Universidade de Aveiro Departamento de Biologia
2009

Patrícia Ribeiro Nogueira

Biomarcadores moleculares em peixes expostos a compostos xenobióticos

Molecular biomarkers in fish exposed to xenobiotics

Universidade de Aveiro Departamento de Biologia
2009

Patrícia Ribeiro Nogueira

Biomarcadores moleculares em peixes expostos a compostos xenobióticos

Molecular biomarkers in fish exposed to xenobiotics

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Sónia Alexandra Leite Velho Mendo Barroso, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro e da Doutora Jeanette Rotchell, Professora Sénior do Departamento de Biologia e Ciências Ambientais da Universidade de Sussex, Reino Unido.

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio.

o júri / the jury

presidente / president

Prof. Doutor Jorge Ribeiro Frade
professor catedrático do Departamento de Engenharia Cerâmica e do Vidro da Universidade de Aveiro

vogais / members

Prof. Doutor Amadeu Mortágua Velho da Maia Soares
professor catedrático do Departamento de Biologia da Universidade de Aveiro

Prof. Doutora Lúcia Maria das Candeias Guilhermino
professora catedrática do Departamento de Estudo de Populações do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Prof. Doutor Eduardo de la Peña de Torres
investigador do Consejo Superior de Investigaciones Cientificas, Centro de Ciencias Medioambientales, Madrid, Espanha

Prof. Doutora Sónia Alexandra Leite Velho Mendo Barroso (orientadora)
professora auxiliar do Departamento de Biologia da Universidade de Aveiro

Prof. Doutora Jeanette Marie Rotchell (co-orientadora)
Senior lecturer in the Department of Biology and Environmental Sciences, School of Life Sciences, University of Sussex, Brighton, Reino Unido

agradecimentos / acknowledgements

I would like to thank my supervisors Dr. Sónia Mendo and Dr. Jeanette Rotchell for accepting me in their laboratories and for all the help, support and advices given during this research. My thanks go also to Dr. Mário Pacheco for his essential support and advices. I am grateful for the financial support received from "Fundação para a Ciência e Tecnologia" through a PhD fellowship (SFRH/BD/16763/2004).

To all my labmates from LBM in University of Aveiro, a huge "thank you" for the patience, help, brainstorming and enjoyable moments spent together. I will miss those late-afternoon periods of craziness. A special thanks to Sónia Ferreira who was there since the beginning, for all her support, availability and friendship; and to Joana who was my right hand and friend during most of the work.

Many thanks should also be given to Mirel, Corina, Ohoud and Elena from the Aquatic Toxicology Laboratory in University of Sussex, for all their support, friendship and wonderful moments.

I am also very grateful to Sr. Aldiro Pereira for his help in the fish sampling and for the pleasant work trips in Ria de Aveiro, and to Sr. Rui Marques for his esential assistance with the histology procedures.

I would like to thank Joana Torres and Engª Olga Mayan from the "Centro de Saúde Ambiental e Ocupacional do Instituto Ricardo Jorge" (Porto, Portugal) for letting me use their equipment for part of the comet assay analysis.

I am as well grateful to Dr. Conceição Santos and her collaborators João and Eleazar for the assistance given during the flow cytometry analysis.

My thanks go also to the friends that were always there, in a paralell world, to keep my sanity by giving me pleasant non-scientific moments.

Finally, but not less important, I would like to thank my family, specially my parents, and João for all their unconditional support, understanding and affection.

palavras-chave

Biomarcador, xenobiótico, hidrocarbonetos policíclicos aromáticos, Ria de Aveiro, contaminação ambiental, genotoxicidade, *Anguilla anguilla*, *Dicentrarchus labrax*, *Liza aurata*.

resumo

Os organismos aquáticos estão expostos a uma grande variedade de contaminantes. Biomarcadores medidos ao nível molecular têm sido propostos como ferramentas sensíveis para a detecção precoce de efeitos biológicos da contaminação, permitindo a aplicação de estratégias de bioremediação antes do aparecimento de danos irreversíveis e com consequências ecológicas. Foram analisadas em três espécies de peixes (*Anguilla anguilla*, *Dicentrarchus labrax* e *Liza aurata*) diferentes respostas moleculares associadas à exposição a diferentes contaminantes com o objectivo de desenvolver novos biomarcadores. Espécimes imaturos juvenis de *A. anguilla* foram expostos em laboratório a dois potentes hidrocarbonetos policíclicos aromáticos, benzo[a]pireno e 7,12-dimetilbenz[a]antraceno (DMBA). Uma análise dos resultados mostrou que células do fígado, brânquias e sangue de *A. anguilla* expostas a estes compostos sofreram um aumento nos níveis de danos no DNA, medidos por "comet assay", embora não tenham sido detectados, por citometria de fluxo, danos cromossomais. Alterações genéticas específicas, incluído mutações no gene *ras* e alterações na expressão dos genes *ras* e *rad1*, foram também analisadas não tendo sido encontradas diferenças. Em resposta à exposição a DMBA foram também detectadas alterações nos níveis de expressão de genes envolvidos no metabolismo de xenobióticos, resposta imunes e dinâmica do citosqueleto. Esta abordagem realçou a complexidade da resposta observada em peixes expostos a compostos genotóxicos e constituiu a base para o desenvolvimento de novos biomarcadores.

Com o intuito de estudar um cenário mais realista, espécimes de *D. labrax* e *L. aurata* foram capturados em diferentes locais contaminados da Ria de Aveiro (Portugal). Biomarcadores usualmente aplicados como, por exemplo, as lesões histopatológicas do fígado e a avaliação da integridade do DNA revelaram o impacto da exposição nas espécies analisadas. Outras respostas moleculares foram também avaliadas e revelaram a ausência de mutações no gene *ras*, enquanto que a expressão do gene *xpf*, envolvido na reparação de DNA, se mostrou diminuída em peixes capturados em locais contaminados. Nestes animais, genes envolvidos na produção de energia, resposta imune e defesa contra o stress oxidativo tiveram também a sua expressão alterada. A expressão destes genes, que estão implicados numa variedade de processos celulares, pode actuar como um indicador precoce de exposição ou de efeito da contaminação em peixes. Estes novos potenciais biomarcadores poderão futuramente ser ancorados a biomarcadores tradicionais em programas de biomonitorização ambiental, contribuindo para uma detecção precoce da exposição a contaminantes e, consequentemente, para a prevenção dos efeitos observados a níveis superiores da organização biológica.

Para além da sua potencial aplicação como biomarcadores, as respostas moleculares estudadas no presente trabalho, contribuíram também para uma melhor compreensão da resposta dos peixes à toxicidade.

keywords

Biomarker, xenobiotic, polycyclic aromatic hydrocarbons, Ria de Aveiro, environmental contamination, genotoxicity, *Anguilla anguilla*, *Dicentrarchus labrax*, *Liza aurata*.

abstract

Aquatic organisms are exposed to a wide variety of contaminants introduced into the environment as a consequence of anthropogenic activities. Biomarkers measured at the molecular level have been proposed as sensitive 'early warning' tools for biological effect measurement in environment quality assessment, allowing the initiation of bioremediation strategies before irreversible environmental damage of high ecological impact occur. Different exposure-related molecular responses were analysed in three fish species (*Anguilla anguilla*, *Dicentrarchus labrax* and *Liza aurata*), with the broad aim of developing novel biomarkers. Juvenile specimens of *A. anguilla* were exposed under well-defined laboratory conditions to two potent polycyclic aromatic hydrocarbons (PAH), benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene (DMBA). Liver, gill and blood cells of *A. anguilla* exposed to PAHs showed increased levels of DNA damage as measured by comet assay, although no permanent chromosomal damage was detected in any of those tissues by flow cytometry. Specific gene alterations, including mutations in *ras* gene and altered expression of *ras* and *rad1* genes, were also analysed however no differences could be detected. Genes involved in xenobiotic metabolism, immune processes and cytoskeleton dynamics were found to be differently expressed in response to DMBA, as revealed by suppression subtractive hybridization (SSH). This approach highlighted the complexity of the response observed in fish exposed to genotoxic compounds and constituted the basis for new biomarker development.

To study a more realistic scenario, specimens of *D. labrax* and *L. aurata* were captured at different contaminated sites in Ria de Aveiro (Portugal) lagoon. Well-established biomarker responses such as liver histopathological lesions and detection of DNA damage revealed the impact of exposure in the species analysed. The molecular responses further studied revealed no alterations in the *ras* gene, while the expression of the DNA repair-related *xpf* gene was found decreased in exposed fish. An altered expression was also found in genes related to energy production, immune response and oxidative stress defence, using the SSH methodology. The expression of these genes, which span a broad range of cellular processes, has the potential to act as early signal of exposure to or effect of contamination in fish. These potential novel molecular biomarker responses could then be anchored to traditional biomarkers in environmental monitoring programs, contributing to an earlier detection of contamination exposure and, consequently, to the prevention of some of the effects observed at higher levels of biological organization.

In addition to their potential biomarker application, the molecular responses studied in the present work, have also contributed to a better understanding of the integrated toxic response in fish.

Contents

1. Introduction

2. Mutation analysis of *ras* gene in the liver of European eel (*Anguilla anguilla* L.) exposed to benzo[a]pyrene

3. A follow-up of late DNA damage in different fish tissues after a benzo[a]pyrene acute exposure

4. Transcript profiling and DNA damage in the European eel (*Anguilla anguilla* L.) exposed to 7,12-dimethylbenz[a]anthracene

5. Anchoring novel biomarker responses to traditional responses in fish exposed to environmental contamination

7. General Discussion

List of Figures

1. Introduction

2. Mutation analysis of *ras* gene in the liver of European eel (*Anguilla anguilla* L.) exposed to benzo[a]pyrene

3. A follow-up of late DNA damage in different fish tissues after a benzo[a]pyrene acute exposure

4. Transcript profiling and DNA damage in the European eel (*Anguilla anguilla* L.) exposed to 7,12-dimethylbenz[a]anthracene

5. Anchoring novel biomarker responses to traditional responses in fish exposed to environmental contamination

6. Conservation of DNA repair-related and cancer genes in aquatic organisms

List of Tables

1. Introduction

4. Transcript profiling and DNA damage in the European eel (*Anguilla anguilla* L.) exposed to 7,12-dimethylbenz[a]anthracene

5. Anchoring novel biomarker responses to traditional responses in fish exposed to environmental contamination

6. Conservation of DNA repair-related and cancer genes in aquatic organisms

List of Abbreviations

Ah	aryl hydrocarbon
AHH	aryl hydrocarbon hydroxylase
AhR	aryl hydrocarbon receptor
ANOVA	analysis of variance
ARNT	AhR nuclear translocator
AhRE	AhR response elements
ATR	ataxia telangiectasia and Rad3-related
BaP	benzo[a]pyrene
BNF	β-naphthoflavone
Bp	base pair
BPDE	BaP-7,8-dihydrodiol-9,10-epoxide
cDNA	complementary deoxyribonucleic acid
CV	coefficient of variation
CYP	cytochrome P450
CYP1A	cytochrome P450 1A
dNTPs	deoxynucleoside triphosphate
DNA	deoxyribonucleic acid
DMBA	7,12-dimethylbenz[a]anthracene
DMSO	dimethyl sulfoxide
EDTA	ethylenediamine tetraacetic acid
EH	epoxide hydrolase
EROD	7-ethoxyresorufin *O*-deethylase
FCM	flow cytometry
GDP	guanosine-5'-diphosphate
GST	glutathione *s*-transferase
GTP	guanosine-5'-triphosphate
Ha-ras	Harvey-ras
HCl	hydrogen chloride
HE	hematoxylin and eosin
HPCV	half-peak coefficient of variation
HSP90	90 kDa heat shock protein
Kb	kilobases
K-ras	Kirsten-ras

KCl	potassium chloride
LB	Luria-Bertani medium
LMPA	low melting point agarose
MMC	melano-macrophages centres
mRNA	messenger RNA
N-ras	neuroblastoma-ras
NAD+	nicotinamide adenine dinucleotide
NER	nucleotide excision repair
NMPA	normal melting point agarose
ORF	open reading frame
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate buffered saline
PCB	polychlorinated biphenyls
PCR	polymerase chain reaction
PI	propidium iodide
qPCR	real-time quantitative PCR
RACE	rapid amplification of cDNA ends
Rb	retinoblastoma gene
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-PCR
SEM	standard error of the mean
SD	standard deviation
Sn	tin
SOD	copper/zinc superoxide dismutase
sqRT-PCR	semi-quantitative reverse transcriptase-PCR
SSH	suppression subtractive hybridization
TAE	tris-acetate-EDTA
TBT	tributyltin
Tris	2-amino 2-hydroxy-methylpropane
UGT	uridine diphosphate glucuronyl-transferases
UV	ultra-violet
XP	xeroderma pigmentosum
XPF	xeroderma pigmentosum, complementation group F

1.

Introduction

1.1. AQUATIC SYSTEMS AND CONTAMINATION

The aquatic environment serves as habitat for interrelated and interacting communities and populations of plants and animals. Moreover, many of the species living in the aquatic systems are important sources of human food. Additionally, it has been estimated that approximately 70% of the human population lives within 60 km of coastal regions and that a significant proportion of the world's largest cities are connected, either directly or indirectly, to the marine environment (Jha 2004). The aquatic environment plays therefore vital roles for ecosystem functioning, human health and civilization.

As a consequence of the various anthropogenic activities, foreign organic chemicals (xenobiotics) are introduced into the aquatic environment leading to the decrease of water and sediment quality. A large proportion of these anthropogenic contaminants are potentially genotoxic and carcinogenic substances, and their presence in the environment has already been linked to the occurrence of malignancies in aquatic organisms (Myers *et al.* 2003; Pacheco and Santos 2002; Simonato *et al.* 2008; Stehr *et al.* 2004).

1.1.1. Ria de Aveiro

Among the aquatic systems, estuaries and lagoons have attracted a significant amount of attention. They are interface ecosystems that link continental and marine environments receiving active inputs from land, rivers and coastal waters. Generally, estuaries are areas of high biological productivity, giving them not only an ecological relevance but also attracting economic interest (Hartl 2002).

Ria de Aveiro, located in the northern west region of Portugal, is a shallow productive estuarine coastal lagoon permanently connected to the Atlantic Ocean. It is 45 km long and 10 km wide covering an area of 83 km^2 and 66 km^2 at high and low tides, respectively (Dias and Lopes 2006). The lagoon is characterized by a complex network of inner channels and by large areas of mud flats and salt marshes (Dias and Lopes 2006). Like other estuaries, Ria de Aveiro offers excellent conditions for colonization by many fish species, which use the lagoon during different phases of their life cycle, especially as a nursery area where juvenile stages of several fish species benefit from abundant food supply and

protection from predators (Rebelo 1992). In addition to its ecological significance, Ria de Aveiro has also important economic development based in fisheries, aquaculture, industry, agriculture and tourism (Lopes *et al*. 2007). These anthropogenic activities have been the main sources of pollution of the lagoon, contributing to the decrease of the water quality in some critical areas of this estuarine system. Chlor-alkali and pulp/paper plants, harbour and dry-dock activities as well as municipal and domestic effluents are the major contributors to the lagoon's contamination. Among the contaminants already identified in Ria de Aveiro (Table 1.1) are polycyclic aromatic hydrocarbons (PAH) (Pacheco *et al.* 2005), heavy metals (Abreu *et al.* 2000; Guilherme *et al.* 2008) and organometallics such as tributyltin (TBT) (Sousa *et al.* 2007).

Table 1.1. Concentrations of various contaminants found in water and sediments of Ria de Aveiro.

Contaminant	Water column	Sediments
PAH [(1)]	0.0010 - 0.8243 µg/l	n.d.
Mercury [(2)]	0.26 - 49.59 µg/l	0.0 - 36.9 µg/g
TBT [(3)]	2.2 - 45.5 ng Sn/l	2.7 - 1780 ng Sn/g

[(1)] Pacheco *et al*, 2005; [(2)] Guilherme *et al.* 2008; [(3)] Sousa *et al.* 2007 ; n.d. – not determined.

1.1.2. Aquatic Contaminants

1.1.2.1. Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs constitute a large group of naturally occurring and synthetic persistent organic chemicals found in the environment, formed by two or more fused aromatic rings. They are distributed widely throughout the environment being introduced into the aquatic systems through biosynthesis, spillage of fossil fuels, discharge of domestic and industrial wastes, atmospheric input and continental runoff (Hartl 2002). PAHs are highly lipophilic, non-polar compounds which, particularly those with high molecular weight, tend to adsorb to organic or inorganic matter in sediments, where they can be trapped for long periods (Johnson *et al.* 2002). Aquatic organisms, such as benthic fish, living in PAH-

contaminated environments may absorb these compounds from the water through gills and skin, or by the ingestion of contaminated sediments or food. Due to their lipophilic properties, PAHs easily diffuse across lipid membranes, and after incorporation into blood, they are rapidly and widely distributed to other organs and tissues. Cellular metabolism of PAHs (biotransformation) results in conversion of these hydrophobic compounds into more polar metabolites that can be readily excreted from the organism. In fish, as in other vertebrates, the metabolism and elimination of PAHs is generally very efficient, and therefore parent PAHs generally do not accumulate in these organisms (Johnson *et al.* 2002; van der Oost *et al.* 2003). However, while metabolism serves mainly as a pathway of detoxification for PAHs, some of the intermediate metabolites of this process have carcinogenic, mutagenic and cytotoxic activity (Bihari and Fafandel 2004; Jha 2004; Johnson *et al.* 2002). Liver neoplasms (Myers *et al.* 2003), immunotoxicity (Johnson *et al.* 2002; Reynaud and Deschaux 2006), reproductive and development toxicity (Johnson *et al.* 2002) among other abnormalities have already been described in fish exposed to PAHs. It is clear that the presence of PAH contamination in the aquatic environment is thus a concerning problem.

In Ria de Aveiro, levels of total PAHs in the water were found to be higher during the Spring/Summer period with a high maximum value of 0.8243 ng/ml at Vagos (Pacheco *et al.* 2005). The high PAH concentrations registered at this site are probably due to municipal effluent treatment plant discharges, which is the most regular source of contamination in this area (Pacheco *et al.* 2005). Among the measured PAHs, naphtalene and benzo[a]pyrene (BaP) were the most abundant.

Two of the PAHs that are most frequently used in laboratory studies are the environmentally-relevant BaP and the prototypical synthetic 7,12-dimethylbenz[a]anthracene (DMBA) (Figure 1.1). Although DMBA is not found in the natural environment, the mechanisms of DMBA toxicity provide important clues about the action of the environmentally prevalent PAHs. Several studies have already reported the strong cytotoxic, mutagenic and immunotoxic properties of BaP and DMBA in aquatic species (Bonacci *et al.* 2003; Maria *et al.* 2002; Regoli *et al.* 2003; Spitsbergen *et al.* 2000). Although both these PAHs are known

carcinogens, DMBA is regarded as one of the most carcinogenic PAHs to mammalian species and fish (Weimer *et al.* 2000).

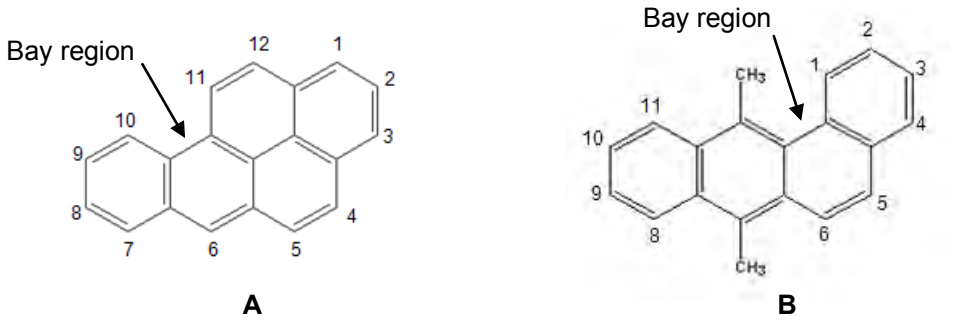

Figure 1.1. Chemical structure of benzo[a]pyrene (A) and 7,12-dimethylbenz[a]anthracene (B).

1.1.2.2. Heavy Metals

Contamination of aquatic habitats with heavy metals (e.g. arsenic, cadmium, lead, mercury and zinc) from various industrial and mineral mining sources has been a problem of concern for many years. Heavy metals enter into aquatic ecosystems mainly through municipal wastes, industrial discharges, surface runoff, damage and weathering of vessel-protective paints, ocean dumpling, and aerial inputs (Carlson and Zelikoff 2008). Exposure of aquatic organisms to toxic metallic pollutants has been shown to induce alterations in hematological parameters, metabolism, reproduction and development and is also related to disturbances in specific immune responses in fish (Sweet and Zelikoff 2001).

Metal contamination in Ria de Aveiro results mainly from industrial effluent discharges in confined areas. One of these areas is the Laranjo basin which has been subjected to mercury contamination from a chlor-alkali plant. In general, the toxic effects of mercury compounds occur because mercury binds to proteins and alters protein production or synthesis (Sweet and Zelikoff 2001). Chronic or sublethal exposures to inorganic mercury or to its most toxic form methylmercury, have been shown to adversely impact reproduction, immune system, growth, behaviour, metabolism, osmoregulation, blood chemistry and oxygen exchange in

aquatic organisms (Sandheinrich and Miller 2006; Sweet and Zelikoff 2001). The genotoxic potential of mercury, including mutagenic, clastogenic and teratogenic effects, has also been reported in humans and in fish (Arabi 2004; Ayllon and Garcia-Vazquez 2000; De Flora *et al.* 1994). Fish from the mercury-contaminated Laranjo basin (Ria de Aveiro) have been shown to accumulate mercury in muscle, liver, gills and stomach (Abreu *et al.* 2000; Guilherme *et al.* 2008), and to be affected by this metal as demonstrated by the elevated erythrocytic nuclear abnormalities observed in fish living in this area (Guilherme *et al.* 2008).

1.1.2.3. Organotin Compounds

Organotin compounds comprise a group of organo-metallic moieties characterized by a tin (Sn) atom covalently bound to one or more organic substituents (e.g. methyl, ethyl, and butyl). These compounds have a broad range of industrial applications being used in antifouling paints in ships, stabilizers of polyvinylchloride, agrochemicals and general biocides, wood preservation, glass treatment and impregnation of textiles (Hoch 2001). With this wide industrial application, considerable amounts of toxic organotin compounds have entered into various ecosystems.

Tributyltin (TBT) is the organotin compound of most concern given its direct introduction into the environment and its high toxicity, even at low concentrations (Thomaidis *et al.* 2007). Impacts of TBT on several marine organisms have been largely documented and include malformation of shells, imposex in gastropods, reduction of mollusc growth and reduced reproduction in oysters (Barroso *et al.* 2002; Hoch 2001; Sonak 2009; Thomaidis *et al.* 2007).

Due to its persistency and biological effects on various organisms, several restrictions were introduced on the usage of TBT and other organotins in antifouling paints in many countries. As a result of these restrictions, TBT contamination, oyster malformations and imposex, declined in many coastal waters (Barroso *et al.* 2002; Hoch 2001; Sousa *et al.* 2007). Nevertheless, despite the ban and regulations on the use of TBT in some countries, contamination continues in the aquatic environment, and environmental concentrations remain high enough to warrant continued concern.

The potential TBT sources of contamination in Ria de Aveiro are the ports, dockyards and marinas. Several studies have already reported the effects of TBT on gastropods of Ria de Aveiro, namely the presence of imposex and female infertility in *Nassarius reticulatus* and *Nucella lapillus* (Barroso *et al.* 2000; Rato *et al.* 2006; Sousa *et al.* 2007). Time comparison studies of the same area showed an obvious decline of TBT environmental levels and a slight decrease of imposex in gastropods between 2000 and 2005, probably as a consequence of the European Union ban of TBT from aquatic environments (Sousa *et al.* 2007). Effects of TBT or other organotin on fish from Ria de Aveiro were not evaluated so far.

1.1.2.4. Pulp and Paper Mill Effluents

The pulp and paper industry is one of the largest polluters, discharging a variety of gaseous, liquid, and solid wastes into the environment. The pollution of water systems is, however, of major concern because large volumes of wastewater are generated for each metric ton of paper produced (Ali and Sreekrishnan 2001). Effluents from pulp and paper mills are complex mixtures of chemical compounds, some of which highly toxic, which are formed and released during various stages of papermaking. Some of the substances included in these effluents are chlorinated compounds (such as dioxins and furans), suspended solids, fatty acids, tannins, resin acids, sulphur compounds and metals (Ali and Sreekrishnan 2001).

Biochemical, physiological and structural effects on fish exposed to pulp and paper mill effluents have been widely documented and include developmental damage, growth disturbance, alterations in organs weight, sexual maturation and reproduction, histopathological effects, liver and blood DNA breakage, and hematologic alterations (Maria *et al.* 2004; Pacheco and Santos 1999; Thompson *et al.* 2001). Many of these effects are chronic with long-term consequences, and therefore aquatic toxicity due to pulp and paper mill effluents is an acute problem that needs to be addressed urgently at a worldwide scale.

In Ria de Aveiro, a pulp mill plant was responsible for continuous hazardous discharges for the past five decades. Several studies assessed the toxicology risk

of these discharges in fish, observing general stress, genotoxic effects and biotransformation activation (Gravato and Santos 2002a; Maria *et al.* 2004; Pacheco *et al.* 2005). In 2000, the paper and pulp mill effluent was deviated to the Aveiro coastal waters (Atlantic Ocean) through a submarine pipe outlet. However, some of the pulp and paper mill effluents are recognized as highly persistent in the environment and therefore the risk for aquatic organisms still remains in the area of the old sewage outlet. In fact, studies conducted after the discharge suspension revealed stress responses and genotoxicity in fish such as eel (Teles *et al.* 2004b) and sea bass (Gravato and Santos 2002a).

1.2. ABSORPTION, DISTRIBUTION, BIOTRANSFORMATION AND EXCRETION OF XENOBIOTICS

In fish, xenobiotic compounds are absorbed through gills, skin or gut. All of these organs possess a large surface area and are separated from the circulatory system by a thin membrane, consisting of a few cells. Once in the blood, xenobiotics, which may exist freely dissolved or bound to plasma proteins, are transported to various tissues. Since absorption from the external environment until the contact with the tissues, the xenobiotics have to cross several cell lipid membranes. The primary pathway for this transport is by passive diffusion, due to the non-polar, lipid-soluble properties of many xenobiotics. However, the transport of polar compounds across lipid membranes requires the action of specialized membrane transport systems, such as multidrug resistance-associated proteins and P-glycoproteins (Bard *et al.* 2002; Sauerborn *et al.* 2004; Zaja *et al.* 2008). After cellular absorption, xenobiotics are metabolized into more water-soluble forms, which can be excreted from the body more easily than the parent compound (van der Oost *et al.* 2003). This metabolism is also referred as biotransformation and requires the action of several enzymes that have a relatively low degree of substrate specificity (van der Oost *et al.* 2003). Following metabolism, xenobiotics are removed from the body mainly through hepatobiliary excretion and are eliminated through faeces or urine. Biotransformation

accelerates the elimination of lipophilic compounds, by converting them into more polar metabolites reducing their biological activity and avoiding their accumulation.

Given its function, position and blood supply, the liver is the major organ involved in the metabolism in fish, playing an important role in the uptake, accumulation, biotransformation and excretion of xenobiotics (Gernhofer *et al.* 2001; van der Oost *et al.* 2003). However, other organs such as gill, gut, kidney, brain and blood vascular lining cells have also the capacity to metabolize xenobiotics (Ortiz-Delgado *et al.* 2005; Sarasquete and Segner 2000).

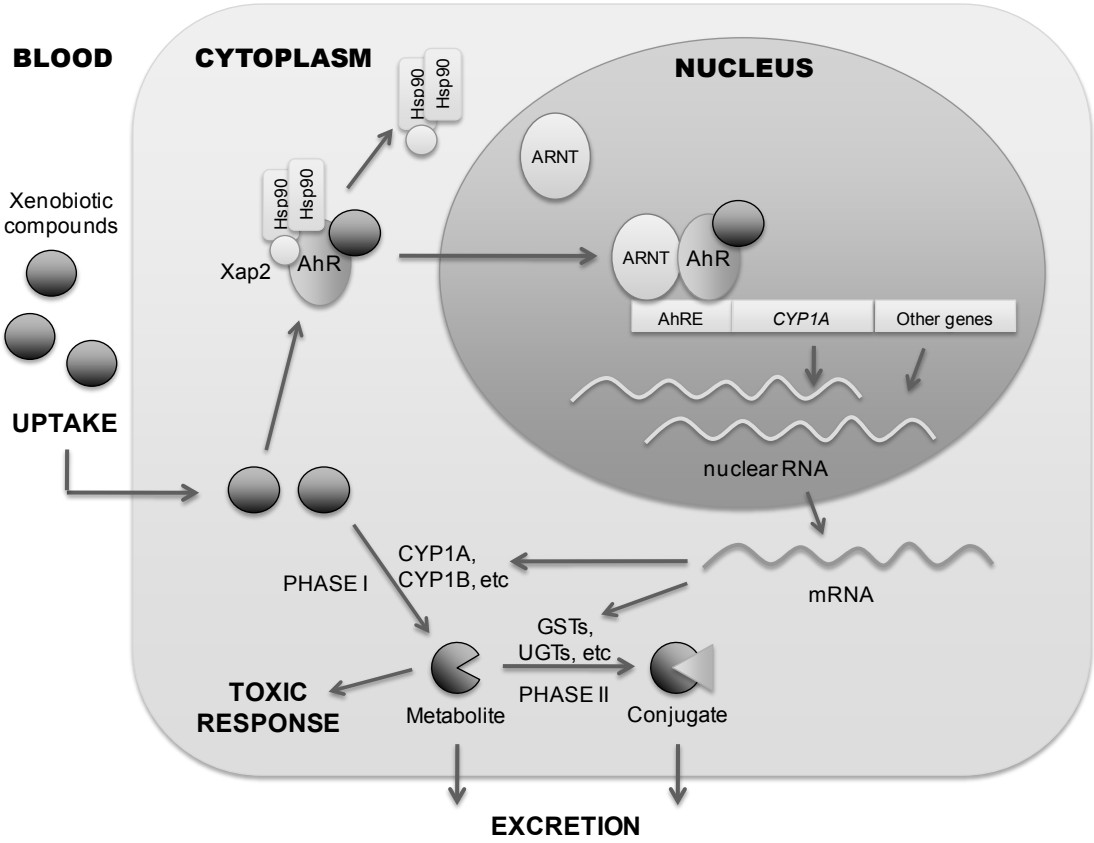

Figure 1.2. Simplified presentation of the fate of xenobiotic compounds in the cell showing possible mechanisms for detoxification/toxication and for enzyme induction. AhR, aryl hydrocarbon receptor; HSP90, 90 kDa heat shock protein; ARNT, Ah receptor nuclear translocator; AhREs, AhR-response elements; CYP, cytochrome P450 isozymes; GSTs, glutathione S-transferases; UGTs, UDPglucuronyl-transferases (adapted from Ma 2007).

1.2.1. Biotransformation

Xenobiotic biotransformation (Figure 1.2) is a two-phase process of enzymatic reactions dominantly leading to detoxification and elimination of the parent compounds. Phase I reactions add or expose chemical functional groups to xenobiotics, which can then be conjugated in phase II to facilitate excretion (Ruddock *et al.* 2003). In this process, highly reactive metabolites are produced (bioactivation), which may interact with biological macromolecules becoming more toxic than the initial parent compounds (Sarasquete and Segner 2000).

1.2.1.1. Phase I Reactions

The first-phase reactions include oxidation, hydroxylation, reduction, hydrolysis, dealkylation and epoxidation. The greatest importance is ascribed to oxidation enzymes involved in the metabolism of the majority of xenobiotics. The most important oxidation enzyme system of the phase I is the cytochrome P450 monooxygenase system (CYP) (Siroka and Drastichova 2004). CYPs comprise a large family of structurally and functionally related heme proteins with broad substrate specificity, being responsible for the oxidative metabolism of many structurally diverse xenobiotic and endogenous compounds. CYPs are membrane-bound proteins which are predominantly located in the endoplasmic reticulum of the liver, although they have also been found in various other fish organelles and tissues such as gills, kidney, gut, brain and vascular endothelium (Ortiz-Delgado *et al.* 2008; Sarasquete and Segner 2000; Siroka and Drastichova 2004).

The cytochrome P4501A (CYP1A) subfamily is particularly important given its role in the biotransformation of many xenobiotic compounds including dioxins, furanes, polychlorinated biphenyls (PCB) and PAHs (Cousinou *et al.* 2000; Sarasquete and Segner 2000; Stegeman and Lech 1991), being the most studied CYP form in fish. The properties and inducibility of fish CYP1A are similar to those found in mammals, being also induced by exposure to many xenobiotics (e.g. PAHs) (Cousinou *et al.* 2000). The inductive response (Figure 1.2) involves the binding of the xenobiotic compound to the cytosolic ligand-activated transcription factor aryl hydrocarbon receptor (AhR), which then undergoes a process that involves the dissociation of two molecules of the heat shock 90 protein that

facilitates nuclear translocation and association with AhR nuclear translocator protein (ARNT) (Carlson and Perdew 2002; Wirgin and Waldman 1998). The AhR/ARNT heterodimer interacts with the DNA through specific AhR-response elements (AhRE) near the promoter region of the *cyp1A* gene or other inducible genes in AhR battery. The *cyp1A* gene transcription is thus initiated followed by increased levels of *cyp1A* messenger RNA, new synthesis of CYP1A protein and, consequently, enhanced CYP1A catalytic activity.

Various other enzymes besides CYP may be involved in the oxidation of xenobiotics. While CYP and other oxygenases catalyse the incorporation of molecular oxygen into molecules, oxidases use water as source of oxygen. Other oxidation phase I reactions include peroxidases which derive oxygen from peroxide cofactors, and dehydrogenases which oxidise substrates transferring electrons to an essential cofactor for catalysis (e.g. NAD^+). Epoxide hydrolase (EH) is another important group of phase I enzymes that catalyses the addition of water to an epoxide or arene oxide, usually formed by CYP oxygenation. It is involved in the detoxification of PAHs, however, in some cases, EH plays a role in the formation of reactive diol epoxide metabolites which may be carcinogenic.

1.2.1.2. Phase II Reactions

The second phase of metabolism involves the conjugation of the xenobiotic parent compound or the metabolites resulting from phase I, with an endogenous polar ligand (Figure 1.2) such as glucuronic acid, sulphate, glutathione or amino acids (van der Oost *et al.* 2003). Phase II reactions are catalysed by enzymes like UDP-glucuronosyl transferase, sulfotransferase and glutathione S-transferase (GST), which play an important role in detoxification and clearance of many xenobiotics, by increasing their polarity and thus facilitating their excretion and elimination.

The mechanism of induction for most forms of phase II enzymes is regulated via the Ah-receptor as well (Köhle and Bock 2007; Sutter and Greenlee 1992). Nevertheless, as compared with phase I systems, the induction responses of phase II enzymes are generally less pronounced (van der Oost *et al.* 2003). In fish, the induction of phase II enzymes may be modulated via exposure to a

variety of compounds such as dioxins (Abalos *et al.* 2008), PAHs (Gravato and Santos 2003), heavy metals (Ahmad *et al.* 2005), organotins (Wang *et al.* 2006) and pesticides (Peixoto *et al.* 2006).

1.2.2. Bioactivation of BaP

Although BaP is not itself carcinogenic, some of the reactive metabolites produced during its biotransformation (Figure 1.3) acquire electrophilic properties and can therefore react with DNA to form stable DNA adducts, mainly on guanine and adenine (Marie *et al.* 2008; Xue and Warshawsky 2005). The generated DNA adducts may then initiate the process of mutagenesis and carcinogenesis (Baird *et al.* 2005). The best known metabolic pathway of BaP activation involves the conversion of BaP to the (-)-7,8-diol by consecutive reactions catalysed by CYP1A and EH, respectively, followed by an additional CYP1A oxygenation producing the ultimate carcinogen, (+)-*anti*-BaP-7,8-diol-9,10-epoxide (BPDE) (Marie *et al.* 2008). Although CYP1A is clearly the predominant enzyme responsible for each oxygenation of BaP, other CYP isoforms are likely contributors (Baird *et al.* 2005; Xue and Warshawsky 2005). Another metabolic pathway involves one-electron oxidation of BaP which is catalysed by peroxidases and CYP enzymes. The BaP radical cations produced during this pathway are also able to form DNA adducts on guanine and adenine (Marie *et al.* 2008). A third BaP activation pathway involves the conversion of BaP diols into catechols which are then converted into *o*-quinones, such as BaP-7,8-dione. These reactive BaP *o*-quinones can give rise to covalent DNA adducts with guanine and adenine bases, as well as establish redox cycles that lead to the generation of reactive oxygen species (ROS) and consequently to oxidative DNA lesions (Park *et al.* 2006; Shen *et al.* 2006). Although BaP may be metabolized to reactive products such as BPDE, radical cations and *o*-quinones, which are able to damage DNA, it should be noted that the majority of the metabolites of BaP are nontoxic, such as phenols, glucuronide, sulfate and glutathione conjugates, and 4,5 and 9,10-diols of BaP.

In fish, the metabolism of BaP has been widely studied showing to be similar to the one observed in higher vertebrates. The enzymes catalysing the different steps of BaP metabolism in mammalian species, such as CYP, EH and GST, have

also been shown to be involved in the BaP biotransformation in fish (Gorbi and Regoli 2004; Miranda *et al.* 2006; Ortiz-Delgado *et al.* 2008).

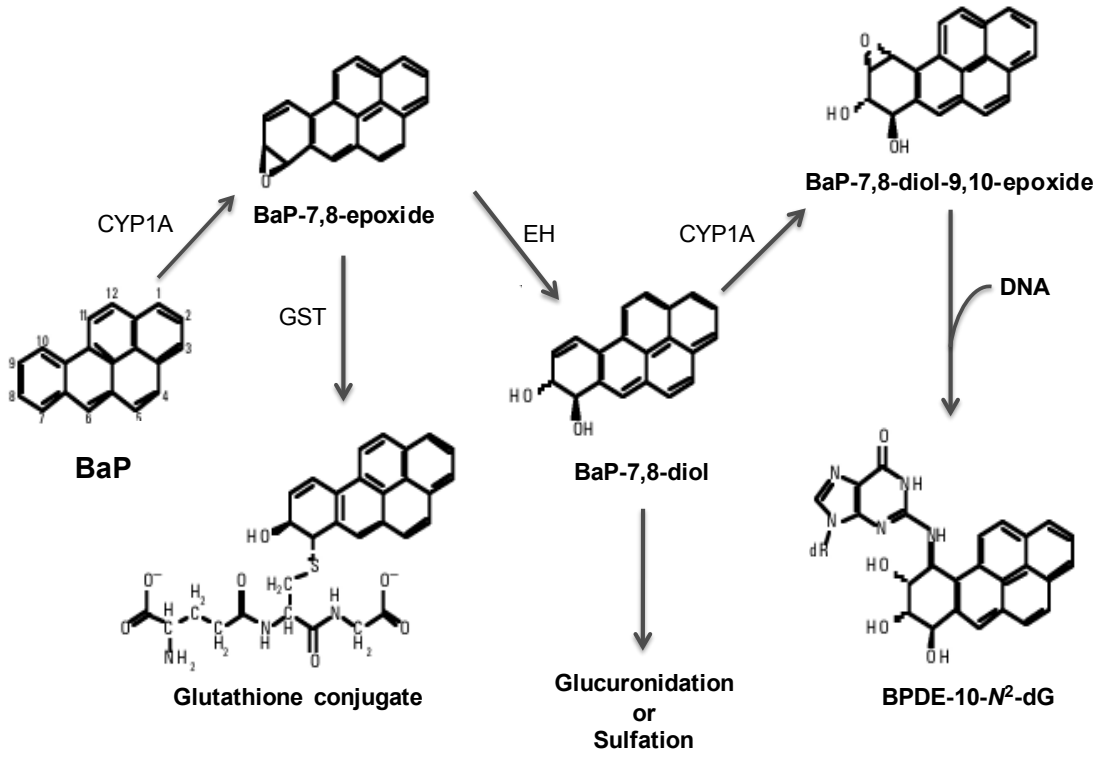

Figure 1.3. Major metabolic pathways for BaP in biological systems. CYP, cytochrome P450; EH, epoxide hydrolase; GST, glutathione S-transferase, BPDE-10-N^2-dG, 10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydro BaP (adapted from Liao *et al.* 2002).

1.2.3. Bioactivation of DMBA

In mammalian cells, the prototypic PAH model compound DMBA, can be metabolized via a sequence of reactions catalyzed by CYP enzymes and EH (Baird *et al.* 2005; Gao *et al.* 2005), to give a variety of metabolites (Figure 1.4). The bay-region diol epoxide DMBA 3,4-diol-1,2-epoxide metabolite, which is able to adduct to adenine and guanine residues in DNA, is thought to be the ultimate carcinogen (Melendez-Colon *et al.* 1999; Miranda *et al.* 1997). Phase I intermediates can be conjugated via phase II reactions (detoxification) to water-

soluble glucuronide, sulfate, and glutathione metabolites. Although recent studies have examined the role of fish CYPs in DMBA metabolism, it is not clear that DMBA-3,4-diol is the proximate carcinogen or the DNA-reactive metabolite in fish. Several studies with fish cells have detected very small amounts of DMBA-3,4-diol among the DMBA metabolites, indicating that other intermediates are the DNA-reactive metabolites in fish (Miranda *et al.* 1997; Weimer *et al.* 2000).

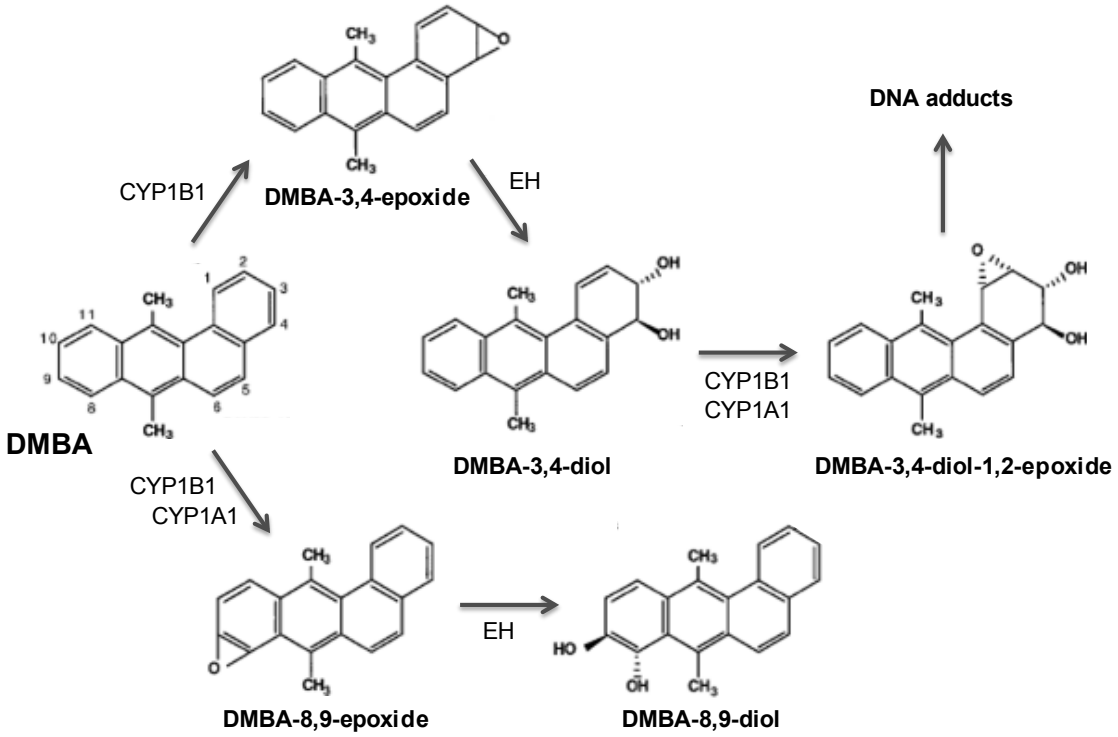

Figure 1.4. Major metabolic pathways for DMBA in biological systems. CYP, cytochrome P450; EH, epoxide hydrolase (adapted from Gonzalez and Kimura 2001).

