plate. Fluorescence was read at the excitation/emission wavelengths pairs of 537/583 using a Gemini XPS SepctraMax Plus microplate reader (Molecular Devices Corporation). Quantification was performed by using a 7-hydroxyresorufin calibration curve and the activity calculated as the amount of 7-hydroxyresorufin (pmol) generated per milligram of protein per minute of reaction time.

2.3.2. UDP-glucuronosyltransferase (UGT)

Hepatic UGT was assayed as described in Fernandes *et al*. (2002). The assay contained 0.25 mg of microsomal protein (pretreated for 15 min with 0.2% TritonX-100 on ice), and the reaction was initiated by the addition of 81 µM *p*-nitrophenol (*p*NP) and run for 30 min at 30 ºC in a shaking water bath. The reaction was stopped by the addition of 0.2 M ice-cold trichloracetic acid (TCA), centrifuged, alkalinized with 0.1 ml of 10 N KOH and the remaining *p*NP was measured spectrophotometrically at 405 nm. Activity was calculated as the amount of *p*NP (nmol) consumed per milligram of protein per minute of reaction time.

2.3.3. Catalase (CAT)

CAT activity was measured in liver cytosol, essentially as described in Livingstone *et al*. (1992). This technique is based in the direct determination of hydrogen peroxide consumption. The assay was performed at room temperature in a final volume of 3 ml, containing 50 mM KH_2PO_4/K_2HPO_4 pH 6.5 and 50 mM 30% H_2O_2 as substrate. After addition of the sample, the decrease in absorbance was measured at 240 nm and the enzyme activity calculated in terms of mmol H_20_2 consumed/min/mg protein using a molar extinction coefficient of 40 $M^{-1}cm^{-1}$.

2.4. Statistical analysis

Biochemical activities were determined individually in 4-6 female organisms per site and run per duplicate. Chemical analyses were conducted in pools of 3-6 individuals per station. Values are presented as means ± SEM. Statistical significance was assessed using one-way ANOVA with Tukey's test using the software package SPSS/PC^{TM} version 12.0 (SPSS Inc., Chicago, IL); *P* value of less than 0.05 was considered as statistically significant. Pearson's correlation coefficients were calculated among selected parameters in order to measure the strength of association between variables. In all instances transformations of the data were performed when the assumption of normality of residuals was not met.

3. Results

3.1. Morphometric data of samples

The morphometric parameters of the sampled fish are listed in Table 1. All individuals were adults and significant differences were observed in weight and length for both species. *L. boscii* from site A1 (71 ± 15 g) were smaller than those collected from sites B7, C9, E13 and E14 (157 – 194 g), whereas *T. luscus* from site D19 (92 ± 29 g) were smaller than those from sites B16 (188 ± 12 g) and E20 (174 ± 13 g). Significant differences were also observed for the hepatosomatic index (HSI). *L. boscii* from site B6 (4.26 ± 0.96%) had higher HSI than those collected from sites A2, C8, C10, E14 and area D (1.71 – 1.90%) (P<0.05). For *T. luscus* the highest HSI was detected in site A15 (4.35 ± 0.50%) and the lowest in sites B17 and D19 (1.69 – 1.73%) (P<0.05). The condition factor (CF) was rather homogenous for *L. boscii* (0.66-0.85) and *T. luscus* (0.93-1.07) indicating a similar nutritional state of fish from all sites, with the exception of *T. luscus* from site B17 (1.33 ± 0.09) that had a higher CF.

3.2. Chemical analysis

The PCB load in the muscle tissue of *L. boscii* and *T. luscus* was estimated on the basis of 14 PCB congeners selected from the GC-ECD profile. These congeners were quantified separately, and results are given as its sum (Figure 2A). A gradient of PCBs was detected along the middle/outer shelf, the highest residues detected in the muscle tissue of *L. boscii* from the Basque Country (site E14: 8.3 ng/g dry weight) and the Asturias area (site C10: 7.2 ng/g dry weight), about 1.5 to 4-fold higher than those

from Galicia (A & B), where PCB concentrations ranged from 2 to 5.5 ng/g dry weight. Meanwhile, for the Cantabria area PCB residues were similar between sites (D: 3.5-4.0 ng/g dry weight). The concentrations of PCBs in the muscle of *T. luscus* (inner shelf)

Table 1. Morphometric data of *Lepidorhombus boscii* and *Trisopterus luscus* collected along the Northern Iberian coast. Values are mean ± SEM. HSI: hepatosomatic index, calculated as (liver weight / body weight) x 100; CF: condition factor, calculated as [body weight / (length)^3] x 100; n: number of individuals analyzed. Distinct letters indicate significant differences between sites according to Tukey's test (p<0.05)

Area	Site	n	Weight (g)	Length (cm)	HSI (%)	CF (g/cm^3)
L. boscii						
A	1	5	71 ± 15^{b}	22 ± 2.0^{b}	3.11 ± 0.95^{ab}	0.70 ± 0.09^{a}
	2	6	103 ± 19^{ab}	24 ± 1.8^{ab}	1.73 ± 0.19^{b}	0.66 ± 0.03^{a}
	3	6	131 ± 4^{ab}	25 ± 0.3^{ab}	2.47 ± 0.27^{ab}	0.81 ± 0.03^{a}
	4	6	109 ± 12^{ab}	24 ± 0.6^{ab}	2.26 ± 0.21^{ab}	0.75 ± 0.03^{a}
B	5	6	106 ± 21^{ab}	24 ± 0.6^{ab}	2.08 ± 0.15^{ab}	0.73 ± 0.03^{a}
	6	6	105 ± 18^{ab}	23 ± 1.5^{ab}	4.26 ± 0.96^{a}	0.85 ± 0.12^{a}
	7	6	167 ± 21^{a}	28 ± 1.0^{a}	2.30 ± 0.15^{ab}	0.76 ± 0.03^{a}
C	8	6	128 ± 5^{ab}	26 ± 0.4^{ab}	1.90 ± 0.25^{b}	0.77 ± 0.03^{a}
	9	6	194 ± 37^{a}	30 ± 1.9^{a}	2.44 ± 0.19^{ab}	0.71 ± 0.05^{a}
	10	6	105 ± 6^{ab}	24 ± 0.5^{ab}	1.74 ± 0.21^{b}	0.72 ± 0.01^{a}
D	11	6	120 ± 16^{ab}	25 ± 0.9^{ab}	1.85 ± 0.17^{b}	0.78 ± 0.01^{a}
	12	4	117 ± 15^{ab}	25 ± 0.9^{ab}	1.73 ± 0.37^{b}	0.78 ± 0.09^{a}
E	13	6	163 ± 31^{a}	26 ± 1.4^{ab}	2.35 ± 0.18^{ab}	0.83 ± 0.03^{a}
	14	6	157 ± 24^{a}	27 ± 1.1^{ab}	1.71 ± 0.16^{b}	0.76 ± 0.02^{a}
T. luscus						
A	15	6	131 ± 9^{ab}	24 ± 0.7^{a}	4.35 ± 0.50^{a}	0.99 ± 0.06^{b}
B	16	6	188 ± 12^{a}	26 ± 0.4^{a}	3.05 ± 0.32^{ab}	1.07 ± 0.03^{ab}
	17	6	128 ± 17^{ab}	21 ± 0.5^{a}	1.69 ± 0.19^{b}	1.33 ± 0.09^{a}
C	18	6	146 ± 11^{ab}	24 ± 0.6^{a}	2.88 ± 0.57^{ab}	1.01 ± 0.02^{b}
D	19	5	92 ± 29^{b}	21 ± 1.7^{a}	1.73 ± 0.26^{b}	0.93 ± 0.06^{b}
E	20	6	174 ± 13^{a}	26 ± 0.7^{a}	3.35 ± 0.19^{ab}	1.03 ± 0.03^{b}

were in the lower range of those detected in *L. boscii*; the highest residues were observed in specimens from site B17 (5.3 ng/g dry weight) and the lowest in site E20 (2.6 ng/g dry weight) (Fig. 2A).

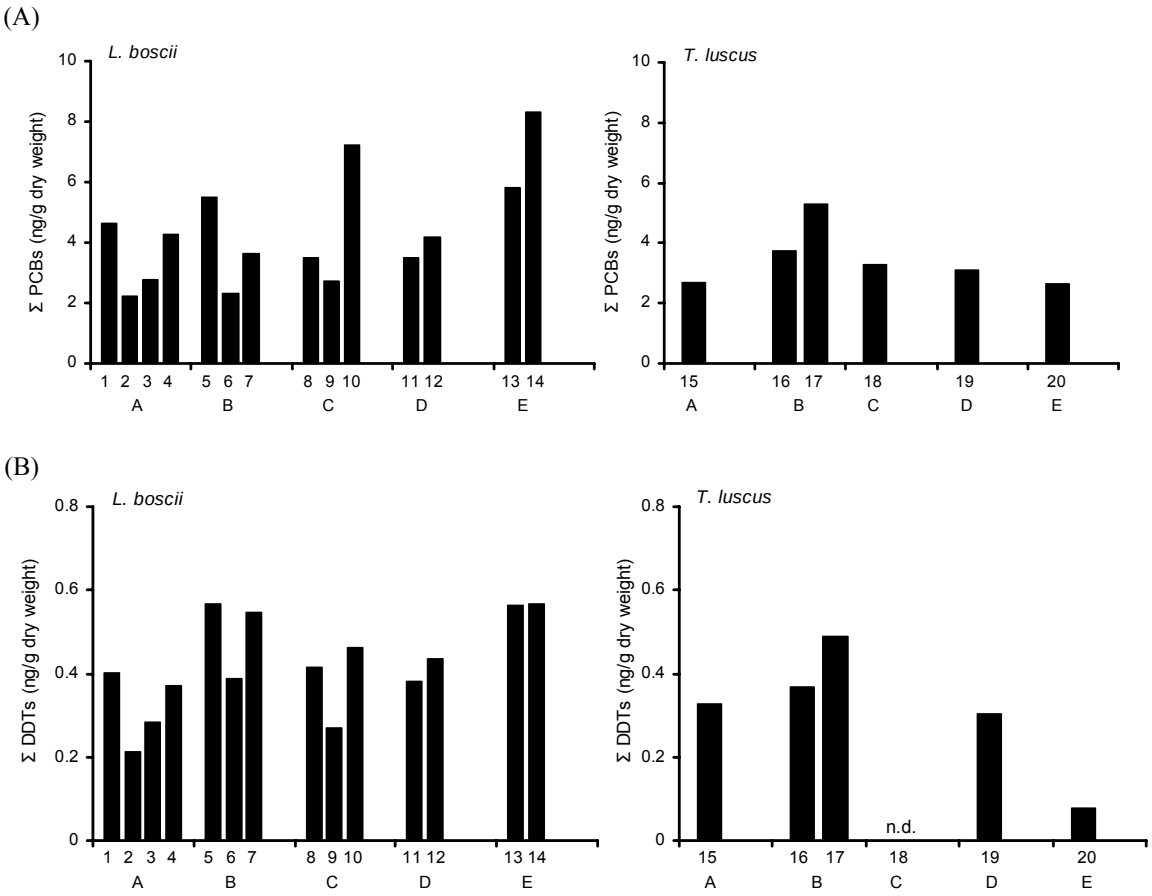

Figure 2. A) Sum of PCBs and B) sum of DDTs in the muscle tissue of *Lepidorhombus boscii* and *Trisopterus luscus* collected along the Northern Iberian coast. n.d.: below detection limit. Data correspond to pools of 4-5 organisms.

Considering all the identified PCB congeners (from tri- to octachlorobiphenyls), hexachlorobiphenyls represented 40-55% of the whole PCB mixture in both species, with the exception to *T. luscus* from site E20 with 61% of tetrachlorobiphenyls. The relative abundance of heptachlorobiphenyl varied among sampling sites; heptachlorinated isomers were relatively more abundant in *L. boscii* from the Basque Country (area E) and site C10 (30-33%) than in the other sites (3-12%).

Regarding the sum of DDTs, the spatial pattern was quite similar to that exhibited by PCBs, although the highest residues were observed in *L. boscii* from N Galicia (area B) and Basque Country (area E) (Fig. 2B). In *T. luscus*, the highest concentration of DDTs were detected in site B17 (0.49 ng/g dry weight) and the lowest in E20 (0.08 ng/g dry weight) and in C18 (below detection limit) (Fig. 2B). The main metabolites detected in the muscle of both species were *p,p'*-DDE and *o,p'*-DDD, although *L. boscii* from sites B5 (N Galicia) and D12 (Cantabria) had residues of *p,p'*-DDT and a ratio *p,p'*-DDE/*p,p'*-DDT of 0.65 and 0.92, respectively.

The concentrations of hydroxylated PAHs in bile are shown in Table 2. For *L. boscii*, the highest concentrations were observed in the Basque Country (E: 66.5 ± 14.5 ng/g of bile) and Cantabria (D: 58.8 ± 8.2 ng/g of bile), whereas the lowest concentrations were detected in specimens from N Galicia (B: 19.1 ± 1.6 ng/g of bile). A similar spatial pattern was detected in *T. luscus*; the highest concentrations detected in Cantabria (D: 94.9 ng/g of bile) and Basque Country (E: 91.9 ng/g of bile), and the lowest in the Galician shelf (areas A & B: 16.7 - 17.7 ng/g of bile). When looking at individual PAHs, additional differences among sampling sites were observed. Thus, 1-naphthol was the dominant PAH metabolite detected (76-100%) in both *T. luscus* and *L. boscii* from Galicia (areas A & B) (Fig. 3). In sites C (both species) and D-E (*L. boscii*), 1-naphthol and 1-pyrenol were equally abundant and they represented 75-100% of the total metabolites detected, whereas in *T. luscus* (sites D-E), pyrenol (75%) dominated over naphthol (25%). 9-Fluorenol was only detected in *L. boscii* from Asturias (25%), Basque Country (14%) and in *T. luscus* from S Galicia (11%); 9-phenanthrol was never detected (Fig. 3).

Table 2. Biliarly levels of hydoxylated PAHs and alkylphenols (ng/g of bile) detected in *Lepidorhombus boscii* and *Trisopterus luscus* collected along the Northern Iberian coast. Values are expressed as mean ± SEM. NP: 4-nonylphenol; OP: 4-*tert*-octylphenol. Data correspond to pools of 3-4 organisms analyzed. Distinct letters indicate significant differences between sites according to Tukey's test ($p<0.05$)

Areas	A	B	C	D	E
L. boscii					
ΣOH-PAHs	26.8 ± 3.9^{ab}	19.1 ± 1.6^{b}	43.7 ± 12.8^{ab}	52.9 ± 8.1^{ab}	66.5 ± 14.5^{a}
NP	157 ± 13^{a}	130 ± 21^{ab}	130 ± 42^{ab}	108 ± 34^{b}	129 ± 18^{ab}
OP	21 ± 4^{a}	12 ± 2^{ab}	11 ± 2^{ab}	5 ± 2^{b}	15 ± 7^{ab}
T. luscus					
ΣOH-PAHs	16.7	17.7	26.0	94.9	91.9
NP	220.3	204.4	148.0	109.6	144.8
OP	13.2	11.0	9.8	9.4	10.3

Additionally, the analysis of bile samples by GC-MS allowed the identification of peaks corresponding to 4-nonylphenol (NP) and 4-*tert*-octylphenol (OP) (Table 2). NP concentrations are reported as the sum of 11 isomers, and their identification was shown unequivocally by the complete match of profiles between samples and the NP standard mixture. NP was above detection limit in all the samples analysed and showed significantly higher concentration than OP. Considering the middle/outer shelf, the highest average concentration of NP (157 ± 13 ng/g of bile) and OP (21 ± 4 ng/g of bile) was detected in the bile of *L. boscii* collected from S Galicia (area A); whereas the lowest was observed in Cantabria (area D) (NP: 111 ± 24 ng/g of bile; OP: 8 ± 3 ng/g of bile). Similarly, *T. luscus* collected from the inner shelf reported higher concentrations of NP (220.3 ng/g of bile) and OP (13.2 ng/g of bile) in S Galicia (area A) and the lowest in Cantabria (area D) (NP: 109.6 ng/g of bile; OP: 9.4 ng/g of bile) (Table 2).

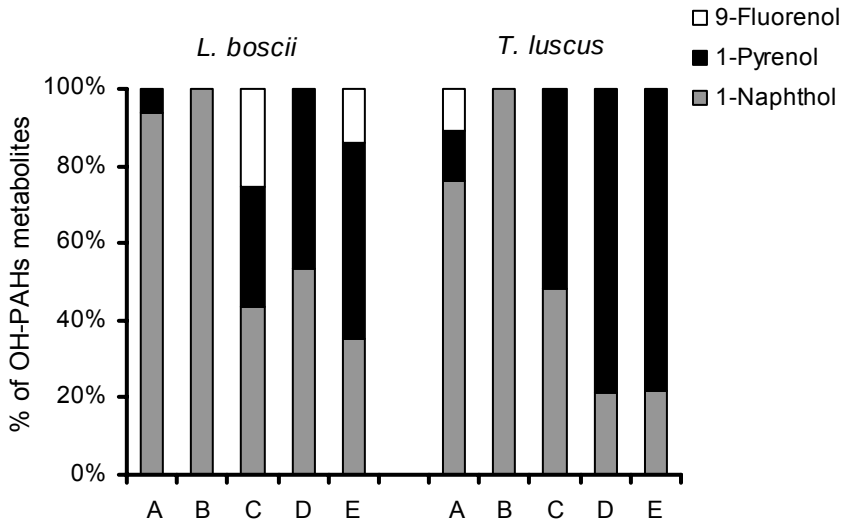

Figure 3. Percentage (%) of hydroxylated-PAH metabolites detected in bile of *Lepidorhombus boscii* and *Trisopterus luscus* collected along the Northern Iberian coast. (A) S Galicia; (B) N Galicia; (C) Asturias; (D) Cantabria; (E) Basque Country.

3.3. Biochemical responses

EROD activity in both species (Fig. 4A) showed a similar pattern to that exhibit by organochlorinated compounds in muscle tissue (Fig. 2). In fact, positive correlations were observed between EROD activity and PCBs residues in *L. boscii* (R^2 = 0.918; P<0.01) and *T. luscus* (R^2 = 0.922; P<0.01). Mean EROD activity was 3 to 5-fold higher in *L. boscii* from S Galicia (site A1), N Galicia (site B5), Asturias (site C10) and Basque Country (site E14) than in Cantabria, confirming the presence of CYP1A inducing agents in those areas. Regarding *T. luscus*, EROD activity was 2 to 4-fold higher in N Galicia (22.5-32.9 pmol/min/mg protein) than elsewhere (Fig. 4A).

