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TOXICITY OF CARCINOGENIC AND NON-CARCINOGENIC POLYCYCLIC AROMATIC HYDROCARBONS AND THEIR MIXTURES TO AQUATIC ORGANISMS UNDER ECOLOGICALLY-RELEVANT SCENARIOS

Dissertação para obtenção do Grau de Doutor em

Ciências do Ambiente

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Toxicity of carcinogenic and non-carcinogenic Polycyclic Aromatic Hydrocarbons and their mixtures to aquatic organisms under ecologically-relevant scenarios

To my lovely daughters Laura and Luísa

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RESUMO

Os hidrocarbonetos aromáticos policíclicos (PAHs) são poluentes ubíquos considerados prioritários, e estão preferencialmente armazenados em sedimentos aquáticos devido à sua elevada hidrofobicidade. No entanto, as diferenças entre os mecanismos e efeitos toxicológicos das diferentes classes de PAHs e suas misturas (como estão presentes no ambiente) não são bem conhecidas. Nesta tese pretendeu-se estabelecer uma ponte entre o estudo das vias metabólicas e a monitorização ambiental de PAH carcinogénicos e não-carcinogénicos, introduzindo relevância ecológica ao estudo. Um primeiro bioensaio, *in situ*, realizado com o bivalve *Mytilus edulis* demonstrou que dragagens em áreas portuárias aumentam a biodisponibilidade de PAHs, gerando efeitos genotóxicos e indicando que os valores de referência estabelecidos pelas normas ambientais subestimam o risco real. Seguidamente foram realizados ensaios laboratoriais testando o carcinogénico, benzo[b]fluoranteno (B[b]F), e o não-carcinogénico, fenantreno (Phe), cuja escolha se baseou nos resultados anteriores, e revelaram que concentrações baixas-moderadas destes compostos em sedimentos induziram efeitos genotóxicos no bivalve *Ruditapes decussatus*. Estes resultados contradizem assim a noção geralmente aceite de que os PAHs são pouco nocivos para os bivalves, comparativamente aos vertebrados, devido a uma menos eficiente bioactivação. Também foi demonstrado que, a utilização de membranas de amostragem passiva permite aferir biodisponibilidade de PAHs, mas não estabelecer uma relação com a sua bioacumulação ou toxicidade. Por seu lado, bioensaios com robalos (*Dicentrarchus labrax*) revelaram padrões complexos de efeitos e respostas, relativamente a genotoxicidade, *stress* oxidativo e produção de metabolitos, especialmente quando estavam expostos à mistura destes PAHs, levando a efeitos supra-aditivos de interacção. Os resultados indicaram também que o Phe causou efeitos genotóxicos, especialmente na presença de B[b]F, apesar das concentrações baixas (realistas) terem diluído a relação tempo- e dose-resposta. O presente trabalho demonstrou que os valores-guia de qualidade ambiental subestimam os efeitos dos PAHs em condições realistas e revelaram que biomarcadores relacionados com *stress* oxidativo ou CYP podem não reflectir os elevados efeitos genotóxicos e histopatológicos causados pelas misturas. Para além de novos aspectos relacionados com o metabolismo de misturas de PAHs, o trabalho revelou a necessidade de se reavaliar os critérios para a avaliação do risco e de se definirem indicadores de potencial toxicológico mais adequados.

Keywords

Genotoxicidade; *Stress* oxidative; Biodisponibilidade de PAHs; Contaminação de sedimentos; Efeitos de interacção; Toxicologia aquática.

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous priority pollutants that tend to be trapped in aquatic sediments due to their high hydrophobicity. Nonetheless, the differential toxicological effects and mechanisms between the various classes of PAHs and their mixtures, as they invariably occur in the environment, are scarcely known, especially under ecologically-relevant scenarios. This thesis aimed at establishing a bridge between the study of mechanistic pathways and environmental monitoring of carcinogenic and non-carcinogenic PAHs, by introducing ecological-relevance in the research with model PAHs. A first bioassay conducted *in situ* with the mussel *Mytilus edulis* demonstrated that, dredging operations in harbours increase PAH bioavailability, eliciting genotoxicity, and showed that established environmental guidelines underestimate risk. Subsequent *ex situ* bioassays were performed with the carcinogenic benzo[b]fluoranthene (B[b]F) and non-carcinogenic phenantrene (Phe), selected following preceding results, and revealed that low-moderate concentrations of these PAHs in spiked sediments induce genotoxic effects to the clam *Ruditapes decussatus,* therefore contradicting the general notion that bivalves are less sensitive to PAHs than vertebrates due to inefficient bioactivation. Also, it was demonstrated that passive samplers permit inferring on PAH bioavailability but not on bioaccumulation or toxic effects. On the other hand, sea basses (*Dicentrarchus labrax*), yielded a complex pattern of effects and responses, relatively to genotoxicity, oxidative stress and production of specific metabolites, especially when exposed to mixtures of the PAHs which led to additive, if not synergistic, effects. It was shown that Phe may elicit significant genotoxicity especially in presence of B[b]F, even though the low, albeit realistic, exposure concentrations diluted dose- and time-independent relationships. The present work demonstrated that environmental quality guidelines underestimate the effects of PAHs in realistic scenarios and showed that the significant genotoxic and histopathological effects caused by mixed PAHs may not be reflected by oxidative stress- or CYP-related biomarkers. Besides important findings on the metabolism of PAH mixtures, the work calls for the need to re-evaluate the criteria for assessing risk and for the disclosure of more efficient indicators of toxicological hazard.

Keywords

Genotoxicity; Oxidative stress; PAH bioavailability; Sediment contamination; Interaction effects, Aquatic toxicology.

ABREVIATIONS

AAS, atomic absorption spectrometry

AHR, aryl hydrocarbon receptor

ARNT, aryl hydrocarbon nuclear translocator

ASE, accelerated solvent extraction

B[a]P, Benzo[a]pyrene

B[b]F, Benzo[b]fluoranthene

BER, base excision repair

BPDE, benzo[a]pyrene dihydrodiol-epoxide

CDNB, chloro-2,4-dinitrobenzene

CYP1A, cytochrome P450 1A

DMSO, dimethylsulfoxide

DNA, deoxyribonucleic acid

dwt - total dry mass (total dry "weight")

ENA, erythrocytic nuclear abnormality

ERA, Environment Risk Assessment

EROD, ethoxyresorufin-O-deethylase

FF, sediment Fine Fraction

GC-ECD , Gas chromatography with electron capture detector

GC-MS, gas chromatography-massspectrometry

GSH, glutathione

GSSG, glutathione disulphide

GST, glutathione *S*-transferase

H&E, Haematoxylin and eosin histological stain

HSP90, heat-shock 90 proteins

IARC, International Agency for Research on Cancer

ICP-MS, inductivity coupled plasma mass spectrometry

Ih, histopathological condition indice

K_{ow}, octanol–water partitioning coefficient

LOE, Line-of-evidence

LMPA, low melting point agarose

LPO, lipid peroxidation

L_s, shell length

MFO, mixed-function oxygenase/oxidase

MN, micronuclei

NA, nuclear abnormalities

NER, nucleotide excision repair

NMPA, normal melting point agarose

PAH, polycyclic aromatic hydrocarbon

PCA, Principal component analysis

PCB, Polychlorinated biphenyls

PBS, phosphate-buffered saline

PEL, probable effects level

PEL-Q, probable effects level quotient

Phe, Phenanthrene

RNA, ribonucleic acid

ROS, reactive oxygen species

SB, strand breakage

SCGE, single cell gel electrophoresis

SIM, selected ion monitoring

SQGs, sediment quality guidelines

SQG-Q, sediment quality guideline quotient

TBARS, thiobarbituric acid reactive substances

TEL, threshold effects level

TOM, total organic matter

VC, variation coefficient

UDPGT, UDP-glucuronyltransferase

USEPA, U.S. Environmental Protection Agency

WFD, Water Framework Directive

WHO, World Health Organization

WOE, weight-of-evidence

ww_t - total wet mass (total wet "weight")

XRE, xenobiotic response element

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CHAPTER 1. GENERAL INTRODUCTION[†]

[†] partially published in Martins and Costa (2014). Mutagenesis. (doi:10.1093/mutage/geu037).

1. Polycyclic aromatic hydrocarbons as priority pollutants

Among the organic environmental toxicants, polycyclic aromatic hydrocarbons (PAHs) are acknowledged priority substances due to their immunotoxic, teratogenic, clastogenic, mutagenic and carcinogenic effects (Miller and Ramos, 2001; Gangar et al., 2010; Yang et al., 2010; Giannapas et al., 2012). Owing also to their high toxicity and ubiquity in the environment, PAHs are allocated within the top of the most aggressive pollutants. As such, these substances are regarded as priority substances by many environmental agencies worldwide, being included in the Priority Substances List attached to the European Water Framework Directive (WFD, updated through the Directive 2008/105/EC), later followed by the Marine Strategy Framework Directive (MSFD, Directive 2008/56/EC). They are also flagged by the U.S. Environmental Protection Agency (USEPA) and the World Health Organization (WHO). Although the toxicity of PAHs is high, their distinct chemical properties render diverse toxicological pathways and effects. With respect to carcinogenic effects alone, these compounds are listed by the International Agency for Research on Cancer (IARC) as non-carcinogenic, potentially carcinogenic and effectively carcinogenic to humans.

Polycyclic aromatic hydrocarbons are usually originated by incomplete combustion of fuel and other organic matter and are characterised by possessing two or more benzenoid rings (Table 1.1). The stereological features of the molecules widely vary and primarily dictate their toxicological and carcinogenic properties. Generally, low molecular weight PAHs (comprising two or three rings), pose higher acute toxicity (due to their higher solubility in water), whereas some of the high molecular weight PAHs (four to seven rings) exert chronic effects more notorious including, mutagenic and carcinogenic. Among the latter, the five-ring PAH benzo[a]pyrene (B[a]P) has extensively studied, since it is highly carcinogenic to a wide range of organisms, including humans, being considered a model PAH in many, if not most studies on environmental toxicology involving carcinogenesis (Baumman et al., 1996). Also, non-alternant PAHs (those with other rings besides benzenoid rings), such as benzo[b]fluoranthene and indeno[1,2,3-cd]pyrene appear to differ in their metabolic activation from the alternant PAHs (those holding structure composed entirely of benzenoid rings), such as phenanthrene and chrysene, which may dictate different toxicological hazard and metabolic pathways (see Phillips and Grover, 1994). Moreover, the presence of a bay- or fjord-region (Fig. 1.1) may also increase the toxicity of a PAH (Xue and Warshawsky, 2005).

Due to the link between PAH toxicity and carcinogenicity, several studies were developed with experimental models *in vivo* and *in vitro*, as well as epidemiological investigations on human occupational health. Most of this research focused on the effects, responses and toxicological mechanisms underlying exposure to single PAH. However, PAHs, occur in the environment in mixtures of both carcinogenic and non-carcinogenic classes, albeit their interaction effects remain

largely unknown. As such, regardless of the importance of undertaking studies with isolated substances, extrapolating findings to natural milieu may lead to an underestimation of risk (e.g. Mayer and Reichenberg, 2006). As an example, two or more substances within a mixture may compete for receptors or actives sites of metabolising enzymes and chaperones, yielding synergistic, antagonistic or additive effects that, in the case of strong mutagens as most PAHs, may result either in cocarcinogenic or chemopreventive effects (Jarvis et al., 2014).

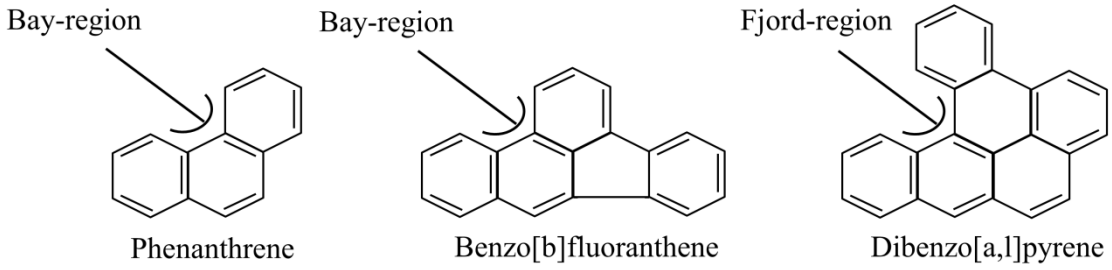


Fig. 1.1. Bay- and Fjord-region PAHs.

