Universidade de Aveiro Departamento de Biologia

2008

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Fish biological alterations induced by the Aveiro Lagoon contaminants

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Dr. Maria Ana Dias Monteiro Santos, Professora Catedrática do Departamento de Biologia da Universidade de Aveiro

Texto Dedico a minha Tese de Mestrado ao meu marido e filha pelo apoio emocional que me prporcionaram.

o júri

presidente

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To my dear husband, who is my day light and my sweet daughter Ayat.

To those who are no longer among us but last forever in our memory, and warm our hearts

Keywords

Environmental contaminants, biomarkers, erythrocytic nuclear abnormalaties, EROD, biotransformation, genotoxicity, cortisol hormone, polycyclic aromatic hydrocarbons, resin acids.

Abstract

The present research work has been focused on the importance and complementarity of field and laboratory *in vivo* and *in vitro* studies. Fish are often used as bioindicator organisms in biomonitorization studies because they play a major role in the aquatic food-webs. Thus, two representative species of Aveiro Lagoon ichthyofauna were adopted in the present work: the sea bass (*Dicentrarchus labrax* L.) and the european eel (*Anguilla anguilla* L.).

The field study (Chapter I) concerned the seasonal genotoxic assessment of a polluted lagoon (Ria de Aveiro), using erythrocytic nuclear abnormalaties (ENAs) of *D. labrax* specimens, collected at six stations of Aveiro lagoon. ENA results demonstrated the existence of a serious risk to fish populations almost at all the sites (except at GAF in spring, RIO in autumn and LAR in winter). Thus, at all the critical sites (BAR, GAF, RIO, LAR and VAG) identified, the results reflected the presence of genotoxic contaminants in the water. In this context, the ENA assay revealed to be a relatively rapid, easy to perform and sensitive genotoxicity test.

The laboratory *in vivo* and *in vitro* components of the present Master thesis (Chapters II, III, IV and V) was carried out using *A. anguilla* L. as a biological model. Anguilla anguilla L. was intraperitoneally injected with β-naphthoflavone, (4mg/kg) which is a synthetic flavonoid compound known as a strong hepatic monooxigenases inducer such as ethoxyresourfin-*O*-deethylase (EROD). *A. anguilla* L. hepatic microsomes were then isolated and used in EROD activity *in vitro* studies. The *in vitro* effects of (i) PAHs - Naphthalene (Naph) and Phenanthrene (Phe); (ii) Resin acid (RAs) – abietic acid (AA) and its derivatives- Retene (Re) on liver microsomal EROD activity were assessed.

A consistent dose dependent liver microsomal EROD activity increase *in vitro* was demonstrated for both Naph and Phe exposure, confirming their strong liver EROD activity *in vitro* inducer *A. anguilla* L. (Chapter II).

Since the endocrine system plays a major role in fish stress mechanism, the *in vitro* effect of a steroid hormone such as cortisol – on liver microsomal EROD activity was also studied, individually and in combination with AA and Re. The cortisol concentration adopted for our studies was 6.9nM, based on previous trials (Chapter III). AA (Chapter IV) and Re (Chapter V) *in vitro* exposure on hepatic microsome showed a significant inhibition of EROD activity at all the concentrations. *In vitro* EROD activity inhibition caused by AA was inversely proportional to the increased concentration of AA whereas the inhibition caused by Re was proportional to its increased concentrations.

The EROD activity inhibition induced in liver microsome by AA and Re *in vitro* exposure was significantly decreased by the addition of cortisol, (except at 0.3μM concentration of Re). The cortisol exerted a protective effect in liver EROD activity, since a significant reduction of the inhibitory effects caused by AA and Re, was observed in its presence.

The data concerning the assessment of *in vitro* hepatic EROD activity under the above conditions confirm the importance of PAHs and RAs structure and molar concentration as well as the organism's physiological conditions such as different hormonal status related with EROD activity *in vivo* induction i.e. either laboratory or field survey.

Palavras-chave

Contaminantes ambientais, biomarcadores, anomalias nucleares eritrocíticas, biotransformação, EROD, genotoxicidade, hormonas cortisol, hidrocarbonetos aromáticos policiclos, ácidos resínicos.

Resumo

O trabalho de investigação que se apresenta foi direccionado para a importância da complementaridade dos trabalhos de campo e laboratoriais *in vivo* e *in vitro*. Os peixes são frequentemente utilizados como organismos bioindicadores nos estudos de biomonitorização devido ao facto de desempenharem um papel fundamental na cadeia alimentar aquática. Deste modo, no presente trabalho foram seleccionadas duas espécies representativas da ictiofauna da Ria de Aveiro: o robalo (*Dicentrarchus labrax* L.) e a enguia europeia (*Anguilla anguilla* L.).

O trabalho de campo (Capítulo I) diz respeito ao estudo da variação sazonal da genotoxicidade, numa Ria poluída tal como a Ria de Aveiro, utilizando o estudo da frequência das anomalias nucleares eritrocíticas (ANEs) em especímens de *D. labrax*, capturados em seis estações da Ria (de Aveiro). Os resultados referentes aos As demonstraram a existência de um sério risco para esta população de peixes, em quase todos os seis locais da Ria, (exceptuando a GAF na primavera, o Rio no outono e o LAR no inverno). Assim, em todos os locais identificados como críticos (BAR, GAF, RIO, LAR and VAG) os resultados reflectem a presença de contaminantes genotóxicos na água. Neste contexto, o método das ANEs revelou ser um ensaio de genotoxicidade relativamente rápido, fácil de realizar e sensível.

A componente laboratorial *in vivo* e in *vitro* da presente Tese de Mestrado, correspondente aos Capítulos II, III, IV and V, foi executada utilizando *A. anguilla* L. como um modelo biológico. *A. anguilla* L. foi injectada intraperitonealmente com β-naftoflavona (BNF) (4mg/kg), um composto flavonóide sintético, conhecido pela sua capacidade como forte indutor das monooxigenases hepáticas tal como a -*O*-desetilase etoxiresourfina (EROD). Os microsomas hepáticos foram isolados, após 24 horas de exposição da enguia ao BNF, e a sua actividade EROD utilizada nos estudos *in vitro*. Os efeitos *in vitro* dos (i) HAPs – Naftaleno (Naph) e Fenantreno (Phe); ácidos resínicos (RAs) – Abietic acid (AA) e seus derivados Reteno (Re) sobre a actividade EROD microsomal hepática foram estudados e analisados.

O aumento da actividade da EROD microsomal hepática, após exposição *in vitro* ao Naph ou Phe, revelou de uma forma consistente ser dependente da respectiva dose, confirmando a sua elevada capacidade de indução da actividade EROD *in vitro* (Capítulo II).

Uma vez que o Sistema endócrino nos peixes desempenha um papel importante nos mecanismo de stress, o efeito *in vitro* de uma hormona esteróide tal como o cortisol sobre a actividade EROD microsomal hepática foi também estudada, individualmente e em combinação com o AA e o Re. A concentração de cortisol seleccionada foi de 6.9 nM, baseada em experiências realizadas para o efeito (Capítulo III). A exposição *in vitro* dos microsomas hepáticos ao AA (Capítulo IV) e ao Re (Capítulo V) demonstrou a sua capacidade de inibição da actividade EROD para todas as concentrações estudadas. *In vitro*, a inibição da actividade EROD induzida pelo AA, é inversamente proporcional ao aumento de concentração do mesmo, enquanto que a inibição provocada pelo Re foi proporcional ao aumento da sua concentração. A exposição prévia dos microsomas hepáticos ao cortisol 6.9 nM diminuiu significativamente a inibição *in vitro* da actividade EROD induzida em pelo AA e Re, (com excepção da concentração 0.3µM de Re). A exposição prévia ao cortisol exerceu uma acção protectora sobre a actividade EROD dos microsomas hepáticos, uma vez que se observou uma redução significativa dos efeitos inibitórios provocados pelo AA e Re.

Os resultados obtidos relativos ao estudo *in vitro* da actividade EROD nos microsomas hepáticos, sob as condições acima referidas, confirmam a importância da estrutura e concentrações molares dos PAHs e RAs, assim como do estado fisiológico do organismo tal como o estado hormonal relacionado com a indução da actividade EROD *in vivo*, tanto no campo como no laboratório.

INDEX

GENERAL INTRODUCTION

1. AQUATIC POLLUTION

Natural and man-made foreign compounds (xenobiotics) enter and are dispersed in aquatic ecosystems by various routes, including direct discharge, direct use, land run-off, atmospheric deposition, abiotic and biotic movement, and food-chain transfer (Livingstone et al., 1992). Subsequently, they may either be broken down to simple forms or remain unaltered for a long time because of their persistent nature thereby posing a great threat to the ecosystem (Stegeman and Hahn, 1994). Furthermore, toxicants can induce alterations in temperature, dissolved oxygen, pH and other physico-chemical properties of water resulting in damage to aquatic life. In most cases depletion through the excessive toxicant load occurs to the extent that the water bodies become incapable of exercising self purification processes of dilution, sedimentation, oxidation, reduction and photo destruction (Livingstone, 1998). The deoxygenation resulting from pollution load may be sufficiently high so as to destroy aquatic life. Thus, it is necessary to proceed an effective ecological risk assessment. Ecological or environmental risk assessment (ERA) is defined as the procedure by which the actual adverse effects of pollutants and other anthropogenic activities on ecosystems and their components are estimated with a known degree of certainty using scientific methodologies (Depledge and Fossi, 1994). ERAs are primarily concerned with establishing the potential relationship between a pollutant source and an ecological effect caused by exposure of organisms to the pollutant (Suter, 1993).

Ria de Aveiro is a coastal lagoon (47 km2 of maximum surface area) permanently connected to the sea, located in northern west region of Portugal surrounded by several urban aggregates and industrial centres (Pacheco *et al.*, 2005). In the context of pollution scenario, this lagoon has been the main receptor of anthropogenic discharges resulting mainly from pulp/paper plants, harbour and dry-dock activities as well as municipal and domestic effluents which leads to the increase level of organic and inorganic contamination. In these environments, organisms are exposed to mixtures of pollutants, whose synergistic/antagonistic effects are hardly interpreted and predicted exclusively

from their chemical characterization. Some contaminants strongly accumulate in tissues without inducing toxic effects, while others are characterized by elevated toxicity at low levels of exposure. Moreover, the impracticality to analyze all the individual chemicals pooled in mixture of contaminants also increases the problem of aquatic pollutants characterization. Thus, during the past two decades, the use of biological responses (biomarkers) on particular test species has become relevant in toxicological assessments since it allows the early detection of overall effects of contaminants, providing information, even at the sublethal level, that reflects eventual chemical interactions (Passino, 1984, Goksøyr and Förlin, 1992, Livingstone, 1993 and Peakall and Shugart, 1993) In this context, fish are often used as bioindicator organisms because of the major role they play by their position at the top of the trophic web, accumulating toxic substances and responding to low concentrations of the toxicants (Cavas and Ergene-Gözükara, 2005). The existence of a great number of fish species occupying different types of habitat makes them useful, particularly in ecotoxicological studies, allowing the evaluation of various types of contaminants under various exposure conditions.

1.1 Major Pollutants

Natural xenobiotics comprise a wide range of chemicals including plant products, animal toxins and natural hydrocarbons, whereas the production of man-made xenobiotics (contaminants) increases daily in variety and quantity in aquatic environment. Many different chemicals are regarded as pollutants, ranging from simple inorganic ions to complex organic molecules. These pollutants are inorganic ion (metals and anions), organic pollutants (polycyclic aromatic hydrocarbons, polychlorinated biphenyls, polychlorinated dibenzodioxins, polychlorinated dibenzofurans, polybrominated biphenyles, organochlorine insecticides, organophosphorus insecticides, carbamate insecticides, detergents and chlorophenols), organometallic compounds and radioactive isotopes. Depending upon their period of exposure they may cause acute and chronic toxicity. Although the environment has a capacity to absorb,

degrade or assimilate the pollutants, many of them are persistent and have various biological effects. Prominent among those chemicals are polycyclic aromatic hydrocarbons (PAHs), heavy metals, organometallic compounds as well as mixture of pollutants.

1.1.1 Polycyclic aromatic hydrocarbon

PAHs are a group of compounds consisting of at least two fused aromatic rings which are ubiquitous in the environmental air, soil and water contaminants (Menzie et al., 1992). PAHs are widely dispersed in the aquatic environments resulting mainly from petrochemical plant's discharges, accidental spills, sewage and urban run-off (Hylland, 2006; Connell 1974) as well as from harbor activities (Pacheco and Santos, 1997). Eisler suggested that approximately 230,000 tons of total PAHs enter into aquatic environments yearly, worldwide (Eisler, 1987). In addition to major accidents related to oil extraction and transportation as well as industrial and urban discharges, navigation activities and associated fuel losses may significantly increase water contamination by hydrocarbons. These type of contaminations constitutes a generalized concern due to their strong bioconcentration capacity (Connell, 1990), persistency in the environment, mutagenic and carcinogenic potential in fish (Gravato e Santos, 2002; Pacheco e Santos, 2002; Maria *et al*., 2002; Busetti *et al*., 2006). The relative insolubility of the PAHs in the water and its strong capacity of adsorption to the particles makes them easily accumulated in the sediments (Seruto et al., 2005). On the other hand they easily cross the lipid membranes due to their lipophilicity, and bioaccumulate in different tissues (Billiard *et al*., 2002). Aquatic organism, namely fish, living in PAH-contaminated environments may absorb these compounds mainly from the water through gill and by the ingestion of contaminated sediments or food (Varasani *et al*., 1989). However, PAHs concentrations in fish tissues can be significantly reduced by metabolism and excretion (Billiard *et al*., 2002). In general, fish populations are exposed in the aquatic ecosystems to a total PAH concentration ranging from 50 up to 410 mg/liter (Smith *et al*., 1991). In this context, β-Naphthoflavone (BNF), a synthetic analogue of a large series of

naturally occurring flavonoid compounds (McKillop and Case, 1991), is an aromatic hydrocarbon (Ah) receptor agonist commonly used to evaluate fish biotransformation responses due to its P450 induction potency, considered similar to that displayed by carcinogenic PAHs (Stegeman et al., 1987; Okey, 1990; Novi, 1998). Like PAHs, its toxicity is dependent on a bioactivation step since it has been correlated with the appearance of BNF epoxide metabolites (Vyas et al., 1983).

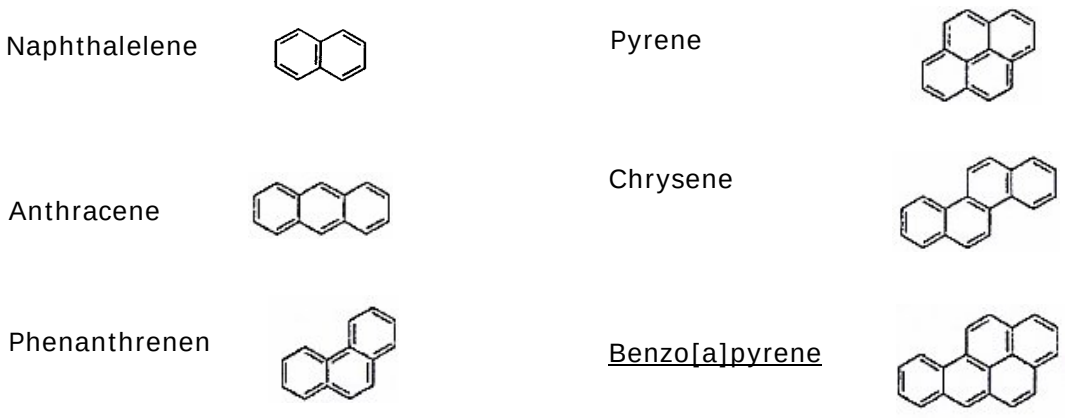

Figure 1 - Representation of the molecular structure of some PAHs. The underlined compound is carcinogenic (modified by Fent and Bätscher, 2000).

1.1.2- Bleached kraft pulp mill effluents (BKPMEs)

During the last 5 decades, the world wide pulp and paper mill industry has undergone a rapid expansion in the utilization of high-yield mechanical and chemi-mechanical pulping processes (O'Conner *et al.*, 1992). The chemi-mechanical processes, which include bleaching, produce large amounts of effluents that are discharged into the aquatic environment, causing serious ecological and toxicological effect in the receiving ecosystems (Pacheco et al., 1997). BKPMEs are complex mixtures of environmentally active substances,

containing about 300 known chemicals (Nestmann et al., 1980); however, not all the BKPME components have been identified (Mather- Mihaich and Di Giulio, 1991). The identified components belong to several classes of organic compounds, namely, unsaturated fatty acids, chlorinated phenols, diterpene alcohols, juvabiones, and resin acids (Walden and Howard, 1977).

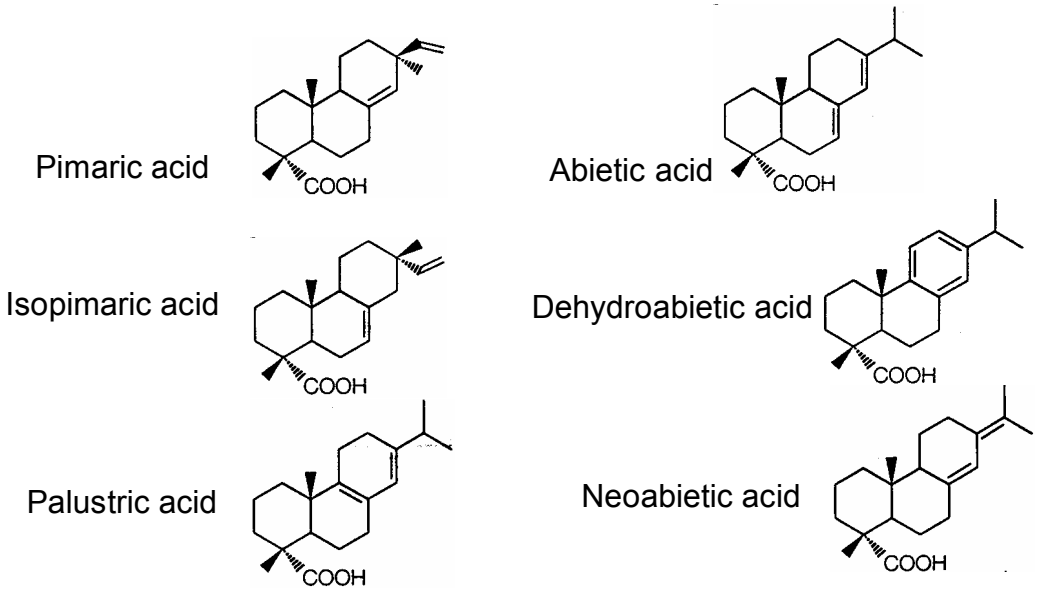

Figure 2- Name and structure of principle resin acids present in bleached kraft pulp mill effluents

Resin acids (RAs) are important components of wood, which are extracted from the cellulose fibers during processing in the kraft pulp activities (Meriläinen and Oikari 2007). These compounds are commonly found at concentrations of 40-2500 µg/liter in the treated effluents and 4-14 µg/liter in receiving waters (Holmbom and Lehtinen, 1980; Kaplin et al., 1997; LepaKnnen et al., 1998) There are seven dominant RAs of which abietic acid (AA) and dehydroabietic acid (DHAA) are among the most abundant (Oikari et al., 1986; Oikari and Hulmbon 1986). RAs constitute an important toxicant group of fish (Nikinmaa and

Oikari, 1982) and can bioaccumulate in liver, bile, and plasma of aquatic animals (Fahraeus-Van Ree and Payne, 1999). An important consequence of high level exposure to RAs is jaundice (Kruzynski, 1979; Matsoff and Oikari, 1987), due to a stimulation of hemoglobin release from erythrocytes (Bushnell *et al.*, 1985) coupled with its subsequent degradation to bilirubin (Pritchard *et al.,* 1991). Moreover, anaerobic microbial biotransformation of RAs in sediments (Koistinen *et al*., 1998) and thermal degradation of abietic-type RAs (Ramdahl, 1983) can form retene (Re) (7-isopropyle-1-methylphenanthrene), a substituted polycyclic aromatic hydrocarbon.

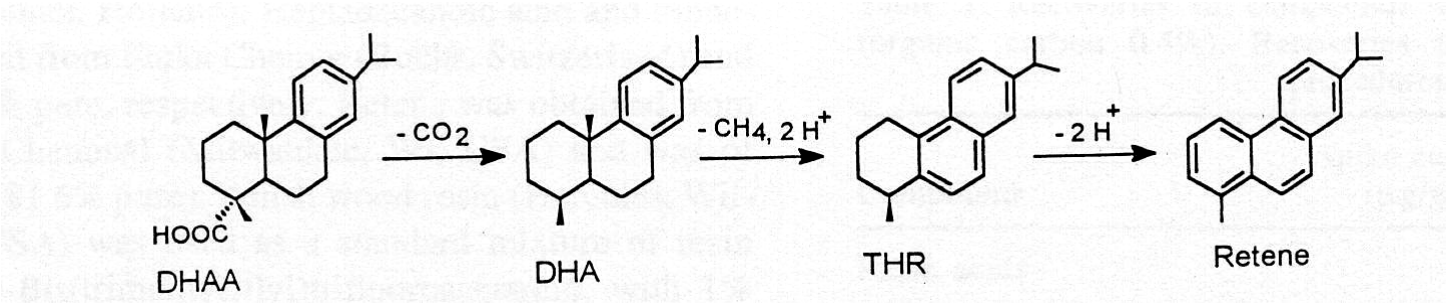

Figure 3 – The anaerobic microbial biotransformation of dehydroabeitic acid into retene. (Adopted by Leppänen and Oikari, 1999a, b).