A different geographical pattern was observed for hepatic UGT activity (Fig. 4B). Considering the middle/outer shelf, the highest UGT activity was recorded in *L. boscii* from S Galicia (sites A3 and A4) (0.25-0.27 nmol/min/mg protein), the area where *L. boscii* had the highest levels of NP and OP in bile; whereas the lowest UGT

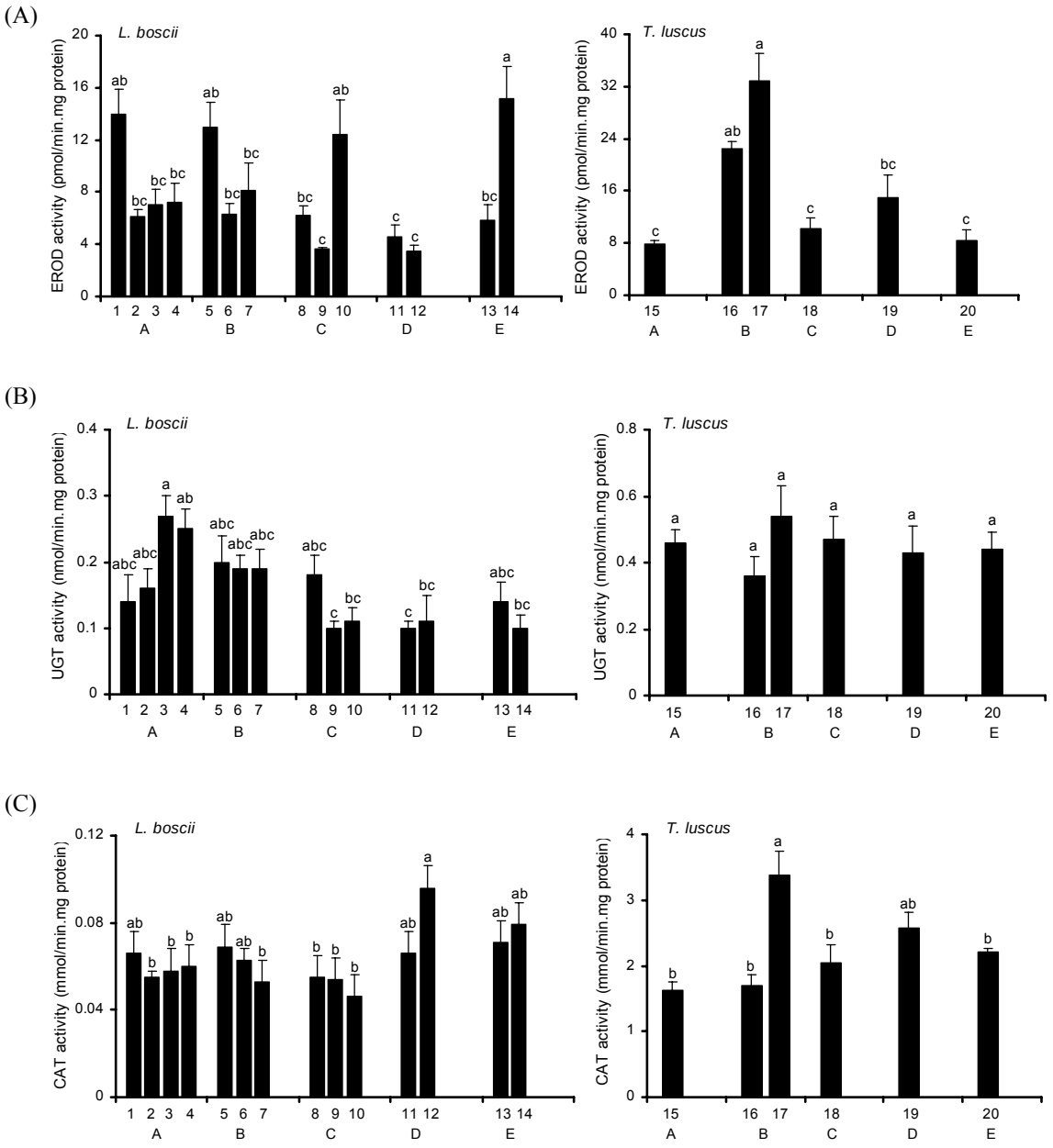

Figure 4. A) 7-Ethoxyresorufin *O*-deethylase; B) UDP-glucuronosyltransferase and C) catalase activities, in liver of *Lepidorhombus boscii* and *Trisopterus luscus* collected along the Northern Iberian coast. Values are expressed as mean ± SEM (n = 4-6). Distinct letters indicate significant differences ($P<0.05$) between sites, according to Tukey's test.

activity was detected in C9 and D11 (0.10 nmol/min/mg protein) in accordance with the lowest EROD activity observed in these individuals. No significant differences among sampling sites were observed for *T. luscus* (0.36-0.54 nmol/min/mg protein) ($P>0.05$).

Pollution and site related differences were observed for CAT activity in both species (Fig. 4C). The highest activities in *L. boscii* were reported in sites D12 (0.10 ± 0.01 mmol/min/mg protein) (P<0.05), and E14 (0.08 ± 0.01 mmol/min/mg protein). As for *T. luscus*, maximum activities were observed in sites B17 (3.5 ± 0.5 mmol/min/mg protein) (P<0.05), and D19 (2.6 ± 0.2 mmol/min/mg protein) (Fig. 4C).

Significant differences in the biochemical activities were observed between the two species; *T. luscus* had significantly higher EROD and UGT activity (about 2-fold) and CAT activity (about 30-fold) than *L. boscii* (Fig. 4).

4. Discussion

The biochemical markers indicated significant differences among sampling sites and different types of stress along the two shelf environments. EROD activity in both species showed a spatial variation along the middle/outer and inner shelf, coinciding with PCB and DDT residues in muscle tissue. Nevertheless such relationship was not observed with bile PAH-metabolites as described previously in other studies (Porte *et al*., 2002). Considering *L. boscii*, EROD activity was about 5-fold higher in E14 (Basque Country), C10 (Asturias), B5 (N Galicia) and A1 (S Galicia) than in the other sites indicating the presence of CYP1A inducing agents in those sampling stations, which were mainly characterized by having urban and industrial settlements nearby.

EROD activity in *L. boscii* (3.5-16 pmol/min/mg protein) was lower than previously reported (20-50 pmol/min/mg protein) by Martínez-Gómez *et al*. (2006) in the same species/area six months after the *Prestige* oil spill. The observed differences

can be attributed to decrease exposure to oil-spill derivated pollutants one year after the accident, but also to seasonal variability on EROD activity. In fact, *L. boscii* from the present survey were all females collected in autumn (pre-spawning) whereas Martínez-Gómez *et al*. (2006) had their fish sampled in spring (post-spawning); it is therefore likely to have a peak of estradiol during the pre-spawning period that will down regulate CYP1A levels (Navas and Segner, 2000), and lead to lower EROD activity.

Regarding *T. luscus*, mean EROD activity was 2 to 4-fold higher in N Galicia than in the other geographical areas, coinciding with the higher residues of PCBs and DDTs. The good correlation between hepatic EROD activity and PCB residues in both species further supports the use of EROD activity as a marker of PCBs exposure in fish, among other compounds (Livingstone *et al*., 1993; Porte *et al*., 2002; Van der Oost *et al*., 2003).

Chemical analysis (PCBs, DDTs, PAHs, APEs) indicated the presence of different pollutants in the region, and significant differences, both qualitative and quantitative, among sampling sites. The most predominant PCB congeners in muscle tissue corresponded to hexachlorobiphenyls, which agrees with most of the authors' findings in aquatic environments (Fernandes *et al*., 2002; Porte *et al*., 2002). Nonetheless, *L. boscii* from the Basque Country (E13 & 14) and Asturias (C10) exhibited higher PCB residues than elsewhere and increased presence of heptachlorobiphenyls, whereas *T. luscus* was comparatively enriched in tetra- and pentachlorinated congeners.

Concerning DDT residues, the main isomers detected were *p,p'*-DDE and *o,p'*-DDD. Detectable residues of *p,p'*-DDT were present in *L. boscii* from N Galicia (B5) and Cantabria (D12), despite of restrictions in DDT use in Spain since 1977.

Those samples presented a ratio *p,p'*-DDE/*p,p'*-DDT <1, suggesting a relatively recent use of the pesticide. When *p,p'*-DDT is introduced into the environment, it is converted to *p,p'*-DDD and *p,p'*-DDE, and despite that DDT has a half-life of 7 years, its metabolite DDE survives much longer and is the predominant form detected in fish and humans (Easton *et al*., 2002). Since elimination occurs slowly, ongoing exposure may lead to an increase in the body burden over time.

Regarding exposure to PAHs, both *T. luscus* and *L. boscii* showed high levels of hydroxylated-PAHs in bile in Cantabria and Basque Country areas. Higher levels of OH-PAHs were reported in *T. luscus* (inner shelf) than in *L. boscii* (middle/outer shelf); this is in agreement with recent studies, which reported higher concentrations of PAHs in sediments from the inner shelf than offshore (IEO, 2003). The obtained results highlight the anthropogenic pressure near coastlines and the chronic pollution of the Cantabria and Basque Country inner shelf due to urban and industrial activities. When considering the OH-PAH metabolites, both species showed a similar geographical pattern. 1-Pyrenol, regarded as the best general indicator of PAH exposure in fish (Ruddock *et al*., 2002), was particularly abundant in fish from Asturias (C), Cantabria (D) and Basque Country (E), while fish from Galicia (A, B) were mainly enriched in 1-naphthol, a marker of recent exposure to petrogenic compounds. In fact, small tar aggregates were still present on the Galician continental shelf almost one year after the *Prestige* accident (Serrano *et al*., 2006), and fish inhabiting the area, particularly benthic fish, might have been exposed to those PAHs. Significant amounts of 1-naphthol (35-53%) were also detected in the bile of fish from areas C, D & E, indicating exposure to PAHs of petrogenic origin. In fact, fish from the present study were characterized by the high relative abundance of 1-naphthol in bile (35-100%) when

compared with elsewhere; e.g. *Mullus barbatus* and *Serranus cabrilla* from the NW Mediterranean coast (2-10%) (Escartín and Porte, 1999a); *Salmo trutta*, *Salvelinus fontianalis* and *Salvelinus alpinus* from European mountain lakes (4-19%) (Escartín and Porte, 1999b).

The washing of oil tanker bottoms is a usual practice along the Galician coast, originating chronic inputs of petroleum and detergents into the marine environment (Salas *et al*., 2006). This was particularly evident when looking at the levels of APEs in fish bile. Both, *L. boscii* and *T. luscus* collected along the Galician coast showed the highest levels of NP and OP in bile, compared to the other areas. APEs are surfactants present in many formulated products, such as herbicides, pesticides, paints, industrial materials, but mainly in certain detergents used for removing oil and their derivatives (Groshart *et al*., 2001).

Among the biomarkers used, UGT activity was less responsive to pollutant exposure than EROD. Low hepatic UGT activity was observed in *L. boscii* exposed to high PCBs and PAHs concentrations, whereas no significant differences were observed between sites for *T. luscus*. Decreases in UGT activity have been previously reported in fish exposed to complex mixtures of pollutants (Soimasuo *et al*., 1995; Tuvikene *et al*., 1999; Fernandes *et al*., 2007). On the other hand, *L. boscii* from S Galicia (sites A3 & A4) indicated high UGT activity together with high concentrations of APEs and 1-naphthol in bile. In fact, several studies have reported 1-naphthol and other phenols to be good substrates for UGTs, indicating that the main metabolic pathway of these compounds is the glucuronidation (Leaver *et al*., 1992; Clarke *et al*., 1992b).

Catalase (CAT) activity was selected for this study because of its inducibility under conditions of oxidative stress and its potential role in adaptation to

pollutant-induced stress. CAT activity in *T. luscus* was 30-fold higher than in *L. boscii*. Different fish species can vary markedly on their antioxidant enzyme activities and thus in their susceptibility to oxidative stress (Hasspieler *et al*., 1994). Significantly high CAT activities were detected in *L. boscii* from site D12 (Cantabria) and *T. luscus* from site B17 (N Galicia), indicating an increase in oxidative stress in these species/areas. Besides the high levels of hydroxylated-PAHs (D12) and PCBs/DDTs (site B17) observed, these individuals also had significantly high residues of Hg (site D12), Cd and Cr (Site B17) in their livers (Fernandes *et al*. submitted). Thus, chronic exposure to certain pollutants may produce an increase in oxyradicals and generate oxidative stress in exposed organisms. The usefulness of CAT as a marker of oxidative stress in fish species has been previously reported (Hasspieler *et al*., 1994; Porte *et al*., 2002). Nonetheless, a number of studies have shown no significant responses of CAT activity to exposure to environmental pollutants (studies reviewed by Van der Oost *et al*., 2003).

Overall, the obtained results indicate significant differences in biological responses and pollutant loads among the selected fish species and along the Northern Iberian shelf and reinforce the importance of selecting different sentinel species from different habitats for the assessment of pollution impact in the marine environment. The set of biomarkers used indicated different levels of stress in *L. boscii* and *T. luscus*, paralleling a coastal pollution gradient. The analysis of PAHs in bile gave an indication of recent exposure to 1-naphthol one year after the *Prestrige* oil-spill, particularly in fish from the Galician area (A & B). The study provides further support for the use of biomarkers in assessing the health of coastal areas, and shows the suitability of *L. boscii* and *T. luscus* as sentinel species for future pollution biomonitoring studies.

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Chapter 4. Mitochondrial Metabolism of 17α-Hydroxyprogesterone in Male Fish

4.

MITOCHONDRIAL METABOLISM OF 17α-HYDROXYPROGESTERONE IN MALE SEA BASS (*DICENTRARCHUS LABRAX*): A POTENTIAL TARGET FOR ENDOCRINE DISRUPTORS

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Resumo

O metabolismo de 17α-hidroxiprogesterona ($17P_4$) foi investigado em diferentes fracções subcelulares, isoladas a partir de gónadas masculinas de robalo (*Dicentrarchus labrax* L.). A existência de reacções catalisadas pela CYP17 (actividade de C17,20-liase) e CYP11B (11β-hidroxilase) foi demonstrada na fracção mitocôndrial, onde a $17P_4$ foi convertida em androstenediona (AD) e posteriormente esta foi metabolizada em 11β-hidroxiandrostenedione (*β*AD). A síntese de *β*AD predominou nas gónadas em fase inicial de maturação, indicando um papel importante na recrudescência testicular em peixes teleostos. Foi ainda, investigado o efeito *in vitro* de compostos químicos considerados modelo de disrupção endócrina (i.e. nonilfenol (NP), *p,p*'-DDE, benzo[a]antraceno (BaA), tributilestanho (TBT), e cetoconazol (KCZ)) no metabolismo mitocôndrial de $17P_4$. De entre os compostos testados, NP a 100 μM inibiu a actividade da enzima CYP17 (C17,20-liase), enquanto que KCZ a 100 μM inibiu ambas as enzimas, CYP17 e CYP11B. Foi demonstrado o potencial destes dois compostos químicos na disrupção do ciclo reprodutivo dos peixes que habitam ambientes poluídos, prejudicando a biosíntese testicular dos esteroides. Estes resultados sugerem ainda, que o metabolismo mitocôndrial de $17P_4$ pode constituir uma nova sonda para avaliar a disrupção endócrina em peixes.

Palavras-chave: robalo, mitocôndria, 17α-hidroxiprogesterona, nonilfenol, C17,20-liase, 11β-hidroxilase

Abstract

The metabolism of 17α-hydroxyprogesterone ($17P_4$) was investigated in different subcellular fractions isolated from male gonads of sea bass (*Dicentrarchus labrax* L). The existence of CYP17 (C17,20-lyase activity) and CYP11B (11β-hydroxylase) catalyzed reactions was demonstrated in the mitochondrial fraction, where $17P_4$ was converted to androstenedione (AD) and further metabolized to 11β-hydroxyandrostenedione (*β*AD). The synthesis of *β*AD predominated in early spermatogenic testis, indicating a role of *β*AD in testicular recrudescence. Additionally, the *in vitro* effect of model endocrine disrupting chemicals (i.e. nonylphenol (NP), *p,p'*-DDE, benzo[a]anthracene (BaA), tributyltin (TBT) and ketoconazole (KCZ)) on the mitochondrial metabolism of $17P_4$ was investigated. Among the tested compounds, 100 μM NP inhibited the activity of CYP17 (C17,20-lyase), whereas 100 μM KCZ inhibited both CYP17 and CYP11B. Both chemicals showed the potential to disrupt the reproductive cycle of fish living in polluted environments due to impairment of testicular steroid biosynthesis. These results suggest that mitochondrial metabolism of $17P_4$ may constitute a new sensitive probe for the assessment of endocrine disruption in fish.

Keywords: sea bass, mitochondria, 17α-hydroxyprogesterone, nonylphenol, C17,20-lyase, 11β-hydroxylase

1. Introduction

A number of both natural and/or man-made chemicals with widespread distribution in the environment may act as endocrine disruptors (EDs). Natural and synthetic hormones (e.g contraceptives), and many synthesised chemicals that are now suspected of unintentionally disrupting the endocrine system (e.g. organochlorine pesticides, PCBs, organotin compounds, PAHs, alklyphenols) may affect the reproduction and development of wildlife and therefore threaten the existence of susceptible species (Colborn *et al*., 1993).

In the last decades, most of the studies on EDs have focused on substances with estrogenic activity. For example, alkylphenolic compounds have been shown to bind to the estrogen receptor (ER) and induce the synthesis of vitellogenin (Vtg) in fish, one of the most sensitive responses to estrogen exposure and the most often used tool for the assessment of endocrine disruption in the aquatic environment (Jobling and Sumpter, 1993; Nilsen *et al*., 1998). Nonetheless, EDs can act through mechanisms other than binding to ERs. Recent studies have demonstrated that chemicals such as tributyltin (TBT) act as nuclear peroxisome proliferators activated receptors/retinoic X receptor (PPARs/RXR) ligands, unchaining a series of responses and alterations in the endocrine system of exposed organisms (Kanayama *et al*., 2005; Grün and Blumberg, 2006). EDs can also act at a non-genomic level, interfering with the synthesis and metabolism of steroids: i.e. nonylphenol (NP) inhibits the glucuronidation of testosterone and estradiol causing a rise in the levels of free active endogenous steroids (Lavado *et al*., 2004; Thibaut and Porte, 2004).