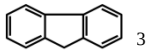
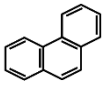
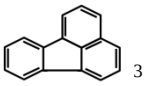
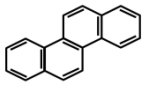
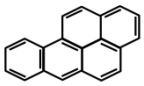
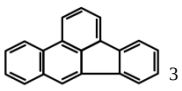
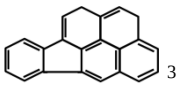
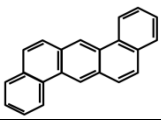
The lack of knowledge about the effects of toxicant mixtures to ecosystems is not, however, the only factor that hinders ecological relevance in the field of environmental toxicology. In order to attain realistic measures of risk, it is also paramount to understand how the local biota is affects (i.e., as opposite to fully laboratorial models); to study realistic routes and vehicles of exposure and to perform research with concentrations of xenobiotics similar to those found in the environment when performing research *ex situ*. These premises apply, of course, to terrestrial, freshwater and brackish or marine ecosystems. However, coastal environments, especially, estuaries and other confined waterbodies, are complex ecosystems that have long been subjected to a myriad of anthropogenic stressors, to which contamination by organic and inorganic substances is considered one of the most important threats to biota and local populations.

2. Polycyclic Aromatic Hydrocarbons in the aquatic environment

Polycyclic aromatic hydrocarbons are primarily present in the aquatic environments as a result of anthropogenic processes. The substances enter the aquatic milieu mostly through oil spills, wastewaters from industrialised and urbanised areas, maritime and riverine traffic and deposition of air- or water-transported particulate matter to which these substances tend to bind to (reviewed in Meador et al., 1995). Due to their hydrophobicity, PAHs, tend to be trapped in the complex

geochemical matrix of sediments, sorbed to fine particles and organic matter. Aquatic sediments are, therefore, long acknowledged potential reservoirs for mixtures of PAHs and may act as a mid/long-term, permanent, source of these and other substances to the biota. As such, the importance of understanding the risk of sediment-bound contaminants, especially those of confined coastal ecosystems is long acknowledged, albeit challenging and ever evolving (see for instance Chapman et al., 2013). In fact, surveying the toxicological hazards of mixed sediment-bound PAH holds many constraints, from the possible effects of toxicant interactions, inter-species differences (related with responses and effects) to factors affecting bioavailability (related with sediment and PAH physico-chemical properties). Regardless of these constraints, guidelines for PAH concentrations in aquatic sediments have been issued for some PAHs, albeit as single compounds and not mixtures, based on large sets of experimental data, such as the Sediment Quality Guidelines derived by MacDonald et al. (1996).

Table 1.1. Selected US EPA priority PAHs and physicochemical properties (adapted from Neff, 2002).

PAH	Chemical structure	IARC classification	Molecular weight (g mol^{-1})	Aqueous solubility (mg L^{-1})	Log K_{ow}
Naphtalene[1]		2B	128	31.7	3.33
Fluorene[1]	 [3]	3	166	1.9	4.18
Phenanthrene[1]		3	178	1.1	4.57
Fluoranthene[2]	 [3]	3	202	0.26	5.23
Chrysene[2]		2B	228	0.0019	5.81
Benzo[a]pyrene[2]		1	252	0.0016	6.13
Benzo[b]fluoranthene[2]	 [3]	2B	252	0.0015	5.80
Indeno[1,2,3-cd]pyrene[2]	 [3]	2B	276	0.0026	7.00
Dibenzo[a,h]anthrancene[2]		2A	278	0.0006	6.75

1 – Carcinogenic to humans; 2A – Probably carcinogenic to humans; 2B – Possibly carcinogenic to humans; 3 - Not classifiable as to its carcinogenicity to humans; K_{ow} – octanol-water partition coefficient; [1] Low molecular weight PAHs; [2] High molecular weight PAHs; [3] Non-alternant PAHs.

The release of sediment-bound PAHs to the water column (either dissolved or bound to resuspended particulate matter) is dependent of the geochemical properties of the sediment (such as the organic matter and fine particle proportions) and the physico-chemical properties of the different PAHs themselves. Desorption of PAHs from particles is chiefly determined by their solubility in water (typically low), which is reflected in the octanol-water partitioning coefficient (K_{ow}) of each compound (Means et al., 1980; Narbonne et al., 1999). As such, the solubility of aromatic compounds decreases as the K_{ow} increases (Table 1.1). Also, molecular weight and number of benzenoid rings, are inversely correlated with desorption rates, i.e., higher molecular weight PAHs exhibit higher tendency to remain adsorbed to particulate matter than to solubilize to water (Narbonne et al., 1999). Moreover, natural processes and anthropogenic activities, such as the remobilization of sediments associated to storm and dredging activities, respectively, may favour the release from adsorbed particles to the water column, potentially rendering them more bioavailable. In the case of PAHs, these processes usually involve oxic/anoxic shifts during sediment disturbance (see Eggleton and Thomas, 2004). Thus, steady-state and disturbed sediments may yield distinct hazards; hence the importance of understanding if and how PAHs are released from sediments in sufficient amounts to elicit adverse effects.

Once bioavailable, PAH uptake, accumulation and detoxification or elimination differs between organisms and is also modulated by the chemical properties of the compounds (Porte and Albaigés., 1993; Meador et al., 1995). Generally, lower molecular PAH are more easily uptaked and eliminated by organisms than higher molecular PAHs due to their higher solubility in water (Djomo et al., 1996). Also, it must be noticed that the uptake of organic xenobiotics from the water column is largely passive across biological barriers, meaning that the uptake rates are generally similar in both invertebrates and fish, hence linear bioconcentration should be predictable from K_{ow} (Zaroogian et al., 1985). Nonetheless, the rates of biotransformation/elimination differ within taxonomic groups and depend on toxicant concentration and availability plus efficiency of biochemical pathways of detoxification (i.e. able to carry, PAHs and their metabolites through phase I and II of detoxification). These biochemical pathways, typically based on the activity of cytochrome P450 (CYP) for many organic toxicants, PAHs included, are acknowledged to be more efficient in vertebrates (Livingstone, 1998). This aids explaining PAH accumulation and bioconcentration is considered to be generally more efficient in invertebrate filter feeders (like bivalve molluscs), for example, than in fish (see Stegeman, 1981; Walker and Livingstone, 1992). On the other hand, fish and other vertebrates, may efficiently biotransform PAHs, yielding reduced bioaccumulation and poor correlations with concentrations of exposure (see van der Oost et al., 2003, for a review). For these reasons, effects-oriented research based may more readily indicate exposure to PAHs, at least in vertebrates, than bioaccumulation *per se*. Nonetheless, there are abiotic techniques, namely recurring to passive sampling devices that are used to determine bioavailability of xenobiotics to organisms that are based,

precisely, on the constancy of uptake rates through biological membranes (Huckins et al., 1993). In this case, hydrophobic contaminants (e.g. PAHs) are accumulated in the device by passive diffusion through a surrogate membrane, permitting the partitioning of compounds between water and the collection phase (which is a lipid in the case of semipermeable membrane devices - SPMD). Passive sampling devices have been recognised as a valuable tool in environmental monitoring, potentially able to detect trace concentrations of contaminants and integrate them in time. Some authors, also state that they are easier to use, less expensive and more reproducible than biological models, avoiding inconveniences related to migration, mortality, metabolism or depuration of contaminants (Vrana et al., 2005). Nonetheless, direct comparisons with organisms to confirm these statements are controversial.

3. Biological pathways of PAH toxicity

As for many other toxicants, the effects and underlying mechanisms triggered by PAHs have been mostly drawn from experimental research where ecological relevance was often omitted, with respect to concentrations, model organisms, and, most importantly, mixtures of PAHs, as they consistently occur in the environment. Also, in spite of the many PAH compounds known to science, research tends to focus on a few representative substances, not necessarily to ones most common in particular natural scenarios. Overall, PAHs are a particular class of pollutants in the sense that most of their toxicity is derived from the cell's own detoxification mechanisms. By other words, the parent compounds are not the main toxic agents *per se* but their metabolites, whose production follows a process commonly designated by bioactivation. This term itself, bioactivation, calls to the action of enzymes that transform hydrophobic, little reactive, substances into more electrophilic, more easily eliminate but more reactive, metabolites.

For many hydrophobic xenobiotics, namely many PAHs, some dioxins, alkylphenols and others, the biotransformation process begins with the addition of an electrophilic group to the molecule, which is typically catalysed by phase I microsomal mixed-function oxygenases (MFOs) of CYPs, like monooxygenases (see for instance Stegeman and Hahn, 1994). In a subsequent reaction, the oxidized metabolite may be conjugated with an endogenous organic ligand such as sugar derivate, an acetate, a peptides (e.g. glutathione) or a sulphate, rendering it inactive and facilitating elimination. These conjugation reactions are catalysed by phase II enzymes such as glutathione-*S*-transferase (GST), UDP-glucuronyltransferase (UDPGT) and sulphotransferases (STs) [Stegeman and Hahn, 1994].

The bioactivation of PAHs by CYP MFOs yields highly reactive and highly hazardous metabolites, such as quinones and diol-epoxides and, simultaneously, generates reactive oxygen species (ROS) as

by products. Depending on parent compound and bioactivation enzyme system or isoform, these metabolites may be genotoxic for being highly reactive with DNA, with which they form bulky adducts, promote nucleotide oxidation and other alterations that may or not be repairable (or even be misrepaires), depending on severity and extension, in the latter case thus being pro-mutagenic (see Conney, 1982, Stegeman and Lech, 1991; Ohnishi and Kawanishi, 2002). It is the case, for instance, of the highly genotoxic PAH-diol epoxides produced from some higher molecular weight PAHs like B[a]P. In addition, ROS may also induce DNA oxidation and affect all levels of the cellular metabolism and structure, from ATP production to DNA repair, phospholipid peroxidation, etc.

The process of PAHs bioactivation may be primarily regulated, at the transcript level, by a positive feedback loop responsible for triggering the expression of CYP genes. This regulatory mechanism is dependent of the aryl hydrocarbon receptor (AHR) pathway, a well-known ligand-activated transcription factor found in vertebrate species, from fish to humans (Schmidt and Bradfield, 1996). Hence, the more AHR-compatible PAHs in cells may imply induction of CYP MFOs, potentially increasing the ability of the cells to metabolise PAHs, which may increase the production of toxic PAH metabolites (Nebert et al., 2004). As such, AHR pathway-related biomarkers, like the contents of CYP1A protein in microsomes, are traditional biomarkers of exposure to PAHs (Nielsen et al., 1998). Although the number of known CYP isozymes keeps increasing, at least for vertebrate model organisms and cell lines (from the zebrafish to murine and human cell models), the CYPs better known to be involved in PAH detoxification processes belong to the CYP1 family, especially subfamilies A, B, C and the CYP2E (see Ioannides and Lewis, 2004; Shimada and Fujii-Kuriyama, 2004; Uno et al., 2012).

Specifically, ligands with affinity to AHR bind to this protein and to two chaperones, namely heat-shock proteins, 90 KDa isoform (HSP90). This complex is then translocated to the nucleus of the cell (loosing then the HSP90 chaperones) and binds with the AHR co-factor, the aryl hydrocarbon receptor nuclear translocator (ARNT). This complex finally binds to a DNA recognition sequence upstream of the CYP genes, also known as the xenobiotic response element (XRE), increasing the transcription of the gene (see for instance Stegeman and Hahn, 1994). Similarly, there are phase II enzymes (e.g. GSTs and UDPGTs) whose regulation is also mediated -via AHR (Owens, 1977; Pickett and Lu, 1989; Rushmore and Pickett, 1990). The affinity of PAHs to AHR depends of their physico-chemical properties. For example, a study that investigated the induction of CYP1A1 and CYP1A2 enzyme activity in human cell lines demonstrated that molecules with aligned rings hold reduced affinity to AHR, thus less potent inducers of CYP1A, compared to molecules with clustered rings. Moreover, the bay-region of the PAH molecule are considered to be important factor to promote CYP1A induction, since it confers higher affinity to the AHR active binding site (Sundberg, et al. 1998; Skupinska et al., 2007). For some PAHs, such as lower molecular weight PAHs that have little or no activity as AHR

ligands (e.g. devoid of a bay-region), toxicity appears to be AHR-independent, thus very distinct, mechanistically, from that of higher molecular weight, AHR-agonist PAHs (Barron et al., 2004).

For instance, the metabolic activation of the model carcinogen B[a]P, for example, has been extensively studied. The first two products of B[a]P metabolism (catalysed by cyt P450 monooxigenases) are either mono-hydroxy-B[a]P (a phenol) and a B[a]P epoxide. Phenol can be conjugated to glucuronic acid or sulphate by the phase II enzymes UDPGT or ST, respectively, and be easily excreted. Alternatively, B[a]P phenols can undergo a second CYP catalysed reaction to form quinones. These, in the presence of cellular reducing agents (such as NADPH or reduced glutathione) may form unsaturated diols, which can be readily oxidized back to the catechol (Flowers-Geary et al., 1992). The catechol can be autooxidized a second time, producing substantial amounts of reactive oxygen species (ROS), which may cause direct nucleobase oxidation (see, e.g. Cavalieri and Rogan, 1995). The resulting B[a]P epoxide can be conjugated to glutathione (GSH), a reaction catalysed by GST, or be transformed by the epoxide hydrolase to a B[a]P dihydrodiol that, on his turn, can be excreted in similar way as phenols, following conjugation to glucuronic acid or sulphate. However, a second oxygenation reaction can occur, catalysed by CYP monooxygenases, producing a B[a]P dihydrodiol-epoxide (BPDE). These metabolites may covalently bind to DNA and form bulky adducts, hence their high mutagenic potential (Wogan et al., 2004).