Reports revealed that Re is bioavailable to fish from sediments of lakes receiving BKME or waterborne particulate matter (Travendale et al., 1996; Leppänen and Oikari, 1999a; Leppänen and Oikari, 1999b; Oikari et al., 2002) since its metabolites were found in *Onchorynchus mykiss* (Fragoso et al., 1999) and *Rutilus rutilus* bile (Leppänen and Oikari, 1999a). There are further experiments in which rainbow trout were exposed to 2 mg/l concentration of RAs for 24 h indicating that RAs mixtures cause a decrease in the arterial P_{O2}, red cell volume, and pH, and an increase in ATP/Hb ratio, causing a respiration impairement (Nikinmaa and Oikari, 1982).

2. BIOMARKERS

Deleterious effects on populations are often difficult to detect in feral organisms since many of these effects tend to manifest only after longer periods of time (Van der Oost *et al*., 2003). When the effect finally becomes clear, the destructive process may have gone beyond the point where it can be reversed by remedial actions or risk reduction (Van der Oost *et al*., 2003) (Figure. 4). Such types of scenarios have triggered the research to establish early-warning signals, or biomarkers, reflecting the adverse biological responses towards anthropogenic environmental toxins. Biomarkers represent the toxicant-induced changes in biological systems that can serve as linkers between environmental contamination (cause) and its effects, providing unique information on the ecosystem health (Bucheli and Fent, 1995).

Several definitions have been given for the term 'biomarker', which is generally used for any measurement reflecting an interaction between a biological system and a potential hazard that 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 behavioral changes) which can be related to exposure to the toxic effects of environmental chemicals (Peakall, 1994). Van Gastel and Van Brummelen (1994) redefined the terms 'biomarker', 'bioindicator' and 'ecological indicator', linking them to different levels of biological organization. They considered a biomarker as any biological response to an environmental chemical at the subindividual level, measured inside an organism or in its products (urine, faeces, hair, feathers, etc.), indicating a deviation from the normal status that cannot be detected in the intact organism. A bioindicator is defined as an organism giving information on the environmental conditions of its habitat by its presence or absence or by its behavior. Effects at higher hierarchical levels are always preceded by earlier changes in biological processes, for that, it was recognized that the biochemical response must be turned to first acknowledgment, being

similar of the integrativa capacity of the livings beings to response the physical and chemical changes of the environmental quality (Bayne et al., 1985). In an environmental context, biomarkers are promising as sensitive indicators demonstrating that toxicants which entered the organisms, have been distributed between tissues, and are eliciting a toxic effect at critical targets (McCarthy and Shugart, 1990).

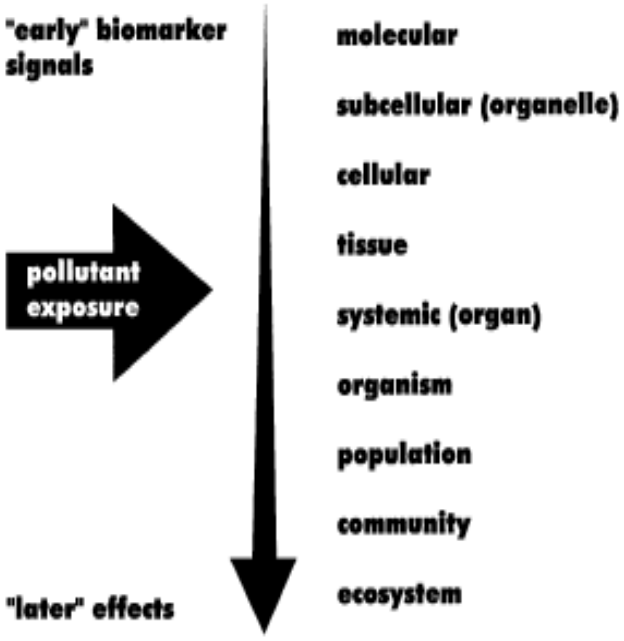

Figure 4- Schematic representation of the sequential order of responses to pollutant stress within a biological system (Modified from Bayne *et al*., 1985).

The most compelling reason for using biomarkers is that they can give information on the biological effects of pollutants rather than a mere quantification of their environmental levels. Biomarkers may also provide insight into the potential mechanisms of contaminant effects. By screening multiple biomarker responses, important information will be obtained about organism toxicant exposure and stress. Normally, a situation of stress caused by the exposition of pollutants triggers a cascade of biological responses, each of which may, in theory, serve as a biomarker (McCarthy et al., 1991). Biomarkers applied in both

the laboratory and the field can provide an important linkage between laboratory toxicity and field assessment (Van der Oost *et al*., 2003). However, improper application or interpretation of biomarker responses, may lead to false conclusions as to pollutant stress or environmental quality. Certain responses established for one species are not necessarily valid for other species (Van der Oost *et al*., 2003).

According to the National Research Council (NRC, 1987), World Health Organization (WHO, 1993), biomarkers can be subdivided into three classes:

- biomarkers of exposure: covering the detection and measurement of an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism;

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

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

In order to evaluate objectively the strength and weaknesses of fish biomarkers, we propose six criteria comprising the most important information that should be available or has to be established for each candidate biomarker as purposed by Van der Oost *et al* (2003) based upon the criteria formulated by Stegeman et al. (1992):

1. The assay to quantify the biomarker should be reliable (with quality assurance (QA)), relatively cheap and easy to perform;

2. The biomarker response should be sensitive to pollutant exposure and/or effects in order to serve as an early warning parameter;

3. Baseline data of the biomarker should be well defined in order to distinguish between natural variability (noise) and contaminant-induced stress (signal);

4. The impact of interfering factors to the biomarker response should be well established;

5. The underlying mechanism of the relationships between biomarker response and pollutant exposure (dosage and time) should be established;

6. The toxicological significance of the biomarker, e.g. the relationships between its response and the (long term) impact to the organism, should be established.

In addition to these criteria, it has been suggested that biomarkers should preferentially be non-invasive or non-destructive, to allow or facilitate environmental monitoring of pollution effects in protected or endangered species (Fossi and Marsili, 1997).

A successful implementation of biomarker in environmental monitoring programs requires, therefore, a good understanding of the underlying response mechanisms. A major challenge of biomarker development with respect to ERA is to define the significance of biomarker responses in terms of ecological effects of the pollutants. Hence, for ERA it is not sufficient to show that biomarker levels differ among sites or even that a biomarker level is abnormally high at a site (Suter, 1990).

2.1- Fish Biomarkers

An important step in promoting aquatic environmental studies is to find an appropriate model organism which can help in clarifying both acute and chronic toxicities of aquatic pollutants with reproducible results and understand the mechanism of toxic action at various levels of biological organizations. Monitoring species should be selected from an exposed community on the basis of their relationship to the assessment endpoint as well as by following some practical considerations (Suter, 1993). Both criteria are met for numerous fish species for

the assessment of the quality of aquatic ecosystems. In addition to the previous criteria, a theoretically ideal fish species to assess toxicity of a type of pollutants should meet the following criteria: (1) widespread in different ecosystems, cosmopolitan if possible, in order to be employed for in situ surveys; (2) sensitive enough to detect genotoxicity of a wide range of pollutants at low doses; (3) adequate for culture conditions in order to carry out laboratory experiments; (4) large natural populations, to allow detraction of individuals without endangering their conservation. (Sanchez-Galan et al., 1999). Therefore, the use of fish biomarkers, indicating the effects caused by pollution increased importance, allows the anticipated detection of problems in aquatic ecosystems.

Most of the biomarker criteria appear to be directly transferable to certain fish biomarkers (Stegeman et al., 1992). However, considerable variation in basic physiological features and the responsiveness of certain biomarkers towards environmental pollution may become apparent among different fish species. Despite their limitations, such as a relatively high mobility, fish are considered to be the most feasible organisms for pollution monitoring in aquatic systems. Most extensively investigated biomarkers include enzymes responsible for the detoxication of xenobiotics and their metabolites (biotransformation enzymes) (Van der Oost *et al*., 2003; Santos and Maria, 2005; Oliveira et al., 2007).

3. BIOLOGICAL RESPONSES TO POLLUTANTS

In the context of contaminants exposure, animal homeostasis initiate an integrated response, i.e. a set of compensating and adaptive biological responses that readjust metabolic processes in order to cope with the effects of pollutants. Compensating for the effects of chemical stressors causes reallocation of metabolic energy away from investment activities and toward the restoration of homeostasis. The restoration of the damaged homeostasis in a stressed fish is associated with an increased metabolic rate in relation to the non-stressed condition (Beyers et al., 1999). The biological responses at the molecular/biochemical level resulting from the contaminants exposure, when not

properly compensated through homeostasis, may be expressed in progressive way at higher levels resulting, for example, in alterations that affect reproduction, growth and the behaviour.

3.1- Biotransformation

In the aquatic environment, the permanent contact of fish with the water maintain the direct absorption of contaminants from water or the sediment, through respiratory systems (by gills) and, in low degree, through the skin. The absorption can equally set through the digestive system via food. The distribution of the contaminants in the tissue after absorption is influenced by some physiological processes and the chemical properties of the contaminants, such as lipophilicity and its capacity to link the macro-molecules. An organism has two major ways of eliminating the chemical: it is either excreted in its original form (the parent compound) or it is biotransformed by the organism. Biotransformation generally leads to the formation of a more hydrophilic compound which is more easily excreted than the parent compound (Vermeulen, 1996). The elimination of xenobiotic can occur through the gills, skin, bile and urine. The organ most commonly involved in the biotransformation of foreign compounds is the liver, because of its functions, position and blood supply (Timbrell, 1991). Kidney, gill, intestine, and skin are also involved to a less extent in biotransformation. In these organs, xenobiotics can undergo a series of sequential reactions conducting to its activation, detoxification, accumulation and excretion.

Biotransformation also alters the toxicity of a compound, which may be either beneficial or harmful to the organism. In case of a detoxification reaction the toxicity of the compound is reduced while the excretion is generally elevated. In case of bioactivation, the compound is transformed into a reactive metabolite, which is more toxic than the parent compound (Van der Oost *et al*., 2003). The toxic effects may manifest themselves when the parent compound or its metabolites bind to cellular macromolecules, which may ultimately lead to

membrane disruption, cell damage and or genotoxic effects that subsequently can lead to development and progression of diseases (e.g. cancer) (figure 5).

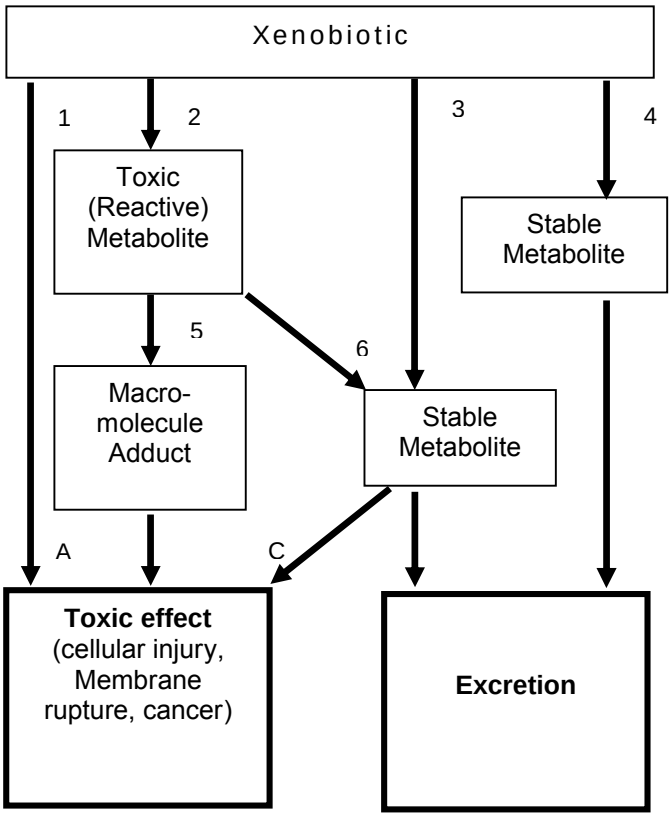

Figure 5- Possible toxication and detoxification pathways of xenobiotic compounds: (1) direct toxic effect (A); (2) metabolic activation; (3) formation of a stable metabolite which may cause a toxic effect (C); (4) detoxification. The reactive metabolite formed by bioactivation (2) may cause a toxic effect (B) through reaction with critical targets (5) or be detoxified through reaction with a protective agent (6). Adapted from Timbrell (1991), slightly modified.

R.T. Williams, in 1959, firstly studied the detoxification mechanism of xenobiotic and divided the entire steps taking the part in enzymatic biotransformation into two phases I and II. Some authors consider the existence of a phase III. Phase I is a non-synthetic alteration (oxidation, reduction or hydrolysis) of the original foreign molecule, which can then be conjugated in phase II and catabolized in

phase III (Commandeur et al., 1995). Phase I reactions precedes Phase II, though not necessarily. During these reactions, polar bodies are either introduced or unmasked, which results in (more) polar metabolites of the original chemicals. In phase I reactions oxidation involves addition of oxygen (forming a negatively charged radical) or removal of hydrogen (forming a positively charged radical). If the metabolites of phase I reactions are sufficiently polar, they may be readily excreted at this point. However, many phase I products are not eliminated rapidly and undergo a subsequent reaction in which an endogenous substrate combines with the newly incorporated functional group to form a highly polar conjugate. Phase II reactions known as conjugation reactions (e.g., with glucuronic acid, sulphonate glutathione or amino acids) which are usually detoxicating in nature and involve the interactions of the polar functional groups of phase I metabolites.

The great majority of xenobiotic is catalyzed by microsomal monooxygenase (MO) enzyme in phase I reaction, which is also known as mixed function oxidase (MFO) system (i.e. cytochrome p450 and NADPH cytochrome dependent). MO has a great capacity to metabolize organic xenobiotic and endobiotic, such as steroids and fatty acid, found especially in the liver (Stegeman and Hahn, 1994), though its activity has also been detected in the kidney, intestinal tract and branchial tissue. Most oxidative phase I biotransformations are catalyzed by cytochrome P450- dependent MOs. Cytochromes P450, comprising a large and still expanding family of heme proteins, are membrane-bound proteins which are predominantly located in the endoplasmic reticulum of the liver (Stegeman et al., 1992; Bucheli and Fent, 1995). The reaction catalyzed by MFO results in the insertion of one oxygen atom from dioxygen to a substrate molecule, increasing its hydrosolubility and facilitating its elimination from the body (Masfaraud et al., 1990). The most important feature of the MFO system is its ability to facilitate the excretion of certain compounds by phase I metabolism, as it transforms lipophilic xenobiotics to more water-soluble compounds (Bucheli and Fent, 1995). Since the MFO system is sensitive to certain environmental pollutants, its activity may serve as a biological monitor for exposure to certain classes of xenobiotic chemicals

(Bucheli and Fent, 1995). In fish, the activity of biotransformation enzymes may be induced or inhibited upon exposure to xenobiotics (Bucheli and Fent, 1995). The enzymatic induction consisting of an increase in the enzymes activity is in question, whereas in case of inhibition, the enzymatic activity is blocked, possibly due to a strong binding or complex formation between the enzyme and xenobiotics. In this context, it has been demonstrated that fish exposure to specific chemicals, induce liver cytochrome P450-dependent enzymatic activities such as ethoxyresorufin-O-deethylation (EROD) (phase I).Thus, liver EROD activity has been adopted as a valuable indicator of the presence of certain chemical pollutants, presumably at toxic levels (Santos and Pacheco 1996).

3.1.1 - Ethoxyresorufin-*O*- deethylase

Ethoxyresorufin-*O*-deethylase (EROD) is a MFO which is found to be very sensitive to induction by several chemical pollutants - namely, PAHs, resin acids and PAH-like substances such as BNF. Thus, the evaluation of the EROD activity in fish has been used as a strong *in vivo* biomarker of the exposure to PAHs, such as benzo[a]pyrene (BaP), and β-naphthoflavone (BNF) a PAH-like compound (Buchelli e Fent, 1995). The mechanism of CYP1A response (cytochrome P-450 related MFO) to the contaminants (such as PAHs) seems to be related with its binding capacity to cytosolic aryl hydrocarbon receptor (AhR) activating the transcription of the gene that codes for CYP1A, thus initiating its synthesis.

The PAHs are easily metabolized by phase I enzymes (MFOs) and biotransformed in more hydrophilic products. Fishes such as rainbow trout, mirror carp, chub and stone loach caught in waters (Chalaronne River- southeast France) contaminated predominantly by PAHs or by complex mixtures (Kosmala et al., 1998) displayed liver EROD activity induction. The EROD activity is measured by following the increase in fluorescence of the reaction product resorufin (Burke and Mayer, 1974).

In various fish species, hepatic CYP1A protein levels seem to be a very sensitive biomarker of exposure to PAHs, which certainly fits well in the procedure necessary for a correct ERA. (Van der Oost *et al*., 2003). CYP1A determinations may be used in various steps of the ERA process, such as quantification of impact and exposure of various organic trace pollutants, environmental monitoring of organism and ecosystem 'health', identifying subtle early toxic effects, triggering of regulatory action, identification of exposure to specific compounds, toxicological screening and the research on toxic mechanisms of xenobiotics (Stegeman et al., 1992).

Naphthalene (Naph) and BNF were reported as potent EROD activity inducers in several fish species. Thus, *A. anguilla* L. 3-day exposure to Naph and BNF (0.1-2.7 μM) significantly increased liver EROD activity, whereas after 6 and 9 days exposure its activity only increased for the highest (0.9 and 2.7 mM) Naph and BNF concentrations (Pacheco and Santos., 2002). Liver EROD activity induction has been also observed in sea bass (*D.labrax*) exposed to BNF a PAH-like substance (Gravato and Santos, 2002b). In *Liza aurata* short-term exposure to phenanthrene (Phe) demonstrated liver EROD activity induction at 0.3, 0.9 and 2.7 μM concentrations (Oliveira et al., 2007). Shailaja and D'Silva (2003) also observed liver EROD activity induction in tropical cichlid (*Oreochromis mossambicus*) exposed to Phe. However, *in vitro* sea bass liver microsomal EROD activity have demonstrated that PAHs such as BaP and a PAH-like compound (BNF) have an inhibitory potential (Gravato and Santos, 2002e) whereas Naph prevented the BaP (0.01 □M) inhibitory effect. The potential of RAs to induce liver EROD activity was also observed either by intraperitoneal or water diluted exposure (Pacheco and Santos, 1997, 1999; 2002). AA increased liver EROD activity in eels after 8h and 16h exposure to 0.9μM, 24h exposure to all the concentrations (0.1-2.7 μM) and 72h exposure to 0.1-0.9 μM (Maria et al., 2004a). DHAA also significantly increased the liver EROD activity after 16h exposure to 0.9, 2.7 □M and after 24h exposure to 0.9 □M in eels (Maria et al., 2004c). However, in juvenile sea bass AA inhibit liver EROD activity at 2h

exposure to 0.05 µM (Gravato and Santos, 2002b). In addition, Re, a derivative of RAs has also been shown to have a high capacity to induce liver EROD activity until 72 hours (8, 16, 24 and 72) following 0.1 up to 2.7 µM of its exposure in *A. anguilla* L.(Maria et al., 2004b). Oikari et al. (2002) also observed that liver EROD activity induction in Rainbow trout exposed to sediments, spiked with Re. Nevertheless Gravato and Santos (2002d) demonstrated inhibited liver EROD activity in sea bass (*D. labrax)* after 6 h exposure to 0.0125 µM Re. Moreover, Santos and Maria (2005) also observed significant eels liver microsomal EROD activity inhibition after *in vitro* exposure to Re (0.01-2.7 µM) and slightly inhibition to AA at all the concentrations (0.001-2.7 µM), however DHAA (0.1-2.7 µM) induced EROD activity.

3.2- Endocrine System

A wide range of synthetic chemicals used for several industrial and household activities have been shown to cause endocrine disturbances in living organisms. The consequences of such perturbation can be important because of the crucial role played by the endocrine system in the coordination of physiological processes and homeostasis maintenance (Hontela, 1998). In this context, cortisol plays an extremely relevant role, as a final product of Hypothalamo-pituitary- interrenal (HPI) tissue, in response to different agents causing stress. Cortisol is a steroid hormone associated with immunosupression, growth retardation and susceptibility to disease (Bowman, W.C., Rand, J., 1981). The role of cortisol in the intermediary metabolism is vital, it mobilizes fuels such as amino acids, converting them into glucose and lipids, thus exerting direct and indirect effects on intermediary metabolism (Van Der Boon et al., 1991) The increase in fish plasma cortisol concentration has been demonstrated after short term exposure to the vast range of contaminants such as heavy metals (De Boeck et al., 2003), resin acids (Teles et al., 2003), PAHs (Pacheco e Santos, 2001a), and pesticides (Waring and Moore, 2004). Plasma Cortisol level is the most commonly used stress indicator in fish since its rapid elevation occurs in

response to various stressors such as handling, confinement, poor water quality, and a wide variety of toxicants (Wendelaar-Bonga, 1997). Several *in vivo* studies, concerning the evaluation of plasma cortisol levels in different fish species such as *A. anguilla* L., (Pacheco and Santos, 2001b) demonstrated its significant increase after 3 hour exposure to diesel oil water-soluble fraction. In the same fish species a significant plasma cortisol increase was also found after 16 h exposure to AA 2.7 μM and after 24 h exposure to 0.1 μM and 2.7 μM as well as after 72 h exposure to 0.9 μM (Teles et al., 2003). In cage studies, *A anguilla* L. plasma cortisol also increased after 48 h exposure to contaminated harbour waters as well as to BKPME contaminated water after 8 h exposure at site 3, which is farthest from the deactivated sewage outlet (Teles et al., 2004b). Oliveira et al. (2007) also observed a significant increase in plasma cortisol induced by Phe 0.3, 0.9 and 2.7 μM in *Liza aurata.* However, a decrease in plasma cortisol levels was also found in S*parus aurata* after 16h exposure to 17β-estradiol 4000 ng/L and to the same 17β-estradiol concentration when mixed with 50,000 ng/L 4-nonylphenol (Teles et al., 2005a). *D. labrax* L. cortisol was not affected by its exposure either to 131nM 17β-estradiol or 4.05 μM 4-nonylphenol (Teles et al., 2004a). According to Hontela et al. (1997), fish sampled at contaminated sites had an impaired capacity to increase their plasma cortisol levels in response to stress. Thus the previous studies reflect the unquestioned relevance of cortisol as stress biomarker in fish.