A complex series of enzymatic systems are responsible for the synthesis of sex steroids in fish. Those systems fall into two major classes of proteins: cytochrome P450 enzymes and hydroxysteroid dehydrogenases, located either in the mitochondrial or the endoplasmatic reticulum membranes (Omura and Morohashi, 1995; Payne and Hales, 2004). Among them, C17,20-lyase, also called cleavage P450c17 or CYP17, is a key enzyme that occurs in fish gonads and plays a key role in the conversion of 17α-hydroxyprogesterone (17P$_4$) to androstenedione (AD), which is a precursor of testosterone (T). In male gonads, both T and AD can be transformed into their respective 11-hydroxylated metabolites by 11β-hydroxylases (CYP11B), and further metabolized to 11-ketoandrostenedione (11-KAD) and 11-ketotestosterone (11-KT) by the 11β-hydroxysteroid dehydrogenase (11β-HSD) (Fig. 1). 11-KT is considered to be

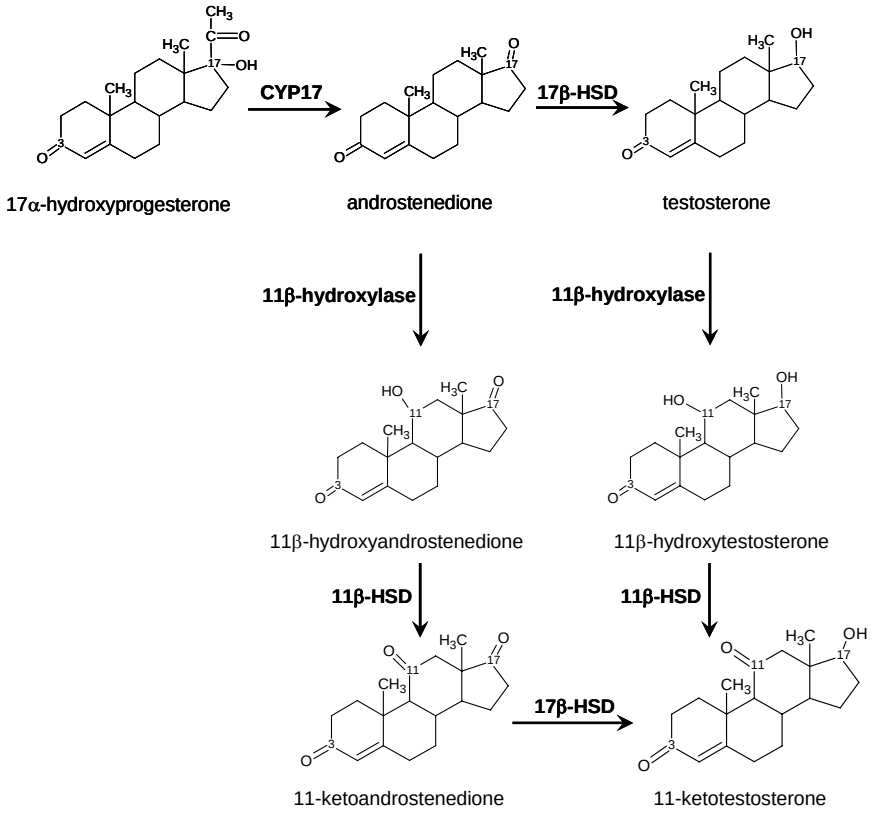

Figure 1. Schematic representation of the synthesis of 11-oxygenated androgens (C19 steroids) and the involved metabolic pathways in male fish gonads. CYP17: C17,20-lyase; 11β-hydroxylase: CYP11B; HSD: hydroxysteroid dehydrogenases.

the main androgen in male teleosts, being more effective than T in stimulating secondary sexual characters, influencing spermatogenesis and stimulating reproductive behaviour (Borg, 1994).

The impairment of steroid synthesis and steroid metabolism by environmental pollutants may alter levels of active hormones within the organism and be a potential mechanism of endocrine disruption. For example, ovarian tissue of flounder (*Platichthys flesus*) exposed to high doses of PAHs showed an inhibition of the steroidogenic enzymes C17,20-lyase (CYP17) and P450 aromatase (CYP19), leading to reduced secretion of AD and 17β-estradiol in vitellogenic ovarian tissue (Monteiro *et al*., 2000). Other studies have shown that NP and certain pesticides interfere with 20β-HSD and/or 17β-HSD activities in carp (*Cyprinus carpio*), these being key enzymes involved in the formation of 17α,20β-dihydroxyprogesterone (17,20βP) and T, respectively (Thibaut and Porte, 2004). Reduced synthesis of 11-oxygenated androgens (e.g. 11β-hydroxyandrostenedione) with a concomitant disappearance of spermatocytes was observed in the testis of sea bream (*Sparus aurata*) treated with estradiol (Condença and Canario, 2001). However, information regarding the interference of pollutants with the synthesis of 11-oxygenated androgens in fish and the consequences in terms of sexual maturation and reproduction is still scarce. The characterization of the enzymatic pathways involved in the synthesis of active androgens (e.g. CYP17, CYP11B, 17β-HSD, 11β-HSD) could offer new complementary tools for the assessment of reproductive disturbance in male fish from polluted environments.

In this context the present study was designed to characterize: (a) the gonadal metabolism of 17α-hydroxyprogesterone ($17P_4$) in male sea bass (*Dicentrarchus*

labrax) by different subcellular fractions (mitochondria, microsomes and 12,000*g* supernatant) and (b) the sensitivity of those enzymatic pathways to perturbation by model endocrine disruptors, namely TBT, NP, *p,p*'-DDE and benzo[a]anthracene (BaA). The antifungal ketoconazole (KCZ), a well known inhibitor of cythochrome P450 steroidogenic enzymes (Gal *et al*., 1994; Monteiro *et al*., 2000), was used as a positive control.

2. Material and Methods

2.1. Chemicals

[^{3}H]17α-Hydroxyprogesterone and [1β-^{3}H]androstenedione (15-30 Ci/mmol) were purchased from Perkin-Elmer Life Sciences (Boston, MA, USA), [4-^{14}C]testosterone (50-60 mCi/mmol) was from Amersham (Buckinghamshire, UK). Unlabelled steroids were obtained from Sigma (Steinheim, Germany) and Steraloids (Wilton, NH, USA). NADPH was obtained from Sigma (Steinheim, Germany). All solvents and reagents were of analytical grade from Merck (Darmstadt, Germany).

2.2. Organisms

Sea bass (*Dicentrarchus labrax*) were sampled from different aquaculture farms located in South of Portugal in March, June and December. Gonads were immediately dissected, deep-frozen in liquid nitrogen, and stored at –80ºC upon arrival at the laboratory. In order to classify the sexual maturation stage (SMS) of each fish, a

subsample from the central part of the gonad was fixed in 10% buffered formalin for histological examination.

2.3. Subcellular fractionation

Individual male gonads (0.5 g) were homogenized in ice-cold 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4 containing 0.15 M KCl, 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM phenylmethylsulfonylfluoride (PMSF). Homogenates were centrifuged at 500*g* for 15 min, the fatty layer removed and the supernatant centrifuged at 12,000*g* for 20 min. The resulting supernatant (S12-fraction) was freshly used for the biochemical assays and the pellet was resuspended in homogenization buffer and further centrifuged at 12,000*g* for 20 min; the resulting pellet, termed mitochondrial fraction, was freshly used for the biochemical assays. When S12-fraction was centrifuged at 100,000*g* for 60 min, the pellet, termed microsomal fraction, was resuspended in a small volume of 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4 containing 0.15 M KCl, 20% (w/v) glycerol, 1 mM DTT, 1 mM EDTA and 0.1 mM PMSF. Microsomes were stored a –80ºC until assays were performed. Protein concentrations were determined by the method described by Lowry *et al*. (1951), using bovine serum albumin as a standard.

2.4. Metabolism of 17α-hydroxyprogesterone

The metabolism of 17α-hydroxyprogesterone ($17P_4$) was assessed by incubating S12, mitochondrial and microsomal fractions (1 mg protein) in 50 mM Tris-HCl buffer

pH 7.4 with 0.2 μM [^{3}H]17α-hydroxyprogesterone (166,500 dpm), 10 mM $MgCl_2$ and 1 mM NADPH in a total volume of 250 μl. The reaction was initiated by the addition of NADPH and incubated in constant shaking for 30 min at 30ºC, and stopped by adding 250 μl of acetonitrile. After centrifugation (1,500*g*, 10 min), 200 μl of supernatant was injected into a reversed-phase HPLC column (RP-HPLC). To evaluate the *in vitro* effect of model pollutants on 17P$_4$ metabolism, the mitochondrial fraction was pre-incubated for 10 min at 25 ºC in the presence of TBT, NP, BaA, *p,p'*-DDE and KCZ at a concentration of 100 μM. The xenobiotics were delivered to the assay solutions in absolute ethanol; the concentration of ethanol in the assay was below 1%, and was kept constant in all the assays. Control mitochondria were incubated with the carrier alone.

2.5. Mitochondrial metabolism of androstenedione and testosterone

Androstenedione (AD) and testosterone (T) metabolism was assessed by incubating mitochondrial proteins (1 mg) in 50 mM Tris-HCl buffer pH 7.4 with 0.2 μM [^{3}H]androstenedione (166,500 dpm) or 1.7 μM [^{14}C]testosterone (111,000 dpm), 10mM $MgCl_2$ and 1mM NADPH in a total volume of 250 μl. The reaction was initiated by the addition of NADPH and incubated in constant shaking for 30 min at 30ºC. Incubations were stopped by adding 250 μl of acetonitrile and after centrifugation (1,500*g*, 10 min), 200 μl of supernatant was injected into a RP-HPLC column.

To evaluate the *in vitro* effect of 100 μM KCZ and NP on AD metabolism, the mitochondrial fraction was pre-incubated for 10 min at 25 ºC, using AD (0.2 μM) as the precursor. The chemicals were delivered to the assay solutions in absolute ethanol; the

concentration of ethanol in the assay was below 1%, and was kept constant in all the assays. Control mitochondria were incubated with the carrier alone.

2.6. HPLC system

HPLC analyses were performed on a Perkin-Elmer Binary 250 LC pump system equipped with a 250 x 4 mm LiChrospher 100 RP-18 (5 μM) reversed-phase column (Merck, Darmstadt, Germany) protected by a guard column LiChrospher 100 RP-18 (5 μM). Separation of metabolites was performed at 1mL/min with a mobile phase composed of A) 80% water, 10% acetonitrile and 10% methanol, and B) 50% acetonitrile and 50% methanol. The run consisted on a 28 min linear gradient from 60% A to 100% B, 28-33 min linear gradient from 100% B to 60% A and 33-43 min 60% A. Radioactive metabolite peaks were monitored by on-line radioactivity detection with a Radioflow detector LB 509 (Berthold Technologies, Bad Wildbad, Germany) using Flo-Scint III (Packard BioScience, Groningen, The Netherlands) as scintillation cocktail. Metabolites were quantified by integrating the area under the radioactive peaks. Identification of the metabolites was based on the comparison of their retention times to those obtained for standards commercially available and monitored at 254 nm using a UV-detector (Knauer 2500 LC-photometer), and further confirmed by gas chromatography-mass spectrometry (GC-MS).

2.7. Gas chromatography-mass spectrometry

Identification of metabolites was conducted by gas chromatography-mass spectrometry (GC-MS) (El+) by comparison of the retention time and the mass spectra with authentic standards. Mass spectra were obtained on a Fissons GC 8000 Series chromatograph coupled to a Fissons MD800 mass spectrometer fitted with a HP-5MS (30 m x 0.25 mm i.d., crosslinked 5% PH ME siloxane) column (Hewlett-Packard). The carrier gas was helium at 1 mL/min. The oven temperature was programmed as follows: 90-140ºC at 12ºC/min and from 140 to 320ºC at 6ºC/min. The injector temperature was 280ºC and the ion source and the analyzer were maintained at 200 and 250ºC, respectively.

2.8. Histological analysis

Gonads were fixed in 10% buffered formalin for 24 hours, dehydrated through a graded ethanol series and embedded in paraffin. Tissue sections (6 μm thick) were stained with hematoxylin-eosin. Sexual maturation stages (SMS) were classified as described in Goodbred *et al*. (1996).

2.9. Data analyses

Enzymatic activities were determined individually in 4 to 8 organisms per assay and run in duplicate. Values are presented as mean ± SEM. Statistical significance was assessed using one-way ANOVA with Tukey's test (for differences between SMS) and

one-way ANOVA Dunnett's test for differences from control (inhibition studies). All statistics were analysed using the software package SPSS/PCTM, version 12.0 (SPSS Inc., Chicago, IL). Level of significance was $P < 0.05$.

3. Results

3.1. $17P_4$ metabolism in different subcellular factions

The metabolism of $17P_4$ was investigated in different subcellular fractions extracted from male gonads of sea bass (mitochondrias, microsomes and S12) (Fig. 2). In the presence of NADPH, mitochondrial fraction readily converted $17P_4$ to an unidentified polar metabolite (UNK1), which represented 38% of the total radioactivity count, eluting at approximately 8.48 min. A second major metabolite (35%) was formed and tentatively identified as androstenedione/testosterone (AD/T) by comparison to the retention time of commercial standards, which were shown to co-elute (RT = 13.60 min) in the chromatographic system. A third metabolite, UNK2 (RT = 19.50 min) represented 12% of the radioactivity count (Fig. 2A). No metabolites were formed in the absence of NADPH.

In order to identify the metabolites derived from the mitochondrial incubations, peaks were monitored and collected from the HPLC/UV_{254} system, and the identification was accomplished by GC-MS. Thus, the AD/T peak was actually identified as androstenedione (AD). The UNK1 metabolite was identified as 11β-hydroxy-4-androstene-3,17-dione (*β*AD). Both metabolites were identified by comparison to the mass spectrum of the authentic steroid standards. Unfortunately the

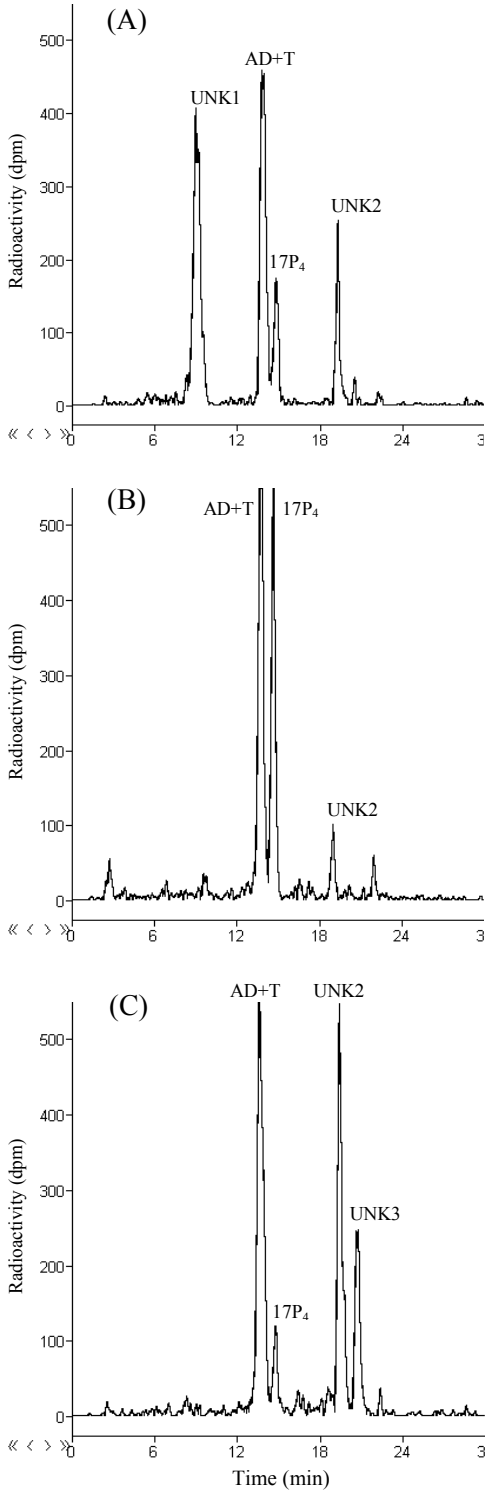

Figure 2. HPLC radiochromatograms of 17α-hydroxyprogesterone (17P$_4$) metabolism by different subcellular fractions isolated from sea bass testis. (A) mitochondria; (B) microsomes; (C) S12 fraction. AD+T: identified as AD; UNK1: identified as *β*AD.

UNK2 metabolite could not be successfully identified. To reinforce these findings, further incubations were performed using AD or T as substrates. When the mitochondrial fraction was incubated in the presence of AD and NADPH, we observed the formation of βAD. However, no metabolites were observed when incubations were performed with testosterone (T).

The metabolism of $17P_4$ in testicular microsomal fractions was less active than in mitochondria. The major metabolite formed, (60% of the total radioactivity count), corresponded to AD/T, whereas UNK2 was formed in a relatively small amount (5%) (Fig. 2B). The AD/T peak formed by the microsomal fraction was also identified as androstenedione (AD).

As for the S12 fraction, $17P_4$ was metabolized to two major metabolites (Fig. 2C), AD/T (41%) and UNK2 (30%). Furthermore, a smaller, but well defined, metabolite UNK3 (RT = 20.70 min), representing 14% of the total radioactivity count, was also formed but could not be identified.

3.2. Mitochondrial metabolism of $17P_4$ in male sea bass

The metabolism of $17P_4$ by mitochondrial fractions isolated from male gonads of sea bass was assessed at different SMS. Representative HPLC chromatograms together with the corresponding histological sections are presented in Figure 3. The major metabolites observed in individuals with undeveloped testes (SMS-0) were AD (39% of the total radioactivity count) and the unknown metabolite UNK2 (28% of the total radioactivity count). The peak corresponding to βAD represented 7% of the total radioactivity count (Fig. 3A). A different metabolic profile was observed in individuals

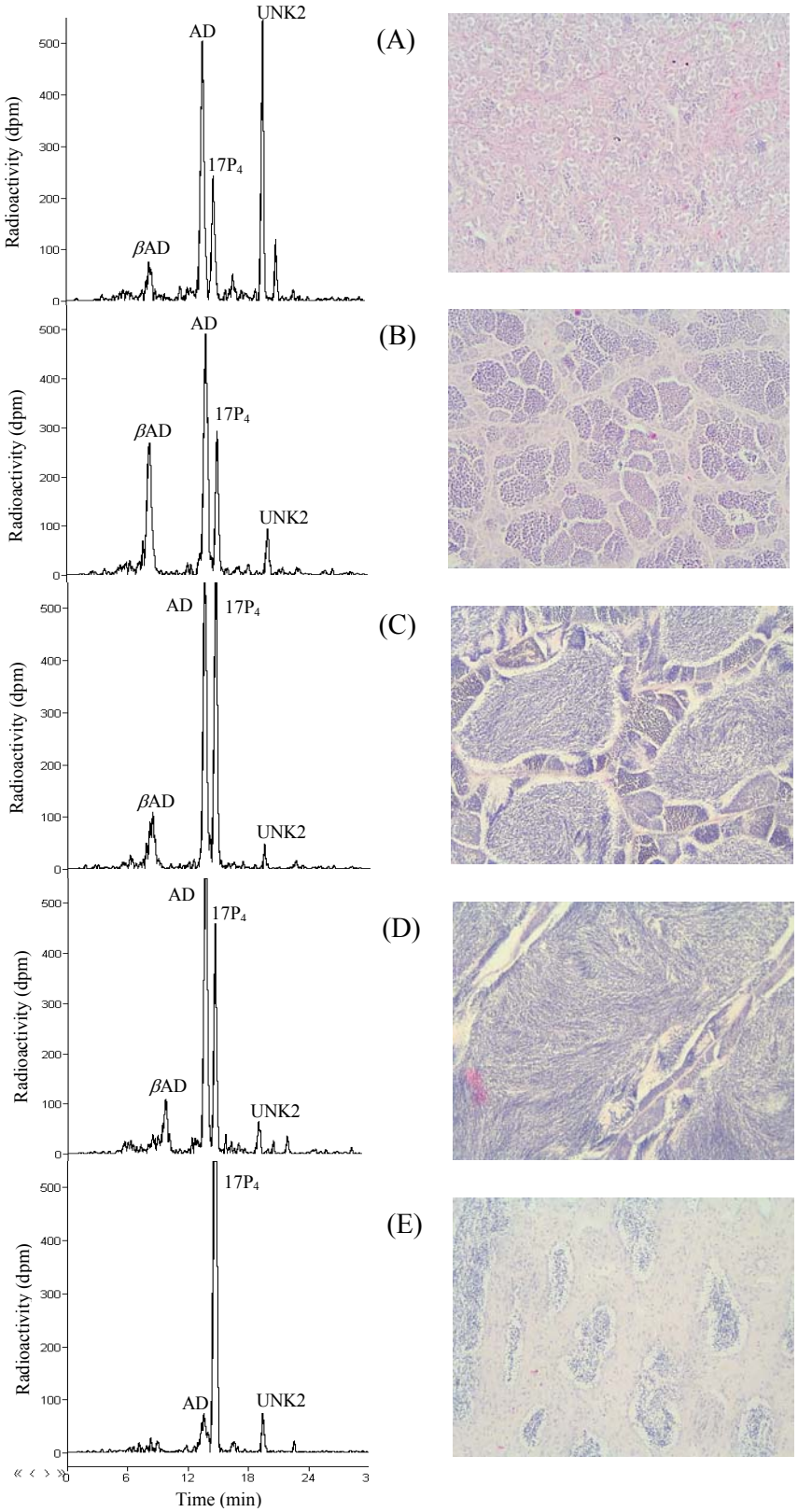

Figure 3. Representative HPLC radiochromatograms of the mitochondrial metabolism of 17P$_4$ in gonads of sea bass sampled at different sexual maturation stages (SMS). (A) undeveloped, SMS-0; (B) early-spermatogenic, SMS-1; (C) mid-spermatogenic, SMS-2; (D) late-spermatogenic, SMS-3; (E) post-spawning, SMS-4 (magnification 100 x). Tissue sections (6μM) were stained with hematoxylin-eosin.

with testes classified as early spermatogenic (SMS-1); those testes were characterized by having thick germinal epithelium and diffuse pronounced proliferation and maturation of spermatozoa (Fig. 3B), and formed a higher amount of βAD (28%) together with AD (39%) and a small amount of UNK2 (5%) than undeveloped tests. Testes classified as mid spermatogenic (SMS-2) that contained a mix of spermatocytes, spermatids and spermatozoa, indicated AD as the prevalent metabolite (42%), whereas both βAD and UNK2 were less abundant, representing 15% and 2% of the total radioactivity count, respectively (Fig. 3C). The same profile was observed in individuals classified as SMS-3 (late-spermatogenic), with mostly thin germinal epithelium and scattered spermatogenic activity, characteristic of full-grown testes (Fig. 3D). Thus, AD was the major metabolite (47%), meanwhile βAD and UNK2 represented only 9% and 4% of the total radioactivity count. For individuals with testes in the regression phase (post-spawning, SMS-4), a low metabolic rate of $17P_4$ was observed with 11% of AD, 5% of UNK2 and no peak of βAD (Fig. 3E).