4. PAHs as genotoxicants, mutagens and carcinogens

Hydrophobic genotoxicants, like PAHs are preferentially adsorbed to suspended particulate matter, and ultimately, incorporated into bottom sediments. As such, sediments are considered a sink for hydrophobic environmental mutagens. Therefore, the burial of mutagenic substances can generate a reservoir of mutagenic hazard since they can be continually reintroduced into the water column via resuspension and trophic transfer, which confers the growing interest in determining the genotoxic effects of these substances to aquatic organisms as a measure of environmental risk (see Chen and White, 2004).

Much research on PAH toxicity has been directed to human health hazards, focusing on the substances' mutagenic and carcinogenic actions. As further detailed below, these two processes are, to some extent, related since there is growing evidence that certain DNA adducts formed by metabolites of carcinogenic PAHs may originate fixed mutations in proto-oncogenes or tumour-suppressor genes, that are found in chemically-induced cancers (Purchase, 1994). In fact, the levels of stable DNA-adducts of many xenobiotics, including PAHs, are highly correlated with the incidence of tumours in laboratory animals (Otteneder and Lutz, 1999). In theory, the presence of an epoxide group in the bay-

or fjord-region (Fig. 1.1) facilitates opening of the saturated benzenoid ring to which it is attached to, facilitating the reaction with DNA (Xue and Warshawsky, 2005). However, the position of the dihydrodiol complex and the epoxide group, as well as the stereochemistry of the PAHs determines the affinity of the metabolite to bind to DNA. Thus, different PAHs may form stronger PAH-DNA adducts than others (Xue and Warshawsky, 2005). In addition to the formation of DNA-adducts, ROS produced during PAH activation, on their turn, can interact with DNA and produce single- or double DNA-strand breakage (DNA-SB), base modifications and DNA crosslinks (Penning et al., 1996; Ohnishi and Kawanishi, 2002), thus increasing the probability of occurring misrepaired and unrepaired damage, leading to mutations. The carcinogenic potential of PAHs, as for other toxicants, may also results from excessive DNA damage or general metabolic failure leading to cell death by necrosis, which implies inflammation that, on its own may promote tumourigenesis (see Sarasin, 2003).

Organisms possess a significant battery of mechanisms to repair DNA damage at the chain level through base excision repair (BER) and nucleotide excision repair (NER) and even backup mechanisms to avoid the dissemination of mutations in case repair fails, namely through cell-cycle arrest (to avoid mitosis) and programmed cell death (apoptosis, in animals). While BER is the major mechanism for repairing oxidative DNA base lesions and single strand breaks, NER is the main repair system for DNA cross-links and bulky adducts generated from exposure to compounds such as PAHs. Nevertheless, increasing DNA damage may increase the genotoxicity of PAHs and the probability of occurring mutagenesis if the DNA lesion is mis- or unrepaired, leading to a permanent change of the genetic information. In fact, if the repair is not complete prior to replication, the presence of adducts can give rise to the misrepair of DNA strand. Also, mis- or unrepaired DNA-SB faulty mitosis may occur, originating chromosome and chromatid fragments and aneugenic events (see Fenech et al., 2011). Mutations, in turn, may lead to teratogenesis, especially if affecting the germ line or if occurring during embryogenesis, leading to malformations. On the other hand, mutations occurring in proto-oncogenes may turn these into active oncogenes whose increased expression may lead to anaplastic (degenerate) or neoplastic (tumour-like) proliferative cells (Payne and Kemp, 2005). The relationship between PAH metabolites produced CYP MFOs, the formation of metabolite-DNA adducts and the activation of *ras*-family oncogenes has long been established in murine models (Nesnow et al., 1995). Loss-of-function mutations occurring in tumour-suppressor genes may also potentiate the formation of tumour-like cells (Payne and Kemp, 2005). Altogether, it appears that PAH-induced mutations are unlikely to be random, rather depending on the affinity of a given metabolite towards a region or motif of the DNA chain which aids explaining, for instance, the high carcinogenic potential of B[a]P diol epoxides (Xue and Warshawsky, 2005).). Nevertheless, the differences regarding the mode-of-action and effects between the distinct classes of PAHs (e.g. carcinogenic/non-carcinogenic) are not fully understood. Also, the pathways underneath PAH metabolism, toxicity and carcinogenicity are acknowledged to be complex and dependent of a

multiplicity of factors, of which PAHs interactions are an example still awaiting to receive adequate focus. In any case, in spite of the many gaps still persisting in the current PAH state-of-the art, DNA damage is a keystone aspect of PAH-induced lesions, especially since carcinogenicity and even mutagenicity are unlikely to occur during the duration of standard bioassays.

5. PAH effects in aquatic vertebrates and invertebrates

Fish and bivalves are, by far, the most common research organisms in aquatic toxicology, whether *in* or *ex situ*. However, the cellular enzymatic machinery of phases I and II of detoxification may vary significantly between the two groups. Overall, the higher rates of *in vivo* biotransformation of B[a]P by fish compared with marine invertebrates are consistent with their higher levels of biotransformation enzyme activities, at least as far as the MFO system is concerned (Livingstone et al., 1998). Regardless of the differences in the underlying mechanisms, many studies have shown that PAHs are toxic to both fish and bivalve (e.g. Canova et al., 1998; Oliveira et al., 2007; Yin et al., 2007; Giannapas et al., 2012). However, there are still many gaps in the knowledge on PAH toxicity in bivalves and other invertebrates.

There are some indications that the metabolism of PAHs, via CYP MFOs is reduced in bivalves when compared to fish (Peters et al., 2002), leading to hampered bioactivation of PAH pro-mutagens. Nevertheless, the existing reports are often contradictory. For instance, the formation of PAH metabolite–DNA adducts has long been reported to occur in mussels (Venier and Canova, 1996; Akcha et al., 2000), as well as the presence of CYP isoforms (e.g. CYP4) may be able to metabolize PAHs (Pan et al., 2011). Still, it has been stated that invertebrate AHR homologues do not bind to ligands similarly to vertebrate AHR, which may decrease CYP induction and the subsequent decrease of PAH metabolization (Butler et al., 2001; Hahn, 2002; Chaty et al., 2004). Also, it is generally acknowledged that PAHs, trigger toxicological effects in bivalves mainly via oxidative stress, either by eliciting direct damage or by exhausting the anti-oxidant defences of the cells, such as the GSH pool (see, e.g., Winston and Di Giulio, 1991; Frenzilli et al., 2001; Livingstone, 2001; Giannapas et al., 2012). Genotoxic damage has been detected and measured in marine invertebrates following chronic exposure to sediments contaminated with various toxicants, including PAHs (Coughland et al., 2002; Hartl et al., 2004; Frenzilli et al., 2009). In fact, some studies detected a significant production of strand breaks (Venier et al., 1997), DNA adducts (Venier and Canova, 1996; Dolcetti et al., 2002), chromosomal aberrations (Al-Sabti and Kurelec, 1985), and micronuclei (Scarpato et al., 1990; Venier et al., 1997) in bivalves associated to B[a]P exposure.

Conversely, fish are known to have a well-developed MFO system that might efficiently detoxify a large number of organic xenobiotics, including PAHs (Uno et al., 2012, for a review). Cytochrome P450, and CYP 1A subfamily, in particular, is predominantly present in the endoplasmatic reticulum of the liver, and play important role in PAH activation. Expression of fish CYP1 family mRNA, like that in mammals, may be induced by PAHs. Although the mechanisms of CYP1A induction in fish are not so well understood as murine models, two putative AHR genes are identified in the mummichog (*F. heteroclitus*) (Morrison et al.,1998), zebrafish (*Danio rerio*) (Andreasen et al., 2002), medaka (*Orizias latipes*), and two species of pufferfish (*Takifugu rubripes* and *Tetraodon fluviatilis*) (Hahn, 2001), which may allow more complex regulation of CYP1A gene expression, in comparison to the single AHR in mammals. It must be noticed that, for instance, the production of hydroxyl radicals, one of the most potent nucleobase oxidating agents, has already been objectively found to be induced by PAHs in fish (Penning et al., 1996; Sun et al., 2006; Hannam et al., 2010), which again confirms the importance of considering DNA damage as endpoint in PAH-related studies with these organisms. Also, in fish, besides a number of reports on genotoxic effects of PAHs, strong links between environmental PAH concentrations and the incidence of liver neoplasms in fish have already been demonstrated (Myers et al., 1991; Baumann et al., 1996, 1998; Vethaak et al., 2009).

The pursuit for ecological relevance in environmental toxicology unavoidably needs to consider ecologically-relevant species of organisms either as sentinels or test subjects, which has, in fact, been one of the main driving forces that led to the employment of fish and bivalves as preferential target subjects. *Ruditapes decussatus* (=*Venerupis decussata*) as has been proposed as a substitute for mussels in biomonitoring studies in areas where these are scarce or absent (Bebianno et al., 2004), since clams also have high economical significance and ecological relevance. Clams burrows in sandy-muddy sediments of coastal ecosystems, especially in enclosed areas such as estuaries and coastal lagoons (see, e.g., Bebianno et al.,2004; Carreira et al., 2013; Costa et al., 2013, and references therein), which are prone to be impacted by multiple anthropogenic pressures. However, most of the toxicological effects and responses to PAH exposure (especially sediment-based) are still lacking, as for most molluscs. The European sea bass (*Dicentrarchus labrax* Linnaeus, 1758, Perciformes: Moronidae) is an eurythermic coastal demersal species that often inhabits estuaries and other confined waters subjected to strong anthropogenic stressors. It is found in waters all around Europe. Fish possess a well-developed MFO system (Uno et al., 2012), which, combined with high ecological and economical importance, besides the ability to act as surrogates for higher-order vertebrates, makes them important models in environmental toxicology.

Thesis objectives and layout

This thesis aims essentially at establishing a bridge between mechanistic studies and environmental monitoring, by introducing ecological-relevance in the research with model PAHs, in order to understand the differences between the modes of action of PAHs considered carcinogenic and non-carcinogenic, and their interactions, towards the biota. Specifically, it is aimed at:

1. Understanding PAHs in sediments, at realistic concentrations, may become bioavailable to organisms as to elicit adverse effects, in the laboratory and in a practical field situation.

2. Disclosing and comparing the main effects and responses triggered by carcinogenic and non-carcinogenic PAHs in sediments to two distinct groups of relevant marine organisms: fish and bivalves.

3. Determining the potential interaction effects of carcinogenic and non-carcinogenic PAHs, under realistic scenarios, to a marine vertebrate, which possesses the molecular machinery to metabolize PAHs, at multiple levels: from tissue- and organ-level lesions to biochemical responses and DNA damage.

4. Inferring on potential pathways of response to chemical challenge imposed by the two types of PAHs aforementioned under realistic conditions while addressing the problem of the suitability of environmental norms and guidelines drawn for these substances, individually.