In the vast majority of living organisms, the induction of phases I and II biotransformation enzymes is a well-known response to organic xenobiotic exposure. However, the knowledge on the linkage between these activations and other biological functions, namely endocrine regulation, is still a challenge to environmental toxicology (Teles et al., 2005b).

3.3 - Genotoxicity Evaluation

The CYP1A induction and its association with the existence of damage at DNA level in fish has been considered as sensible and adequate in the

evaluation of the genotoxicity caused by the exposition of the pollutants (Maria et al., 2002). The PAHs reactive intermediates have potential to interact with any nucleophilic site within the cell, including those on proteins and DNA, inducing genetic damage (Stegeman and Lech, 1991). The study of DNA damage, at the level of chromosome, is an essential part of the genetic toxicology since the chromosomal mutation is an important event in the carcinogenesis (Fenech, 2000). The observation that DNA damage can be caused by exposure to ionizing radiation or carcinogenic chemicals was among the first reasonable evidence that physical and chemical agents can cause major alterations at the level of genetic material in eukaryotic cells (Evans, 1977). In 1970, Miller established a relationship between the alteration of genetic material and carcinogenesis, demonstrating that the development of a short -term test for mutagens can serve a simple indicator for the cancer risk assessment. In this context, genotoxicity test, such as erythrocytic nuclear abnormalities detection, allied with biotransformation enzyme studies, performed on juvenile specimens, deserve a special attention from an ecotoxicological point of view. Moreover, the demonstration that industrial wastes can induce genotoxic effects underlines the urgent need for sensitive assays for the assessment of the genotoxic potential of the industrial wastes (Kohlpoth *et al*., 1999).

Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosomes lagging behind in the anaphase stage of cell division. Their presence in cells is a reflection of structural and/or numerical chromosomal aberrations arising during mitosis (Fenech *et al*., 1999). In recent years, various studies have described the presence of nuclear abnormalaties, other than micronuclei, in cells of fish exposed to genotoxic substance (Cavas and Ergene-Gözükara, 2003). In general, these abnormalities are considered to be indicators of genotoxic damage and, therefore, they may complement micronuclei scoring in studies of genotoxicity (Cavas and Ergene-Gözükara, 2005a). The erythrocytic nuclear abnomalaties assay, based on the detection of micronuclei and other nuclear anomalies, has been regarded as a simple, rapid, inexpensive and sensitive test, applied with success in different fish

species such as *A. anguilla* (Pacheco and Santos, 1997, 1998, 1999; 2001a,b), *D. labrax* (Gravato and Santos, 2002a) and *Oncorhynchus mykiss* (Ayllón and Garcia-Vasquez, 2001), exposed to various classes of genotoxins, and thus frequently used as the genotoxicity biomarker (Pacheco et al., 2005).

In this direction studies based on the effects of toxic compounds such as, BaP on *A. anguilla* L. showed that ENA was increased by BaP at 0.9 μM (Pacheco and Santos, 1997). Pacheco and Santos (1998) also studied ENA after treatment with PAH (BaP), PAH-like compound (BNF) and an alkylating agent-cyclophosphamide (CP) (14.7 □M/Kg) in *A. anguilla* L..Among these chemicals, only CP induces a significant increase in the ENA frequency after 3 days exposure. The effect of Naph and BNF on *A. anguilla* L. ENA frequency was investigated over 3, 6 and 9 day exposure (0.1-2.7 μM) by Pacheco and Santos (2002) showing that BNF has a considerable genotoxic potential by measuring ENA. However, no alteration was found in ENA when exposed to Naph. Moreover, a short term study (0 – 8 h) done by Gravato and Santos (2002a) on *D. labrax*, taking BNF (0 and 2.7 □M), B(a)P (0, 0.1, 0.3, 0.9 and 2.7 □M) and Naph (0, 0.1, 0.3, 0.9 and 2.7 □M) demonstrated that B(a)P is the most genotoxic compound in terms of ENA frequency, followed by Naph and BNF. An additional PAH having three rings such as Phe has also been studied in *Liza aurata* demonstrating its genotoxic potential in liver and blood, measured as increased DNA strand breaks and ENA frequency respectively (Oliveira et al., 2007).

Genotoxicity studies, in the context of pollutants mixture, were carried out on BKPME by Pacheco and Santos (1999). In the previous study, a progressive ENA increase was observed up to 12.5% BKPME and a decline in its frequency over 25% BKPME after 3 day of exposure. The ENA frequency observed at 50% BKPME was significantly lower than the ENA 25% of BKPME for the same exposure length. Among various organic and inorganic pollutants, AA and DHAA are the most abundant RAs in BKPME receiving waters. Pacheco and Santos (1997) demonstrated that is able to induce an increased ENA frequency after *A. anguilla* short term exposure to 2.7μM DHAA. Maria et al (2004a) also found that

AA is a weak ENA inducer in *A. anguilla,* but considering DNA strand breaks AA is more genotoxic to liver than to blood in the same fish species. In addition, has been demonstrated (Maria et al, 2004b) to have a capacity to induce genotoxicity by decreasing liver DNA integrity, in *A. anguilla* L.

4. ORIENTATION AND OBJECTIVES OF THE THESIS

In vitro toxicity assays have advantages over traditional *in vivo* system for sacrifice a small number of organisms, the best control of the variable environment, the possibility of simultaneous or repeated sampling over time, the use of minute amounts of chemical compounds (xenobiotics), allowing the study of a specific biotransformation reaction under strict controlled conditions (Moore and Simpson, 1992). However, besides the previous advantages, the possibility of the occurance of overestimation and underestimation of the laboratory results generate a need to validate laboratory (mainly *in vitro*) observations on biomarkers responses by field research work. Keeping in view, the relevance of both types of studies (*in vivo* and *in vitro*), the present dissertation has been divided into five (I through V) chapters.

Chapter I present the seasonal assessment of **erythrocytic nuclear abnormalities** (ENAs) in *D. labrax* L. caught from different sampling sites in Aveiro lagoon (Portugal), a polluted coastal lagoon. In this study the *D. labrax* was chosen as bioindicator due to its ecological relevance, abundance and geographical distribution, as well as easy to capture and manipulation.

Chapter II describes the *in vitro* effect of two PAHs - **naphthalene** and **phenanthrene** on *A. anguilla* L. liver microsomal EROD activity.

Chapter III describes the *in vitro* effect of Cortisol with/without DMSO on *A. anguilla* L. liver microsomal EROD activity.

Chapter IV and V focuses on the *in vitro* effect of **abietic acid** (resin acid) and **retene** (substituted PAH) with or without a steroid hormone – **cortisol** on hepatic microsomal EROD activity of *A. anguilla* L. previously induced *in vivo* by β-napthoflavone (BNF).

One of the methodologies frequently used in fish *in vitro* studies is the use of microsomes, obtained in stock from the liver with homogenous characteristics which turned easy if great dimensional animal is sacrificed, previously exposed by β-naphthoflavone either intraperitoneally or through water. This methodology, allows the preservation of the microsomal stock at -80 ºC during months and have demonstrated the reliability and reproducibility for the EROD activity (Yamazaki et al., 1997). The choice of the conger-eel is base on the fact that it has previously been proven its sensitivity for PAHs and BNF administration either intraperitoneally or through water exposure, by inducing hepatic EROD activity (Pacheco and Santos., 1998; Santos and Maria., 2005). Due to the previous fact, this species warranted its suitability to achieve the desired microsomal stock from a single animal having previously raised enzymatic activity. In the end we discuss the general results obtained from chapter I, II, III, IV and V.

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CHAPTER I

SEASONAL ASSESSMENT OF A CONTAMINATED COASTAL LAGOON (AVEIRO LAGOON, PORTUGAL) USING *Dicentrarchus labrax* L. ERYTHROCYTIC NUCLEAR ABNORMALITIES

Fresenius Environmental Bulletin (in press)

ABSTRACT

Aveiro Lagoon, a coastal lagoon, located in the northern west region of Portugal has been the main receptor of anthropogenic discharges resulting mainly from industries (chlor-alkali and pulp/paper plants), harbours and dry-dock activities as well as municipal effluents. In this perspective, the European sea bass (*Dicentrarchus labrax* L.) erythrocytic nuclear abnormalities (ENA) assay, as a measure of genetic damage, was applied to monitor the presence of genotoxic contaminants in this complex water pollution. Sea bass capture was carried out between spring 2006 to winter 2007, at six locations (Torreira-TOR, Barra-BAR, Gafanha-GAF, Rio Novo do Príncipe-RIO, Laranjo-LAR, Vagos-VAG) along the lagoon. Among all the study sites, TOR was selected as a reference site due to the absence of known input point sources and because it is far from the main polluting sources.

In spring, a significant ENA increase was observed at BAR, RIO, LAR and VAG in comparison to TOR. In summer, the ENA increase was pronounced at all the study sites. In autumn, ENA induction was significant only at GAF, LAR and VAG. In winter, BAR, GAF and RIO were the sites showing a significant ENA increase when compared to TOR. The present investigation findings provide a rational use of ENA applied to sea bass and can be successfully employed for biomonitoring anthropogenic contamination in both Atlantic and Mediterranean European coastal waters.

INTRODUCTION

Aveiro lagoon is a coastal lagoon located in northern west region of Portugal. Historically, this lagoon has been the main receptor of anthropogenic discharges resulting from chlor-alkali and pulp/paper plants, harbour and dry-dock activities as well as municipal and domestic effluents. In the lagoon, fish are exposed to pollutant's mixtures, whose synergistic/antagonistic effects are hardly

interpreted and predicted from the chemical analyses (Regoli et al., 2004). Moreover, the impracticality to analyze each individual chemical in a mixture of contaminants also increases the problem of aquatic pollutant characterization. Thus, during the past decades, biomarkers utility has become relevant in toxicological assessments since it allows the early detection of overall contaminants effects (Passino, 1984, Peakall and Shugart, 1993).

Genotoxicity biomarkers have received particular attention towards the use of piscine micronuclei (MN) and erythrocytic nuclear abnormalities (ENA) assays (Pacheco et al, 2005). The ENA, based on the MN detection and other nuclear anomalies, has been regarded as a simple, rapid, inexpensive and sensitive test, applied with success in different fish species including European sea bass (*Dicentrarchus labrax* L.)((Pacheco and Santos, 1997 and 2001; Gravato and Santos, 2002; Ayllón and Garcia-Vasquez 2001). In the case of abnormal nucleus, apoptotic cells are formed by actively dying cells including cell shrinkage, membrane blebbing and chromatin condensation (Murakawa et al., 2001), while necrotic cells are formed due to cytoplasm swelling, lysis of organelles and entire cells (Kirsh-Volder and Fenech, 2001). Like apoptosis and necrosis, another abnormal nucleus such as notch nucleated and bi-nucleated cells are indicators of abnormal cell divisions (Cavas et al., 2005)

Therefore, to investigate the ENA efficacy in environmental biomonitoring programmes, *D. labrax* were captured from different lagoon sites. Previous chemical analyses displayed the presence of polycyclic aromatic hydrocarbons (PAHs), organometallic compounds and heavy metal, as an indication of water quality deterioration in some critical areas of this estuarine system (Sousa et al., 2007). In addition, it was also intended to observe the fish suitability for environmental health assessment in a multi-pollution context.

MATERIAL AND METHODS

Chemicals

Chemicals and reagents (analytical grade) used for this study were purchased from Sigma.

Sampling – Animals and Stations

D. labrax L seasonal samplings (spring, summer, autumn and winter) were carried out between May 2006 and March 2007 using a fishing stick. As per the biology of fish, *D. labrax* is a eurythermic (5-28°C) and euryhaline (3‰ to full strength sea water) fish species inhabiting a variety of environments from coastal waters to estuaries and brackish water lagoons, and occasionally rivers. Estuaries are nursery areas where juvenile sea bass usually predominate. Its karyotype presents 24 pairs of chromosomes (2n = 48) (Sola et al., 1993).
There is only one breeding season per year, which takes place in winter in the Mediterranean population, and up to June in Atlantic populations. *D. labrax* are predators and their feeding range includes small fish, prawns, crabs and cuttlefish (Stickney, 2000)

The sampling sites in Aveiro lagoon were selected on a geographic distribution basis (Fig. 1) along the main channels and at the lagoon entrance, taking into account the various type and sources of contamination as well as the selection of a (theoretically) unpolluted reference point. The sampling sites were: Torreira (TOR) as a reference point at the stretch of the longest channel (S. Jacinto-Ovar channel) having non-point pollution inputs and located far from the main polluting sources; Barra (BAR) at the initial part of Mira channel, close to the sea-side lagoon entrance and subjected to a considerable naval traffic; Gafanha (GAF) situated in the vicinity of a deep-sea fishing port and dry-docks, also connected with the main channel coming from Aveiro city receiving domestic discharges; Rio Novo do Principe (RIO) located at the terminal area of the Vouga river and the main lagoons' freshwater source, 6.5 km distant from a pulp/paper mill effluent outlet, that was exposed during nearly five decades to its discharges;

Laranjo (LAR) close to a chlor-alkali plant, an important source of heavy metals contamination (mainly $HgCl_2$); Vagos (VAG) at the terminal part of the Ílhavo channel, receiving municipal and domestic effluents. Biometrical measurements, such as fish weight and length ranged from 18.71 - 41.96 g and 12.9 – 16.5 cm. Hydrological abiotic parameters were also measured (Table 1).

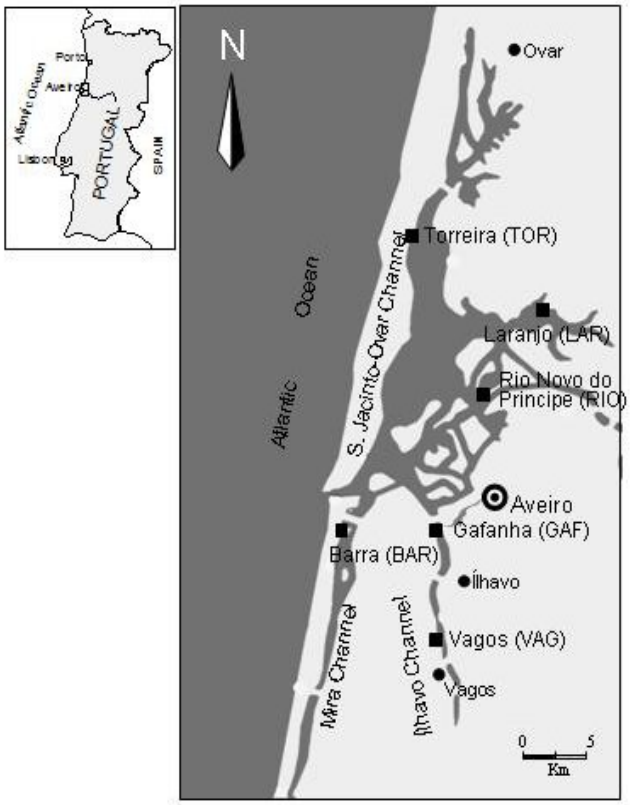

Figure 1. Map of the sampling stations (■) in the Ria de Aveiro. The res
cordinates are: reference site (TOR)- 40˚44'02 N, 008°41'44 W; BAR- 4(
008°44'35 W; GAF- 40°38'38 N, 008°41'42 W; RIO- 40°41'08 N , 008°
LAR- 40°43'30 N, 008°37'43 W; and VAG- 40°33'59 N, 008°40'55 W.

Table 1. Hydrological characteristics determined seasonally on different study sites (Torreira-TOR, Barra- BAR, Gafanha-GAF, Rio Novo do Príncipe-RIO, Larango-LAR and Vagos-VAG at Ria de Aveiro: nd- not determined

Sites	Depth (m)	Turbidity (m)	Dissolved O_2 (mg/L)	Temperature (° C)	pH	Salinity
Spring 2006						
TOR	1.1	0.3	3.51	23.3	8.351	nd
BAR	0.8	0.4	3.52	22.3	8.351	30
GAF	3	1	4.1	21.4	8.207	28
RIO	4.2	0.8	3.1	22.1	7.765	0
LAR	1.1	0.6	3.28	23.4	7.742	17
VAG	2.8	0.65	nd	24.6	7.655	11
Summer 2006						
TOR	1.2	0.5	5.03	25.1	8.396	26
BAR	2.7	2.5	1.8	22.4	8.205	24
GAF	5.6	1	3.64	20.1	8.249	30
RIO	0.9	0.6	2.27	24.1	8.056	25
LAR	1.2	0.3	1.07	22	7.446	25
VAG	3.6	0.5	2.21	24	7.842	26
Autumn 2006						
TOR	1	50 cm	17.7	17.7	8,122	23
BAR	2.35	30 cm	15.75	15.6	8,022	4.5
GAF	1.5	15 cm	12.16	14.5	7,865	8
RIO	4.75	70 cm	4.26	16.3	7,947	0
LAR	1.3	30 cm	3.52	16.6	7,511	6
VAG	nd		6.19	16.3	7,441	0
Winter 2007						
TOR	nd	nd	9.4	14.5	8.132	15
BAR	2.55	50	9.2	15.9	7.748	nd
GAF	4.1	40	9.44	14.9	8.195	21
RIO	1.5	0.5	8.31	14	8.322	0
LAR	1	0.2	8.58	12	7.184	22
VAG	2	0.15	7.5	14	7.253	0

ENAs assay

Blood smears were performed at the sampling sites and were fixed in the laboratory with methanol during 10 min, stained with Giemsa (5%) during 30 min. ENA were scored in 1000 mature erythrocytes sample per fish, according to the criteria of Schmid (1996), Carrasco et al. (1990) and Smith (1990) as adapted by Pacheco and Santos (1996), considering the following nuclear lesions categories: dumbbell shaped or kidney shaped nuclei (K), lobed nuclei (L), segmented nuclei (S), micronuclei (MN). In addition, notched nuclei (N) were also scored as

suggested by Ayllón and Garcia-Vasquez (2001). Different types of ENA have been depicted in Fig. 2. The final results, in each group, were expressed as the mean value (‰) of the sums (K+L+S+MN+N) for all the individual lesions observed.

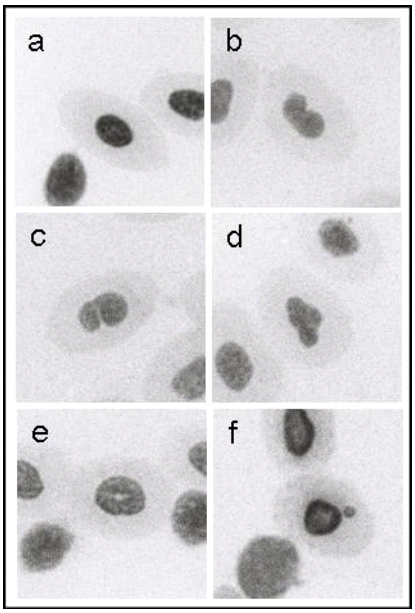

Figure 2- Mature erythrocytes of juvenile *D. labrax* L. with nuclear normal shape (a) and nuclear abnormalities: kidney-shaped nuclei (b), segmented nuclei (c), lobed nuclei (d), notched nuclei (e) and micronuclei (f). Giemsa stain, 2200×.

Statistical analysis

SigmaStat software (SPSS Inc.) was used for statistical analyses. Data were first tested for normality and homogeneity of variance to meet statistical demands. ANOVA analysis was used to compare the results of the various fish groups, followed by the Tukey's test in order to compare results between fish groups (Zar, 1996) and the significance of the results was ascertained at $p < 0.05$.