1.3. GENOTOXICITY

A large proportion of the aquatic contaminants are potentially genotoxic and carcinogenic substances (Jha 2004). Reactive xenobiotics and the reactive metabolites that are not detoxified, as well as ROS generated as a by-product of metabolism of xenobiotics may interact with macromolecules such as proteins, lipids and DNA. In the last decades, a concern regarding the genotoxic/carcinogenic hazards of anthropogenic contaminants has been

increasing, and a large number of studies reporting an association between environmental contaminants and damage at molecular level has been published (Akcha *et al.* 2003; Lyons *et al.* 2004; Mitchelmore and Chipman 1998). Damage at the DNA level has been linked with perturbations in fecundity, longevity and growth of affected organisms (Moustacchi 2000; Theodorakis *et al.* 2000), as well as to the occurrence of neoplasias, being involved in the initiation steps of the process (Rotchell *et al.* 2001b; Shugart 2000).

Several structural modifications on the DNA may occur after exposure to the genotoxicant, such as the formation of DNA adducts when the reactive compound binds covalently to DNA (Shugart 2000). BaP metabolites are a classical example of bulky DNA adduct-inducers (Machella *et al.* 2005; Malmström *et al.* 2000; Moustacchi 2000). The occurrence of DNA adducts in fish has already been associated with the presence of environmental contamination (Lyons *et al.* 2004; Myers *et al.* 1998a; Reichert *et al.* 1998). Toxic chemical exposure may also cause other types of DNA lesions such as single- and double-strand breaks, base modifications and base loss (with the production of apurinic and apyrimidic sites), DNA-DNA cross-links and DNA-protein cross-links. Genotoxicants may also indirectly cause other types of damage such as DNA oxidation and prevention of correct methylation of DNA (Shugart 2000).

Nevertheless, all cells have a complex, highly preserved, and well-regulated system which routinely checks for and repairs DNA damage before DNA replication establishes the change permanently (Miller III *et al.* 2001). Any defect in the cell cycle control and/or DNA repair capacity leads to unrepaired DNA lesions, fixation of mutations and thus genomic instability which may contribute to the occurrence of malignancies as severe as cancer. In fact, alterations in key genes such as oncogenes and tumour suppressor genes induced after environmental contamination exposure have been implicated in fish carcinogenesis process (Peck-Miller *et al.* 1998; Vincent *et al.* 1998).

Although some progress has been made in recent years, DNA damage detection, repair mechanisms and tumour induction in fish are still not as well defined as those in humans and other mammals. A lack of information on genes involved in these processes, such as oncogenes and tumour suppressor genes

still exists for aquatic species, including DNA sequences, functioning and the way they might be affected following exposure to environmental contamination. The information available on fish oncogenes, tumour suppressor genes and DNA repair genes, suggests an evolutionary conservation of these genes among the vertebrates (Bhaskaran *et al.* 1999; Du Corbier *et al.* 2005; Rotchell *et al.* 2001b).

1.3.1. *ras* Proto-oncogene

The *ras* gene is regarded as one of the most important genes involved in multistep carcinogenesis, being its activation one of the first steps of the process. *ras* genes encode proteins that function as GDP/GTP regulated switches and which play a central role in cell growth, differentiation and apoptosis signalling cascades, by transducing extracellular ligand-mediated stimuli into the cytoplasm (Malumbres and Barbacid 2003).

The three *ras* genes found in mammalian species: Ha-*ras*, K-*ras* and N-*ras,* encode four 188-189 amino acid proteins. The fish *ras* genes characterized to date have a high degree of nucleotide sequence and deduced amino acid similarity with the mammalian *ras* gene counterparts (Rotchell *et al.* 2001b). The functional domains found in these proteins include the effector domain, the nucleotide binding sites, interaction with guanine exchange factors and the post-translational modification site, responsible for the protein cellular location. The most frequent sites for *ras* mutations found in mammalian species and fish species, are located in exons I and II, especially in codons 12, 13, 59 and 61 (Rotchell *et al.* 2001b). These transforming products are defective in GTPase activity and thus lead to constitutive, deregulated activation of Ras function. Several field and laboratory studies have described the incidence of *ras* mutations in fish exposed to environmental pollutants (McMahon *et al.* 1990; Roy *et al.* 1999; Vincent *et al.* 1998), although the pattern and incidence of *ras* gene mutations in environmentally-induced tumours appear to be species specific.

1.3.2. Cell Cycle Checkpoint Genes

In response to genotoxic exposure, cells initiate biochemical signalling pathways called cell cycle checkpoints that sense damage on the DNA structure or impaired chromosome function and elicit complex cellular repair responses. These checkpoints rapidly induce cell cycle delay, generally at the G1, S, and G2/M phases, allowing time for the action of DNA repair proteins, and initiating cell cycle progression once repair is complete (Wu *et al.* 2005). If the DNA cannot be adequately repaired, the cell then undergoes permanent cell cycle arrest and apoptosis. Individuals with mutations in cell cycle checkpoint genes, such as *p53* and *Rb* genes, are prone to cancer, highlighting the importance of checkpoint signalling pathways as tumour-suppressive mechanisms (Bi *et al.* 2006). Genes with the ability of controlling the progression of tumours have been called tumour suppressor genes. The *Rb* and *p53* genes are among the most studied tumour suppressor genes.

1.3.2.1. *rad1* Gene

In mammals, bulky adducts such as those induced by PAHs, elicit an S-phase checkpoint pathway involving the heterotrimeric Rad9-Rad1-Hus1 (9-1-1) complex and the ATR protein. The 9-1-1 complex has been characterized as a DNA damage sensor targeted to the nucleus and damaged DNA following genotoxic stress (Gembka *et al.* 2007; Helt *et al.* 2005). The ATR and 9-1-1 complex are recruited separately to affected DNA where they attract specialized DNA polymerases and other DNA repair effectors (Helt *et al.* 2005). Studies with the fission yeast, *Schizosaccharomyces pombe,* showed that *rad1* mutants are extremely sensitive to DNA damaging agents and fail to invoke cell arrest, yet they are DNA repair-competent, indicating a loss in the ability to recognize the presence of damaged DNA (Parker *et al.* 1998).

The *rad1* gene is completely characterized in humans (Bluyssen *et al.* 1998). It comprises 849 nucleotides and encodes a protein of 282 amino acids. In fish species, very few sequences are available for this gene. The existing sequences have a shorter open reading frame with 840 nucleotides, encoding 279 amino-acid residues. The conserved domains observed in the human gene are also present in

fish *rad1* gene sequences, namely the exonuclease domain and the leucine zippers.

1.3.2.2. *Retinoblastoma (Rb)* Tumour Suppressor Gene

In vertebrates, the *retinoblastoma* tumour suppressor gene (*Rb*) product is a nuclear phosphoprotein that regulates normal cell cycle progression (G1 checkpoint) by binding to transcription factors and interacting with kinases (Burkhart and Sage 2008; Weinberg 1995). In addition to its role as a cell cycle gatekeeper, Rb protein is also thought to play other important cellular roles including control of cellular differentiation, regulation of cell apoptosis, maintenance of permanent cell cycle arrest and preservation of chromosomal stability (Dannenberg and te Riele 2006; Zheng and Lee 2001). The importance of Rb as a central mechanism to arrest cell proliferation is manifested by the frequent loss of function of the *Rb* gene, either by mutation or deletion, in the formation of many human cancers, including retinoblastoma and hepatocellular carcinoma (Brunelli and Thorgaard 1999; Zhang *et al.* 1994).

Human *Rb* gene consists of 27 exons encoding a phosphoprotein of 928 amino acids that can be detected in all human tissues. Many of the human Rb structural motifs show high homology with the few fish *Rb* genes sequenced to date (Brunelli and Thorgaard 1999; Du Corbier *et al.* 2005; Rotchell *et al.* 2001a). The most conserved regions include two binding domains, a leucine zipper, a nuclear targeting site, CDK2 kinase phosphorylation sites, and a myc binding domain (Brunelli and Thorgaard 1999). Polymorphisms and variants within the *Rb* gene have been reported in human (Lohmann *et al.* 1996; Mateu *et al.* 1997) and in fish samples (Du Corbier *et al.* 2005; Rotchell *et al.* 2001c). Some of these polymorphisms are important factors in determining individual susceptibility to cancer and may potentially explain population differences in liver tumour incidences observed in fish from field studies (Du Corbier *et al.* 2005). The pattern of mutational alterations is also similar in humans and fish *Rb* genes, being exons 12-23 the preferentially altered.

1.3.3. DNA Repair Genes

DNA repair enzymes maintain the integrity of the genetic information by minimizing replication errors caused by damaged or rearranged DNA templates and by removing damaged DNA segments, and thus protecting the genome from mutations (Miller III *et al.* 2001). Depending on the type of damage sustained, eukaryotic cells undergo different types of DNA repair, including base excision repair, nucleotide excision repair (NER), mismatch repair, or recombinatorial repair. Most of the DNA repair mechanisms involve the concerted action of a number of enzymes (Moustacchi 2000).

Bulky DNA adducts that distorts the DNA helix such as bulky xenobiotic adducts and UV photoproducts are removed by NER enzymes (Miller III *et al.* 2001). NER pathway has four steps involving initial damage recognition, incision on either side of the lesion, gap-filling and DNA ligation (Moustacchi 2000). This multiple step process removes small sections of adducted DNA involving the assembly of numerous proteins at the site of DNA damage (Notch *et al.* 2007). In humans, defects in NER are associated with the inherited condition xeroderma pigmentosum (XP), which is characterised by photosensitivity and a predisposition to cancer (Miller III *et al.* 2001).

The repair factor XPF (also know as ERCC4), studied in the present work, is one of the proteins involved in NER mechanism. The XPF-ERCC1 complex functions as a structure-specific endonuclease that cleaves on the 5' side of bubble structures containing damaged DNA (Wu *et al.* 2007). The XPF-ERCC1 complex plays also a role in crosslink repair and homologous recombination (Niedernhofer *et al.* 2004), which further highlights its importance in the maintenance of genome integrity. Alterations in conserved nuclease domains of XPF are associated to no or a residual enzymatic activity, leading to a defected NER (Wu *et al.* 2007). In humans, alterations in XPF have been related with the repair disorder XP (Kraemer *et al.* 1994).

The fish *xpf* gene sequences (homolog to *rad1* in *Saccharomyces cerevisiae*) available to date are limited to *Danio rerio* (Notch *et al.* 2007) and to the fish species which genome is already sequenced (*Gasterosteus aculeatus, Oryzias latipes, Takifugu rubripes* and *Tetraodon nigroviridis)*. As human *xpf* gene, also

fish *xpf* has 11 exons, with a high homology with the human gene. The same conserved domains are present across species, including fish: helicase domain, endonuclease domain and double helix-hairpin-helix motifs.

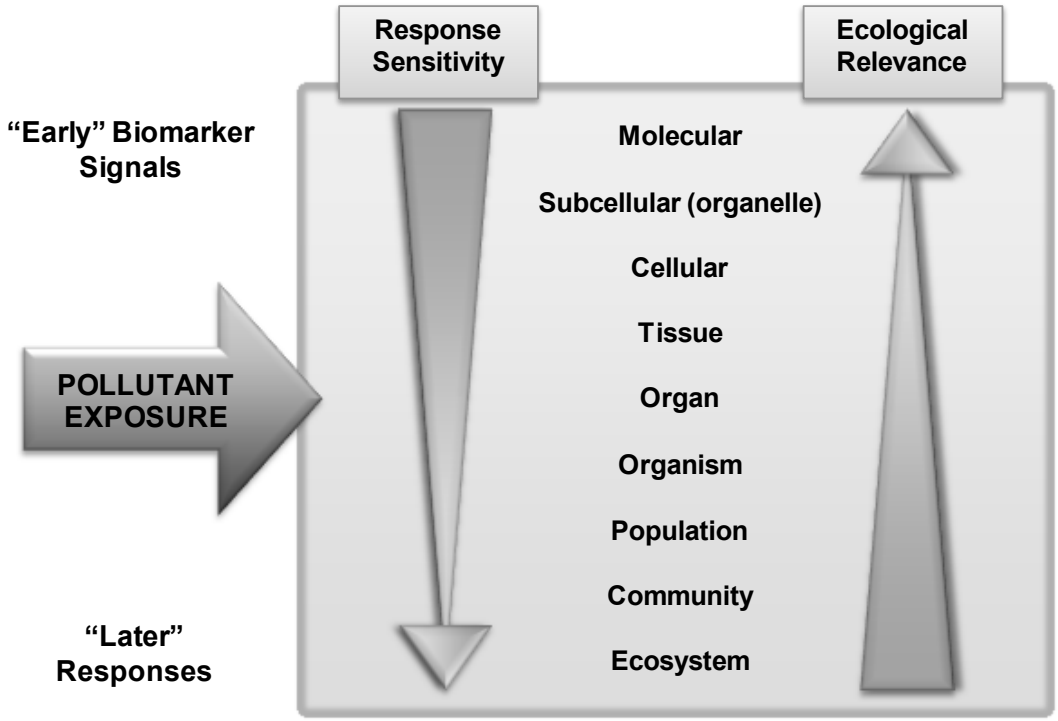

Figure 1.5. Schematic representation of the sequential order of responses to pollutant stress within a biological system, showing the relationship between response sensitivity and ecological relevance (adapted from Van der Oost *et al.*, 2003).

1.4. BIOMARKERS

The growing concern regarding the risks posed by chemical pollutant discharges to the environment and natural resources has led to the development of different methods to identify, estimate, assess and manage those risks. The measurement of the biological effects of pollutants has become of major importance for the assessment of the quality of the aquatic environment, providing an indication of deleterious effects of contaminants on the biota, and thus complementing the chemical analysis of environmental samples (Cajaraville *et al.*

2000). In this context, sensitive early-warning biochemical responses, or biomarkers, reflecting the adverse biological responses towards anthropogenic environmental toxins, have been proposed as tools for biological effect monitoring in environmental quality assessment (Cajaraville *et al.* 2000; van der Oost *et al.* 2003).

A biomarker is defined as a change in a biological response (ranging from molecular through cellular and physiological responses to behavioural changes) which can be related to exposure to (biomarkers of exposure) or toxic effects of (biomarkers of effect) environmental chemicals (Figure 1.5) (van der Oost *et al.* 2003). Effects at higher hierarchical levels are preceded by earlier changes in biological processes and therefore, changes at molecular or cellular levels have the potential to anticipate changes at higher levels of biological organization (population, community or ecosystem). These effects may be readily detected in a limited period of time and the biological response manifested at a low concentration of exposure. Thus, the use of molecular and cellular biomarkers, which are considered to be intermediates between pollutant sources and higher-level effects, may permit intervention before irreversible environmental damage becomes inevitable. Molecular-level responses may not have readily apparent ecological relevance, in contrast to population- or community-level responses which have a great amount of ecological relevance (Figure 1.5). However, by the time that adverse effects at such levels (population or community) are detected, significant ecological damage may have occurred. Therefore, the use of molecular biomarkers is important to identify early-onset changes, which predict increased risk of adverse effects following exposure to environmental chemicals. An additional advantage of the biomarker approach is that by screening multiple biomarker responses, important information will be obtained concerning the mechanism of contaminant stress response.

The exposure of an organism to pollutants normally triggers a cascade of biological responses, each of which have the potential to serve as a biomarker. Enzymes involved in the detoxification of xenobiotics and their metabolites, such as CYP1A and other biotransformation enzymes have been extensively employed as biomarkers (e.g. Ahmad *et al.* 2008; Fenet *et al.* 1998; Pacheco and Santos

1999). If the xenobiotic circumvents the cell protective barriers, it may interact with the DNA molecule, and a suite of other biological responses may be expressed shortly after exposure which may also represent potential biomarkers. These include detection of DNA damage and the induction of cellular repair machinery and other genes involved in the stress/damage response.

1.4.1. Induction of Cytochrome P450 1A

Alteration of biochemical defence systems is typically the initial response to any toxic insult by a xenobiotic, and thus, the measurement of response by these systems can act as extremely sensitive indicators of altered cell function. In this context, the inducibility of CYP1A by xenobiotic exposure has led to its application as a biomarker in pollution monitoring studies of the aquatic environment. In fish, induction of CYP1A protein has been determined at the mRNA (Rees *et al.* 2003), protein (Livingstone *et al.* 2000), and at its associated enzyme activity levels: 7-ethoxyresorufin O-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH) (Fenet *et al.* 2006; Myers *et al.* 1998b; Pacheco *et al.* 2005). Several laboratory-controlled and field experiments have shown that CYP1A induction in fish may be related to contaminant levels, such as PAHs and pulp/paper mill effluents (Billiard *et al.* 2004; Gorbi and Regoli 2004; Pacheco and Santos 1999). Nonetheless, in the natural environment, a number of confounding factors such as environmental temperature, season, presence of inhibitors (e.g. metals and organotin compounds) and endogenous factors (e.g. hormone levels and the physiological condition of the fish), may overlay the induction response, and therefore, a direct relation between the concentration of an inducing contaminant and CYP1A induction may not be observed (Cajaraville *et al.* 2000; Sarasquete and Segner 2000). On the other hand, elevated expression should not be equated with toxicity, once that induction may or may not lead to toxicity. As a consequence, interpretation of isolated biomarker data should be done cautiously (Goksoyr and Forlin 1992).

1.4.2. Biomarkers of genotoxicity

The analysis of DNA damage in aquatic organisms has been shown to be a suitable method for evaluating the genotoxic contamination of environments (Jha 2004; Shugart 2000), with the advantage of detecting and quantifying the genotoxic impact without requiring a detailed knowledge of the identity and the physical/ chemical properties of the contaminants present (Frenzilli *et al.* 2009). Some of the methodologies commonly adopted in the assessment of genotoxic impact include the detection of structural damage into DNA such as DNA adducts (e.g. Akcha *et al.* 2004; Lyons *et al.* 1999) and DNA strand breaks (Mitchelmore and Chipman 1998).

The alkaline single cell gel electrophoresis (comet) assay has become a common technique for detection of DNA strand breaks. This technique relies on the electrophoretic mobility of relaxed or broken strands of DNA following denaturation, to detect damage. The comet assay has some advantages over other methodologies designed for the assessment of strand breaks: (i) does not require DNA extraction and purification from tissues, (ii) allows measurements of strand-breaks in individual cells, requiring only a small sample size of cells, (iii) is generally faster and more sensitive than other available methods for the assessment of strand breaks (Frenzilli *et al.* 2009; Hartmann *et al.* 2004). The assay has already proved a useful and effective tool to the assessment of the genotoxic potential of organic contaminants in a range of invertebrate and vertebrate aquatic organisms, including fish captured from contaminated environments (e.g. Brown and Steinert 2004; Costa *et al.* 2008; Hartl *et al.* 2007) or following laboratory exposures (e.g. Çavaş and Könen 2007; Nogueira *et al.* 2006).

The structural perturbations occurring into the DNA molecule that do persist and do not result in the death of cell may interfere with the fidelity of DNA replication resulting in abnormal DNA. This abnormal DNA has been associated with clastogenic events such as chromosomal aberrations, micronuclei formation and aneuploidy, which have also been widely used as biomarkers of genotoxicity in aquatic organisms (Akcha *et al.* 2004; Custer *et al.* 2000; Frenzilli *et al.* 2008). Flow cytometry (FCM) is one of the techniques used to evaluate chromosomal

damage induced by genotoxic agents, by detecting variations in nuclear DNA content. This technique allows a rapid analysis of a large number of samples, applicable to any organism or tissue from which cellular or nuclear suspensions can be obtained (Bickham *et al.* 1998). FCM has been used successfully to evaluate the genotoxic effects of environmental pollutants in fish from laboratory exposures and in field experiments (Easton *et al.* 1997; Goanvec *et al.* 2008; Marchand *et al.* 2003).

The interference with the fidelity of DNA replication may also result in the activation of oncogenes (e.g. *ras*) or deactivation of tumour suppressor genes (e.g. *p53* and *Rb*), either by mutations in important functional domains or by altered expression of the normal gene. The *ras* gene has been found mutated in fish populations exposed to environmental contamination such as in winter flounder, tomcod and dragonet exposed to PAHs and PCBs (McMahon *et al.* 1990; Vincent *et al.* 1998) and in pink salmon embryos exposed to crude oil (Roy *et al.* 1999). Alterations in *p53* gene have been observed in liver tumours of flounders dwelling in highly contaminated coastal areas of Northern Europe (Cachot *et al.* 2000) and in fish exposed to environmental relevant contaminants such as BaP (Sueiro *et al.* 2000) and endocrine disrupting chemicals (Lee *et al.* 2008). Structural alterations in the coding region of the *Rb* gene were reported in methylene chloride- and in environmental induced liver tumours in medaka and in dab (Du Corbier *et al.* 2005; Rotchell *et al.* 2001c; Rotchell *et al.* 2009). Alterations in these key genes are early events in fish tumourigenesis, preceding histopathological cellular damage, and thus their detection could be a potential biomarker of contaminant-induced genotoxic damage. In fact, activation of *ras* gene (Peck-Miller *et al.* 1998; Rotchell *et al.* 2001b), alterations in *p53* (Bhaskaran *et al.* 1999; Sueiro *et al.* 2000) and in *Rb* genes (Du Corbier *et al.* 2005) in fish have already been proposed as biomarkers of exposure to genotoxic chemicals.

Many studies have focused in alterations in single or in a few genes following genotoxicant exposure, but the continuous developments in genomics, nanotechnology and biotechnology areas have permitted the application of gene expression profiling to screen large numbers of genes in response to environmental changes, employing tools such as DNA microarrays or suppression

subtractive hybridization (SSH). SSH identifies differences between two transcriptomes (control *vs.* experimental) being a useful technique to employ when the available sequence data of an organism is limited. This technique involves the hybridization of cDNA from one sample population (tester) to an excess of mRNA from another sample population (driver), followed by separation of the unhybridized fraction (target transcripts differently expressed between two transcriptomes) from hybridized common sequences (Diatchenko *et al.* 1996). SSH has been used to identify genes that are induced (upregulated) or repressed (downregulated) in fish from environmental impacted areas (Sheader *et al.* 2004; Wang *et al.* 2007; Williams *et al.* 2003) and in fish exposed to individual compounds (Auslander *et al.* 2008; Hagenaars *et al.* 2008). Changes in gene expression are thought to precede the manifestation of strong functional alterations, giving expression profiling a great potential for early detection of alterations (Schulte 2001; Steiner and Anderson 2000).

1.4.3. Liver Histopathology

Sublethal exposure to environmental chemicals may result in changes in the structure of cells and the occurrence of pathologies which can significantly modify the function of tissues and organs. Histopathological changes are thus medium-term responses to sublethal stressors that have been used as valuable tools to evaluate the toxic effects of contaminant exposure. Several studies have used histopathological biomarkers in fish to identify and evaluate the impact of aquatic pollutants (e.g. Bernet *et al.* 2004; Handy *et al.* 2002; Stehr *et al.* 2004; Stentiford *et al.* 2003; Teh *et al.* 1997). Fish liver, being the primary organ for biotransformation of xenobiotics and where many contaminants tend to accumulate, has been the target of many of these studies. A relation between exposure to pollutants and the development of hepatic lesions has been demonstrated in fish (Stehr *et al.* 2004; Stentiford *et al.* 2003), making toxicopathic liver lesions effective biological markers of chemical exposures. Some of the liver lesions found in fish exposed to chemical contamination such as PAHs and PCBs include neoplasms, foci of cellular alteration, hepatocellular nuclear pleomorphism, hydropic vacuolation and non-specific necrotic lesions (e.g. Lyons *et al.* 2004;

Myers *et al.* 2003; Stehr *et al.* 2004; Stentiford *et al.* 2003). In general, liver histopathological lesions are not specific to pollutants, and certain lesions appear to be species specific (Au 2004).

An advantage of histopathology as a biomarker lies in its intermediate location in the hierarchy of biological organization. However, as reported for other biomarkers, histological alterations may be influenced by factors other than chemical exposure such as age, diet, environmental factors, seasonal variation, and reproductive cycle (Au 2004). These factors may induce several structural states which may represent normality and could act as potentially confounding issues when attempting to use histological criteria as biomarkers of aquatic pollution.

1.5. FISH AS INDICATORS OF ENVIRONMENTAL POLLUTION

In the aquatic environment, fish are usually regarded as organisms of choice for assessing the biological effects of environmental pollution on aquatic ecosystems. Fish can be found virtually everywhere in the aquatic environment, they occupy the top of the aquatic food chain, are a highly visible resource, and are known to accumulate toxicants (Gernhofer *et al.* 2001; van der Oost *et al.* 2003). In addition, they are in contact with pollutants either via the direct uptake from water by their gills and their body surface, or via ingestion of contaminated sediments. For these reasons fish are frequently used as bioindicator species, by giving information about the environmental conditions of its habitat.

In the present work, three representative species of teleost fish in Ria de Aveiro were selected: *Anguilla anguilla* L. (European eel), *Dicentrarchus labrax* L. (European sea bass) and *Liza aurata* R. (golden grey mullet), based on their abundance in the lagoon and also in their sensitivity to the toxic effects of a wide range of pollutants.

1.5.1. *Anguilla anguilla*

The European eel, *A. anguilla* (L., 1758) (Figure 1.6) is a catadromous fish species with a high-economic importance, which is widely distributed in numerous European estuaries and freshwater courses. The life cycle of eel is unique and complex being characterized by two major migrations: the up-river migration of elvers and the spawning sea-migration of silver eels. After hatching in the Sargasso Sea, the larvae are swept towards Europe where they turn into glass eels. As juveniles, they penetrate freshwater upstream until they reach sexual maturity. As they grow, glass eels metamorphose into yellow eels and later into silver eels. The adult silver eels then migrate to the Sargasso Sea, where they eventually spawn and die (Bonacci *et al.* 2003; Doyotte *et al.* 2001).

Despite its description as a bottom-dwelling fish, eels can move along the entire water column (Costa *et al.* 1992) although within the estuary their movements have been shown to be limited (Doyotte *et al.* 2001). For these reasons allied with their easiness to capture and handling in laboratory, their tolerance to fluctuations in salinity, temperature, food availability and dissolved oxygen level (Bonacci *et al.* 2003; Doyotte *et al.* 2001; Fenet *et al.* 1998), and also their sensitivity to the presence of water contaminants (Maria *et al.* 2002; Pacheco and Santos 2001), eels have been used as versatile indicator species in biomonitoring studies (e.g. Doyotte *et al.* 2001; Pacheco and Santos 1999; Ruddock *et al.* 2003). Several laboratory studies have also characterized the main responses induced by pollutants in this species (Bonacci *et al.* 2003; Pacheco and Santos 2002; Regoli *et al.* 2003).

Figure 1.6. *Anguilla anguilla* (source: www.fishbase.org).

1.5.2. *Dicentrarchus labrax*

D. labrax (L. 1758) (Figure 1.7) is a European estuarine representative species with a high commercial value in fish farming and as the basis of professional and recreational fishing activities. It is a top predator species feeding on small fishes, prawns and crabs. Adults migrate to coastal areas during the spawning season and larvae and juveniles develop in the coast. Then they migrate to sheltered coastal areas or estuarine nurseries where they spend their first years of life (Vinagre *et al.* 2009).

D. labrax has already demonstrated to be a very sensitive species for assessing pollutant-induced effects, either in field monitoring studies (Ahmad *et al.* 2008; Stien *et al.* 1998) as in laboratory-controlled exposures to PAH compounds, pulp mill contaminants and contaminated harbour waters, among others (Gravato and Santos 2002b; Gravato and Santos 2002c; Teles *et al.* 2004a).

Figure 1.7. *Dicentrarchus labrax* (source: www.fishbase.org).

1.5.3. *Liza aurata*

L. aurata (R. 1810) (Figure 1.8) is widely distributed in both Atlantic and Mediterranean coastal waters. Its feeding behaviour is characterized by a regular contact with the sediment being often extensive to the whole water column (Pacheco *et al.* 2005). Grey mullets form transient populations of fish in Ria de Aveiro whose numbers are regulated by the immigration of 0+ group fish from the sea and the emigration of older groups to the sea where they remain at least 1 year before spawning (Arruda *et al.* 1991).

L. aurata has been regarded as a good bioindicator species for monitoring estuarine water contamination by organic xenobiotics, once it already demonstrated to be sensitive enough to the detection of the effects induced by the exposure to different pollutants such as mercury, pulp/paper mill effluents and PAHs (Guilherme *et al.* 2008; Oliveira *et al.* 2009; Pacheco *et al.* 2005).

Figure 1.8. *Liza aurata* (source: www.fishbase.org).

1.6. SCOPE AND OBJECTIVES OF THIS THESIS

The global aim of the present PhD thesis was the study of the biological effects of xenobiotics in fish, particularly the molecular events imposed by contaminants and that might be involved in the toxic response. Because the molecular changes constitute the earliest signals of response to the toxicant exposure and also because their detection may allow the intervention before irreversible damaging effects become inevitable, the present work was directed to the search of new molecular responses to xenobiotics in different fish species, which could be potentially employed as molecular biomarkers. These molecular responses also contributed for a better understanding of the molecular events involved in fish response to aquatic contamination.

The xenobiotics studied (BaP and DMBA) belong to the PAH class, which is one of the most commonly found in aquatic environments. Both laboratorial and field experiments were carried out with the sexually immature stages of the studied fish species to reduce the interference of the metabolic processes associated to reproduction with the metabolic processes under study. The use of juvenile specimens also minimized the background of previous exposures to pollutants.

To achieve the proposed objective, the following points were investigated:

- Study of the genotoxic potential of individual PAHs (BaP and DMBA) in *A. anguilla* (Chapters 2, 3 and 4), focusing on:
  - ✓ detection of DNA damage in different tissues using comet assay and flow cytometry;
  - ✓ analysis of *ras* gene mutational profile and alterations in *ras* gene expression induced by BaP;
  - ✓ analysis of *cyp1A* and *rad1* gene expression levels in DMBA-exposed eels;
  - ✓ identification of DMBA-responsive genes by SSH.

- Study of new molecular responses induced by environmental contamination in *D. labrax* and *L. aurata* from Ria de Aveiro lagoon, in parallel with well-established biomarkers (Chapter 5), including:
  - ✓ analysis of liver histopathological alterations;
  - ✓ detection of DNA damage by comet assay;
  - ✓ study of the *cyp1A* gene expression levels;
  - ✓ analysis of *ras* gene mutations and alterations in *ras* gene expression levels;
  - ✓ analysis of *xpf* gene expression in *D. labrax* from a selected contaminated site;
  - ✓ identification of differently expressed genes by SSH in *D. labrax* from a selected contaminated site.

- Isolation and characterization of several novel cancer-related genes in aquatic species that can potentially be used as molecular biomarkers in biomonitoring studies (Chapter 6):
  - ✓ *ras, rad1, xpf, Rb* and *p53* genes.

1.7. REFERENCES

Abalos M, Abad E, Estevez A, Sole M, Buet A, Quiros L, Pina B, Rivera J. 2008. Effects on growth and biochemical responses in juvenile gilthead seabream *Sparus aurata* after long-term dietary exposure to low levels of dioxins. Chemosphere 73(1):S303-S310.

Abreu SN, Pereira E, Vale C, Duarte AC. 2000. Accumulation of mercury in sea bass from a contaminated lagoon (Ria de Aveiro, Portugal). Marine Pollution Bulletin 40(4):293-297.

Ahmad I, Oliveira M, Pacheco M, Santos MA. 2005. *Anguilla anguilla* L. oxidative stress biomarkers responses to copper exposure with or without β-naphthoflavone pre-exposure. Chemosphere 61(2):267-275.

Ahmad I, Maria VL, Oliveira M, Serafim A, Bebianno MJ, Pacheco M, Santos MA. 2008. DNA damage and lipid peroxidation vs. protection responses in the gill of *Dicentrarchus labrax* L. from a contaminated coastal lagoon (Ria de Aveiro, Portugal). Science of the Total Environment 406(1-2):298-307.

Akcha F, Hubert FV, Pfhol-Leszkowicz A. 2003. Potential value of the comet assay and DNA adduct measurement in dab (*Limanda limanda*) for assessment of in situ exposure to genotoxic compounds. Mutation Research, Genetic Toxicology and Environmental Mutagenesis 534(1-2):21-32.

Akcha F, Leday G, Pfohl-Leszkowicz A. 2004. Measurement of DNA adducts and strand breaks in dab (*Limanda limanda*) collected in the field: effects of biotic (age, sex) and abiotic (sampling site and period) factors on the extent of DNA damage. Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis 552(1-2):197-207.

Ali M, Sreekrishnan TR. 2001. Aquatic toxicity from pulp and paper mill effluents: a review. Advances in Environmental Research 5(2):175-196.

Arabi M. 2004. Analyses of impact of metal ion contamination on carp (*Cyprinus carpio* L.) gill cell suspensions. Biological Trace Element Research 100(3):229-245.

Arruda LM, Azevedo JN, Neto AI. 1991. Age and growth of the grey mullet (Pisces, Mugilidae) in Ria de Aveiro (Portugal). Scientia Marina 55(3):497-504.

Au DWT. 2004. The application of histo-cytopathological biomarkers in marine pollution monitoring: a review. Marine Pollution Bulletin 48(9-10):817-834.

Auslander M, Yudkovski Y, Chalifa-Caspi V, Herut B, Ophir R, Reinhardt R, Neumann PM, Tom M. 2008. Pollution-affected fish hepatic transcriptome and its expression patterns on exposure to cadmium. Marine Biotechnology 10(3):250-261.

Ayllon F, Garcia-Vazquez E. 2000. Induction of micronuclei and other nuclear abnormalities in European minnow *Phoxinus phoxinus* and mollie : An assessment of the fish micronucleus test. Mutation Research, Genetic Toxicology and Environmental Mutagenesis 467(2):177-186.

Baird WM, Hooven LA, Mahadevan B. 2005. Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. Environmental and Molecular Mutagenesis 45(2-3):106-114.

Bard SM, Bello SM, Hahn ME, Stegeman JJ. 2002. Expression of P-glycoprotein in killifish (*Fundulus heteroclitus*) exposed to environmental xenobiotics. Aquatic Toxicology 59(3-4):237-251.

Barroso CM, Moreira MH, Gibbs PE. 2000. Comparison of imposex and intersex development in four prosobranch species for TBT monitoring of a southern European estuarine system (Ria de Aveiro, NW Portugal). Marine Ecology Progress Series 201:221-232.

Barroso CM, Reis-Henriques MA, Ferreira MS, Moreira MH. 2002. The effectiveness of some compounds derived from antifouling paints in promoting imposex in *Nassarius reticulatus*. Journal of Marine Biology 82:249-255.

Bernet D, Schmidt-Posthaus H, Wahli T, Burkhardt-Holm P. 2004. Evaluation of two monitoring approaches to assess effects of waste water disposal on histological alterations in fish. Hydrobiologia 524(1):53-66.

Bhaskaran A, May D, Rand-Weaver M, Tyler C. 1999. Fish *p53* as a possible biomarker for genotoxins in the aquatic environment. Environmental and Molecular Mutagenesis 33(3):177-84.

Bi XH, Barkley LR, Slater DM, Tateishi S, Yamaizumi M, Ohmori H, Vaziri C. 2006. Rad18 regulates DNA polymerase kappa and is required for recovery from S-phase checkpoint-mediated arrest. Molecular and Cellular Biology 26(9):3527-3540.

Bickham J, Mazet J, Blake J, Smolen M, Lou Y, Ballachey B. 1998. Flow cytometric determination of genotoxic effects of exposure to petroleum in mink and sea otters. Ecotoxicology 7:191-199.

Bihari N, Fafandel M. 2004. Interspecies differences in DNA single strand breaks caused by benzo(a)pyrene and marine environment. Mutation Research 552:209-217.

Billiard SM, Bols NC, Hodson PV. 2004. In vitro and in vivo comparisons of fish-specific CYP1A induction relative potency factors for selected polycyclic aromatic hydrocarbons. Ecotoxicology and Environmental Safety 59(3):292-299.

Bluyssen HAR, Van Os RI, Naus NC, Jaspers I, Hoeijmakers JHJ, De Klein A. 1998. A human and mouse homolog of the *Schizosaccharomyces pombe rad1*+ cell cycle checkpoint control gene. Genomics 54(2):331-337.

Bonacci S, Corsi I, Chiea R, Regoli F, Focardi S. 2003. Induction of EROD activity in european eel (*Anguilla anguilla*) experimentally exposed to benzo[a]pyrene and β-naphthoflavone. Environment International 29:467-473.

Brown JS, Steinert SA. 2004. DNA damage and biliary PAH metabolites in flatfish from Southern California bays and harbors, and the Channel Islands. Ecological Indicators 3(4):263-274.

Brunelli J, Thorgaard G. 1999. Sequence, expression and genetic mapping of a rainbow trout retinoblastoma cDNA. Gene 226:175-180.

Burkhart DL, Sage J. 2008. Cellular mechanisms of tumour suppression by the retinoblastoma gene. Nature Reviews Cancer 8(9):671-682.

Cachot J, Cherel Y, Galgani F, Vincent F. 2000. Evidence of *p53* mutations in an early stage of liver lesions cancers in European flounder, *Platichthys flesus* (L.). Mutation Research 464:279-287.

Cajaraville MP, Bebianno MJ, Blasco J, Porte C, Sarasquete C, Viarengo A. 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Science of the Total Environment 247(2-3):295-311.

Carlson DB, Perdew GH. 2002. A dynamic role for the Ah receptor in cell signaling? Insights from a diverse group of Ah receptor interacting proteins. Journal of Biochemical and Molecular Toxicology 16(6):317-325.

Carlson E, Zelikoff JT. 2008. The immune system of fish: a target organ of toxicity. In: Di Giulio RT, Hinton DE, editors. The toxicology of fishes. Boca Raton: CRC Press. p 489-530.

Çavaş T, Könen S. 2007. Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (*Carassius auratus*) exposed to a glyphosate formulation using the micronucleus test and the comet assay. Mutagenesis 22(4):263-268.

Costa JL, Assis CA, Almeida PR, Moreira FM, Costa MJ. 1992. On the food of the European eel, *Anguilla anguilla* (L.), in the upper zone of the Tagus Estuary, Portugal. Journal of Fish Biology 41(5):841-850.

Costa PM, Lobo J, Caeiro S, Martins M, Ferreira AM, Caetano M, Vale C, DelValls TÁ, Costa MH. 2008. Genotoxic damage in *Solea senegalensis* exposed to sediments from the Sado Estuary (Portugal): effects of metallic and organic contaminants. Mutation Research, Genetic Toxicology and Environmental Mutagenesis 654(1):29-37.

Cousinou M, Nilsen B, Lopez-Barea J, Dorado G. 2000. New methods to use fish cytochrome P4501A to assess marine organic pollutants. Science of the Total Environment 247:213-225.

Custer T, Custer C, Hines R, Sparks D, Melancon M, Hoffman D, Bickam J, Wickliffe J. 2000. Mixed-function oygenases, oxidative stress, and chromosomal damage measured in lesser scaup wintering on the Indiana Harbor Canal. Archives of Environmental Contamination and Toxicology 38:522-529.

Dannenberg JH, te Riele HP. 2006. The retinoblastoma gene family in cell cycle regulation and suppression of tumorigenesis. In: Richter D, Tiedge H, editors. Results and Problems in Cell Differentiation. Berlin: Springer Verlag. p 183-225.

De Flora S, Bennicelli C, Bagnasco M. 1994. Genotoxicity of mercury compounds. A review. Mutation Research, Reviews in Genetic Toxicology 317(1):57-79.

Dias JM, Lopes JF. 2006. Implementation and assessment of hydrodynamic, salt and heat transport models: the case of Ria de Aveiro Lagoon (Portugal). Environmental Modelling and Software 21(1):1-15.

Diatchenko L, Lau YFC, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED and others. 1996. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proceedings of the National Academy of Sciences of the United States of America 93(12):6025-6030.

Doyotte A, Mitchelmore CL, Ronisz D, McEvoy J, Livingstone DR, Peters LD. 2001. Hepatic 7-ethoxyresorufin O-deethylase activity in eel (*Anguilla anguilla*) from the Thames Estuary and comparisons with other United Kingdom Estuaries. Marine Pollution Bulletin 42(12):1313-1322.

Du Corbier FA, Stentiford GD, Lyons BP, Rotchell JM. 2005. Isolation of the retinoblastoma cDNA from the marine flatfish dab (*Limanda limanda*) and evidence of mutational alterations in liver tumors. Environmental Science and Technology 39(24):9785-9790.

Easton MDL, Kruzynski GM, Solar, II, Dye HM. 1997. Genetic toxicity of pulp mill effluent on juvenile chinook salmon (*Oncorhynchus tshawytscha*) using flow cytometry. Water Science and Technology 35(2-3):347-355.

Fenet H, Casellas C, Bontoux J. 1998. Laboratory and field-caging studies on hepatic enzymatic activities in European eel and rainbow trout. Ecotoxicology and Environmental Safety 40:37-143.

Fenet H, Gomez E, Rosain D, Casellas C. 2006. Polycyclic aromatic hydrocarbon metabolites and 7-ethoxyresorufin O-deethylase activity in caged European

eels. Archives of Environmental Contamination and Toxicology 51(2):232-236.

Frenzilli G, Falleni A, Scarcelli V, Del Barga I, Pellegrini S, Savarino G, Mariotti V, Benedetti M, Fattorini D, Regoli F and others. 2008. Cellular responses in the cyprinid *Leuciscus cephalus* from a contaminated freshwater ecosystem. Aquatic Toxicology 89(3):188-196.

Frenzilli G, Nigro M, Lyons BP. 2009. The Comet assay for the evaluation of genotoxic impact in aquatic environments. Mutation Research, Reviews in Mutation Research 681(1):80-92.

Gao J, Lauer FT, Dunaway S, Burchiel SW. 2005. Cytochrome P450 1B1 is required for 7,12-dimethylbenz(a)-anthracene (DMBA) induced spleen cell immunotoxicity. Toxicological Sciences 86(1):68-74.

Gembka A, Toueille M, Smirnova E, Poltz R, Ferrari E, Villani G, Hubscher U. 2007. The checkpoint clamp, Rad9-Rad1-Hus1 complex, preferentially stimulates the activity of apurinic/apyrimidinic endonuclease 1 and DNA polymerase β in long patch base excision repair. Nucleic Acids Research 35(8):2596-2608.

Gernhofer M, Pawert M, Schramm M, Müller E, Triebskorn R. 2001. Ultrastructural biomarkers as tools to characterize the health status of fish in contaminated streams. Journal of Aquatic Ecosystem Stress and Recovery 8(3-4):241-260.

Goanvec C, Theron M, Lacoue-Labarthe T, Poirier E, Guyomarch J, Le-Floch S, Laroche J, Nonnotte L, Nonnotte G. 2008. Flow cytometry for the evaluation of chromosomal damage in turbot *Psetta maxima* (L.) exposed to the dissolved fraction of heavy fuel oil in sea water: a comparison with classical biomarkers. Journal of Fish Biology 73(2):395-413.

Goksoyr A, Forlin L. 1992. The cytochrome P-450 system in fish, aquatic toxicology and environmental monitoring. Aquatic Toxicology 22(4):287-312.

Gonzalez FJ, Kimura S. 2001. Understanding the role of xenobiotic-metabolism in chemical carcinogenesis using gene knockout mice. Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis 477(1-2):79-87.