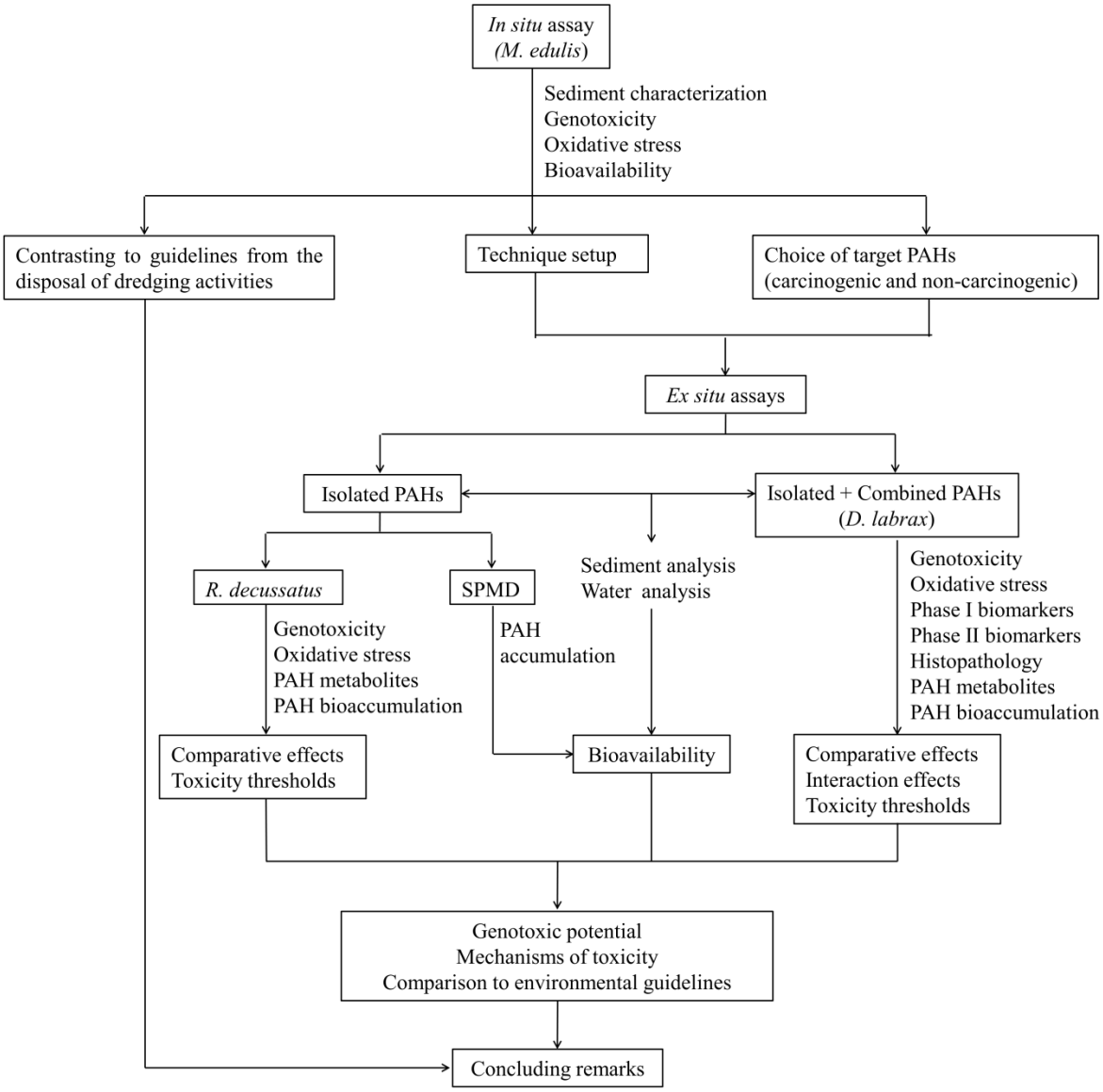


Fig. 1.2. General organization of the research and thesis highlighting the main analytical techniques and goals.

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CHAPTER 2. IMPACT OF REMOBILIZED CONTAMINANTS IN *MYTILUS EDULIS* DURING DREDGING OPERATIONS IN A HARBOUR AREA: BIOACCUMULATION AND BIOMARKER RESPONSES[†]

[†] Martins et al. (2012). *Ecotoxicol. Environ. Safe.* 85, 96-103 (doi: 10.1016/j.ecoenv.2012.08.008).

Abstract

Dredging operations in harbours are recurrent to maintain accessibility and navigational depths. One of the main environmental risks of these operations is the remobilization of contaminants trapped in the sediments, rendering them more bioavailable to the biota. However, regulatory policies regarding the contamination risk of dredging chiefly apply to the disposal of dredged materials rather than the direct impact of the procedure itself. In order to assess the ecotoxicological risk of harbour dredging operations in a polluted estuary (the Tagus, W Portugal), the present study compared bioaccumulation and biomarker responses in field-deployed mussels before and after the beginning of operations, complemented by sediment characterization and risk analysis based on standardized sediment quality guidelines. The results revealed a very significant increase in genotoxicity and oxidative stress from the beginning of dredging onwards, which was accompanied by increased bioaccumulation of toxicants, especially polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). Overall, the results indicate the importance of surveying the direct impacts of these procedures on local contamination, especially considering these sediments had been previously classified as ‘‘trace contaminated’’, according to normative guidelines, and therefore safe for disposal. This study shows the importance of obtaining both chemical and biological data in standard monitoring procedures and that the remobilization of contaminants by dredging operations may be grossly underestimated, which calls for caution when assessing the impact of these activities even in low to moderately polluted areas.

Keywords

Dredging; Sediment contamination; Mussel; Bioavailability; Sediment Quality Guidelines

1. Introduction

The fate of dredged materials and the impact of dredging operations in harbours are acknowledged factors in coastal and estuarine environmental quality since sediments from impacted areas tend to store hazardous concentrations of organic and inorganic contaminants that may be desorbed following disturbance, therefore promoting bioavailability and speciation to more toxic forms (Roberts, 2012). However, determining the ecotoxicological risk of aquatic sediments retains many acknowledged constraints, in particular those that relate to the likely presence of complex mixtures of contaminants, their possible within organism interaction effects and to their intricate geochemical matrix (e.g., Costa et al., 2012). Therefore, researchers in the field recognize nowadays the need to enforce integrative approaches to determine the potential hazard of sediment-bound contaminants. The Weight-of-Evidence (WOE) approach, for instance, has been developed to provide a multidisciplinary characterization of environmental quality of sediments which combine different lines of evidence (LOEs), in essence integrating the levels of contamination to the ecological changes it may trigger (Chapman et al., 2002; Chapman, 2007; Dagnino et al., 2008). Recent research suggest, after validation through modelling, that WOE approaches should indeed include key LOEs such as sediment chemistry, contaminant bioavailability, bioassays and biomarkers, the later including genotoxicity assessment (Piva et al., 2011; Benedetti et al., 2012). The WOE approach is a key of many Ecological Risk Assessment (ERA) strategies and is also in line with the updated European Water Framework (Directive 2008/105/EC) which requires member states to evaluate and classify the ecological status of water bodies integrating different quality indicators.

Through the Oslo-Paris (OSPAR) convention, consensus guidelines for the control of the disposal of dredged materials have already been proposed, being based on preceding sediment quality assessment approaches that take into account LOEs such as sediment chemistry, analysis of benthic communities and toxicity tests. Although the combination of multiple LOEs represents an additional value to monitoring and management protocols, the regulatory frameworks related to dredged material still rely on the chemical characterization relative to Sediment Quality Guidelines (SQGs). However, these guidelines do not represent directly any measure of the toxicological risk of dredging to the sites where these operations are to occur. The Portuguese guidelines for regulating the disposal options for dredged materials assign sediments to five different classes of quality (1-clean to 5-highly contaminated) on the basis of their chemical characterization. Although these guidelines recommend toxicity and bioaccumulation tests, such information stands mostly for indicative purposes. In fact, much research focusing on the risk of aquatic sediment contamination include contrasts to available SQGs and confirms, in most cases, that although of importance as an *a priori* measure of risk, they often do not predict the full extent of adverse effects to the biota, thus confirming the need to add LOEs besides sediment contaminant determination (e.g., Benedetti et al., 2012; Costa et al., 2012).

The use of appropriate marine sediment biotests, coupled to a suitable battery of biomarkers may permit inferring the long term or even irreversible adverse effects that the exposure to contaminated dredged material may lead to (Martín-Díaz et al., 2008). Sediment-directed bioassays, performed either in the laboratory or *in situ*, are generally regarded as simple tests to evaluate the toxicity of sediment-bound toxicants (Urban et al., 2010). However, bioassays commonly designed to provide measurements of risk do not necessarily reflect the risk associated to the release of hazardous substances during dredging activities since most strategies are performed in the laboratory and tend to either underestimate or overestimate toxicity (Maycock et al., 2003; Martín-Díaz et al., 2004; Costa et al., 2012). Most of the studies concerning the application of biomarkers in dredged sediments focus on determining the activity of biotransformation enzymes and biological indicators of oxidative damage (e.g., Regoli et al., 2002; Martín-Díaz et al., 2004; Bocchetti et al., 2008). Organisms can metabolize some organic xenobiotics (e.g., some PAHs) into a more water-soluble form which is more easily excreted than the parent compound (Vermeulen, 1996). Some xenobiotic derivatives like aromatic diols and quinones, nitroaromatics, aromatic hydroxylamines, bipyridyls and certain transformation metal chelatesomatic diols may be accompanied by a burst in the production of reactive oxygen species (ROS) as a consequence of biotransformation (Winston and Di Giulio, 1991). When ROS exceeds the cellular defence systems, alterations like DNA damage, lipid peroxidation and enzyme inactivation can occur, since the redox state in cells is defined as the balance between reducing (antioxidants) and oxidizing (hazardous) agents.

The Tagus estuary is one of the largest estuaries in Western Europe with an approximate area of 320 km^2. This estuary is mainly supplied by the freshwater from the Tagus river and consists of a mesotidal environment with several channels and intertidal areas. The Tagus estuary is located in the most populated metropolitan area of Portugal, which includes Lisbon and its large industrial belt plus shipyard and port facilities. Several pollution hotspots have been identified, from industrial discharges (Canário et al., 2005; Vale et al., 2008) to domestic effluents and diffuse sources (Silva et al., 2004). In order to maintain navigation infrastructures and enlarge or deepen existing channels, dredging operations have been carried out regularly in the Tagus estuary. However, few studies have been published concerning these activities and their direct consequences to the environment and none in this specific estuary, which is affected by two important constraints to any ERA approach; the contamination by multiple xenobiotics and moderate levels of contamination.

The present paper aims at testing the potential risk of sediment-bound contaminants to a bivalve species (M. *edulis*) deployed *in situ* before and during harbour dredging activities. The assessment strategy is based on the integration of sediment characterization, contaminant bioaccumulation and biomarker responses related to oxidative stress and DNA lesions. Ultimately, it is intended to contrast the biological effects of sediment contamination elicited by dredging operations to regulatory and

reference sediment quality guidelines in order to enlighten the changes between disturbed and steady-state sediments.

2. Materials and methods

2.1. Study area

The study area consists of an enclosed navy harbour located in the Tagus estuary (Fig. 2.1). Approximately 140,000 m^3 of sediment was dredged from the harbour, in a three-month operation. As mandatory, the sediments were analysed for arsenic and metals (Cd, Cr, Cu, Ni, Pb, Zn, Hg) and organic compounds (PAHs and PCBs) prior to the dredging operations and classified according to the Portuguese guidelines for the management and disposal of dredged material as "trace contaminated sediments", therefore without risk for disposal.

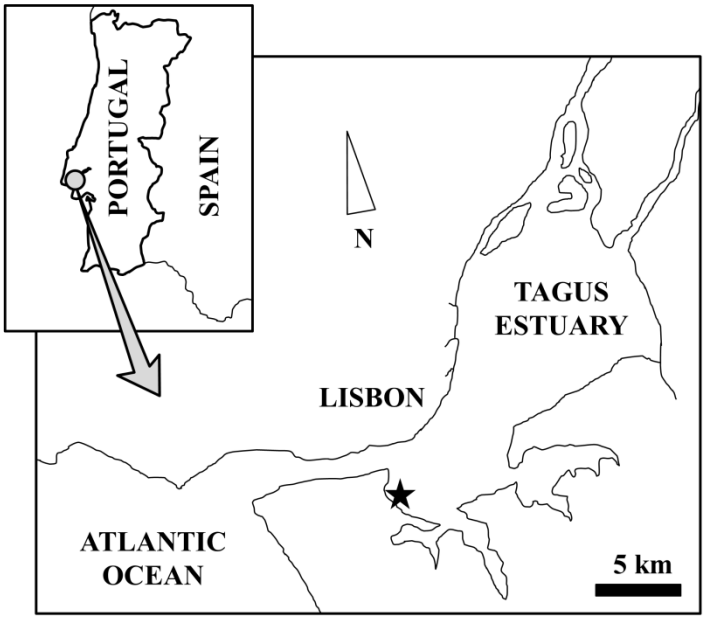

Fig. 2.1. Map of the study area pointing the harbour where dredging was performed (★

2.2. Experimental design

Adult M. *edulis* (53.47±3.3 mm shell length, 3.577±0.1 g whole-body wet weight [ww]) were collected in a coastal area north of the Tagus estuary with low contamination by inorganic and organic pollutants. The *in situ* bioassay arrangement consisted of five plastic cages (40 × 40 × 8 cm frame lined by a 5 mm plastic mesh) placed 0.5 m above bottom sediments, at a mooring pillar contiguous to

the operation area, to ensure direct influence of the dredging operations without compromising the integrity of the experimental apparatus. Approximately 50 randomly-selected individuals were placed in each cage to account for any potential mortality and assuring a minimum of forty two individuals for biological analyses. Animals were acclimatized *in situ* for 30 day before dredging started. Reference mussels (termed REF) were collected immediately before the beginning of dredging operations. Sampling was done at days 30 (T_{30}), 45 (T_{45}) and 60 (T_{60}) after the beginning of operations. At each sampling time forty two randomly-selected mussels were retrieved from cages and transported alive to the laboratory in cold containers. All individuals were measured for total length and weight before processing. Twelve individual mussels were sacrificed and the digestive gland and gills were excised for biomarker analyses. For bioaccumulation analysis, whole mussel soft tissue was excised from thirty specimens and pooled into three independent samples comprising ten animals each, in order to obtain the required ≈3.5 g for all analyses. Sediments were sampled before dredging with a grab and transported cold to the laboratory for subsequent contaminant analyses.