RESULTS AND DISCUSSION

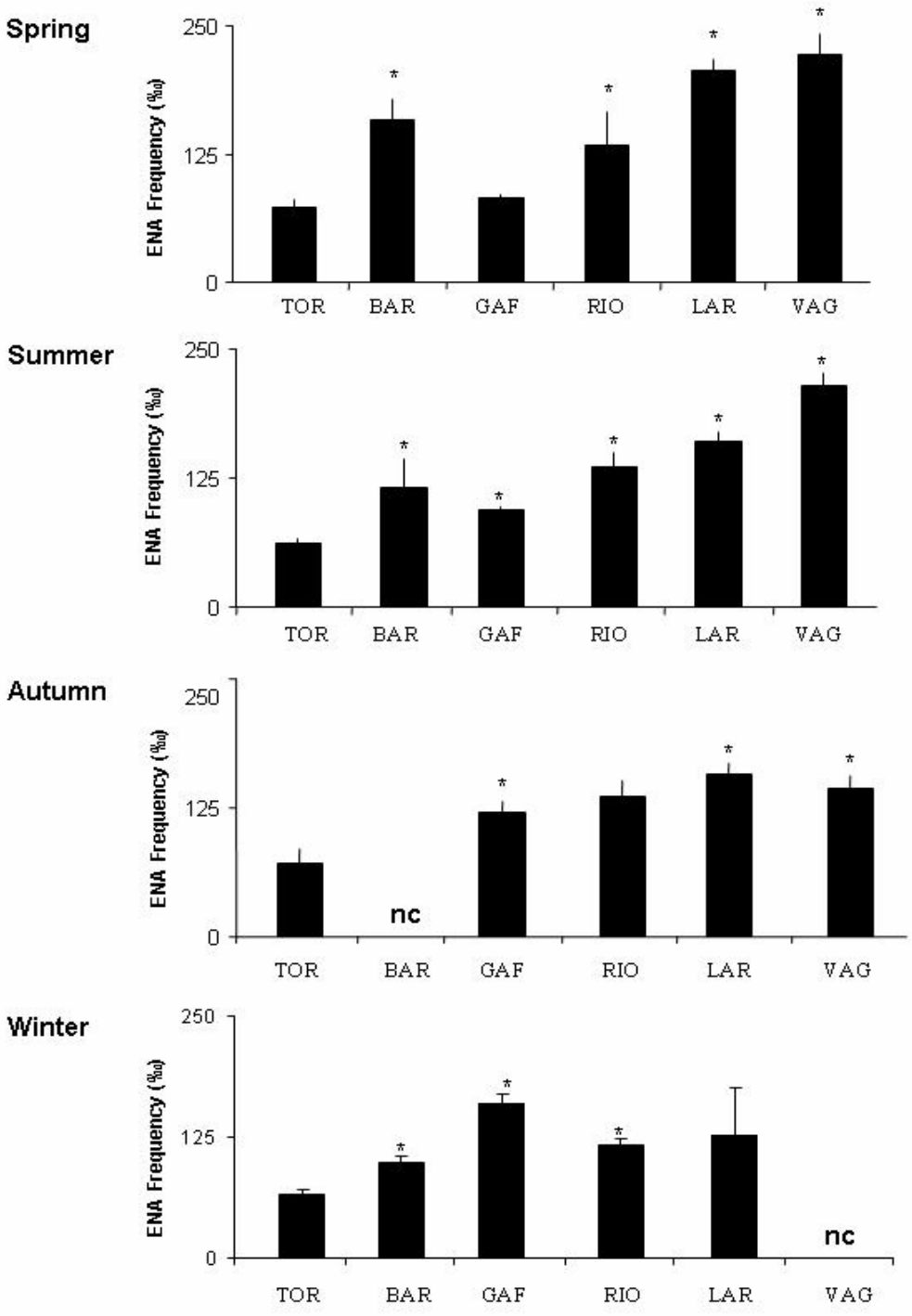

Figure 3. Mean ENA frequency in indigenous *Dicentrarchus labrax* collected seasonally from Torreira (TOR), Barra (BAR),Gafanha (GAF), Rio (RIO), Laranjo (LAR) and Vagos (VAG) sites of Ria de Aveiro. The symbol (*) denotes statistically significant differences ($p < 0.05$, n=5) with respect to reference (TOR) site within each season. Error bars represent the standard error.; nc=not collected.

This is the first fish survey carried out on the Aveiro lagoon seasonal assessment using *D. labrax* ENA as a tool to evaluate the ecosystem health.

Concerning *D. labrax* sensitivity to detect effects of a wide range of pollutants, phase I and Phase II enzymes activities (Gravato and Santos, 2003; Vaccaro et al., 2005), ENAs (Gravato and Santos, 2003), DNA strand breaks (Maria et al., 2008), plasma cortisol, lactate and glucose content (Teles et al., 2006), vitellogenin (VTG) synthesis (Teles, et al., 2004), have been used as biomarkers for the evaluation of environmental water contamination. Taking into account the previous description and the goals of the present study, the European *D. labrax* justify its selection as a sentinel. Moreover, juvenile specimens were surveyed in order to aside the gender interference and diminish the background of previous exposures to pollutants.

In spring season (Fig. 3), a significant ENA increase was observed at BAR, RIO, LAR and VAG sites when compared to TOR. Previous Aveiro lagoon survey, based on *Liza aurata* carried out on 2000 (five years earlier), showed no genotoxicity induction (Pacheco et al, 2005), however the present survey observed ENA increase and thus suggesting a water quality deterioration in last half decade. The observed differences may also be related to the biology (e.g. feeding behaviour) and susceptibility to DNA damage (e.g. defence mechanisms and DNA repair) of the adopted fish species. However, the possibility of the presence of different pollutants as causative agents for these observed genotoxic effects should also be considered as discussed following. Preliminary studies based on chemical characterization indicated the presence of different classes of pollutant in different sites of Aveiro lagoon. Thus, Sousa et al. (2007) reported the tributyltin (TBT) levels above the predicted no effect concentration level (0.0074 ng TBT-Sn/L) (EPA, 2003) to BAR site, indicating that the risk is high. In addition, Pacheco et al. (2005) also showed the moderate levels of PAHs presence. Moreover, due to its location, the occurrence of xenoestrogens at BAR site must be also considered. Considering the previous pollutants presence, the observed genotoxic responses can be reinforced to the combination of different classes of compounds instead of individual chemicals. Further validation of this explanation

came by Micael et al. (Micael et al., 2007), who reported that chronic exposure to binary mixtures of low levels of TBT plus ethinylestradiol and triphenyltin plus ethinylestradiol are genotoxic to zebrafish. No significant ENA increase was observed at GAF (Fig. 3) in the present spring survey despite being a highly polluted site having a variety of contaminants such as PAHs and TBTs (Micael et al., 2007; Pacheco and Santos, 2002). Moreover, *in situ* caging experiments with European eel (*Anguilla anguilla* L.) carried out in 2001 at the same site (Maria et al., 2003) displayed that GAF water has potential to induce DNA integrity loss. Taking into account the recent (Sausa et al., 2007) chemical characterization, the current results are in agreement to the statements of Pacheco and Santos (2002) suggesting that ENA assay may be inadequate for situations of intense contamination since some disturbances of genotoxicity expression may occur. These disturbances were confirmed to depend on the balance between an increased splenic erythrocytic catabolism and reduced erythropoiesis rate (Pacheco and Santos, 2002). The observed ENA increase at RIO (Fig. 3) may be correlated with the presence of chemicals that have been identified in pulp/paper mill effluents viz. resin acids (Gravato and Santos, 2002) since this site is located at the terminal area of the Vouga river, 6.5 km distant from a pulp/paper mill effluent outlets where the effluent discharge was continue during five decades. Reinforcing this idea Maria et al. (Maria et al., 2004) also observed a genotoxicity induction as measured by liver and blood DNA strand breaks as well as ENA increase in *A. anguilla* caged at RIO area on 2002. In relation to LAR (Fig. 3), a significant ENA increase was observed when compared to TOR. The current observations are not unexpected and seem to be in agreement with the findings of Guilherme et al. (2008) who showed ENA increase in *L. aurata* captured in the same area and its relation with the presence of mercury as a result of an effluent discharge from a chlor-alkali plant (Guilherme et al., 2008; Perelra et al., 1997). A significant ENA increase was also observed at VAG in comparison to TOR (Fig. 3). Previously, a study done by et al. (2005) based on PAHs analysis reflected benzo(a)pyrene (BaP) high levels at VAG station. Thus, the observed effects may be directed towards the presence of PAHs at this particular area.

Table 2- Mean frequency (%) of each nuclear abnormality category (±SE) in peripheral blood erythrocytes of *D. labrax* L. captured at TOR, BAR, GAF, RIO, LAR and VAG sites of Ria de Aveiro in spring 2006.

Site	**Kidney Shaped (K)**	**Lobed (L)**	**Segmented (S)**	**Micronuclei (MN)**	**Notched (N)**	**TOTAL (K+L+S+MN+N)**
TOR	9.10 ± 2.34	42.00 ± 5.73	1.90 ± 0.88	0.00 ± 0.00	8.30 ± 2.43	67.00 ± 10.78
BAR	11.00 ± 2.25	81.00 ± 20.91*	1.80 ± 0.60	1.20 ± 0.44	17.00 ± 5.74	158.00 ± 24.03
GAF	19.60 ± 1.43*	52.60 ± 2.05	3.60 ± 1.37	2.80 ± 0.67	4.00 ± 2.54	83.00 ± 9.54
RIO	11.00 ± 1.93	103.20 ± 11.95*	1.40 ± 0.67	0.40 ± 0.22	17.80 ± 2.55	134 ± 19.37
LAR	27.50 ± 4.15*	164.00 ± 11.11*	5.00 ± 1.12	0.5 ± 0.18	8.7 ± 1.68	206 ± 31.12
VAG	23.20 ± 2.34	183.50 ± 21.61*	4.30 ± 1.32	1.30 ± 0.51	9.20 ± 1.68	222 ± 35.00

* Statistically significant difference ($p<0.05$) from reference (TOR) site when compared to BAR, GAF, RIO, LAR, VAG.

Table 3- Mean frequency (%) of each nuclear abnormality category (±SE) in peripheral blood erythrocytes of *D. labrax* L. captured at TOR, BAR, GAF, RIO, LAR and VAG sites of Ria de Aveiro in summer 2006.

Site	**Kidney Shaped (K)**	**Lobed (L)**	**Segmented (S)**	**Micronuclei (MN)**	**Notched (N)**	**TOTAL (K+L+S+MN+N)**
TOR	1.88 ± 0.477	42.00 ± 4.16	0.33 ± 0.19	1.00 ± 0.40	15.67 ± 2.15	61.00 ± 7.98
BAR	3.25 ± 0.84	81.00 ± 22.84	0.33 ± 0.20	0.75 ± 0.39	30.00 ± 9.32	116.00 ± 17.128
GAF	8.60 ± 2.33*	68.00 ± 5.45*	1.20 ± 0.88	1.00 ± 0.00	14.00 ± 4.78	93.00 ± 12.71
RIO	3.40 ± 0.68	108.20 ± 7.79*	0.20 ± 0.18	0.80 ± 0.34	23.80 ± 4.25	136.00 ± 20.69
LAR	5.00 ± 1.04	136.20 ± 5.44*	0.60 ± 0.22	0.60 ± 0.365	18.00 ± 4.53	160.00 ± 26.22
VAG	8.00 ± 0.76*	158.60 ± 8.21*	1.00 ± 0.40	0.60 ± 0.22	46.60 ± 4.44*	215.00 ± 30.13

* Statistically significant difference ($p<0.05$) from reference (TOR) site when compared to BAR, GAF, RIO, LAR, VAG.

Table 4- Mean frequency (%) of each nuclear abnormality category (±SE) in peripheral blood erythrocytes of *D. labrax* L. captured at TOR, GAF, RIO, LAR and VAG sites of Ria de Aveiro in autumn 2006.

Site	**Kidney Shaped (K)**	**Lobed (L)**	**Segmented (S)**	**Micronuclei (MN)**	**Notched (N)**	**TOTAL (K+L+S+MN+N)**
TOR	2.20 ± 0.58	51.40 ± 10.08	0.6 ± 0.40	0.40 ± 0.17	16.60 ± 3.72	71.00 ± 9.77
BAR	nc	nc	nc	nc	nc	nc
GAF	3.60 ± 0.465	80.00 ± 8.52	6.00 ± 0.36	0.60 ± 0.36	35.20 ± 4.24	120.00 ± 15.41
RIO	3.70 ± 0.70	96.30 ± 16.07*	1.17 ± 0.16	1.50 ± 0.77	33.00 ± 1.29	135.00 ± 18.31
LAR	5.80 ± 0.60*	123.00 ± 13.28*	1.67 ± 0.33	0.83 ± 0.30	25.50 ± 2.88	156.00 ± 23.34
VAG	4.00 ± 1.15	116.30 ± 9.38*	1.00 ± 0.57	1.00 ± 0.57	21.6 ± 8.29	144.00 ± 22.21

*Statistically significant difference ($p<0.05$) from reference (TOR) site when compared to GAF, RIO, LAR, VAG in autumn. nc= not collected

Table 5- Mean frequency (%) of each nuclear abnormality category (±SE) in peripheral blood erythrocytes of *D. labrax* L. captured at TOR, BAR, GAF, RIO and LAR sites of Ria de Aveiro in winter 2006.

Site	**Kidney Shaped (K)**	**Lobed (L)**	**Segmented (S)**	**Micronuclei (MN)**	**Notched (N)**	**TOTAL (K+L+S+MN+N)**
TOR	3.50 ± 0.39	52.00 ± 2.68	0.50 ± 0.31	0.80 ± 0.30	9.00 ± 2.22	66.00 ± 9.76
BAR	3.00 ± 0.40	75.00 ± 4.37*	0.80 ± 0.34	1.20 ± 0.53	20.00 ± 2.98*	100.00 ± 14.20
GAF	6.80 ± 1.53	129.00 ± 5.43*	1.80 ± 0.67	3.20 ± 1.33	19.20 ± 3.89	161.00 ± 24.56
RIO	5.00 ± 0.40	91.20 ± 4.47*	1.80 ± 0.60	1.40 ± 0.46	18.00 ± 3.61	117.00 ± 17.19
LAR	3.00 ± 1.03	93.00 ± 17.55*	0.00 ± 0.00	1.0 ± 0.00	31.50 ± 6.45*	129.00 ± 17.81
VAG	nc	nc	nc	nc	nc	nc

*Statistically significant difference ($p<0.05$) from reference (TOR) site when compared to BAR, GAF, RIO, LAR in winter; nc= not collected

In summer (Fig. 3), a significant ENA increase was observed at all the study sites whereas in autumn (Fig. 3) and winter (Fig. 3), the increase was punctual viz. only GAF, LAR and VAG presented a significant ENA increase in autumn; and BAR, GAF and RIO displayed a significant increase in winter. Thus, the above given pollutants combined effect at different study sites can also be suggested here as a probable cause for the ENA increase through out the year. However, the absence of ENA increase (in autumn at RIO; in winter at LAR) may reflect the involvement of seasonal changes and pollutants coming from non point sources affecting on the lagoon contamination pattern. According to Gorbi et al. (2005) the same chemical disturbance can produce a different biological impact, depending on the seasonal susceptibility to stress.

The analysis of each nuclear lesion category individually in the different seasons (Tables 2-5) revealed that the lobed nuclei frequencies were significantly higher in spring (at BAR, RIO, LAR and VAG sites); in summer and winter (at all the study sites); and in autumn (at RIO, LAR and VAG sites) with respect to TOR, exhibiting the robustness of this biomarker for genotoxicity assessment. Analyzing the literature, the lack of uniform criteria for nuclear abnormalities scoring is evident, making difficult the comparison of the results from different laboratories. Despite some discrepancies, it seems to be uncontroversial that problems in segregating twisted and attached chromosomes or gene amplification via the breakage–fusion–bridge cycle could cause nuclear buds (NB) (lobed nuclei and blebbed nuclei) during the removal of amplified DNA from the nucleus (Tolbert et al., 1992; Shimizu et al., 1998 and 2000). According to Serrano-Garcia and Montero-Montoya (2001), the phenomena of budding cell nuclei and bi-nucleated cells have a similar origin as MN and are supposed to be genotoxic occurrences. Moreover, a positive and significant relationship between MN and NB induction was found by different authors (Bolognesi et al., 2006; Ergene et al., 2007), suggesting that NB formations in erythrocytes may be a useful complementary assay for genotoxicity assessment in fish. Considering the previous statements, the analysis of current results (Tables 2-5) based on the frequency of each nuclear lesion category clearly demonstrates that the lobed

nuclei (budding nuclei) frequency display a pattern of response similar to that one provided by the jointly scoring of all abnormalities categories.

As per the aspect of seasonal changes, besides other effects such as pollutants pattern, hydrodynamics, sediment suspension, biomarker basal levels also represents a suitable approach for environmental risk assessment (Collier et al., 1995; Eggens et al., 1996: Soborowiki et al., 1996; Roniz et al., 1999). In the current study no significant differences on ENA basal levels were observed in fish collected from TOR along the year, proving its robustness as control for future studies on Aveiro lagoon biomonitoring. Moreover in the context of genotoxicity, water temperature may influence cell replication rates and DNA repair of poikilothermal organisms (Venier et al., 1997). Brunetti et al. (1992) also suggested a considerable influence of temperature on MN frequency. In the same way, salinity, water levels, rain and storm events in field exposure have also been reported to influence DNA damage (Rank et al., 2007) by affecting the availability of contaminants as well as the fish physiological processes. According to the previous explanation, the highest temperature recorded at LAR and highest salinity recorded at BAR in spring seems to be one of the contributory factors for the increased ENA levels in current study. However, no changes in abiotic factors (mainly temperature, salinity and dissolved oxygen) recorded at VAG and GAF in spite of increased ENA level suggest the possibility of genotoxic contaminants presence coming from non point sources. According to Livingstone (Livingstone et al., 1993), the correct use of biological responses as biomarkers requires knowledge of their natural variability. Thus, assessed natural variability of ENA in the current field conditions represents an important aspect to improve the ecotoxicological use of ENA in biomonitoring programs.

CONCLUSIONS

The current ENA results demonstrate that a serious risk to fish populations exists almost at all the sites (except at GAF in spring, RIO in autumn and LAR in winter). Thus, at all the critical sites (BAR, GAF, RIO, LAR and VAG) identified, the results reflected contamination by a mixture of pollutants from point and non

point sources. However, the circumstantial absence of significant ENA increase in some sites should be interpreted with some precaution. The current findings reflect the ability of *D. labrax* for biomonitoring aquatic anthropogenic contaminants on the basis of its ENA assessment and can be successfully employed in both Atlantic and Mediterranean European coastal waters. *D. labrax* revealed to be a particularly appropriate species for genotoxicity monitoring of heterogeneous genotoxic contamination in coastal ecosystems.

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CHAPTER II

NAPHTHALENE AND PHENANTHRENE *IN VITRO* EFFECTS ON *Anguilla anguilla* L. LIVER MICROSOMAL EROD ACTIVITY

ABSTRACT

Liver microsome isolated from eels (*Anguilla anguilla* L.) after 24 hours of exposure to 4mg/kg β-napthoflavone (BNF) were *in vitro* exposed to naphthalene (Naph) and phenanthrene (Phe) 0.001, 0.01, 0.1, 0.3, 0.9, 2.7μM. *In vitro* liver microsomal ethoxyresorufin-O-deethylase (EROD) activity was significantly increased by Naph at all the concentration (0.001-2.7 μM). Concerning Phe, a significant EROD activity increase was observed at 0.001, 0.01, 0.1, 0.3, 0.9 and 2.7 μM concentrations except 0.001 μM concentration in comparison to DMSO control. A consistent dose dependent EROD activity increasing effect was demonstrated for both Naph and Phe exposure confirming them strongest *in vitro* inducer of *A. anguilla* L. liver microsomal EROD activity. Both PAHs increased *in vitro* liver microsomal EROD activity may be due to its microsomal membrane disruptive effects.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the aquatic environment both as natural products and as environmental contaminants. Aquatic organisms, namely fish, living in PAH-contaminated environments may absorb these compounds mainly from the water through gill and by the ingestion of contaminated sediments or food (Varanasi et al., 1989). PAHs easily cross lipid membranes, due to their lipophilicity, and bioaccumulate in different tissues. Among PAHs, two-ring compound, naphthalene (Naph) is ubiquitous pollutant in the aquatic environment mainly as the result of discharges from coal tar production and distillation processes (ATSDR, 1995) as well as from petroleum products and by-products spillages (Irwin, 1997; Pacheco and Santos, 2001). Fish readily take up Naph from the environment and possess a variety of cellular mechanisms for protection against the deleterious effects of such chemicals (Peters et al., 1997; Gravato and Santos, 2002a). A limited number of studies have been conducted to assess Naph toxicity to aquatic species, namely fish.

The early literature on Naph toxicity to teleosts concerns histopathological (DiMichele and Taylor, 1978), physiological (Levitan; Varanasi and Krahm) and structure–toxicity relationship (Black et al., 1983) studies. More recently, cytotoxicity (Schirmer et al., 1998), time-to-death (Gert-Jan de Maagd et al., 1997) and oxidative stress (Ahmad et al., 2003) were assessed. In the context of cytochrome P450 isoforms such as cytochrome P450 1A1 as well as associated activities like EROD, Pacheco and Santos (2002) observed Naph effects on *A. nguilla* L. hepatic metabolism and found Naph as strong EROD activity inducer.

In the context of PAH's toxicity, another PAH such as phenanthrene (Phe), limited information on Phe toxicity has been reported in fish, despite its abundance and ubiquity in the aquatic environment. Phe, a three ring PAH has been included in the 16 US EPA priority PAHs (Smith et al., 1989) as a result of both petrogenic and pyrogenic sources. Phe water levels in polluted areas ranged from 14.6 µg/l (Vrana et al., 2001) to 1460 µg/l (Anyakora et al., 2005) in a fishing settlement near a crude oil exploration area and considerable levels were detected in fish (Hellou and Warren, 1997).

PAHs concentrations in fish tissues can be significantly reduced by metabolism and excretion (Billiard et al., 2002). A limited number of studies are available on Phe CYP1A modulation showing divergent results. Thus, Bols et al. (1999) observed no induction of CYP1A activity in a rainbow trout liver cell line. Moreover, *in vitro* and *in vivo* EROD (ethoxyresorufin-*O*-deethylase) induction by other PAHs was inhibited by Phe (Hawkins et al., 2002). On the other hand, a significant CYP1A induction was observed in cod, flounder (Goksøyr et al., 1986), scup (Stegeman et al., 1998) and tilapia (Shailaja and D'Silva, 2003). Furthermore, Pangrekar et al. (1995), Pangrekar et al. (2003) reported that brown bullhead liver microsomes metabolize Phe.

The above *in vivo* studies revealed fish mixed function oxidase system as a useful tool for monitoring PAHs or other structurally similar compounds in

polluted aquatic environment (Connell, 1990; Pacheco and Santos, 1998). As regards *in vitro* studies, which have advantages such as the utilization of a small number of animals, controlled experimental conditions, genetic heterogeneity removal, small quantities of test chemicals and amount of toxic wastes (Baksi and Frazier, 1990), few studies have been offered in this perspective. Previously, Gravato and Santos (2002c) demonstrated that PHAs (BaP and Naph) and PAH-like compounds β-napthoflavon (BNF) *in vitro* exposure have an inhibitory potential for *Dicentrarchus labrax* liver microsomal EROD activity. *A. anguilla* and *Dicentrarchus labrax* liver microsomal EROD activity was also found to be very sensitive to *in vitro* heavy metal exposure (Oliveira et al., 2005; 2004). In the present study, findings on *in vitro* effects of 0.001, 0.01, 0.1, 0.3, 0.9 and 2.7μM Naph and Phe concentrations on the EROD activity of liver microsomes isolated from *A. anguilla* L. previously exposed to 4mg/kg BNF during 24 hours have been reported.