Gorbi S, Regoli F. 2004. Induction of cytochrome P4501A and biliary PAH metabolites in European eel *Anguilla anguilla*: Seasonal, dose- and time-response variability in field and laboratory conditions. Marine Environmental Research 58(2-5):511-515.

Gravato C, Santos MA. 2002a. Juvenile sea bass liver biotransformation and erythrocytic genotoxic responses to pulp mill contaminants. Ecotoxicology and Environmental Safety 53(1):104-112.

Gravato C, Santos MA. 2002b. Juvenile sea bass liver biotransformation induction and erythrocytic genotoxic responses to resin acids. Ecotoxicology and Environmental Safety 52(3):238-247.

Gravato C, Santos MA. 2002c. Juvenile sea bass liver P450, EROD induction, and erythrocytic genotoxic responses to PAH and PAH-like compounds. Ecotoxicology and Environmental Safety 51(2):115-127.

Gravato C, Santos MA. 2003. Genotoxicity biomarkers' association with B(a)P biotransformation in *Dicentrarchus labrax* L. Ecotoxicology and Environmental Safety 55(3):352-358.

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

Hagenaars A, Knapen D, Meyer IJ, van der Ven K, Hoff P, De Coen W. 2008. Toxicity evaluation of perfluorooctane sulfonate (PFOS) in the liver of common carp (*Cyprinus carpio*). Aquatic Toxicology 88(3):155-163.

Handy RD, Runnalls T, Russell PM. 2002. Histopathologic biomarkers in three spined sticklebacks, *Gasterosteus aculeatus*, from several rivers in Southern England that meet the freshwater fisheries directive. Ecotoxicology 11(6):467-479.

Hartl MGJ. 2002. Benthic fish as sentinel organisms of estuarine sediment toxicity. In: Bright M, Dworschak P, Stachowitsch M, editors. The Vienna School of Marine Biology: A Tribute to Jörg Ott. Facultas Universitätsverlag, Wien p89-100.

Hartl MGJ, Kilemade M, Sheehan D, Mothersill C, O'Halloran J, O'Brien NM, van Pelt FNAM. 2007. Hepatic biomarkers of sediment-associated pollution in juvenile turbot, *Scophthalmus maximus* L. Marine Environmental Research 64(2):191-208.

Hartmann A, Schumacher M, Plappert-Helbig U, Lowe P, Suter W, Mueller L. 2004. Use of the alkaline in vivo Comet assay for mechanistic genotoxicity investigations. Mutagenesis 19(1):51-59.

Helt CE, Wang W, Keng PC, Bambara RA. 2005. Evidence that DNA damage detection machinery participates in DNA repair. Cell Cycle 4(4):529-532.

Hoch M. 2001. Organotin compounds in the environment - an overview. Applied Geochemistry 16(7-8):719-743.

Jha AN. 2004. Genotoxicological studies in aquatic organisms: an overview. Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis 552(1-2):1-17.

Johnson LL, Collier TK, Stein JE. 2002. An analysis in support of sediment quality thresholds for polycyclic aromatic hydrocarbons (PAHs) to protect estuarine fish. Aquatic Conservation: Marine and Freshwater Ecosystems 12(5):517-538.

Köhle C, Bock KW. 2007. Coordinate regulation of Phase I and II xenobiotic metabolisms by the Ah receptor and Nrf2. Biochemical Pharmacology 73(12):1853-1862.

Kraemer KH, Lee MM, Andrews AD, Lambert WC. 1994. The role of sunlight and DNA repair in melanoma and nonmelanoma skin cancer: The xeroderma pigmentosum paradigm. Archives of Dermatology 130(8):1018-1021.

Lee Y-M, Rhee J-S, Hwang D-S, Kim I-C, Raisuddin S, Lee J-S. 2008. p53 gene expression is modulated by endocrine disrupting chemicals in the hermaphroditic fish, *Kryptolebias marmoratus*. Comparative Biochemistry and Physiology Part C Toxicology & Pharmacology 147(2):150-157.

Liao KH, Dobrew ID, Dennison JE, Andersen ME, Reisfeld B, Reardon KF, Campain JA, Wei W, Klein MT, Quann RJ and others. 2002. Application of biologically based computer modeling to simple or complex mixtures. Environmental Health Perspectives 110:957-963.

Livingstone D, Mitchelmore C, Peters L, O'Hara S, Shaw J, Chesman B, Doyotte A, McEvoy J, Ronisz D, Larsson D and others. 2000. Development of hepatic CYP1A and blood vitellogenin in eel (*Anguilla anguilla*) for use as biomarkers in the Thames Estuary, UK. Marine Environmental Research 50(1-5):367-71.

Lohmann DR, Brandt B, Höpping W, Passarge E, Horsthemke B. 1996. The spectrum of RB1 germ-line mutations in hereditary retinoblastoma. American Journal of Human Genetics 58(5):940-949.

Lopes CB, Pereira ME, Vale C, Lillebø AI, Pardal MA, Duarte AC. 2007. Assessment of spatial environmental quality status in Ria de Aveiro (Portugal). Scientia Marina 71(2):293-304.

Lyons BP, Stewart C, Kirby MF. 1999. The detection of biomarkers of genotoxin exposure in the European flounder (*Platichthys flesus*) collected from the River Tyne Estuary. Mutation Research, Genetic Toxicology and Environmental Mutagenesis 446(1):111-119.

Lyons BP, Stentiford GD, Green M, Bignell J, Bateman K, Feist SW, Goodsir F, Reynolds WJ, Thain JE. 2004. DNA adduct analysis and histopathological biomarkers in European flounder (*Platichthys flesus*) sampled from UK estuaries. Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis 552(1-2):177-186.

Ma Q. 2007. Aryl hydrocarbon receptor degradation-promoting factor (ADPF) and the control of the xenobiotic response. Molecular Interventions 7(3):133-137.

Machella N, Regoli F, Santella RM. 2005. Immunofluorescent detection of 8-oxo-dG and PAH bulky adducts in fish liver and mussel digestive gland. Aquatic Toxicology 71(4):335-343.

Malmström CM, Miettinen S, Bylund G. 2000. DNA adducts in liver and leukocytes of flounder (*Platichthys flesus*) experimentally exposed to benzo[a]pyrene. Aquatic Toxicology 48(2-3):177-184.

Malumbres M, Barbacid M. 2003. *ras* oncogenes: the first 30 years. Nature Reviews Cancer 3(6):459-65.

Marchand J, Tanguy A, Laroche J, Quiniou L, Moraga D. 2003. Responses of European flounder *Platichthys flesus* populations to contamination in different estuaries along the Atlantic coast of France. Marine Ecology Progress Series 260:273-284.

Maria VL, Correia AC, Santos MA. 2002. *Anguilla anguilla* L. biochemical and genotoxic responses to benzo[a]pyrene. Ecotoxicology and Environmental Safety 53(1):86-92.

Maria VL, Teles M, Pacheco M, Correia AC, Santos MA. 2004. Biomarker responses in a polluted river: Effects of pulp and paper mill contaminants on caged *Anguilla anguilla* L. Fresenius Environmental Bulletin 13(4):317-325.

Marie C, Maître A, Douki T, Gateau M, Tarantini A, Guiraud P, Favier A, Ravanat JL. 2008. Influence of the metabolic properties of human cells on the kinetic of formation of the major benzo[a]pyrene DNA adducts. Journal of Applied Toxicology 28(5):579-590.

Mateu E, Sánchez F, Nájera C, Beneyto M, Castell V, Hernández M, Serra I, Prieto F. 1997. Genetics of retinoblastoma: A study. Cancer Genetics and Cytogenetics 95(1):40-50.

McMahon G, Huber LJ, Moore MJ, Stegeman JJ, Wogan GN. 1990. Mutations in c-Ki-*ras* oncogenes in diseased livers of winter flounder from Boston Harbor. Proceedings of the National Academy of Sciences of the United States of America 87(2):841-845.

Melendez-Colon V, Luch A, Seidel A, Baird W. 1999. Cancer initiation by polycyclic aromatic hydrocarbons results from formation of stable DNA adducts rather than apurinic sites. Carcinogenesis 20(10):1885-1891.

Miller III MC, Mohrenweiser HW, Bell DA. 2001. Genetic variability in susceptibility and response to toxicants. Toxicology Letters 120(1-3):269-280.

Miranda CL, Henderson MC, Williams DE, Buhler DR. 1997. In vitro metabolism of 7,12-dimethylbenz[a]anthracene by rainbow trout liver microsomes and trout P450 isoforms. Toxicology and Applied Pharmacology 142(1):123-132.

Miranda CL, Chung WG, Wang-Buhler JL, Musafia-Jeknic T, Baird WM, Buhler DR. 2006. Comparative in vitro metabolism of benzo[a]pyrene by

recombinant zebrafish CYP1A and liver microsomes from β-naphthoflavone-treated rainbow trout. Aquatic Toxicology 80(2):101-108.

Mitchelmore C, Chipman J. 1998. DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. Mutation Research 399(2):135-47.

Moustacchi E. 2000. DNA damage and repair: Consequences on dose-responses. Mutation Research, Genetic Toxicology and Environmental Mutagenesis 464(1):35-40.

Myers MS, Johnson LL, Hom T, Collier TK, Stein JE, Varanasi U. 1998a. Toxicopathic hepatic lesions in subadult English sole (*Pleuronectes vetulus*) from Puget Sound, Washington, USA: Relationships with other biomarkers of contaminant exposure. Marine Environmental Research 45(1):47-67.

Myers MS, Johnson LL, Olson OP, Stehr CM, Horness BH, Collier TK, McCain BB. 1998b. Toxicopathic hepatic lesions as biomarkers of chemical contaminant exposure and effects in marine bottomfish species from the Northeast and Pacific Coasts, USA. Marine Pollution Bulletin 37(1-2):92-113.

Myers MS, Johnson LL, Collier TK. 2003. Establishing the causal relationship between polycyclic aromatic hydrocarbon (PAH) exposure and hepatic neoplasms and neoplasia-related liver lesions in English sole (*Pleuronectes vetulus*). Human and Ecological Risk Assessment 9(1):67-94.

Niedernhofer LJ, Odijk H, Budzowska M, Van Drunen E, Maas A, Theil AF, De Wit J, Jaspers NGJ, Beverloo HB, Hoeijmakers JHJ and others. 2004. The structure-specific endonuclease Ercc1-Xpf is required to resolve DNA interstrand cross-link-induced double-strand breaks. Molecular and Cellular Biology 24(13):5776-5787.

Nogueira PR, Lourenco J, Mendo S, Rotchell JM. 2006. Mutation analysis of *ras* gene in the liver of European eel (*Anguilla anguilla* L.) exposed to benzo[a]pyrene. Marine Pollution Bulletin 52(12):1611-1616.

Notch EG, Miniutti DM, Mayer GD. 2007. 17α-Ethinylestradiol decreases expression of multiple hepatic nucleotide excision repair genes in zebrafish (*Danio rerio*). Aquatic Toxicology 84(3):301-309.

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

Ortiz-Delgado JB, Segner H, Sarasquete C. 2005. Cellular distribution and induction of CYP1A following exposure of gilthead seabream, *Sparus aurata*, to waterborne and dietary benzo(a)pyrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin: an immunohistochemical approach. Aquatic Toxicology 75(2):144-161.

Ortiz-Delgado JB, Behrens A, Segner H, Sarasquete C. 2008. Tissue-specific induction of EROD activity and CYP1A protein in *Sparus aurata* exposed to B(a)P and TCDD. Ecotoxicology and Environmental Safety 69(1):80-88.

Pacheco M, Santos MA. 1999. Biochemical and genotoxic responses of adult Eel (*Anguilla anguilla* L.) to resin acids and pulp mill effluent: laboratory and field experiments. Ecotoxicology and Environmental Safety 42(1):81-93.

Pacheco M, Santos MA. 2001. Tissue distribution and temperature-dependence of *Anguilla anguilla* L. EROD activity following exposure to model inducers and relationship with plasma cortisol, lactate and glucose levels. Environment International 26(3):149-155.

Pacheco M, Santos MA. 2002. Biotransformation, genotoxic, and histopathological effects of environmental contaminants in European eel (*Anguilla anguilla* L.). Ecotoxicology and Environmental Safety 53(3):331-347.

Pacheco M, Santos MA, Teles M, Oliveira M, Rebelo JE, Pombo L. 2005. Biotransformation and genotoxic biomarkers in mullet species (*Liza* sp.) from a contaminated coastal lagoon (Ria de Aveiro, Portugal). Environmental Monitoring and Assessment 107(1-3):133-153.

Park JH, Troxel AB, Harvey RG, Penning TM. 2006. Polycyclic aromatic hydrocarbon (PAH) o-quinones produced by the Aldo-Keto-Reductases (AKRs) generate abasic sites, oxidized pyrimidines, and 8-Oxo-dGuo via reactive oxygen species. Chemical Research in Toxicology 19(5):719-728.

Parker AE, Van De Weyer I, Laus MC, Oostveen I, Yon J, Verhasselt P, Luyten WHML. 1998. A human homologue of the *Schizosaccharomyces pombe* rad1+ checkpoint gene encodes an exonuclease. Journal of Biological Chemistry 273(29):18332-18339.

Peck-Miller K, Myers M, Collier T, Stein J. 1998. Complete cDNA sequence of the Ki-*ras* proto-oncogene in the liver of wild English sole (*Pleuronectes vetulus)* and mutation analysis of hepatic neoplasms and other toxicopathic liver lesions. Molecular Carcinogenesis 23(4):207-16.

Peixoto F, Alves-Fernandes D, Santos D, Fontainhas-Fernandes A. 2006. Toxicological effects of oxyfluorfen on oxidative stress enzymes in tilapia *Oreochromis niloticus*. Pesticide Biochemistry and Physiology 85(2):91-96.

Rato M, Sousa A, Quinta R, Langston W, Barroso C. 2006. Assessment of inshore/offshore tributyltin pollution gradients in the northwest Portugal continental shelf using *Nassarius reticulatus* as a bioindicator. Environmental Toxicology and Chemistry 25(12):3213-3220.

Rebelo J. 1992. The ichthyofauna and abiotic hydrological environment of the Ria de Aveiro, Portugal. Estuaries and Coasts 15(3):403-413.

Rees CB, McCormick SD, Vanden Heuvel JP, Li W. 2003. Quantitative PCR analysis of CYP1A induction in Atlantic salmon (*Salmo salar*). Aquatic Toxicology 62(1):67-78.

Regoli F, Winston GW, Gorbi S, Frenzilli G, Nigro M, Corsi I, Focardi S. 2003. Integrating enzymatic responses to organic chemical exposure with total oxyradical absorbing capacity and DNA damage in the European eel *Anguilla anguilla*. Environmental Toxicology and Chemistry 22(9):2120-2129.

Reichert WL, Myers MS, Peck-Miller K, French B, Anulacion BF, Collier TK, Stein JE, Varanasi U. 1998. Molecular epizootiology of genotoxic events in marine fish: Linking contaminant exposure, DNA damage, and tissue-level alterations. Mutation Research, Reviews in Mutation Research 411(3):215-225.

Reynaud S, Deschaux P. 2006. The effects of polycyclic aromatic hydrocarbons on the immune system of fish: a review. Aquatic Toxicology 77(2):229-238.

Rotchell JM, Blair JB, Shim JK, Hawkins WE, Ostrander GK. 2001a. Cloning of the Retinoblastoma cDNA from the Japanese medaka (*Oryzias latipes*) and preliminary evidence of mutational alterations in chemically-induced retinoblastomas. Gene 263(1-2):231-237.

Rotchell JM, Lee JS, Chipman JK, Ostrander GK. 2001b. Structure, expression and activation of fish *ras* genes. Aquatic Toxicology 55(1-2):1-21.

Rotchell JM, Ulnal E, van Beneden RJ, Ostrander GK. 2001c. Retinoblastoma gene mutations in chemically induced liver tumor samples of Japanese medaka (*Oryzias latipes*). Marine Biotechnology 3:S44-S49.

Rotchell JM, du Corbier FA, Stentiford GD, Lyons BP, Liddle AR, Ostrander GK. 2009. A novel population health approach: using fish retinoblastoma gene profiles as a surrogate for humans. Comparative Biochemistry and Physiology Part C Toxicology & Pharmacology 149(2):134-140.

Roy N, Stabile J, Habicht C, Seeb J, Wirgin I. 1999. High frequency of K-*ras* mutations in Pink Salmon embryos experimentally exposed to Exxon Valdez oil. Environmental Toxicology and Chemistry 18(7):1521-1528.

Ruddock P, Bird D, McEvoy J, Peters L. 2003. Bile metabolites of polycyclic aromatic hydrocarbons (PAHs) in European eels *Anguilla anguilla* from United Kingdom estuaries. Science of the Total Environment 301:105-117.

Sandheinrich MB, Miller KM. 2006. Effects of dietary methylmercury on reproductive behavior of fathead minnows (*Pimephales promelas*). Environmental Toxicology and Chemistry 25(11):3053-3057.

Sarasquete C, Segner H. 2000. Cytochrome P4501A (CYP1A) in teleostean fishes: a review of immunohistochemical studies. Science of the Total Environment 247:313-332.

Sauerborn R, Polancec DS, Zaja R, Smital T. 2004. Identification of the multidrug resistance-associated protein (mrp) related gene in red mullet (*Mullus barbatus*). Marine Environmental Research 58(2-5):199-204.

Schulte PM. 2001. Environmental adaptations as windows on molecular evolution. Comparative Biochemistry and Physiology Part B Biochemistry & Molecular Biology 128(3):597-611.

Sheader DL, Gensberg K, Lyons BP, Chipman K. 2004. Isolation of differentially expressed genes from contaminant exposed European flounder by suppressive, subtractive hybridisation. Marine Environmental Research 58(2-5):553-557.

Shen YM, Troxel AB, Vedantam S, Penning TM, Field J. 2006. Comparison of *p53* mutations induced by PAH o-quinones with those caused by anti-benzo[a]pyrene diol epoxide in vitro: role of reactive oxygen and biological selection. Chemical Research in Toxicology 19(11):1441-1450.

Shugart LR. 2000. DNA damage as a biomarker of exposure. Ecotoxicology 9(5):329-340.

Simonato JD, Guedes CLB, Martinez CBR. 2008. Biochemical, physiological, and histological changes in the neotropical fish *Prochilodus lineatus* exposed to diesel oil. Ecotoxicology and Environmental Safety 69(1):112-120.

Siroka Z, Drastichova J. 2004. Biochemical markers of aquatic environment contamination - Cytochrome P450 in fish. A review. Acta Veterinaria Brno 73(1):123-132.

Sonak S. 2009. Implications of organotins in the marine environment and their prohibition. Journal of Environmental Management 90(Supplement 1):S1-S3.

Sousa A, Matsudaira C, Takahashi S, Tanabe S, Barroso C. 2007. Integrative assessment of organotin contamination in a southern European estuarine system (Ria de Aveiro, NW Portugal): tracking temporal trends in order to evaluate the effectiveness of the EU ban. Marine Pollution Bulletin 54(10):1645-1653.

Spitsbergen JM, Tsai HW, Reddy A, Miller T, Arbogast D, Hendricks JD, Bailey GS. 2000. Neoplasia in zebrafish (*Danio rerio*) treated with 7,12-dimethylbenz[a]anthracene by two exposure routes at different developmental stages. Toxicologic Pathology 28(5):705-715.

Stegeman J, Lech J. 1991. Cytochrome P-450 monooxygenase systems in aquatic species: carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. Environmental Health Perspectives 90:101-9.

Stehr CM, Myers MS, Johnson LL, Spencer S, Stein JE. 2004. Toxicopathic liver lesions in English sole and chemical contaminant exposure in Vancouver Harbour, Canada. Marine Environmental Research 57(1-2):55-74.

Steiner S, Anderson NL. 2000. Expression profiling in toxicology - potentials and limitations. Toxicology Letters 112-113:467-471.

Stentiford GD, Longshaw M, Lyons BP, Jones G, Green M, Feist SW. 2003. Histopathological biomarkers in estuarine fish species for the assessment of biological effects of contaminants. Marine Environmental Research 55(2):137-159.

Stien X, Percic P, Gnassia-Barelli M, Roméo M, Lafaurie M. 1998. Evaluation of biomarkers in caged fishes and mussels to assess the quality of waters in a bay of the NW Mediterranean Sea. Environmental Pollution 99(3):339-345.

Sueiro R, Jenkins G, Lyons B, Harvey J, Parry J. 2000. Genotypic mutation analysis in the *p53* gene of benzo[a]pyrene-treated European flounder (*Platichthys flesus*). Mutation Research 468(1):63-71.

Sutter TR, Greenlee WF. 1992. Classification of members of the Ah gene battery. Chemosphere 25(1-2):223-226.

Sweet LI, Zelikoff JT. 2001. Toxicology and immunotoxicology of mercury: a comparative review in fish and humans. Journal of Toxicology and Environmental Health Part B 4(2):161 - 205.

Teh SJ, Adams SM, Hinton DE. 1997. Histopathologic biomarkers in feral freshwater fish populations exposed to different types of contaminant stress. Aquatic Toxicology 37(1):51-70.

Teles M, Gravato C, Pacheco M, Santos MA. 2004a. Juvenile sea bass biotransformation, genotoxic and endocrine responses to β-naphthoflavone, 4-nonylphenol and 17β-estradiol individual and combined exposures. Chemosphere 57(2):147-158.

Teles M, Santos MA, Pacheco M. 2004b. Responses of European eel (*Anguilla anguilla* L.) in two polluted environments: In situ experiments. Ecotoxicology and Environmental Safety 58(3):373-378.

Theodorakis CW, Swartz CD, Rogers WJ, Bickham JW, Donnelly KC, Adams SM. 2000. Relationship between genotoxicity, mutagenicity, and fish community structure in a contaminated stream. Journal of Aquatic Ecosystem Stress and Recovery 7(2):131-143.

Thomaidis N, Stasinakis A, Gatidou G, Morabito R, Massanisso P, Lekkas T. 2007. Occurrence of organotin compounds in the aquatic environment of Greece. Water Air and Soil Pollution 181(1):201-210.

Thompson G, Swain J, Kay M, Forster CF. 2001. The treatment of pulp and paper mill effluent: a review. Bioresource Technology 77(3):275-286.

van der Oost R, Beyer J, Vermeulen NPE. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology 13(2):57-149.

Vinagre C, Ferreira T, Matos L, Costa MJ, Cabral HN. 2009. Latitudinal gradients in growth and spawning of sea bass, *Dicentrarchus labrax*, and their relationship with temperature and photoperiod. Estuarine Coastal and Shelf Science 81(3):375-380.

Vincent F, de Boer J, Pfohl-Leszkowicz A, Cherrel Y, Galgani F. 1998. Two cases of *ras* mutation associated with liver hyperplasia in dragonets (*Callionymus lyra*) exposed to polychlorinated biphenyls and polycyclic aromatic hydrocarbons. Molecular Carcinogenesis 21(2):121-7.

Wang CG, Zhao Y, Zheng RH, Ding X, Wei W, Zuo ZH, Chen YX. 2006. Effects of tributyltin, benzo[a]pyrene, and their mixture on antioxidant defense systems in *Sebastiscus marmoratus*. Ecotoxicology and Environmental Safety 65(3):381-387.

Wang J, Wei Y, Li X, Xu M, Dai J. 2007. Identification of differentially expressed genes from contaminant and thermal exposed goldfish *Carassius auratus* in Gaobeidian Lake in Beijing, China. Ecotoxicology 16(7):525-532.

Weimer TL, Reddy AP, Harttig U, Alexander D, Stamm SC, Miller MR, Baird W, Hendricks J, Bailey G. 2000. Influence of β-naphthoflavone on 7,12-dimethylbenz(a)anthracene metabolism, DNA adduction, and tumorigenicity in rainbow trout. Toxicological Sciences 57(2):217-228.

Weinberg RA. 1995. The retinoblastoma protein and cell cycle control. Cell 81(3):323-330.

Williams TD, Gensberg K, Minchin SD, Chipman JK. 2003. A DNA expression array to detect toxic stress response in European flounder (*Platichthys flesus*). Aquatic Toxicology 65(2):141-157.

Wirgin I, Waldman J. 1998. Altered gene expression and genetic damage in North American fish populations. Mutation Research 399:193-219.

Wu X, Roth JA, Zhao H, Luo S, Zheng YL, Chiang S, Spitz MR. 2005. Cell cycle checkpoints, DNA damage/repair, and lung cancer risk. Cancer Research 65(1):349-357.

Wu Y, Zacal NJ, Rainbow AJ, Zhu X-D. 2007. XPF with mutations in its conserved nuclease domain is defective in DNA repair but functions in TRF2-mediated telomere shortening. DNA Repair 6(2):157-166.

Xue W, Warshawsky D. 2005. Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: a review. Toxicology and Applied Pharmacology 206(1):73-93.

Zaja R, Munić V, Klobučar RS, Ambriović-Ristov A, Smital T. 2008. Cloning and molecular characterization of apical efflux transporters (ABCB1, ABCB11 and ABCC2) in rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Aquatic Toxicology 90(4):322-332.

Zhang X, Xu HJ, Murakami Y, Sachse R, Yashima K, Hirohashi S, Hu SX, Benedict WF, Sekiya T. 1994. Deletions of chromosome 13q, mutations in retinoblastoma 1, and retinoblastoma protein state in human hepatocellular carcinoma. Cancer Research 54(15):4177-4182.

Zheng L, Lee WH. 2001. The retinoblastoma gene: a prototypic and multifunctional tumor suppressor. Experimental Cell Research 264(1):2-18.

2.

Mutation analysis of ras gene in the liver of European eel (Anguilla anguilla L.) exposed to benzo[a]pyrene

---

Chapter published as original article:

Nogueira P, Lourenço J, Mendo S and Rotchell JM. 2006. Mutation analysis of *ras* gene in the liver of European eel (*Anguilla anguilla* L.) exposed to benzo[a]pyrene. Marine Pollution Bulletin 52: 1611-1616

2.1. ABSTRACT

ras is regarded as one of the most important genes involved in carcinogenesis. Such genes have been characterised in several fish species and the presence of *ras* mutations has already been described in fish populations from hydrocarbon contaminated areas and following experimental exposure to specific contaminants. The aims of this study were to evaluate the DNA integrity by comet assay, to isolate the normal *ras* gene of *Anguilla anguilla* and analyse for the presence of *ras* gene mutations or changes in gene expression levels induced after one month of benzo[a]pyrene (BaP) experimental exposure. The *A. anguilla ras* cDNA isolated revealed a 189 amino acid protein and alignment with other vertebrate Ras proteins revealed conservation of functionally important regions. Following experimental exposure to BaP, an increase in DNA damage was found by comet assay. However, no point mutations or changes in *ras* gene expression levels were detected when compared to control samples. In contrast to the majority of fish *ras* gene sequences, a high degree of polymorphic variation was detected in the *A. anguilla ras* gene.

Keywords

ras, mutation, *Anguilla anguilla*

2.2. INTRODUCTION

The *ras* oncogene is regarded as one of the most important genes involved in multistep carcinogenesis. A large proportion and a wide variety of mammalian tumours possess mutant forms of *ras*. In such cases, the location of *ras* mutations has been restricted to exons 1 and 2, and to codons 12, 13 and 61, and are observed in both spontaneous and chemically-induced tumours in a variety of species. These transforming products are defective in GTPase activity and thus lead to constitutive, deregulated activation of Ras function (Bos 1989).

ras genes have been characterised in several fish species (for review see Rotchell *et al.* 2001) and they all have a high degree of nucleotide sequence and deduced amino acid similarity with the mammalian *ras* gene counterparts. Several studies have described the presence of *ras* gene mutations in fish populations from areas of high hydrocarbon contamination (Roy *et al.* 1999; Vincent *et al.* 1998).

The loss of DNA integrity, such as oncogenes mutations, can be induced by various contaminants that are capable of interacting with the DNA and cause genotoxic effects. Among the different techniques used to detect genotoxic damage, the comet assay (or single cell gel electrophoresis) is a simple, rapid and sensitive method able to detect strand breaks at single cell level (Hartmann *et al.* 2003). The analysis of the individual cell, the use of small cell samples and the applicability to virtually any eukaryotic cell population constitute the main advantages of this technique. The comet assay has already revealed useful for monitoring of DNA damage in laboratory and field studies with various fish species (Akcha *et al.* 2003; Frenzilli *et al.* 2004; Jha 2004).

A. anguilla is widely distributed in estuaries and freshwater courses and, despite its description as a bottom dwelling fish, can move throughout the entire water column being thus a versatile indicator species with a high economic value, that is easy to capture and to maintain in a laboratory (Costa *et al.* 1992). However, since the 1980s, a decline in *A. anguilla* and *A. rostrata* populations have been recorded. Because of their unusual life cycle, they are considered particularly susceptible to pollutant exposure and this is postulated as one of the causes for their population decline (Robinet and Feunteun 2002).

Experimental exposure of *A. anguilla* to the carcinogenic benzo[a]pyrene (BaP) have been conducted previously and the genotoxic potential of this compound demonstrated. A decrease in blood and liver DNA integrity and an increase in the frequency of erythrocytic nuclear abnormalities have been reported in *A. anguilla* following exposure to BaP (Maria *et al.* 2002). Nigro *et al.* (2002) also observed elevated DNA damage, as measured by comet assay, and a significant induction of apoptosis after exposure to a relatively high dose of BaP (50 mg/kg). A field short-term exposure of *A. anguilla* to harbour waters from Aveiro Lagoon, Portugal, was carried out by Maria and co-workers (2003) and revealed genotoxic and/or progenotoxic compounds among harbour water contaminants, which induced early genotoxicity as DNA strand breaks in *A. anguilla* blood cells and a delayed genotoxicity in liver and kidney.

Here, we isolate the normal *ras* gene of *A. anguilla* and analyse for liver *ras* mutations caused by BaP experimental exposure. There are currently few studies reporting liver tumours in *A. anguilla* (Nakatsuru *et al.* 2000; Oliveira Ribeiro *et al.* 2005). Nevertheless, experimental exposures have detected evidence of DNA damage occurring in *A. anguilla* (Maria *et al.* 2003; Pacheco and Santos 2002) and this suggests that mutations in key genes, such as *ras*, may be involved in the aetiology of such abnormalities observed as a result of carcinogen exposure.

2.3. MATERIALS AND METHODS

2.3.1. Experimental exposure

A. anguilla with an average weight of 50 g were collected in the Aveiro Lagoon (Portugal), from a clean site. The fish were transported and acclimated to laboratory conditions in aerated, filtered, and dechlorinated tap water in 200 l aquaria for 1 week, at 20 ºC, under a natural photoperiod (light/dark hours = 12). Fish were exposed to 0 (control), 0.1 µM and 0.3 µM BaP over a period of 4 weeks. BaP (Sigma Chemical Co.) was dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Co.) and added to the aquaria water. The DMSO concentration in water (0.0033 %) has no detectable cytogenetic or toxic effect. The same DMSO level was maintained in the control aquarium. Two thirds of the test solution in each aquarium was renewed weekly. Five fish were sacrificed every week and

their blood collected in microcentrifuge tubes containing 10 µl of heparin. The livers were excised and immediately frozen in liquid nitrogen for storage at –80 ºC.

2.3.2. Evaluation of DNA integrity by comet assay

The comet assay was conducted with eel whole blood under yellow light to prevent UV induced DNA damage and performed with slight modifications on the method described by Speit and Hartmann (2005). In general, aliquots of 5 µl of 30 times diluted blood mixed with 0.5 % (w/v) low melting point agarose (LMPA) were placed on 0.8 % (w/v) normal melting point agarose (NMPA) precoated microscope slides. After polymerization of agarose at 4 ºC, a final layer of 0.8 % (w/v) LMPA was added and left to solidify at 4 ºC. The slides were immersed into a freshly made lysing solution (Speit and Hartmann 2005) for at least 1 h at 4 ºC in the dark. The slides were then placed on a horizontal electrophoresis tray previously filled with freshly prepared cold alkaline buffer and left for 15 min to allow DNA unwinding. Electrophoresis was performed at 0.7 V/cm and 300 mA for 10 min. After electrophoresis, the slides were neutralised in ice cold Tris (0.4 M, pH 7.5) for 3 × 5 min, rinsed with ice cold distilled water and drained. One hundred microliters of ethidium bromide solution (20 µg/ml) was added and overlaid with a coverslip. Slides were placed in a humid dark box at 4 ºC until analysis. A LEICA DMLS fluorescence microscope (400× magnification) was used for slide analysis. Fifty cells per individual were randomly scored using a public domain NIH-Image program (Helma and Uhl 2000). The tail moment (tail length × DNA density) and the percentage of DNA in tail were the primary measurements used to quantify DNA damage. For statistical analysis all data were tested for normality and homogeneity of variances and, when these assumptions were met, the data were compared using ANOVA (SigmaStat 2.0 Software). If necessary, raw data were mathematically transformed to normalise distribution of the measured parameter. Each time an effect was found to be significant ($P < 0.05$), a Tukey test was performed (SigmaStat 2.0 Software).

2.3.3. Isolation of total RNA and RT-PCR

RNA extractions were carried out with RNeasy reagents (Qiagen Ltd., UK). First strand cDNA was obtained using 1 µg of total RNA and oligo d(T) primers. cDNA was used as template to amplify exon 1 and part of exon 2 of the *A. anguilla ras* gene. A 50 µl PCR reaction was performed in reaction buffer (200 mM Tris-HCl (pH 8.4) and 500 mM KCl), 400 µM each deoxynucleoside triphosphate, 50 pmol each primer (Ras1F 5'GAATATAAGCTGGTGGTG3'/ Ras2R 5'CCTGTCCTCATGTACTG3'), 3 µl of synthesized cDNA and 1 U platinum Taq DNA polymerase (Invitroge, UK). The 224 bp *ras* fragment was amplified in a BioRad iCycler^{TM} using 35 sequential cycles at 94 ºC for 40 s, 52 ºC for 40 s, 72 ºC for 50 s, followed by a final 2 min extension at 72 ºC. The sequence obtained from the cloned *ras* fragment subsequently served as a starting point for 3' RACE primer design.

2.3.4. RACE isolation of 3' end *ras* cDNA

The mRNA was purified from one control *A. anguilla* liver total RNA (1 µg) using SMART^{TM} RACE cDNA amplification reagents and protocol (BD Biosciences Clontech, UK). The 3' end of the *A. anguilla ras* gene was obtained using a gene specific primer: 5'AGACGTGCCTGCTGGACATCCTGGACAC3'. Amplification was performed in 50 µl reactions using a BioRad iCycler^{TM} for 5 cycles at 94 ºC for 30 s, 72 ºC for 3 min, 5 cycles at 94 ºC for 30 s, 70 ºC for 30 s, 72 ºC for 3 min, followed by 25 cycles at 94 ºC for 30 s, 68 ºC for 30 s, 72 ºC for 3 min. The RACE products obtained were analysed on an agarose gel, excised and purified using a Qiaquick spin column (Qiagen, UK). Purified cDNA was ligated into a TA cloning vector (Invitrogen, UK). Recombinant plasmids were transformed, and selected using kanamycin LB plates. Plasmid DNA was purified for DNA sequence analysis using commercial sequencing (MWG, Germany) to verify the identity of the product.

2.3.5. *ras* gene mutation analysis

cDNA from control and BaP-exposed samples were used as template to amplify exons 1 and 2 of the *A. anguilla ras* gene. For each reaction, 1 µl of template cDNA was used in 50 µl reaction mixture containing 400 µM each deoxynucleoside triphosphate, 50 pmol each primer (Ras1F2 5'ATGACCGAGTACAAGCTTGT3' and Ras2R2 5'GTTGATGGCGAAGACGCA3'), and 1 U Taq DNA polymerase (Invitrogen, UK) in reaction buffer (200 mM Tris-HCl (pH 8.4) and 500 mM KCl). After a 2 min denaturation step at 94 ºC, 35 cycles of denaturation at 94 ºC for 40 s, primer annealing at 55 ºC for 40 s and primer extension at 72 ºC for 30 s were conducted using a BioRad iCycler^{TM}. A final extension step at 72 ºC for 1 min was performed after the last cycle. The 255 bp fragment amplified was directly sequenced (by MWG, Germany) in both directions in order to identify and characterise any mutations present.

2.3.6. Determination of *ras* gene expression levels

Expression levels of *ras* gene were analysed by semi-quantitative RT-PCR. The amount of isolated RNA was measured by UV-spectroscopy at 260 nm and 1 µg of total RNA from each sample was used for the reverse transcription reaction. In order to normalize differences in efficiency during amplification, 28S rRNA primers were used to amplify a 100 bp fragment as internal standard (5'GCCTGAGTCCTTCTGATCGAG3' and 5'TCCCAAACAACCCGACTCCGAG3'). Amplifications were performed with a BioRad iCycler ^{TM} in 50 µl reaction volumes, for 35 sequential cycles at 94 ºC for 40 s, 52 ºC for 40 s and 72 ºC for 50 s, followed by a final 2 min extension at 72 ºC. Ten microliters of each PCR product was taken for agarose gel electrophoresis (1.5 % agarose, TAE buffer).

2.4. RESULTS

2.4.1. Evaluation of DNA integrity by comet assay

The level of DNA damage is revealed by the tail moment defined as the product of tail length and fraction of DNA in the tail (Helma & Uhl, 2000). Figure 2.1 represents the comet assay results for whole blood cells from eels exposed to two doses of BaP for different exposure times up to 1 month. After 14 and 28 days

of experimental exposure, eels showed a significantly increased tail moment ($p < 0.05$) at the highest concentration tested (0.3 µM BaP) when compared to the untreated controls. At the lowest concentration exposure, there was no significant alteration in the levels of DNA damage along the exposure period.

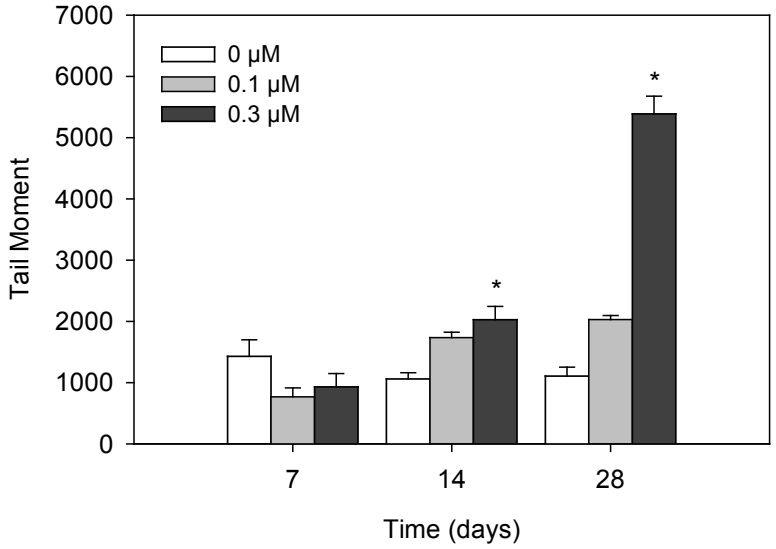

Figure 2.1. DNA damage (based on tail moment) in eel after a 7-, 14- and 28-day exposure to two doses of benzo[a]pyrene. Results are expressed as mean ± SEM (n= 3-5 individual). An asterisk (*) represents significant differences from control (Tukey test, $P < 0.05$).

2.4.2. Isolation of the normal *A. anguilla ras* gene

Primers derived from previously published sequences for several fishes *ras* exons 1 and 2 were used for the amplification of the *ras* oncogene from eel liver cDNA. RT-PCR produced one band of expected size (224 bp). This fragment was sequenced and the obtained sequence was used to design a specific primer and to generate a 636 bp sequence with a 3'RACE reaction. The *A. anguilla ras* cDNA isolated contained a complete open reading frame (GenBank accession no. DQ195224) and the predicted amino acid sequence revealed a 189 amino acid protein. Multiple alignment of the eel *ras* deduced amino acid sequence with Ras proteins from different fish species and with the three human Ras homologues was performed with ClustalW. The analysis revealed a strong homology between the *A. anguilla ras* predicted amino acid sequence and the Ki-Ras homologous proteins. (Figure 2.2). Amplification of *ras* exons 1 and 2 cDNAs from livers of

untreated *A. anguilla* samples produced fragments of 255 bp, which were cloned and sequenced. Several *ras* gene sequence variations were identified, comprising polymorphic substitutions occurring predominantly at the third base position within each codon (Figure 2.3). Two base substitutions were observed in position 1 for codons 41 and 56, though again, these did not result in a change in the encoded amino acid sequence. The remainder of the coding region was identical.

```
A.anguilla          MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCLLDILDTAG 60
C.carpio K-ras      MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCLLDILDTAG 60
D.rerio N-ras       MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCLLDILDTAG 60
P.flesus K-ras a    MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCLLDILDTAG 60
P.flesus K-ras b    MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCLLDILDTAG 60
P.vetulus K-ras b   MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCLLDILDTAG 60
H.sapiens K-ras     MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCLLDILDTAG 60
H.sapiens H-ras     MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCLLDILDTAG 60
H.sapiens N-ras     MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCLLDILDTAG 60
                    ************************************************************

A.anguilla          QEEYSAMRDQYMRTGEGFLCVFAINNTKSFEDIHHYREQIKRVKDSEDVPMVLVGNKCDL 120
C.carpio K-ras      QEEYSAMRDQYMRTGEGFLCVFAINNTKSFEDIHHYREQIKRVKDSEDVPMVLVGNKCDL 120
D.rerio N-ras       QEEYSAMRDQYMRTGEGFLCVFAINNSKSFADVHLYREQIKRVKDSDDVPMVLVGNICDL 120
P.flesus K-ras a    QEEYSAMRDQYMRTGEGFLCVFAINNIKSFEDVHLYREQINRVKDSDSVPMVLVGNKSDL 120
P.flesus K-ras b    QEEYSAMRDQYMRTGEGFLCVFAINNTKSFEDIHHYREQIKRVKDSEDVPMVLVGNKCDL 120
P.vetulus K-ras b   QEEYSAMRDQYMRTGEGFLCVFAINNTKSFEDIHHYREQIKRVKDSEDVPMVLVGNKCDL 120
H.sapiens K-ras     QEEYSAMRDQYMRTGEGFLCVFAINNTKSFEDIHHYREQIKRVKDSEDVPMVLVGNKCDL 120
H.sapiens H-ras     QEEYSAMRDQYMRTGEGFLCVFAINNTKSFEDIHQYREQIKRVKDSDDVPMVLVGNKCDL 120
H.sapiens N-ras     QEEYSAMRDQYMRTGEGFLCVFAINNSKSFADINLYREQIKRVKDSDDVPMVLVGNKCDL 120
                    ************************** *** *:: *****:*****:.******** .**

A.anguilla          PSRTVDTKQAQDLARNYGIPFIETSAKTRQGVDDAFYTLVREIRKHK-EKTSKEGRKKKK 180
C.carpio K-ras      PSRSVDTKQAQDLARSYGIPFIETSAKTRQGVDDAFYTLVREIRKHK-EKMSKEG-KKKK 180
D.rerio N-ras       -ARTVDTKQAQELARSYGIEFVETSAKTRQGVEDAFYTLVREIRHYRMKKLNSREDRKQG 179
P.flesus K-ras a    GTRNVESRQAQELARSYGVPFVETSAKTRQGVEEAFYSLVREIRKYK--ETNRSNKKSKK 180
P.flesus K-ras b    PSRTVDTKQAQDLARSYGIPFIETSAKTRQGVDDAFYTLVREIRKHK-EKMSKEG-KKKK 180
P.vetulus K-ras b   PFRTVDTKQAQDLALSYGIPFIETSAKTRQGVDDAFYTLVREIRKHK-EKMSKEG-KKKK 180
H.sapiens K-ras     PSRTVDTKQAQDLARSYGIPFIETSAKTRQGVDDAFYTLVREIRKHK-EKMSKDG-KKKK 180
H.sapiens H-ras     AARTVESRQAQDLARSYGIPYIETSAKTRQGVEDAFYTLVREIRQHKLRKLNPPDESGPG 180
H.sapiens N-ras     PTRTVDTKQAHELAKSYGIPFIETSAKTRQGVEDAFYTLVREIRQYRMKKLNSSDDGTQG 180
                      *.*::::**::** .**: ::***********::***:******:::    .   .

A.anguilla          KKSKAKCVVM 189
C.carpio K-ras      KKSKTKCVLM 188
D.rerio N-ras       CLG-VSCEVM 188
P.flesus K-ras a    NTQKRRCLIL 188
P.flesus K-ras b    KKSKTKCSLM 188
P.vetulus K-ras b   KKSKTKCSLM 188
H.sapiens K-ras     KKSKTKCVIM 188
H.sapiens H-ras     CMS-CKCVLS 189
H.sapiens N-ras     CMG-LPCVVM 189
                          * :
```

Figure 2.2. Comparison of the deduced *A. anguilla ras* sequence with human and other fish Ras sequences: *Homo sapiens* Ki –Ras (GenBank accession no. 0909262B); *H. sapiens* Ha-Ras (P01112); *H. sapiens* N-Ras (P01111), *Cyprinus carpio* (AAD10839.1), *Danio rerio* (NP_571220.1), *Platichthys flesus* (K-Ras-a: CAA76679.1 and K-Ras-b: CAA76678.1), *Pleuronectes vetulus* (AAC25633.1). Areas showing homology are highlighted with an asterisk and slashes indicate a deletion.