2.3. Sediment analyses

Sediments were surveyed for the metals cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), lead (Pb), nickel (Ni) and zinc (Zn). Approximately 100 mg of dried sediment was mineralized with 6 mL HF (40%, v/v) and 6 mL of Aqua-Regia (36% HCl and 60% HNO_3; 3/1 v/v) in closed Teflon vials (Caetano et al., 2007). Element quantification was achieved by inductivity coupled plasma mass spectrometry (ICP-MS) with a Thermo Elemental X-Series equipment. Mercury was determined by atomic absorption spectrometry (AAS) using a silicon UV diode detector (AMA-254 model, from Leco), after pyrolysis of each sample in a combustion tube at 750 ºC under an oxygen atmosphere and collection on a gold amalgamator (Costley et al., 2000). Certified sediment reference materials PACS-2 and MESS-3 (NRC, Canada), were analysed to validate the methodology and the determination were observed within the certified range. The recoveries of metals were found between 94 and 106%. Results are given in mg g^{-1} sediment dry weight (dw).

To determine polycyclic aromatic hydrocarbons (PAH), about 10 g of dry sediment samples were mixed with diatomaceous earth, transferred to Dionex standard 33 mL stainless steel cells and spiked with 1 mL surrogate standards (from Supelco) containing acenaphthene-d_{10} (0.408 mg mL^{-1}), pyrene-d_{10} (0.397 mg mL^{-1}), chrysene-d_{12} (0.397 mg mL^{-1}) and perylene-d_{12} (0.433 mg mL^{-1}). The samples were extracted using a mixture of acetone/hexane (v/v) with an ASE (accelerated solvent extraction) 200 system (Dionex, USA) and concentrated with a N_2 stream. Each cell was preheated for 5 min to reach the set temperature (100 ºC) and pressure (1500 psi), followed by a static extraction step of 5 min. Each extraction was performed with one cycle and the purge time was 150 s and the flush volume of 60% of the extraction cell volume. The extracts were concentrated, fractionated with a

silica/alumina (g/g) glass column and concentrated to 0.5mL under a gentle stream of N_2 for prior analysis (Martins et al., 2008). The determination of sixteen individual PAHs (3 to 6-ring) was performed on a Thermo DSQ gas chromatography-mass spectrometry (GC-MS) system in selected ion monitoring (SIM) mode. Identification of the PAH compounds was based on the comparison of their GC-retention times and mass spectrum with appropriate individual standards. Concentrations of individual PAHs were measured by the internal standard peaks area method and a 9-point calibration curve for each compound. Polychlorinated biphenyls (PCB) were analysed from dry sediment samples Soxhlet-extracted with *n*-hexane for 16 h (Ferreira et al., 2003). The extracts were fractionated with a Florisil chromatographic column and purified with sulphuric acid. Seventeen PCB congeners (tri- to hepta-CB) were quantified by gas chromatography (Hewlett-Packard 6890) with an electron-capture detector and a capillary column (DB5, J&W, 60m). Quantification was obtained by the external standard method, using a seven-point calibration curve for each compound. Certified sediment SRM 1941b (NIST, USA) analysis yielded values within the certified range and recoveries of 80-118% for PAHs and 93-110% for PCBs. Results are expressed as ng g^{-1} sediment dw.

2.4. Determination of the potential sediment contamination impact

The probable effects level quotient (*PEL-Q*) was calculated for each contaminant to evaluate the potential for causing adverse biological effects of the tested sediment based on the published guideline values for coastal waters, namely the Threshold Effects Level (*TEL*) and the Probable Effects Level (*PEL*), according to MacDonald et al. (1996). This indice was calculated according to the formula described by Long et al. (1998):

$$PEL - Q_i = \frac{C_i}{PEL} \quad [1]$$

where *PEL* is the guideline value for the contaminant *i* and C_i the measured concentration of the same contaminant. In essence, the *PEL-Q* attributes a weight according to the relative toxicity of each surveyed compound within a mixture of xenobiotics. The Sediment Quality Guideline Quotient (*SQG-Q*), developed to compare sites affected by contaminant mixtures was calculated for tested sediment (according to Long et al., 1998) as:

$$SQG - Q = \frac{\sum_{i=1}^{n} PEL{-}Q_i}{n} \quad [2]$$

where *PEL-*Q_i derives from formula [1] for the contaminant *i* and *n* the number of surveyed contaminants. Although prone to be affected by the removal or introduction of a toxicant (for constituting a mean value of *PEL-Qs*), this effect is diluted when integrating a large number of pollutants and considering the relative toxicity of substances, which renders this indice as one of the

most employed global indices of sediment contamination risk. The tested sediment was classified according to its potential to produce biological effects: *SQG-Q* < 0.1-unimpacted; 0.1 ≤ *SQG-Q* ≤ 1-moderately impacted*; SQG-Q* > 1-highly impacted (MacDonald et al., 2004).

2.5. Biological analyses

2.5.1. Bioaccumulation

Metal bioaccumulation was determined from vacuum-dried mussel samples, digested with a mixture of HNO_3 (supra-pur grade, 65% v/v) and H_2O_2 (supra-pur grade, 30% v/v) at different temperatures according to the method described by Ferreira et al. (1990). The concentrations of six metals (Cd, Cr, Cu, Ni, Pb and Zn) were determined by ICP-MS. Total Hg was determined by atomic absorption spectrometry as described for sediments. The reference biological materials DORM-1, DOLT-4, CRM 278 (NRC, Canada) were analysed by the same procedures and the values were consistent with the certified range, the recoveries ranging between 93 and 107%. Metal concentrations are given in mg g^{-1} whole-mussel soft tissue dw.

PAHs were determined in mussel samples spiked with surrogate standards (from Supelco), after extraction by ASE. Quantification was performed similarly to the procedure described for sediments, adapted to biological tissue by Martins et al. (2008). PCB congeners were Soxhlet-extracted from wet mussel samples with hexane, for 6 h (Ferreira et al., 1990). Quantification was performed similarly to the procedure described in the previous section. Reference mussel tissue SRM 2977 (NIST, USA) was analysed to validate the procedure and the obtained PAH and PCB levels were found within certified range, with the recoveries being 73-112% and 80-110%, respectively. Results are expressed in ng g^{-1} whole mussel soft tissue dw.

2.5.2. Genotoxicity assessment

The alkaline comet assay was applied in mussel gills mainly based on the protocol described by Costa et al. (2008), adapted from Singh et al. (1988). In brief: individual gills were excised and then chopped in 100 µL cold Kenny's salt solution (KSS: 0.4 M NaCl, 9 mM KCl, 0.7 mM K_2HPO_4, 2 mM $NaHCO_3$). After centrifugation to precipitate cellular debris (1500 × g, 4 ºC for 2 min), 10 µL of the supernatant was diluted in 180 mL of liquid (35-40 ºC) 1% (w/v) low-melting-point agarose (LMPA, Sigma) prepared with KSS. The cells suspended in LMPA were then transferred (2 × 75 µL) to slides pre-coated with 1% (w/v) normal melting-point agarose in TAE buffer. Afterwords, slides were dipped for 1 h (in the dark), at 4 ºC in lysis solution (2.64% NaCl (w/v), 3.72% EDTA (w/v) and 5 mM TRIS) to which was added 10% (v/v) DMSO and 1% (v/v) Triton-X 100 just before use. Slides

were then placed in cold (4 ºC) electrophoresis solution (pH 13) for 40 min to promote DNA unwinding and enhanced expression of alkali-labile sites. Electrophoresis was run at 25 V, for 30 min, at 4 ºC, in the dark. Finally, the slides were neutralized in 0.1 N Tris-HCl buffer (pH 7.5) for 15 min. Solutions and electrophoretic apparatus were kept in the dark and in the cold to reduce accessory DNA damage. Slides were stained with ethidium bromide (0.02 mg mL^{-1}) for 5 min and examined with a DMLB microscope adapted for epifluorescence equipped with an EL6000 light source for mercury short-arc reflector lamps and a N2.1 filter (Leica Microsystems). Approximately 100 random comets were analysed per slide using CometScore (TriTek, USA). The percentage of DNA in the tail was employed as a direct measure of DNA-strand breakage (Lee and Steinert, 2003). The results are expressed as average percentage of DNA in tail per individual.

2.5.3. GSH/GSSG ratio

The reduced/oxidized glutathione ratio (GSH/GSSG ratio) was measured in mussel digestive gland using a commercial kit (Cayman), following manufacturer instructions, based on the enzymatic recycling method (using glutathione reductase). The assay permit the estimation of total glutathione and oxidized glutathione (GSSH) by derivatizing GSH in samples with 2-vinilpyridine (Sigma-Aldrich). Total GSH and GSSG were calculated from a calibration curve obtained with GSH. The GSH/GSSG ratio was calculated as GSH/(GSSG/2).

2.6. Statistical analysis

After failing to comply with parametric ANOVA assumptions, non-parametric statistics were performed, namely the Mann-Whitney *U* test, in order to compare the concentrations of contaminants and the biomarkers determined in caged mussels in the sampling periods (REF, T_{30}, T_{45} and T_{60}), and the Spearman correlation *R* statistic between bioaccumulation and biomarker responses. Correlation-based principal component analysis (PCA) was performed to survey the relations between contaminant bioaccumulation and biomarker responses. Statistics were performed with the software Statistica (Statsoft), following Zar (1996).

3. Results

No significant mortality occurred during the bioassay procedure. Animals lost during the bioassay accounted for ~2% by the end of the experiment.

3.1. Sediment contamination

Sediments collected before the beginning of dredging operations were globally moderately impacted by contaminants ($SQG\text{-}Q_t$ = 0.33, Table 2.1). Cr, Cu, Pb, Ni, Zn, acenaphthylene, acenaphtene, fluorene, fluoranthene, pyrene, benzo[a]anthracene and dibenzo[*a*,*h*]anthracene showed levels above TEL guideline value. Total mercury (organic plus inorganic species) is the only contaminant reaching PEL levels. Four- and five-ring PAH were the predominant organic compounds.

3.2. Bioaccumulation

The average concentrations of metals in the whole soft tissue of caged mussels is presented in Fig. 2.2. In general, the concentrations of metals in caged mussels were low; however, patterns differed between metals. Concentrations of Ni and Pb in mussels deployed during dredging were significantly higher (Mann-Whitney U, $p < 0.05$) than in mussels exposed before the beginning of the operations (REF). However, no significant increase in bioaccumulation was observed during the dredging period, except for a moderate increment in Pb and Ni. On the other hand, the concentrations of Cu decreased significantly with the beginning of dredging. Total Hg concentrations were always observed below the detection limit.

Concentrations of PAH in caged mussels revealed significant differences between REF and the dredging period (Fig. 2.3). Before the beginning of dredging operations (REF), 3-ring PAH concentrations were higher (83.8 ng g^{-1}) than 4- and 5-ring PAH (64.5 ng g^{-1}), whereas 6-ring compound presented values below the detection limit (< 0.6 ng g^{-1}). Still, thirty days after the beginning of dredging (T_{30}), the accumulation of 4-, 5- and 6- ring compounds were clearly higher in caged mussels reaching 1.5-, 2- and 9-fold higher levels than REF, respectively. However, the maximum concentrations were observed after 45 day of the assay for 4-ring (175.7 ng g^{-1}) and 5-ring-PAH (216.9 ng g^{-1}) and after 60 day for 6-ring PAH (10.6 ng g^{-1}). At T_{60} the 4-ring PAH levels returned to similar values observed at T_{30}, while the 5- and 6-ring PAH levels increased. Three-ring PAHs presented a different pattern: lower levels were observed in caged mussels exposed to dredging operations than those exposed before dredging; no significant differences were recorded during the dredging period.

Table 2.1. Contaminant concentrations measured in the sediments and comparison to *TEL* and *PEL* guidelines (following MacDonald et al., 1996).