MATERIAL AND MATHODS

Reagents

Napthelene (Naph), Phenanthrene (Phe) was purchased from Sigma Chemical Co. (USA). All the other chemicals were of analytical grade and were obtained from Sigma Chemical Co. (USA), Boehringer (Germany) and E. Merck-Darmstadt (Germany).

Fish

One adult eel (*A. anguilla* L.) with an average weight 500 gram were collected from Aveiro Lagoon (Portugal). The eel was acclimatized to laboratory conditions in a 50 L aquarium containing aerated and filtered freshwater at 20^{o}C during 7 days. After acclimatization, the eel was intraperitoneally (i.p.) injected with 4 mg/kg β–napthoflavon (BNF) and killed 24 hours later. The liver was

immediately removed, frozen in liquid nitrogen and stored at -80^{o}C until homogenization. The fish was neither fed during recovery nor during the experimental period.

Liver microsaomal fraction preparation

Liver microsomes were prepared according to the methods of Lange et al. (1992) and Monod and Vindimian (1991) as adapted by Pacheco and Santos (1998). The liver was removed, frozen in liquid nitrogen, and stored at -20^{o}C until homogenization. Each liver was homogenized in a 1: 4 w/v ratio (liver weight: buffer volume) of 0.1M Tris-HCl (pH 7.4) containing 0.15MKCl and 20% glycerol, by using ±5 up-and-down strokes of a Potter glass-Teflon homogenizer at 2500 rpm. Microsomes were obtained by differential centrifugation, at 4ºC, in a Beckman Optima TL Ultracentrifuge (TLA-100.4 fixed angle rotor). The homogenate was first centrifuged at 15,000 rpm for 20 min to remove cell debris, nuclei, mitochondria, and lisosomes. Then, the resultant supernatant was collected and recentrifuged at 50,000 rpm for 75 min to isolate the microsomes. The resulting microsomal pellet was resuspended in 200 µl of the previous bu¤er, frozen in liquid nitrogen, and stored at -20^{o}C until use.

Protein concentration measurement

Microsomal protein concentrations were determined according to the Biuret method (Gornall et al., 1949) using bovine serum albumin as a standard.

EROD activity assay

Liver microsomal EROD activity was measured as describe by Burke and Mayer (1974). Briefly, liver microsomal EROD assay was carried out at 25^{o}C, in the fluorometer cuvette containing 1.09 ml of 0.5 µM ethoxyresorufin in 0.1 M

Tris-HCl pH 7.4, containing 0.15 M KCl and 20% glycerol and 5 µl of liver microsomal suspension from BNF induced eel. Five µl of DMSO (0.45%) were used to dissolve the xenobiotics tasted (0.001, 0.01, 0.1, 0.3, 0.9, 2.7 µM Phe, Naph) and added to incubation mixture as a vehicle. The same DMSO volume was added to the incubation mixture in all control tests. The reaction was initiated by adding 10 µl of NADPH (10 µM) and the progressive increase in fluorescence, resulting from the resourfin formation, was measured for 3 min (excitation wavelength 530 nm, emission wavelength 585 nm). Liver microsomal EROD activity was expressed as picomoles per minutes per milligram of microsomal protein. Experiment was carried out using three replicates (n=3).

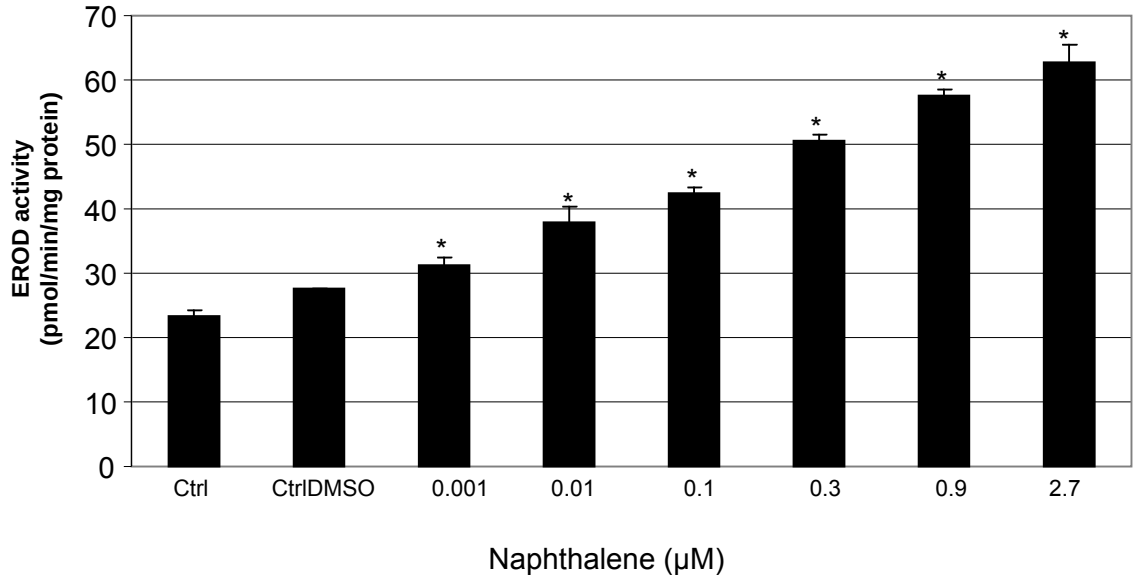
Statistical analysis

Statistica software (SigmaStat 2.03) was used for statistical analyses. All the data were first tested for normality and homogeneity of variance to meet statistical demands. Variance analysis was used to compare results between fish groups, followed by the Tukey test (Zar, 1996). Differences between means were considered significant when $p<0.05$.

RESULTS

A.anguilla L. previously exposed to 4 mg/kg BNF for 24 hrs demonstrated that the 0.45% DMSO vehicle has no significant effect on liver EROD activity when compared to control (Figure 1). However, fish liver microsome in vitro exposed to Naph showed a similar significant ($p < 0.05$) EROD activity increase at all Naph concentrations (0.001, 0.01, 0.1, 0.3, 0.9 and 2.7 µM) when compared to DMSO control. Concerning Phe, a significant EROD activity increase was observed at 0.001, 0.01, 0.1, 0.3, 0.9 and 2.7 µM concentrations except 0.001 µM concentration in comparison to DMSO control.

(A)



(B)

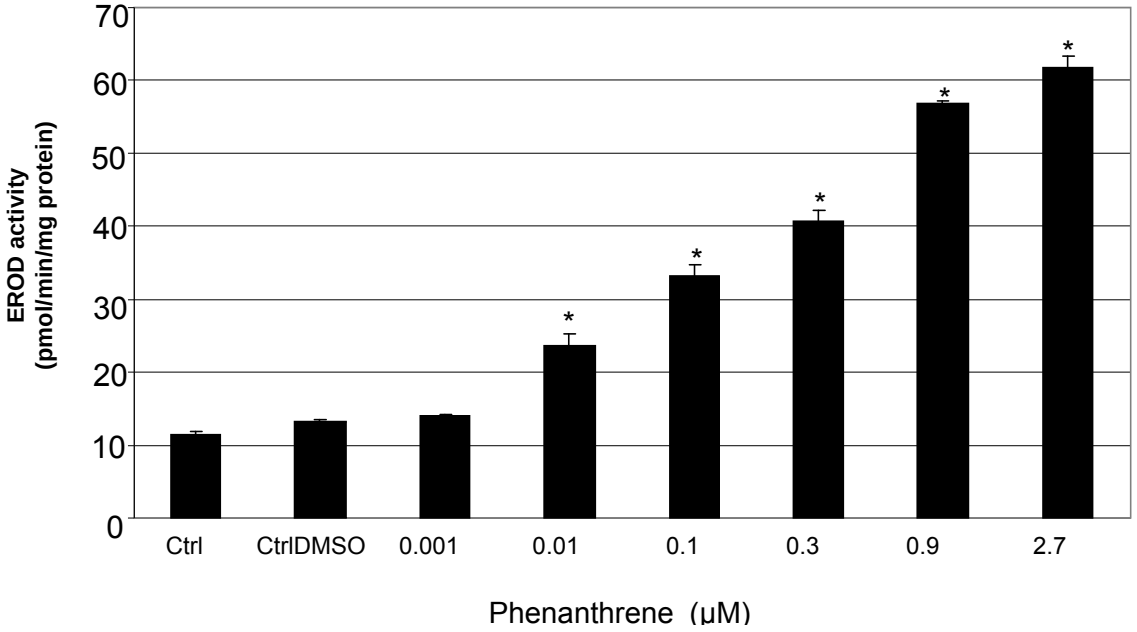


Figure 1- *In vitro* effects of 0.001, 0.01, 0.1, 0.3, 0.9 and 2.9µM naphthalene (A) and phenanthrene (B) on EROD activity from eel's (*Anguilla anguilla* L.) liver microsome, *in vivo* induced by 4mg/kg BNF for 24 hours exposure. Statically different from DMSO exposure condition: * $p<0.005$

DISCUSSION

Naph and its alkyl homologous series are especially hazardous PAHs to the aquatic environment due to their particular combination of mobility and toxicity (Irwin et al., 1997). The adopted Naph concentration range (12.8–346.1 µg/l) can be considered environmentally realistic since Naph concentrations detected in environmental waters range from 0.005 to 66,000 µg/l (Environment Canada, 1993; ATSDR, 1995). Naph *in vitro* exposure induced a significant hepatic microsomal EROD activity at all the studied concentrations reflecting a clear Naph potential to activate phase I biotransformation reactions in the present study. Moreover the increase was dosed dependent showing its EROD activity inducing potential at each and every concentration ranging from 0.001 - 2.7µM. In this context the explanation based on the membrane breakdown can be suggested for Naph as suggested for dehydroabietic acid by Santos and Maria (2005) since both findings are based on *in vitro* studies in the same fish species. The results are in agreement with the findings of Maria et al., (2002) and Pacheco and Santos, (2002) who observed the hepatic microsomal EROD activity induction in *A. anguilla L*., after the *in vivo* Naph exposure.

Concerning Phe, the concentration range (0.001 up to 2.7 µM) used in current study can be considered environmentally realistic since it falls within the level (up to 8.2 µM) detected in natural waters (Anyakora et al., 2005).The available data on fish CYP1A induction by Phe provided non-consensual results being those responses species dependant (Shailaja and D'Silva, 2003). However, the current results showed that liver microsomal EROD activity was induced by 0.01-2.7 µM Phe exposure. The Phe no observed effect concentration (NOEC) for liver microsome in vitro exposure was 0.001 µM. Thus, the current results are in the agreement of the previous *in vivo* results on different fish species including *L. aurata* confirming Phe CYP1A inducing potential (Goksøyr et al. 1986; Stegeman et al. 1998; Shailaja and D'Silva 2003 and Oliveira et al., 2007). Moreover, another PAH such as fluoranthene also reflected

similar hepatic microsomal EROD activity increase at 0.1 and 0.3 □M *in vitro* exposure (Ferreira, 2007).

Over all the results similarity in both Naph and Phe may be directed to the fact that naphthalene and phenanthrene share common metabolic pathways as suggested by Kiyohara H, et al. (1994). Phe is degraded through one of two different routes. In one route, 1-hydroxy-2-naphthoic acid is oxidized to 1,2-dihydroxynaphthalene, which is further degraded via the Naphthalene Pathway to salicylate which can be further metabolized. In the other pathway, the ring of 1-hydroxy-2-naphthoic acid is cleaved and further metabolized via the Phthalate Pathway. Thus, the previous toxicological responses suggest that these two PAHs may interact in similar way with liver EROD activity, causing its increase.

CONCLUSION

- a consistent dose dependent EROD activity increasing effect was demonstrated for both Naph and Phe exposure confirming them strongest *in vitro* inducer of *A. anguilla* L. liver microsomal EROD activity.

- the increase was dose dependent, Naph showed its potential to increase EROD activity at each and every concentration ranging from 0.001 - 2.7μM, whereas Phe no observed effect concentration (NOEC) for liver microsome in vitro exposure was 0.001 μM.

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CHAPTER III

CORTISOL *IN VITRO* EFFECTS ON *Anguilla anguilla* L. LIVER MICROSOMAL EROD ACTIVITY

ABSTRACT

An *Anguilla anguilla* L. weighing 500 g was injected intraperitoneally (i.p.) with 4 mg/Kg β-napthoflavone (BNF). The liver was excised after 24 hours and *in vitro* exposed to different concentrations of cortisol (6.9, 13.8 and 27.7 nM) without DMSO and with DMSO. Liver microsomal ethoxyresorufin-O-deethylase (EROD) activity showed a significant increase at all the tested concentrations of cortisol without DMSO. Nevertheless, in the presence of DMSO, microsomal EROD activity significantly increased only at 6.9 nM concentration of cortisol among all the studied concentrations. Thus, cortisol 6.9 nM was the only concentration, able to induce significant liver microsomal EROD activity, under both conditions i.e. without DMSO and with DMSO.

INTRODUCTION

Cortisol, the predominant corticosteroid in teleosts, is a key mediator of physiological processes and homeostasis maintenance (Hontela, 1998) that is thought to be crucial for supporting the increased energy demand associated with stress (Mommsen et al., 1999; Saplosky et al., 2000; Vijayan et al 1997). The role of cortisol as an intermediary in metabolism is vital, since it is the major glucocorticosteroid secreted by the teleost's interrenal tissue in response to adrenocorticotropic hormone stimulation (Pottinger *et al.*, 2000). Moreover, cortisol also plays a significant role in osmoregulation, growth and reproduction. Cortisol plasma levels are the most commonly used stress indicator in fish because of the rapid elevation that occurs in response to various stressors such as handling, confinement, poor water quality, and a wide variety of toxicants (Wendelaar-Bonga, 1997).

An elevation in plasma cortisol levels has been observed in fish after short-term exposure to pesticides (Bennet and Wolke, 1987), heavy metals (Bleau *et al.*, 1996), polycyclic aromatic hydrocarbons and crude oil (Thomas *et al.*, 1993), as well as pulp mill effluent (Kennedy *et al.*, 1995) and resin acids (Teles *et al.*, 2003a). Moreover, a decrease in plasma cortisol levels was also found in S*parus aurata* after 16h exposure to 17β-estradiol and 17β-estradiol

concentration when mixed with 4-nonylphenol (Teles et al., 2005a). However, *D. labrax* L. cortisol was not affected by its exposure either to 17β-estradiol or 4-nonylphenol (Teles et al., 2004a). The exposure of *A. anguilla* L. to diesel water soluble fraction revealed an inhibition in plasma cortisol (Pacheco and Santos, 2001). Moreover *A. anguilla* L. short-term exposure to secondary treated bleached kraft pulp mill effluent depresses plasma cortisol uptake during recovery from capture, anoxia and transport. On the other hand, short-term exposure after recovery from capture, and transport prevents intrarrenal cortisol release to the blood, decreasing its plasma concentration (Santos and Pacheco, 1996) and significantly increasing liver EROD activity (Pacheco and Santos, 1999).

In the context of *in vitro* investigations, few studies reflect the cellular physiological level of cortisol and its effects on isolated hepatic microsomal EROD activity. Interactions between cortisol and toxicants may be the key to the physiology of this hormone. In this direction, an important step is to determine the concentration of cortisol and dose-response relationship leading to more meaningful results during final stages of toxicity testing. Effects of cortisol may occur within a narrow range of concentrations. Previous study done by Santos et al., (2000) exhibit a significant enhancement of *A. anguilla* L. liver EROD activity under *in vitro* condition by minimum essential media (MEM) containing 1.4 □M concentration of cortisol from 0 up to 4 and 24 hours. Ferreira (2007) also observed the *in vitro* protective effects of cortisol at 22.02 nM concentration in combination with benzo[a]pyrene and fluoranthene (0.1-2.7□M) on *A. anguilla* L. hepatic microsomal EROD activity. Thus, keeping in view the previous scenario, the experimental setup was intended to find out, how and to what extent, the *in vitro* exposure to different concentrations of cortisol (6.9, 13.8 and 27.7 nM) will affect the *A anguilla* L. hepatic microsomal EROD activity.

MATERIALS AND METHODS

Chemicals

β-naphthoflavone (BNF), dimethyl sulfoxide (DMSO) and resorufin (Sigma Chemical Co., USA); NADPH and 7-ethoxyresorufin (Roche); cortisol (Merck Sharp & Dohme).

Fish

One adult eel (*A. anguilla* L.) with an average weight 650 gram were brought from local fish market. The eel was acclimatized to laboratory conditions in a 50 L aquarium containing aerated and filtered freshwater at 20oC during 48 hours. After acclimatization, the eel was intraperitoneally (i.p.) injected with 4 mg/kg β-napthoflavon (BNF) and killed 24 hours later and the liver removed, immediately frozen in liquid nitrogen and stored at -80oC until homogenization. The fish was neither fed during recovery nor during the experimental period.

Liver microsomal fraction preparation

Liver microsomes were prepared according to the methods of Lange et al. (1993) and Monod and Vindimian (1991) as adapted by Pacheco and Santos (1998). The liver was homogenized in a 1: 4 w/v ratio (liver weight: buffer volume) of 0.1M Tris-HCl (pH 7.4) containing 0.15MKCl and 20% glycerol, by using ±5 up-and-down strokes of a Potter glass-Teflon homogenizer at 2500 rpm. Microsomes were obtained by differential centrifugation, at 4ºC, in a Beckman Optima TL Ultracentrifuge (TLA-100.4 fixed angle rotor). The homogenate was first centrifuged at 15,000 rpm for 20 min to remove cell debris, nuclei, mitochondria, and lisosomes. Then, the resultant supernatant was collected and recentrifuged at 50,000 rpm for 75 min to isolate the microsomes. The resulting microsomal pellet was resuspended in 200 µl of the previous buffer, frozen in liquid nitrogen, and stored at -20oC until use.

Protein concentration measurement

Microsomal protein concentrations were determined according to the Biuret method (Gornall et al., 1949) using bovine serum albumin as a standard.

EROD activity assay

Liver microsomal EROD activity was measured as describe by Burke and Mayer (1974). Briefly, liver microsomal EROD assay was carried out at 25^{o}C, in a fluorometer cuvette. The reaction was initiated by adding 10 µl of NADPH (10 mM) and the progressive increase in fluorescence, resulting from the resourfin formation, was measured for 3 min (excitation wavelength 530 nm, emission wavelength 585 nm). Liver microsomal EROD activity was expressed as picomoles per minutes per milligram of microsomal protein.

Experimental Protocol

Buffers used during the experimental setup are given in Table 1 and the whole experimental procedure is given in Table 2.

Table 1 – Buffers and solvent used during the experimental setup.

Buffers	
B	Tris-HCl 0,1 M pH 7.4 with KCl 0,15M and 20% glycerol
BS	0.5 µM ethoxyresorufin in Tris-HCl 0,1 M pH 7.4 with KCl 0,15M and 20% glycerol
BSC	**BS** with cortisol (6.9, 13.8 and 27.7 nM concentrations)
Solvent	
DMSO	Dimethyl sulfoxide

Table 2 – Experimental procedure for evaluation of the hepatic microsomal EROD activity. The numbers (1, 2, 3 and 4) indicates the order by which compounds have been added in the cuvette.

Assays	**Control - DMSO**	**Control + DMSO**	**Cortisol - DMSO**	**Cortisol + DMSO**
5µl microsomes	1	1	1	1
1090µl **BS**	2	2		
1090µl **BSC (6.9,13.8 & 27.7 nM)**			2	2
5µl **B**	3		3	
5µl **DMSO**		3		3
10µl **NADPH**	4	4	4	4

Hepatic microsomes are exposed to the **cortisol** under the following conditions: to a suspension of 5 µl of hepatic microsomes was added;

(1) 1090 µl of buffer substrate **(BS)** and 5 µl of buffer **(B)** without substrate are assigned as **Control- DMSO.**

(2) 1090 µl of **BS** and 5 µl of dimethy sulfoxide **(DMSO),** is assigned as **Control + DMSO.**

(3) 1090 µl **BSC** (6.9, 13.8 and 27.7 nM concentrations) and 5 µl of **B,** is assigned as **Cortisol- DMSO.**

(4) 1090 µl **BSC** (6.9, 13.8 and 27.7 nM concentrations) and 5 µl of **DMSO**, is assigned as **Cortisol + DMSO.**

RESULTS

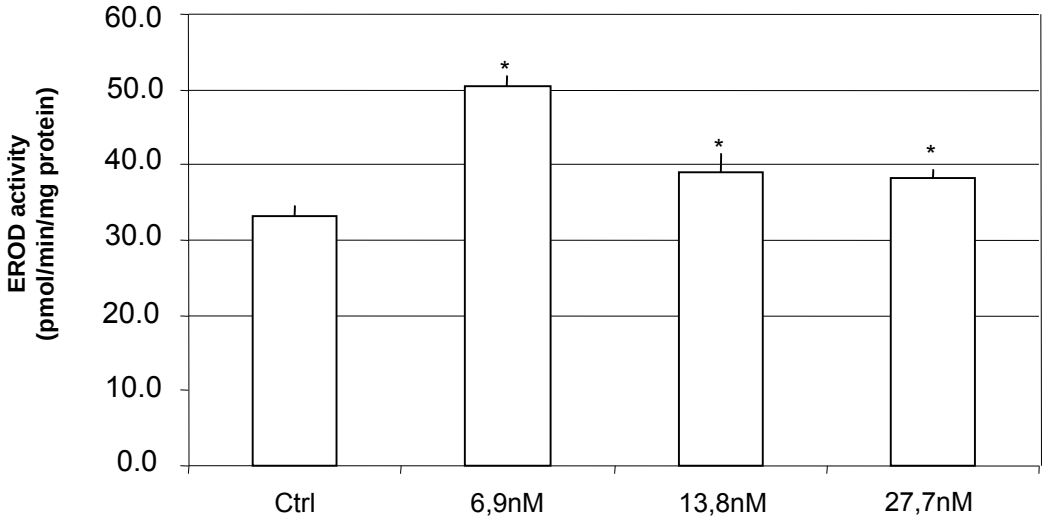

Figure 1 - *In vitro* effects of cortisol 6.9, 13.8 and 27.7 nM without DMSO. The values represent means ± SE (n=4). The significant difference ($p<0.05$) is: * *vs* Control

In **Figure 1**, Hepatic microsomal EROD activity determined in *A. anguilla* L. after 24 hour intraperitoneal exposure to BNF 4mg/kg, was 33.16 pmol.min^{-1}.mg protein^{-1}. A significant increase in hepatic microsomal EROD activity was observed after *in vitro* exposure to cortisol 6.9, 13.8 and 27.7 nM when compared with Control.