5′exon 1 ATG ACC GAG TAC AAG CTT GTG GTG GTG GGA(G)
GCT GGT(A)12 GGT(C)13 GTG GGG(A) AAG AGT(C) GCG CTG(C)
ACC ATC CAG CTC(G) ATC CAG AAC CAC TTT(C) GTG GAC GAG
TAT(C) GAC CCC ACC ATC GAG
exon 2 GAC TCG TAC(T) A(C)GG AAG CAG GTG GTG ATC GAC
GGC GAG ACG TGC CTG CTG GAC ATC C(T)TG GAC ACT(GA) GCG(A) GGT
CAG61 GAG GAG TAC(T) AGC GCC ATG CGG GAC CAG TAC ATG AGG ACC
GGA(G) GAG GGC TTC CTC TGC GTC TTC GCC ATC AAC 3′

Figure 2.3. Nucleotide sequence of normal *A. anguilla ras* gene from coding nucleotides 1 to 255 with parenthesis showing polymorphic variations.

2.4.3. *ras* gene mutation and expression analysis

BaP-exposed *A. anguilla* liver samples were screened for *ras* mutations by direct sequencing of *ras* cDNA. No point mutations were found at the traditional mutation hot spots, codons 12, 13 and 61, in control or BaP-exposed samples. The analysis of *ras* gene expression levels in the same samples revealed no difference between control and exposed fish (Figure 2.4).

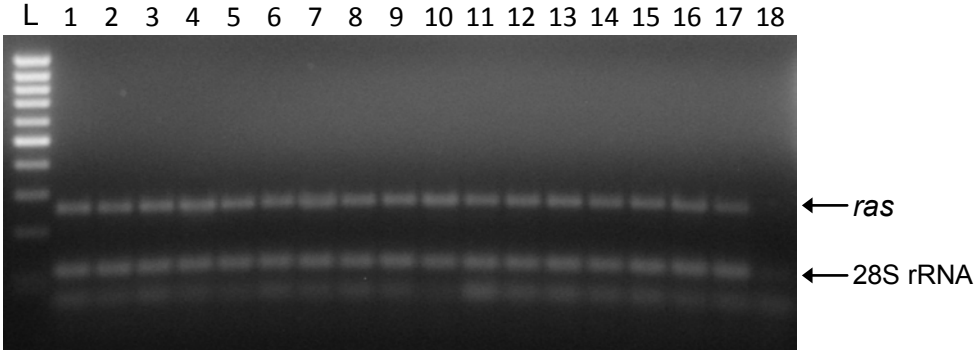

Figure 2.4. RT-PCR amplification of *ras* and 28S rRNA genes from control and 0.3 µM BaP-exposed eel samples. L: molecular ladder (GeneRuler 100bp, MBI Fermentas); 1-2: environmental reference samples; 3-5: control samples; 6-8: 1 week exposure; 9-11: 2 weeks exposure; 12-14: 3 weeks exposure; 15-17: 4 weeks exposure; 18: control without DNA.

2.5. DISCUSSION

Here, we report the cDNA sequence of the *ras* gene in *A. anguilla*. The predicted amino acid sequence of *A. anguilla ras* gene displays conserved structural domains suggesting that the functional role may also be conserved. Direct sequencing of *ras* exons 1 and 2 (255 bp fragment) of different *A. anguilla* control samples revealed several nucleotide variations within this fragment. The variations occurred mainly in the third base position and none of them led to amino acid alteration in the predicted amino acid sequence. *ras* polymorphism has previously been reported in *O. latipes* (Liu *et al.* 2003) and *O. gorbuscha (*Cronin *et al.* 2002). The level of polymorphic variation observed in the *A. anguilla ras* gene is significantly higher and may indicate greater genome instability in this fish species compared with others.

A. anguilla, like many others species of fish that inhabit estuaries and coastal zones, are exposed to urban and industrial pollutants such as PAHs. In fish, PAH biotransformation occurs mainly in the liver, where they are metabolised by phase I enzymes of the mixed function oxygenase system to more hydrophilic products (van der Oost *et al.* 2003). These metabolites are very reactive and can covalently bind to DNA forming bulky, hydrophobic DNA adducts that may result in DNA single strand breaks or gene mutations (Roy *et al.* 1999). *ras* gene mutations in fish have been related with PAH exposure (Fong *et al.* 1993; Vincent *et al.* 1998) and are thought to be an early event in the carcinogenesis process. The identification of a gene that is mutated early in the carcinogenesis process could be used as biomarker of contaminant exposure by mutation analysis before obvious tumour development (Peck-Miller *et al.* 1998).

In previous studies, increases in CYP1A protein levels, EROD activity and PAH metabolites in bile were observed in *A. anguilla* exposed to BaP (Gorbi and Regoli 2004; Maria *et al.* 2002), indicating that this xenobiotic is bioavailable and biotransformed by *A. anguilla*. Furthermore, Maria and co-workers (2002) have demonstrated that the *A. anguilla* liver has the capacity to convert BaP into a mutagenic compound. The ability of BaP to cause DNA damage in exposed eels was evaluated by comet assay. This method detects DNA strand-breaks and alkali-labile sites in DNA by measuring the migration of DNA from immobilized

nuclear DNA (Hartmann *et al.* 2003). Eels exposed at the low experimental BaP dose (0.1 µM) had no significant alteration in DNA damage, as measured by tail moment, when compared to the control eels. On the other hand, a significant increase in DNA damage was found at 0.3 µM BaP after 14 and 28 days of exposure. The results were similar using a calculation of the percentage of DNA in tail (data not shown).

These results suggest that eel DNA may be damaged by BaP or its metabolites. If the potential lesions are not correctly repaired they may lead to the induction of mutations. To complete the cause and effect relationship between PAH exposure and genotoxic damage at the molecular level, the ability of BaP to cause *ras* gene DNA sequence alterations in *A. anguilla* was evaluated in this study. Using experimental exposures of 0.1 and 0.3 µM BaP over a period of one month, no mutations were observed in the *ras* gene mutational hotspot codons 12, 13 and 61. The dose of BaP exposure is one thousand times higher than that proven to induce *ras* gene mutations in mammalian models over a 10 week period (Wang *et al.* 2005). Other researchers have also reported a lack of *ras* mutations in livers of exposed fish. Peck-Miller *et al.* (1998) reported a lack of *ras* gene mutations in hepatic lesions of *Pleuronectes vetulus* from contaminated waterways in Puget Sound. No *ras* gene mutations were detected in *O. gorbuscha* from streams impacted by the *Exxon Valdez* oil spill (Cronin *et al.* 2002). This suggests that some fish species, including *A. anguilla*, may be less susceptible to *ras* gene mutational activation by PAHs than others. A second mechanism of *ras*-implicated carcinogenesis involves overexpression of the gene. RT-PCR analysis revealed no differences in the *ras* gene expression levels of *A. anguilla* exposed to two different concentrations of BaP compared with control samples. The lack of *ras* gene mutations and no apparent change in *ras* gene expression levels following BaP exposure, suggests that hydrocarbon genotoxic end points in *A. anguilla* involve a molecular aetiology that does not involve the *ras* gene. The apparent lack of point mutations in the eel *ras* gene does not rule out other potential detrimental effects. The BaP-induced increase in DNA strand breaks could, for instance, lead to the induction of chromosomal aberrations, which are also associated with initiation and promotion of cancer (Jha 2004). Secondly, induction

of heritable mutations in germ cells could have long term detrimental effects on population survival (Jha 2004).

2.6. REFERENCES

Akcha F, Hubert FV, Pfhol-Leszkowicz A. 2003. Potential value of the comet assay and DNA adduct measurement in dab (*Limanda limanda*) for assessment of in situ exposure to genotoxic compounds. Mutation Research, Genetic Toxicology and Environmental Mutagenesis 534(1-2):21-32.

Bos JL. 1989. ras Oncogenes in human cancer: a review. Cancer Research 49(17):4682-4689.

Costa JL, Assis CA, Almeida PR, Moreira FM, Costa MJ. 1992. On the food of the European eel, *Anguilla anguilla* (L.), in the upper zone of the Tagus Estuary, Portugal. Journal of Fish Biology 41(5):841-850.

Cronin M, Wickliffe J, Dunina Y, Baker R. 2002. K-*ras* oncogene DNA sequences in pink salmon in streams impacted by the Exxon Valdez oil spill: no evidence of oil-induced heritable mutations. Ecotoxicology 11(4):233-41.

Fong A, Dashwood R, Cheng R, Mathews C, Ford B, Hendricks J, Bailey G. 1993. Carcinogenicity, metabolism and Ki-*ras* proto-oncogene activation by 7,12-dimethylbenz[a]anthracene in rainbow trout embryos. Carcinogenesis 14(4):629-35.

Frenzilli G, Scarcelli V, Del Barga I, Nigro M, Forlin L, Bolognesi C, Sturve J. 2004. DNA damage in eelpout (*Zoarces viviparus*) from Goteborg harbour. Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis 552(1-2):187-195.

Gorbi S, Regoli F. 2004. Induction of cytochrome P4501A and biliary PAH metabolites in European eel *Anguilla anguilla*: Seasonal, dose- and time-response variability in field and laboratory conditions. Marine Environmental Research 58(2-5):511-515.

Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V and others. 2003. Recommendations for conducting the in vivo alkaline Comet assay. Mutagenesis 18(1):45-51.

Helma C, Uhl U. 2000. A public domain image-analysis program for the single-cell gel- electrophoresis (comet) assay. Mutation Research, Genetic Toxicology and Environmental Mutagenesis 466(1):9-15.

Jha AN. 2004. Genotoxicological studies in aquatic organisms: an overview. Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis 552(1-2):1-17.

Liu Z, Kullman SW, Bencic DC, Torten M, Hinton DE. 2003. *ras* oncogene mutations in diethylnitrosamine-induced hepatic tumors in medaka (*Oryzias latipes*), a teleost fish. Mutation Research 539:43–53.

Maria VL, Correia AC, Santos MA. 2002. *Anguilla anguilla* L. biochemical and genotoxic responses to benzo[a]pyrene. Ecotoxicology and Environmental Safety 53(1):86-92.

Maria VL, Correia AC, Santos MA. 2003. Genotoxic and biochemical responses in caged eel (*Anguilla anguilla* L.) after short-term exposure to harbour waters. Environment International 29(7):923-9.

Nakatsuru Y, Minami K, Yoshikawa A, Zhu J, Oda H, Masahito P, Okamoto N, Nakamura Y, Ishikawa T. 2000. Eel WT1 sequence and expression in spontaneous nephroblastomas in Japanese eel. Gene 245(2):245-51.

Nigro M, Frenzilli G, Scarcelli V, Gorbi S, Regoli F. 2002. Induction of DNA strand breakage and apoptosis in the eel *Anguilla anguilla*. Marine Environmental Research 54:517-520.

Oliveira Ribeiro CA, Vollaire Y, Sanchez-Chardi A, Roche H. 2005. Bioaccumulation and the effects of organochlorine pesticides, PAH and heavy metals in the Eel (*Anguilla anguilla*) at the Camargue Nature Reserve, France. Aquatic Toxicology 74(1):53-69.

Pacheco M, Santos MA. 2002. Biotransformation, genotoxic, and histopathological effects of environmental contaminants in European eel (*Anguilla anguilla* L.). Ecotoxicology and Environmental Safety 53(3):331-347.

Peck-Miller K, Myers M, Collier T, Stein J. 1998. Complete cDNA sequence of the Ki-*ras* proto-oncogene in the liver of wild English sole (*Pleuronectes vetulus)* and mutation analysis of hepatic neoplasms and other toxicopathic liver lesions. Molecular Carcinogenesis 23(4):207-16.

Robinet T, Feunteun E. 2002. Sublethal effects of exposure to chemical compunds: a cause for the decline in Atlantic Eels? Ecotoxicology 11:265-277.

Rotchell JM, Lee JS, Chipman JK, Ostrander GK. 2001. Structure, expression and activation of fish *ras* genes. Aquatic Toxicology 55(1-2):1-21.

Roy N, Stabile J, Habicht C, Seeb J, Wirgin I. 1999. High frequency of K-*ras* mutations in Pink Salmon embryos experimentally exposed to Exxon Valdez oil. Environmental Toxicology and Chemistry 18(7):1521-1528.

Speit G, Hartmann A. 2005. The comet assay: a sensitive genotoxicity test for the detection of DNA damage. Methods in molecular biology (Clifton, N.J.) 291:85-95.

van der Oost R, Beyer J, Vermeulen NPE. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology 13(2):57-149.

Vincent F, de Boer J, Pfohl-Leszkowicz A, Cherrel Y, Galgani F. 1998. Two cases of *ras* mutation associated with liver hyperplasia in dragonets (*Callionymus lyra*) exposed to polychlorinated biphenyls and polycyclic aromatic hydrocarbons. Molecular Carcinogenesis 21(2):121-7.

Wang Y, Gao D, Atencio DP, Perez E, Saladi R, Moore J, Guevara D, Rosenstein BS, Lebwohl M, Wei H. 2005. Combined subcarcinogenic benzo[a]pyrene and UVA synergistically caused high tumor incidence and mutations in H-ras gene, but not p53, in SKH-1 hairless mouse skin. International Journal of Cancer 116(2):193-199.

3.

A follow-up of late DNA damage in different fish tissues after a benzo[a]pyrene acute exposure

---

Chapter submitted as original article:
Nogueira P, Lourenço J, Loureiro J, Pacheco M, Santos C, Mendo S. 2009.
A follow-up of late DNA damage in different fish tissues after a benzo[a]pyrene acute exposure.
Ecotoxicology and Environmental Safety

3.1. ABSTRACT

The genotoxicity of an acute exposure (24 h) to 0.39 and 3.9 µM benzo[a]pyrene (BaP) was evaluated in the post-exposure period (at regular intervals up to 28-day post-exposure) in different *Anguilla anguilla* tissues. Damage was assessed at DNA and chromosomal levels using the comet assay and flow cytometry, respectively. Liver and blood cells showed higher DNA damage after 24-h exposure, while damage in gill cells was only detected at 1- and 7-day post-exposure. DNA damage was additionally detected at 14- and 28-day post-exposure in liver cells (0.39 µM). Chromosomal damages were only detected in gill and blood cells at 3-day post-exposure. The presence of DNA and chromosomal damages in these important tissues, even after the removal of the toxicity source, suggests the potential of this compound for the disruption of critical functions, leading to detrimental effects at the organism level. Both techniques employed revealed to be very useful in the detection of genetic damage induced by BaP.

Keywords

Anguilla anguilla, comet assay, flow cytometry, genotoxicity, PAH

3.2. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) constitute a large group of organic chemical compounds that can be found in the aquatic environment as a result of spillage and seepage of fossil fuels, discharge of domestic and industrial wastes, atmospheric input and continental runoff (Hartl 2002). PAHs are known cytotoxic, carcinogenic and mutagenic agents (Bihari and Fafandel 2004; Johnson *et al.* 2002) and exposure to these chemicals has already been correlated with the development of neoplasms and other abnormalities in fishes (Jha 2004). Benzo[a]pyrene (BaP), an environmental PAH pollutant frequently used as model toxicological compound, has been shown to induce marked molecular, cellular and carcinogenic effects in fishes (e.g. Maria *et al.* 2002; Regoli *et al.* 2003). As other PAHs, BaP requires metabolic activation in order to produce potentially damaging effects. The biotransformation process occurs mainly in the liver of fishes and results in more water-soluble products facilitating excretion and detoxification. Nevertheless, during the metabolism of PAHs, reactive intermediates are produced, including free radicals, which are known to affect DNA (Jha 2004). The genotoxic effects of BaP have been studied in fishes after water-borne exposure, through diet or after intra-peritoneal injections (Maria *et al.* 2002; Nigro *et al.* 2002). Although detrimental effects of BaP may be observed after a few hours of exposure (Maria *et al.* 2002), the post-exposure period is often considered more critical. For example, DNA breaks and apoptosis were observed in eel erythrocytes, 7 days after exposure to BaP (Nigro *et al.* 2002) and maximal levels of DNA adducts were detected in the blood, liver, kidney and spleen cells of *Fundulus heteroclitus*, 32 days after an injection of BaP, persisting for at least 96 days (Rose *et al.* 2001).

The use of molecular biomarkers as early warning tools may permit intervention before the onset of irreversible damage in the organisms (van der Oost *et al.* 2003). Biomarkers directed to detect DNA and chromosomal damage resulting from the exposure to genotoxic agents have been used in aquatic species (for a review see Jha 2004). The comet assay is a simple and rapid technique to detect DNA damage in cells and a valuable tool for monitoring DNA integrity in laboratory and field studies with fishes (reviewed in Frenzilli *et al.*

2009). Also, as a consequence of structural perturbations to the DNA molecule (such as adducts and strand breaks), permanent lesions, detected as chromosomal aberrations and variations in nuclear DNA amount, are prone to occur. Flow cytometry (FCM) is a valuable tool to analyse chromosomal damage induced by genotoxic agents, having been successfully applied in the evaluation of the effects of environmental pollutants in *Psetta maxima* (Goanvec *et al.* 2008) and *Platichthys flesus* (Marchand *et al.* 2003), as examples.

The aim of this study was to evaluate the long-term late effects on DNA integrity of three different tissues of the European eel (*Anguilla anguilla* L.), following an acute exposure to BaP. *A. anguilla* is a well suited system for toxicological assays in the laboratory, due to its adaptability and ease of handling, together with a high sensitivity to xenobiotics (Maria *et al.* 2002; Regoli *et al.* 2003). Eels metabolize BaP through the cytochrome P450 system (Maria *et al.* 2002), producing reactive metabolites, such as epoxides, that can bind covalently to DNA. In this work we assessed the genotoxic effects of BaP in eels using two methods (comet assay and FCM) in three different tissues: liver, as the principal organ for xenobiotic metabolism; gills, as an organ in close contact with the contaminated water; and blood, as a vehicle for the parent compound and metabolites between organs.

3.3. MATERIALS AND METHODS

3.3.1. Experimental exposure

Juvenile specimens of European eel (*A. anguilla*) with an average weight of 0.2 g (glass eels) were caught at the Mondego River mouth, Figueira da Foz (Portugal). Fish were acclimatized to laboratory conditions for a period of 2 weeks in recirculating, filtered, well aerated and dechlorinated tap water, at 20 ºC, under a 12 h light/12 h darkness photoperiod. Animals were fed three times a week during the acclimation period. Eels were exposed in 40 l aquaria to BaP (Sigma, USA) previously dissolved in dimethyl sulfoxide (DMSO, Sigma, USA). The DMSO concentration in water (0.0033 %) has no detectable cytogenetic or toxic effect in juvenile eels (Pacheco and Santos 2002). The same DMSO volume was added to the control aquarium. Groups of at least 150 animals were exposed to an

initial/nominal concentration of 0.39 and 3.9 µM of BaP for 24 h, and afterwards maintained in clean water and fed as before. Control fish were also transferred to clean water.

Eels were sampled before the exposure (T0), immediately after the exposure and at days 1, 3, 7, 14 and 28 in the post-exposure period. At each sampling date, five fish were killed by decapitation and the blood directly collected from the heart using heparinized capillary tubes, suspended in cold phosphate buffered saline (PBS) 1 X and kept on ice until use. Liver and gills were also collected and kept on cold PBS 1 X/EDTA 100 mM/DMSO 10 % buffer until used for comet assay.

All studies were conducted in accordance with international guidelines for the protection of animal welfare.

3.3.2. Evaluation of DNA integrity by the comet assay

The comet assay was conducted in eel whole blood, liver and gill tissue, under yellow light to prevent UV-induced DNA damage. Liver and gill cell suspensions were obtained by homogenizing the tissues in PBS 1X/EDTA 100 mM/DMSO 10 % buffer to reduce DNase activity and prevent oxidant-induced DNA damage (Hartmann *et al.* 2003). The comet assay protocol was performed as described in Nogueira *et al.* (2006). Slides were examined in a fluorescence microscope (Leica DMLS, Germany). Fifty randomly selected cells per slide (2 slides per animal) were analysed using the image analysis software Comet Assay IV (Perceptive Instruments, UK). DNA damage was expressed as percentage tail DNA (% tDNA) and presented as mean of medians ± standard error of the mean (SEM).

3.3.3. Evaluation of DNA content variation by flow cytometry

Blood samples used in the comet assay were further preserved in citrate buffer (sucrose 250 mM, trisodium citrate 40 mM, pH 7.6) with 10 % DMSO, frozen in liquid nitrogen and stored at -70 ºC, before being used for flow cytometry analysis. Liver and gill cell suspensions were also frozen in liquid nitrogen and stored at -70 ºC.

Frozen samples of blood, liver and gill suspensions were thawed and suspended in a nuclear isolation buffer (LB01, Doležel *et al.* 1989). Isolated nuclei were treated with 50 mg/ml RNase (Fluka, Buchs, Switzerland) and stained with 50 mg/ml propidium iodide (PI, Fluka). Samples were incubated for 10 min on ice before the analysis. The fluorescence intensity of, at least, 5000 nuclei was measured per sample with a Beckman-Coulter EPICS-XL flow cytometer (Hialeah, FL, USA). The instrument was equipped with an air-cooled argon ion laser (15 mW, operating at 488 nm) and PI fluorescence was collected through 645 nm dichroic long-pass and 620 nm band-pass filters. In each sample, the half-peak coefficient of variation (HPCV) of the G_0/G_1 peak of isolated nuclei was registered (SYSTEM II software v. 3.0). This dispersion measurement of nuclear fluorescence has been previously shown to be positively correlated with chromosomal damage, as a result of cell-to-cell variation in nuclear DNA content (Matson *et al.* 2004).

3.3.4. Statistical analyses

For statistical analyses (SigmaStat 3.11 Software), all data were tested for normality and homogeneity of variances. Since an invalidation of normality and variances homogeneity was observed, data were analysed using a non-parametric Kruskal-Wallis ANOVA on ranks, followed by a Mann–Whitney *U* pairwise comparison test to detect which groups were significantly different (SigmaStat 3.11 Software).

3.4. RESULTS

3.4.1. Evaluation of DNA integrity by comet assay

DNA integrity was evaluated in liver, gill and blood cells of *A. anguilla* after acute exposure to two different concentrations of BaP (Figure 3.1). Liver cells showed a significant increase ($P < 0.05$) in DNA damage levels, higher than 5-fold, after 1 day of exposure to both BaP concentrations; however these levels decreased ($P < 0.05$) at 1 day post-exposure. High levels of DNA damage were also observed in hepatic tissue from eels exposed to the lowest concentration (0.39 µM) at days 14 and 28 post-treatment. In gill cells, significantly higher levels

of DNA damage were observed at days 1 ($P < 0.01$) and 7 ($P < 0.05$) of post-exposure to the lowest BaP concentration. Blood cells revealed significantly higher levels of DNA damage after 1 day exposure to BaP ($P < 0.01$). In the post-exposure period, no significant differences in comparison with the control group were observed in the blood cells.

3.4.2. Evaluation of DNA content variation by flow cytometry

The BaP exposure did not induce any major changes in ploidy level (e.g., polyploidy) in any of the tested tissues (data not shown). For putative chromosomal damage evaluation, mean HPCV values of the G_0/G_1 peak were calculated varying between 2.6 and 3.6 % for the liver, 2.7 and 6.6 % in the gill, and 2.2 and 3.9 % in blood cells. No significant differences in HPCV values ($P > 0.05$) were found in liver cells of exposed eels. Gill cells from eels sampled at day 3 of post-exposure to the highest BaP concentration showed a two-fold increase in HPCV, but after the seventh day of post-exposure, these values decreased, being similar to those observed in the control eels (Figure 3.2). Blood cells of eels exposed to 0.39 µM BaP showed a small but significant increase ($P < 0.01$) in HPCV values at day 3 of post-treatment, while those in contact with the highest concentration of BaP presented a small decrease at day 14 of the same period.

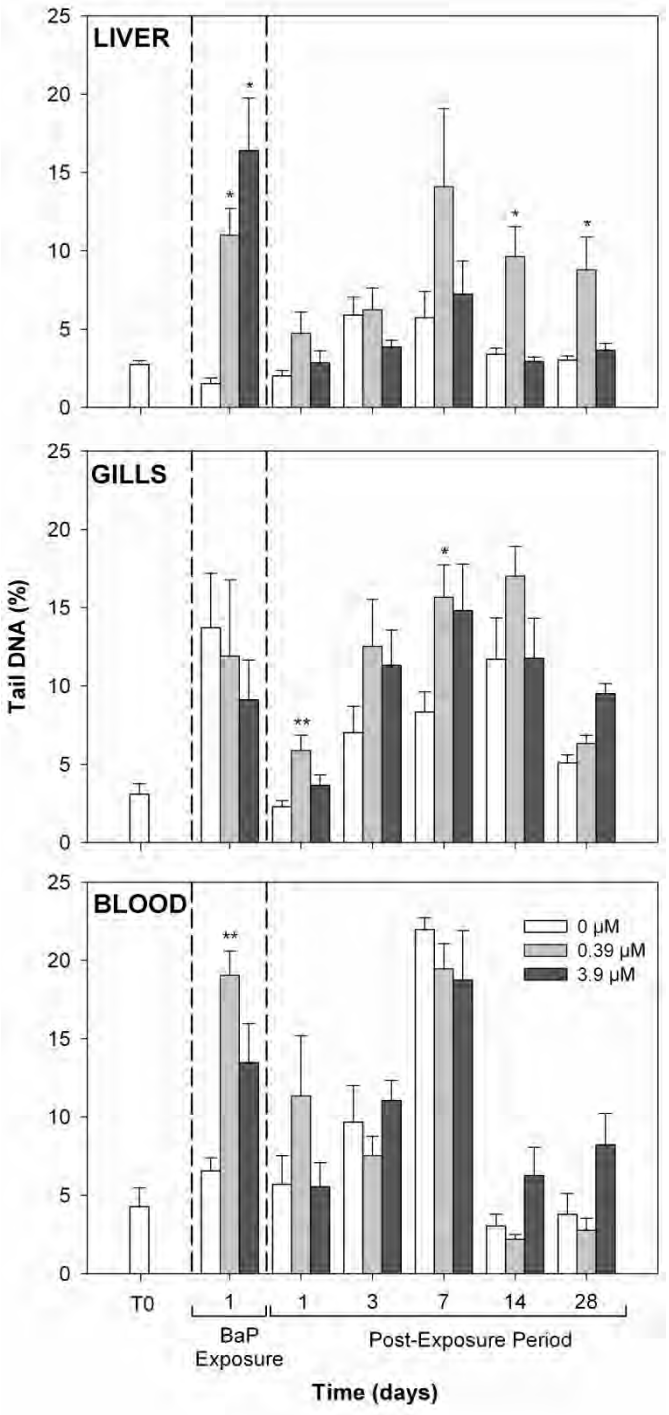

Figure 3.1. DNA damage detected by the comet assay and expressed as tail length (%) in liver, gill and blood cells isolated from European eel (*A. anguilla*) exposed for 1 day to two different concentrations of BaP (0.39 and 3.9 µM) and sampled before the exposure (T0), after 1 day of exposure (sampling time = 1) and at 1, 3, 7, 14 and 28 days post-exposure. Data are expressed as mean of medians ± SEM (n = 5 animals). Asterisks denote statistically significant differences from the control group: * $P < 0.05$; ** $P < 0.01$.

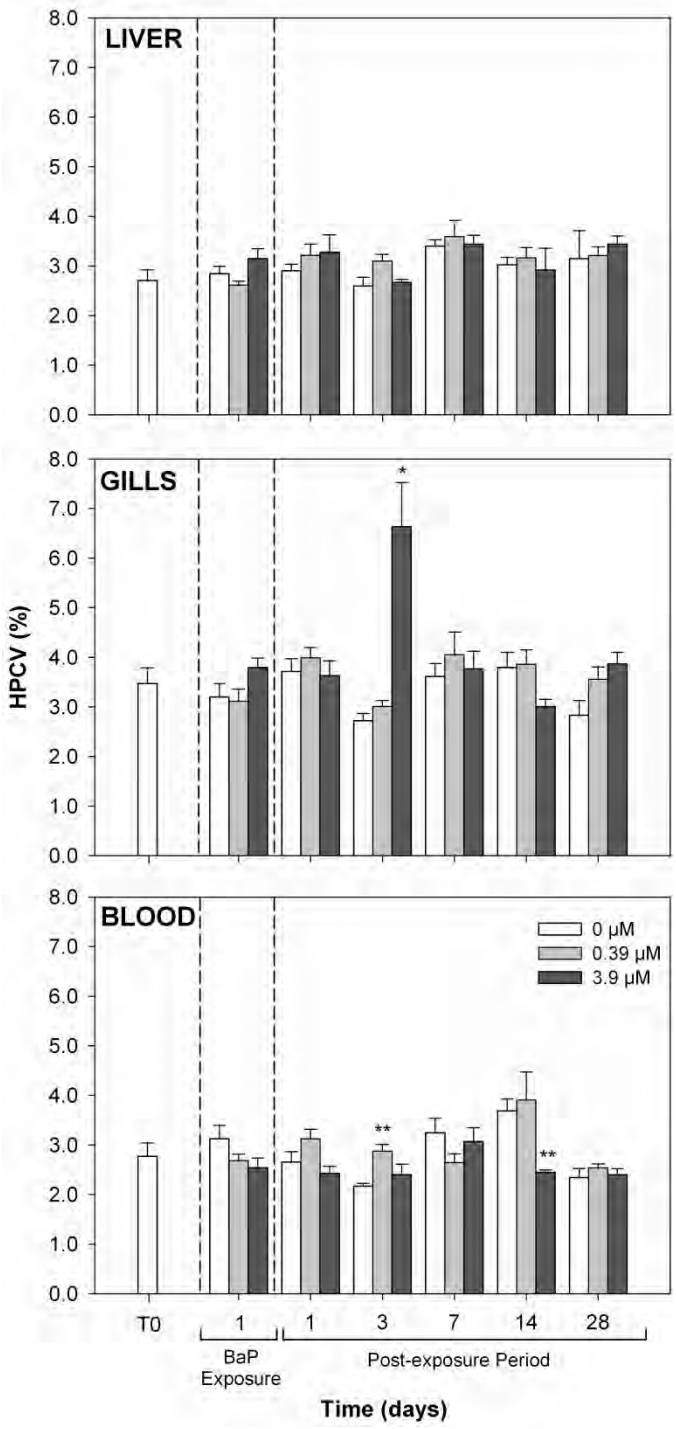

Figure 3.2. Variation in DNA content detected by flow cytometry and expressed as HPCV (%) in liver, gill and blood cells isolated from European eel (*A. anguilla*) exposed for 1 day to two different concentrations of BaP (0.39 and 3.9 µM) and sampled before the exposure (T0), after 1 day of exposure (sampling time = 1) and at 1, 3, 7, 14 and 28 days post-exposure. Data are expressed as means ± SEM (n = 5 animals). Asterisks denote a statistically significant difference from the control group: * $P < 0.05$; ** $P < 0.01$.

3.5. DISCUSSION

Here we report that BaP induces DNA strand-breaks and chromosomal damage in the gills, blood and, to a lesser degree, liver of juvenile eel, even in the post-exposure period to low concentrations of compound, after the removal of the contamination source. The presence of DNA damage suggests putative disruptions of the main functions of these tissues, which can lead to detrimental effects at the organism level.

The strong induction of DNA damage in the liver cells after a 24h exposure to BaP, probably resulted from the interaction of the reactive electrophiles and reactive oxygen species (ROS), produced during the metabolism of BaP by the P450 cytochrome system, as shown previously in other studies using the same species and tissue (e.g. Maria *et al.* 2002; Regoli *et al.* 2003). The absence of DNA damage at day 1 of post-exposure may indicate that the phase II enzymes were able to efficiently conjugate the reactive metabolites produced during phase I. Furthermore, the rapid decrease in the DNA damage levels in comparison with the values registered for the day of BaP exposure suggests that eel's liver cells present efficient mechanisms of repair of the DNA lesions. Nevertheless, the DNA defence and maintenance systems, such as antioxidant and DNA repair mechanisms, seem to have been affected somehow, as at 14 and 28 days post-exposure, DNA breaks started to appear and accumulate in liver cells. The apparently higher genotoxic effect of the lowest BaP concentration (0.39 µM) may be explained by a decrease in the metabolism rate of the parental BaP to reactive intermediates in the high concentration-exposed eels, due to the saturation of metabolism enzymes or inhibition/inactivation of cytochrome P450 1A (CYP1A). Studies with different fish species exposed to BaP and to other PAH compounds showed an inhibition in the 7-ethoxyresorufin O-deethylase (EROD) activity at high concentrations of PAH (Gravato and Santos 2002; Scholz and Segner 1999). Alternatively, as suggested by other authors (Ching *et al.* 2001; Siu *et al.* 2003), the levels of DNA damage induced by the higher BaP concentration might have elicited faster and more efficient rates of DNA repair than the levels of DNA damage induced by the lowest concentration.

Gills are organs that are in close contact with the water, thus, waterborne chemicals can easily pass the relatively thin epithelia before entering the blood. Given that low levels of CYP1A activity have been detected in eel gill cells (Pacheco and Santos 2001), the high DNA damage levels observed in this study are possibly a consequence of the oxidative stress originated by the exposure to BaP (Machella *et al.* 2005), rather than due to the reactive metabolites (e.g., BaP diol epoxide) generated during the biotransformation process. The low antioxidant defence capacity of gill cells (Ahmad *et al.* 2004; Levine and Oris 1999), further contributes to the accumulation of DNA damage. Moreover, the absence of DNA damage after 1 day of exposure to BaP, when the toxic compound is in direct contact with the gill cells, confirms that the parental compound is not itself the DNA damage inducer.

DNA damage was also observed in blood cells immediately after BaP exposure. The activated BaP and ROS produced during the hepatic and gill biotransformation processes and that entered the blood stream were probably the primary cause of the DNA damage observed in these cells. The endothelial cells of the blood vascular lining and mature erythrocytes of fish have also shown to possess xenobiotic metabolizing capacity, although at low levels (Mitchelmore and Chipman 1998; Ortiz-Delgado *et al.* 2005), and might further contribute to the increase in the DNA damage levels. During the post-exposure period, no DNA damage was observed in blood cells of exposed eels. The removal of damaged erythrocytes from circulation together with the production of new blood cells are one possible explanation for the absence of DNA damage during that period.

Variations in the dispersion of nuclear fluorescence were evaluated in the present study using FCM. Previous works have already reported a positive correlation between the coefficient of variation of DNA peaks and chromosomal damage in organisms exposed to clastogenic contaminants (Goanvec *et al.* 2008; Matson *et al.* 2004). Here, no significant variations in this measure were detected in the liver cells after BaP exposure. In contrast, gill cells showed increased values of HPCV at day 3 post-exposure to the highest BaP concentration (3.9 µM). The presence of chromosomal damage in gill and not in liver cells may be a consequence of their different abilities to protect DNA from damage, particularly

from oxidative stress (Ahmad *et al.* 2004; Levine and Oris 1999). The apparent recovery to HPCV values similar to those of control group might reflect the regenerative proliferation of gill cells, which are known to have a high mitotic rate (Al-Sabti and Metcalfe 1995), after the degenerative effects induced by the BaP exposure.

A significant increase in HPCV values was observed in blood cells at day 3 of post-exposure in eels exposed to the lowest BaP concentration. This is in accordance with previous studies (Maria *et al.* 2002; Pacheco and Santos 1997), where nuclear abnormalities were detected in eel erythrocytes exposed to similar concentrations of BaP. A rapid and efficient elimination of abnormal erythrocytes from the circulating system of peripheral blood and their replacement by new cells may have contributed to the observed recovery to the values similar to the control.

3.6. CONCLUSIONS

The results herein presented, demonstrated that DNA damage is a consequence of BaP exposure, even in the post-exposure period when the source of contamination is removed. Moreover, genotoxicity was observed in different tissues, being the damage levels related to organ location and function, tissue regeneration rate, capacity of protection against DNA damaging agents and DNA repair ability. Chromosomal damage given by the variation in HPCV values was not observed in liver cells and the damage detected in gill and blood cells seemed to be only temporary. The rapid cell recovery in these organs was probably due to the high rate and efficacy of cell renewal that is associated with the juvenile stages. Nevertheless, other problems besides clastogenic events are associated with structural perturbations of the DNA molecule that do persist, including the activation of oncogenes and/or the expression of protein dysfunction. These endpoints were not the focus of this study, but it should be emphasized that they may also have detrimental effects at later life stages of the animal. Further studies should, therefore, be aimed at these endpoints to complete the cause-effect relationship between PAH exposure and genotoxic damage at the molecular level.

3.7. REFERENCES

Ahmad I, Pacheco M, Santos MA. 2004. Enzymatic and nonenzymatic antioxidants as an adaptation to phagocyte-induced damage in *Anguilla anguilla* L. following in situ harbor water exposure. Ecotoxicology and Environmental Safety 57(3):290-302.

Al-Sabti K, Metcalfe CD. 1995. Fish micronuclei for assessing genotoxicity in water. Mutation Research, Genetic Toxicology and Environmental Mutagenesis 343(2-3):121-135.

Bihari N, Fafandel M. 2004. Interspecies differences in DNA single strand breaks caused by benzo(a)pyrene and marine environment. Mutation Research 552:209-217.

Ching EWK, Siu WHL, Lam PKS, Xu L, Zhang Y, Richardson BJ, Wu RSS. 2001. DNA adduct formation and DNA strand breaks in Green-lipped mussels (*Perna viridis*) exposed to benzo[a]pyrene: dose- and time-dependent relationships. Marine Pollution Bulletin 42(7):603-610.

Doležel J, Binarová P, Lucretti S. 1989. Analysis of nuclear DNA content in plant cells by flow cytometry. Biologia Plantarum 31(2):113-120.

Frenzilli G, Nigro M, Lyons BP. 2009. The Comet assay for the evaluation of genotoxic impact in aquatic environments. Mutation Research, Reviews in Mutation Research 681(1):80-92.

Goanvec C, Theron M, Lacoue-Labarthe T, Poirier E, Guyomarch J, Le-Floch S, Laroche J, Nonnotte L, Nonnotte G. 2008. Flow cytometry for the evaluation of chromosomal damage in turbot *Psetta maxima* (L.) exposed to the dissolved fraction of heavy fuel oil in sea water: a comparison with classical biomarkers. Journal of Fish Biology 73(2):395-413.

Gravato C, Santos MA. 2002. Liver phase I and phase II enzymatic induction and genotoxic responses of β-naphthoflavone water-exposed sea bass. Ecotoxicology and Environmental Safety 52(1):62-68.

Hartl MGJ. 2002. Benthic fish as sentinel organisms of estuarine sediment toxicity. In: Bright M, Dworschak P, Stachowitsch M, editors. The Vienna School of Marine Biology: A Tribute to Jörg Ott. Facultas Universitätsverlag, Wien p89-100.

Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V and others. 2003. Recommendations for conducting the in vivo alkaline Comet assay. Mutagenesis 18(1):45-51.

Jha AN. 2004. Genotoxicological studies in aquatic organisms: an overview. Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis 552(1-2):1-17.

Johnson LL, Collier TK, Stein JE. 2002. An analysis in support of sediment quality thresholds for polycyclic aromatic hydrocarbons (PAHs) to protect estuarine fish. Aquatic Conservation: Marine and Freshwater Ecosystems 12(5):517-538.

Levine S, Oris J. 1999. CYP1A expression in liver and gill of rainbow trout following waterborne exposure: implications for biomarker determination. Aquatic Toxicology 46:279-287.

Machella N, Regoli F, Santella RM. 2005. Immunofluorescent detection of 8-oxo-dG and PAH bulky adducts in fish liver and mussel digestive gland. Aquatic Toxicology 71(4):335-343.

Marchand J, Tanguy A, Laroche J, Quiniou L, Moraga D. 2003. Responses of European flounder *Platichthys flesus* populations to contamination in different estuaries along the Atlantic coast of France. Marine Ecology Progress Series 260:273-284.

Maria VL, Correia AC, Santos MA. 2002. *Anguilla anguilla* L. biochemical and genotoxic responses to benzo[a]pyrene. Ecotoxicology and Environmental Safety 53(1):86-92.

Matson C, Franson J, Hollmen T, Kilpi M, Hario M, Flint P, Bickam J. 2004. Evidence of chromosomal damage in common eiders (*Somateria mollissima*) from the Baltic Sea. Marine Pollution Bulletin 49:1066-1071.

Mitchelmore C, Chipman J. 1998. Detection of DNA strand breaks in brown trout (*Salmo trutta*) hepatocytes and blood cells using the single cell gel electrophoresis (comet) assay. Aquatic Toxicology 41:161-182.

Nigro M, Frenzilli G, Scarcelli V, Gorbi S, Regoli F. 2002. Induction of DNA strand breakage and apoptosis in the eel *Anguilla anguilla*. Marine Environmental Research 54:517-520.

Nogueira PR, Lourenco J, Mendo S, Rotchell JM. 2006. Mutation analysis of *ras* gene in the liver of European eel (*Anguilla anguilla* L.) exposed to benzo[a]pyrene. Marine Pollution Bulletin 52(12):1611-1616.

Ortiz-Delgado JB, Segner H, Sarasquete C. 2005. Cellular distribution and induction of CYP1A following exposure of gilthead seabream, *Sparus aurata*, to waterborne and dietary benzo(a)pyrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin: An immunohistochemical approach. Aquatic Toxicology 75(2):144-161.

Pacheco M, Santos MA. 1997. Induction of EROD activity and genotoxic effects by polycyclic aromatic hydrocarbons and resin acids on the juvenile eel (*Anguilla anguilla* L.). Ecotoxicology and Environmental Safety 38(3):252-259.

Pacheco M, Santos MA. 2001. Tissue distribution and temperature-dependence of *Anguilla anguilla* L. EROD activity following exposure to model inducers and relationship with plasma cortisol, lactate and glucose levels. Environment International 26(3):149-155.

Pacheco M, Santos MA. 2002. Biotransformation, genotoxic, and histopathological effects of environmental contaminants in European eel (*Anguilla anguilla* L.). Ecotoxicology and Environmental Safety 53(3):331-347.

Regoli F, Winston GW, Gorbi S, Frenzilli G, Nigro M, Corsi I, Focardi S. 2003. Integrating enzymatic responses to organic chemical exposure with total oxyradical absorbing capacity and DNA damage in the European eel *Anguilla anguilla*. Environmental Toxicology and Chemistry 22(9):2120-2129.