					SQGs	
Contaminant				**Csed**	***TEL***	***PEL***
Metallic ($\mu g\ g^{-1}$ dw)	**Metals**		Cd	0.5 ± 0.1	0.68	4.21
			Cr	56.4 ± 6.2 [a]	52.3	160
			Cu	45.6 ± 2.6 [a]	18.7	108
			Pb	78.6 ± 10.7 [a]	30.2	112
			Ni	23.4 ± 2.4 [a]	15.9	42.8
			Zn	257.2 ± 34.5 [a]	124	271
			Hg	0.9 ± 0.00 [b]	0.13	0.7
		SQG-Q metallic		***0.62***		
	PAH		acenaphthylene	7.3 ± 0.9 [a]	5.87	88.9
			Acenaphthene	13.9 ± 1.6 [a]	6.71	128
		3-ring	Fluorene	11.7 ± 1.0 [a]	21.2	144
			Phenanthrene	72.4 ±13.1	86.7	544
			Anthracene	12.3 ±1.1	46.9	245
			Fluoranthene	402.1 ± 53.4 [a]	113	1494
		4-rings	Pyrene	325.3 ± 32.8 [a]	153	1398
			Benzo[a]anthracene	87.7 ± 11.5 [a]	74.8	693
			Chrysene	37.6 ± 6.0	108	846
			Benzo[b]fluoranthene	79.6 ± 3.4	[ng]	[ng]
			Benzo[k]fluoranthene	34.0 ± 2.8	[ng]	[ng]
		5-rings	Benzo[e]pyrene	62.3 ± 4.1	[ng]	[ng]
			Benzo[a]pyrene	70.2 ± 5.6	88.8	763
			Perylene	273.6 ±9.2	[ng]	[ng]
			Dibenzo[a,h]anthracene	71.8 ± 4.7 [a]	6.22	135
		6-ring	Indeno[1,2,3-cd]pyrene	11.9 ± 0.5	[ng]	[ng]
			Benzo[g,h,i]perylene	69.8 ± 9.0	[ng]	[ng]
			∑3-ring	105.3 ± 16.1	[ng]	[ng]
			∑4-ring	852.7 ± 85.9	[ng]	[ng]
			∑5-ring	531.5 ± 23.7	[ng]	[ng]
			∑6-ring	141.5 ± 13.7	[ng]	[ng]
			PAHtotal	**1643.3 ± 129.8**	**1684**	**16770**
Organic (ng g^{-1} dw)	**PCB**		PCB-18	0.11 ± 0.01	[ng]	[ng]
		Tri-CB	PCB-26	0.03 ± 0.01	[ng]	[ng]
			PCB-31	0.13 ± 0.03	[ng]	[ng]
			PCB-44	0.19 ± 0.05	[ng]	[ng]
		Tetra-CB	PCB-49	0.19 ± 0.05	[ng]	[ng]
			PCB-52	0.26 ± 0.07	[ng]	[ng]
			PCB-101	0.38 ± 0.10	[ng]	[ng]
		Penta-CB	PCB-105	0.16 ± 0.06	[ng]	[ng]
			PCB-118	0.35 ± 0.05	[ng]	[ng]
			PCB-128	0.49 ± 0.19	[ng]	[ng]
			PCB-138	0.64 ± 0.18	[ng]	[ng]
		Hexa-CB	PCB-149	0.35 ± 0.06	[ng]	[ng]
			PCB-151	0.16 ± 0.04	[ng]	[ng]
			PCB-153	0.61 ± 0.15	[ng]	[ng]
			PCB-170	0.14 ± 0.01	[ng]	[ng]
		Hepta-CB	PCB-180	0.37 ± 0.11	[ng]	[ng]
			PCB-187	0.44 ± 0.11	[ng]	[ng]
			PCB-194	0.03 ± 0.01	[ng]	[ng]
			∑tri-CB	0.27 ± 0.04	[ng]	[ng]
			∑tetra-CB	0.65 ± 0.17	[ng]	[ng]
			∑penta-CB	1.04 ± 0.34	[ng]	[ng]
			∑hexa-CB	1.82 ± 0.17	[ng]	[ng]
			∑hepta-CB	0.96 ± 0.15	[ng]	[ng]
			PCBtotal	**4.73 ± 0.86**	**21.6**	**189**
		SQG-Q organic		***0.11***		
		SQG-Q total		***0.33***		

[ng] – no guideline available; a Value above *TEL*; b Value above *PEL*.

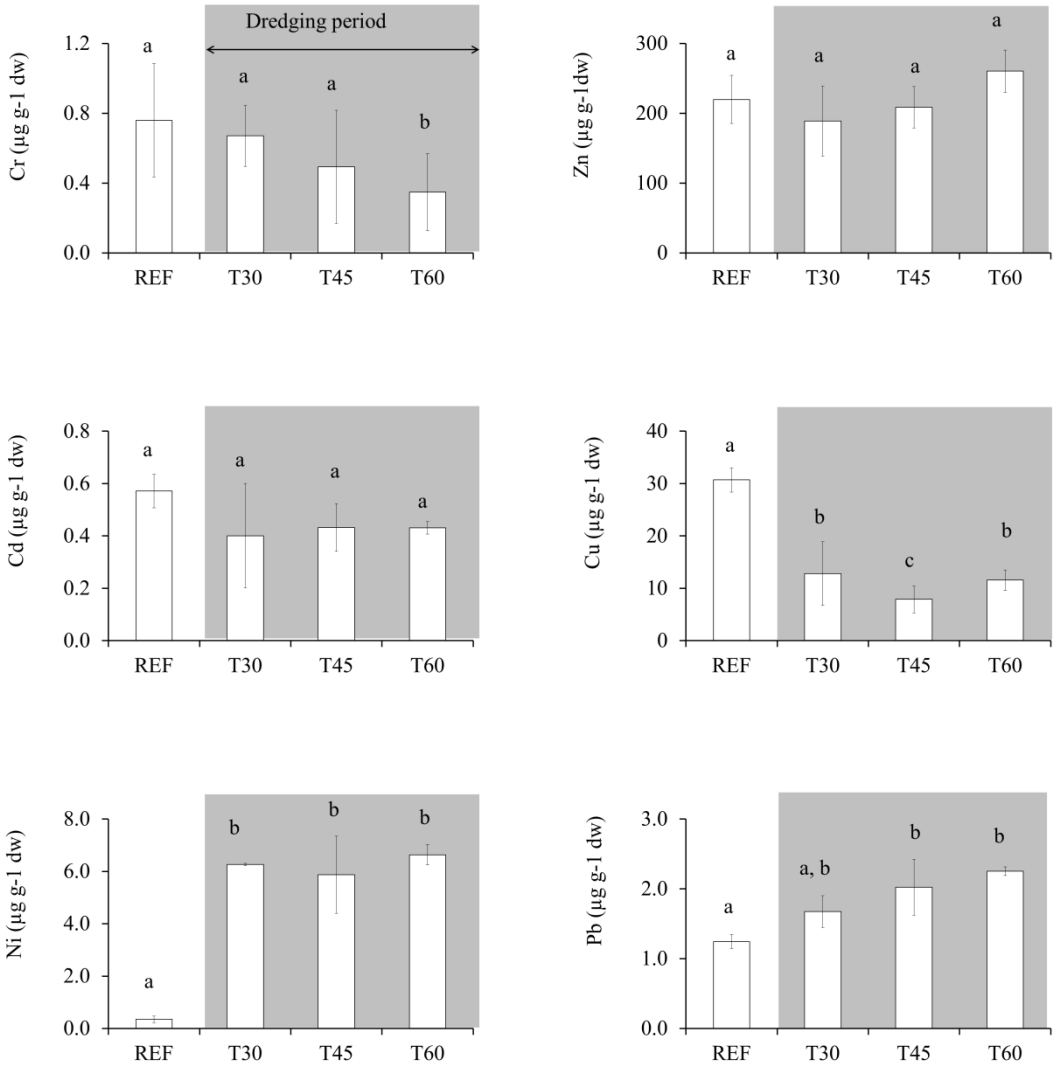

Fig. 2.2. Average concentrations (µg g^{-1} whole-mussel soft tissue dw, ± SD) of Cr, Zn, Cd, Cu, Ni, Pb in mussels sampled before (REF) and after 30 (T_{30}), 45 (T_{45}) and 60 (T_{60}) days of the beginning of dredging operations. The grey area indicates the dredging period. Different letters indicate significant differences ($p < 0.05$, Mann-Whitney *U* test).

Concentrations of PAH in caged mussels revealed significant differences between REF and the dredging period (Fig. 2.3). Before the beginning of dredging operations (REF), 3-ring PAH concentrations were higher (83.8 ng g^{-1}) than 4- and 5-ring PAH (64.5 ng g^{-1}), whereas 6-ring compound presented values below the detection limit (< 0.6 ng g^{-1}). Still, thirty days after the beginning of dredging (T_{30}), the accumulation of 4-, 5- and 6- ring compounds were clearly higher in caged mussels reaching 1.5-, 2- and 9-fold higher levels than REF, respectively. However, the maximum concentrations were observed after 45 day of the assay for 4-ring (175.7 ng g^{-1}) and 5-ring-PAH (216.9 ng g^{-1}) and after 60 day for 6-ring PAH (10.6 ng g^{-1}). At T_{60} the 4-ring PAH levels returned to similar values observed at T_{30}, while the 5- and 6-ring PAH levels increased. Three-ring

PAHs presented a different pattern: lower levels were observed in caged mussels exposed to dredging operations than those exposed before dredging; no significant differences were recorded during the dredging period.

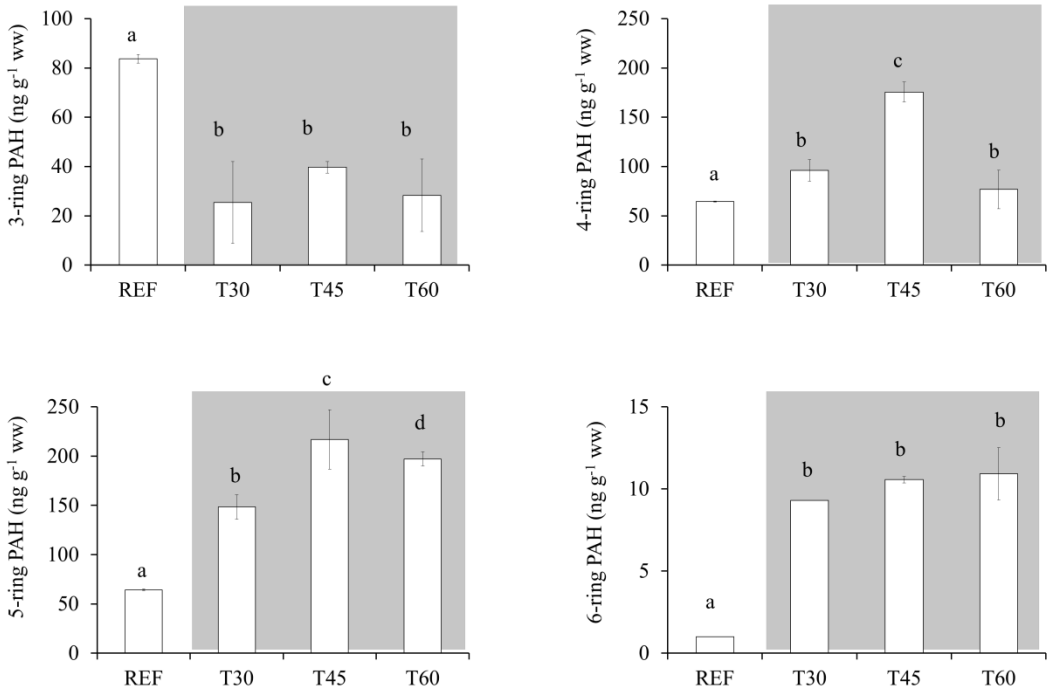

Fig. 2.3. Average concentrations (ng g^{-1} whole-mussel soft tissue ww, ± SD) of three- to six-ring PAHs in mussels sampled before (REF) and after 30 (T_{30}), 45 (T_{45}) and 60 (T_{60}) days of the beginning of dredging operations. The grey area of the graph indicates the dredging period. Different letters indicate significant differences ($p < 0.05$, Mann-Whitney *U* test).

As observed for PAH compounds, significant differences were registered between PCB bioaccumulation at REF and mussels collected during dredging period (Fig. 2.4). The major increments were observed at T_{30} for all the CB groups but hexa-CB depicted the highest bioaccumulation, accounting for up to 55% of total PCBs. No significant differences were found between T_{30} and T_{45} for tri-, tetra- and penta-CB, however a significant decrease was observed for hexa- and hepta-CB concentrations. The concentrations of the sum of CB groups in caged mussels collected at T_{60} were similar to those registered in REF mussels.

3.3. Biomarkers analyses

The average percentage of DNA in tail and average GSH/GSSG ratio analysed in the gills of mussels are presented in Fig. 2.5. The caged mussels exposed during dredging operations showed significantly higher DNA damage (i.e., higher proportion of DNA in the nucleoids' tail) than REF animals,

especially at T_{30} and, moreover, at T_{60}, reaching to levels up to ~70% of DNA in tail. The GSH/GSSG ratio was similar between REF and T_{30} mussels. At T_{45}, however, the ratio significantly decreased in comparison with T_{30}, with no differences being observed between T_{45} and T_{60}. A significant negative correlation between DNA damage and GSH/GSSG ratio (Spearman $R = 0.73$, $p < 0.001$) was observed. DNA damage was positively correlated to Ni, Pb and benzo[k]fluoranthene bioaccumulation (Spearman $R = 0.9$, $p < 0.05$) and negatively correlated with Cr and phenanthrene (Spearman $R = 0.9$, $p < 0.05$). No significant correlations were observed between GSH/GSSG ratio and bioaccumulation.