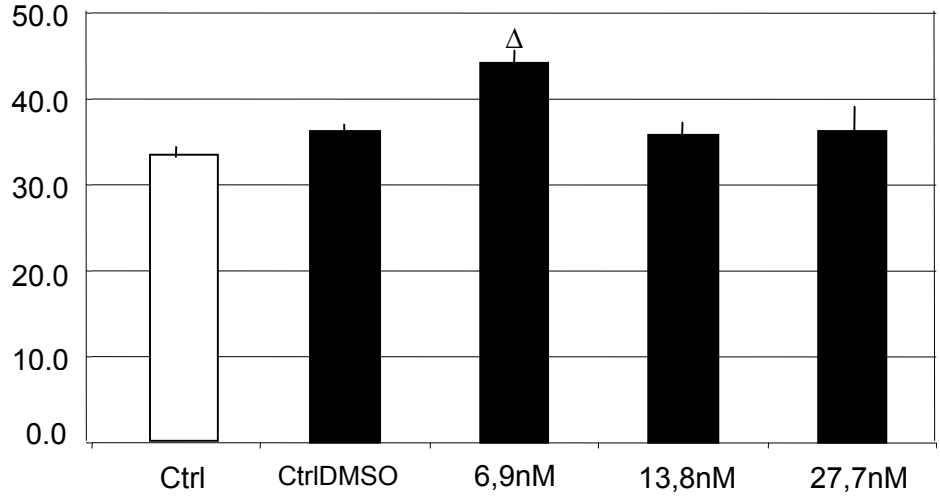

Figure 2- *In vitro* effects of BS with cortisol 6.9, 13.8 and 27.7 nM and DMSO. The values represent means ± SE (n=4). The significant difference ($p<0.05$) is: Δ *vs* CtrlDMSO

In **Figure 2,** the addition of 5µl of DMSO in Control induced EROD activity was 36.29 pmol.min^{-1}.mg protein^{-1}, though not significantly different from Control without DMSO. Moreover, a significant increase in hepatic microsomal EROD activity was observed only in the presence of 6.9nM cortisol with 5µl DMSO among all the studied cortisol concentrations when compared with Control DMSO.

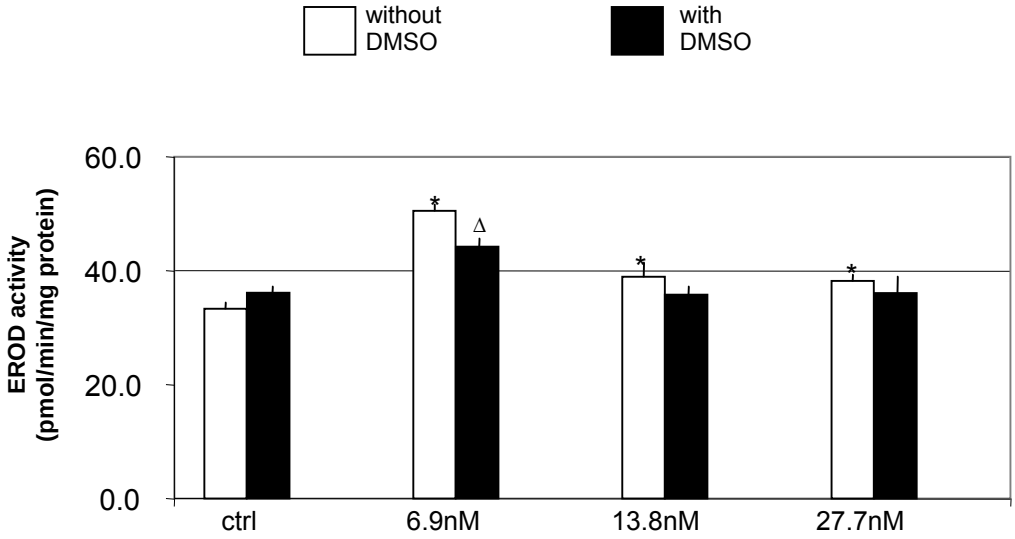

Figure 3 -*In vitro e*ffects of cortisol 6.9 nM, 13.8nM and 27.7 nM. The values represent means ± SE (n=4). The significant difference ($p<0.05$) is: * *vs* Ctrl without DMSO; Δ *vs* Ctrl with DMSO.

Finally, the hepatic microsomal EROD activity is significantly increased at 6.9nM concentration of cortisol under both conditions, i.e. without DMSO and with DMSO (**Figure 3**).

DISCUSSION

Hepatic microsomal EROD activity determined in *A. anguilla* L. after 24 hour intraperitoneal exposure to BNF 4mg/kg was 33.16 pmol.min^{-1}.mg protein^{-1}. A significant increase in hepatic microsomal EROD activity was observed after *in vitro* exposure to buffer-substrate cortisol 6.9, 13.8 and 27.7 nM when compared with Control. The addition of 5µl DMSO in control induced EROD activity, though

not significantly different from Control without DMSO. A significant increase in hepatic microsomal EROD activity was observed only in the presence of 6.9nM cortisol with 5µl DMSO among all the studied cortisol concentrations when compared with Control DMSO. Thus, hepatic microsomal EROD activity is significantly increased at 6.9nM concentration of cortisol under both the conditions i. e. without DMSO and with DMSO. *A. anguilla* L. also exhibited a significant increase in liver EROD activity due to MEM containing 1.4 □M (1400 nM) cortisol concentration, in liver organ culture (Santos et al., 2000). According to Ferreira (2007) Cortisol 22.02 nM combined with PAHs also showed a protective effect on hepatic microsomal EROD activity inhibition when compared to the same exposure condition without cortisol.

CONCLUSION

A. anguilla L. hepatic microsomes, exposed *in vitro* to Cortisol, demonstrated a significant increase in hepatic EROD activity at all the studied concentrations without DMSO. Though, our results reveal that 6.9nM cortisol is the concentration which caused the highest increase in hepatic EROD activity (with or without DMSO). Thus, the optimum concentration for further *in vitro* studies in combination with different xenobiotics, such as polycyclic aromatic hydrocarbons and resin acids, seems to be 6.9nM cortisol.

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CHAPTER IV

ABIETIC ACID AND CORTISOL *IN VITRO* EFFECTS ON *Anguilla anguilla* L. LIVER MICROSOMAL EROD ACTIVITY.

ABSTRACT

One adult eel (*Anguilla Anguilla* L) was injected intraperitoneally with β-naphthoflavone 4mg/kg and killed after 24 hours exposure. The eel's liver was removed. Liver microsomes were isolated and exposed in a cuvette to abietic acid (AA) 0.1, 0.3, 0.9, 2.7 μM. The protective effect of cortisol (6.9nM) on hepatic microsomal EROD activity was also tested through its previous inclusion in the buffer substrate, before exposure to the previously tested AA concentrations. Hepatic microsomes, exposed *in vitro* to AA, showed a significant EROD activity inhibition at all the concentrations. Moreover, the previous inhibition of EROD activity was inversely proportional to the AA increasing concentration. The liver microsome EROD activity inhibition, induced by *in vitro* exposure to AA, was significantly decreased by the cortisol (6.9 nM) presence, clearly showing its protection.

INTRODUCTION

Pollution of water resources is a serious and growing problem despite the existence of relevant legislation. Contamination of the aquatic environment by toxic chemical pollutants continues to occur, with domestic and industrial effluents being the main responsible sources (Matsumoto et al., 2006). In this context, one of the main sources of environmental pollution, affecting the Aveiro lagoon, has been the pulp and paper mill industry. Industrial effluents resulting from wood processing are highly complex mixtures of organic compounds, toxic to aquatic organisms, namely fish (Walden, C., 1976). The most important are the chlorinated and non-chlorinated small-molecular weight fractions of these pulp and paper mill effluents, the so-called resin acid (RAs) and chlorophenolics (CPs).

RAs are a class of extractives commonly found in the oleoresin of coniferous trees such as pines, spruces and firs (Fengel and Wegner 1985). These compounds are released into processing waters during the chemical and mechanical pulping of soft wood and are usually present in the pulp and paper industry effluent-receiving water (Leppänen et al., 1998) as well as in adjusent

sediments (Leppänen and Oikari 1999). Biochemical, physiological, and structural effects such as developmental damage, growth disturbance, altered weight of organs, liver biotransformation enzyme induction or inhibition, liver and blood DNA breakage, carbohydrate and protein metabolism changes, osmoregulation, and hematologic alteration on fish exposed to BKPMEs as a whole or to their compounds have been widely documented (Andersson et al., 1987; Lindström-Seppä and Oikari, 1989, 1990a, b; Bengtsson, 1991; Owens, 1991; Södergren, et al., 1992; Räbergh et al., 1992, Pacheco and Santos, 1999; Gravato e Santos, 2002a; Maria et al., 2003a). Moreover, biochemical, physiological, and structural effects on fish exposed to BKPMEs as a whole or to their compounds have been widely documented (Owens, 1991). Abietic acid (AA) and dehydroabietic acid (DHAA) are among the most abundant RAs in BKPMEs effluents (Oikari et al., 1980). These diterpene acids are also known to be relevant contributors to the toxicity of BKPMEs and strong toxicants to fish (Nikinmaa and Oikari, 1982; Oikari et al., 1983., Maria et al., 2003). RAs when incorporated into an organism have their toxicological effects regulated by two general mechanisms, namely the specific binding to the ligands and excretion. Thus, RAs constitute an important toxicant group to fish (Oikari et al 1983), bioaccumulating in the liver, bile and plasma.

Several *in vivo* investigations concerning ethoxysesorufin-O-deethylase (EROD) induction have been performed in fish. Hence, Pacheco and Santos (1997) observed a significant total EROD activity induction in glass eels (*A. anguilla* L.) after 3 days exposure to 0.3 μM AA. In adult eel, liver EROD activity induction was also reported after 3 days exposure to 0.1, 0.3, and 0.9 μM AA (Pacheco and Santos 1999). Maria et al., (2004) observed a significant increase in hepatic EROD activity of *A. anguilla* L. after 24h exposure to AA ranging from 0.1 up to 2.7 □M. A highly significant liver EROD increase was also observed after 8, 16, 24, and 72 h exposure to 0.9 μM AA. However, a significant eel liver microsomal EROD activity decrease was found, after *in vitro* exposure to AA 0.001, 0.01, 0.1, 0.3, 0.9, 2.7 □M (Santos and Maria, 2005). Gravato and Santos

(2002a) also demonstrated that AA inhibits liver EROD activity, in juvenile sea bass (Dicentrarchus labrax), after 2 h exposure to 0.05 mM.

The endocrine system plays an extremely important role in fish stress mechanisms since biochemical alterations and consequent effects on specific hormonal functions may constitute important stress biomarkers (Hontela, 1997). In this context, changes in interrenal function, measured as plasma cortisol, are recognized as important fish stress biomarkers (Santos and Pacheco, 1996). Cortisol is the main, most abundant and active corticosteroid in fish synthesized in the cells of interrenal tissue. A cortisol secretion dysfunction reduces the fish physiological competence, growth, and survivorship since this hormone is required for a wide range of important homeostatic mechanisms, including fuel reserves mobilization. The main target-organ of cortisol is the gill, liver and intestine, also sharing its important action on the skeletal muscles (Hontela, 1997). The increase in the concentration of cortisol plasma in fish was demonstrated after short term exposure to a vast range of contaminants such as heavy metals (De Boeck *et al.*, 2003), resin acids (Teles *et al*., 2003), PAHs (Pacheco e Santos, 2001a), and pesticides (Waring and Moore, 2004). Cortisol plasma levels are the most commonly used stress indicator in fish because of the rapid elevation that occurs in response to various stressors such as handling, confinement, poor water quality, and a wide variety of toxicants (Wendelaar-Bonga, 1997). Despite the existence of sufficient scientific information concerning the importance of endocrine system of fish, mainly cortisol response to stress situations, less is known about the cellular physiological level of hormone and their effects on isolated hepatic microsomal EROD activity, *in vitro*.

The present work concerns the *in vitro* effect of AA and cortisol+AA on the EROD activity of isolated liver microsomes previously induced by β-naphthoflavone.

MATERIALS AND METHODS

Chemicals

β-naphthoflavone (BNF), dimethyl sulfoxide (DMSO), abietic acid (AA) and resorufin (Sigma Chemical Co., USA); NADPH and 7-ethoxyresorufin (Roche); cortisol (Merck Sharp & Dohme).

Fish

One adult eel (*A. anguilla* L.) with an average weight 650 gram was caught at the Aveiro Lagoon and brought it to the laboratory. The eel was acclimatized to laboratory conditions in a 50 L aquarium containing aerated and filtered freshwater at 20oC during 48 hours. After acclimatization, the eel was intraperitoneally (i.p.) injected with 4 mg/kg □-napthoflavon (BNF) and killed 24 hours. The liver was immediately removed, frozen in liquid nitrogen and stored at -80oC until homogenization. The fish was neither fed during recovery nor during the experimental period.

Liver microsomal fraction preparation

Liver microsomes were prepared according to the methods of Lange et al. (1993) and Monod and Vindimian (1991) as adapted by Pacheco and Santos (1998). The liver was homogenized in a 1:4 w/v ratio (liver weight: buffer volume) of 0.1M Tris-HCl (pH 7.4) containing 0.15MKCl and 20% glycerol, by using ±5 up-and-down strokes of a Potter glass-Teflon homogenizer at 2500 rpm. Microsomes were obtained by differential centrifugation, at 4ºC, in a Beckman Optima TL Ultracentrifuge (TLA-100.4 fixed angle rotor). The homogenate was first centrifuged at 15,000 rpm for 20 min to remove cell debris, nuclei, mitochondria, and lisosomes. Then, the resultant supernatant was collected and recentrifuged at 50,000 rpm for 75 min to isolate the microsomes. The resulting microsomal pellet was resuspended in 200 µl of the previous buffer, frozen in liquid nitrogen, and stored at -20^{o}C until use.

Protein concentration measurement

Microsomal protein concentrations were determined according to the Biuret method (Gornall et al., 1949) using bovine serum albumin as a standard.

EROD activity assay

Liver microsomal EROD activity was measured as describe by Burke and Mayer (1974). Briefly, liver microsomal EROD assay was carried out at 25^{o}C, in a fluorometer cuvette. The reaction was initiated by adding 10 µl of NADPH (10 mM) and the progressive increase in fluorescence, resulting from the resourfin formation, was measured for 3 min (excitation wavelength 530 nm, emission wavelength 585 nm). Liver microsomal EROD activity was expressed as picomoles per minutes per milligram of microsomal protein.

Experimental Protocol

Experiment was carried out using four replicates. Buffers, solvent and solution used during the experimental setup are given in Table 1 and the whole experimental procedure is given in Table 2. Cortisol concentration used in the experiment is based on the dose finding experiment (chapter III) where 6.9 nM concentration of cortisol with buffer and with DMSO showed a significant increase in EROD activity.

Table 1 – Buffers, solvent and solution used during the experimental setup.

	Buffers
B	Tris-HCl 0,1 M pH 7.4 with KCl 0,15M and 20% glycerol
BS	0.5 µM ethoxyresorufin in Tris-HCl 0,1 M pH 7.4 with KCl 0,15M and 20% glycerol
BSC	**BS** with cortisol (C) (final concentration in cuvette = 6.9nM)
	Solvent
DMSO	Dimethyl sulfoxide
	Solution
AA	AA (0.1, 0.3, 0.9 and 2.7µM) in DMSO

Table 2 – Experimental procedure for evaluation of the hepatic microsomal EROD activity. The numbers (1, 2, 3 and 4) indicates the order by which compounds have been added in the cuvette.

Assays	**Control**	**Control DMSO**	**Control C + DMSO**	**AA (0.1,0.3,0.9 & 2.7 µM)**	**C + AA (0.1,0.3,0.9 & 2.7 µM)**
5µl microsomes	1	1	1	1	1
1090µl **BS**	2	2		2	
1090µl **BSC**			2		2
5µl **B**	3				
5µl **DMSO**		3	3		
5 µl **AA** (with DMSO)				3	3
10µl **NADPH**	4	4	4	4	4

Hepatic microsomes are exposed to the **AA** and **cortisol** under the following conditions: to a suspension of 5 µl of hepatic microsomes was added;

(1) 1090 µl of buffer substrate **(BS)** and 5 µl of buffer **(B)** without substrate are assigned as **Control.**

(2) 1090 µl of **BS** and 5 µl of dimethy sulfoxide **(DMSO),** is assigned as **Control DMSO.**

(3) 1090 µl **BS** and 5 µl of **AA** 2.7, 0.9, 0.3, 0.1µM**,** dissolved in DMSO, is assigned as **AA.**

(4) 1090 µl **BS** contain 6.9 nM of cortisol **(BSC)** and 5 µl of **DMSO**, is assigned as **Control C+DMSO**

(5) 1090 µl **BSC** and 5 µl of **AA**, is assigned as **C+AA**

Statistical analysis

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Statistica software (SigmaStat 2.03) was used for statistical analyses. All the data were first tested for normality and homogeneity of variance to meet statistical demands. Variance analysis was used to compare results between fish groups, followed by the Tukey test (Zar, 1996). Differences between means were considered significant when $p<0.05$.

RESULTS

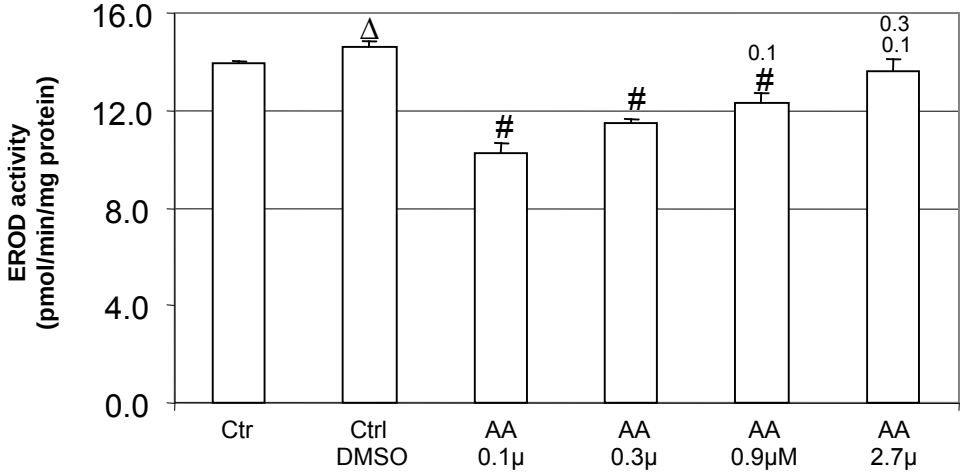

Figure 1- *In vitro e*ffects of AA 0.1, 0.3, 0.9, 2.7µM. The values represent means ± SE (n=4). The significant difference ($p<0.05$) is: Δ *vs* control; # *vs* ctrl DMSO; 0.1 *vs* AA 0.1µM ; 0.3 *vs* AA 0.3µM

In **figure 1**, *A. anguilla* L. hepatic microsomal EROD activity determined after the interperitoneal exposition of BNF 4mg/kg, was 13.96 $pmol.min^{-1}.mg\ protein^{-1}$. The addition of 5µl of DMSO in control induced significant EROD activity increase when compared with control. A significant inhibition in hepatic microsomal EROD activity was observed after the exposition of AA 0.1, 0.3, 0.9 µM when compared with control DMSO. The previous inhibition of EROD activity is inversely proportional to the increased concentration of AA.

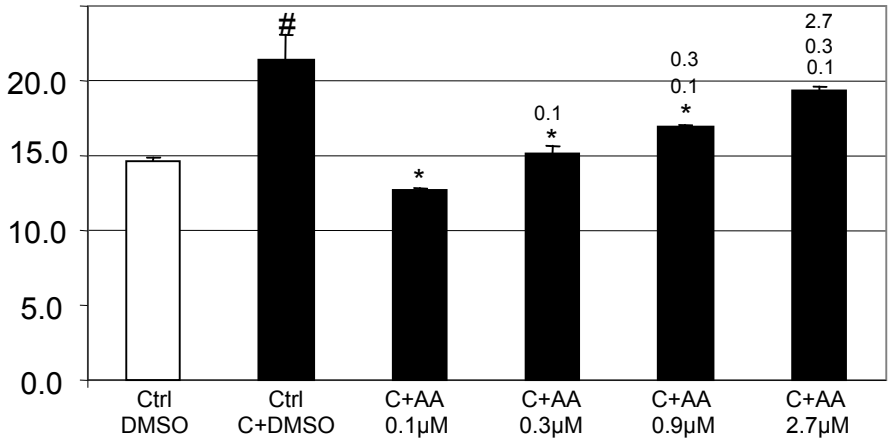

Figure 2- *In vitro e*ffects of AA 0.1, 0.3, 0.9, 2.7µM in presence of cortisol(6.9nM).The values represent means ± SE. (n =4)
The significant difference ($p<0.05$) is: # *vs* control DMSO; ***** *vs* control - C (cortisol)+DMSO; 0.1 *vs* C+ AA 0.1µM ; 0.3 *vs* C+ AA 0.3µM ; 0.9 *vs* C+ AA 0.9µM.