Table 1 summarizes the formation rate of each metabolite with respect to SMS. The highest formation of 11β-hydroxy-4-androstene-3,17-dione (βAD) was observed in individuals at SMS-1 (14.6 ± 2.2 pmol/h/mg protein), whereas those at SMS-0 indicated a higher formation of the metabolite UNK2 (18.7 ± 5.5 pmol/h/mg protein) (P <0.05). Meanwhile, low amounts of androstenedione (AD) were formed by individuals with testes in the regression phase (SMS-4). Overall, the metabolism of $17P_4$ decreased significantly with increasing maturation of the gonads, going from 70-77% (SMS-0 and 1) to 24% in SMS-4.

Table 1. Metabolism of 17P$_4$ by the mitochondrial fraction isolated from sea bass testis at different sexual maturation stages: undeveloped (SMS-0), early-spermatogenic (SMS-1), mid-spermatogenic (SMS-2), late-spermatogenic (SMS-3), post-spawning (SMS-4); formation rate of the different metabolites in pmol/h/mg protein. Values are mean ± SEM (n = 4). *Significant differences between SMS ($P < 0.05$).

Male gonad (sea bass)	SMS-0	SMS-1	SMS-2	SMS-3	SMS-4
11β-hydroxy-4-androstene-3,17-dione	4.1 ± 1.0	14.6 ± 2.2*	5.6 ± 0.1	3.1 ± 0.8	n.d.
Androstenedione	16.7 ± 1.7	18.6 ± 4.2	20.2 ± 4.5	14.0 ± 3.2	5.1 ± 0.1*
UNK2 metabolite	18.7 ± 5.5*	3.8 ± 0.6	1.2 ± 0.2	2.0 ± 0.1	2.8 ± 0.2
Total metabolism	39.6 ± 4.7	35.6 ± 6.5	27.0 ± 4.2	19.1 ± 2.3	7.8 ± 0.4*

n.d.: not detected

3.3. Effects of model pollutants on 17P$_4$ metabolism

The *in vitro* effect of model pollutants on 17P$_4$ metabolism is shown in Figure 4. BaA, TBT and *p,p'*-DDE (tested at a concentration of 100 μM) did not significantly alter the metabolism of 17P$_4$ in male gonads, although TBT lead to a slight decrease in the formation of AD (10%) and *β*AD (18%). In contrast, 100 μM nonylphenol (NP) and ketoconazole (KZC) had a strong inhibitory effect; the synthesis of AD was decreased by 50% and 74%, respectively, indicating an inhibition of the CYP17 enzyme (C17,20-lyase activity). Regarding *β*AD, KCZ completely inhibited its formation whereas NP led to a 56% inhibition which might be due to the observed decrease in the formation of AD. In fact, when AD was used as precursor instead of 17P$_4$ and incubated in the presence of KCZ and NP, we observed that KCZ completely inhibited the formation of *β*AD whereas no significant effect was observed for NP. Thus, these

results further indicate a specific inhibition of CYP11B by KCZ but not by the other compounds tested. None of the selected pollutants altered the synthesis of the UNK2 metabolite, although *p,p*'-DDE lead to a slight decrease in its formation (10%).

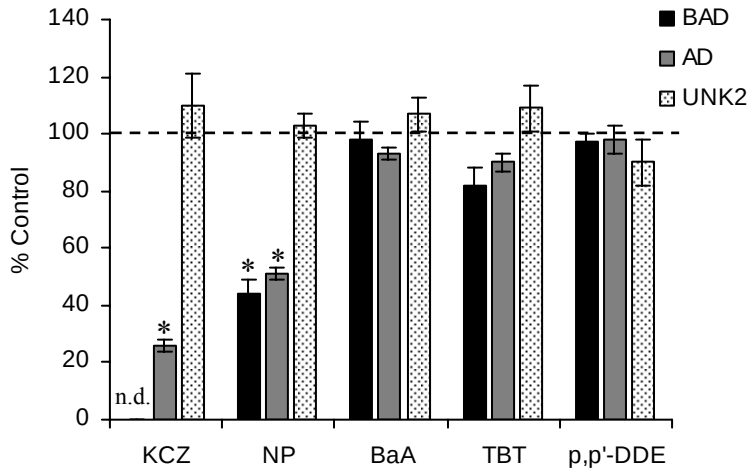

Figure 4. Effect of KCZ, NP, BaA, TBT and *p,p'*-DDE (100 µM) on the mitochondrial metabolism of $17P_4$ in male gonads of sea bass. Values are mean ± SEM (n = 8). n.d.: not detected. *Significant differences relative to the control ($P < 0.05$).

4. Discussion

The rate-limiting step in the biosynthesis of steroid hormones is the transport of free cholesterol from the cytoplasm into the mitochondria. Within mitochondria, cholesterol is converted by the P450-associated side chain cleavage enzyme (P450scc) into pregnenolone, the starting precursor for the synthesis of all of the steroid hormones (Devlin and Nagahama, 2002). Pregnenolone diffuses across the mitochondrial membranes and may then be further metabolized by two other cytochrome P450-associated enzymes, 17α-hydroxylase/C17,20-lyase (CYP17) and 11β-hydroxylase (CYP11B), that catalyse key steps in the further metabolism, of pregnenolone to the 11-oxygenated androgens (C19 steroids) that are characteristic of teleost fish

(Schulz *et al*., 2001). The catalysis of each of these reactions requires a pair of electrons, which is donated by the flavoprotein NADPH transfer system (Miller, 2002).

Although in the literature the CYP17 enzymes are often associated with membranes of the endoplasmatic reticulum (Omura and Morohashi, 1995; Schulz *et al*., 2001; Miller, 2002; Payne and Hales, 2004), in the present study enzymes leading to the biosynthesis of active steroids were detected in the mitochondrial fraction, where 17P$_4$ was metabolized to androstenedione (C17,20-lyase catalyzed pathway) and further converted to 11β-hydroxy-4-androstene-3,17-dione (CYP11B). CYP17 (C17,20-lyase activity) was also detected in the microsomal fraction with a higher specific activity (57.7 ± 3.2. pmol/h/mg protein) than in mitochondria (17-33 pmol/h/mg protein). No metabolism occurred in the absence of NADPH.

On the other hand, CYP11B or 11β-hydroxylase activity was only detected in the mitochondrial fraction. This enzyme catalyses the hydroxylation of androgen steroids, in this case AD, at their C_{11} position, in the presence of molecular oxygen, giving rise to the formation of *β*AD. The formation of *β*AD was significantly high at the initial stages of maturation (SMS-1), which is consistent with the role of 11-oxygenated androgens in testicular recrudescence and spermatogenesis in many teleost fish (Borg, 1994; Miura *et al*., 1996). The formation rate of both *β*AD and AD significantly decreased at the later stages of maturation (post-spawning), which may indicate that, even though androgens are necessary, later stages of spermatogenesis do not demand very high levels of androgens. This is in agreement with data by Schulz and Brüm (1990) that found the highest production of androgen per weight of testes at very early stages of spermatogenesis in rainbow trout. *β*AD was the major 11-oxygenated male

androgen synthesised in fragments of gonadal tissue by several other teleost species (Guiguen *et al*., 1995; Cavaco *et al*., 1997; Condença and Canario, 2001).

No synthesis of 11-KT, the main androgen in male teleosts, could be detected when $17P_4$ was incubated with either mitochondrial or microsomal fractions of male gonads. However, the formation of a peak coinciding with the retention time of commercial 11-KT standard was observed when liver microsomal fractions were incubated with βAD; the synthesis of this peak was not dependent on the SMS contrary to that previously observed for βAD (data not shown). The formation of 11-KT involves the presence of hydroxysteroid dehydrogenses (11β-HSD; 17β-HSD) found as membrane-bound enzymes (Payne and Hales, 2004). It has been often hypothesised that βAD maybe converted to 11-KT at extra-testicular sites. Thus, 11B-HSD activity has been detected in the liver of rainbow trout (Schulz, 1986) and in blood cells of different fish species (Mayer *et al.*, 1990). *In vitro* incubations with labelled βAD showed that catfish liver tissue produced significant amounts of 11-KT (Cavaco *et al*., 1997).

EDs can exert their effects at a variety of sites on the hypothalamus–pituitary–gonadal axis and alter the reproductive development and/or behaviour of susceptible organisms. Among the compounds tested in this study, only KCZ and NP inhibited the metabolism of $17P_4$ in gonads of sea bass. This is in contrast with data by Monteiro *et al*. (2000) that reported a strong inhibition of C17,20-lyase in ovary of flounder exposed *in vitro* to several PAHs (phenanthrene, chrysene, benzo[a]pyrene) at a concentration of 15 μM. In the present study, C17,20-lyase activity in testicular tissue of sea bass was not affected by BaA (100 μM). Nevertheless, the use of different tissues and/or subcellular fractions must be taken into account, since Monteiro *et al*. (2000) worked with minced ovarian tissue while we used testicular mitochondrial fraction.

Inhibition of both CYP17 and CYP11B enzymes was observed in incubations treated with KCZ, while NP inhibited the CYP17 enzyme (C17,20-lyase pathway) and had no detectable effect on CYP11B. This is in agreement with other findings that report KCZ to inhibit C17,20-lyase activity in human adrenals (tests and ovary), in flounder ovary and in rat testis (Weber *et al*., 1991; Ahmed *et al*., 1995; Monteiro *et al*., 2000). Moreover, inhibitory effects of KCZ on other cytochrome P450 enzymes (CYP19; P450scc; CYP1; CYP2; CYP3) in fish and humans has also been reported (Gal *et al*., 1991; Miranda *et al*., 1998; Monteiro *et al*., 2000). Regarding NP, it has been shown to inhibit CYP17 (17α-hydroxylase) in human adrenocortical cells (H295R) (Nakajin *et al*., 2001), and to induce the activity of 20β-HSD in carp ovaries, increasing the synthesis of 17,20βP, which is known to act as maturation-inducing hormone and as a pheromone (Scott and Sorensen, 1994; Nagahama, 1997; Thibaut and Porte, 2004). Thibaut and Porte (2004) also reported that NP affected the metabolic clearance pathway of hormones by inhibiting the glucuronidation of estradiol and testosterone. Furthermore, the inhibition and/or induction of several cytochrome P450 monooxygenases (e.g. CYP1; CYP2 and CYP3) by NP and octylphenol (OP) has been reported (Lee *et al*., 1996a,b; Hanioka *et al*., 1999).

In summary, this study showed the occurrence of key enzymatic steps for the biosynthesis of active steroids in the mitochondrial fraction of male sea bass gonads, where 17P$_4$ was readily converted to βAD in the presence of NADPH. The metabolism of 17P$_4$ varied according to the SMS of the organism; the synthesis of βAD was elevated at the initial stages of sexual maturation (SMS-1), which confirms its importance in testicular recrudescence in male sea bass. Furthermore, the synthesis of oxysteroids was greatly reduced in the presence of KCZ and NP, both inhibited

C17,20-lyase activity, but only KCZ had a strong inhibitory effect on CYP11B. To our knowledge this is the first report on the effect of NP on C17,20-lyase activity in fish gonads; the inhibition of this key step on the synthesis of androgens may lead to disturbances in spermatogenesis, reproductive behaviour and development of secondary sexual characters. Finally, the assessment of the mitochondrial metabolism of $17P_4$ may constitute a new complementary probe for the assessment of endocrine alteration in male fish.

Acknowledgements

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Chapter 5. General Discussion

5.1. Assessment of pollution in cultured and wild sea bass

Marine fish culture is increasing in the Atlantic and Mediterranean regions, working in parallel with traditional fisheries. Sea bass (*Dicentrarchus labrax*) is one of the main species cultured, and in the last years has achieved high production levels and high economic value in European Countries (FAO, 2003). Although, both cultured fish and wild fish are important components of the Atlantic and Mediterranean diet, the extensive use of chemicals (i.e. therapeutants, antibiotics, antifoulants) in current aquaculture practices have raised concerns over the quality of cultured fish in comparison to wild fish. Despite of these concerns, information on the levels of pollutants to which cultured fish are exposed to, their bioaccumulation and their toxicological consequences is still limited. Therefore, in this chapter a particular emphasis was placed to investigate pollutant exposure to a broad range of contaminants together with several biochemical responses in both wild (from the Arade Estuary) and cultured sea bass, from different aquaculture facilities located in Southern Europe, as screening tools in aquaculture practises.

5.1.1. Metal exposure and MT

In general, levels of hepatic Cu were higher in cultured sea bass than in wild specimens, with the exception of those collected in the area influenced by the marina (A1) of the Arade Estuary (Fig. 1A). Moreover, residues of Cu in the liver of cultured sea bass (104-1000 μg/g dry weight) were 2-3 orders of magnitude higher than those measured in the two commercial wild species, *T. luscus* and *L. boscii*, from the

Northern Iberian shelf (5-30 μg/g dry weight) (Chapter 3). Although essential metals (e.g. Cu & Zn) are present in pellet feed and may be a source of metal exposure due to solid waste and degradation, the high concentrations of Cu detected in cultured fish from sites FF (ponds) and RC (net cages) suggest an additional exposure to Cu in the aquaculture facility. Therapeutants and antifoulants based on Cu are common in the aquaculture industry. Copper sulphate ($CuSO_4$) is indicated for the treatment of algae and various ectoparasitic infestations (Straus, 1993), while copper-based antifouling paints are used to treat cage netting in order to prevent the attachment of fouling organisms (Katranitsas *et al*., 2003). Although the main source of contamination in cultured fish from RC remains unknown, exposure to $CuSO_4$ (three weeks before sampling) to treat an ectoparasitic infection of *Amydodinium ocellatum* is the source of Cu in FF. Although, Cu is essential for fish metabolism; when its intake is excessively high, toxic or adverse effects in the organism occur. Several studies have shown Cu to be immunosuppressive in fish (Zelikoff, 1993; Schlenk *et al*., 1999), and to act through a general stress response increasing the production of cortisol (Carballo *et al*., 1995).

Interestingly, both cultured (FF, RC) and wild sea bass (A1) with high Cu residues in the liver showed an infection in gonads by the myxosporean parasite *Sphaerospora testicularis*. Pollutant exposure can lead to immunodepression, which could result in increased susceptibility of polluted fish to parasitic infections (Hecker and Karbe, 2005). However, further studies are needed to determine whether therapeutic treatment of cultured fish with $CuSO_4$ and/or exposure to antifouling paints may impede resistance to parasites or diseases. Furthermore, macrophage aggregates were observed in the gonads of fish from RC, and this proliferation in several fish tissues has been associated with adverse factors, among them: nutritional imbalances,

infectious diseases and parasite infestations, toxicant-induced hemolytic anemia's, heat stress and sediment contamination (Wolf and Wolfe, 2005).

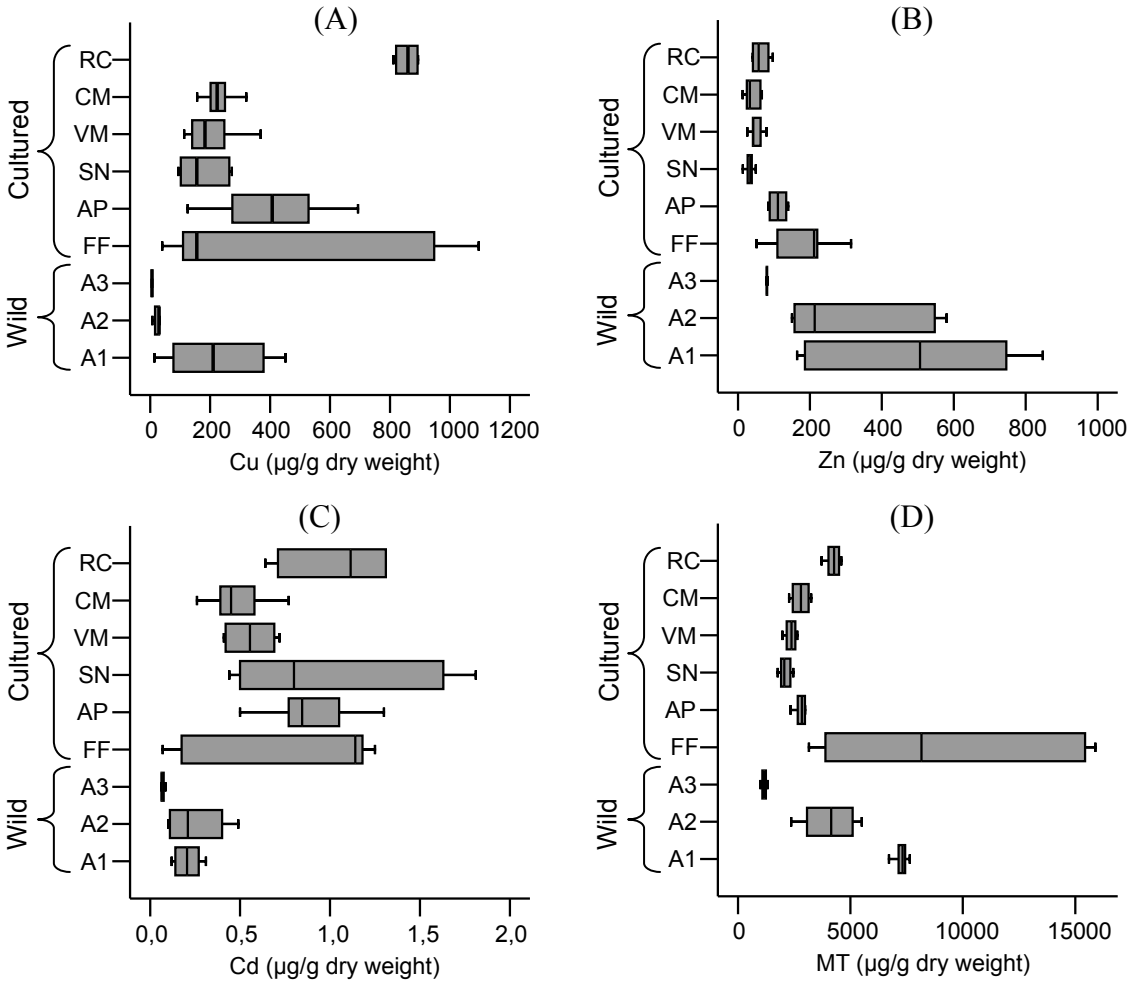

Figure 1. Range levels of metals (Cd, Cu and Zn) and metallothionein (MT) detected in liver of cultured and wild sea bass. Cultured sea bass were sampled from several aquacultures located in Southern Europe and wild sea bass were collected from the Arade Estuary. (A) Copper; (B) Zinc; (C) Cadmium and (D) Metallothionein, (Chapter 2).