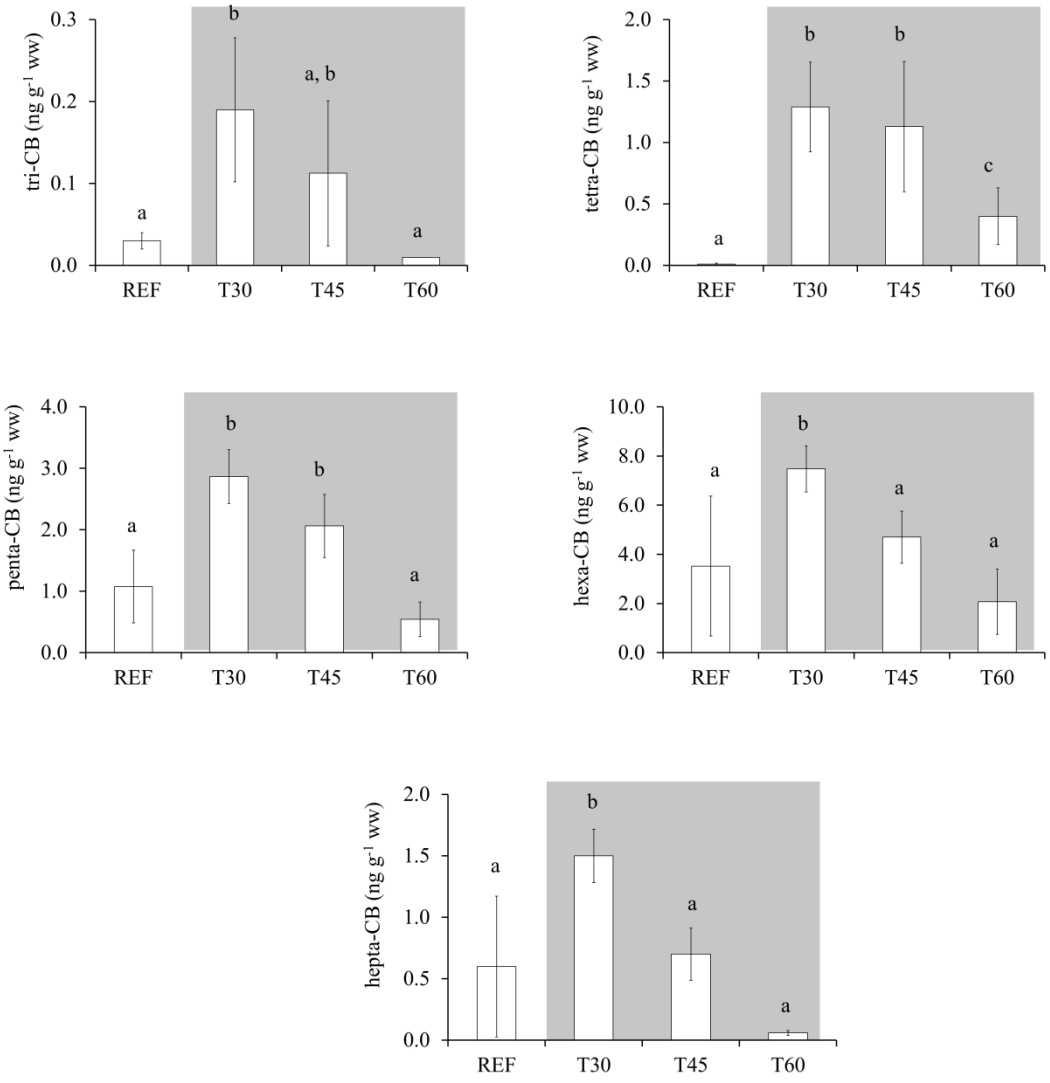

Fig. 2.4. Average concentrations (ng g^{-1} whole-mussel soft tissue ww, ± SD) of three- to hepta-CB in mussels sampled before (REF) and after 30 (T_{30}), 45 (T_{45}) and 60 (T_{60}) days of the beginning of dredging operations. The grey area of the graph indicates the dredging period. Different letters indicate significant differences ($p < 0.05$, Mann-Whitney *U* test).

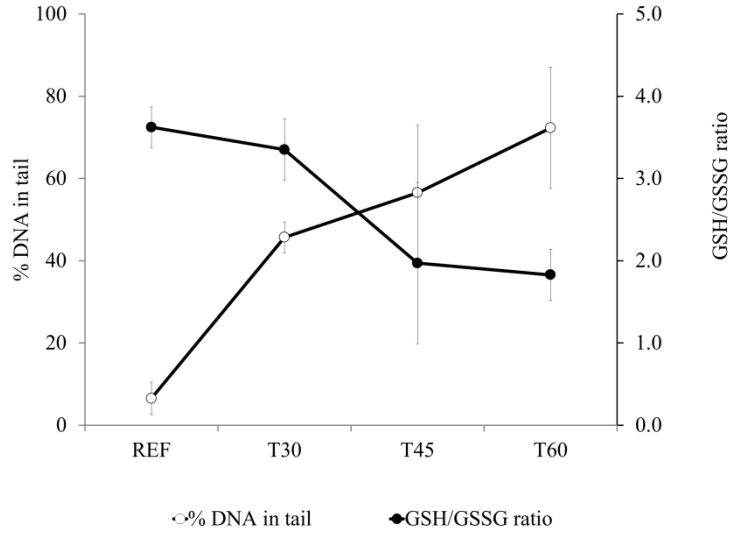

Fig. 2.5. Average percentage of DNA in tail and average reduced/oxidized glutathione ratio (GSH/GSSG) analysed in the gills of mussels sampled before (REF) and after 30 (T_{30}), 45 (T_{45}) and 60 (T_{60}) days of the beginning of dredging operations. Error bars indicate standard deviation. Different letters indicate significant differences ($p < 0.05$, Mann-Whitney *U* test).

Principal component analysis (Fig. 2.6) integrating biomarker responses with bioaccumulation data of those contaminants for which significant changes relative to REF were observed yielded two factors that combined explain in excess of 84% cumulative variation between the assay time points (REF to T_{60}). The main variables contributing for factor 1 were the tissue concentrations of Ni and Pb, 4- to 6-ring PAHs, tetra-chlorinated CBs and DNA-strand breakage (DNA-SB), the remaining, especially the GSH/GSSG ratio (although with opposite trend to DNA-SB, together with Cu and 3-ring PAH accumulation), being significant for factor 2. Unlike for the accumulation of 5- and 6-ring PAHs plus Ni and Pb, PCB accumulation failed to reveal any distinct link with DNA-SB.

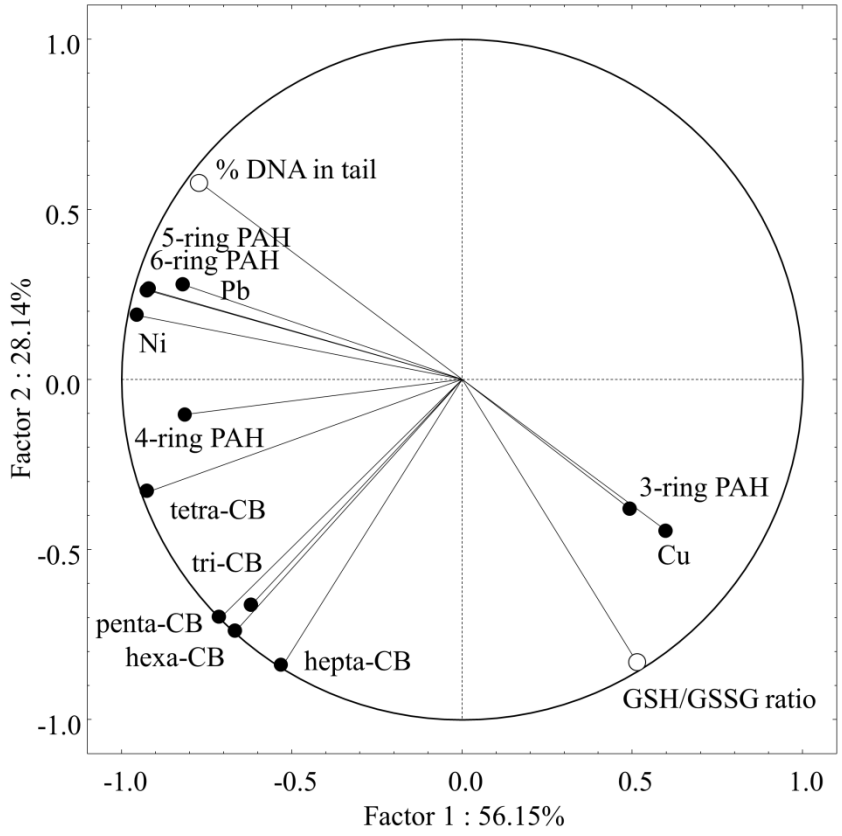

Fig. 2.6. Principal component analysis (PCA) scatterplot integrating biomarker responses (total DNA-strand breakage and the GSH/GSSG ratio) plus contaminant concentrations in whole mussel soft tissue, grouped by classes of organic (PCBs and PAHs) or individual metallic contaminants. Only the contaminants revealing significant changes to REF animals were included in the model, for the sake of parsimony. Eigenvalues for PCA factors 1 and 2 are 7.86 and 3.84, respectively.

4. Discussion

According to the analysed *SQG-Qs*, the sediments collected at the bioassay site can be considered moderately impacted by contaminants. In accordance, the surveyed sediments revealed much lower levels of metals and organic contaminants than recognized pollution hotspots of Tagus estuary, which presents one or two orders higher levels, depending of the contaminant (Canário et al., 2005; Vale et al., 2008). In addition, when contrasting the measured levels of contamination to the Portuguese guidelines for the management and disposal of dredged material it may be inferred that the surveyed sediments are allocated within the category of "trace contaminated".

The concentrations of contaminants in dredged materials may not necessarily reflect the potential ecological risk, since the remobilization of sediments changes their oxic/anoxic status, which can greatly influence the mobility of contaminants and, therefore, its bioavailability. In fact, xenobiotics sorbed to organic matter and fine fraction may be transferred to the water column, especially if a positive change in redox potential occurs as consequence of re-oxygenation (e.g., Eggleton and

Thomas, 2004; Du Laing et al., 2009). On the other hand, organic and inorganic substances have different desorption and remobilization behaviours: while the rate of metal desorption during resuspension is mostly influenced by sediment properties (Cantwell et al., 2008), desorption of organic contaminants from particulates to the dissolved state is chiefly determined by the solubility of the contaminant, on its turn determined by its molecular structure (Goossens and Zwolsman, 1996). In the present study, the analysis of metals, PAHs and PCBs in caged mussels revealed a contrast between the bioaccumulation before and after the beginning of dredging operations, mainly for organic compounds. The significant bioaccumulation of PAH and PCB compounds during dredging indicated increased xenobiotic bioavailability. However, for metals, this was only observed for Ni and Pb at the beginning of the dredging operations; however, without significant bioaccumulation from this point onwards. In fact, some authors reported only a brief remobilization of metals at the onset of dredging operations since metallic ions may immediately form hydroxide sulphide complexes, rendering them more insoluble (Hall, 1989; Urban et al., 2010). Additionally, if sediments are not sufficiently oxidized during disturbance, or if pH is not affected, remobilization to dissolved phases will be negligible (Eggleton and Thomas, 2004; Atkinson et al., 2007; Maddock et al., 2007). In general terms, metal contents in caged mussels are close to background levels recorded in mussels from other Portuguese areas (Bebianno and Machado, 1997).

It was also observed that the beginning of dredging activities changed the qualitative pattern of PAH distribution in soft mussel tissues. Before dredging, the predominance of 3-ring PAHs in caged mussels indicated a tendency to accumulate PAH of low molecular weight, quite typical for harbour areas where these compounds are present as dissolved or colloidal forms or just loosely bound to suspended matter (Zhou et al., 1998). The enrichments of high molecular weight PAHs observed in caged mussels exposed to dredging indicate that remobilization of sediments influenced the bioavailability of these compounds, which are usually strongly bound to sediments (Zhou et al., 1998). Bocchetti et al. (2008) also observed higher levels of high molecular weight PAHs in mussels affected by dredging operations, however, the levels of PAHs present in the area are much higher than those reported here (by at least 2-fold). In addition, the caged mussels also presented enrichments of PCB with low water solubility (hexa-CBs) from the beginning of dredging onwards. Since mussels are exposed both to dissolved and particulate forms of contaminants present in the water column (Baumard et al., 1999), it is plausible that the dredging operations resulted mostly in increased bioavailability of organic contaminants, most of which probably bound to particulate matter. This premise reinforces the importance of surveying the risk associated to this increased bioavailability, given the known toxic (especially genotoxic) hazards of these substances.