Rose WL, French BL, Reichert WL, Faisal M. 2001. Persistence of benzo[a]pyrene-DNA adducts in hematopoietic tissues and blood of the mummichog, *Fundulus heteroclitus*. Aquatic Toxicology 52(3-4):319-328.

Scholz S, Segner H. 1999. Induction of CYP1A in primary cultures of rainbow trout (*Oncorhynchus mykiss*) liver cells: concentration-response relationships of four model substances. Ecotoxicology and Environmental Safety 43(3):252-260.

Siu WHL, Hung CLH, Wong HL, Richardson BJ, Lam PKS. 2003. Exposure and time dependent DNA strand breakage in hepatopancreas of green-lipped mussels (*Perna viridis*) exposed to Aroclor 1254, and mixtures of B[a]P and Aroclor 1254. Marine Pollution Bulletin 46(10):1285-1293.

van der Oost R, Beyer J, Vermeulen NPE. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology 13(2):57-149.

4.

Transcript profiling and DNA damage in the European eel (Anguilla anguilla L.) exposed to 7,12-dimethylbenz[a]anthracene

---

Chapter published as original article:
Nogueira P, Lourenço J, Rodriguez E, Pacheco M, Santos C, Rotchell JM, Mendo S. 2009. Transcript profiling and DNA damage in the European eel (*Anguilla anguilla* L.) exposed to 7,12-dimethylbenz[a]anthracene. Aquatic Toxicology 94: 123-130

4.1. ABSTRACT

The molecular responses induced during and after an acute exposure to 7,12-dimethylbenz[a]anthracene (DMBA) were analysed in liver, gill and blood cells of juvenile *Anguilla anguilla* with the aim of developing molecular biomarkers of environmental PAH pollution. Changes in the mRNA expression levels of the cell cycle checkpoint-related *rad1* gene and the mRNAs of differentially expressed genes identified by suppression subtractive hybridization (SSH) were analysed in the liver, and related to well-established biomarkers: *cyp1A1* mRNA expression and assessment of the DNA integrity using the comet assay and flow cytometry. DMBA exposure resulted in increased *cyp1A1* mRNA levels, suggesting that CYP1A1 might be involved in the metabolism of DMBA. Global DNA damage, detected by the comet assay, was observed in the three tissues analysed but only blood cells showed chromosomal lesions as analysed by flow cytometry. Although DNA damage was found in the liver, no induction in *rad1* gene was observed in this organ. The global SSH approach revealed that mRNAs of genes related to xenobiotic metabolism, immune processes and cytoskeleton dynamics were differentially expressed in DMBA-exposed eel livers, highlighting the complexity in the response observed in fish exposed to a genotoxic agent and providing directions for new biomarker development.

Keywords

Anguilla anguilla, biomarker; SSH; genotoxicity; PAH

4.2. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are contaminants that may enter aquatic systems through spillage and seepage of fossil fuels, discharge of domestic and industrial wastes, atmospheric input and continental runoff (Hartl 2002). Their presence in the environment is of concern since they induce acute toxicity in organisms and have been linked to liver neoplasms and other abnormalities in fish species (Jha 2004). 7,12-dimethylbenz[a]anthracene (DMBA) is a prototype synthetic PAH, cytotoxic, mutagenic and immunotoxic (Miyata *et al.* 2001). It is regarded as one of the most carcinogenic PAHs in mammalian and fish species (Miyata *et al.* 2001; Weimer *et al.* 2000). Like other PAHs, DMBA requires metabolic activation in order to produce potentially damaging effects. PAHs biotransformation is most effective in the liver of fish, where they are easily metabolized by the phase I enzymes of the cytochrome P450 monooxygenase system (e.g. CYP1A1 enzyme) to more hydrophilic products (van der Oost *et al.* 2003). Most of the resultant metabolites are excreted after conjugation by phase II enzymes. However, some of the intermediates produced during the biotransformation process, especially epoxides and dihydrodiols, are highly reactive and may bind to cellular macromolecules such as DNA, forming covalent bulky DNA adducts responsible for mutagenicity and carcinogenicity.

Cells have evolved a variety of systems to maintain the genomic stability by detecting and repairing damaged DNA. In response to genotoxic exposure, cells initiate cell cycle checkpoints that halt replication to allow time to repair damaged DNA. The mammalian Rad9-Rad1-Hus1 (9-1-1) checkpoint complex has been characterized as a sensor of DNA damage (Helt *et al.* 2005). Bulky adducts such as those induced by DMBA, elicit a checkpoint pathway involving the 9-1-1 complex (Bi *et al.* 2006), which is recruited to affected sites where it may attract specialized DNA polymerases and other DNA repair effectors (Helt *et al.* 2005). In fish, the role of Rad1 and other mechanisms of detecting DNA damage are yet to be elucidated.

Exposure of organisms to chemicals may also result in complex changes in gene expression levels as a direct response or as a compensatory response to molecular damage or cellular dysfunction. Suppression subtractive hybridization

(SSH) is a technique used to detect mRNAs of differentially expressed genes (Diatchenko *et al.* 1996), being particularly useful when the available sequence data of an organism is limited, which is most often the case for aquatic species. In the toxicology field, advances in transcript analysis have led to the recognition that altered gene expression is potentially an early, rapid and sensitive means of stress response detection (Williams *et al.* 2003).

The aim of this study was to investigate global and specific molecular responses to DMBA exposure in juvenile *Anguilla anguilla* (European eel), with the broad aim of being able to develop these as molecular biomarkers of environmental PAH pollution. *A. anguilla* is widely distributed in estuaries and freshwater courses and, despite its description as a bottom dwelling fish, can move throughout the entire water column being thus a versatile indicator species with a high economic value, that is easy to capture and to maintain in a laboratory (Costa *et al.* 1992). However, since the 1980s, a decline in *A. anguilla* and *A. rostrata* populations have been recorded. Because of their unusual life cycle, they are considered particularly susceptible to pollutant exposure and this is postulated as one of the causes for their population decline (Robinet and Feunteun 2002).

To develop such biomarkers, changes in the mRNA expression levels of the cell cycle checkpoint-related *rad1* gene and the detection of mRNAs of differentially expressed genes by SSH were analysed in the liver of DMBA-exposed eels. To anchor the novel molecular biomarkers described herein, well-established biomarkers were studied in parallel. These included hepatic *cyp1A1* gene expression and assessment of DNA integrity by the comet assay and flow cytometry (FCM).

4.3. MATERIALS AND METHODS

4.3.1. Experimental exposure

Juvenile specimens of European eel (*A. anguilla*) with an average weight of 0.2 g (glass eels) were caught at the Mondego River mouth, Figueira da Foz (Portugal). Fish were acclimatised to laboratory conditions for a period of 2 weeks in recirculating, filtered, well aerated and dechlorinated tap water, at 20 ºC, under a 12 h light/12 h darkness photoperiod. Animals were fed three times a week

during the acclimation period. Eels were exposed in 40 l aquaria to DMBA (Sigma, USA) previously dissolved in dimethyl sulfoxide (DMSO, Sigma, USA). The DMSO concentration in water (0.0033 %) has no detectable cytogenetic or toxic effect in juvenile eels (Pacheco and Santos 2002). The same DMSO volume was added to the control aquarium. Groups of at least 150 animals were exposed to a nominal concentration of 0.39 and 3.9 µM (0.1 and 1 ppm, respectively) of DMBA for 24 h, and afterwards maintained in clean water and fed three times a week. Control fish were also transferred to clean water. DMBA concentrations were selected basing on previous studies which showed the ability of DMBA to bind to fish DNA and to induce neoplasic lesions (El-Zahr *et al.* 2002).

Eels were sampled before the exposure (T0), immediately after the exposure and at 1, 3, 7, 14, 28 and 90 days during the post-exposure period. At each sampling point, five fish were sacrificed by decapitation and the blood directly collected from the heart using heparinised capillary tubes, suspended in cold phosphate buffered saline (PBS) 1× and kept on ice until use. Liver and gills were also collected and kept in cold PBS 1×/EDTA 100 mM/DMSO 10 % buffer until use in the comet assay. The liver from 15 other eels were collected, kept in RNAlater RNA Stabilization Reagent (Qiagen, UK) and frozen at -70 ºC.

4.3.2. Traditional biomarkers

4.3.2.1. Evaluation of DNA integrity by comet assay

The comet assay, which detects DNA strand breaks and alkali-labile sites, was conducted with eel whole blood, liver and gill tissue, under yellow light to prevent UV induced DNA damage. Liver and gill cell suspensions were obtained by homogenizing the tissues in the presence of PBS 1×/EDTA 100 mM/DMSO 10 % buffer, to reduce DNase activity and prevent oxidant-induced DNA damage (Hartmann *et al.* 2003). The comet assay protocol was performed as described in Nogueira *et al.* (2006). Slides were examined using a fluorescence microscope (Leica DMLS, Germany). Fifty randomly selected cells per slide (2 slides/animal) were analysed using the image analysis software Comet Assay IV (Perceptive Instruments, UK). DNA damage was expressed as percentage tail DNA (% tDNA) and presented as mean of medians ± standard error of the mean (SEM). The

extent of DNA migration (% tDNA) is positively correlated with the amount of DNA damage in the cell (Hartmann *et al.* 2003).

4.3.2.2. Evaluation of DNA integrity by flow cytometry

One volume of citrate buffer (sucrose 250 mM, trisodium citrate 40 mM, pH=7.6) with 10 % DMSO was added to blood samples before frozen in liquid nitrogen and stored at -70 ºC. Liver and gill cell suspensions (obtained as described in section 4.3.2.1.) were also frozen in liquid nitrogen and stored at -70 ºC for flow cytometry analysis.

Frozen samples were thawed and suspended in LB01 buffer (Doležel *et al.* 1989), treated with 50 mg/ml RNase (Fluka, Buchs, Switzerland) and stained with 50 mg/ml propidium iodide (PI, Fluka). Samples were incubated for 10 min on ice before analysis. The fluorescence intensity of, at least, 5000 nuclei/sample was measured with a Coulter EPICS-XL flow cytometer (Beckman-Coulter®, USA). The instrument was equipped with an air-cooled argon-ion laser (15 mW, operating at 488 nm) and PI fluorescence was collected through 645 nm dichroic long-pass and 620 nm band-pass filters. The results were acquired using the SYSTEM II software (v. 3.0, Beckman Coulter®) in the form of three graphs: fluorescence integral (FL) histogram (to analyse the FL intensity of nuclei); FL *vs.* time cytogram (to analyse the nuclei fluorescence stability during sample acquisition); and FL pulse integral *vs.* FL pulse height cytogram (to detect and eliminate, through region gating, doublets and other debris). Prior to data acquisition, the instrument was checked for linearity and resolution with fluorescent check beads (Beckman-Coulter®), and the amplification was adjusted so that the peak corresponding to sample's nuclei was positioned approximately at channel 200 of a 1024 scale. This setting was kept constant throughout the whole experiment. An increase in the amount of chromosomal damage is reflected by an increase in cell-to-cell variation in DNA content (Matson *et al.* 2004), so the half-peak coefficient of variation (HPCV) of the G_0/G_1 peak was calculated for each sample.

4.3.2.3. Determination of mRNA expression of *cyp1A1* gene

The expression of *cyp1A1* gene was determined by real-time quantitative PCR (qPCR) in the liver of control (non-exposed) and DMBA-exposed eels sampled during the post-exposure period at 3, 7 and 28 days, in order to have a temporal response overview. Total RNA was isolated from pools of two livers (5 pools/group) using RNeasy reagents (Qiagen, UK) and treated with RNA-free DNase I (Qiagen, UK) to remove genomic DNA. RNA concentrations were measured with the Quant-iT RNA assay kit (Invitrogen, UK) using a Qubit fluorometer (Invitrogen, UK). Reverse transcription of 1 µg of total RNA samples was carried out using SuperScript® Vilo™ cDNA synthesis kit (Invitrogen, UK). Real-time PCR reactions were performed in duplicate, in a final volume of 25 µl containing 12.5 µl of Express SYBR® GreenER™ qPCR SuperMix (Invitrogen, UK), 5 µl of diluted cDNA (1/100), 3.75 µM primers (Table 4.1) and 0.1 µl of ROX reference dye. A control lacking cDNA template was included in qPCR analysis to determine the specificity of target cDNA amplification. Amplification was detected with a Mx3005P real-time PCR system (Stratagene, UK) and with the following cycling parameters: 50 ºC for 2 min, 95 ºC 10 min, 40 cycles of 30 s at 95 ºC, 1 min at 60 ºC and 30 s at 72 ºC. Melting curves were determined following the instrument instructions to identify the presence of primer dimers and analyse the specificity of the reaction. The amplification efficiency of each primer pair was calculated using a dilution series of cDNA. Eel acidic ribosomal protein (ARP) gene was used as the internal reference (Weltzien *et al.* 2005). The relative mRNA expression levels of target genes were calculated according to the efficiency-corrected method described by Pfaffl (2001), using the non-treated group as calibrator.

Table 4.1. Real-time PCR primer sequences.

Primer name	GenBank accession No.	Primer forward (5' – 3')	Primer reverse (5'-3')
ARP	AY63793	GTGCCAGCTCAGAACACTG	ACATCGCTCAAGACTTCAATGG
CYP	AF420257	GTGTGGGACTGGAGCGTACT	CAGTGCGGTGTCTTTAGTGG
RAD1	FJ438472	GGGAAGCACTACACCAGGAG	AATGGGATCCTCTGGCTCTT
ST	GO096062	ACCTGGAAGCAAGGGAAGAT	ACTCTATCAGCGGTGGCATC
VLIG	GH717880	CTGCTGTTTGGGTCATCTCA	AGTTGGGAGAAAACCCTGCT
COMT	GH717900	TGTGAGCAAAGGTGGCATTA	GCGAGTAAGCTCAGGTCGAT
ITIH3	GH717895	TTTCGCTTTGCCCTAACTGT	CCGTGCTGAAGTTTGTGATG

4.3.3. Development of novel biomarkers

4.3.3.1. Determination of mRNA expression of *rad1* gene

For the isolation of *rad1* gene cDNA, RNA was extracted from the pool of two livers of non-exposed eels using RNeasy reagents (Qiagen, UK) and treated with RNA-free DNase I (Qiagen, UK). First strand cDNA was synthesized from 1 µg of total RNA in the presence of random hexamer primers using Superscript First Strand cDNA Synthesis System for RT-PCR (Invitrogen, UK). This cDNA was the template for the amplification of a *rad1* gene fragment using the degenerated primers Rad1F (5'-GAAGAAGGWGGWGTGGTG-3') and Rad1R (5'-TACTGTARDGAGAGGAAGCCTC-3'), designed basing on published sequences of fish species. The PCR reaction was performed in reaction buffer with 400 µM each dNTP, 50 pmol each primer and 1 U platinum Taq DNA polymerase (Invitrogen, UK). The *rad1* fragment was amplified in a Techne^{TM} thermal cycler using 35 sequential cycles at 94 ºC for 40 s, 50 ºC for 40 s, 72 ºC for 60 s, followed by a final 2 min extension at 72 ºC. The sequence obtained from the cloned fragments subsequently served for specific primer design. The isolation of the complete 5' and 3' ends of *rad1* gene was performed with control *A. anguilla* liver total RNA (1 µg) using SMART^{TM} RACE cDNA amplification reagents (Clontech, France) and the gene-specific primers RACE5rad1 (5'-CCTTGTTGGTGACATTGGTGCTGCAG-3') and RACE3rad1 (5'-CTTTGGGGGAAGCACTACACCAGGA-3'). The RACE products obtained were analysed on agarose gel, excised and purified using a Qiaquick spin columns (Qiagen, UK). Purified DNA was ligated into a pGEM®-T Easy vector (Promega, UK). Recombinant plasmids were transformed and selected using ampicilin LB plates. Plasmid DNA was purified for DNA sequence analysis using commercial sequencing (Eurofins MWG Operon, Germany) to verify the identity of the products.

The mRNA expression of *rad1* gene was determined by real-time qPCR in control (non-exposed) and in DMBA-exposed eels sampled during the recovery period at 3, 7 and 28 days, as described in section 4.3.2.3, and using the RAD1 primers (Table 4.1). Data were normalized to eel ARP gene and expressed as the fold difference from the control group.

4.3.3.2. Suppression subtractive hybridization (SSH)

The SSH procedure was used to isolate and enrich for gene sequences with mRNAs differentially expressed between the liver of control (non-exposed) and 3.9 µM DMBA-exposed eels sampled at day 7 of the post-treatment period, when the highest levels of DNA damage were observed in the liver, as measured by comet assay. For each group, equal amounts of the RNA extracted in section 4.3.2.3 were pooled (five RNA samples in each group). The synthesis of cDNA was carried out with the SuperSMART PCR cDNA Synthesis kit (Clontech, France) and the generated cDNA was directly used for SSH. The forward and reverse-subtracted libraries were produced using the PCR-Select cDNA Subtraction kit (Clontech, France) according to the manufacturer's protocol. The differential PCR products generated by SSH were inserted in a pGEM®-T Easy vector (Promega, UK) and cloned into JM109 bacterial cells. Sixty randomly selected colonies were inoculated in LB broth and screened for inserts by PCR using vector-based primers. A total of 20 clones per library were randomly selected for sequencing (Eurofins MWG Operon, Germany) directly from the PCR amplified product. Sequence identities were obtained by BLAST searches against the NCBI nucleic acid and protein databases. Sequence reads with *E*-value higher than 10^{-5} were filtered out. The differential screening result was confirmed by qPCR using four candidate genes: sulfotransferase (ST), similar to very large inducible GTPase 1 (VLIG), inter-alpha globulin inhibitor H3 (ITIH3) and catechol-O-methyltransferase (COMT), as described in the section 4.3.2.3. Primer pairs used in qPCR are listed in Table 4.1.

4.3.4. Statistical analysis

For statistical analysis (SigmaStat 3.11 Software), all data were tested for normality and homogeneity of variances. Differences between control and exposed fish were determined using analysis of variance (ANOVA), followed by a Tukey test ($P<0.05$). If an invalidation of normality and variances homogeneity was observed, data were analysed using a non-parametric Kruskal-Wallis ANOVA on ranks, followed by a Mann–Whitney *U* pairwise comparison test (SigmaStat 3.11 Software). A $P<0.05$ was considered significant.

4.4. RESULTS

4.4.1. Traditional biomarkers

4.4.1.1. Evaluation of DNA integrity by comet assay

DNA damage was analysed in liver, gill and blood cells of *A. anguilla* exposed for 24 h to two different concentrations of DMBA and sampled at different times post-exposure. Levels of damage were compared with the respective control group (Figure 4.1). The highest level of DNA damage in liver cells was observed in eels exposed to 3.9 µM DMBA at 7-day post-exposure. A significant increase ($P < 0.05$) was also observed in liver cells at 28-day post-exposure. The gill cells were affected by both concentrations of DMBA in eels sampled at different days post-exposure. The group exposed to the higher concentration showed significant increases in DNA damage levels at 7- (near fivefold) and 28-day post-exposure. The DNA damage detected in the gill cells of eels exposed to 0.39 µM was observed at 1- and 28-day post-exposure. Interestingly, the values observed in this exposed group following 1 day in clean water were significantly higher than those observed in the group subjected to the higher concentration. Blood cells showed increased levels of DNA damage during the first week post-exposure, in both DMBA-treated groups. DNA damage in these cells was also observed 28- and 90-day post-exposure for the highest DMBA concentration. A significant ($P < 0.05$) decrease in tDNA values of blood cells was observed at 28-day post-exposure in eels exposed to 0.39 µM DMBA.

4.4.1.2. Evaluation of DNA integrity by flow cytometry

The DMBA exposure did not induce significant changes in the cell cycle or any major ploidy changes (e.g. aneuploidy) in any of the tested tissues. For putative chromosomal damage evaluation, mean HPCV values were calculated for liver, gill and blood cells and varied between 2.7 and 5.7 % (Figure 4.2). The variations in the mean HPCV of liver and gill cells were not statistically significant ($P > 0.05$) between the eels exposed and non-exposed to DMBA. Blood cells showed higher variation during the experiment than liver and gill cells. Significant ($P < 0.05$) increases in the mean HPCV value of blood cells were found in eels exposed to the highest concentration and analysed after 1 day of exposure and at 28-day post-exposure.

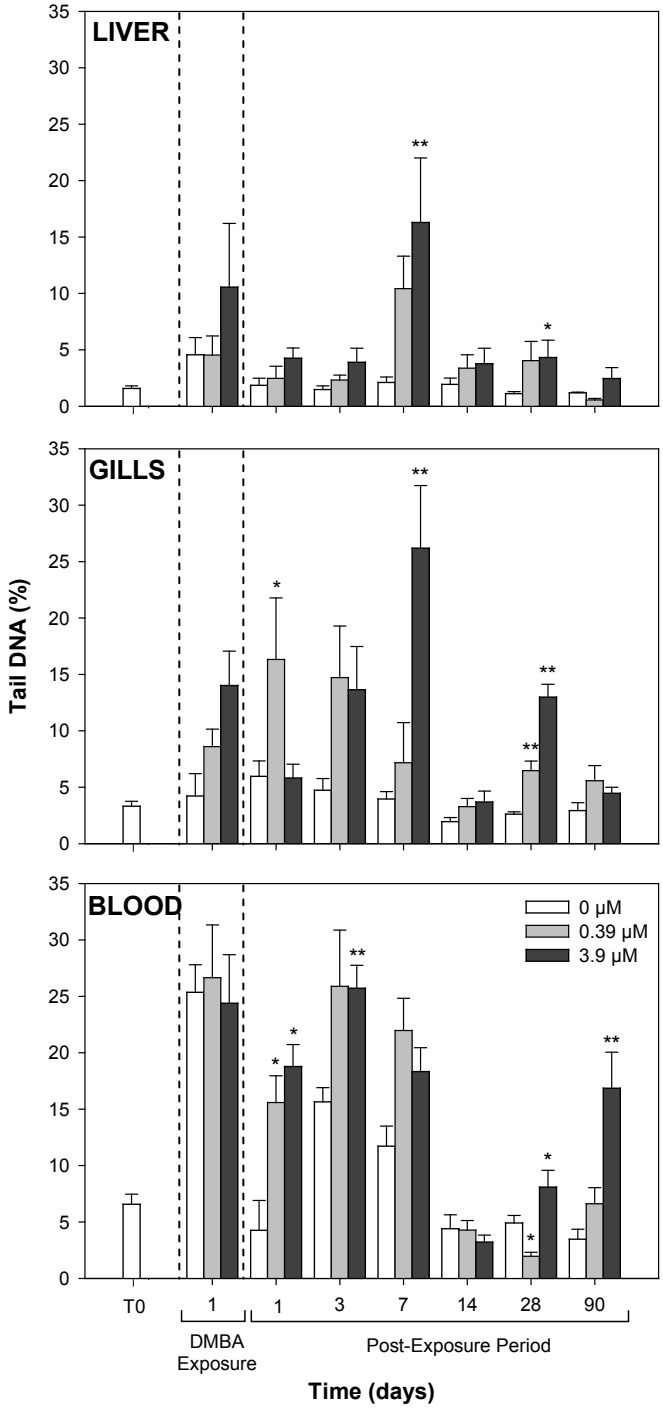

Figure 4.1. DNA damage detected by the comet assay and expressed as tail length (%) in liver, gill and blood cells isolated from *A. anguilla* exposed for 1 day to two different concentrations of DMBA (0.39 and 3.9 µM) and sampled before the exposure (T0), after 1 day of exposure (sampling time = 1) and at 1-, 3-, 7-, 14-, 28- and 90-day post-exposure. Data are expressed as mean of medians ± SEM (n = 5 animals). Asterisks denote statistically significant differences from the control group: * $P < 0.05$; ** $P < 0.01$.

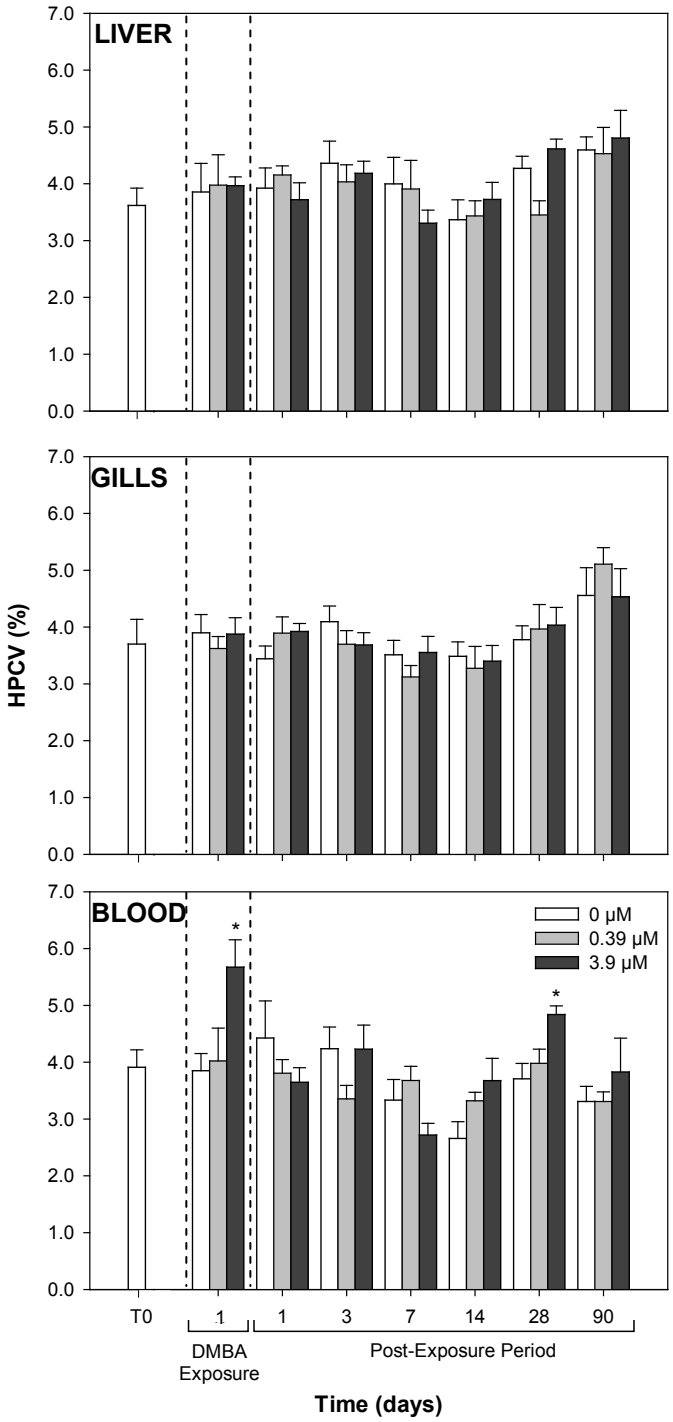

Figure 4.2. DNA damage detected by flow cytometry and expressed as HPCV (%) in liver, gill and blood cells isolated from *A. anguilla* exposed for 1 day to two different concentrations of DMBA (0.39 and 3.9 µM) and sampled before the exposure (T0), after 1 day of exposure (sampling time = 1) and at 1-, 3-, 7-, 14-, 28- and 90-day post-exposure. Data are expressed as means ± SEM (n = 5 animals). Asterisks denote statistically significant differences from the control group: * $P < 0.05$.

4.4.1.3. Determination of mRNA expression of *cyp1A1* gene

The relative mRNA expression of *cyp1A1* gene was determined in liver cells exposed to 3.9 µM DMBA and sampled at 3, 7 and 28 days during the post-exposure period. The hepatic *cyp1A1* transcript levels significantly ($P < 0.05$) increased nearly sevenfold at 7 days, and remained close to the control values at days 3 and 28 (Table 4.2).

Table 4.2. Relative expression of *cyp1A1* and *rad1* genes in the liver of *A. anguilla* exposed to 3.9 µM DMBA and sampled 3-, 7- and 28-day post-exposure. Data were normalized to eel ARP gene and expressed as the fold difference from the control group. Asterisk (*) denotes a statistically significant difference from the control group ($P<0.05$).

Gene	Condition	Relative expression
cyp1A1	3 days	0.90
	7 days	6.90 *
	28 days	0.92
rad1	3 days	1.17
	7 days	1.06
	28 days	1.19

4.4.2 Development of novel biomarkers of exposure

4.4.2.1. Determination of mRNA expression of *rad1* gene

The isolated *A. anguilla rad1* cDNA with a nucleotide sequence of 1281 bp (excluding the polyA tail) included an ORF of 840 nucleotides, encoding 279 amino-acids (GenBank accession no. FJ438472). The predicted amino-acid sequence showed conserved exonuclease domains and leucine zippers found in *rad1* genes (Figure 4.3). The alignment of the *A. anguilla* deduced amino-acid sequence with other fish species and human Rad1 sequences is shown in Figure 4.3, revealing a high identity between eel and other fish species (from 86 % to 91 %) and with the human *rad1* sequence (75 %). The relative mRNA expression of *rad1* gene was studied in the same samples used for mRNA expression levels of *cyp1A1* but no alterations were observed in the relative *rad1* transcript levels (Table 4.2).

```
A.anguilla      MPLSTQSEAG-DERYVLVASLDNVRNLSNILKAITFKDHALFNATPNGLKVTVEDSKCLQ 59
D.rerio         MPLSTQSQSD-VDQYILIASLDNARNLSNILKAISFKDHAIFNATQNGLKVTVEDSKCLQ 59
G.aculeatus     MPLSTQSQAD-DEQYVLVASLDNARNLSNLLKAITFKDHAIFSATPNGLKVTVEESKCLQ 59
H.sapiens       MPLLTQQIQDEDDQYSLVASLDNVRNLSTILKAIHFREHATCFATKNGIKVTVENAKCVQ 60
                **: **   .  ::* * .****.****.:**** *::**   ** **:*****::**:*

                                        I                       II
A.anguilla      ANAFIQAEIFQEFTIQEDSVSFQVNLTVLLDCLTIFGGSTTPGVTTALRMCYNGYGYPLT 119
D.rerio         ANAFIQADIFQEYIIKEDAVGFQVNLTVLLDCLTIFGGSTVPGVCTALRMCYNGYGYPLT 119
G.aculeatus     ANAFIQAEIFQEFTIREDSVGFQVNLTVLLDCLSIFGGSTVPGVSTALRMCYRGYGYPLT 119
H.sapiens       ANAFIQAGIFQEFKVQEESVTFRINLTVLLDCLSIFGSSPMPGTLTALRMCYQGYGYPLM 120
                ******* ****: ::*::* *::*********:***.*. **. ***:***.***:**

                                                        III
A.anguilla      LFLEEGGVVTVCKINTEEPEDPIDFEFCSTNVTNKVILQSDSLREAFSELDMTSEILQIT 179
D.rerio         LFLEEGGVVTVCKINTQEPEEPIDFDFCSTNVTNKVILQSDSLKEAFSELDMTSEVLQIT 179
G.aculeatus     LFLEEGGVVTVCKINTQEPEEPVDFEFCSTNVTNKVILQSESLKEAFSELDMTSEVLQIT 179
H.sapiens       LFLEEGGVVTVCKINTQEPEETLDFDFCSTNVINKIILQSEGLREAFSELDMTSEVLQIT 180
                **************:*:***:.:**:****** **:****:.*:***:******:.***

                                                                IV
A.anguilla      MSPSQPYFRLSTFGNSGNAHYDYPKDSDMMELFQCTKTQTNRYKMSLLKPSTKALALSCK 239
D.rerio         MSPSHPYFRLSTFGNSGNAHYDYPKDSDMMELFQCTKIQTNRYKMSLLKPSTKALALSCK 239
G.aculeatus     MSPSQPYFRLSTFGNSGNAHYDYPKDSDMMELFRCDKTQTNRYKMSLLKPSTKALALSCK 239
H.sapiens       MSPDKPYFRLSTFGNAGSSHLDYPKDSDLMEAFHCNQTQVNRYKISLLKPSTKALVLSCK 240
                ***.:**********:*.:* *******::* *:* : *.****:**********.****

A.anguilla      VSVRTDTRGFLSLQYLVRNDDGQICFVEYYCCPDEEVEEE-- 279
D.rerio         VSVRTDSRGFLSLQYLVRNDDGQICFVEYYCCPDEEVEEE-- 279
G.aculeatus     VSVRTDSRGFLSLQYLVRNDDGQICFVEYFCCPDEEVEEE-- 279
H.sapiens       VSIRTDNRGFLSLQYMIRNEDGQICFVEYYCCPDEEVPESES 282
                **:***.********::**:*********:******* *
```

Figure 4.3. Comparison of the deduced *A. anguilla rad1* sequence (GenBank accession no. FJ438472) with selected vertebrate Rad1 sequences: *Danio rerio* (GenBank accession no. AAH49464); *Gasterosteus aculeatus* (Ensembl gene ENSGACP00000021769) and *Homo sapiens* (GenBank accession no. AAC95466). Areas showing homology are marked with an asterisk. The conserved exonuclease domains (I, II, III) and leucine zippers (III and IV) are overlined.

4.4.2.2. Suppression subtractive hybridization (SSH)

SSH methodology was employed in liver cells to screen differential mRNA expression of genes, between non-exposed group and the group exposed to 3.9 µM DMBA and sampled 7-day post-exposure, where the DNA damage in the liver was higher as revealed by the comet assay. Twenty randomly selected clones were sequenced for each subtracted library and compared to publicly available sequences. Ten sequences from the forward library (genes with upregulated mRNAs, Table 4.3) and nine from the reverse (genes with

downregulated mRNAs, Table 4.3) could be matched to genes from different organisms, mainly from other fish species. The remaining sequenced clones showed homology to unidentified hypothetical or novel proteins or showed no homology with other sequences deposited in the database.

The SSH results were validated by real-time qPCR, normalized to ARP gene, using two of the genes spotted for each library (Table 4.3). mRNA upregulation of sulfotransferase and very large inducible GTPase 1 genes, and down regulation of inter-alpha globulin inhibitor H3 and catechol-O-methyltransferase genes were confirmed by qPCR (Table 4.3).

Table 4.3. Genes differentially expressed in liver of *A. anguilla* exposed to 3.9 µM DMBA detected by SSH analysis (sequences with no similarity to genes with known functions are not included). Asterisk (*) denotes a statistically significant difference from the control group ($P < 0.05$).

Clone access. no.	Species	Putative identity	E-value	Relative Expres.
Upregulated genes				
GO096062	*Danio rerio*	Sulfotransferase	5E-93	4.61*
GH717880	*Danio rerio*	Similar to very large inducible GTPase 1	1E-79	2.52*
GH717881	*Ctenopharyngodon idella*	Beta-actin gene	4E-45	
GH717890	*Danio rerio*	Similar to cystine/glutamate transporter	7E-60	
GH717889	*Danio rerio*	Similar to ribosomal protein S8	7E-19	
GH717888	*Paralichthys olivaceus*	Tributyltin binding protein type 1	3E-12	
GH717887	*Oncorhynchus mykiss*	CCAAT/enhancer binding protein beta2	1E-34	
GH717886	*Crassostrea gigas*	Bindin precursor 5 repeat variant	9E-15	
GH717885	*Salmo salar*	Ribosomal protein L37a-1 mRNA	8E-14	
GH717883	*Danio rerio*	Similar to enteropeptidase-2	2E-27	
GH717884	*Salmo salar*	Membrane-spanning 4-domains subfamily A member 12	6E-53	
Downregulated genes				
GH717891	*Danio rerio*	Similar to vertebrate NCK-associated protein 1	7E-104	
GH717892	*Oncorhyncus mykiss*	Pentraxin	3E-36	
GH717894	*Tamia sibiricus*	Alpha 1-antitrypsin-like protein	5E-41	
GH717895	*Danio rerio*	Similar to inter-alpha-trypsin inhibitor heavy chain 3	1E-59	0.35*
GH717897	*Danio rerio*	Serpin peptidase inhibitor, clade A, member 7	5E-49	
GH717898	*Platichthys flesus*	Serine proteinase inhibitor	1E-33	
GH717900	*Danio rerio*	Catechol-O-methyltransferase domain containing 1	2E-59	0.46*
GH717901	*Anguilla anguilla*	Cytochrome b	7E-131	
GH717903	*Anguilla anguilla*	Mitochondrial 16S rRNA gene	0.0	

4.5. DISCUSSION

Here, we have observed that DMBA exposure significantly increases DNA damage (as measured by comet assay) in the gills, blood and, to a lesser extent, liver of juvenile eel, in a 1- to 7-day post-exposure time frame. We also observe that mRNA expression of *cyp1A1* gene is similarly increased at 7-day post-exposure in the liver of exposed eels. In contrast however, the flow cytometry and mRNA expression of *rad1* gene, a component of the DNA repair mechanism in mammals, display no such significant increases in the liver following DMBA exposure. These targeted specific biomarkers of DMBA exposure thus provide contrasting results. On adopting an alternate global approach in parallel with the same samples, we observed that numerous genes have their mRNA expression changed after exposure to DMBA, including a number of novel genes that may in future serve as possible biomarkers of hydrocarbon exposure.

The interaction of DMBA metabolites with DNA leads to the formation of adducts, contributing to the genotoxicity and carcinogenicity of this compound in fish (Weimer *et al.* 2000). Here we observe that DMBA exposure in *A. anguilla* resulted in an increase in DNA damage, and thus has similar genotoxic potential. CYP1A enzymes catalyse the first step of the DMBA detoxification process (van der Oost *et al.* 2003), and, again, the increase in the mRNA expression of *cyp1A1* gene observed is consistent with that observed in the liver of different fish species after PAH exposure (Levine and Oris 1999). Elevated *cyp1A1* and DNA damage both peak in the liver at day 7 suggesting that reactive metabolites take a few days to accumulate and result in actual DNA damage.

Little is known about the DNA repair efficiency of hepatic cells in fish. The possibility that the DNA repair *rad1* gene is inducible was investigated yet no alterations in its mRNA expression levels were found in hepatic cells of eels exposed to the highest concentration of DMBA at any sampling time point, including the day 7 post-exposure where a significant increase in the DNA damage level was observed. Similarly, Weimer *et al.* (2000) also reported a lack repair of DMBA-DNA adducts in trout liver cells, 48 h after DMBA exposure. Both findings seem to be in agreement that teleosts have a low DNA damage repair rate (Wirgin and Waldman 1998).

In terms of tissue-specific effects, gill tissue showed high levels of DNA damage compared to the other tissues, suggesting that either unmetabolized DMBA or its' metabolites are affecting gill DNA. Pacheco and Santos (2001) observed no significant increases in the gill EROD activity levels in eels exposed to β-naphthoflavone (a PAH-type chemical), suggesting that the metabolism capacity of eel gill cells is reduced. Subsequently, the production of reactive oxygen species due to DMBA exposure and a low antioxidant defence capacity of gill cells (Levine and Oris 1999) are, possibly, the main contributors for the DNA damage observed in these cells during the first 7-day post-exposure. This hypothesis, however, does not explain the DNA damage levels detected at 28-day post-exposure since the elimination of PAHs is generally very efficient in fish and no bioaccumulation of these compounds has generally been demonstrated (van der Oost *et al.* 2003). Genome instability and vulnerabilities in the DNA detection/repair systems may have possibly occurred in gill cells due to DMBA exposure and could explain the delayed damage observed in these cells. The replacement of damaged cells by new ones could have occurred after that period, as a consequence of the high mitotic rate of gill cells (Al-Sabti and Metcalfe 1995), explaining the absence of DNA damage at 90-day post-exposure. The apparent higher genotoxic effect at the lowest DMBA concentration tested (0.39 µM) observed in gill cells after 1-day post-exposure, is possibly explained by a decrease in the gill metabolism of the parental DMBA to reactive intermediates in the high concentration-exposed eels, due to the saturation of metabolic enzymes or inhibition/inactivation of CYP1A.

Blood cells showed increased levels of DNA damage throughout the experiment even in control eels. The temporal variation of the damage baseline is dependent on non-contaminant related factors. Blood is a "buffered" tissue which receives and transports many substances, including contaminants and metabolites of exogenous and endogenous compounds produced in different tissues. The analysed response may thus be a result and reflection of the global organism condition rather than of the blood cells isolated. The blood DNA damage observed at 28 and 90 days may constitute the late effects of DMBA exposure resulting from the conjugation of different factors in blood and other tissues, in particular

hematopoietic tissues (kidney and spleen). These factors include the toxicant accumulation/depuration ratio, DNA repair rate, antioxidant levels and cell turnover rate.

Structural perturbations on the DNA molecule, such as adducts and DNA strand breaks detected by comet assay, may result in genotoxic lesions that become permanent, including chromosomal aberrations or variations of nuclear DNA content. FCM is a useful method to analyse changes in nuclear DNA content induced by genotoxic agents and has already been used in fish to evaluate the effects of environmental pollutants (Goanvec *et al.* 2008). Here, the chromosomal damage induced by DMBA exposure was evaluated using FCM in liver, gill and blood cell nuclei. Liver and gill cells showed no statistically significant differences between exposed and non-exposed eels. Gill cells have a high mitotic rate (Al-Sabti and Metcalfe 1995) and may have eliminated and replaced the cells with high levels of damage. Liver cells, in contrast, have a lower mitotic rate and, therefore, the DNA repair capacity of cells may have a more determinant role in the removal of lesions that may have led to irreversible chromosomal damage.

In exposed organisms, genes that are differentially expressed may be useful as early warning molecular biomarkers to indicate exposure to toxicants. Here, DMBA-responsive gene transcripts were isolated from eel liver cells by SSH. The results represent a preliminary approach to the analysis of gene expression change in DMBA-exposed eels. Of particular note, and their relevance discussed in turn, are genes encoding a sulfotransferase, a number of protein translation machinery proteins, and immune response proteins.

mRNA expression of sulfotransferase gene was upregulated in the liver of DMBA-exposed eels. The reactions of sulfonation catalysed by this enzyme play a key role by facilitating the inactivation and elimination of potent endogenous chemicals and xenobiotics (Martin-Skilton *et al.* 2006). In mammalian liver, it is involved in metabolic activation of DMBA producing reactive metabolites that may react with DNA bases (Watabe 1983). This result suggests that the sulfotransferase is also involved in the metabolism of DMBA in fish, possibly contributing to the DNA damage observed.

mRNA of genes involved in protein translation machinery (ribosomal protein S8 and L37a-1) were upregulated in the liver of exposed eels. This suggests that new proteins are required to cope with the stress response. Specifically, mRNA of cysteine/glutamate transporter gene was upregulated in the exposed eels. This plasma membrane transporter is important for the uptake of cysteine used for the synthesis of glutathione which is essential for cellular protection from oxidative stress (Lo *et al.* 2008) and an important endogenous polar ligand for the biotransformation phase II reactions which contribute for the elimination of xenobiotics (van der Oost *et al.* 2003). Moreover, there is evidence that this transporter plays an important role in the growth and progression of cancers (Lo *et al.* 2008). As no tumours were observed (data not shown), the role of cysteine/glutamate transporter in DMBA-exposed eels is likely to be related to the DMBA phase II detoxification and/or the antioxidant defence, and consequently, protection of liver cells against oxidative damage. This result is in accordance to the low levels of DNA damage observed in liver cells when compared to those detected in gill and blood cells.

mRNA of genes involved in the inflammatory and innate immune response were also upregulated in exposed eels. The presence of DNA strand breaks in mammalian cells has been related to immune suppression (O'Connor *et al.* 1996). In fish, although the PAH-induced immunotoxicity has been widely described (reviewed in Reynaud and Deschaux 2006), little is known about the mechanism of induction. Here, mRNAs of the immune-related pentraxin, antitrypsin and serine protease genes were found down-regulated in DMBA-exposed eels supporting the PAHs-mediated immunosuppression. Other upregulated mRNAs included the very large inducible GTPase1 (VLIG), tributyltin (TBT) binding protein and CCAAT/enhancer-binding protein beta2 (C/EBP).