The increasing anthropogenic use of Cu, Zn and other metals may certainly contribute to an increase in its environmental concentrations (Schiff *et al*., 2004; Sánchez-Bayo and Goka, 2005). Levels of hepatic Zn were high in wild fish than in cultured specimens (Fig. 1B). Residues of Zn and Cu were particularly higher in sea bass from site A1, probably associated with the use of these metal ions in

antifouling paints on vessels hulls and in harbour infrastructures. In fact, high levels of these essential trace metals have been reported in water and sediments from harbours and marinas (Osman *et al*., 1995; Schiff *et al*., 2004). While Cu and Zn are essential metals, others such as Cd are potentially toxic and pose a serious risk for human health when entering into the food web (Rojas *et al*., 1999). Intriguingly, residues of hepatic Cd were higher in cultured sea bass than in wild specimens, particularly in those cultured in net cages (RC & SN), indicating the importance of water pollution in the process of metal accumulation in the cultured specimens (Fig. 1C). The levels of Cd in cultured sea bass were in the high range of those detected in the liver of the two wild commercial species (0.35 and 1.5 μg/g dry weight) collected along the Northern Iberian shelf (Chapter 3). Nonetheless, with regard to food consumption, the metal concentrations in the muscle tissue of cultured sea bass were below the recommended FAO maximum limits for commercial species (Cd: 0.5 mg/kg, Cu: 30 mg/kg, and Zn: 30 mg/kg).

Regarding tissue distribution, the highest concentration of each metal, was observed in the liver, followed by the kidney, and the lowest in the muscle tissue. Liver tissue appears to be the major storage site for metals, therefore hepatic metal concentrations may be reflective of recent accumulation and may be a useful indicator of recent exposure. In agreement with hepatic metal residues, MT levels were significantly high in the liver of specimens from FF, RC and A1 that showed the highest Cu residues (Fig. 1D). In fact, hepatic MTs were positively correlated with Cu residues in the liver (r = 0.971; r = 0.987; P<0.01). These results support the use of MT as a biomarker of exposure to relatively high concentrations of Cu, and reveal once more the role of MT in metal detoxification in fish.

5.1.2. Estrogenic compounds and Vtg

Although concentrations of nonylphenol (NP) were present in the bile of cultured specimens, indicating recent exposure to alkylphenolic compounds (APEs), the highest levels of NP were observed in bile of wild specimens, particularly in the upper sites (A2 & A3) of the Estuary, subjected to the proximity of the sewage treatment plant (STP) (Fig. 2). Alkylphenol polyethoxylates (APEs) are an important class of non-ionic surfactants that are widely used in many detergent formulations and plastic products for industrial and domestic use, being NP the main breakdown product of APEs occurring in STPs and much more toxic and persistent than the parent compound (White *et al*., 1994; Tyler *et al*., 1998). NP and other APEs have raised reasonable concern because they represent a potential health risk to fish and other aquatic biota and also to humans. NP exerts an estrogenic effect at environmentally realistic concentrations and has been recognized as an endocrine disruptor chemical (Purdom *et al*., 1994; Jobling *et al*., 1996; Lye *et al*., 1999).

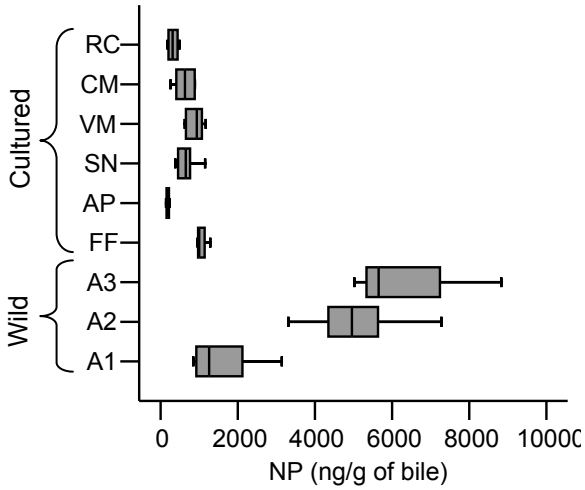

Figure 2. Range levels of nonylphenol (NP) detected in bile of cultured and wild sea bass. Cultured sea bass were sampled from several aquacultures located in Southern Europe and wild sea bass were collected from the Arade Estuary, (Chapter 2).

APEs have been shown to bind to the estrogen receptor and induce the synthesis of vitellogenin (Vtg) in fish (Jobling and Sumpter, 1993). Unfortunately, the measurement of Vtg was not assessed in wild specimens in this thesis. Nonetheless, Vtg levels in sea bass kept under laboratory conditions (BC) indicated significantly higher concentrations of NP in bile (1554 ± 57 ng/g) than cultured sea bass (360-898 ng/g). Thus, the detection of high concentrations of Vtg circulating in the plasma of male sea bass from BC (see Fig 3 in chapter 2, pag. 89) strongly suggested the presence of estrogenic compounds in the water of the laboratory facility, pumped from the Barcelona's coast. Low background levels of Vtg are likely to be normal in males (Gross, *et al*., 2003) and only significantly increases above background levels (0.2 μg/ml in cultured sea bass), as observed in specimens from BC (100 μg/ml), are indicative of estrogenic exposure. Interestingly, the levels of NP previously reported in wild specimens were significantly higher (up to 5-fold) than those detected in the bile of laboratory fish, which leads us to suspect that wild sea bass from the Arade Estuary may be suffering from endocrine alterations. However, future research will be necessary to confirm this hypothesis.

5.1.3. Organochlorinated compounds (OCs) and EROD activity

In previous studies higher levels of PCBs (26-36 ng/g d.w.) and DDTs (22-39 ng/g d.w.) in cultured sea bass than in wild specimens from Northern Portugal, was reported (Antunes and Gil, 2004). However, in the present study levels of PCBs (7.0 -19 ng/g d.w.) and DDTs (1.4 – 8.6 ng/g d.w.) were in the same range as those reported in wild sea bass from Northern Portugal. On a consumer point of view, fish

from aquaculture CM were the most contaminated, thus showing the highest residues of organochlorine compounds (PCB, DDTs, HCB) in muscle tissue. Nonetheless, these levels were lower than environmental agencies and FAO/WHO recommended values for commercial species (see Annex).

Commercial pellets have been suggested as the major source of PCBs and other OCs in cultured specimens, this together with the higher lipid content of cultured in comparison to wild specimens can lead to a rather high bioaccumulation of lipophilic persistent pollutants in the former (Easton *et al*., 2002; Hites *et al*., 2004). In the present study, when OCs were analysed in commercial pellets, no significant differences in OCs residues between pellets feed from the different aquacultures facilities were observed, and levels were lower (8-10 fold) than those reported in the muscle tissue. Moreover, cultured specimens from RC with a higher CF indicated the lowest levels of OCs in muscle, suggesting that water (aquaculture location) does account as well as a source of contaminants for the analyzed specimens. These findings were reinforced by those specimens kept under laboratory conditions (BC), and fed with the same commercial pellet as the others, that indicated the highest levels of DDTs, OH-PAHs and APEs, reflecting the poor quality of the coastal water of the city of Barcelona (Escartín and Porte, 1999a; Porte *et al*., 2002). Therefore, the relatively high PCBs residues in fish from CM might mirror the water quality of this specific aquaculture rather than a specific input of PCBs through contaminated food. In fact, this aquaculture does drain water from an estuary system pressured by urban and industrial effluents.

Interestingly, individuals from CM had significantly low hepatic EROD activity despite of the relatively high PCBs residues in their tissue (Fig. 3). Despite that EROD activity is commonly used as a biomarker of exposure to PCBs and PAHs,

positive correlations between piscine EROD activity and those pollutants can not always be expected, since in the natural environment fish are exposed to complex mixtures of contaminants that can act both as inducers and inhibitors of CYP1A (Burton *et al*., 2002). Meanwhile, in agreement with PAHs exposure data, high EROD activity was recorded in the liver of wild sea bass, mainly in those from the area influenced by the marina (A1), which confirms the presence of CYP1A inducing agents in the Estuary (Fig. 3).

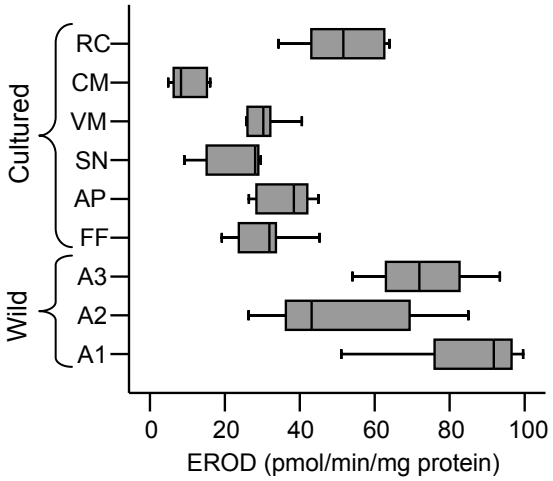

Figure 3. Range levels of hepatic EROD activity detected in cultured and wild sea bass. Cultured sea bass were sampled from several aquacultures located in Southern Europe and wild sea bass were collected from the Arade Estuary, (Chapter 2).

5.1.4. Other biological responses

Considering the other biochemical markers analysed in this study, it is worth mentioning that phase II enzymes were less responsive to pollutant exposure than EROD, and this is in agreement with several studies that have reported no changes in GST activities or a decrease in UGT activity of different species exposed to complex mixtures of pollutants (Soimasuo *et al*. 1995; Fenet *et al*., 1998; Tuvikene *et al*., 1999; Teles *et al*., 2004; Lavado *et al*., 2006). A significant decrease of CAT activity was

observed in cultured sea bass from site FF, possibly related to high metal exposure (e.g Cu & Cd). CAT has been shown to be either induced or inhibited by metals, depending on the dose, the species and/or the route of exposure (Palace *et al*., 1992; Paris-Palacios *et al*., 2000; Dautremepuits *et al*., 2002). A 50% inhibition of AChE activity was reported in muscle tissue of both wild (A1) and cultured (FF) sea bass, whereas no differences were observed in the brain. These results suggest that AChE in sea bass is more sensitive in muscle tissue than in the brain, and that not only pesticides, but metals may have an inhibitory effect on AChE activity. Other studies have reported depressed AChE activity in the muscle of sea bass (*D. labrax*) caged in a harbour area (Stien *et al*., 1998), and in the muscle of wild carp (*C. carpio*) exposed to high levels of Cd (Lavado *et al*., 2006), or experimentally exposed to $CuSO_4$ (Szabó *et al*., 1992).

5.2. Assessment of pollution along the Northern Iberian shelf

The analysis of contaminants in two different fish species provided data on pollutants bioavailability/exposure in different shelf environments (70-120 m and 200-500 m depth). The data indicated the presence of different pollutants in the region, and significant differences, both qualitative and quantitative, among sampling sites, that will be discussed below.

5.2.1. Metals

The analysis of metals indicated high residues in the liver of *L. boscii* (up to 10-fold) in comparison to *T. luscus*, which might be associated to its benthic

habitat but also to a high bioavailability of metals in the middle/outer shelf (200-500 m). In fact, it is often stated that sediments constitute the most important reservoir or sink of metals and other pollutants in the aquatic environment, and *L. boscii* lives in close contact with the sediment. However, the effectiveness of metal uptake varies in relation to contamination gradients of water, sediment and food, as well as ecological needs, swimming behaviour and metabolic activity of species (Kalay *et al*., 1999; Filipović and Raspor, 2003; Çoğun *et al*., 2006). Moreover, the results evidenced different geographical patterns of metal inputs along the studied area (see Fig. 4).

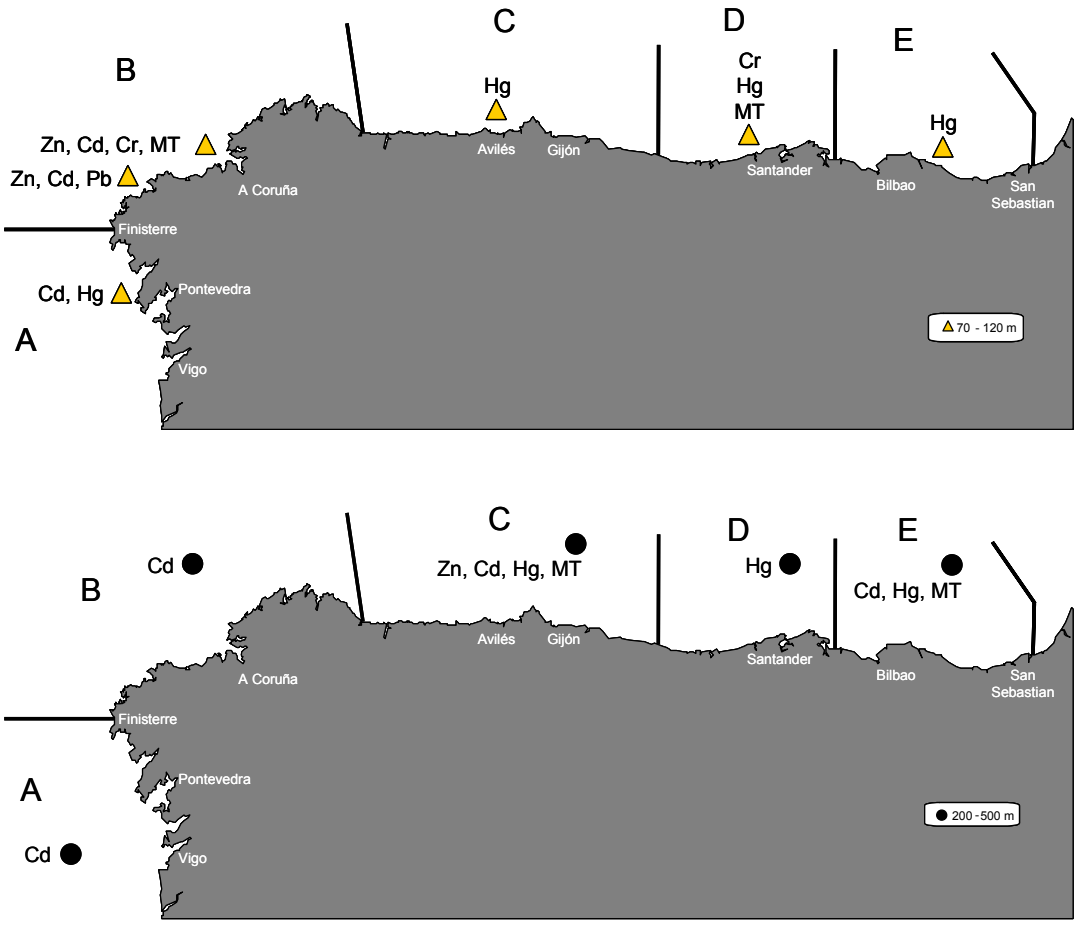

Figure 4. Map depicting significant levels ($P<0.05$) of metals and MT in liver of (△) *Trisopterus luscus* and (●) *Lepidorhombus boscii* collected along the Northern Iberian shelf. A – S Galicia; B – N Galicia; C – Asturias; D – Cantabria; E – Basque Country, (Chapter 3).

While *L. boscii* reported high residues of Cd in the liver, all along the Northern Iberian middle/outer shelf (areas A, B, C, E), in *T. luscus* (inner shelf) the highest levels of Cd were mostly found in the Galician areas (A & B), up to 15-fold higher than in area E (Basque Country). Furthermore, high concentrations of Pb (up to 25-fold) were detected in *T. luscus* from N Galicia, which was not observed in *L. boscii*, thus suggesting a decrease in the concentration of Pb with depth and evidencing the anthropogenic origin of Pb from coastal urban settlements. These findings are in agreement with Cd and Pb enrichment, previously reported, in water and sediments of some Galician Rias and associated to industrial activities and mining sewage inputs (Prego and Cobelo-Garcia, 2003). In this context, although Cd and Pb were among the trace metals present in the *Prestige* fuel oil, which wrecked in the vicinity of the Galician coast, the levels of Pb and Cd detected in the present study cannot be directly associated to the *Prestige* accident.

Meanwhile, elevated residues of Cr were reported in *T. luscus* from N Galicia (site B16) and Cantabria (site D18), indicating a higher availability of Cr in the surrounding medium. Significant negative correlations were observed between the concentration of Cr in the liver and biometric parameters such as HSI (R^2 = -0.754; P <0.01) and length (R^2 = -0.650; P<0.01), suggesting a negative impact of Cr on fish health (Maes *et al*., 2005). In fact, it has been stated by several authors that, a decrease in liver size as a consequence of the loss of hepatic glycogen and/or lipid is a common morphologic response of fish liver to metal toxicity, and that high concentrations of either essential or non-essential metals in water can retard fish development and lead to potential alterations in fish size (Canli and Atli, 2003; Wolf and Wolfe, 2005).

In contrast, measurements of total Hg – recommended as the standard for regulatory monitoring (Bloom, 1992) – indicated significantly high levels in the liver of both species from Asturias (site C9, C17), Cantabria (site D11, D18) and Basque Country (site E13, E19) suggesting a chronic exposure to Hg in those areas. Meanwhile, *T. luscus* from S Galicia also reported high Hg residues, that was consistent with data on sediment contamination and Hg enrichment in mussels from the Pontevedra Ria (S Galicia, near station A14) (Prego and Cobelo-Garcia, 2003).