In **figure 2**, the hepatic EROD activity is significantly increased in Control C+DMSO (6.9nM) when compared with control DMSO. After the exposition of AA, there is a significant inhibition in EROD activity at 0, 1, 0.3, 0.9 µM concentrations when compared with Control C+DMSO. In presence of C, inhibitory effect of AA on EROD activity, were also inversely proportional to the concentrations of AA.

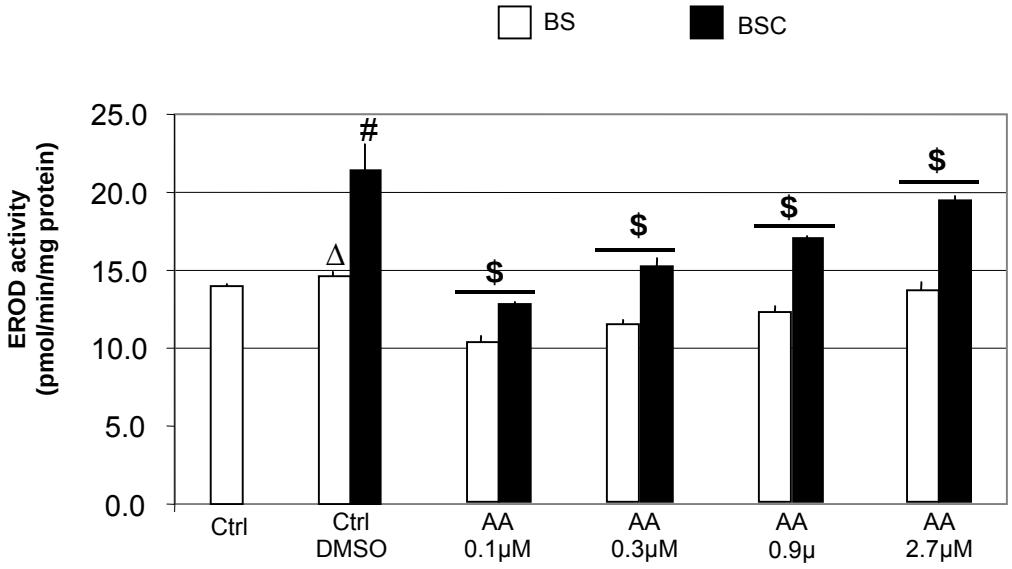

Figure 3- *In vitro e*ffects of AA 0.1, 0.3, 0.9, 2.7µM without (BS) and with (BSC) inclusion of cortisol (6.9nM) in buffer substrate. The values represent means ± SE. The significant differences ($p<0.05$) are: Δ *vs* control; # *vs* ctrl DMSO; $ is the difference between in absence and presence of cortisol for each concentration of AA

In **figure 3**, hepatic microsomal EROD activity is significantly increased in control DMSO when compared with control and the significant difference is exist between Control C+DMSO and control with DMSO only. The comparison of microsomal EROD activity with exposition of AA (0.1, 0.3, 0.9, 2.7 µM), with or without C reveals in both the cases inhibition, which was inversely proportional to the respective concentrations of the AA. However, inhibition caused by different concentrations of AA, was significantly reduced by the exposition of C contained in the buffer substrate.

DISSCUSSION

Abietic acid constitutes a major class of environmental toxic compounds, and a potential health hazard to animal wildlife (Aranda and Villalain, 1997). As amphipatic molecule, it interacts with the phospholipid membranes with its carboxyl group in the close proximity to the phospholipid ester groups being placed in the upper part of the membrane palisade structure (Vilialain, 1997). In the current study, AA *in vitro* liver microsomal EROD activity inhibitory response to all the exposure concentrations was observed. Surprisingly, an incremental trend of EROD activity recovery was found from the lowest to the highest concentration. These findings are in the agreement of the previous *in vitro* observation of Santos and Maria (2005) who found the same EROD activity pattern for the concentrations higher than 0.01 µM in same fish hepatic microsome. The currents results may be suggested considering the AA amphipathic properties as well as the possibility of its biotransformation into DHAA by the liver microsomal membranes and their consequent breakdown that may elicit a significant slight recovery due to an enlarged cytochrome P450 multi-function oxidases (MFOs) exhibition before the substrate, namely EROD. Gravato and Santos (2002a) also demonstrated that AA *in vivo* exposure inhibites liver EROD activity, in juvenile sea bass (*Dicentrarchus labrax*), after 2 h exposure to 0.05 mM.

The comparison of microsomal EROD activity with exposition of AA with or without cortisol reveals in both the cases inhibition, which was inversely proportional to the respective concentrations of the AA. However, inhibition caused by different concentrations of AA, was significantly reduced by the exposition of cortisol contained in the buffer substrate. In this context, no study is available based on the AA and cortisol in vitro exposure. However, few *in vivo* studies demonstrated the elevated plasma cortisol level by the short term exposure of pollutants (Pacheco and Santos, 2001; Teles et al 2004 and 2007), namely, PAHs (Oliveira et al., 2007), AA (Teles et al., 2003). The present work verified the EROD activity increase by *in vitro* exposition of cortisol as well as

inhibitory effect of AA after previous exposition to hepatic microsome to relate hormone. In this perspective, a study done by Santos et al. (2000) on eel's organ culture also reflected a significant enhancement of liver EROD activity under *in vitro* condition by minimum essential media (MEM) containing 1.4 µM cortisol from 0 up to 4 and 24 hours. The current results obtained from *in vitro* study showed that the cortisol exerts the protective effect on hepatic microsomal EROD activity when exposed to inhibitor contaminants.

CONCLUSION

AA *in vitro* exposure on the *A. anguilla* L. hepatic microsome, previously induced by β-naphthoflavone, demonstrate an inhibition of EROD activity. The previous inhibition of EROD activity was inversely proportional to the increased concentration of AA. Moreover, the inhibition induced by AA *in vitro* exposure was significantly decreased by the addition of cortisol in liver microsome, clearly showing its protection against AA induced liver microsomal EROD activity inhibition.

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CHAPTER V

RETENE AND CORTISOL IN VITRO EFFECTS ON *Anguilla anguilla* L. LIVER MICROSOMAL EROD ACTIVITY.

ABSTRACT

An adult eel (*Anguilla Anguilla* L) was injected intraperitoneally with β-naphthoflavone 4mg/kg and killed after 24 hours exposure. The liver was removed. Liver microsomes were isolated and exposed in cuvette to retene (Re) 0.1, 0.3, 0.9, 2.7 μM. The protective effect of cortisol (6.9nM) on hepatic microsomal EROD activity was tested through its previous inclusion in the buffer substrate, when exposed to Re tested concentrations. Hepatic microsomes, exposed *in vitro* to Re, showed a significant EROD activity inhibition at all the concentrations. Moreover, EROD activity inhibition was proportional to the increased concentrations of Re. Hepatic microsomal EROD activity inhibition by *in vitro* exposure to Re, was significantly decreased by the presence of cortisol, except at 0.3 μM concentration, revealing its protective capacity against Re induced liver microsomal EROD activity inhibition.

INTRODUCTION

Aquatic contamination by industrial and domestic sewage outlets are a constant source of public health concern. These types of contamination vary, between organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) from harbour areas, resin acids (RAs) from pulp and paper mill industries, alkylphenols deriving from domestic sewage, and heavy metals either from industry or leisure activities. Among various pollutants, Retene (Re) (7-isopropyl-1-methylphenanthrene), which is a subsitutional PAH, was found in aquatic sediments affected by kraft pulp mill effluents (Travendale et al., 1995; Maria et al., 2004). Moreover, Re is a chemical indicator of the terrestrial influence of catchment areas, and is also used as a molecular marker of coniferous wood combustion (Ramdahl, 1983; Mazurek and Cass, 1991), as it can be formed by thermal degradation of abietic-type resin acids (Ramdahl, 1983). High Re concentrations (up to 3300 mg/g dry wt) found in lake sediments downstream of kraft pulp mill effluents rich in resin acids (Leppänen and Oikari, 2001) were

formed by anaerobic microbial degradation of dehydroabietic (DHAA) or abietic acid (AA) (Wakeham et al., 1980; Zender et al., 1994; Travendale et al., 1995; Judd et al., 1995, 1996; Koistinen et al., 1998). During effluent treatment, anaerobic zones within aeration ponds can also generate Re (Zender et al., 1994; Stuthrige and Tavendale, 1996).

Reports revealed that Re is bioavailable to fish under natural circumstances, from sediments of lakes receiving kraft pulp mill effluents or waterborne particulate matter (Travendale et al., 1996; Leppänen and Oikari, 1999a and Leppänen and Oikari, 1999b; Oikari et al., 2002) since its metabolites were found in *Onchorynchus mykiss* (Fragoso et al., 1999) and *Rutilus rutilus* bile (Leppänen and Oikari, 1999a). In laboratory experiments by Brumley et al. (1997), Re was also detected in liver, muscle and bile of rainbow trout. Other reports also showed the Re potential of liver EROD activity induction in different teleost species including *A. anguilla* L. (Fragoso et al. 1999; Billiard et al., 1999; Oikari et al., 2002; Maria et al., 2002 and 2005). Thus, Re capacity to induce rainbow trout (*Onchorynchus mykiss*) liver cytochrome P450 (CYP1A) monooxygenases is a proof of its bioactivity as an arylhydrocarbon receptor (AhR) agonist (Billiard et al., 2002) and also of its bioavailability in the environment (Fragoso et al., 1999). However, continuous rainbow trout exposure to Re is necessary to maintain high EROD induction since depuration experiments demonstrated a rapid loss of EROD activity, suggesting that Re is labile and rapidly eliminated in the previously reported fish species (Fragoso et al., 1998). Despite eel (*A. anguilla* L.) significant liver EROD induction by Re (Maria et al., 2002), its inhibition has also been demonstrated by Gravato and Santos (2002b) in sea bass *(Dicentrarchus labrax)*. Moreover, *in vitro* condition Santos and Maria (2005) found a significant eel liver microsomal EROD activity decrease after its *in vitro* exposure to Re (0.01-2.7μM).

The endocrine system plays a central role in fish stress mechanisms. Plasma cortisol levels are the most commonly used stress indicators in fish

because of the rapid elevation in response to various stressors viz. handling, confinement, poor water quality and a wide variety of toxicants (Vijayan et al., 1996; Wendelaar-Bonga, 1997; Teles et al., 2004, 2007). The plasma cortisol level has been widely studied *in vivo* in *A. anguilla* L. (Santos and Pacheco, 1996; Pacheco and Santos, 2001a and 2001b; Teles *et al*., 2004a), *Sparus aurata* L. (Teles *et al*., 2005), *Liza aurata* (Oliveira et al., 2007) and in *Dientrarchus labrax* L. (Teles *et al*., 2004b) under diverse environmental conditions, and also *in vitro* condition in *A. anguilla* L. (Santos et al., 2000) but in less exhausted form. In the vast majority of living organisms, the induction of phases I and II biotransformation enzymes is a well-known response to organic xenobiotic exposure. The relation between the hepatic EROD activity (Pacheco and Santos 1998) and plasmatic and cellular level of determined steroid hormone such as cortisol is not very well known in fish. In this perspective, a study has done by Santos et al (2000) shows a strong enhancement of *A. Anguilla* L. liver EROD activity under *in vitro* condition by minimum essential media (MEM) containing cortisol from 0 up to 4 and 24 hours.

The recent literature reveals that there is a scarcity of information about the cellular physiological hormone levels such as cortisol and their effects on isolated hepatic microsomal EROD activity, in a situation of stress.. The present work concern the *in vitro* effect of Re separately and also with cortisol, to study the effect of cortisol on EROD activity previously induced by β-naphthoflavone (BNF).

MATERIALS AND METHODS

Chemicals

β-naphthoflavone (BNF), dimethyl sulfoxide (DMSO), Re and resorufin (Sigma Chemical Co., USA); NADPH and 7-ethoxyresorufin (Roche); cortisol (Merck Sharp & Dohme).

Fish

One adult eel (A. anguilla L.) with an average weight 650 gram was caught at the Aveiro Lagoon and brought it to the laboratory. The eel was acclimatized under laboratory conditions in a 50 L aquarium containing aerated and filtered freshwater at 20oC during 48 hours. The eel was then intraperitoneally (i.p.) injected with 4 mg/kg β-napthoflavon (BNF) and killed 24 hours later and the liver removed, immediately frozen in liquid nitrogen and stored at -80oC until homogenization. The fish was neither fed during recovery nor during the experimental period.

Liver microsaomal fraction preparation

Liver microsomes were prepared according to the methods of Lange et al. (1992) and Monod and Vindimian (1991) as adapted by Pacheco and Santos (1998). The liver was homogenized in a 1: 4 w/v ratio (liver weight: buffer volume) of 0.1M Tris-HCl (pH 7.4) containing 0.15MKCl and 20% glycerol, by using ±5 up-and-down strokes of a Potter glass-Teflon homogenizer at 2500 rpm. Microsomes were obtained by differential centrifugation, at 4ºC, in a Beckman Optima TL Ultracentrifuge (TLA-100.4 fixed angle rotor). The homogenate was first centrifuged at 15,000 rpm for 20 min to remove cell debris, nuclei, mitochondria, and lisosomes. Then, the resultant supernatant was collected and recentrifuged at 50,000 rpm for 75 min to isolate the microsomes. The resulting microsomal pellet was resuspended in 200 µl of the previous buffer, frozen in liquid nitrogen, and stored at -20^{o}C until use.

Protein concentration measurement

Microsomal protein concentrations were determined according to the Biuret method (Gornall et al., 1949) using bovine serum albumin as a standard.

EROD activity assay

Liver microsomal EROD activity was measured as describe by Burke and Mayer (1974). Briefly, liver microsomal EROD assay was carried out at 25°C, in a fluorometer cuvette. The reaction was initiated by adding 10 µl of NADPH (10

mM) and the progressive increase in fluorescence, resulting from the resourfin formation, was measured for 3 min (excitation wavelength 530 nm, emission wavelength 585 nm). Liver microsomal EROD activity was expressed as picomoles per minutes per milligram of microsomal protein. Experiment was carried out using five replicates (n=4).

Experimental Protocol

Experiment was carried out using four replicates. Buffers, solvent and solution used during the experimental setup are given in Table 1 and the whole experimental procedure is given in Table 2. Cortisol concentration (6.9nM) used in the experiment is based on the concentration ranging experiment described in previous chapter III.

Table 1 – Buffers, solvent and solution using during the experimental setup.

	Buffers
B	Tris-HCl 0,1 M pH 7.4 with KCl 0,15M and 20% glycerol
BS	0.5 µM ethoxyresorufin in Tris-HCl 0,1 M pH 7.4 with KCl 0,15M and 20% glycerol
BSC	**BS** with cortisol (C) (final concentration in cuvette = 6.9nM)
	Solvent
DMSO	Dimethyl sulfoxide
	Solution
Re	Re(0.1, 0.3, 0.9 and 2.7µM) in DMSO

Table 2 – Experimental procedure for evaluation of the hepatic microsomal EROD activity. The numbers (1, 2, 3 and 4) indicates the order by which compounds have been added in the cuvette.

Assays	**Control**	**Control DMSO**	**Control C + DMSO**	**Re (0.1,0.3,0.9 & 2.7 µM)**	**C + Re (0.1,0.3,0.9 & 2.7 µM)**
5µl microsomes	1	1	1		1
1090µl **BS**	2	2		2	
1090µl **BSC**			2		2
5µl **B**	3				
5µl **DMSO**		3	3		
5 µl **Re** (with DMSO)				3	3
10µl **NADPH**	4	4	4	4	4

Hepatic microsomes are exposed to the **Re** and **cortisol** under the following conditions: to a suspension of 5 µl of hepatic microsomes was added;

(1) 1090 µl of buffer substrate **(BS)** and 5 µl of buffer without substrate **(B)** and assigned as **Control.**

(2) 1090 µl of **BS** and 5 µl of dimethy sulfoxide **(DMSO),** is assigned as **Control DMSO.**

(3) 1090 µl **BS** and 5 µl of **Re** 2.7, 0.9, 0.3, 0.1µM**,** dissolved in DMSO, is assigned as **Re.**

(4) 1090 µl **BS** contain 6.9 nM of cortisol **(BSC)** and 5 µl of **DMSO**, is assigned as **Control C + DMSO**

(5) 1090 µl **BSC** and 5 µl of **Re**, is assigned as **C + Re**.

Statistical analysis

Statistica software (SigmaStat 2.03) was used for statistical analyses. All the data were first tested for normality and homogeneity of variance to meet statistical demands. Variance analysis was used to compare results between fish

groups, followed by the Tukey test (Zar, 1996). Differences between means were considered significant when $p<0.05$.

RESULTS

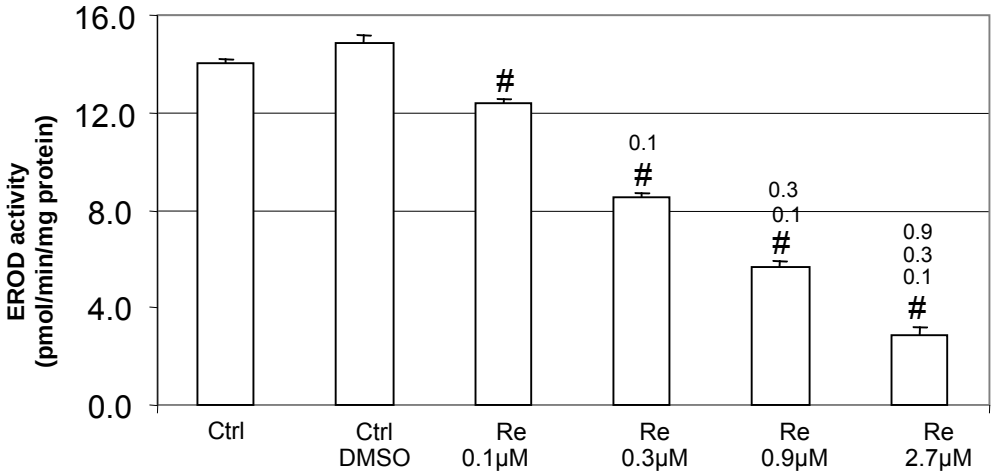

Figure 1- *In vitro e*ffects of Retene 0.1, 0.3, 0.9, 2.7µM. The values represent means ± SE (n =4).
The significant difference ($p<0.05$) is: # *vs* Ctrl DMSO; 0.1 *vs* retene 0.1µM ; 0.3 *vs* retene 0.3µM ; 0.9 *vs* retene 0.9µM ; 2.7 *vs* retene 2.7µM.

In **figure 1**, the hepatic microsomal EROD activity in *Anguila anguilla* L., after 24 hour intraperitoneal exposure of BNF 4mg/kg, was 14.01 pmol.min^{-1}.mg protein^{-1}. The addition of 5µl of DMSO in Control did not affect significant EROD activity, when compared with Control without DMSO.

Liver microsomal exposed to increasing concentrations of Re (0.1, 0.3, 0.9, 2.7 µM) had their EROD activity significantly inhibited when compared with Control DMSO. Liver microsomal inhibition of EROD activity is proportional to the increased concentration of Re.

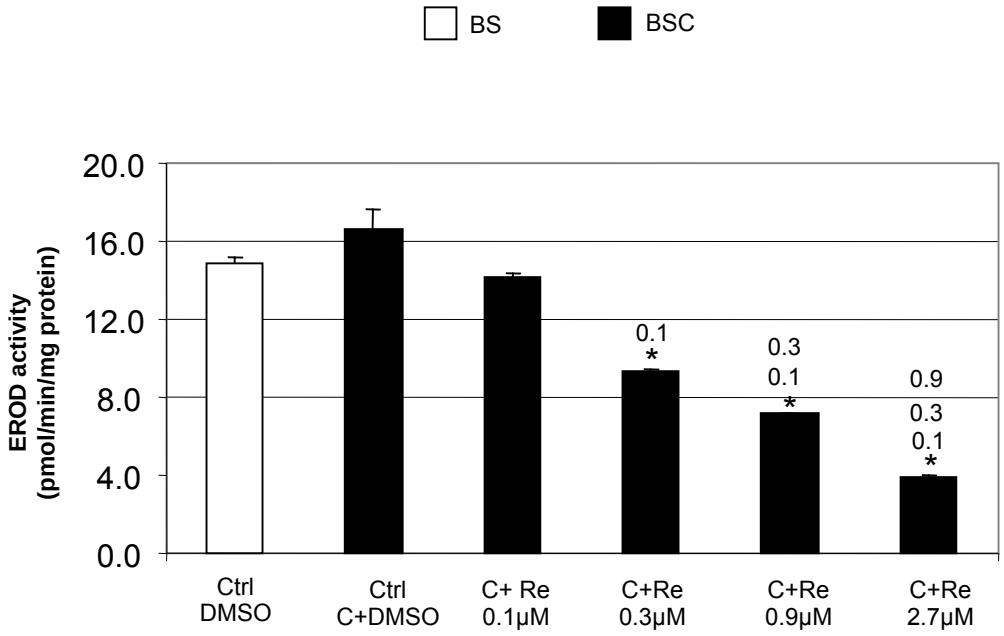

Figure 2- *In vitro e*ffects of Retene 0.1, 0.3, 0.9, 2.7µM in presence of cortisol(6.9nM).The values represent means ± SE. (n =4)
The significant difference ($p<0.05$) is: ***** *vs* Control+ C (cortisol); 0.1 *vs* C+retene 0.1µM; 0.3 *vs* C+retene 0.3µM ; 0.9 *vs* C+retene 0.9µM.

In **figure 2**, Microsomal EROD activity was not significantly affected by its exposure to Control DMSO with Cortisol (C, 6.9 nM). However, liver microsomal exposure to Re 0.3, 0.9, 2.7 µM concentrations, resulted in a significant inhibition in EROD activity at when compared to control C+DMSO. Moreover, EROD activity inhibition was also proportional to the increased concentrations of Re.