The expression of NCK-associated protein 1 (Nap1) transcript was lower in DMBA-exposed eels. In humans, Nap1 protein plays an essential role in the regulation of actin cytoskeleton dynamics and its reduced expression has been linked to the induction of apoptosis of neuronal cells (Suzuki *et al.* 2000). Nap1 expression has also been found reduced in the liver and brain of benzo[a]pyrene-

exposed fish (Brzuzan *et al.* 2007) suggesting that PAHs may affect the cytoskeleton dynamics of the cell or induce apoptosis in the affected fish.

In conclusion, DMBA exposure induced a toxic response in eels, as indicated by the study of traditional biomarker responses alongside with novel early-warning molecular biomarkers. The tissue-specific genotoxic responses obtained in the present work are related to organ location and function, tissue regeneration rate, capacity of protection against DNA damaging agents and DNA repair ability. The additional analysis performed with liver, revealed that the transcription of the checkpoint *rad1* gene, involved in the detection and repair of DNA lesions, is not induced by the levels of DNA damage observed in the hepatic cells. Additionally, no irreversible damage was detected by FCM in liver throughout the 90-day post-exposure, although important cell activities seemed to respond to DMBA exposure, including immune processes, protein translation and cytoskeleton dynamics. Alterations in these important cell processes may lead to future repercussions at organism-level of biological organization. The SSH approach also provided directions for new molecular biomarkers development. Finally, the application of novel molecular biomarkers allowed for a better understanding of the genotoxic implications of DMBA exposure in fish.

4.6. REFERENCES

Al-Sabti K, Metcalfe CD. 1995. Fish micronuclei for assessing genotoxicity in water. Mutation Research, Genetic Toxicology and Environmental Mutagenesis 343(2-3):121-135.

Bi XH, Barkley LR, Slater DM, Tateishi S, Yamaizumi M, Ohmori H, Vaziri C. 2006. Rad18 regulates DNA polymerase kappa and is required for recovery from S-phase checkpoint-mediated arrest. Molecular and Cellular Biology 26(9):3527-3540.

Brzuzan P, Jurczyk L, Foks T. 2007. Differential gene expression in benzo[a]pyrene-exposed whitefish (*Coregonus lavaretus*). Advances in Limnology 60:149-157.

Costa JL, Assis CA, Almeida PR, Moreira FM, Costa MJ. 1992. On the food of the European eel, *Anguilla anguilla* (L.), in the upper zone of the Tagus Estuary, Portugal. Journal of Fish Biology 41(5):841-850.

Diatchenko L, Lau YFC, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED and others. 1996. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proceedings of the National Academy of Sciences of the United States of America 93(12):6025-6030.

Doležel J, Binarová P, Lucretti S. 1989. Analysis of nuclear DNA content in plant cells by flow cytometry. Biologia Plantarum 31(2):113-120.

El-Zahr CR, Zhang Q, Hendricks JD, Curtis LR. 2002. Temperature-modulated carcinogenicity of 7,12-dimethylbenz[a]anthracene in rainbow trout. Journal of Toxicology and Environmental Health Part A 65(11):787 - 802.

Goanvec C, Theron M, Lacoue-Labarthe T, Poirier E, Guyomarch J, Le-Floch S, Laroche J, Nonnotte L, Nonnotte G. 2008. Flow cytometry for the evaluation of chromosomal damage in turbot *Psetta maxima* (L.) exposed to the dissolved fraction of heavy fuel oil in sea water: a comparison with classical biomarkers. Journal of Fish Biology 73(2):395-413.

Hartl MGJ. 2002. Benthic fish as sentinel organisms of estuarine sediment toxicity. In: Bright M, Dworschak P, Stachowitsch M, editors. The Vienna School of Marine Biology: A Tribute to Jörg Ott. Facultas Universitätsverlag, Wien p89-100.

Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V and others. 2003. Recommendations for conducting the in vivo alkaline Comet assay. Mutagenesis 18(1):45-51.

Helt CE, Wang W, Keng PC, Bambara RA. 2005. Evidence that DNA damage detection machinery participates in DNA repair. Cell Cycle 4(4):529-532.

Jha AN. 2004. Genotoxicological studies in aquatic organisms: an overview. Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis 552(1-2):1-17.

Levine S, Oris J. 1999. CYP1A expression in liver and gill of rainbow trout following waterborne exposure: implications for biomarker determination. Aquatic Toxicology 46:279-287.

Lo M, Wang YZ, Gout PW. 2008. The xc-cystine/glutamate antiporter: A potential target for therapy of cancer and other diseases. Journal of Cellular Physiology 215(3):593-602.

Martin-Skilton R, Coughtrie MWH, Porte C. 2006. Sulfotransferase activities towards xenobiotics and estradiol in two marine fish species (*Mullus barbatus* and *Lepidorhombus boscii*): characterization and inhibition by endocrine disrupters. Aquatic Toxicology 79(1):24-30.

Matson C, Franson J, Hollmen T, Kilpi M, Hario M, Flint P, Bickam J. 2004. Evidence of chromosomal damage in common eiders (*Somateria mollissima*) from the Baltic Sea. Marine Pollution Bulletin 49:1066-1071.

Miyata M, Furukawa M, Takahashi K, Gonzalez FJ, Yamazoe Y. 2001. Mechanism of 7,12-dimethylbenz[a]anthracene-induced immunotoxicity: role of metabolic activation at the target organ. Japanese Journal of Pharmacology 86(3):302-309.

Nogueira PR, Lourenco J, Mendo S, Rotchell JM. 2006. Mutation analysis of *ras* gene in the liver of European eel (*Anguilla anguilla* L.) exposed to benzo[a]pyrene. Marine Pollution Bulletin 52(12):1611-1616.

O'Connor A, Nishigori C, Yarosh D, Alas L, Kibitel J, Burley L, Cox P, Bucana C, Ullrich S, Kripke M. 1996. DNA double strand breaks in epidermal cells cause immune suppression in vivo and cytokine production in vitro. Journal of Immunology 157(1):271-278.

Pacheco M, Santos MA. 2001. Tissue distribution and temperature-dependence of *Anguilla anguilla* L. EROD activity following exposure to model inducers and relationship with plasma cortisol, lactate and glucose levels. Environment International 26(3):149-155.

Pacheco M, Santos MA. 2002. Biotransformation, genotoxic, and histopathological effects of environmental contaminants in European eel (*Anguilla anguilla* L.). Ecotoxicology and Environmental Safety 53(3):331-347.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29(9):2002-2007.

Reynaud S, Deschaux P. 2006. The effects of polycyclic aromatic hydrocarbons on the immune system of fish: a review. Aquatic Toxicology 77(2):229-238.

Robinet T, Feunteun E. 2002. Sublethal effects of exposure to chemical compunds: a cause for the decline in Atlantic Eels? Ecotoxicology 11:265-277.

Suzuki T, Nishiyama K, Yamamoto A, Inazawa J, Iwaki T, Yamada T, Kanazawa I, Sakaki Y. 2000. Molecular cloning of a novel apoptosis-related gene, human *Nap1* (NCKAP1), and its possible relation to Alzheimer disease. Genomics 63(2):246-254.

van der Oost R, Beyer J, Vermeulen NPE. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology 13(2):57-149.

Watabe T. 1983. Metabolic activation of 7,12-dimethylbenz[a]anthracene (DMBA) and 7-methylbenz[a]anthracene (7-MBA) by rat liver P-450 and sulfotransferase. Journal of Toxicological Sciences 8(2):119-131.

Weimer TL, Reddy AP, Harttig U, Alexander D, Stamm SC, Miller MR, Baird W, Hendricks J, Bailey G. 2000. Influence of β-naphthoflavone on 7,12-dimethylbenz(a)anthracene metabolism, DNA adduction, and tumorigenicity in rainbow trout. Toxicological Sciences 57(2):217-228.

Weltzien FA, Pasqualini C, Vernier P, Dufour S. 2005. A quantitative real-time RT-PCR assay for European eel tyrosine hydroxylase. General and Comparative Endocrinology 142(1-2 SPEC. ISS.):134-142.

Williams TD, Gensberg K, Minchin SD, Chipman JK. 2003. A DNA expression array to detect toxic stress response in European flounder (*Platichthys flesus*). Aquatic Toxicology 65(2):141-157.

Wirgin I, Waldman J. 1998. Altered gene expression and genetic damage in North American fish populations. Mutation Research 399:193-219.

5.

Anchoring novel molecular biomarker responses to traditional responses in fish exposed to environmental contamination

---

Chapter submitted as original article:
Nogueira P, Pacheco M, Pereira ML, Mendo S, Rotchell JM. 2009.
Anchoring novel molecular biomarker responses to traditional responses in fish exposed to environmental contamination.
Environmental Pollution

5.1. ABSTRACT

The responses of *Dicentrarchus labrax* and *Liza aurata* to aquatic pollution were assessed in a contaminated coastal lagoon, using both traditional and novel biomarkers combined. DNA damage, assessed by comet assay, was higher in both fish species from the contaminated sites, whereas levels of the cytochrome P450 1A1 gene expression were not significantly altered. The liver histopathological analysis also revealed significant lesions, in fish from contaminated sites. Alterations in *ras* and *xpf* genes, and identification of pollutant-responsive gene transcripts were additionally analysed. While no alterations were found in *ras* gene, a down-regulation of *xpf* gene was observed in *D. labrax* from a contaminated site. Suppression subtractive hybridization applied to *D. labrax* collected at a contaminated site, revealed altered expression in genes involved in energy metabolism, immune system activity and antioxidant response. The approach and results reported herein demonstrate the utility of anchoring traditional biomarker responses alongside novel biomarker responses.

Keywords

Pollution, biomarker, toxicology, fish

5.2. INTRODUCTION

Aquatic organisms are exposed to a wide variety of environmental contaminants and it is usual to employ a suite of biomarkers to assess the possible biological impacts. Such biomarkers act as an early warning of a specific detrimental biological endpoint and the mechanistic cause and effect relationship is known. Histopathological analysis, DNA integrity and detoxification enzyme status in fish tissues, combined and singly, have frequently been employed as such biomarkers, each regarded as established early warnings of tumour development, particularly in the liver.

Fish liver plays a major role in the uptake, biotransformation and excretion of pollutants (Gernhofer *et al.* 2001). Cytochrome P450 1A (CYP1A) protein converts lipophilic xenobiotics to more water-soluble compounds promoting excretion and detoxification (van der Oost *et al.* 2003). However, some metabolites produced are highly reactive and ultimately more toxic, affecting the structural integrity of DNA (Shugart 2000). DNA damage has been associated with the carcinogenesis process and perturbations in fecundity, longevity and growth of affected organisms that may have repercussions on population-level effects (Theodorakis *et al.* 2000). In environmental monitoring, such biomarkers have been routinely used as an early warning, yet the specific and ultimate impact are rarely established. Here we add two molecular biomarkers to fill that gap in knowledge by determining whether the damaged DNA is repaired or leads to mutations, the first of many steps in the possible progression to cancer. We also adopt an exploratory approach in parallel to scan the horizon for complementary molecular biomarkers in the same samples.

To conserve genomic stability, cells have mechanisms to detect and repair damaged DNA (Bi *et al.* 2006). Bulky adducts, such as those induced by polycyclic aromatic hydrocarbons (PAHs), are repaired by the nucleotide excision repair (NER) mechanism. This pathway removes sections of adducted DNA via a multiple step process involving numerous proteins (Notch *et al.* 2007), including Xeroderma Pigmentosum group F (XPF). In fish, DNA repair mechanisms are less well studied, compared to mammals, beyond sequencing of a small number of relevant genes (Rotchell *et al.* 2008), and two investigations of NER gene

expression in aquaria-held *Danio rerio* (Notch *et al.* 2007) and *Xiphophorus* sp. (David *et al.* 2004).

Any defect in DNA repair mechanisms leads to genomic instability, either as mutations or other genetic alterations. The *ras* gene, one of the most important genes involved in carcinogenesis, has been found to be mutated in fish from areas of high PAH contamination (Rotchell *et al.* 2001). These mutations often occur at 'hot spots' in codons 12, 13 and 61 producing altered proteins that lead to deregulated activation of Ras protein (Rotchell *et al.* 2001). Ras protein is critical in processing external messages to the cell, playing a central role in transduction pathways involved in cell growth, differentiation and apoptosis (Rotchell *et al.* 2001).

Here we combine traditional (histopathology, *cyp1A* expression and Comet assay) with further molecular biomarker responses (*xpf* and *ras*), in an integrated approach to environmental biological effects monitoring, in two species of feral fish (*L. aurata* and *D. labrax*) living in a contaminated coastal lagoon, Ria de Aveiro. The former has a feeding behaviour characterized by regular contact with sediment (Pacheco *et al.* 2005). *D. labrax*, in contrast, is a top predator which spends most of its time in the water column. Both species have high economic importance and have demonstrated to be very sensitive species for assessing genotoxicity and biotransformation responses to several aquatic contaminants (Gravato and Santos 2003; Pacheco *et al.* 2005). Ria de Aveiro is a shallow estuarine coastal lagoon that receives multiple anthropogenic discharges, being contaminated with PAHs, organometallic compounds and heavy metals (Mieiro *et al.* 2009; Pacheco *et al.* 2005; Sousa *et al.* 2007). We also adopt an exploratory approach using suppression subtractive hybridization (SSH) to isolate differentially expressed transcripts (Diatchenko *et al.* 1996). The aim is to identify novel molecular responses from as yet uncharacterised complex changes that occur at the level of gene expression, either as a direct or compensatory response, to molecular damage or cellular dysfunction in the same fish samples, and in doing so, anchoring any newly characterised responses to traditional biomarker responses.

5.3. MATERIALS AND METHODS

5.3.1 Study area and sampling

Juvenile specimens (n=5) of *D. labrax* of average length 15.4 ± 1.8 cm and weight 47.5 ± 14.8 g, and *L. aurata* with an average length of 13.1 ± 1.7 cm and a weight of 25.9 ± 12.7 g, were collected in May 2005 from Ria de Aveiro, Portugal. The sampling sites were located along the main channels and at the lagoon entrance (Figure 5.1), and included various types and sources of contamination: Laranjo (LAR) close to a chlor–alkali plant, an important source of mercury contamination; Rio Novo do Principe (RIO) , 6 km from a pulp/paper mill effluent outlet; Gafanha (GAF) situated in the vicinity of a deep-sea fishing port and dry-docks, also receiving domestic discharges; and Vagos (VAG) receiving municipal and domestic effluents. S. Jacinto (SJA) located close to the lagoon entrance and far from the main pollution sources was used as reference site. Immediately after sampling, fish were sacrificed and blood was collected. Livers were removed and a portion fixed in Bouin's solution for histopathological analysis. The remaining liver was immediately frozen in liquid nitrogen and stored at -70 ºC until further analysis.

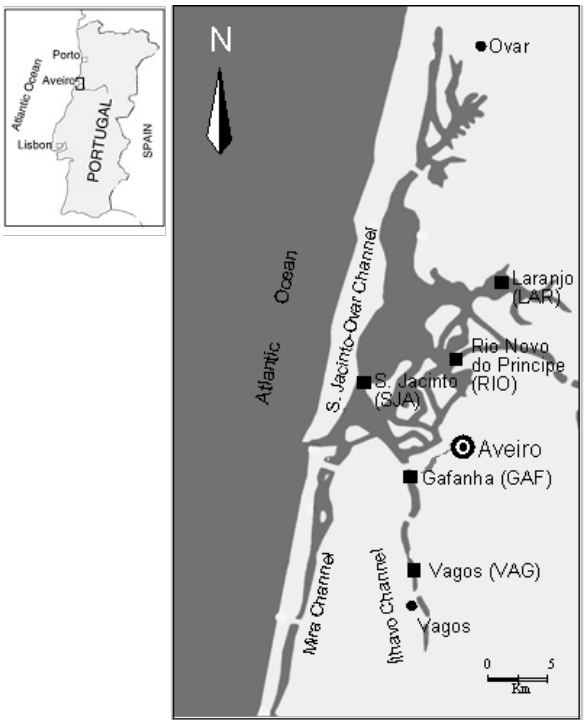

Figure 5.1. Map of the sampling stations (■) in the Ria de Aveiro.

5.3.2. Traditional biomarker responses

5.3.2.1. Liver histopathology

The liver samples fixed in Bouin's solution were embedded in paraffin for light microscopy. Sections of 3-5 µm thick were stained with hematoxylin and eosin (HE). Histopathological alterations of the liver were evaluated qualitatively and semiquantitatively using a modified version of the protocol described by Bernet *et al.* (1999).

5.3.2.2. Evaluation of DNA integrity by comet assay

The alkaline version of comet assay was conducted with whole blood under yellow light to prevent UV-induced DNA damage and as described in Nogueira *et al.* (2006). Slides were examined under a fluorescence microscope (Leica DMLS, Germany) and fifty randomly selected cells per slide (2 slides/animal) were analysed using the image analysis software Comet Assay IV (Perceptive Instruments, UK). DNA damage was expressed as percentage tail DNA (% tDNA) and presented as mean of medians ± standard error of the mean (SEM).

5.3.2.3. Determination of *cyp1A1* gene expression levels

cyp1A1 gene expression levels were analysed in *D. labrax* and *L. aurata* liver samples by semiquantitative reverse transcriptase-PCR (sqRT-PCR). Total RNA was extracted from liver using RNeasy reagents and treated with RNA-free DNase I (Qiagen, UK). First strand cDNA was synthesised from 1 µg of total RNA in the presence of oligo d(T) primers using Superscript reagents (Invitrogen, UK). The cDNAs were amplified by platinum®*Pfx* DNA polymerase (Invitrogen, UK) using *cyp1A1* gene specific primers (Cyp1AF/Cyp1AR, Table 5.1) producing a 295 bp fragment. To normalize for initial variations in sample concentration and differences in efficiency during amplification, 28S rRNA primers (Table 5.1, primers 28SF/28SR) were also included in each reaction to amplify a 100 bp fragment as internal standard. Amplifications were performed with a BioRad iCycler ™ in 25 µl reaction volumes, for 30 cycles at 94 ºC for 20 s, 58 ºC for 20 s and 68 ºC for 20 s, followed by a final 2 min extension at 68 ºC. The number of cycles was optimized in order to stop the reaction in the logarithmic phase of

amplification. Ten microliters of each PCR product were used for agarose gel electrophoresis (1.5 % agarose, TAE buffer). cDNA amounts were quantified by densitometric analysis using Quantity One software (BioRad, USA). The results were expressed in arbitrary densitometric units after background subtraction. The ratio between the sample RNA to be determined and 28S was calculated, and the results presented as mean ± SEM for each sampling site.

Table 5.1. PCR primer sequences.

Gene	Primer	Sequence (5´- 3')	GenBank Access. No.
ras	RasF1	ATGACNGAATATAAGCTGG	Various
	RasR1	GTTGATGGCGAAGACGCA	Various
	RACE3ras	CGGAGAGACGTGTCTGCTGGACATCC	FJ529376
	RACE5ras	GGATGTCCAGCAGACACGTCTCTCCG	FJ529376
	RasF2	ATGACCGAGTATAAGCTGGT	FJ529376
	RasR2	CCTGTCCTCATGTACTG	FJ529376
	RASmulF	CCCTCACCATCCAGCTCA	FJ529377
	RASsbF	GGCAGAGCGCACTTACTA	FJ529376
xpf	XpfF1	GTTTTGTYCGCCAGCTGG	Various
	XpfR1	TGACGGGCTCGATGTCCA	Various
	RACE3xpf	CGGAGGCTCGACAGAAGAACAGA	FJ438473
	RACE5xpf	AGGTCCAGGTTGGTGTCTTCTCGTC	FJ438473
28S	28SF	GCCTGAGTCCTTCTGATCGAG	AY141637
	28SR	TCCCAAACAACCCGACTCCGAG	AY141637
cyp1A	Cyp1AF	GTCTTCATCAATCAGTGGCAG	U78316
	Cyp1AR	CAGCGTTTGTGCTTCATTGTGA	U78316
Real-time PCR primer sequences			
18S		For: TGTGCCGCTAGAGGTGAAAT Rev: GCATCGTTTATGGTCGGAAC	AM419038
XPF		For: GGAAAGCCACTCAGGGTGTA Rev: CCTCCCTCTCCTCTGGTATG	FJ438473
A2MG		For: GCTGACCCCAGCTATCAAAG Rev: GTTTCCTGCTCCTGATCCAA	GH196448
HGF		For: GGACCAGACCATCCCAGTAA Rev: GGGTCTCCTTGGCACAGTAA	GH196449
ITIH2		For: TGAGTTCCGGGACGATGTAA Rev: GCTGGCAAAAGGACTACAGG	GH196468
BDH		For: CCTGACCCAGAACAGGCTTA Rev: CAGTCACATAGGCCGACTCA	GH196471

5.3.3. Development of novel biomarkers of exposure in *D. labrax* and *L. aurata*

5.3.3.1. Analysis of *ras* gene mutations

The normal *D. labrax* and *L. aurata ras* gene sequences were isolated from the liver of animals collected at a relatively clean site (SJA). cDNA synthesised as described in section 5.3.2.3 was the template for the amplification of *ras* gene fragments using 1 U of platinum®*Taq* DNA polymerase (Invitrogen, UK) and the primer pairs RasF1/RasR1 (Table 5.1), which in turn were designed based on other fish published sequences. The 255 bp fragments were amplified in a BioRad iCycler™ thermal cycler using 35 cycles at 94 ºC for 30 s, 50 ºC for 40 s, 72 ºC for 60 s, followed by a final 2 min extension at 72 ºC. The resulting nucleotide sequence served as a starting point for gene specific primer design. The isolation of the complete 5' and 3' ends of *ras* genes was performed using liver total RNA (1 µg) and SMART™ RACE cDNA amplification reagents and protocol (Clontech, France). The gene specific primers (Table 5.1) used in the procedure were RACE3ras (3' end) and RACE5ras (5' end). The RACE products obtained were analysed on an agarose gel, excised and purified with Qiaquick columns (Qiagen, UK). DNA was ligated into a pGEM® T-Easy cloning vector (Promega, UK). Recombinant plasmids were transformed and selected using ampicillin LB plates. Plasmid DNA was purified for DNA sequence analysis using commercial sequencing (Eurofins MWG Operon, Germany) to verify the identity of the products.

The analysis of *ras* gene mutations was carried out for all *D. labrax* and *L. aurata* liver samples. cDNA was used as template to amplify exon 1 and part of exon 2 of *D. labrax* and *L. aurata ras* genes. The 50 µl PCR reaction was performed in reaction buffer with 400 µM each deoxynucleoside triphosphate, 50 pmol each specific primer (RasF2/RasR2, Table 5.1), 3 µl of cDNA and 1 U platinum®*Taq* DNA polymerase (Invitrogen, UK). The 224 bp *ras* fragment was amplified in a BioRad iCycler™ using 35 cycles at 94 ºC for 30 s, 55 ºC for 40 s, 72 ºC for 30 s, followed by a final 2 min extension at 72 ºC. Amplified fragments were directly sequenced (Eurofins MWG Operon, Germany) in both directions in order to identify and characterise any mutations present.

5.3.3.2. Determination of *ras* gene expression levels

The expression levels of *ras* gene were analysed for all liver samples using the sqRT-PCR method as described in section 5.3.2.3. Primers RASmulF/RasR2 for *L. aurata* samples and RASsbF/RasR2 for *D. labrax* (Table 5.1) were used in the PCR reaction producing 203 bp and 213 bp *ras* fragments respectively. 28S rRNA was used as an internal standard.

5.3.3.3. Determination of *xpf* gene expression levels

The isolation of *xpf* gene was carried out as described in section 5.3.3.1. The primers used in the amplification of the first *xpf* fragment were XpfF1/XpfR1 (Table 5.1). The sequenced *xpf* fragments for both species were the basis for the design of specific primers used in RACE amplification (RACE3xpf/RACE5xpf, Table 5.1).

The analysis of *xpf* expression levels was performed in *D. labrax* samples from the sampling sites SJA and GAF, using real-time quantitative PCR (qPCR). *D. labrax* 18S rRNA *(*18S*)* was used as an internal control. First-strand cDNA was synthesized from 1 µg of RNA (obtained as described in section 5.3.2.3.) using SuperScript® Vilo™ cDNA synthesis kit (Invitrogen, UK). qPCR reactions were performed in a final volume of 25 µl containing 12.5 µl of Express SYBR® GreenER™ qPCR SuperMix (Invitrogen, UK), 5 µl of diluted (1:100) cDNA, 3.75 µM primers (Table 5.1) and 0.1 µl of ROX reference dye. A control lacking cDNA template was included in qPCR analysis to determine the specificity of target cDNA amplification. Amplification was detected using the Mx3005P real time PCR system (Stratagene, UK) and the following cycling parameters: 50 ºC for 2 min, 95 ºC 10 min, 40 cycles of 30 s at 95 ºC, 1 min at 60 ºC and 30 s at 72 ºC. Melting curves were performed to identify the presence of primer dimers and analyse the specificity of the reaction. The amplification efficiency of each primer pair was calculated using a dilution series of cDNA. The relative expression levels of target genes were calculated according to the efficiency-corrected method described by Pfaffl (2001). The reference site group (SJA) was used as calibrator.

5.3.3.4. Suppression subtractive hybridization (SSH)

The SSH procedure was used to isolate and enrich for genes differentially-expressed between *D. labrax* captured at the reference site and at a contaminated site (GAF). For each group, equal amounts of RNA were pooled (5 fish in each group). cDNA was synthesised using SuperSMART PCR cDNA Synthesis kit (Clontech, France). The forward- and reverse-subtracted libraries were produced using PCR-Select cDNA Subtraction reagents (Clontech, France) according to the manufacturer's protocol. The differential PCR products generated were inserted in a pGEM®-T Easy vector (Promega, UK) and cloned into JM109 bacterial cells (Promega, UK). Sixty randomly selected colonies from each subtracted library were inoculated in LB broth and screened by PCR for inserts using vector-based primers. A total of 20 clones per library were randomly selected for sequencing (Eurofins MWG Operon, Germany) directly from the PCR product. Sequence identities were obtained by BLAST searches against the NCBI nucleic acid and protein databases. Sequence reads with *E*-value > 10^{-5} were filtered out. The differential screening result was confirmed by qPCR as described in section 5.3.3.3, using four candidate genes (Table 5.1): alpha-2-macroglobulin (A2MG), HGF activator like protein (HGFA), inter-alpha globulin inhibitor H2 (ITIH2) and 3-hydroxybutyrate dehydrogenase (BDH).

5.3.4. Statistical analysis

For statistical analysis (SigmaStat 3.11 Software), all data were tested for normality and homogeneity of variances and, when these assumptions were met, differences between control and exposed fish were determined using analysis of variance (ANOVA), followed by Tukey's test ($P < 0.05$).

5.4. RESULTS

5.4.1. Traditional biomarkers

5.4.1.1. Liver histopathology

A macroscopic examination of liver samples was carried out *in situ*. Alterations in the general appearance of the liver, the presence of any macroscopically visible nodules or other lesions were not observed in any fish.

Signs of parasite infection were also not evident. The histopathological liver injury indices varied between 12.6 and 21.8 in *D. labrax*, and from 15.0 to 39.0 in *L. aurata* (Figure 5.2.A). A significant difference in liver injury index was observed in *L. aurata* from all the contaminated sites when compared to the reference site (Figure 5.2.A). Statistically different liver indices were also observed in *D. labrax* specimens from RIO and GAF (Figure 5.2.A). Both species from the reference site showed a normal liver architecture with hepatocytes presenting a homogeneous cytoplasm and a large spherical nucleus containing one nucleolus (Figure 5.3.A and D). *L. aurata* also presented bile ducts scattered irregularly throughout the parenchyma, while these elements were more rarely seen in the analysed liver sections of *D. labrax*. The main alterations found in *L. aurata* liver were karyomegaly and pyknosis, leukocyte infiltration, haemorrhage and areas of necrosis (Figure 5.3.E and F). Melano-macrophages centres (MMC) were also more abundant in *L. aurata* collected at contaminated sites than in the reference site. Additionally, specimens from GAF and VAG showed alterations in the bile ducts, such as pyknosis, inflammation, presence of MMC, and, in the worst cases, destruction of some bile ducts (Figure 5.3.E). In general, the liver of *D. labrax* was less damaged than *L. aurata* liver. The main alterations in *D. labrax* included nuclear changes (karyomegaly and pyknosis), some haemorrhage and small areas of necrosis (Figure 5.3.B and C). Vacuolar degeneration was also observed in *D. labrax* from RIO and VAG, and, to a smaller extent, GAF. A strong leukocyte infiltration was additionally observed in specimens from RIO and GAF.

5.4.1.2. Evaluation of DNA integrity by comet assay

DNA damage levels measured in blood cells by comet assay are shown in Figure 5.2.B. Of note, *D. labrax* from the GAF sampling site showed a 3-fold significant increase in DNA damage compared with the reference site (Figure 5.2.B). *D. labrax* from RIO and *L. aurata* from GAF and RIO also presented significant higher levels of DNA damage (Figure 5.2.B). The levels measured in *L. aurata* from contaminated sites were higher than those observed in *D. labrax*. Nevertheless, the differences observed between contaminated and reference site animals were higher in *D. labrax*.

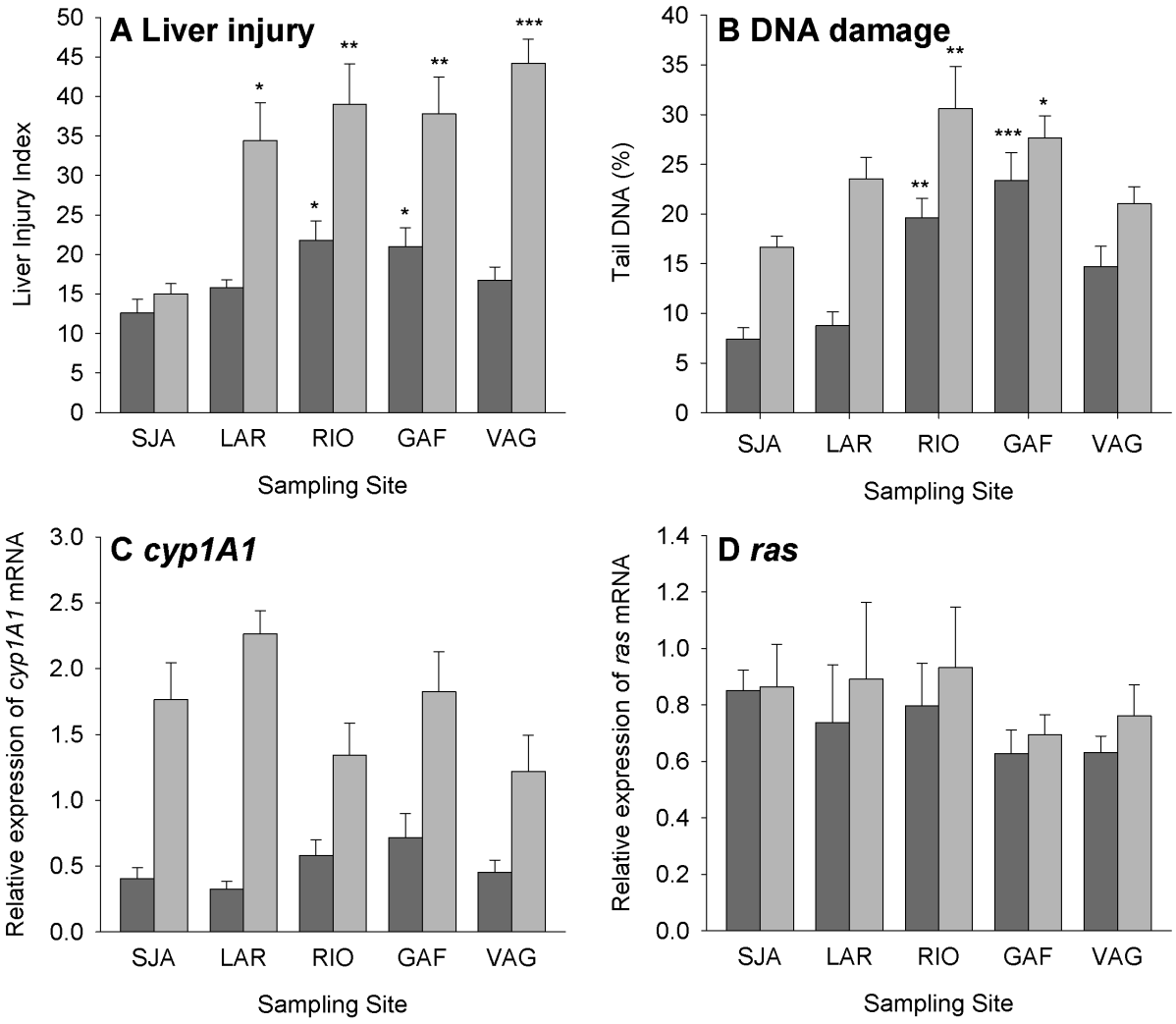

Figure 5.2. Responses measured in *D. labrax* (dark grey bar) and *L. aurata* (light grey bar) captured from contaminated sites in Ria de Aveiro. Data are expressed as means ± SEM (n = 5 animals). **A:** Liver injury index; **B:** DNA damage expressed as tail length (%) in blood cells; **C:** Relative hepatic *cyp1A1 g*ene expression; **D:** Relative hepatic *ras* gene expression. Asterisks denote a statistically significant difference from the reference site group: * P < 0.05; ** P < 0.01; *** P < 0.001.

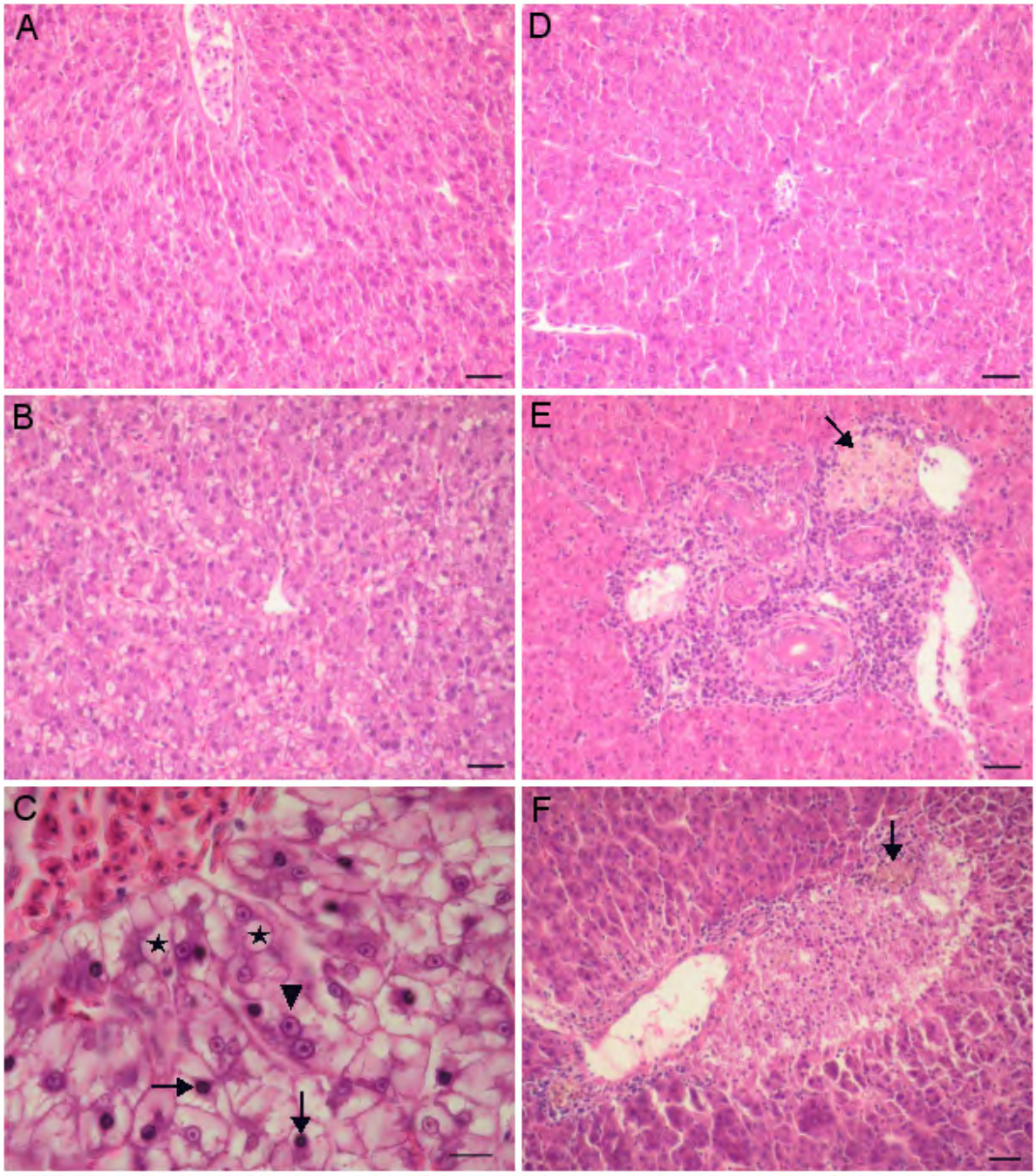

Figure 5.3. Histopathology analysis of the liver of *D. labrax* (**A, B, C**) and *L. aurata* (**D, E, F**): (**A,D**) normal hepatic tissue without pathological alterations; (**B**) Cytoplasmic vacuolization of hepatocytes; (**C**) Hepatocyte vacuolization (star) with nuclear pyknosis (black arrow) or karyomegaly (arrow head); (**E**) Inflammation and degeneration of bile ducts associated with a melano-macrophages centre (black arrow); (**F**) Hepatic tissue degeneration associated with a melano-macrophages centre (black arrow). HE stain, scale bars = 20 µM (A, B, D-F); 10 µM (C).

5.4.1.3. Determination of *cyp1A1* gene expression levels

The analysis of *cyp1A1* gene expression levels in individual samples using sqRT-PCR revealed no significant variation between animals from the contaminated and reference sites (Figure 5.2.C). A high inter-individual variability was observed in both species.

5.4.2. Development of novel biomarkers of exposure in *D. labrax* and *L. aurata*

5.4.2.1. Analysis of *ras* gene mutations and expression levels

The *D. labrax ras* cDNA contained a complete open reading frame (ORF) of 567 nucleotides (GenBank accession no. FJ529376) encoding a 188 amino acid protein. For *L. aurata,* two *ras* cDNAs were isolated corresponding to the two alternative spliced *Ki-ras* genes: *K-ras-1* (GenBank accession no. FJ529377) with an ORF of 567 nucleotides encoding 188 amino acids, and *K-ras-2* (GenBank accession no. FJ529378) containing a complete ORF of 570 nucleotides encoding a 189 predicted amino acid sequence. No mutations were found in the *ras* fragments screened in either species though polymorphic variation was observed. No differences in *ras* gene expression levels were observed between fishes from different sites (Figure 5.2.D).

5.4.2.2. Determination of *xpf* gene expression levels

xpf genes from *D. labrax* and *L. aurata* contained an ORF of 2700 and 2694 nucleotides, respectively (GenBank accession no. FJ438473 and FJ438474), revealing a predicted amino acid sequence of 899 and 897 residues. Both genes showed a high homology between them (91 %) and more than 75 % identity with other fish sequences. *xpf* gene expression was studied in the liver samples of *D. labrax* from the sampling site GAF, where the most significant increase of DNA damage was observed as measured by comet assay. The qPCR method using individual, not-pooled, samples revealed a statistically significant ($P < 0.05$) downregulation of this gene (0.44) when compared to the expression levels in *D. labrax* from the reference site (SJA).

Table 5.2. Genes differentially expressed in liver of *D. labrax* from contaminated (GAF) and reference areas (sequences showing no similarity to genes with known functions are not included). Asterisk (*) denotes a statistically significant difference from the reference site group ($P < 0.05$).

Clone Access. No.	Species	Putative Identity	Blast *E*-value	Relative Expression
Upregulated genes				
GH196447	*Paralichthys olivaceus*	Complement component C3	6E-69	-
GH196448	*Sparus aurata*	Alpha-2-macroglobulin	6E-81	3.57 *
GH196449	*Ornithorhynchus anatinus*	Similar to HGF activator like protein	5E-38	4.39 *
GH196450	*Oncorhynchus mykiss*	Secreted phosphoprotein 24	2E-29	-
GH196451	*Catostomus commersonii*	Cytochrome c oxidase subunit III	3E-51	-
GH196452	*Lepidocybium flavobrunneum*	Cytochrome c oxidase subunit I	9E-71	-
GH196454	*Danio rerio*	Immune-related, lectin-like receptor 2	3E-10	-
GH196453	*Danio rerio*	Electron-transfer-flavoprotein alpha polypeptide	1E-64	-
GH196456	*Danio rerio*	Cytochrome c oxidase subunit VIIc	1E-27	-
GH196457	*Salmo salar*	Apolipoprotein B	6E-6	-
GH196458	*Zu cristatus*	NADH dehydrogenase subunit 1	2E-51	-
Downregulated genes				
GH196461	*Salmo salar*	Ribosome biogenesis protein RLP24	8E-70	-
GH196463	*Epinephelus coioides*	Copper/zinc superoxide dismutase	9E-78	-
GH196466	*Zebrasoma flavescens*	Mitochondrial DNA	4E-138	-
GH196467	*Salmo salar*	Serum lectin isoform 2 precursor	3E-19	-
GH196460	*Oplegnathus fasciatus*	Apolipoprotein A-I (apoA-I) mRNA	6E-178	-
GH196462	*Pagrus major*	Transferrin mRNA	2E-59	-
GH196468	*Danio rerio*	Inter-alpha globulin inhibitor H2 mRNA	6E-18	0.73 *
GH196469	*Xenopus laevis*	Stromal cell derived factor 4, mRNA	4E-10	-
GH196471	*Salmo salar*	3-hydroxybutyrate dehydrogenase, type 2	6E-29	0.85 *

5.4.2.3. Suppression subtractive hybridization

Eleven sequences from each forward (up-regulated genes) and reverse (down-regulated genes) libraries could be matched to genes from different organisms, mainly fish species (Table 5.2). The remaining sequenced clones showed homology to unidentified hypothetical or novel proteins or showed no homology with the sequences deposited in the database.

The SSH results were validated by qPCR, normalized to 18S rRNA, using two of the genes highlighted for each library (Table 5.2). Up-regulation of A2MG and HGFA-like genes, and down-regulation of ITIH2 and BDH genes were confirmed by qPCR (Table 5.2).

5.5. DISCUSSION

In summary, we have reported liver cellular damage; gross DNA damage; and changes, though no obvious trend, in detoxification enzyme *cyp* gene expression in two species of fish from selected sites (Fig. 1. RIO and GAF) of environmental contamination. Based on those traditional biomarker responses one may conclude that the fish at RIO and GAF sampling sites are impacted detrimentally by the contamination present. At the next level of biological effects, which probes the actual significance of this damage, no *ras* gene mutations or changes in *ras* gene expression were observed, yet a decrease in *xpf* gene expression was observed (at GAF).

The actual significance, in terms of future repercussions at higher levels of biological organisation of the apparent contaminant-induced biological effects thus becomes less clear. Based on the *ras* gene expression and mutation results, admittedly only one of many genes implicated in the carcinogenesis process, yet usually a key player, one would have to conclude that the gross DNA damage observed at RIO and GAF, is either repaired or is not necessarily affecting important genes involved in cell cycle control and regulation. The targeted, mechanistic approach combining traditional biomarkers with two new specific gene biomarkers adopted herein, thus provided contrasting results.

The exploratory global approach using SSH also highlights the complexity in response observed in fish sampled from a contaminated sampling site compared with fish sampled from a reference site. Detoxification and cell growth genes were highlighted as differentially expressed in impacted fish, yet genes from other processes, particularly the immune response, also appear to be important in considering the wider implications of contaminant exposure.