Despite of the observed differences in bioaccumulation, both species had higher levels of essential than non-essential metals, and indicated a similar pattern in the accumulation of essential metals (Fe>Zn>Cu>Cr), which might be related to their key role in the regulation of enzymatic and respiratory processes in both organisms. In contrast, the different bioaccumulation pattern detected for non-essential metals in *L. boscii* (Cd>Hg>Pb) and *T. luscus* (Cd>Pb>Hg) may be related to differences in metal speciation/bioavailability in the two shelf environments. For instance, as mentioned above, levels of Pb seem to decrease with depth, however, for Hg although it occurs in both organic and inorganic forms in marine water and sediments, concentrations of organic mercury (e.g. methylmercury) tend to increase with depth and in this form Hg is more rapidly bioaccumulated and more slowly released from tissues of marine organisms (Neff, 2002).

Metal concentrations in the liver of the studied fish are in the range or lower than those previously reported in wild fish from coastal areas, with the exception of the levels of Cr in both species, and Zn, Cd and Hg in *L. boscii*, which were higher than some of the values reported in the literature (see Table 2 in chapter 3, pag. 112).

5.2.2. Organochlorinated compounds

The levels of organochlorine compounds (OCs) in muscle tissue were above detection limit in all the samples analyzed, with the exception of DDTs in *T. luscus* collected from the Asturias area (C18). Results evidenced a different pollution gradient and/or bioavailability of PCBs and DDTs along the inner and middle/outer shelf (see Fig.5). The highest levels of PCB residues were observed in *L. boscii* from the Basque Country (E13 & 14) and Asturias (C10). Nonetheless, levels of OCs detected in this study were in the low range of those reported for other commercial species from coastal environments (Lewis *et al*., 2002; Porte *et al*., 2002). Even more, levels of PCBs and DDTs detected in the muscle tissue of the present wild fish were respectively, 3-fold and 14-fold lower than those previously detected in cultured sea bass (Chapter 2). Differences in the amount of lipids and uptake of different congeners in the diet and/or from sediments, as well as biotransformation could explain the inter- and intra-species distribution of PCB congeners (Porte and Albaigés, 1994).

Concerning the pesticide residues, the main isomers detected were *p,p'*-DDE and *o,p'*-DDD. However, detectable levels of *p,p'*-DDT were also present in *L. boscii* from N Galicia (B5) and Cantabria (D12), despite that the widespread use of DDT has been banned in Spain (Bordajandi *et al*. 2003). In fact, the ratio *p,p'*-DDE/*p,p'*-DDT <1 suggests a recent use of this pesticide. When *p,p'*-DDT is introduced into the environment, it is converted to *p,p'*-DDD and *p,p'*-DDE, and despite that DDT has a half-life of 7 years, its metabolite DDE survives much longer and is the predominant form detected in fish and humans (Easton *et al*., 2002). Since elimination occurs slowly, ongoing exposure may lead to an increase in the body burden over time.

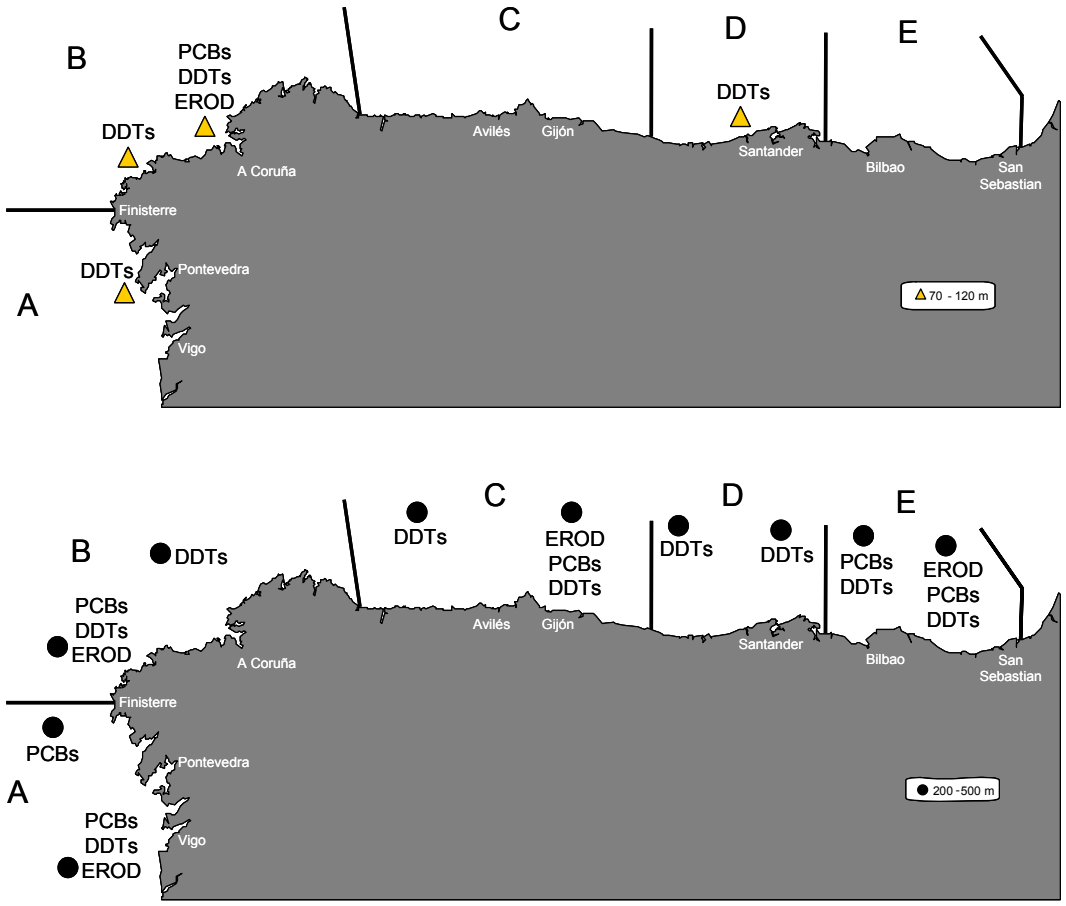

Figure 5. Map depicting high levels of organochlorinated compounds in muscle tissue together with significant levels of EROD activity ($P<0.05$) in liver of (△) *Trisopterus luscus* and (●) *Lepidorhombus boscii* collected along the Northern Iberian shelf. A – S Galicia; B – N Galicia; C – Asturias; D – Cantabria; E – Basque Country, (Chapter 3)

5.2.3. Hydroxylated-PAHs and the Prestige fingerprint

Regarding exposure to PAHs, results highlighted the anthropogenic pressure near coastlines and the chronic pollution of the Asturias, Cantabria and Basque Country inner shelf due to urban and industrial activities (see Fig. 6). Considering the OH-PAH metabolite profile, both species showed a similar geographical pattern. 1-Pyrenol, regarded as the best general indicator of PAH exposure in fish (Ruddock *et al*., 2002), was particularly abundant in fish from Asturias (area C), Cantabria (area D) and Basque

Country (area E). In contrast, fish from Galicia (area A & B) were mainly enriched in 1-naphthol (80-100%), a marker of recent exposure to petrogenic compounds. In fact, small tar aggregates were still present on the Galician continental shelf almost one year after the *Prestige* accident (Serrano *et al*., 2006), and it is plausible that fish inhabiting the area, particularly benthic fish, might have been exposed to those PAHs.

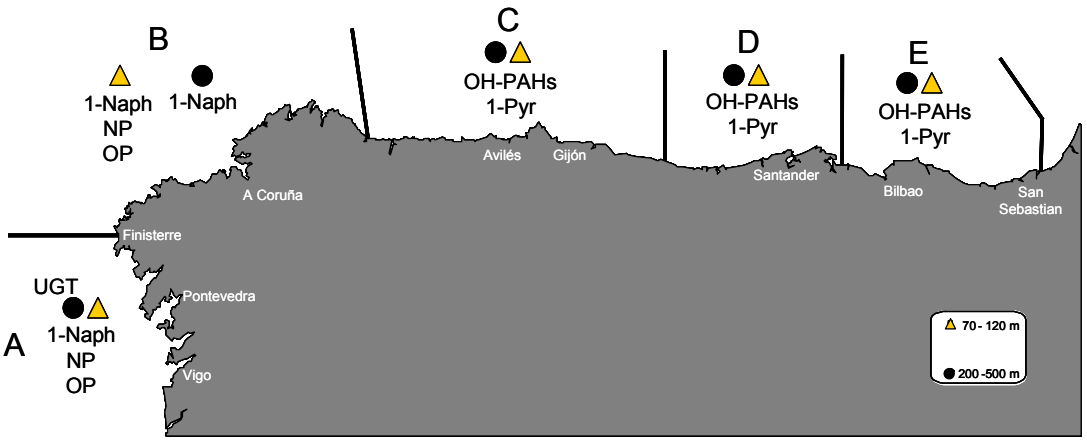

Figure 6. Map depicting significant levels (P<0.05) of hydroxylated-PAHs and alkylphenolic compounds in bile, together with UGT activity in liver of (△) *Trisopterus luscus* and (●) *Lepidorhombus boscii* collected along the Northern Iberian shelf. A – S Galicia; B – N Galicia; C – Asturias; D – Cantabria; E – Basque Country. (Chapter 3).

The *Prestige* fuel oil was particularly rich on PAHs, it represented 50% of the total hydrocarbon content in the fuel, with a clearly predominance of naphthalene and alkyl-naphthalenes (González *et al*., 2006). Nonetheless, elevated amounts of 1-naphthol (35-53%) were also detected in the bile of fish from areas C, D & E, indicating significant exposure to PAHs of petrogenic origin. In fact, fish from the present study were characterized by the high relative abundance of 1-naphthol in bile (35-100%) when compared with elsewhere; e.g. *Mullus barbatus* and *Serranus cabrilla* from the

NW Mediterranean coast (2-10%) (Escartín and Porte, 1999a); *Salmo trutta*, *Salvelinus fontianalis* and *Salvelinus alpinus* from European mountain lakes (4-19%) (Escartín and Porte, 1999b).

5.2.4. Alkylphenolic compounds in bile

Apart from the higher levels of 1-naphthol, both species collected along the Galician coast reported the highest levels of NP (157-220 ng/g) and OP (13-21 ng/g) in bile, compared to the other areas, probably due to the use of detergents in washing of oil tanker bottoms (Salas *et al*., 2006) (see Fig. 6). The levels of NP, detected in *L. boscii* and *T. luscus*, were however significantly lower than those previously reported for both wild and cultured sea bass (Chapter 2), which in a way highlights the higher pressure of sewage effluents in Estuarine systems, and further reflects the existence of water pollution in aquacultures, as well as its importance in the process of pollutant accumulation in cultured sea bass tissues.

5.2.5. Biochemical markers

The biochemical markers analysed (MT, EROD, UGT, CAT) indicated significant differences among sampling sites and different types of stress along the two shelf environments. Often the degree of enzyme induction is assessed by comparing the biological responses with those observed in reference areas. However, in the present

study, no area could be selected as a reference due to the lack of previous information, the wide anthropogenic pressure along the Spanish coast and the occurence of the *Prestige* accident one year before the sampling.

The elevation of hepatic MT is a measure of the biologically available metal in the environment, and its induction in the liver has been recognized as the main form of storage and detoxification of metals in fish. Hence, hepatic MT was elevated in both species showing high residues of Zn, Cd, Hg and Cr in their livers (see Fig. 4). Positive correlations were observed for Zn-MT (0.537) and Cd-MT (0.573) in liver of *L. boscii* and Cr-MT (0.784) in liver of *T. luscus* (Fig. 7). It is well established, that MT have a role in Zn homeostasis and Zn is a constituent element of MT. George and Olsson (1994) and Roméo *et al*. (1997) report that hepatic Zn and MT display linear relationship in most species because basal levels of Zn are sequestred by MT. Therefore, the basis for MT synthesis has been attributed to Zn, displaced from Zn-metalloproteins by other metals (Roesijadi, 2000). On the other hand, Cd and Hg are known to be toxic pollutants, whose deleterious effects are minimized when they are bound to MT. Nonetheless, it has been demonstrated that toxic metal binding by hepatic MT is more efficient with Cd than with Hg (Cosson, 1994), inferring that Cd has higher affinity for cysteinic residues, which can explain the observed correlations in the liver of *L. boscii*. However, binding of metals on MT sites is not only dependent on their respective affinity for cysteinic residues, but also related to their relative amount in the cell (Cosson, 1994). *T. luscus* indicated a positive correlation between MT and Cr, whereas no relation exited for the other analyzed metals. Several authors have

interpreted good correlations between metal and MT content as an indication of metal sequestration by MTs (Roméo *et al*., 1999; Rotchell *et al*., 2001; Filipović and Raspor, 2003).

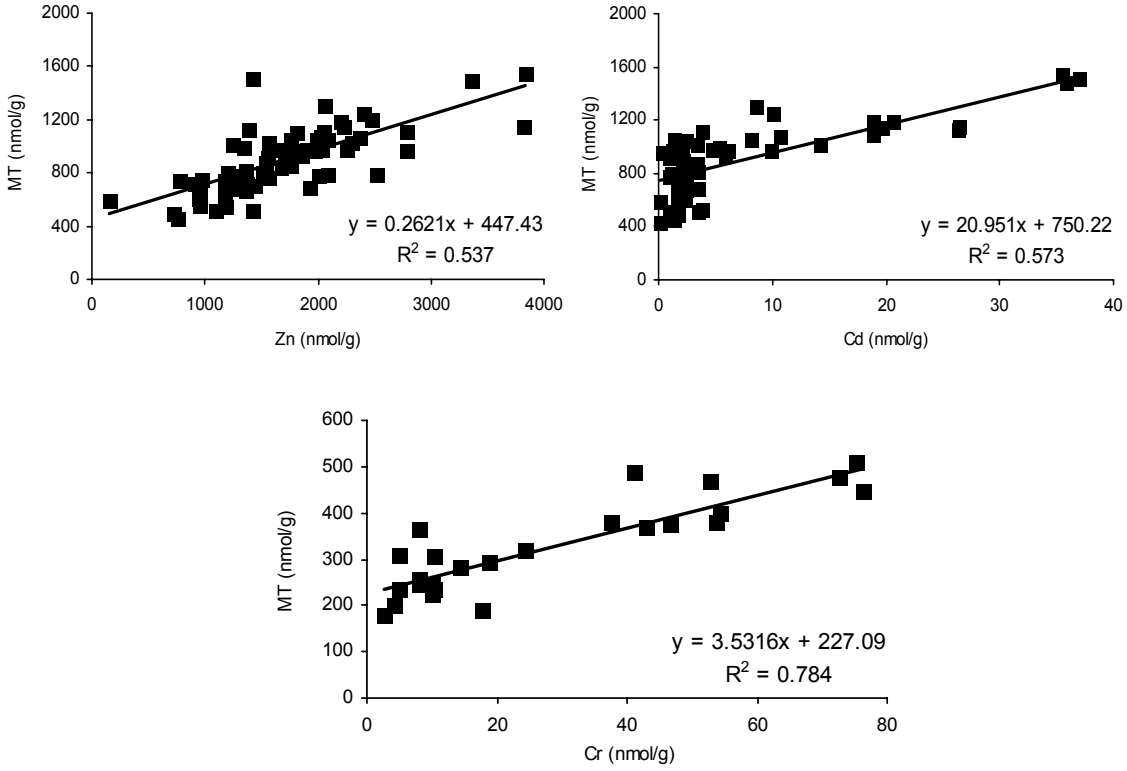

Figure 7. Relationship between Zn-MT and Cd-MT in the liver of *L. boscii* (n = 64) and Cr-MT in the liver of *T. luscus* (n = 25), collected along the Northern Iberian shelf, (Chapter 3).

Meanwhile, a good correlation between hepatic EROD activity and PCB residues in muscle tissue of both species was recorded (Fig. 8; $P<0.01$). In fact, EROD activity has been widely demonstrated as a marker of PCBs exposure in fish, among other compounds (Livingstone *et al*., 1993; Porte *et al*., 2002; Van der Oost *et al*., 2003). Considering *L. boscii*, EROD activity was about 5-fold higher in sites E14 (Basque Country), C10 (Asturias), B5 (N Galicia) and A1 (S Galicia), whereas for

T. luscus it was 2 to 4-fold higher in N Galicia, indicating higher exposure to CYP1A inducing agents of urban and industrial origin in those sites (see Fig. 5).

Regarding UGT activity, it showed once more to be less responsive to pollutant exposure than EROD. Nonetheless, an increase in UGT activity was observed in *L. boscii* from S Galicia (sites A3 & A4), showing high concentrations of APEs and 1-naphthol in bile (See Fig. 6). In fact, several studies have reported 1-naphthol and other phenols to be good substrates for UGTs, indicating that the main metabolic pathway of these compounds is the glucuronidation (Leaver *et al*., 1992; Clarke *et al*., 1992).

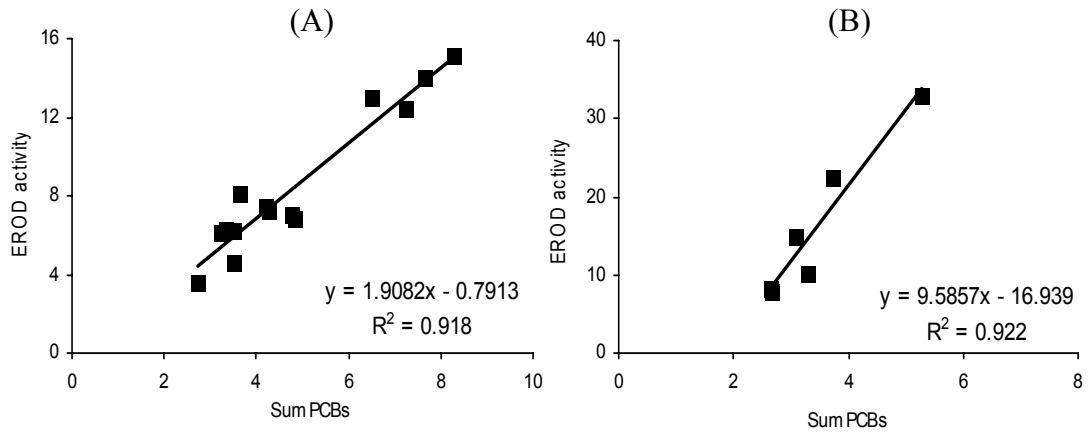

Figure 8. Relationship between the Sum of PCBs in muscle and mean EROD activity in liver of (A) *L. boscii* (N = 14) and (B) *T. luscus* (N = 6)**,** collected along the Northern Iberian shelf, (Chapter 3).

Meanwhile, catalase (CAT) activity was selected for this study because of its inducibility under conditions of oxidative stress and its potential role in adaptation to pollutant-induced stress. Significantly high CAT activities were detected in *L. boscii* from site D12 (Cantabria) and *T. luscus* from site B17 (N Galicia), indicating an increase in oxidative stress in these species/areas. Chronic exposure to certain pollutants

(e.g. trace metals or organic compounds) may produce an increase in oxyradicals and generate oxidative stress in exposed organisms. In fact, besides the high levels of OH-PAHs (D12) and PCBs/DDTs (site B17) these individuals also indicated significantly high residues of Hg (site D12), Cd and Cr (Site B17) in their livers. The usefulness of CAT as a marker of oxidative stress in fish species has been previously reported (Hasspieler *et al*., 1994; Porte *et al*., 2002). Although, a number of studies have also shown no significant responses of CAT activity to exposure to environmental pollutants (studies reviewed by Van der Oost *et al*., 2003).