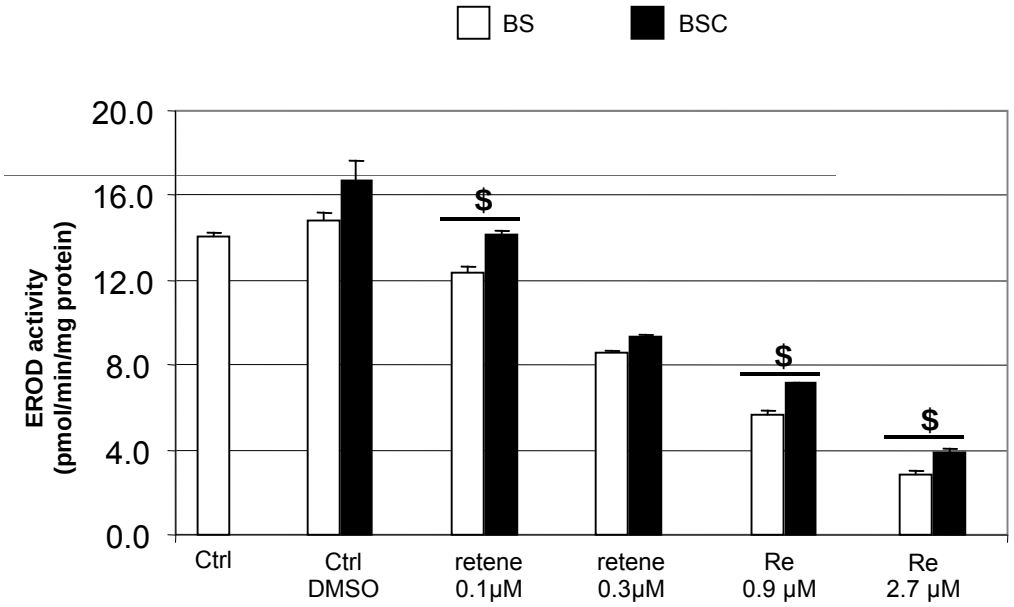

Figure 3- *In vitro e*ffects of Retene 0.1, 0.3, 0.9, 2.7µM without (BS) and with inclusion of cortisol (6.9nM) (BSC) in buffer substrate. The values represent means ± SE. (n =4)
$ $p<0.05$ significant difference between in absence and presence of cortisol for each concentration of retene.

In **figure 3**, hepatic microsomal EROD activity showed no significant change by the addition of 5µl DMSO in control and also there is no significance difference between Control C+DMSO. The presence of C did not affect hepatic microsomal EROD activity significantly at 0.3 µM Re exposure. However, EROD inhibition caused by Re 0.1, 0.9, 2.7 µM was significant reduced by the exposition of C contained in the buffer substrate.

DISCUSSION

Previous *in vivo* studies performed in *A. Anguilla* L., showed liver EROD activity increase until 72 hours (8, 16, 24 and 72) following 0.1 up to 2.7 µM Re exposure (Maria et al., 2004). In *in vitro* condition, Santos and Maria (2005) observed an inhibition of the liver EROD activity after 0.01-2.7µM Re exposure in *A. Anguilla* L. The current results are also showing an *in vitro* liver microsomal EROD activity inhibition at all the studied Re concentrations. Therefore, our experimental results showed that Re, a potent *in vivo* inducer of *A. anguilla* P450-dependent

monooxygenases, is an *in vitro* inhibitor of liver microsomal EROD activity since it inhibits from 0.1 up to 2.7μM. These findings are in accordance with Fragoso et al. (1999) who demonstrated an *in vitro* rainbow trout EROD activity inhibition by Re concentrations above 2.5 $\mu g.ml^{-1}$ (approximately 10μM) reducing by 88% at the highest Re tested concentration (250 $\mu g.ml^{-1}$, approximately 1mM). Gravato and Santos (2002b) also observed in juvenile *Dicentrarchus labrax* L. that other PAHs such as BaP, within a concentration range between 0.001μM up to 2.7 μM, also inhibits liver EROD activity, which coincided with the present Re concentration range *in vitro* liver microsomal inhibition. High BaP concentrations (above 100 nM), also abruptly decrease EROD activity in a trout liver cell line, suggesting enzymatic inhibition rather than xenobiotic-induced cytotoxicity (Bols et al., 1999).Thus, the previous toxicological responses suggest that these two compounds may interact in similar way with liver EROD activity, causing its inhibition.

Steroid hormones are included in the endogenous factors that can regulate CYP1A expression, however their interaction mechanisms are not well established (Navas and Segner, 2000). The vast majoirity of studies concerning cortisol, showed a fast elevation in plasma cortisol level after an *in vivo* exposure of different contaminats including PAHs (Pacheco and Santos, 2001b; Teles et al., 2004; Oliveira et al., 2007). In the current study, the comparison of microsomal EROD activity exposed to Re concentration with or without the presence of cortisol reveals a significant concentration dependent inhibition in both cases except at the lowest concentration of Re (0.1μM) with cortisol Moreover, EROD inhibition caused by different concentrations of Re, was significantly reduced by its exposure to the cortisol contained in the buffer substrate, except at 0.3μM concentration, suggesting a protective effect of the *in vitro* exposure to cortisol. Santos et al. (2000) also found in *A. Anguilla* L. liver organ culture, an additive effect of minimum essential media (MEM) with cortisol on liver EROD increase after BNF *in vivo* exposure. Moreover, in this *in vitro* study, no significant difference between control DMSO and control without DMSO was observed, thus, the present results on the EROD activity inhibition caused by

different concentrations of Re and its inverse relation to cortisol clearly indicates a stimulatory/stabilizing role of cortisol in a significant increase in hepatic EROD activity.

CONCLUSIONS

Re *in vitro* exposure on the *A. anguilla* L. hepatic microsome, previously induced by β-naphthoflavone, demonstrated an inhibition of EROD activity. The previous EROD activity inhibition was proportional to the increased concentration (0.1, 0.3, 0.9, 2.7 μM) of Re. The inhibition induced by Re *in vitro* exposure was significantly decreased by the addition of cortisol in liver microsome revealing its protective role against Re induced liver microsomal EROD activity inhibition.

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GENERAL DISCUSSION

The general discussion has been made taking into account the critical analysis of the over all thesis as well as specific discussion of each chapter.

1. Genotoxic evaluation in Aveiro Lagoon

This point corresponds the seasonal genotoxic alterations induced on fish by Aveiro lagoon enviromental contaminants (chapter 1), fulfilling one of the objectives whose advantage had previously been presented in point 5 of the general introduction. In spring season a significant ENA increase was observed at BAR, RIO, LAR and VAG sites when compared to TOR. Previous Ria de Aveiro survey, based on *Liza aurata* carried out on 2000 (five years earlier), showed no genotoxicity induction (Pacheco et al, 2005), however the present survey observed ENA increase and thus suggesting an water quality deterioration in last half decade. Despite the possibility of different pollutants presence such as tributyltin (TBT) (Sousa et al., 2007), PAHs (Pacheco et al., 2005) and as well as their combined effects, the observed differences may also be due to species differences, related to the biology (e.g. feeding behaviour) and DNA susceptibility to damage (e.g. defence mechanisms and DNA repair) of the adopted fish species. No significant ENA increase was observed at GAF in the present spring survey despite being a highly polluted site having a variety of contaminants such as PAHs and TBTs (Micael et al., 2007; Pacheco and Santos, 2002) suggesting that ENA assay may be inadequate for situations of intense contamination since some disturbances of genotoxicity expression may occur depending on the balance between an increased splenic erythrocytic catabolism and reduced erythropoiesis rate (Pacheco and Santos, 2002). However, considering also this fish species feeding behaviour there is a possibility of lack of food capture under these environmental conditions. The increase in ENA observed at RIO may be correlated with the presence of chemicals that have been identified in pulp/paper mill effluents viz. resin acids (Gravato and Santos, 2002a) since this site is located 6.5 km distant from a pulp/paper mill effluent outlets where the continuous effluent discharge lasted five decades. In relation to LAR, observation on ENA increase, is not unexpected, and seems to be in

agreement with Guilherme et al. (2007) findings who showed ENA increase in *L. aurata* captured in the same area and its relation with the presence of mercury as a result of an effluent discharge from a chlor-alkali plant (Guilherme et al., 2007; Pereira et al., 1997). A significant ENA increase at VAG may be directed towards the presence of PAHs since Pacheco et al. (2005) analysed benzo(a)pyrene (BaP) high levels at VAG station.

The above given pollutants combined effect at different study sites can also be suggested for a significant increase observed in summer at all the studied sites, in autumn for GAF, LAR and VAG as well as in winter for BAR, GAF and RIO and a probable cause for the ENA increase through out the year. However, the absence of ENA increase (in autumn at RIO; in winter at LAR) may reflect the involvement of seasonal changes and pollutants coming from non point sources affecting the lagoon contamination pattern and thus resulting in a change of their antagonism/synergism. According to Gorbi et al. (2005) the same chemical disturbance can produce a different biological impact, depending on the seasonal susceptibility to stress.

The analysis of each nuclear lesion category individually in the different seasons revealed that the lobed nuclei frequencies were significantly higher in spring (at BAR, RIO, LAR and VAG sites); in summer and winter (at all the study sites); and in autumn (at RIO, LAR and VAG sites) with respect to TOR, exhibiting the robustness of this biomarker for genotoxicity assessment. Besides other effects such as pollutants pattern, hydrodynamics, sediment suspension, biomarker basal levels also represents a suitable approach for environmental risk assessment (Collier et al., 1995; Eggens et al., 1996: Soborowiki et al., 1996; Roniz et al., 1999). In the current study no significant differences on ENA basal levels were observed in fish collected from TOR along the year, proving its robustness as control for future studies on Aveiro lagoon biomonitoring. Moreover in the context of genotoxicity, water temperature may influence cell replication rates and DNA repair of poikilothermal organisms (Venier et al., 1997). Brunetti et al. (1992) also suggested a considerable influence of temperature increase on MN frequency increase. In the same way, salinity, water levels, rain

and storm events in field exposure have also been reported to influence DNA damage (Rank et al., 2007) by affecting the availability of contaminants as well as the fish physiological processes. According to the previous explanation, the highest temperature recorded at LAR and highest salinity recorded at BAR in spring seems to be one of the contributory factors for the increased ENA levels in current study. However, no changes in abiotic factors (mainly temperature, salinity and dissolved oxygen) recorded at VAG and GAF in spite of increased ENA level suggest the possibility of genotoxic contaminants presence coming from non point sources. According to Livingstone (Livingstone et al., 1993), the correct use of biological responses as biomarkers requires knowledge of their natural variability. Thus, assessed natural variability of ENA in the current field conditions represents an important aspect to improve the ecotoxicological use of ENA in biomonitoring programs. Over all, the data from Aveiro lagoon field study provided the strong indication of degree of genotoxic contaminants in this ecosystem.

2. *In vitro* laboratory study

The works analysis in this point includes chapters II, III, IV, V and correspond the *in vitro* laboratory studies in *A anguilla* L. Studied pollutants were: Polycyclic aromatic hydrocarbon (PAHs) – naphthalene (Naph) and phenanthrene (Phe) (Chapter II); Cortisol (Chapter III); Resin acid (RAs) – abietic acid (AA), and its derivative – retene (Re); as well as AA and Re in combination with a steroid hormone – cortisol (Chapters IV and V).

2.1 Liver microsomes *in vitro* exposure to naphthalene and phenanthrene

The *in vitro* tested Naph and Phe concentrations (0.001 up to 2.7 μM) used in these studies were environmentally realistic since the Naph concentrations detected in environmental waters ranged from 0.000039 – 515.6 μM (Environment Canada, 1993; ATSDR, 1995) and Phe concentration falls within the level (up to 8.2 μM) in natural waters (Anyakora et al., 2005). Liver

microsomes *in vitro* exposure to Naph demonstrated a significant increase in its EROD activity at all the studied concentrations reflecting a clear Naph potential to activate phase I biotransformation reactions (Chapter II). Moreover the increase was dose-dependent showing its EROD activity inducing potential at each and every concentration. The current *in vitro* results are in agreement with the findings of Maria et al., (2002) and Pacheco and Santos, (2002) who observed the hepatic microsomal EROD activity induction in *A anguilla* L., after the *in vivo* Naph exposure. However, the current *in vitro* results showed that liver microsomal EROD activity was induced by 0.01-2.7 μM Phe exposure. The Phe no observed effect concentration (NOEC) for liver microsome in vitro exposure was 0.001 μM. Thus, the current results are in the agreement with the previous *in vivo* results on fish species such as *L. aurata* confirming Phe CYP1A inducing potential (Oliveira et al., 2007). Moreover, liver microsomal *in vitro* exposure to another PAH such as fluoranthene 0.1 and 0.3 μM, also induced a similar EROD activity increase (Ferreira, 2007).

The overall results similarity in both Naph and Phe may be directed to the fact that naphthalene and phenanthrene share common metabolic pathways as suggested by Kiyohara *et al*. (1994). Phe is degraded through one of two different routes. In one route, 1-hydroxy-2-naphthoic acid is oxidized to 1, 2-dihydroxynaphthalene, which is further degraded via the Naphthalene Pathway to salicylate which can be further metabolized. In the other pathway, the ring of 1-hydroxy-2-naphthoic acid is cleaved and further metabolized via the Phthalate Pathway. Thus, the previous toxicological responses suggest that these two PAHs may interact in a similar way with liver EROD activity, causing its increased activity.

2.2 Liver microsomes *in vitro* exposure of abietic acid and retene

In vitro exposure of AA causes a significant inhibition in hepatic microsomal EROD activity at 0.1, 0.3, 0.9 μM concentrations in *A anguilla* L. The previous inhibition of EROD activity is inversely proportional to the increased concentration of AA. Surprisingly, an incremental trend of EROD activity recovery

was found from the lowest to the highest concentration. These findings are in agreement with previous *in vitro* observation of Santos and Maria (2005) who found the same EROD activity pattern for the concentrations higher than 0.01 μM AA in the same species *in vitro* hepatic microsomes. The current results may be suggested considering the AA amphipathic properties, as well as the possibility of its biotransformation into DHAA by the liver microsomal membranes and their consequent breakdown eliciting a significant slight recovery due to an enlarged cytochrome P450 multi-function oxidases (MFOs) exhibition before the substrate, namely EROD. Gravato and Santos (2002a) also demonstrated that AA *in vivo* exposure inhibits liver EROD activity, in juvenile sea bass (*Dicentrarchus labrax*), after 2 h exposure to 0.05 mM.

Hepatic microsomal exposure to different Re concentrations (0.1, 0.3, 0.9, 2.7 μM) significantly inhibits its EROD activity. The previous inhibition of EROD activity is proportional to the increased concentration of Re. These results agree with previous findings of Santos and Maria, (2005) showing an inhibition of the liver EROD activity after 0.01-2.7μM Re *in vitro* exposure in *A. anguilla* L. However, the previous *in vivo* studies performed in *A. anguilla*, showed liver EROD activity increase until 72 hours (8, 16, 24 and 72) under Re exposure concentrations ranging from 0.1 up to 2.7 μM (Maria et al., 2004). Therefore, our experimental results showed that Re, a potent *in vivo* inducer of *A. anguilla* P450-dependent monooxygenases, is an *in vitro* inhibitor of liver microsomal EROD activity since its inhibition occurred from 0.1 up to 2.7μM Re. These findings are in accordance with Fragoso et al. (1999) who demonstrated an *in vitro* rainbow trout EROD activity inhibition at Re concentrations above 2.5 $\mu g.ml^{-1}$ (approximately 10μM) reducing by 88% at the highest Re tested concentration (250 $\mu g.ml^{-1}$, approximately 1mM). Gravato and Santos (2002b) also observed in juvenile *Dicentrarchus labrax* L. that other PAHs such as BaP, within a concentration range between 0.001μM up to 2.7 μM, inhibits liver EROD activity, which coincided with the present Re concentration range *in vitro* liver microsomal inhibition. High BaP concentrations (above 100 nM), also abruptly decrease EROD activity in a trout liver cell line, suggesting enzymatic inhibition rather than

xenobiotic-induced cytotoxicity (Bols et al., 1999).Thus, the previous toxicological responses suggest that these two compounds may interact in a similar way with liver EROD activity, causing its inhibition.

2.3 Liver microsomes *in vitro* exposure to cortisol

The endocrine system plays an extremely important role in fish stress mechanisms since biochemical alterations and consequent effects on specific hormonal functions may constitute important stress biomarkers (Hontela, 1997). Despite the existence of sufficient scientific information concerning the importance of the endocrine system of fish, mainly the steroid hormone response in the situation of stress, less is known about the cellular physiological level of hormone and their effects on isolated hepatic microsomal EROD activity, *in vitro*. In the vast majority of living organisms, the induction of phases I and II biotransformation enzymes is a well-known response to organic xenobiotic exposure. However, the knowledge of the linkage between these activations and endocrine regulation is still a challenge to environmental toxicology. The relation between the hepatic EROD activity (Pacheco and Santos 1998) and plasmatic and cellular level of specific steroid hormone is less known in fish.

Plasma cortisol levels are the most commonly used stress indicators in fish because of its rapid elevation in response to various stressors viz. handling, confinement, poor water quality and a wide variety of toxicants (Vijayan and Moon, 1992; Vijayan et al., 1996; Santos and Pacheco, 1996; Wendelar-Bonga, 1997). Plasma cortisol levels have been widely studied in *A anguilla* L. (Santos and Pacheco, 1996; Pacheco and Santos, 2001a and 2001b; Teles et al., 2004a), *Sparus aurata* L. (Teles *et al*., 2005), *Liza aurata* (Oliveira et al., 2007) and in *Dientrarchus labrax* L. (Teles *et al*., 2004b) under diverse environmental conditions.

The majority of laboratory (Santos and Pacheco, 1996) and field studies (Pacheco and Santos, 2001b; Teles et al., 2004a, Oliveira et al., 2007) described the elevation in plasma cortisol level as a characteristic response to the exposure of chemical stressor isolated or in chemical mixture (Teles et al., 2004a),

including PAHs and paper pulp mill effluents, and due to this reason cortisol is frequently used as a stress indicator. The eels' interrenal cortisol release, to the blood plasma, is prevented by the 50% secondary treated bleached kraft pulp mill effluent (STBKPME) exposure. Therefore, the STBKPME may contain toxic substances that might affect the normal interrenal cortisol release to the blood (Pacheco and Santos, 1996).

It is recognized that the steroid hormones are included in the endogenous factors that can regulate CYP1A expression; however their interaction mechanisms are not well established (Navas and Segner, 2000). A previous study carried out by Santos et al. (2000) concerning the effect of cortisol on eel's liver organ culture reflected an increase in hepatic microsomal EROD activity, whenever cortisol was added to the culture medium. Thus, the previous results suggested that the cortisol capability of regulating the CYP 1A expression.

2.4 Liver Microsomes *in vitro* exposure to abietic acid and retene in combination with cortisol

In vitro pre exposure of hepatic microsomes to cortisol followed by AA, causes its EROD activity significant increase when compared with *in vitro* exposure to AA. The present work verified the inhibitory effect of AA upon hepatic microsome EROD activity and its increased activity by *in vitro* pre exposure to cortisol. In this perspective, Ferreira (2007) observed that cortisol exerted a protective effect in liver EROD, since a significant reduction of the inhibitory effects caused by benzo[a]pyrene and fluoranthene (0.9 and 2.7 μM), was observed in their presence. Santos et al. (2000) also observed a significant enhancement of liver EROD activity in *A. anguilla* L. liver organ cultures containing 1.4 μM cortisol in minimum essential media (MEM) from 0 up to 4 and 24 hours.

The current *in vitro* exposure with Re 0,1, 0,3, 0,9 and 2,7 μM after being previously pre exposed to cortisol, also leads to a significant increase in EROD activity when compared with Re exposure only, strenghtening the cortisol protective effect on hepatic microsomal EROD activity when exposed to inhibitor

contaminants. Moreover, in this *in vitro* study, no significant difference between control DMSO and control without DMSO was observed. Thus, the present results on the EROD activity inhibition caused by different concentrations of Re and its relation to cortisol clearly indicates a stimulatory/stabilizing role of cortisol in increasing significantly its hepatic EROD activity.

Over all the results demonstrated that Re and AA both, inhibited eels in vitro liver microsomal EROD activity whereas cortisol showed its significant protective/stabilizing role concerning the two xenobiotics isolated effects.

3. Final considerations and future perspectives

Globally, in the present thesis, an enhanced knowledge of different environmental relevant compounds potentially toxic became visible in respect to the studied parameters. The ENA assessment reflected contamination by a mixture of pollutants from point and non point sources in the Aveiro lagoon, and thus recommended *in situ* and laboratory experiments for further field data corroboration. The *in vitro* effects of PAHs, resin acid and cortisol upon EROD activity, in isolated *A. anguilla* L. liver microsomes, showed its utility as an excellent data generation concerning these and other xenobiotic contaminants probable *in vivo* effects. Moreover, cortisol confirms its protection ability against AA and Re induced EROD activity decrease. In addition, the data concerning the *in vitro* hepatic EROD activity assessment also confirms the importance of structure and molar concentration of the studied compounds as well as the organism's physiological conditions for EROD activity *in vivo* induction. Thus, there is still a need to underline the necessity of additional *in vitro* studies which will make easier to understand the protective effect of steroid hormone applying enzyme activity and to allow an establishment of a linkage between the endocrine regulation and the response of a specific enzyme involved in the biotransformation process, mainly EROD. Moreover, the additional studies evaluating the influence of the steroid hormone such as cortisol and β-estradiol, on hepatic microsomal activity taking other compounds i.e. PAHs, resin acid and heavy metal (individually and/or in mixture) should also be considered. In this

direction, the established condition in chapters IV and V is equally interesting and applicable for the proceeding of additional studies with different concentrations of cortisol and β-estradiol.

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