5.5.1. Fish liver histopathological responses to contamination

Two of the main alterations observed in both species were necrotic foci and inflammatory processes. These alterations have also been described in other species of fish living in contaminated environments (Nero *et al.* 2006) suggesting that these alterations might be related to the exposure to environmental chemicals present. Nuclear alterations such as karyomegaly and pyknosis were also found to

be more pronounced in the fish captured from LAR, GAF and RIO. Nuclear changes have been viewed as early toxicopathic lesions resulting from exposure to toxic and carcinogenic chemicals (Nero *et al.* 2006). MMCs, focal accumulations of pigmented macrophages associated with a degenerative-necrotic condition, have been used as nonspecific indicators of stress that can reflect starvation, contaminant exposure, environmental stress or age of an organism (Teh *et al.* 1997). Here, the number and size of MMCs increased in *L. aurata* collected from all the contaminated sites, consistent with reports for other fish species captured from contaminated areas (Teh *et al.* 1997). Necrotic foci, inflammation, nuclear alterations and MMC, were also present in the liver of *D. labrax*, but less pronounced than in *L. aurata*. In addition to these alterations, a vacuolar degeneration was observed in *D. labrax* from RIO, VAG and GAF, again consistent with other fish species living in harbour areas and contaminated rivers (Teh *et al.* 1997).

The analysis of the histopathology of the liver suggests that *D. labrax* liver is being less affected by aquatic contamination than *L. aurata*. While *D. labrax* is a predator of small fishes and feeds in the water column, *L. aurata* is feeding on benthic organisms and is in a regular contact with the sediments where the majority of the contaminants accumulate. In this way, *L. aurata* may be in contact with higher concentrations of contaminants which may lead to an increased risk of damage to the liver as observed in this study.

5.5.2. Targeted molecular responses to contamination

Both species of fish collected at GAF and RIO sampling sites showed significant increases of DNA damage when compared to the reference populations (SJA) (Figure 5.2.B). One cause for the significant levels of DNA damage observed may be the action of highly reactive xenobiotic metabolites following liver metabolism.

No significant differences in *cyp1A* gene expression were found between the fish populations analysed. However, a high inter-individual variability was observed, which may be due to natural variation (Cajaraville *et al.* 2000). Another possible explanation is that, in the natural environment, a mixture of both inducers

and inhibitors of CYP1A system may act simultaneously, and any CYP1A regulation (up or down) is likely to be the net result of additive, synergistic or antagonist chemical interactions (Hartl *et al.* 2007). Anderson et al (2003) also observed an absence of CYP1A induction in smallmouth bass collected from a polychlorinated biphenyl contaminated area.

The induction of DNA damage in fish from contaminated environments may lead to mutations, which contribute towards the multistage carcinogenesis process. Oncogenes, such as *ras*, activated by point mutations, have been identified as genetic targets both in feral fish from heavy contaminated areas as in laboratory-exposed fish (Rotchell *et al.* 2001). The *ras* sequences obtained displayed all the conserved structural domains of the gene and showed a high degree of similarity with other fish *ras* genes characterized to date. Polymorphic variation was observed in the *ras* of *D. labrax* and *L. aurata*, mainly in the third position of the codons, without changing the predicted amino-acid sequence and is consistent with *Anguilla anguilla* (Nogueira *et al.* 2006), *Oryzias latipes* (Liu *et al.* 2003) and *Oncorhynchus gorbuscha* (Cronin *et al.* 2002).

Screening for *ras* mutations in hot spots of the gene revealed no mutations in the fish collected from the contaminated sites. Alteration in the expression of this gene was analysed using sqRT-PCR but no statistically significant differences were detected. Nevertheless, neither of the species used in this study is prone to tumour formation and few studies have demonstrated *ras* mutations or altered gene expression in normal tissues from species that are not susceptible to neoplasia (Roy *et al.* 1999). *D. labrax* and *L. aurata* may have also efficiently repaired any mutation in the *ras* gene or in any other part of their genome, through mechanisms that did not involve the induction of *xpf* gene expression.

Relevantly, a general lack of information on DNA repair mechanisms still exists for aquatic species, including DNA sequences and the way they might be affected after exposure to environmental contamination. The *xpf* gene was isolated from *D. labrax* and *L. aurata* and the sequences obtained revealed a high homology with the human gene. The conservation of the gene between species highlights the likely importance of XPF protein in the cell. Decreased *xpf* gene expression was observed in the fish sampled at the contaminated site (GAF)

compared with those from the reference site (SJA), suggesting that contamination may reduce the repair capacity of fish and in this way increase the risk for genomic instability. Nevertheless, a correlation between transcriptional and protein changes needs to be verified, to confirm this result. Notch and colleagues (2007) also reported a significant decrease in gene expression in several hepatic NER components, including *xpf*, in zebrafish liver after exposure to environmentally relevant concentrations of 17α-ethinylestradiol. Both findings seem to suggest that *xpf* gene is a good candidate for biomarker development.

5.5.3. Global molecular responses to contamination

The SSH approach revealed differently expressed genes (Table 5.2) some of which share roles in common physiological processes. Genes related to energy production and ATP metabolism (cytochrome c oxidase, electron-transfer-flavoprotein, and NADH dehydrogenase genes) and genes involved in the inflammatory and innate immune response (e.g. complement component C3, alpha-2-macroglobulin and lectin-like receptor 2 genes) have been found to be upregulated in the liver of *D. labrax* from the contaminated sampling site (GAF). This result is consistent with the literature in that genes involved in these processes were also found induced in fish exposed to contamination (Marchand *et al.* 2006; Williams *et al.* 2003). The hepatocyte growth factor activator (HGFA) was also found upregulated. This gene encodes a protein important for the activation of the hepatocyte growth factor which is involved in the regeneration of injured liver tissue. The upregulation of HGFA gene observed in *D. labrax* from the contaminated site suggests an injured liver associated with the exposure to contamination, a finding supported by the liver histopathology alterations observed in this study. HGFA gene has also been found induced in the liver of other fish species following contaminant-induced tissue injury (Marchand *et al.* 2006; Roling *et al.* 2004), and seems thus to be a good indicator of liver tissue injuries. Some of the genes found downregulated in the exposed *D. labrax* include transferrin and copper/zinc superoxide dismutase (SOD) genes which share a role in the antioxidant defence system.

Although these transcriptional changes may not give accurate information of functional changes at the protein level and thus do not allow a mechanistic interpretation, they are a response of the organism to environmental stress. In this respect, gene expression biomarkers may be used to indicate exposure to toxicants, thus acting as early warning sensors.

5.6. CONCLUSIONS

Here, novel early-warning molecular biomarkers, alongside traditional biomarker responses, were studied in *D. labrax* and *L. aurata* exposed to environmental contamination. The *xpf* and *ras* biomarkers added important information regarding genome stability, while the SSH allowed a global approach and provided directions for new biomarker response development. Both, in turn, facilitated a better understanding of the pollutant-induced biological effects and potential future repercussions at higher levels of biological organisation.

5.7. REFERENCES

Anderson MJ, Cacela D, Beltman D, Teh SJ, Okihiro MS, Hinton DE, Denslow N, Zelikoff JT. 2003. Biochemical and toxicopathic biomarkers assessed in smallmouth bass recovered from a polychlorinated biphenyl-contaminated river. Biomarkers 8(5):371-393.

Bernet D, Schmidt H, Meier W, Burkhardt-Holm P, Wahli T. 1999. Histopathology in fish: proposal for a protocol to assess aquatic pollution. Journal of Fish Diseases 22(1):25-34.

Bi XH, Barkley LR, Slater DM, Tateishi S, Yamaizumi M, Ohmori H, Vaziri C. 2006. Rad18 regulates DNA polymerase kappa and is required for recovery from S-phase checkpoint-mediated arrest. Molecular and Cellular Biology 26(9):3527-3540.

Cajaraville MP, Bebianno MJ, Blasco J, Porte C, Sarasquete C, Viarengo A. 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Science of the Total Environment 247(2-3):295-311.

Cronin M, Wickliffe J, Dunina Y, Baker R. 2002. K-*ras* oncogene DNA sequences in pink salmon in streams impacted by the Exxon Valdez oil spill: no evidence of oil-induced heritable mutations. Ecotoxicology 11(4):233-41.

David WM, Mitchell DL, Walter RB. 2004. DNA repair in hybrid fish of the genus *Xiphophorus*. Comparative Biochemistry and Physiology Part C Toxicology & Pharmacology 138(3):301-309.

Diatchenko L, Lau YFC, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED and others. 1996. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proceedings of the National Academy of Sciences of the United States of America 93(12):6025-6030.

Gernhofer M, Pawert M, Schramm M, Müller E, Triebskorn R. 2001. Ultrastructural biomarkers as tools to characterize the health status of fish in contaminated streams. Journal of Aquatic Ecosystem Stress and Recovery 8(3-4):241-260.

Gravato C, Santos MA. 2003. *Dicentrarchus labrax* biotransformation and genotoxicity responses after exposure to a secondary treated industrial/urban effluent. Ecotoxicology and Environmental Safety 55(3):300-306.

Hartl MGJ, Kilemade M, Sheehan D, Mothersill C, O'Halloran J, O'Brien NM, van Pelt FNAM. 2007. Hepatic biomarkers of sediment-associated pollution in juvenile turbot, *Scophthalmus maximus* L. Marine Environmental Research 64(2):191-208.

Liu Z, Kullman SW, Bencic DC, Torten M, Hinton DE. 2003. *ras* oncogene mutations in diethylnitrosamine-induced hepatic tumors in medaka (*Oryzias latipes*), a teleost fish. Mutation Research 539:43–53.

Marchand J, Tanguy A, Charrier G, Quiniou L, Plee-Gauthier E, Laroche J. 2006. Molecular identification and expression of differentially regulated genes of the European flounder, *Platichthys flesus*, submitted to pesticide exposure. Marine Biotechnology 8(3):275-294.

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

Nero V, Farwell A, Lister A, Van Der Kraak G, Lee LEJ, Van Meer T, MacKinnon MD, Dixon DG. 2006. Gill and liver histopathological changes in yellow perch (*Perca flavescens*) and goldfish (*Carassius auratus*) exposed to oil sands process-affected water. Ecotoxicology and Environmental Safety 63(3):365-377.

Nogueira PR, Lourenco J, Mendo S, Rotchell JM. 2006. Mutation analysis of *ras* gene in the liver of European eel (*Anguilla anguilla* L.) exposed to benzo[a]pyrene. Marine Pollution Bulletin 52(12):1611-1616.

Notch EG, Miniutti DM, Mayer GD. 2007. 17α-Ethinylestradiol decreases expression of multiple hepatic nucleotide excision repair genes in zebrafish (*Danio rerio*). Aquatic Toxicology 84(3):301-309.

Pacheco M, Santos MA, Teles M, Oliveira M, Rebelo JE, Pombo L. 2005. Biotransformation and genotoxic biomarkers in mullet species (*Liza* sp.) from a contaminated coastal lagoon (Ria de Aveiro, Portugal). Environmental Monitoring and Assessment 107(1-3):133-153.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29(9).

Roling JA, Bain LJ, Baldwin WS. 2004. Differential gene expression in mummichogs (*Fundulus heteroclitus*) following treatment with pyrene: comparison to a creosote contaminated site. Marine Environmental Research 57(5):377-395.

Rotchell JM, Lee JS, Chipman JK, Ostrander GK. 2001. Structure, expression and activation of fish *ras* genes. Aquatic Toxicology 55(1-2):1-21.

Rotchell JM, Miller MR, Hinton DE, DiGuilio R, Ostrander GK. 2008. Chemical carcinogenesis in fishes. In: DiGuilio RT, Hinton DE, editors. Toxicology of Fishes: CRC Press. p 531-596.

Roy N, Stabile J, Habicht C, Seeb J, Wirgin I. 1999. High frequency of K-*ras* mutations in Pink Salmon embryos experimentally exposed to Exxon Valdez oil. Environmental Toxicology and Chemistry 18(7):1521-1528.

Shugart LR. 2000. DNA damage as a biomarker of exposure. Ecotoxicology 9(5):329-340.

Sousa A, Matsudaira C, Takahashi S, Tanabe S, Barroso C. 2007. Integrative assessment of organotin contamination in a southern European estuarine system (Ria de Aveiro, NW Portugal): tracking temporal trends in order to evaluate the effectiveness of the EU ban. Marine Pollution Bulletin 54(10):1645-1653.

Teh SJ, Adams SM, Hinton DE. 1997. Histopathologic biomarkers in feral freshwater fish populations exposed to different types of contaminant stress. Aquatic Toxicology 37(1):51-70.

Theodorakis CW, Swartz CD, Rogers WJ, Bickham JW, Donnelly KC, Adams SM. 2000. Relationship between genotoxicity, mutagenicity, and fish community structure in a contaminated stream. Journal of Aquatic Ecosystem Stress and Recovery 7(2):131-143.

van der Oost R, Beyer J, Vermeulen NPE. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology 13(2):57-149.

Williams TD, Gensberg K, Minchin SD, Chipman JK. 2003. A DNA expression array to detect toxic stress response in European flounder (*Platichthys flesus*). Aquatic Toxicology 65(2):141-157.

6.

Conservation of DNA repair-related and cancer genes in aquatic organisms

6.1 ABSTRACT

Different DNA repair and cancer-related genes were isolated from three fish species and one mollusc. *Dicentrarchus labrax* and *Liza aurata* checkpoint-related *rad1* genes were isolated in the present work for the first time. The *D. labrax* and *L. aurata rad1* cDNAs encode predicted proteins of 278 and 281 amino acids, respectively. DNA sequence analysis with vertebrate *rad1* sequences demonstrates that *D. labrax* and *L. aurata rad1* cDNAs are highly conserved in regions of functional importance. A partial cDNA sequence of the homologue to the human *retinoblastoma* (*Rb*) tumour suppressor gene was also isolated in three fish species: *D. labrax*, *L. aurata* and *Anguilla anguilla*. The partial cDNAs, which corresponded to the final part of the gene and included one of the conserved binding domains, showed a high identity with other vertebrate *Rb* sequences. Hitherto, the proto-oncogene *ras* gene is still poorly studied in invertebrate species. Here, we isolated the homologue of the vertebrate *ras* gene from the gastropod mollusc *Nassarius reticulatus*. The *N. reticulatus ras* cDNA encodes a predicted protein of 187 amino acids that shows a high conservation of the functional domains found in vertebrate Ras proteins, including mutational hot spots regions. The cancer gene sequences characterized herein will allow the development of specific biomarkers of genotoxic damage in different groups of aquatic organisms, namely fish and molluscs.

Keywords

Aquatic organisms, *Rb* gene, *rad1* gene, *ras* gene, biomarker

6.2 INTRODUCTION

As a consequence of anthropogenic activities, the levels of contaminants in the aquatic environment continually increases leading to a diminished water and sediment quality. Many of these environmental contaminants are known genotoxic agents which have already been related with the occurrence of different malignancies in aquatic organisms (Pacheco and Santos 2002; Simonato *et al.* 2008; Stehr *et al.* 2004). Sensitive early-warning biochemical responses (biomarkers) reflecting the adverse biological responses towards environmental contaminants, have been developed and used as tools for biological effect monitoring in environmental quality assessment (van der Oost *et al.* 2003). In this context, the molecular and cellular biomarker responses have gained increased interest, being able to identify early-onset changes following exposure to environmental chemicals, especially to genotoxic agents.

The cellular response to genotoxic stress is a highly complex process which is essential to ensure the stability of cell genome. This process involves repair and checkpoint signalling pathways which most of the time provide an effective response to DNA damage. However, disruption of these mechanisms leads to an ineffective response to genotoxic damage, leading to the persistence of damage and accumulation of mutations, which may contribute to the initiation and progression of tumours.

Among the genes most frequently implicated in vertebrate tumourigenesis are *ras* gene and two checkpoint genes, the *retinoblastoma* (*Rb*) and *p53* tumour suppressor genes (Hanahan and Weinberg 2000). *ras* gene encodes a protein that function as a GDP/GTP regulated switch that plays a central role in the cell growth, differentiation and apoptosis signalling cascades, by transducing extracellular ligand-mediated stimuli into the cytoplasm. The *ras* gene of fish and other aquatic organisms characterized to date, have a high degree of similarity with the mammalian *ras* gene counterparts, suggesting that the function of this protein is evolutionary conserved (Rotchell *et al.* 2001b). Alterations in the *ras* gene, particularly mutations in codons 12, 13, 59 and 61, have been implicated in a large proportion and wide variety of experimentally or environmentally-induced

vertebrate tumours, including fish (Roy *et al.* 1999; Vincent *et al.* 1998; Watzinger *et al.* 2001).

Rb gene product is a nuclear phosphoprotein that regulates normal cell cycle progression by binding to transcription factors and interacting with kinases (Burkhart and Sage 2008; Rotchell *et al.* 2009). In addition to its role as a gatekeeper to cell proliferation, Rb protein is also involved in the control of cellular differentiation, regulation of apoptotic cell death, maintenance of permanent cell cycle arrest and preservation of chromosomal stability (Dannenberg and te Riele 2006; Zheng and Lee 2001). The importance of Rb as a central mechanism in controlling cell proliferation is evidenced by the frequent loss of function of the *Rb* gene by mutation or deletion in the formation of a diverse set of vertebrate cancers (Brunelli and Thorgaard 1999; Zhang *et al.* 1994). To date, few *Rb* genes have been isolated from fish species (Brunelli and Thorgaard 1999; Butler *et al.* 2007; Du Corbier *et al.* 2005; Merson *et al.* unpublished; Rotchell *et al.* 2001a). The available sequences reveal conservation between fish and other vertebrate *Rb* genes. Structural alterations in the coding region of *Rb* gene observed in human tumours (Lohmann *et al.* 1996; Mateu *et al.* 1997), also occur in some fish tumours (Du Corbier *et al.* 2005; Rotchell *et al.* 2001c), being exons 12 to 23 preferentially altered.

The *rad1* gene encodes a cell cycle checkpoint protein involved in the detection of DNA damage following genotoxic stress. Rad1 forms a trimeric complex with Hus1 and Rad9 proteins (9-1-1 complex) which is recruited to DNA affected sites where it may attract specialized DNA polymerases and other DNA repair effectors (Helt *et al.* 2005). The importance of *rad1* gene is further highlighted by the extremely sensitivity to DNA damaging agents observed in Rad1 mutants, which fail to invoke cell arrest (Parker *et al.* 1998). Like other checkpoint genes, *rad1* is conserved from the yeasts to humans. The few *rad1* sequences available for aquatic organisms also show the conserved domains observed in human gene.

In the present study, we isolated and characterized the cancer genes *ras, Rb* and *rad1* from different aquatic species that can potentially be used in the development of biomarkers of genotoxic response. The aquatic organisms here

analysed included three fish species: European eel (*Anguilla anguilla),* European sea bass *(Dicentrarchus labrax)* and golden grey mullet *(Liza aurata)*, and one gastropod mollusc, the netted whelk *Nassarius reticulatus*. All these species are environmentally and economically important.

6.3 MATERIALS AND METHODS

6.3.1. Isolation of total RNA and RT-PCR

The fish species *D. labrax*, *L. aurata* and *A. anguilla*, and the gastropod *N. reticulatus* were captured from non-polluted sites at Ria de Aveiro, Portugal. Fish livers and muscle tissue from *N. reticulatus* were snap frozen in liquid nitrogen and stored at -80 ºC. Total RNA was extracted with the RNeasy Kit (Qiagen, U.K.) and treated with RNA-free DNase I (Qiagen, U.K.). First-strand cDNAs were synthesized from 1 µg of total RNA in the presence of random hexamer primers using Superscript First Strand cDNA Synthesis System for RT-PCR (Invitrogen, UK). cDNAs were used as template in subsequent PCRs. The primers for each gene (Table 6.1) were designed based on available published sequences. Fish cDNAs were used as template for the amplification of *Rb* and *rad1* fragments. *N. reticulatus* cDNA acted as template for *ras* gene fragment amplification. Amplifications were performed in a BioRad iCycler in 50 µl reaction volume using 35 sequential cycles at 94 ºC for 40 s, 50 ºC for 40 s, 72 ºC for 60 s, followed by a final 2 min extension at 72 ºC. Purified PCR products were ligated into a pGEM®-T Easy vector (Promega, UK). Recombinant plasmids were transformed and selected on LB+ampicilin plates. Plasmid DNA was purified for further DNA sequence analysis using commercial sequencing (Eurofins MWG Operon, Germany) to verify the identity of the products.

Table 6.1. PCR primer sets used for the isolation of *Rb*, *rad1* and *ras* genes from *A. anguilla*, *D. labrax*, *L. aurata* and *N. reticulatus* (R = A or G; W = A or T; D = A, T or G).

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Species
Rb	CTGGACTCTGTTCCAGCACAC	GGGAGTTGGGGAACTTGTA	*A. anguilla*
	CTGGACTCTGTTCCAGCACAC	CTCTGCAAGTTTCTGTATCAG	*D. labrax* and *L. aurata*
rad1	GAAGAAGGWGGWGTGGTG	TACTGTARDGAGAGGAAGCCTC	*D. labrax* and *L. aurata*
ras	GAATATAAGCTGGTGGTG	CCTGTCCTCATGTACTG	*N. reticulatus*

6.3.2. RACE isolation of cDNAs

The isolation of the complete 5' and 3' ends of fish *Rb* and *rad1* genes and *N. reticulatus ras* gene was performed with 1 µg total RNA using SMART™ RACE cDNA amplification reagents and protocol (Clontech, France) and gene-specific primers (Table 6.2). Resulting RACE products were analysed on agarose gel, excised and purified using a Qiaquick spin columns (Qiagen, UK). Purified DNA was ligated into a pGEM®-T Easy vector (Promega, UK). Recombinant plasmids were transformed and selected using LB+ampicilin plates. To confirm the identity of the products, plasmid DNA was purified for DNA sequence analysis using commercial sequencing (Eurofins MWG Operon, Germany).

Table 6.2. PCR primers used in RACE reactions.

Gene	Primer Name	Primer Sequence (5' to 3')	Species
Rb	RACE5Rb1	GATGGAGTCGTAGTGGCCCTCAGTG	*D. labrax* and *L. aurata*
	RACE5Rb2	GACATCATCAACTGGTCGAGGTGGC	*D. labrax* and *L. aurata*
	RACE5Rb3	GGCGGCCTGGTAGACGCATACTGCAG	*D. labrax* and *L. aurata*
	RACE5RbEel	GGAGGCCTTGGAGAGGCATACTGTAGG	*A. anguilla*
rad1	RACE5Rad1	GTATGGCTGGCTGGGGGACATGG	*D. labrax* and *L. aurata*
	RACE3Rad1	GGAGGAGGGTGGGGTAGTGACTG	*D. labrax* and *L. aurata*
ras	RACE5rasNass	CCAGGATGTCCAGCAGGCAGGTCTCTCC	*N. reticulatus*
	RACE3rasNass	CTGGTAGTGGTTGGAGCGGGAGGTGTGG	*N. reticulatus*

6.4 RESULTS

Degenerate primers and RT-PCR techniques were employed to isolate *Rb, rad1* and *ras* genes in the selected aquatic species. Primers were designed for the most conserved regions of each gene based on published sequences for other species.

An *Rb* initial fragment corresponding to the 3' end of the *Rb* gene was obtained for each fish species studied: 621 bp for *D. labrax* (GenBank accession no. FJ705225), 671 bp for *L. aurata* (GenBank accession no. FJ705226) and 309 bp for *A. anguilla* (GenBank accession no. FJ705227). These fragments were used to design gene-specific primers which were used in RACE reactions. However, the specific primers failed to amplify the cDNA ends of *Rb* gene in the

three fish species analysed. The alignment of the *Rb* fragments of *D. labrax, L. aurata* and *A. anguilla* with published *Rb* genes (Figure 6.1) revealed more than 76 % identity with other fish species and more than 68 % with the human counterpart.

```
D.labrax        ------------------------------------------------------------
L.aurata        ------------------------------------------------------------
A.anguilla      ------------------------------------------------------------
O.latipes       MPPK--KRNSGTAQSKEPKPSSDKSFADKKGSPEFS----------LNKHTEKDVEFVTL 48
O.mykiss        MPPK--KRNSGAAQNKEMKPSIKNASPDKE-NPDLS----------IEKHQEKDADFMTL 47
H.sapiens       MPPKTPRKTAATAAAAAAEPPAPPPPPPPEEDPEQDSGPEDLPLVRLEFEETEEPDFTAL 60
                ****  ::.:.:*     :*.   . .  : .*: .          :: .  :: :* :*

D.labrax        ------------------------------------------------------------
L.aurata        ------------------------------------------------------------
A.anguilla      ------------------------------------------------------------
O.latipes       CKSLHATDSVCERAWSLWK---TVQDVMEDVDANQKRLWGSCLFVTATDMDATCFTLTEV 105
O.mykiss        CQSLQVIDAVCDRAWTIWK---AVQASVDKVLDTQKKLWAACLFVAVIDLEIASFTFTQI 104
H.sapiens       CQKLKIPDHVRERAWLTWEKVSSVDGVLGGYIQKKKELWGICIFIARVDLDEMSFTLLSY 120
                *:.*:  * * :***  *:   :*:  :     .:*.**. *:*::  *::  .**: .

D.labrax        ------------------------------------------------------------
L.aurata        ------------------------------------------------------------
A.anguilla      ------------------------------------------------------------
O.latipes       LKAVNINVKQFVDLVRQLDVNLDTISTKVNSALMRVEKKYDVMLALYQRFEKTCKKIFAS 165
O.mykiss        LKAVDLNVKQFLGLLKKMDVNLDTISTKVNSAVTRLEKKYDVSRALYQRFEKTCSKIYAE 164
H.sapiens       RKTYEISVHKFFNLLKEIDT-----STKVDNAMSRLLKKYDVLFALFSKLERTCELIYLT 175
                 *: ::.*::*..*::::*.     ****:.*: *: *****  **:.::*:**. *:

D.labrax        ------------------------------------------------------------
L.aurata        ------------------------------------------------------------
A.anguilla      ------------------------------------------------------------
O.latipes       TP------DKKERATMRTCWTMFLLAKGRALQMEDDLVISFQLLLCTLELFIKRSSPELL 219
O.mykiss        DS------EAKGKEILRSCWTMFLLAKGRALQMEDELVISLHLMVCVLEFFIRRCPPSLL 218
H.sapiens       QPSSSISTEINSALVLKVSWITFLLAKGEVLQMEDDLVISFQLMLCVLDYFIKLSPPMLL 235
                 .      : :    :: .*  ******..*****:****::*::*.*: **: ..* **

D.labrax        ------------------------------------------------------------
L.aurata        ------------------------------------------------------------
A.anguilla      ------------------------------------------------------------
O.latipes       LPLYKSVVSKAQSP-TRTSRRNQSKAKSRVVESEVNVELLETLCKDNECNAEEVKNVYQT 278
O.mykiss        QPLYQSVISTAQSPPTRTSRRNQSKAKPRQAPPEVDVQLLETLCKENDCSVEEVKSVYQT 278
H.sapiens       KEPYKTAVIPINGS-PRTPRRGQNRSARIAKQLENDTRIIEVLCKEHECNIDEVKNVYFK 294
                   *::.:   :.. .**.**.*.::       * :..::*.***:::*. :***.** .

D.labrax        ------------------------------------------------------------
L.aurata        ------------------------------------------------------------
A.anguilla      ------------------------------------------------------------
O.latipes       SLSAFLDLMDLPIPDFL--GTDVSKQYQEHYLKSGDIDGRLFFDGDETVLAPKVDISQVG 336
O.mykiss        SFSAFLDSMSLSGTRDLPQATDLSKQYEELYFKSRDFDSRLFLDNDETLLTPKVEPMPVE 338
H.sapiens       NFIPFMNSLGLVTSNGLPEVENLSKRYEEIYLKNKDLDRRLFLDHDKTLQTDSIDSFETQ 354
                .: .*:: :.*    .:    ::**:*:* *:*. *:* ***:* *:*: : .::   .

                                                                 ==========
D.labrax        ------------------------------------------------------------
L.aurata        ------------------------------------------------------------
A.anguilla      ------------------------------------------------------------
O.latipes       RTPKKTSPDDDGPLIPPQTPIRAAMNSIKMLRGDLPSSGDQPSSNLEIYFKNCTVDPTQG 396
O.mykiss        RTPRKNMP-EDVVLIPPQTPVRAAMNSIAQLRVDLITSGDQPSSNLAVYFKNCTVDPTEE 397
H.sapiens       RTPRKSNLDEEVNIIPPHTPVRTVMNTIQQLMMILNSASDQPSENLISYFNNCTVNPKES 414
                ***:*.   ::  :***:**:*:.**:*  *   * ::.****.**  **:****:*.:
```

```
D.labrax        ------------------------------------------------------------
L.aurata        ------------------------------------------------------------
A.anguilla      ------------------------------------------------------------
O.latipes       IQS-LEMLG-LFSQKFGEAVGPHCIVYGRERFTLGVRLYYKVMEAMLESKKKRLFVQNFS 454
O.mykiss        VLQRVESLGLLFSQRFGQAVGPRCVGLGKQRFTLGIRLYYRVMESMLKSEEKRLSVQNFS 457
H.sapiens       ILKRVKDIGYIFKEKFAKAVGQGCVEIGSQRYKLGVRLYYRVMESMLKSEEERLSIQNFS 474
                 :: :: :* :*.::*.:***  *:  * :*:.**:****:***:**:*:::** :****

D.labrax        ------------------------------------------------------------
L.aurata        ------------------------------------------------------------
A.anguilla      ------------------------------------------------------------
O.latipes       KLLNDSTFHMSLLACALEVVMVTYEESSFKNRGCSHGEGDQAVRDLCFPWILDVFDLSAF 514
O.mykiss        KLLNDSTFHTSLLACALEVAMATYGGSTFKNGGYSNG-GVPVETDLCFPWILDVFQLTAF 516
H.sapiens       KLLNDNIFHMSLLACALEVVMATYSRSTSQN--------LDSGTDLSFPWILNVLNLKAF 526
                *****. ** *********.*.**  *: :* . .         **.*****:*::*.**

D.labrax        ------------------------------------------------------------
L.aurata        ------------------------------------------------------------
A.anguilla      ------------------------------------------------------------
O.latipes       DFYKVIESFIKAEPTLSKDIIKHLETCENLIMEKIAWRTGSPLFELLKREHE-AGATEQA 573
O.mykiss        DFYKVIESFIKAEPSLSKDIVKHLERCEHLIMETIAWREDSPLFELLKRTRE-EGPVEQA 575
H.sapiens       DFYKVIESFIKAEGNLTREMIKHLERCEHRIMESLAWLSDSPLFDLIKQSKDREGPTDHL 586
                ************* .*::::::**** **: ***.:**  .****:*:*: ::  *..::

D.labrax        ------------------------------------------------------------
L.aurata        ------------------------------------------------------------
A.anguilla      ------------------------------------------------------------
O.latipes       EPPASFSLPLQHNHTAVDLYLSPIRPGLRVLP-PESPATPSSQASSQPTPQAAGQAPRQP 632
O.mykiss        EPPATLNQPLQHNHTAADLYLSPVRPSSRTLPAPESTAPPS----SQPSAPAPHQTPRHP 631
H.sapiens       ESACPLNLPLQNNHTAADMYLSPVRSPKKKGSTTRVNSTAN----AETQATSAFQTQKPL 642
                *....:. ***:****.*:****:*.  :  . ..  :...    ::. . :. *: :

D.labrax        ----------------------------------------------TLQHEHELMRGRHLDQ 16
L.aurata        -------------------------LTCCPPTLNWSIIWTLFQHTLQHEYELMRDRHLDQ 35
A.anguilla      ----------------------------------------------TLQHEYELMRDRHLDQ 16
O.latipes       KSNSLSLFYKKLYRLAYTRLKTLCSCLLSNHPDLEHVIWTLFQHTLQYEYDLMRDRHLDQ 692
O.mykiss        KSNSLSLFYKKLYRLAYMRLKMLYTQLLTSHPELEPIMWTLFQHTLQNEYELMRDRHLDQ 691
H.sapiens       KSTSLSLFYKKVYRLAYLRLNTLCERLLSEHPELEHIIWTLFQHTLQNEYELMRDRHLDQ 702
                **.********:***** **: *        *      ********* *::***.*****

D.labrax        LMMSAMYAICKVKSVDLRFKTIVTAYKNMPNTNQETFKHVLITEGHYDSIIVFYNQVFMQ 76
L.aurata        LMMSAMYAICKVKSVDLRFKTIVTSYKNMPNTSQETFKHVLITEGHYDSIIIFYNQVFMQ 95
A.anguilla      LMMSAMYAICKVKNIDLRFKTIVTAYKNLPNTNQETFKRVLIRDGQYDSIIVFYNLVFMQ 76
O.latipes       LMMSAMYAICKVKSVDLRFKLIVSAYKNMPNTSQETFKHVLITEGQYDSIIVFYNQVFMQ 752
O.mykiss        LMMSAMYAICKVKNVDLRFKTIVTAYKNMPNTNQETFKHVLIREGQYDSIIVFYNLVFMQ 751
H.sapiens       IMMCSMYGICKVKNIDLKFKIIVTAYKDLPHAVQETFKRVLIKEEEYDSIIVFYNSVFMQ 762
                :**.:**.*****.:**:** **::**::*:: *****:*** : .*****:*** ****

D.labrax        KLKTNILQYASTRPPTLSPIPQIPRSPYKFPNSPLRVPGSNNVYISPLKSP-RMSPG--- 132
L.aurata        RLKTNILQYASTRPPTLSPIPQIPRSPYKFPNSPLRVPGSNNVYISPMKSP-RMSPS--- 151
A.anguilla      RLKTNILQYASPRPPTLSPIPNIPCSP-------------------------------- 103
O.latipes       KLKTNILQYASTRPPTLSPIPQIPCSPYKFPNSPLRVPGSNNVYISPLKNS-RLSAG--- 808
O.mykiss        KLKTNILQYASPRPPTLSPIPHIPRSPYKFPNSPLRVPGSNNVYISPMKSPTRMSP---- 807
H.sapiens       RLKTNILQYASTRPPTLSPIPHIPRSPYKFPSSPLRIPGGN-IYISPLKSPYKISEGLPT 821
                :**********.*********:** ******.****:**.* :****:*.. ::* *

D.labrax        --IMTPRSRMLVSIGESFGPSNRFQKINQMVNSSDRSFKRTLELGSTPKPLKRLRFDVDG 190
L.aurata        --LMTPRTRMLVSIGESFGQSNRFQKINQMVNSSDRTFKRTLDMGPTPKPLKRLRFDVDG 209
A.anguilla      ------------------------------------------------------------
O.latipes       --MMAPRSRMLVSIGESFGFANRFQKINQMVNSGDRAHKRTLDGGSALKPLKRLRFDVDG 866
O.mykiss        --VMTPRTRILVSIGESFGTSDKFQKINAMVNSSDRSLKRSLDMSSAPKPLKRLRFDVEG 865
H.sapiens       PTKMTPRSRILVSIGESFGTSEKFQKINQMVCNSDRVLKRSAEGSNPPKPLKKLRFDIEG 881
                   *:**:*:********* :::***** ** ..**  **: : ... ****:****::*
```

```
D.labrax        QDEADGSLIYSKSGGDS---------------------------------- 207
L.aurata        QDEADG----SKPGEDST-------------------------------- 223
A.anguilla      ---------------------------------------------------
O.latipes       QDEADG----SKSGGESALIQKLAEMSSTRSRMQEQKMKEDAESRKKT-- 910
O.mykiss        QDESDE----SKPGEESRLIQKLAEIGSTRTRMQEQKMKDDAESEKDKP- 910
H.sapiens       SDEADGS---KHLPGESKFQQKLAEMTSTRTRMQKQKMNDSMDTSNKEEK 928
                .**:*     .:   :* : *****: ***:***:***::. :: :.
```

Figure 6.1. The predicted amino acid sequence of the *D. labrax* (GenBank accession no. FJ705225), *L. aurata* (FJ705226), and *A. anguilla* (FJ705227) partial *Rb* gene and comparison with *Oryzias latipes* (AAG21826), *Oncorhynchus mykiss* (AAD13390) and *Homo sapiens* (NP_000312) Rb protein sequences. Regions where all sequence match are indicated by an asterisk. The functionally conserved adenovirus E1A-binding domains are overlined.

The same methodology was used to isolate *rad1* gene from *D. labrax* and *L. aurata*. Specific RACE primers were designed based on the sequences of the initial *rad1* fragments and were used to amplify the 5' and 3' cDNA ends of the gene. The *rad1* gene isolated from *D. labrax* (GenBank accession no. FJ705223) and *L. aurata* (GenBank accession no. FJ705224) contained open reading frames (ORF) of 834 and 843 nucleotides, encoding proteins of 278 and 281 amino acids, respectively. The alignment of these predicted amino acid sequences with that of other organisms revealed more than 86 % homology with the available sequence of Rad1 protein of other fish species and more than 73 % to the human protein (Figure 6.2).

A *ras* gene fragment representing the conserved exon 1 and part of exon 2 was isolated from *N. reticulatus* using primers derived from previously published sequences. The specific primers designed based on the sequence of this fragment, were used to amplify the complete *N. reticulatus ras* gene. The *ras* cDNA isolated contained a complete ORF of 564 bp (GenBank accession no. DQ198150) encoding a predicted protein of 187 amino acids. Multiple alignment of the *N. reticulatus ras* deduced amino acid sequence with Ras proteins from different species including the three human Ras homologues (Figure 6.3), revealed 79 % identity with the human Kb-Ras and H-Ras, and more than 78 % with the fish counterparts. The highest identity was found to be with the mussel

Mytilus edulis (86 %) however the homology was lower between *N. reticulatus* and the other molluscs, 68 % with *A. californica* and 62 % with *L. forbesi*.

```
D.labrax        MPLSTQS-QADDEQYVLVASLDNARCLSNILKAITFKDHAIFSATPNGLKVTVEDSKCLQ 59
L.aurata        MPLSTQS-EGDDEQYVLMACLDNARNLSNILKAITFKDHAIFTATTNGLKVTVEESKCLQ 59
A.anguilla      MPLSTQS-EAGDERYVLVASLDNVRNLSNILKAITFKDHALFNATPNGLKVTVEDSKCLQ 59
D.rerio         MPLSTQS-QSDVDQYILIASLDNARNLSNILKAISFKDHAIFNATQNGLKVTVEDSKCLQ 59
M.musculus      MPLLTQYNEEEYEQYCLVASLDNVRNLSTVLKAIHFREHATCFATKNGIKVTVENAKCVQ 60
H.sapiens       MPLLTQQIQDEDDQYSLVASLDNVRNLSTILKAIHFREHATCFATKNGIKVTVENAKCVQ 60
                *** **  :   ::* *:*.***.* **.:**** *::**   ** **:*****::**:*

                                      I                          II
D.labrax        ANAFIQAEIFQEFTIREDLVGFQVNLTVLLDCLNIFGGSTVPGVSTALRMCYRGYGYPLT 119
L.aurata        ANAFIQAEIFQEFTIKEDMVGFQINLTVLMDCLNIFGGSNVPGISTTVRMCYKGYGYPLT 119
A.anguilla      ANAFIQAEIFQEFTIQEDSVSFQVNLTVLLDCLTIFGGSTTPGVTTALRMCYNGYGYPLT 119
D.rerio         ANAFIQADIFQEYIIKEDAVGFQVNLTVLLDCLTIFGGSTVPGVCTALRMCYNGYGYPLT 119
M.musculus      ANAFIQADVFQEFVIQEESVTFRINLTILLDCLSIFGSSPTPGTLTALRMCYQGYGHPLM 120
H.sapiens       ANAFIQAGIFQEFKVQEESVTFRINLTVLLDCLSIFGSSPMPGTLTALRMCYQGYGYPLM 120
                ******* :***: ::*: * *::***:*:***.***.*  **  *::****.***:**

                                                    III
                LFLEEGGVVTVCKINTQEPEEPIDFEFCSTNVTNKVILLSESLKEAFSELDMTSEVLQIT 179
L.aurata        LFLEEGGVVTVCKINTQEPEEPIDFEFCSANVTNKVILQSESLKEAFSELDMTSEVLQIT 179
A.anguilla      LFLEEGGVVTVCKINTEEPEDPIDFEFCSTNVTNKVILQSDSLREAFSELDMTSEILQIT 179
D.rerio         LFLEEGGVVTVCKINTQEPEEPIDFDFCSTNVTNKVILQSDSLKEAFSELDMTSEVLQIT 179
M.musculus      LFLEEGGVVTVCKITTQEPEETLDFDFCSTNVMNKIILQSEGLREAFSELDMTGDVLQIT 180
H.sapiens       LFLEEGGVVTVCKITTQEPEETLDFDFCSTNVINKIILQSEGLREAFSELDMTSEVLQIT 180
                **************.*:***:.:**:***:** **:** *:.*:*********.::****

                                                              IV
D.labrax        MSPSQPYFRLSTFGNSGNAHYDYPKDSDMMELFRCTKTQTNRYKMSLLKPSTKALALSCK 239
L.aurata        MSPNQPYFRLSTFGNAGNAHYDYSNDSDMMELFRCSETQTNRYKMSLLKPSTKALALSCK 239
A.anguilla      MSPSQPYFRLSTFGNSGNAHYDYPKDSDMMELFQCTKTQTNRYKMSLLKPSTKALALSCK 239
D.rerio         MSPSHPYFRLSTFGNSGNAHYDYPKDSDMMELFQCTKIQTNRYKMSLLKPSTKALALSCK 239
M.musculus      VSPDKPYFRLSTFGNAGSSHLDYPKDSDLVEAFHCDKTQVNRYKLSLLKPSTKALALSCK 240
H.sapiens       MSPDKPYFRLSTFGNAGSSHLDYPKDSDLMEAFHCNQTQVNRYKISLLKPSTKALVLSCK 240
                :**.:**********:*.:* **.:***::* *:* : *.****:**********.****

                VSVRTDSRGFLSLQYLVRNDDGQICFVEFYCCPDEEVE---- 277
L.aurata        VSVRTDNRGFLSLQYLVRNDDGQICFVEYYCCPDEEVDEEL- 280
A.anguilla      VSVRTDTRGFLSLQYLVRNDDGQICFVEYYCCPDEEVEEE-- 279
D.rerio         VSVRTDSRGFLSLQYLVRNDDGQICFVEYYCCPDEEVEEE-- 279
M.musculus      VSIRTDNRGFLSLQYMIRNEDGQICFVEYYCCPDEEVPES-- 280
H.sapiens       VSIRTDNRGFLSLQYMIRNEDGQICFVEYYCCPDEEVPESES 282
                **:***.********::**:********:********
```

Figure 6.2. Comparison of the *rad1* deduced amino acid sequence of *D. labrax* (GenBank accession no. FJ705223) and *L. aurata* (FJ705224) with selected vertebrate Rad1 sequences: *A. anguilla* (FJ438472), *Danio rerio* (AAH49464); *Mus musculus* (AAC95465); and *Homo sapiens* (AAC95466). Regions showing homology are indicated by an asterisk. The conserved exonuclease domains (I, II, III) and leucine zippers (III and IV) are overlined.