Overall, the obtained results indicate significant differences in biological responses and pollutant loads among the selected fish species and along the Northern Iberian shelf. The study provides further support for the use of biomarkers in assessing the health of coastal areas, and results reinforce the importance of selecting different sentinel species from different habitats for the assessment of pollution impact in the marine environment.

5.3. Mitochondrial metabolism of $17P_4$ in male sea bass

CYP17 is the qualitative regulator of steroidogenesis, determining which class of steroid will be produced. When CYP17 remains inactive the products are mineralcorticoids (C21) such as aldosterone, when its 17α-hydroxylase activity is present glucocorticoids (C21) such as cortisol are produced, and when both 17α-hydroxylase and C17,20-lyase activities are present, C19 precursors of sex steroids are produced, being the C17,20-lyase activity essential for the biosynthesis of androgens

(Miller, 2002). The activity of CYP17 (C17,20-lyase) is often associated with the membranes of endoplasmatic reticulum (microsomes) (Omura and Morohashi, 1995; Schulz *et al*., 2001; Miller, 2002; Payne and Hales, 2004), however in the present study its activity was also detected in the mitochondrial membrane. In fact, enzymes leading to the biosynthesis of active steroids were detected in the mitochondrial fraction (Fig. 9), where $17P_4$ was metabolized to androstenedione (C17,20-lyase catalyzed pathway), and further metabolized to 11β-hydroxy-4-androstene-3,17-dione (*β*AD) by the CYP11B enzyme (11β-hydroxylase); responsible for catalysing the hydroxylation of AD, at its C_{11} position, in the presence of molecular oxygen. Moreover, NADPH

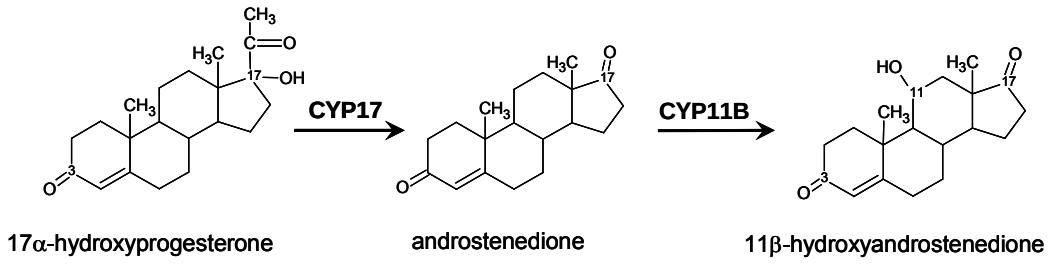

Figure 9. Mitochondrial metabolism of 17α-hydroxyprogesterone ($17P_4$) in male gonads of sea bass (*Dicentrarchus labrax*). CYP17: C17,20-lyase; CYP11B: 11β-hydroxylase; (Chapter 4).

indicated its importance in the catalysis of these reactions, since no metabolites were observed in its absence. The identification of the mitochondrial metabolites was accomplished by GC-MS and the mass spectrum of AD and *β*AD is shown in Figure 10. The formation of *β*AD was significantly higher at the initial stages of maturation (SMS-1) (Fig. 11), which is consistent with the role of 11-oxygenated androgens in testicular recrudescence and spermatogenesis in many teleost fish (Borg, 1994; Miura *et al*., 1996). The formation rate of *β*AD and AD significantly decreased in the later

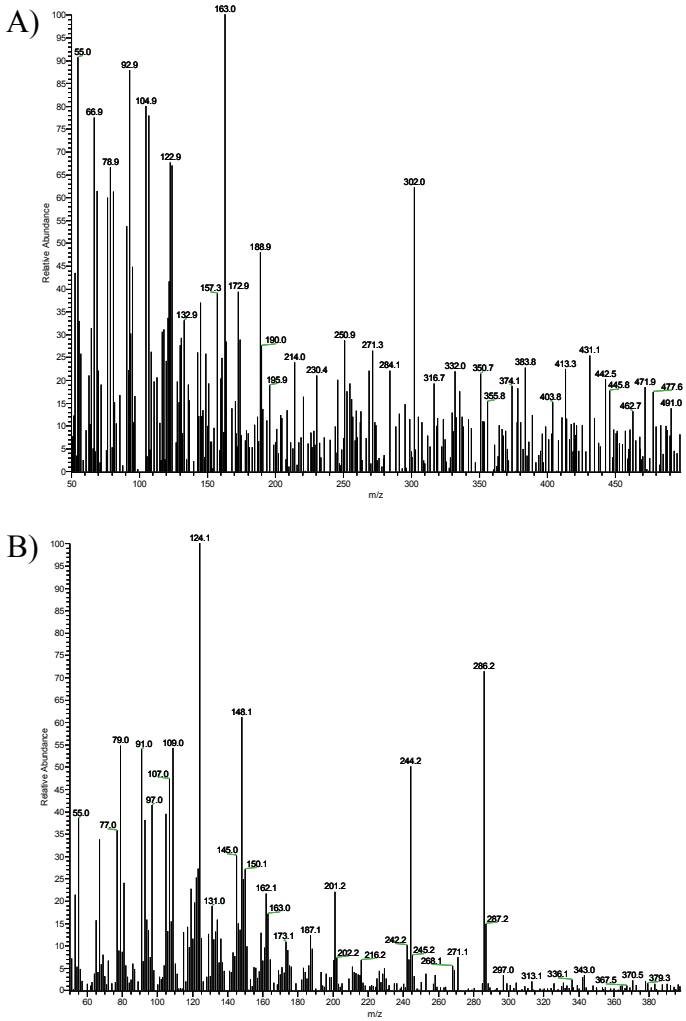

Figure 10. Mass spectrum of mitochondrial metabolites from male gonads of sea bass, identified as: A) 11β-hydroxy-4-androstene-3,17-dione (*β*AD); B) 4-androstene-3,17-dione (AD); (Chapter 4).

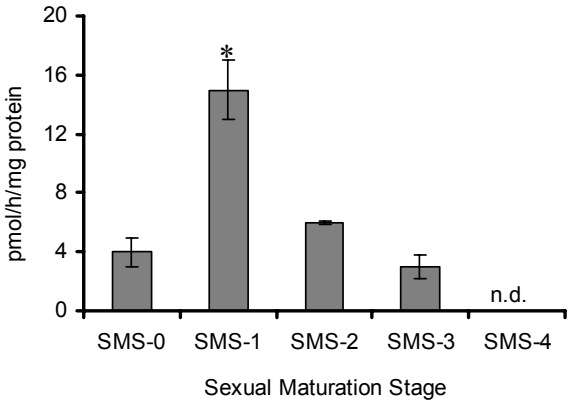

Figure 11. Formation rate of 11β-hydroxy-4-androstene-3,17-dione (*β*AD) in male gonad of sea bass at different sexual maturation stages: undeveloped (SMS-0), early-spermatogenic (SMS-1), mid-spermatogenic (SMS-2), late-spermatogenic (SMS-3), post-spawning (SMS-4). * Significant differences between SMS ($P < 0.05$); (Chapter 4).

stage of maturation, which indicates that even though androgens are necessary, later stages of spermatogenesis do not demand very high levels of androgens. This agrees with other studies, which also found the highest production of androgen per weight of testes at the very early stages of spermatogenesis in a teleost fish (Schulz and Brüm, 1990). Therefore, the mitochondrial metabolism of $17P_4$ in sea bass testes showed a significant decreased (from 77% to 24%) with increasing maturation of the gonads (Fig. 12).

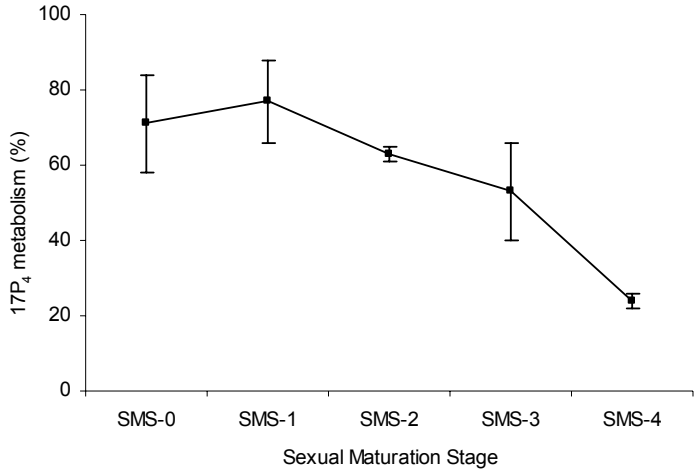

Figure 12. Percentage of total mitochondrial metabolism of $17P_4$ in male gonad of sea bass at different sexual maturation stages: undeveloped (SMS-0), early-spermatogenic (SMS-1), mid-spermatogenic (SMS-2), late-spermatogenic (SMS-3), post-spawning (SMS-4); (Chapter 4).

5.3.1. In vitro effects of endocrine disruptors on steroidogenic enzymes

Among the compounds tested (BaA, NP, TBT, *p,p'*-DDE, KCZ), only KCZ and NP inhibited the metabolism of $17P_4$ in gonads of sea bass. Inhibition of both enzymes was observed in incubations treated with 100 μM KCZ, although CYP11B was more sensitive to KCZ than C17,20-lyase. The antifungal ketoconazole (KCZ) was used as a positive control in this study, since it's a well known inhibitor of cytochrome P450 steroidogenic enzymes (Gal *et al*., 1994; Monteiro *et al*., 2000). Hence, sea bass

testicular steroidogenic enzyme system was shown to be as sensitive as those from mammalian tissues and other fish species to KCZ (Weber *et al*., 1991; Ahmed *et al*., 1995; Monteiro *et al*., 2000).

In contrast, 100 μM NP only inhibited the CYP17 enzyme (C17,20-lyase pathway) and had no visible effect on testes CYP11B. Recently, induction of 20β-HSD by NP (at 1mM) in gonads of female carp was reported, this being the key enzyme involved in the formation of 17,20βP (Thibaut and Porte, 2004), which is known to act as a maturation-inducing hormone and as a pheromone (Scott and Sorensen, 1994; Nagahama, 1997). Inhibition of CYP17 (17α-hydroxylase pathway) by NP in human adrenocortical cells (H295R) has also been reported, this pathway being important for cortisol synthesis (Nakajin *et al*., 2001). Nonetheless, to our knowledge this is the first study that reports the effect of NP on C17,20-lyase pathway in fish gonads; the inhibition of this key step on the synthesis of androgens may lead to disturbances in spermatogenesis, reproductive behaviour and development of secondary sexual characters.

It has been evidenced in this thesis (Fig. 2 and Chapter 3) that NP is widely distributed in the environment, being detected in bile of both wild and cultured fish from different ecosystems (i.e. ponds, marine and estuary systems). Exposure to relatively high levels of NP in the environment is indeed capable of disrupting the endocrine system of male fish by binding to estrogenic receptors (ERs) and inducing the synthesis of Vtg (Chapter 2). Moreover, Chapter 4 reports that NP is capable of impairing the reproductive successes of male fish through mechanisms other than binding to ERs, and that the assessment of mitochondrial metabolism of $17P_4$ may

constitute a new sensitive tool for detecting endocrine disruption in both wild and cultured stocks.

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Chapter 6. Final Conclusions

6. Conclusions of the thesis

The final conclusions of this dissertation can be summarized as follows:

I. The integrated use of chemical analysis and biochemical responses in biota is a useful tool for assessing the impact of anthropogenic pollutants in both wild and cultured fish populations. The applied biomarkers (MT, EROD, CAT, AChE, Vtg) might be used as a first screening method to discriminate between basal and high levels of exposure to certain chemical compounds.

II. The elevated EROD activity and depressed AChE detected in wild sea bass from the Arade Estuary (marina area) suggests the presence of CYP1A inducing agents in the water, apart from other agents having an inhibitory effect on AChE. Chemical analyses in tissues indicated rather high levels of OH-PAHs, APEs and metals (Cu and Zn).

III. A clear response to $CuSO_4$ treatment in cultured sea bass was observed, this response consisted on the induction of MT and depressed CAT and AChE activity. Hepatic MT was induced in both cultured and wild specimens (i.e. FF, RC and A1) showing the highest Cu residues in the liver, which supports the role of MT in metal detoxification in fish. The liver appears to be the major storage site for metals and may be a useful indicator of recent exposure.

IV. Pollutant exposure can lead to immunodepression, which would result in increased susceptibility of polluted fish to parasitic infections; the correspondence between Cu contamination and the infestation with *Sphaerospora testicularis* in cultured and wild sea bass may support this hypothesis.

V. Aquaculture location (i.e. water quality) does have an influence on pollutant exposure and tissue residues in cultured fish (e.g. Cd & PCBs). Sea bass kept under laboratory conditions indicated the highest levels of DDTs, OH-PAHs and APEs together with high levels of EROD and Vtg, reflecting the poor quality of the coastal water of the city of Barcelona.

VI. Although, levels of certain pollutants were higher in cultured fish than in wild fish, with regard to food consumption the detected concentrations in the edible tissue of cultured stocks were below the threshold limits recommended for commercial species, suggesting that consumption of cultured sea bass from the studied areas does not pose potential risks for human health.

VII. *Lepidorhombus boscii* and *Trisopterus luscus* are suitable sentinel species for future biomonitoring programmes in the Northern Iberian coast. The results reinforce the importance of selecting different sentinel species from different habitats for the assessment of pollution impact in the marine environment.

VIII. Both *Lepidorhombus boscii* and *Trisopterus luscus*, highlighted a chronic exposure to Hg in the Asturias, Cantabria and Basque Country areas. Nonetheless, metal pollution in the middle/outer shelf was mostly concentrated in the Asturias and Basque Country area, whereas in the inner shelf it displayed a gradient along the coast

IX. The analysis of PAHs in the bile of *Lepidorhombus boscii* and *Trisopterus luscus* gave an indication of recent exposure to compounds of petrogenic origin, particularly in the Galician area, one year after the *Prestige* accident.

X. The biomarker approach used indicated different levels of stress in *Lepidorhombus boscii* and *Trisopterus luscus*, paralleling a coastal pollution gradient. Hepatic MT was induced in fish with higher levels of Zn, Cd, Hg and Cr in their livers. EROD induction was related with PCB residues in the muscle of both species, whereas UGT was less responsive to pollutant exposure; high UGT activity was detected in *Lepidorhombus boscii* with high levels of APEs and 1-naphthol in bile.

XI. The mitochondrial synthesis of βAD was elevated in gonads of sea bass at the initial stages of sexual maturation, confirming its role in testicular recrudescence and spermatogenesis in male sea bass.

XII. Among the EDs tested, NP showed that it can inhibit C17,20-lyase activity, a key steroidogenic enzyme involved in the synthesis of androgens, while no significant effects were detected for TBT, *p,p'*-DDE and B(a)A. NP has therefore the potential to disrupt the reproductive cycle of fish living in polluted environments due to impairment of testicular steroid biosynthesis.

6.1. Future perspectives

- Environmental problems such as the high levels of Cu in cultured sea bass livers and NP in the bile of wild sea bass from the Arade Estuary have been pin-pointed and deserve further investigation in the near future. Strategies must be adopted to reduce sources of pollution (e.g. the use of chemicals in current aquaculture practises) and to develop methodologies that would allow continuous monitoring of cultured fish quality, among them the use of biomarkers can offer a great potential.

- Regarding biomonitoring aspects, this work has provided background information on pollutant residues and biochemical responses in two areas, the Arade Estuary and the Northern Iberian coast. The existence of basal data on those ecosystems is of valuable information that will allow future assess of the general health of both ecosystems as well as of new environmental threats (e.g. accidental spills).

- The mitochondrial metabolism of $17P_4$ might be a sensitive tool for the assessment of endocrine alterations in male fish and deserves further research. *In vitro* screening of known and suspected EDs will allow the detection of those compounds that have the ability of impairing testicular steroid biosynthesis. Furthermore, *in vivo* exposure experiments are needed to validate the *in vitro* findings.

ANNEX:
Risk-Based Consumption Limit Tables

This Annex provides risk-based consumption limits for chemical contaminants measured in muscle tissue of fish species and have been extracted from www.epa.gov/ost/fishadvice/volume2/index.html.

Tables 1 through 8 are consumption limit tables for carcinogenic and chronic systemic health endpoints for each of the target analytes in this thesis. Readers using the tables as a basis for fish consumption advisories should note that the values given in the tables are valid **only** for single contaminants in single-species diets (see Note). Each consumption table lists, by chemical, the maximum number of fish meals per unit time (monthly) that may be safely eaten. Readers may use these tables by determining the chemical contaminant concentration in fish surveyed and reading the value for the maximum number of meals per month that may be safely eaten for each contaminant for noncancer and cancer endpoints. For those contaminants with monthly fish consumption limits calculated for both the noncancer and cancer endpoints, EPA recommends using the more conservative of the two values.

Seven variables and two required equations are involved in calculating the values in the consumption limit tables (see Equations (A) through (C) presented after the tables):

- Maximum acceptable risk level (ARL)
- Cancer slope factor (CSF)
- Chronic reference dose (RfD)
- Consumer body weight (BW)
- Fish meal size (MS)
- Contaminant concentration in edible fish tissue (C_m)
- Time-averaging period (30-d period)

Note: Individuals often eat several species of fish in their diets. However, equations used for the present tables are based on contaminant concentrations in a single species of fish. Where multiple species of contaminated fish are consumed by a single individual, such limits may not be sufficiently protective. If several fish species are contaminated with the same chemical, then doses from each of these species must first be summed across all species eaten in proportion to the amount of each fish species eaten.

Table 1. Monthly Fish Consumption Limits for Noncarcinogenic Health Endpoint - Cadmium

Risk Based Consumption Limit [a]	Noncancer Health Endpoints [b]
Fish Meals/Month	Fish Tissue Concentrations (*ppm, wet weight*)
Unrestricted (>16)	0 - 0.088
16	>0.088 - 0.18
12	>0.18 - 0.23
8	>0.23 - 0.35
4	>0.35 - 0.70
3	>0.7 - 0.94
2	>0.94 - 1.4
1	>1.4 - 2.8
0.5	>2.8 - 5.6
None (<0.5)	>5.6

[a] The assumed meal size is 8-oz (0.227 kg). The ranges of chemical concentrations presented are conservative, e.g., the 12-meal-per-month levels represent the concentrations associated with 12 to 15.9 meals.

[b] Chronic, systemic effects.

Notes:

1. Consumption limits are based on an adult body weight of 70 kg and an RfD of $1x10^{-3}$ mg/kg-d.
2. None = No consumption recommended.
3. In cases where >16 meals per month are consumed, refer to Equations (A) and (C), for methods to determine safe consumption limits.
4. The detection limit for cadmium is $5x10^{-3}$ mg/kg.
5. Instructions for modifying the variables in this table are found at: www.epa.gov/ost/fishadvice/volume2/index.html (in section 3.3).
6. Monthly limits are based on the total dose allowable over a 1-month period (based on the RfD). When the monthly limit is consumed in less than 1-month (e.g. in a few large meals), the daily dose may exceed the RfD.

Table 2. Monthly Fish Consumption Limits for Noncarcinogenic Health Endpoint - Methylmercury

Risk Based Consumption Limit [a]	Noncancer Health Endpoints [b]
Fish Meals/Month	Fish Tissue Concentrations (*ppm, wet weight*)
Unrestricted (>16)	0 - 0.029
16	>0.029 - 0.059
12	>0.059 - 0.078
8	>0.078 - 0.12
4	>0.12 - 0.23
3	>0.23 - 0.31
2	>0.31 - 0.47
1	>0.47 - 0.94
0.5	>0.94 - 1.9
None (<0.5)	>1.9

[a] The assumed meal size is 8-oz (0.227 kg). The ranges of chemical concentrations presented are conservative, e.g., the 12-meal-per-month levels represent the concentrations associated with 12 to 15.9 meals.

[b] Chronic, systemic effects.

Notes:

1. Consumption limits are based on an adult body weight of 70 kg and an interim RfD of $1x10^{-4}$ mg/kg-d.
2. None = No consumption recommended.
3. In cases where >16 meals per month are consumed, refer to Equations (A) and (C), for methods to determine safe consumption limits.
4. The detection limit for methylmercury is $1x10^{-3}$ mg/kg.
5. Instructions for modifying the variables in this table are found at: www.epa.gov/ost/fishadvice/volume2/index.html (in section 3.3).
6. Monthly limits are based on the total dose allowable over a 1-month period (based on the RfD). When the monthly limit is consumed in less than 1-month (e.g. in a few large meals), the daily dose may exceed the RfD.
7. Because most Hg in fish and shelfish tissue is present primarly as methylmercury and because of the relatively high cost of analyzing for methylmercury, it is recommended that total Hg be analyzed and the conservative assumption be made that all Hg is present as methylmercury. This approach is deemed to be most protective of human health and most cost-effective.

Table 3. Monthly Fish Consumption Limits for Noncarcinogenic Health Endpoint - Dicofol

Risk Based Consumption Limit [a]	Noncancer Health Endpoints [b]
Fish Meals/Month	Fish Tissue Concentrations (*ppm, wet weight*)
Unrestricted (>16)	0 - 0.088
16	>0.088 - 0.18
12	>0.18 - 0.23
8	>0.23 - 0.35
4	>0.35 - 0.70
3	>0.7 - 0.94
2	>0.94 - 1.4
1	>1.4 - 2.8
0.5	>2.8 - 5.6
None (<0.5)	>5.6

[a] The assumed meal size is 8-oz (0.227 kg). The ranges of chemical concentrations presented are conservative, e.g., the 12-meal-per-month levels represent the concentrations associated with 12 to 15.9 meals.

[b] Chronic, systemic effects.

Notes:

1. Consumption limits are based on an adult body weight of 70 kg and an RfD of $4x10^{-4}$ mg/kg-d.
2. None = No consumption recommended.
3. In cases where >16 meals per month are consumed, refer to Equations (A) and (C), for methods to determine safe consumption limits.
4. The detection limit for dicofol is $1x10^{-3}$ mg/kg.
5. Instructions for modifying the variables in this table are found at: www.epa.gov/ost/fishadvice/volume2/index.html (in section 3.3).
6. Monthly limits are based on the total dose allowable over a 1-month period (based on the RfD). When the monthly limit is consumed in less than 1-month (e.g. in a few large meals), the daily dose may exceed the RfD.

Table 4. Monthly Fish Consumption Limits for Carcinogenic and Noncarcinogenic Health Endpoints - DDT

Risk Based Consumption Limit [a]	Noncancer Health Endpoints [b]	Cancer Health Endpoints [c]
Fish Meals/Month	Fish Tissue Concentrations (*ppm, wet weight*)	Fish Tissue Concentrations (*ppm, wet weight*)
Unrestricted (>16)	0 - 0.015	0 - 0.0086
16	>0.015 - 0.029	>0.0086 - 0.017
12	>0.029 - 0.039	>0.017 - 0.023
8	>0.039 - 0.059	>0.023 - 0.035
4	>0.059 - 0.12	>0.035 - 0.069
3	>0.12 - 0.16	>0.069 - 0.092
2	>0.16 - 0.23	>0.092 - 0.14
1	>0.23 - 0.47	>0.14 - 0.28
0.5	>0.47 - 0.94	>0.28 - 0.55
None (<0.5)	>0.94	>0.55

[a] The assumed meal size is 8-oz (0.227 kg). The ranges of chemical concentrations presented are conservative, e.g., the 12-meal-per-month levels represent the concentrations associated with 12 to 15.9 meals.

[b] Chronic, systemic effects.

[c] Cancer values represent tissue concentrations at a 1 in 100,000 risk level

Notes:

1. Consumption limits are based on an adult body weight of 70 kg, an RfD of $5x10^{-4}$ mg/kg-d, and a cancer slope factor (CSF) of 0.34 $(mg/kg\text{-}d)^{-1}$. This CSF value considers total DDT (sum of DDT, DDE and DDD).
2. None = No consumption recommended.
3. In cases where >16 meals per month are consumed, refer to Equations (A) and (C), for methods to determine safe consumption limits.
4. The detection limit for DDT is $1x10^{-4}$ mg/kg.
5. Instructions for modifying the variables in this table are found at: www.epa.gov/ost/fishadvice/volume2/index.html (in section 3.3).
6. Monthly limits are based on the total dose allowable over a 1-month period (based on the RfD). When the monthly limit is consumed in less than 1-month (e.g. in a few large meals), the daily dose may exceed the RfD.

Table 5. Monthly Fish Consumption Limits for Carcinogenic and Noncarcinogenic Health Endpoints – Hexachlorobenzene (HCB)

Risk Based Consumption Limit [a]	Noncancer Health Endpoints [b]	Cancer Health Endpoints [c]
Fish Meals/Month	Fish Tissue Concentrations (*ppm, wet weight*)	Fish Tissue Concentrations (*ppm, wet weight*)
Unrestricted (>16)	0 - 0.23	0 - 0.0018
16	>0.23 - 0.47	>0.0018 - 0.0037
12	>0.47 - 0.63	>0.0037 - 0.0049
8	>0.63 - 0.94	>0.0049 - 0.0073
4	>0.94 - 1.9	>0.0073 - 0.015
3	>1.9 - 2.5	>0.015 - 0.020
2	>2.5 - 3.8	>0.02 - 0.029
1	>3.8 - 7.5	>0.029 - 0.059
0.5	>7.5 - 15	>0.059 - 0.12
None (<0.5)	>15	>0.12

[a] The assumed meal size is 8-oz (0.227 kg). The ranges of chemical concentrations presented are conservative, e.g., the 12-meal-per-month levels represent the concentrations associated with 12 to 15.9 meals.

[b] Chronic, systemic effects.

[c] Cancer values represent tissue concentrations at a 1 in 100,000 risk level

Notes:

1. Consumption limits are based on an adult body weight of 70 kg, an RfD of $8x10^{-4}$ mg/kg-d, and a cancer slope factor (CSF) of 1.6 $(mg/kg\text{-}d)^{-1}$.
2. None = No consumption recommended.
3. In cases where >16 meals per month are consumed, refer to Equations (A) and (C), for methods to determine safe consumption limits.
4. The detection limit for hexachlorobenzene is $1x10^{-4}$ mg/kg.
5. Instructions for modifying the variables in this table are found at: www.epa.gov/ost/fishadvice/volume2/index.html (in section 3.3).
6. Monthly limits are based on the total dose allowable over a 1-month period (based on the RfD). When the monthly limit is consumed in less than 1-month (e.g. in a few large meals), the daily dose may exceed the RfD.

Table 6. Monthly Fish Consumption Limits for Carcinogenic and Noncarcinogenic Health Endpoints – Lindane (γ-HCH)

Risk Based Consumption Limit [a]	Noncancer Health Endpoints [b]	Cancer Health Endpoints [c]
Fish Meals/Month	Fish Tissue Concentrations (*ppm, wet weight*)	Fish Tissue Concentrations (*ppm, wet weight*)
Unrestricted (>16)	0 - 0.088	0 - 0.0023
16	>0.088 - 0.18	>0.0023 - 0.0045
12	>0.18 - 0.23	>0.0045 - 0.0060
8	>0.23 - 0.35	>0.0060 - 0.0090
4	>0.35 - 0.7	>0.0090 - 0.018
3	>0.7 - 0.94	>0.018 - 0.024
2	>0.94 - 1.4	>0.024 - 0.036
1	>1.4 - 2.8	>0.036 - 0.072
0.5	>2.8 - 5.6	>0.072 - 0.14
None (<0.5)	>5.6	>0.14

[a] The assumed meal size is 8-oz (0.227 kg). The ranges of chemical concentrations presented are conservative, e.g., the 12-meal-per-month levels represent the concentrations associated with 12 to 15.9 meals.

[b] Chronic, systemic effects.

[c] Cancer values represent tissue concentrations at a 1 in 100,000 risk level

Notes:

1. Consumption limits are based on an adult body weight of 70 kg, an RfD of $3x10^{-4}$ mg/kg-d, and a cancer slope factor (CSF) of 1.6 $(mg/kg\text{-}d)^{-1}$.
2. None = No consumption recommended.
3. In cases where >16 meals per month are consumed, refer to Equations (A) and (C), for methods to determine safe consumption limits.
4. The detection limit for lindane is $1x10^{-4}$ mg/kg.
5. Instructions for modifying the variables in this table are found at: www.epa.gov/ost/fishadvice/volume2/index.html (in section 3.3).
6. Monthly limits are based on the total dose allowable over a 1-month period (based on the RfD). When the monthly limit is consumed in less than 1-month (e.g. in a few large meals), the daily dose may exceed the RfD.

Table 7. Monthly Fish Consumption Limits for Carcinogenic and Noncarcinogenic Health Endpoints – PCBs

Risk Based Consumption Limit [a]	Noncancer Health Endpoints [b]	Cancer Health Endpoints [c]
Fish Meals/Month	Fish Tissue Concentrations (*ppm, wet weight*)	Fish Tissue Concentrations (*ppm, wet weight*)
Unrestricted (>16)	0 - 0.0059	0 - 0.0015
16	>0.0059 - 0.012	>0.0015 - 0.0029
12	>0.012 - 0.016	>0.0029 - 0.0039
8	>0.016 - 0.023	>0.0039 - 0.0059
4	>0.023 - 0.047	>0.0059 - 0.012
3	>0.047 - 0.063	>0.012 - 0.016
2	>0.063 - 0.094	>0.016 - 0.023
1	>0.094 - 0.19	>0.023 - 0.047
0.5	>0.19 - 0.38	>0.047 - 0.094
None (<0.5)	>0.38	>0.094

[a] The assumed meal size is 8-oz (0.227 kg). The ranges of chemical concentrations presented are conservative, e.g., the 12-meal-per-month levels represent the concentrations associated with 12 to 15.9 meals.

[b] Chronic, systemic effects.

[c] Cancer values represent tissue concentrations at a 1 in 100,000 risk level

Notes:

1. Consumption limits are based on an adult body weight of 70 kg, an RfD of $2x10^{-5}$ mg/kg-d, and a cancer slope factor (CSF) of 2 $(mg/kg\text{-}d)^{-1}$.
2. None = No consumption recommended.
3. In cases where >16 meals per month are consumed, refer to Equations (A) and (C), for methods to determine safe consumption limits.
4. The detection limit for PCBs (Sum of Aroclors) is $2x10^{-2}$ mg/kg.
5. Instructions for modifying the variables in this table are found at: www.epa.gov/ost/fishadvice/volume2/index.html (in section 3.3).
6. Monthly limits are based on the total dose allowable over a 1-month period (based on the RfD). When the monthly limit is consumed in less than 1-month (e.g. in a few large meals), the daily dose may exceed the RfD.

Table 8. Monthly Fish Consumption Limits for Carcinogenic Health Endpoint – PAHs

Risk Based Consumption Limit [a]	Noncancer Health Endpoints [b]	Cancer Health Endpoints [c]
Fish Meals/Month	Fish Tissue Concentrations (*ppm, wet weight*)	Fish Tissue Concentrations (*ppm, wet weight*)
Unrestricted (>16)	NA	0 - 0.0004
16	NA	>0.0004 - 0.0008
12	NA	>0.0008 - 0.0011
8	NA	>0.0011 - 0.0016
4	NA	>0.0016 - 0.0032
3	NA	>0.0032 - 0.0043
2	NA	>0.0043 - 0.0064
1	NA	>0.0064 - 0.013
0.5	NA	>0.013 - 0.026
None (<0.5)	NA	>0.026

[a] The assumed meal size is 8-oz (0.227 kg). The ranges of chemical concentrations presented are conservative, e.g., the 12-meal-per-month levels represent the concentrations associated with 12 to 15.9 meals.

[b] Chronic, systemic effects. An RfD is not available (NA) for this compound.

[c] Cancer values represent tissue concentrations at a 1 in 100,000 risk level

Notes:

1. Consumption limits are based on an adult body weight and a cancer slope factor (CSF) of 7.3 $(mg/kg\text{-}d)^{-1}$. No RfD was available (June 1999).
2. None = No consumption recommended.
3. In cases where >16 meals per month are consumed, refer to Equations (A) and (C), for methods to determine safe consumption limits.
4. The detection limit for PAHs is $1x10^{-6}$ mg/kg.
5. Instructions for modifying the variables in this table are found at: www.epa.gov/ost/fishadvice/volume2/index.html (in section 3.3).
6. Monthly limits are based on the total dose allowable over a 1-month period (based on the RfD). When the monthly limit is consumed in less than 1-month (e.g. in a few large meals), the daily dose may exceed the RfD.

EQUATIONS

-Calculation of Daily Consumption Limits-

Equation (A) calculates an allowable daily consumption of contaminated fish based on a contaminant's carcinogenicity, expressed in kilograms of fish consumed per day:

$$CR_{lim} = \frac{ARL \bullet BW}{CSF \bullet C_m} \qquad (A)$$

Where

CR_{lim} = maximum allowable fish consumption rate (kg/d)
ARL = maximum acceptable individual lifetime risk level. Value 10^{-5} (unitless)
BW = consumer body weight (kg)
CSF = cancer slope factor, usually the upper 95 percent confidence limit on the linear term in the multistage model used by EPA [$(mg/kg\text{-}d)^{-1}$]
C_m = measured concentration of chemical contaminant *m* in a given species of fish (mg/kg)

The calculated daily consumption limit (CR_{lim}) represents the amount of fish (in kilograms) expected to generate a risk no greater than the maximum ARL used, based on a lifetime of daily consumption at that consumption limit.

Equation (B) calculates an allowable daily consumption (CR_{lim}) of contaminated fish, based on a contaminant's noncarciongenic health effects, and is expressed in kilograms of fish per day:

$$CR_{lim} = \frac{RfD \bullet BW}{C_m} \qquad (B)$$

Where

CR_{lim} = maximum allowable fish consumption rate (kg/d)
RfD = chronic reference dose (mg/kg-d)
BW = consumer body weight (kg)
C_m = measured concentration of chemical contaminant *m* in a given species of fish (mg/kg)

CR_{lim} represents the maximum lifetime daily consumption rate (in kilograms of fish) that would not be expected to cause adverse noncarcinogenic health effects. Most RfDs are based on chronic exposure studies (or subchronic studies used with an additional uncertainty factor). Because the contaminant concentrations required to produce chronic health effects are generally lower than those causing acute health effects, the use of chronic RfDs in developing consumption limits is expected to also protect consumers against acute health effects. They are designed to protect the most sensitive individuals.

To calculate weekly fish meal consumption limits, Equation (B) was modified as follows:

$$C_m = \frac{RfD \times BW}{CR_{lim}}$$

Using this equation, one can calculate the level of chemical contamination (Cm) in a given species of fish assuming that 70-kg adult consumes a maximum of one 8-oz (0.227-kg) meal/wk.

-Calculation of Meal Consumption Limits-

Daily consumption limits may be more conveniently expressed as the allowable number of fish meals of a specified meal size that may be consumed over a given time period. The consumption limit is determined in part by the size of the meal consumed. An 8-oz (0.227-kg) meal size was assumed. Equations (A) and (C) can be used to convert daily consumption limits, the number of allowable kilograms per day –calculated using Equation (A)–, to the number of allowable meals per month:

$$CR_{mm} = \frac{CR_{lim} \bullet T_{ap}}{MS} \qquad (C)$$

Where

CR_{mm} = maximum allowable fish consumption rate (meals/mo)
CR_{lim} = maximum allowable fish consumption rate (kg/d)
MS = meal size (0.227 kg fish/meal)
T_{ap} = time averaging period (365.25 d/12 mo = 30.44 d/mo)

Equation (C) was used to convert daily consumption limits, in kilograms, to meal consumption limits over a given time period (month) as a function of meal size. Other consumption rates, such as meals per week, could also be calculated using this equation by substituting, for example, 7 d/wk for 30.44 d/mo. In using Equation (C) in the table calculations presented, the reader should note that 1 month was expressed as 365.25 d/12 mo or 30.44 d/mo (monthly limit).

Complete information is available at: www.epa.gov/ost/fishadvice/volume2/index.html

Aquatic environments offer and sustain important habitats for many fish species, but also attract an ever-increasing level of human activities. These environments are the ultimate sink for many of the chemical contaminants released from anthropogenic activities, which can represent a great threat to organisms living in these ecosystems, as to human health. In recent years, there has been a growing awareness of the need to detect and assess the adverse effects of contaminants in organisms exposed to different levels of pollutants. Among the available techniques, the integrated use of chemical analysis and biochemical responses to pollutants is a sound procedure for detecting impact of anthropogenic contaminants in aquatic systems. Besides wild fish consumption, the aquaculture industry is an expanding activity, due to the world population demand of fish however, the intensive production and the extensive use of chemicals in current practises, has raised concerns over the quality of cultured fish in comparison to wild fish. Although, both wild and cultured fish are important components of the Atlantic and Mediterranean diet, there is little information regarding pollutant levels in farmed fish and the risks associated to consumption. In the present thesis the impact of pollutants were investigated in both wild and cultured fish from different aquatic ecosystems, by the combined analysis of chemical residues in fish tissues together with biochemical responses. Specific biological responses (MT, phase I and II enzymes, CAT, AChE, Vtg) to a range of environmental stressors (metals, OCs, PAHs, APEs) were assessed in wild sea bass (*Dicentrarchus labrax* L.) from the Arade Estuary (S Portugal) and in cultured sea bass from different aquaculture facilities located in Southern Europe. Furthermore, the impact of pollution along the Northern Iberian coast was assessed using two commercial fish species, the four-spotted-megrim (*Lepidorhombus boscii*) and the pouting (*Trisopterus luscus*) as sentinel organisms. Finally, since the interference of xenobiotics with steroid synthesis and steroid metabolism in fish may alter the bioavailable amounts of active hormones within the organism, the metabolism of 17α-hydroxyprogesterone ($17P_4$) in male sea bass gonads was investigated, as a potential tool for the detection of endocrine alterations in male fish, by evaluating the *in vitro* effect of model endocrine disrupters (NP, BaA, TBT, *p,p*'-DDE, KCZ)