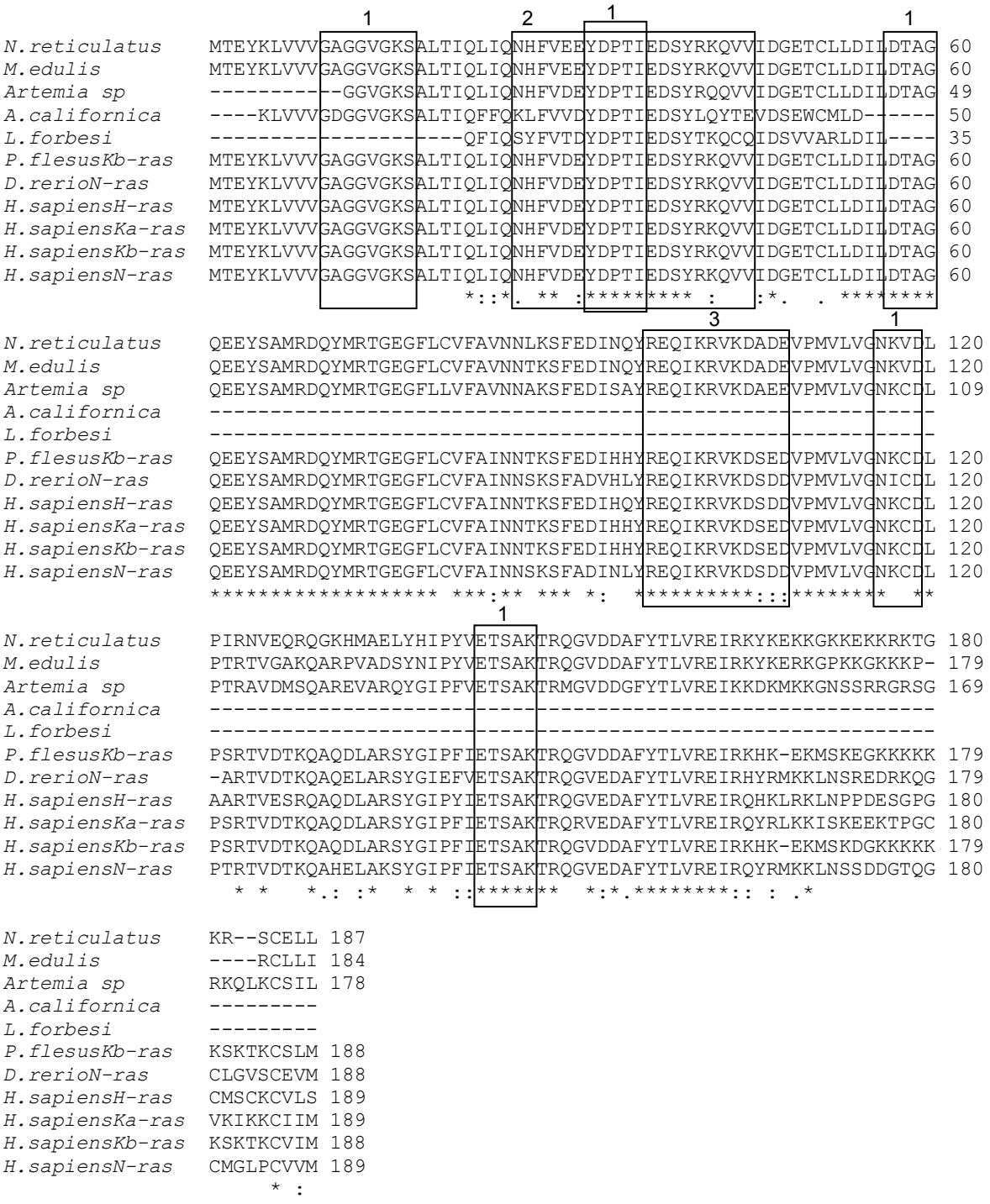

Figure 6.3. Comparison of the *ras* deduced amino acid sequence of *N. reticulatus* (DQ198150) with selected invertebrate and vertebrate Ras sequences: *Mytilus edulis* (AAT81171); *Artemia* (AAC83399); *Aplysia californica* (AAA19441); *Loligo forbesi* (AAA64420); *Danio rerio* (AAB40625); *Platichthys flesus* Kb-Ras (CAA76678); *Homo sapiens* Ka-Ras (P01116); *H. sapiens* Kb-Ras (0909262B); *H. sapiens* Ha-Ras (P01112); *H. sapiens* N-Ras (PO1111). Regions showing homology are indicated by an asterisk. The conserved domains are boxed: guanine nucleotide binding domains (1), effector domain (2), and interaction with guanine exchange factors domain (3).

6.5 DISCUSSION

6.5.1. Cancer-related genes in fish: *Rb* and *rad1*

In the present study, we report the cDNA sequences of two cancer-related genes, *Rb* and *rad1,* in the teleosts *D. labrax, L. aurata* and *A. anguilla*. The partial *Rb* cDNAs isolated from *D. labrax, L. aurata* and *A. anguilla* correspond to exons 19 to 24, 19 to 25 and 19 to 22, respectively, and include one of the two conserved binding domains (Figure 6.1). A high identity was found between the human Rb and the three species analysed: 68 % with *L. aurata*, 70 % with *D. labrax* and 81 % with *A.anguilla*, suggesting a conserved function of the Rb protein among species. A higher identity ranging from 76 % (between *D. labrax* and *Danio rerio*) up to 92 % (between *A. anguilla* and *Onchorhynchus mykiss*) was also found with the other fish *Rb* genes (Brunelli and Thorgaard 1999; Butler *et al.* 2007; Du Corbier *et al.* 2005; Merson *et al.* unpublished; Rotchell *et al.* 2001a). The conservation of the gene sequence between species highlights the importance of this protein as a cell cycle gatekeeper.

The role of the checkpoint protein Rad1 in fish has not been studied yet, and the few *rad1* gene sequences available have resulted from the genome sequencing of *Danio rerio, Takifugu rubripes, Gasterosteus aculeatus* and *Oryzias latipes*. The *rad1* cDNA sequences of *D. labrax* and *L. aurata* are here reported for the first time. The predicted amino acid sequences display a high identity with the available fish Rad1 (86 % to 96 %) and with the human counterpart (73 % to 74 %). The conserved exonuclease domains and leucine zippers are also conserved in fish *rad1* genes, suggesting a similar function of this gene in lower and higher vertebrates.

6.5.2. *N. reticulatus ras* gene

Several *ras* gene sequences are now available for fish species, but the number is still limited for aquatic invertebrates. To date, *ras* gene has been sequenced from the aquatic invertebrates *Mytilus edulis*, *Artemia* sp. and a small fragment from *Loligo forbesi* and *Aplysia californica*. Herein we report for the first time, the cDNA sequence of the aquatic mollusc *N. reticulatus ras* gene. The predicted amino acid sequence of *N. reticulatus ras* gene displays all the

conserved structural domains of the gene, showing a high degree of similarity with *M. edulis ras* gene (86 %) and more than 78 % identity with the fish and human counterparts. The first 85 amino acids of the *N. reticulatus ras* gene, which contain the functionally important nucleotide binding site and effector domains, are similar to *M. edulis* and vertebrate sequences, differing at only two codons, 30 and 84. The similarity with the *ras* fragments available for the molluscs *L. forbesi* and *A. californica* was, however, lower (62 % and 68 %, respectively).

Relevant for ecotoxicologists is the fact that the mutational hot spot residues of the *ras* gene, which affect the activator function (codons 12, 13, 59 and 61), are conserved in *N. reticulatus*. The fact that this gene is conserved, including the mutational hot spot regions, suggests its potential as early-warning biomarker of genotoxic damage and carcinogenesis in the aquatic environment. Molluscs have assumed a major role in monitoring contaminants world-wide, mainly due to their ease of sampling, widespread distribution, relatively sedentary life style, suitable size and, often, economic or ecological importance. The netted whelk *N. reticulatus* is a ubiquitous gastropod in European marine waters and has also been used as a bioindicator species of organometals pollution, especially TBT (Rato *et al.* 2008). The conservation of cancer-related genes such as *ras* gene *in N. reticulatus* may increase its usefulness in environmental monitoring programs as a bioindicator species of genotoxic contaminants.

6.6 CONCLUSIONS

The increasing concern regarding the risks posed by chemical pollutant discharges to the environment and natural resources has conducted to the development of different methodologies to identify, estimate, assess and manage those risks. The molecular responses to pollutant exposure are among the most sensitive and earliest detectable. Fish cancer-related genes such as *ras* and *Rb* here studied have already been proposed as molecular biomarkers of exposure to genotoxic chemicals (Du Corbier *et al.* 2005; Rotchell *et al.* 2001b). Determining if these genes are altered by environmental contaminants is essential to their establishment as early-warning biomarkers of genotoxic damage and carcinogenesis in the aquatic environment.

The usefulness of fishes and molluscs in aquatic pollution monitoring studies has been demonstrated (Minier *et al.* 2000; Morcillo *et al.* 1999). The commercial important *D. labrax*, *L. aurata*, *A. anguilla* and *N. reticulatus* are widely distributed in European marine waters and in numerous European estuaries, and are therefore good candidates as bioindicator species of European coastal waters. In fact, these species have already proved their usefulness in the assessment of the biological effects of aquatic contamination (Ahmad *et al.* 2008; Barroso *et al.* 2005; Oliveira *et al.* 2008; Ruddock *et al.* 2003), however few studies have been focused on gene-specific alterations by the action of environmental genotoxic compounds in these species. In future work, it is our aim to screen *D. labrax, L. aurata, A. anguilla* and *N. reticulatus* from different contaminated sites to investigate if there is altered expression and/or mutational alterations of the *Rb*, *rad1* and *ras* genes, and thus establish the value of these genes as molecular biomarkers.

6.7 REFERENCES

Ahmad I, Maria VL, Oliveira M, Serafim A, Bebianno MJ, Pacheco M, Santos MA. 2008. DNA damage and lipid peroxidation vs. protection responses in the gill of *Dicentrarchus labrax* L. from a contaminated coastal lagoon (Ria de Aveiro, Portugal). Science of the Total Environment 406(1-2):298-307.

Barroso CM, Reis-Henriques MA, Ferreira M, Gibbs PE, Moreira MH. 2005. Organotin contamination, imposex and androgen/oestrogen ratios in natural populations of *Nassarius reticulatus* along a ship density gradient. Applied Organometallic Chemistry 19(11):1141-1148.

Brunelli J, Thorgaard G. 1999. Sequence, expression and genetic mapping of a rainbow trout retinoblastoma cDNA. Gene 226:175-180.

Burkhart DL, Sage J. 2008. Cellular mechanisms of tumour suppression by the retinoblastoma gene. Nature Reviews Cancer 8(9):671-682.

Butler AP, Trono D, Coletta LD, Beard R, Fraijo R, Kazianis S, Nairn RS. 2007. Regulation of CDKN2A/B and Retinoblastoma genes in *Xiphophorus* melanoma. Comparative Biochemistry and Physiology Part C Toxicology & Pharmacology 145(1):145-155.

Dannenberg JH, te Riele HP. 2006. The retinoblastoma gene family in cell cycle regulation and suppression of tumorigenesis. In: Richter D, Tiedge H, editors. Results and Problems in Cell Differentiation. Berlin: Springer Verlag. p 183-225.

Du Corbier FA, Stentiford GD, Lyons BP, Rotchell JM. 2005. Isolation of the retinoblastoma cDNA from the marine flatfish dab (*Limanda limanda*) and evidence of mutational alterations in liver tumors. Environmental Science and Technology 39(24):9785-9790.

Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. Cell 100(1):57-70.

Helt CE, Wang W, Keng PC, Bambara RA. 2005. Evidence that DNA damage detection machinery participates in DNA repair. Cell Cycle 4(4):529-532.

Lohmann DR, Brandt B, Höpping W, Passarge E, Horsthemke B. 1996. The spectrum of RB1 germ-line mutations in hereditary retinoblastoma. American Journal of Human Genetics 58(5):940-949.

Mateu E, Sánchez F, Nájera C, Beneyto M, Castell V, Hernández M, Serra I, Prieto F. 1997. Genetics of retinoblastoma: a study. Cancer Genetics and Cytogenetics 95(1):40-50.

Merson RR, Karchner SI, Hahn ME. unpublished. GenBank accession number AAS80140.

Minier C, Levy F, Rabel D, Bocquene G, Godefroy D, Burgeot T, Leboulenger F. 2000. Flounder health status in the Seine Bay. a multibiomarker study. Marine Environmental Research 50(1-5):373-377.

Morcillo Y, Albalat A, Porte C. 1999. Mussels as sentinels of organotin pollution: bioaccumulation and effects on P450-mediated aromatase activity. Environmental Toxicology and Chemistry 18(6):1203-1208.

Oliveira M, Pacheco M, Santos MA. 2008. Organ specific antioxidant responses in golden grey mullet (*Liza aurata*) following a short-term exposure to phenanthrene. Science of the Total Environment 396(1):70-78.

Pacheco M, Santos MA. 2002. Biotransformation, genotoxic, and histopathological effects of environmental contaminants in European eel (*Anguilla anguilla* L.). Ecotoxicology and Environmental Safety 53(3):331-347.

Parker AE, Van De Weyer I, Laus MC, Oostveen I, Yon J, Verhasselt P, Luyten WHML. 1998. A human homologue of the *Schizosaccharomyces pombe* rad1+ checkpoint gene encodes an exonuclease. Journal of Biological Chemistry 273(29):18332-18339.

Rato M, Gaspar MB, Takahashi S, Yano S, Tanabe S, Barroso C. 2008. Inshore/offshore gradients of imposex and organotin contamination in *Nassarius reticulatus* (L.) along the Portuguese coast. Marine Pollution Bulletin 56(7):1323-1331.

Rotchell JM, Blair JB, Shim JK, Hawkins WE, Ostrander GK. 2001a. Cloning of the Retinoblastoma cDNA from the Japanese medaka (*Oryzias latipes*) and preliminary evidence of mutational alterations in chemically-induced retinoblastomas. Gene 263(1-2):231-237.

Rotchell JM, Lee JS, Chipman JK, Ostrander GK. 2001b. Structure, expression and activation of fish *ras* genes. Aquatic Toxicology 55(1-2):1-21.

Rotchell JM, Ulnal E, van Beneden RJ, Ostrander GK. 2001c. Retinoblastoma gene mutations in chemically induced liver tumor samples of Japanese medaka (*Oryzias latipes*). Marine Biotechnology 3:S44-S49.

Rotchell JM, du Corbier FA, Stentiford GD, Lyons BP, Liddle AR, Ostrander GK. 2009. A novel population health approach: using fish retinoblastoma gene profiles as a surrogate for humans. Comparative Biochemistry and Physiology Part C Toxicology & Pharmacology 149(2):134-140.

Roy N, Stabile J, Habicht C, Seeb J, Wirgin I. 1999. High frequency of K-*ras* mutations in Pink Salmon embryos experimentally exposed to Exxon Valdez oil. Environmental Toxicology and Chemistry 18(7):1521-1528.

Ruddock P, Bird D, McEvoy J, Peters L. 2003. Bile metabolites of polycyclic aromatic hydrocarbons (PAHs) in European eels *Anguilla anguilla* from United Kingdom estuaries. Science of the Total Environment 301:105-117.

Simonato JD, Guedes CLB, Martinez CBR. 2008. Biochemical, physiological, and histological changes in the neotropical fish *Prochilodus lineatus* exposed to diesel oil. Ecotoxicology and Environmental Safety 69(1):112-120.

Stehr CM, Myers MS, Johnson LL, Spencer S, Stein JE. 2004. Toxicopathic liver lesions in English sole and chemical contaminant exposure in Vancouver Harbour, Canada. Marine Environmental Research 57(1-2):55-74.

van der Oost R, Beyer J, Vermeulen NPE. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology 13(2):57-149.

Vincent F, de Boer J, Pfohl-Leszkowicz A, Cherrel Y, Galgani F. 1998. Two cases of *ras* mutation associated with liver hyperplasia in dragonets (*Callionymus lyra*) exposed to polychlorinated biphenyls and polycyclic aromatic hydrocarbons. Molecular Carcinogenesis 21(2):121-7.

Watzinger F, Mayr B, Gamerith R, Vetter C, Lion T. 2001. Comparative analysis of *ras* proto-oncogene mutations in selected mammalian tumors. Molecular Carcinogenesis 30(4):190-8.

Zhang X, Xu HJ, Murakami Y, Sachse R, Yashima K, Hirohashi S, Hu SX, Benedict WF, Sekiya T. 1994. Deletions of chromosome 13q, mutations in retinoblastoma 1, and retinoblastoma protein state in human hepatocellular carcinoma. Cancer Research 54(15):4177-4182.

Zheng L, Lee WH. 2001. The retinoblastoma gene: a prototypic and multifunctional tumor suppressor. Experimental Cell Research 264(1):2-18.

7.

General Discussion

Aquatic organisms are exposed to a wide variety of environmental contaminants. To evaluate the possible biological impacts of pollution it is usual to employ a suite of biomarkers, ideally at different levels of the biological organization (Hagger *et al.* 2006). Changes at the molecular and cellular level have the potential to anticipate changes at higher levels, and therefore their identification may permit time to take measures before irreversible biological damage becomes inevitable. In the present study, molecular responses to xenobiotic exposure were assessed in the European eel (*Anguilla anguilla*), European sea bass (*Dicentrarchus labrax*) and golden grey mullet (*Liza aurata*) with the broad aim of developing novel molecular biomarkers of environmental pollution.

7.1. CONSERVATION OF CANCER GENES IN AQUATIC ORGANISMS

The isolation and characterization of the cancer-related genes *ras*, *rad1, xpf* and *Rb*, are herein reported for the first time in the fish species *A. anguilla* (Chapters 2, 4 and 6), *D. labrax* (Chapters 5 and 6) and *L. aurata* (Chapters 5 and 6). These genes encode proteins which play important roles in the cell. Ras plays an important role in many cellular signaling cascades, being involved in the transduction of external stimuli to the nucleus. The cell-cycle checkpoint protein Rad1 is a component of the Rad9-Hus1-Rad1 complex which is able to detect and recruit repair machinery to DNA damaged sites. XPF, a DNA repair factor, is involved in the nucleotide excision repair mechanism. Rb acts as a gatekeeper to cell proliferation, being also involved in the control of cellular differentiation, regulation of apoptotic cell death, maintenance of permanent cell cycle arrest and preservation of chromosomal stability. The high homology of *ras*, *rad1*, *xpf* and *Rb* genes observed between vertebrate species highlights their importance in the normal cellular functioning. The newly identified genes from *A. anguilla*, *D. labrax* and *L. aurata* showed an homology to the human counterpart higher than 96 % for *ras* gene, 73 % for *rad1*, 64 % for *xpf*, and 68 % for the isolated *Rb* fragment.

While a number of studies have already focused on the fish cancer-related genes *ras* and *Rb* and also in the alterations induced by contaminants over these genes, very few studies have been centered in the DNA damage detection and

repair genes such as *rad1* and *xpf* in fish. Not many studies have been published on the mechanism of detection and repair of DNA damage in fish (David *et al.* 2004; Notch *et al.* 2007) and the nucleotide sequences of these genes are only available for a reduced number of fish species. The newly identified *rad1* and *xpf* genes thus contribute to this knowledge.

Fish *ras* and *Rb* genes have already been proposed as molecular biomarkers of exposure to genotoxic chemicals (Du Corbier *et al.* 2005; Rotchell *et al.* 2001). Similarly, *A. anguilla*, *D. labrax* and *L. aurata ras* and *Rb* genes have also the potential to be employed as biomarkers of exposure, due to their high homology with the human and fish available sequences and, more relevant, due to the conservation of the mutational hotspots residues of these genes. In spite of the little information on fish *rad1* and *xpf* genes, the high homology between species, their important role in the cell and their association to important disorders such as cancer and xeroderma pigmentosum, respectively, suggest that these are target genes for biomarker development.

While several *ras* gene sequences are now available for fish species, the number is still reduced for aquatic invertebrates. The *ras* gene of the gastropod *Nassarius reticulatus* was also identified for the first time in the present study. High homologies were found between *N. reticulatus ras* gene and the bivalve *Mytilus edulis*, fish and human counterparts. The mutational hotspot residues of the *ras* gene that affect the activator function (codons 12, 13, 59 and 61) are also conserved in *N. reticulatus*. As for the newly-identified fish *ras* genes, also *N. reticulatus ras* gene can potentially be used as biomarker of genotoxic damage.

7.2. MOLECULAR RESPONSES IN *A. ANGUILLA* EXPOSED TO PAH MODEL COMPOUNDS

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants in aquatic systems which have been linked to the presence of anomalies in fish (Jha 2004; Johnson *et al.* 2002). To better understand the molecular responses in fish exposed to PAHs, two prototypic PAHs, the environmental-relevant benzo[a]pyrene (BaP) and the synthetic 7,12-dimethylbenz[a]anthracene (DMBA), were used in laboratory-controlled

experiments. Several studies have demonstrated that both compounds are highly cytotoxic, mutagenic and immunotoxic in fish (Maria *et al.* 2002; Regoli *et al.* 2003; Weimer *et al.* 2000).

Although laboratory conditions may not reflect the exact exposure routes in the environment, they can be useful for interpreting data obtained in field conditions, to determine mechanisms of action induced by pollutants and to develop applicable methods. The control of abiotic (water temperature, salinity, dissolved oxygen and photoperiod) and biotic variables (life stage of the selected species) provides a smaller degree of variability than the field exposures, where confounding factors are present. Furthermore, laboratory-controlled exposures using a single compound with a particular concentration, duration and exposure route permit the evaluation of the biological effects induced by that particular compound and represent a starting point to the study of the impact of contaminant mixtures as it is usually found in the environment.

The molecular responses of *A. anguilla* to PAHs were studied in two laboratory controlled experiments: (i) adult eels were chronically exposed to water-borne BaP for 28 days, and (ii) juvenile eels were acutely exposed to BaP and DMBA, and the responses analysed at the molecular level at regular times during a 90 day post-exposure period. The chronic exposure partially simulates the type of exposure that adult eels may endure when dwelling in the estuaries and rivers, while the acute exposure using a juvenile stage of European eel simulates, to some extent, the short-term exposures that may occur during the migration of glass eels to European estuaries and rivers, thus allowing for the study of the effects of such temporary exposures. The biological effects of PAHs were evaluated in three tissues which were selected based on their location and function in the organism: liver, gills and blood. The harmful effects of PAH exposure in fish are mainly linked to CYP system-mediated metabolism, with the balance between the activities of Phase I and Phase II reactions determining the amount of reactive intermediates that is available for macromolecular binding and thus cellular damage (Sandvik *et al.* 1998). The significant increase ($P < 0.05$) observed for c*yp1A* mRNA in the liver of PAH-exposed eels (Chapter 4), confirms the involvement of CYP system in the metabolism of PAHs in eels.

In both experiments, increased levels of DNA damage, measured by the comet assay, were observed in eels exposed to BaP concentrations higher than 0.3 µM. The exposure to lower concentrations of this compound did not induce a significant damage of DNA, even after 28 days of continuous exposure (Chapter 2). A balance between the production of reactive metabolites and their detoxification, and an efficient DNA repair of the putative lesions caused by the transient reactive intermediates may explain the absence of DNA damage at low BaP concentrations in eel. The acute exposure to higher concentrations of BaP and DMBA, on the other hand, significantly increased DNA damage in the gills, blood, and liver of juvenile eel, during the post-exposure period from 1 to 7 days (Chapters 3 and 4). The DNA damage observed in liver cells was generally lower than that of the other tissues, reflecting the high efficiency of liver detoxification system (van der Oost *et al.* 2003). In addition to the differences in the efficiency of the detoxification system, the tissue specific genotoxic responses observed in eels exposed to BaP and DMBA are also related to organ location and function, tissue regeneration rate, capacity of protection against DNA damaging agents and ultimately DNA repair ability.

The presence of DNA lesions, if not efficiently repaired, may lead to detrimental effects at the organism level by inducing mutations, chromosomal aberrations or protein dysfunction which are associated with the initiation and promotion of cancer and other abnormalities (Shugart 2000). To complete the cause-effect relationship between PAH exposure and genotoxic damage at the molecular level, different endpoints were evaluated. In the chronic exposure to BaP (Chapter 2), the ability of this PAH to cause *ras* gene mutations or to alter *ras* gene expression in *A. anguilla* was evaluated, yet no alterations were observed even in the group of animals exposed to the higher concentration (0.3 µM). Although the presence of *ras* gene alterations has been reported in other fish species exposed to PAHs (Rotchell *et al.* 2001), the present results suggest that the molecular aetiology of hydrocarbon genotoxicity in *A. anguilla* does not involve the *ras* gene, at least using the exposure regime employed. In the acute exposure to DMBA (Chapter 4), changes in the expression levels of the cell cycle checkpoint-related *rad1* gene and the detection of differentially expressed genes

were analysed in liver cells at day 7 of the post-exposure period to this compound (3.9 μM), when the levels of DNA damage were higher. The transcription of *rad1* gene did not seem to be induced as a response to the DNA damage observed in the liver cells. Yet, genes involved in important cell activities responded to PAH exposure, including genes involved in the xenobiotic metabolism, immune processes, protein translation and cytoskeleton dynamics, as revealed by suppression subtractive hybridization (SSH). This gene expression global approach highlighted the complexity of the mechanisms behind the response to genotoxic agents, indicating some of the processes that are involved in such response and the cellular activities that are affected by the exposure to PAHs. The alteration in the expression levels of different genes associated with important cellular processes and the presence of DNA damage may be early indicators of possible future repercussions at organism-level of biological organization. The presence and persistence of these alterations in a post-exposure period indicate that punctual sources of contamination, such as accidental spillage of fuels in the aquatic environment, may have the potential for disruption of some vital functions of the organisms even after the removal of the contamination source.

7.3. MOLECULAR RESPONSES IN *D. LABRAX* AND *L. AURATA* EXPOSED TO ENVIRONMENTAL CONTAMINANTS

D. labrax and *L. aurata* were employed as bioindicator species of aquatic pollution in the Ria de Aveiro coastal lagoon (Portugal). Both species have previously demonstrated their usefulness in the detection of the biological effects of contaminated waters (Ahmad *et al.* 2008; Oliveira *et al.* 2009). Nevertheless, few studies have focused on gene-specific alterations induced by aquatic pollution on these species. The biological effects of the lagoon contaminants were evaluated using novel molecular responses in parallel with well-established biomarkers. Five areas were selected in Ria de Aveiro due to their differences in the type and sources of contamination (Chapter 5).

Both *D. labrax* and *L. aurata* from contaminated areas showed several histopathological lesions in the liver and gross DNA damage (assessed by comet assay), indicating the presence of genotoxic and cytotoxic agents among the pollutants of Ria de Aveiro. The absence of changes detected in the expression of the detoxification enzyme c*yp1A* gene is probably the net result of the action of inducers and inhibitors present in each of the contaminated sites. To further study the cause-effect relationship between contaminant exposure and cellular/genotoxic damage, *ras* and *xpf* gene alterations were analysed and pollutant-responsive gene transcripts identified in *D. labrax* from GAF, a site typically contaminated by PAHs, heavy metals and organotin compounds (Pacheco *et al.* 2005). No *ras* gene mutations or changes in *ras* gene expression were detected, yet a decrease in *xpf* gene expression was observed, possibly indicating impaired DNA repair capacity in those fish. The SSH global approach revealed an increased expression of genes involved in the energy metabolism, suggesting a need for high levels of energy to cope with the conditions imposed by the exposure to the contaminant. However, genes involved in other cellular processes seemed to be affected by pollution, such as those responsible for antioxidant response and immune system activity, indicating a wider implication of contaminant exposure.

The approach employed highlighted the utility of anchoring traditional biomarker responses alongside novel biomarker responses, since it facilitates the appreciation of the confounding factors involved in attempting to assess the biological effect impacts of contaminants in natural environments. *D. labrax* and *L. aurata* confirmed their usefulness as bioindicator species of environmental contamination. Nevertheless, considering the biomarkers analysed, *D. labrax* showed higher differences between individuals from the reference and the contaminated sites thus demonstrating a higher sensitivity to the contaminants. For this reason, *D. labrax* seems to be a more suitable species for biomonitoring estuarine ecosystems.

7.4. APPLICATION OF THE TOXICOLOGICAL RESPONSES AS BIOMARKERS

The set of methodologies employed in the present work proved useful for the detection of the biological responses to contaminant exposure. The analysis of histopathological lesions has been widely employed in environmental biomonitoring programs (Stentiford *et al.* 2003; Teh *et al.* 1997), and the alterations found in this study have been observed in other fish species living in contaminated waters (Nero *et al.* 2006; Schwaiger *et al.* 1997). Nevertheless, several other factors such as diet and environmental temperature may induce structural states which may represent normality and could not be a consequence of the chemical exposure *per se*. The parallel use of different biomarkers is, thus, of major importance to minimize the influence of natural variation contributing to a better evaluation of the toxicant exposure impact.

At the molecular level, different endpoints were evaluated, including the increase of *cyp1A* transcript levels. Alterations in the activity or gene expression of the enzymes involved in the xenobiotic metabolism are typically the initial response to any toxic insult by a xenobiotic, and for that reason measurement of the activity of these enzymes have been employed as biomarkers of pollution exposure (Fenet *et al.* 1998; Livingstone *et al.* 2000; Maria *et al.* 2004). In the present work, PCR-based techniques were used to quantify the *cyp1A* mRNA level in *A. anguilla* exposed to DMBA and in feral fish (*D. labrax* and *L. aurata*) living in different contaminated sites. This marker revealed useful in the laboratory controlled experiment where the expression of *cyp1A* was found induced in response to DMBA exposure (Chapter 4). Yet, no changes in the expression levels of this gene were found in *D. labrax* and *L. aurata* captured from the natural environment (Chapter 5). As with the histopathological alterations, also CYP1A activity is modulated by different factors, such as the physiological condition of the fish, genetics, environmental temperature and the presence of inhibitors in water. The occurrence of these confounding factors in the natural environment may thus mask the induction response imposed by contaminants such as PAHs.

The genotoxic potential of xenobiotics was assessed using two methodologies: comet assay and flow cytometry (FCM). Both approaches have

previously proved their usefulness in the detection of gross DNA damage induced by aquatic contamination (Frenzilli *et al.* 2009; Marchand *et al.* 2003). While comet assay was used to measure breaks in the DNA molecule, FCM was employed to evaluate damage at the chromosomal level (variations in the nuclear DNA content), and therefore the two analyses were complementary. The assessment of DNA and chromosome integrity using these methods revealed to be suitable biomarkers of toxicant exposure in the three fish species studied.

At the next level of biological effects, which probes the actual significance of the DNA damage found by the comet assay and FCM, different liver gene-specific alterations were evaluated and applied as novel biomarkers of exposure to contaminant exposure. These included the analysis of *ras* gene mutations and expression in *A. anguilla, D. labrax* and *L. aurata*, changes in *rad1* gene expression in *A. anguilla* exposed to DMBA, alterations in *xpf* gene expression in *D. labrax* from a selected contaminated site, and the identification of pollutant-responsive transcripts in *A. anguilla* exposed to DMBA and in *D. labrax* captured from a contaminated site. The *ras* and *xpf* biomarkers gave important information about genome stability. SSH analysis, besides allowing a global approach, further provided directions for the development of new biomarkers.

The study of these novel molecular responses revealed the wide implication of contaminant exposure and, anchored to well-established biomarker approaches, contributed to a better understanding of the pollutant-induced effects and the potential future repercussions at higher levels of biological organization. The results herein presented also demonstrate that, as supported by other authors (Cajaraville *et al.* 2000; Hagger *et al.* 2006), environmental biomonitoring programs should employ a suite of biomarkers, ideally at a number of different levels of organization, in order to assess the possible biological impacts minimizing misinterpretations induced by confounding factors.

7.5. DEVELOPMENT OF NOVEL BIOMARKERS

In environmental monitoring, biomarkers such as liver histopathology and DNA damage detection have been routinely used as an early warning, yet the specific and ultimate impact are rarely established. In the present work, novel molecular responses were analysed to fill that gap by determining whether the damaged DNA is repaired or leads to mutations in key genes. The use of these markers, which reflect early molecular changes, combined to biomarkers at higher levels of biological organization, would also allow a more integrated approach to the assessment of the adverse effects of pollutants.

The molecular responses studied in the laboratory exposures to single PAHs are difficult to extrapolate to field experimental exposures where a complex mixture of compounds and factors is present. The responses analysed in this study should be, therefore, tested in fish from the natural environment before accepted as useful biomarkers in monitoring studies to predict potential effects under complex mixture scenarios.

In the assessment of aquatic pollution in Ria de Aveiro, different responses were analysed. Some of the examined molecular responses showed potential to be used as indicators of exposure and of biological response to contamination. In that study, few individuals in a limited number of fish species were investigated. Before the inclusion of these molecular responses as biomarkers in environmental surveys of contaminated sites, further work is necessary to define more thoroughly the range of responses considered normal for different sites and to identify seasonal influences.

Complementary molecular biomarkers were explored using the SSH approach. This approach revealed differentially expressed genes involved in different cellular processes that have the potential to be used as markers of contaminant exposure. Genes involved in the energy production (cytochrome c oxidase, electron-transfer-flavoproteins and NADH dehydrogenase) are good candidates as they were found upregulated in exposed fish (Chapter 5). HGF activator-like protein may also be a potential marker of liver injury resulting from toxicant exposure. Some of the genes found downregulated in exposed *D. labrax* (transferrin, copper/zinc superoxide dismutase, stromal cell derived factors 4 and

3-hydroxybutyrate dehydrogenase) may also be further studied. The DMBA-responsive transcripts that showed potential to be employed as biomarkers of exposure to DMBA in particular, and PAH in general include: very large inducible GTPase1, TBT binding protein 1, CCAAT/enhancer binding protein beta 2, NCK-associated protein 1 and catechol-O-methyltransferase (Chapter 4). A gene of particular interest is cysteine/glutamate transporter because it is involved in the cellular protection from oxidative stress and has been related to the development of tumours in mammals (Lo *et al.* 2008), and is thus a strong candidate for biomarker development.

In addition to genes identified via SSH approach, also the newly isolated cancer genes (Chapter 6) are targets for biomarker development. Determining if these genes are altered by environmental contaminants is essential to their establishment as early-warning biomarkers of genotoxic damage in the aquatic environment.

The systematic use of multiple biomarkers is increasing in environmental monitoring studies. The inclusion of novel, more sensitive molecular and genetic biomarkers could represent a viable contribution to these ecological studies.

7.6. CONCLUSIONS

The major conclusions that can be withdrawn from the present work are the following:

A) Study of the genotoxic potential of individual PAHs (BaP and DMBA) in *A. anguilla* (Chapters 2, 3 and 4):
   - Chronic exposure to BaP and acute exposures to BaP and DMBA induce DNA damage in *A. anguilla,* in concentrations higher than 0.3 µM;
   - Gill and blood cells seem to be more affected by the exposure to BaP and DMBA than the liver cells;
   - CYP1A seems to be involved in PAH metabolism in *A. anguilla*;
   - BaP genotoxic endpoint in *A. anguilla,* under the tested conditions, does not involve alterations in *ras* gene;

- Expression of *rad1* gene is not increased in DNA damaged cells after DMBA exposure, indicating that other genes/proteins are involved in the detection and repair of the lesions induced by PAHs in eels;
- The identified DMBA-responsive genes are involved in xenobiotic metabolism, immune processes, protein translation and cytoskeleton dynamics, showing a wide implication of PAH exposure.

B) Study of new molecular responses induced by environmental contamination in *D. labrax* and *L. aurata* from Ria de Aveiro lagoon, in parallel with well-established biomarkers (Chapter 5):
   - *D. labrax* and *L. aurata* captured from contaminated sites in Ria de Aveiro show histopathological lesions of the liver related to the type of contaminants present at each studied site;
   - Both species captured at the contaminated sites presented DNA damage as measured by the comet assay indicating, as expected, the presence of genotoxic agents among the contaminants of the estuary; the presence of DNA damage may be associated with the presence of the histopathological lesions, since fish possessing DNA damage also showed significant levels of histopathological lesions;
   - Expression of *cyp1A* gene was found unaltered in exposed *D. labrax*, suggesting the presence of inhibitors or other confounding factors in the selected contaminated sites;
   - The genotoxic endpoints of Ria de Aveiro contamination do not involve *ras* gene mutations or altered *ras* gene expression;
   - *xpf* gene expression is decreased in *D. labrax* exposed to contaminants, suggesting impaired DNA repair capacity in those fish;
   - The SSH global approach revealed alterations in the expression of genes involved in the energy metabolism, antioxidant response and immune system activity, indicating that vital cellular processes are affected by contaminant exposure.

C) Isolation and characterization of several novel cancer-related genes in aquatic species (Chapter 6):

- *ras*, *rad1*, *xpf* and *Rb* genes from *A. anguilla*, *D. labrax* and *L. aurata* show a high homology with those of other fish species and higher vertebrates counterparts, suggesting a conserved function between species and their potential application as novel molecular biomarkers in biomonitoring studies.

Finally, and to summarize, the expression of *xpf, Rb*, *rad1* and of genes identified by SSH are potential novel molecular biomarker responses. The study of novel molecular responses anchored to well-established biomarker approaches, were useful in the assessment of the contamination impact on fish. This integrated approach contributed to a better understanding of the pollutant-induced biological effects and of the potential future repercussions at higher levels of the biological organization.

7.7. FUTURE WORK

- Study the impact of the contamination in key genes identified by the SSH in order to develop new molecular biomarkers of contaminant exposure;
- Investigate the effects of xenobiotic impact in the novel fish cancer-related genes isolated in the present work, aiming the development of new molecular biomarkers of genotoxic pollution;
- Analyse the genotoxic potential of other environmental relevant xenobiotics, under laboratory controlled conditions, in fish, targeting to the development of new molecular biomarkers;
- Isolate and characterize different DNA repair genes in fish species, and study of the effects of xenobiotic exposure on those important genes;
- Analyse possible *ras* gene alterations in *N. reticulatus* either exposed to selected xenobiotics or to environmental contamination, with the purpose of studying the potential of employing the *ras* gene from *N. reticulatus* as molecular biomarker of contaminant exposure.

7.8. REFERENCES

Ahmad I, Maria VL, Oliveira M, Serafim A, Bebianno MJ, Pacheco M, Santos MA. 2008. DNA damage and lipid peroxidation vs. protection responses in the gill of *Dicentrarchus labrax* L. from a contaminated coastal lagoon (Ria de Aveiro, Portugal). Science of the Total Environment 406(1-2):298-307.

Cajaraville MP, Bebianno MJ, Blasco J, Porte C, Sarasquete C, Viarengo A. 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Science of the Total Environment 247(2-3):295-311.

David WM, Mitchell DL, Walter RB. 2004. DNA repair in hybrid fish of the genus *Xiphophorus*. Comparative Biochemistry and Physiology Part C Toxicology & Pharmacology 138(3):301-309.

Du Corbier FA, Stentiford GD, Lyons BP, Rotchell JM. 2005. Isolation of the retinoblastoma cDNA from the marine flatfish dab (*Limanda limanda*) and evidence of mutational alterations in liver tumors. Environmental Science and Technology 39(24):9785-9790.

Fenet H, Casellas C, Bontoux J. 1998. Laboratory and field-caging studies on hepatic enzymatic activities in European eel and rainbow trout. Ecotoxicology and Environmental Safety 40:37-143.

Frenzilli G, Nigro M, Lyons BP. 2009. The Comet assay for the evaluation of genotoxic impact in aquatic environments. Mutation Research, Reviews in Mutation Research 681(1):80-92.

Hagger JA, Jones MB, Leonard DR, Owen R, Galloway TS. 2006. Biomarkers and integrated environmental risk assessment: are there more questions than answers? Integrated environmental assessment and management 2(4):312-329.

Jha AN. 2004. Genotoxicological studies in aquatic organisms: an overview. Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis 552(1-2):1-17.

Johnson LL, Collier TK, Stein JE. 2002. An analysis in support of sediment quality thresholds for polycyclic aromatic hydrocarbons (PAHs) to protect estuarine fish. Aquatic Conservation: Marine and Freshwater Ecosystems 12(5):517-538.

Livingstone D, Mitchelmore C, Peters L, O'Hara S, Shaw J, Chesman B, Doyotte A, McEvoy J, Ronisz D, Larsson D and others. 2000. Development of hepatic CYP1A and blood vitellogenin in eel (*Anguilla anguilla*) for use as biomarkers in the Thames Estuary, UK. Marine Environmental Research 50(1-5):367-71.

Lo M, Wang YZ, Gout PW. 2008. The xc-cystine/glutamate antiporter: A potential target for therapy of cancer and other diseases. Journal of Cellular Physiology 215(3):593-602.

Marchand J, Tanguy A, Laroche J, Quiniou L, Moraga D. 2003. Responses of European flounder *Platichthys flesus* populations to contamination in different estuaries along the Atlantic coast of France. Marine Ecology Progress Series 260:273-284.

Maria VL, Correia AC, Santos MA. 2002. *Anguilla anguilla* L. biochemical and genotoxic responses to benzo[a]pyrene. Ecotoxicology and Environmental Safety 53(1):86-92.

Maria VL, Teles M, Pacheco M, Correia AC, Santos MA. 2004. Biomarker responses in a polluted river: Effects of pulp and paper mill contaminants on caged *Anguilla anguilla* L. Fresenius Environmental Bulletin 13(4):317-325.

Nero V, Farwell A, Lister A, Van Der Kraak G, Lee LEJ, Van Meer T, MacKinnon MD, Dixon DG. 2006. Gill and liver histopathological changes in yellow perch (*Perca flavescens*) and goldfish (*Carassius auratus*) exposed to oil sands process-affected water. Ecotoxicology and Environmental Safety 63(3):365-377.

Notch EG, Miniutti DM, Mayer GD. 2007. 17α-Ethinylestradiol decreases expression of multiple hepatic nucleotide excision repair genes in zebrafish (*Danio rerio*). Aquatic Toxicology 84(3):301-309.

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

Pacheco M, Santos MA, Teles M, Oliveira M, Rebelo JE, Pombo L. 2005. Biotransformation and genotoxic biomarkers in mullet species (*Liza* sp.) from a contaminated coastal lagoon (Ria de Aveiro, Portugal). Environmental Monitoring and Assessment 107(1-3):133-153.

Regoli F, Winston GW, Gorbi S, Frenzilli G, Nigro M, Corsi I, Focardi S. 2003. Integrating enzymatic responses to organic chemical exposure with total oxyradical absorbing capacity and DNA damage in the European eel *Anguilla anguilla*. Environmental Toxicology and Chemistry 22(9):2120-2129.

Rotchell JM, Lee JS, Chipman JK, Ostrander GK. 2001. Structure, expression and activation of fish *ras* genes. Aquatic Toxicology 55(1-2):1-21.

Sandvik M, Horsberg TE, Skaare JU, Ingebrigtsen K. 1998. Comparison of dietary and waterborne exposure to benzo[a]pyrene: Bioavailability, tissue disposition and CYP1A1 induction in rainbow trout (*Oncorhynchus mykiss)*. Biomarkers 3(6):399-410.

Schwaiger J, Wanke R, Adam S, Pawert M, Hönnen W, Triebskorn R. 1997. The use of histopathological indicators to evaluate contaminant-related stress in fish. Journal of Aquatic Ecosystem Stress and Recovery 6(1):75-86.

Shugart LR. 2000. DNA damage as a biomarker of exposure. Ecotoxicology 9(5):329-340.

Stentiford GD, Longshaw M, Lyons BP, Jones G, Green M, Feist SW. 2003. Histopathological biomarkers in estuarine fish species for the assessment of biological effects of contaminants. Marine Environmental Research 55(2):137-159.

Teh SJ, Adams SM, Hinton DE. 1997. Histopathologic biomarkers in feral freshwater fish populations exposed to different types of contaminant stress. Aquatic Toxicology 37(1):51-70.

van der Oost R, Beyer J, Vermeulen NPE. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology 13(2):57-149.

Weimer TL, Reddy AP, Harttig U, Alexander D, Stamm SC, Miller MR, Baird W, Hendricks J, Bailey G. 2000. Influence of β-naphthoflavone on 7,12-dimethylbenz(a)anthracene metabolism, DNA adduction, and tumorigenicity in rainbow trout. Toxicological Sciences 57(2):217-228.