Although sediments were previously classified as “trace contaminated”, the dredging operations induced oxidative challenge and genotoxicity in caged mussels. Bocchetti et al. (2008), in the few

studies reporting the direct impact of dredging activities, also observed genotoxic effects on mussels caged in an Italian harbour, however, the overall sediment contamination profiles (PAHs plus the metals Cd, Pb, Zn and Hg) were much higher than those reported here, being above the normative guidelines for the disposal of dredged materials, therefore constituting a different scenario to that hereby presented. The present findings showed an imbalance in the GSH/GSSG ratio, which corroborates the link between genotoxicity and oxidative challenge. Glutathione is predominantly present in cells in its active and reduced form (GSH) but, as a consequence of oxidizing conditions, forms oxidized glutathione (GSSG). The redox state in cells is often accessed via GSH/GSSG ratio and, normally, cells maintained a high ratio. The decrease of this ratio could mean that reactive oxygen species (ROS) production are increasing during dredging exposure. In fact, both organic and metal contaminants can stimulate ROS production (Livingstone et al., 1990; Di Giulio et al., 1995). At the same time, an increase of DNA-SB was also observed during this period and a negative correlation with the GSH/GSSG ratio was observed. Previous studies have demonstrated the interactions between ROS and DNA, as well as with macromolecules, resulting in DNA damage and lipid peroxidation (Winston and Di Giulio, 1991).

DNA strand breaks were observed in aquatic organisms exposed in field conditions to various classes of contaminants, including PAH, PCB, dioxins, herbicides and even metals (Steinert et al., 1998; Costa et al., 2008; Frenzilli et al., 2009). In addition, mussels exposed to oil spills revealed a positive relationship between DNA damage in gills and content of PAHs (Pérez-Cadahía et al., 2004). In the present research, the PAH compounds presented the highest difference in mussels bioaccumulation deployed before and during dredging operations, although there were observed correlations between DNA-SB and benzo[b]fluranthene (a 5-ring PAH) bioaccumulation. Furthermore, PCA analysis (Fig. 2.6) yielded a very clear link between 5- and 6-ring PAH bioaccumulation with DNA-SB, whereas none was observed regarding PCBs or lower molecular weight PAHs. However, it is plausible that some metals, especially Ni and, moreover, the oxidative stress-induced genotoxic agent Pb (see, e.g., Avery et al., 1996, for a study on bivalves), contributed to the cumulative increment of DNA damage. PAHs are known to cause genetic damage in organisms, including marine mussels, through the formation of xenobiotic-DNA adducts (Venier and Canova, 1996) and to generate reactive oxygen species (Mitchelmore et al., 1998) which are, themselves, directly genotoxic. Since PAHs have low water solubility, cells promote detoxification by activating these compounds, converting them into more soluble molecules. In particular, *Mytilus* sp. can activate B[a]P to mutagenic compounds and to produce ROS directly, e.g., through the 2-electron oxidation step to form reactive epoxides, which can produce bulky DNA adducts and DNA-SB (Livingstone et al., 1990; Venier and Canova, 1996). Another acknowledged mechanism involves a 1-electron oxidation step to form cation radicals, quinones and semi-quinones radicals, giving rise to further potential DNA adducts and production of ROS via redox cycling (Venier and Canova, 1996). ROS involvement in DNA strand breaks has been

previously indicated by a 75% inhibition in % tail DNA by a free radical scavenger (*N-N-t*-butyl-*α*-phenylnitrone) in B[a]P exposed M. *edulis* digestive gland cells (Mitchelmore et al., 1998). ROS can also be generated from the active site of the haeme moiety during metabolic reaction of some CYP isoforms, e.g., with planar PCB, and through redox cycling. This may, at least in part, explain the observed negative correlation between strand-breakage and GSH/ GSSG ratio.

In previous research with estuarine sediments (Costa et al., 2012) it has been proposed that biomarker responses that reflect some measure of damage, such as genotoxicity, are very efficient in recognising risk situations, regardless of their low or null specificity to toxicants. The present findings appear to confirm this statement, since a clear genotoxicity threshold could be identified at the beginning of the dredging operations, albeit the initially low-risk value attributed to the sediments or even the sediment's physico-chemical characteristics.

To summarize, the present research demonstrated that even sediments regarded as moderately contaminated can induce toxicological stress during dredging operations in an active harbour. Therefore, it has been verified that radical sediment disturbance during such operations greatly enhances risk. Also, it must be stressed that the results oppose the low potential to cause adverse effects to the biota originally attributed to the sediments before dredging, which strengthens the premise that steady-state and disturbed sediments may yield different measures of risk, hence the importance of obtaining both chemical and biological data in standard monitoring and management procedures at the onset of dredging operations, in order to determine the toxicological risk of the disposed material and to the dredging site's biota *per se*.

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CHAPTER 3. DIFFERENTIAL UPTAKE AND ACCUMULATION OF SEDIMENT-BOUND PHENANTHRENE AND BENZO[b]FLUORANTHENE: A COMPARISON BETWEEN SEMI-PERMEABLE MEMBRANE DEVICES AND FILTER-FEEDING ORGANISMS†

† Martins et al. (submitted).

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are priority pollutants that, because of their high hydrophobicity, tend to be trapped and stored in aquatic sediments. However, their bioavailability, is modulated by the physicochemical properties of both sediments and PAHs. In a comparative study employing two different approaches to determine PAH bioavailability in aquatic environments, semi-permeable membrane devices (SPMD) and a marine clam were exposed, in the laboratory, to sediments spiked with two distinct PAHs, phenanthrene and benzo[b]fluoranthene. The sediments contaminated with ecologically-relevant concentrations released significant amounts of either PAH to the water column. The bioaccumulation of phenanthrene was similar between SPMDs and clams and yielded good correlation with the contaminants' concentrations in water, albeit non-integratively. However, the accumulation of the more hydrophobic benzo[b]fluoranthene was different between devices and clams, the latter being consistent with the ability to biotransform and eliminate this compound, which may bias estimates for bioavailability. Conversely, accumulation of benzo[b]fluoranthene in SPMDs was mainly governed by a simple partitioning of the compound between devices and water. The findings thus suggest significant differences between the two indicators of waterborne PAHs (either dissolved or bound to particulate matter) and, moreover, distinct release between low and high molecular weight PAHs. As such, caution is mandatory when developing biomonitoring procedures to assess the release of sediment-bound marine pollutants whose effective bioavailability is ultimately modulated by complex geo- and biochemical processes that depend on the contaminats' intrinsic chemical behavior and the model organisms' own ability to store, transform and eliminate the toxicants.

Keywords

Polycyclic aromatic hydrocarbons; *Ruditapes decussatus*; Bioaccumulation; Passive samplers; K_{ow}.

1. Introduction

In the marine environment, hydrophobic compounds like polycyclic aromatic hydrocarbons (PAHs) tend to be adsorbed on particles and to be deposited into the underlying sediments (De Luca et al., 2004). The highest concentrations of PAHs in marine ecosystems are generally found in coastal areas, particularly in estuaries, with total PAH concentrations up to 8.5 ug L^{-1} for the water phase and up to 6 mg kg^{-1} (dry weight) for sediments (OSPAR, 2002). The intrinsic properties of sediments, namely organic matter and fine particle proportion, greatly influence the sequestration of these compounds (Narbonne et al., 1999). However, sediment-bound PAHs can became bioavailable to aquatic organisms, depending on the partitioning behavior of different PAHs between aqueous (pore water, overlying water) and solid phases (sediment and suspended particulate matter). Commonly, the octanol-water partition coefficient (K_{ow}) is used as a model to determine the affinity of a compound to biota and, generally, it is acknowledged that toxicant bioavailability decreases, due to the increase of K_{ow} (Jonsson et al., 2004).

Approaches that employ organisms as indicators of exposure to toxicants such as PAHs have been developed and widely applied to the monitoring of marine pollution. Specifically, biomonitoring procedures using filter-feeding organisms, such as bivalves, are chiefly based on their ability to more directly retrieve chemical substances from the water column and accumulate them in tissues, providing a time-integrated information of pollutant occurrence, bioavailability and distribution (Pereira et al., 1996; Hagger et al., 2008). Bivalves are ubiquitously employed due to their feeding behavior and limited mobility, which render them particularly exposed to contaminants in both water column (dissolved or adsorbed to particulate matter) and sediments, either directly or after resuspension (Li et al., 2006; Wang et al., 2010). However, the use of bivalves may hold some limitations in contaminant monitoring since these organisms, as others, may have several uptake and elimination routes, to which is added the fact that contaminant accumulation depends on environmental variables such as temperature, salinity, food availability and other factors that affect the overall physiological status and therefore the functioning of apical entry, accumulation and detoxification mechanisms (Gunther et al., 1999; Huckins et al., 2004).

Monitoring by passive samplers has some advantages over the use of aquatic organisms, since these devices do not metabolize contaminants or endure acute affects from exposure, as well as their ability for deployment under various environmental conditions (Huckins et al., 1990). However, *in situ* parameters like temperature and hydrodynamics may also play a role in the uptake rates, leading to inconsistencies between the concentrations of toxicants in water and those expected to be recorded in the device (Vrana and Schüürmann, 2002; Booij et al., 2003). The most widely used passive sampling devices for the monitoring of organic hydrophobic contaminants are semi-permeable membrane

devices (SPMDs) (Huckins et al., 1990). Comparisons of SPMDs and mussels have already been reported during *in situ* monitoring studies (Booij et al 2006; Bourgeault and Gourlay-Francé, 2013), risk assessment studies following oil spills (Boehm et al., 2005) and *ex situ* studies with crude oil (Baussant et al., 2001). Similar trends are usually identified in mussels and SPMDs in terms of spacial variability; however, the accumulation patterns differ, not only due to the difference in kinetic and thermodynamic parameters, but also because, unlike SPMDs, mussels also may accumulate chemical substances associated with particulate matter. In addition, there has been little effort to validate and compare the deployment of SPMDs and bivalves under realistic exposure scenarios, meaning ecologically–relevant toxicant concentrations and, moreover, in the case of hydrophobic compounds such as PAHs, to perform surveys when the vehicle of the pollutant are sediments, which constitute the single most important storage of such substances in the aquatic milieu.

The present work aims primarily at comparing the uptake and accumulation of sediment-bound PAHs between SPMDs and a filter-feeding organism. For the purpose, two distinct PAHs usually present in contaminated sediments were selected: phenanthrene (Phe) and benzo[b]fluoranthene (B[b]F), both acknowledge as toxic to aquatic wildlife, albeit the latter being regarded as carcinogenic PAH, meaning distinct toxicological pathways. The two PAHs have different solubility in water: while for phenanthrene, with three fused benzene rings, the solubility is 1.1 mg L^{-1}, the five-ring benzo[b]fluoranthene's solubility is 0.0015 mg L^{-1} (Neff et al., 2002), which indicates higher hydrophobicity. The clam *Ruditapes decussatus* (=*Venerupis decussata*) was selected as the model organism for this study for being a soft–sediment burrowing filter–feeder organism, therefore in close contact with sediments and the overlying water column, unlike mussels, which attach to outcrops in rocky shores.

2. Materials and methods

2.1. Sediment-spiking procedure

The artificial sediments containing 5-10% total organic matter (TOM) and ≈ 50% fine fraction (FF) were obtained, by mixing sandy and muddy sediments collected from an unpolluted area in SW Portugal, the Mira estuary (Vasconcelos et al., 2007). After combustion at 450 ± 50 ºC the sediment final TOM was 6% sediment dry weight (dw), as estimated from total carbon loss-on-ignition. Final sediment FF was determined by hydraulic sieving after digestion with H_2O_2 and disaggregation with pyrophosphate, yielding the value of 46.2% of sediment dw.

Concentrations of phenanthrene and benzo[b]fluoranthene in sediments were defined according to available sediment quality guidelines (MacDonald et al., 1996) in order to achieve ecologically-relevant concentrations of each compound. Two concentrations were set to each compound (hereafter termed C1 and C2). The concentration C1 was targeted between the Threshold Effects Level (TEL) and the Probable Effects Level (PEL), whereas C2 was intended to attain a value immediately above PEL. The TEL and the PEL values are, respectively, 86.7 and 544 ng g^{-1} for Phe. In absence of a guideline available for B[b]F, the guideline used referred to benzo[a]pyrene, considering the chemical similarities between the two PAHs, with the values of 88.8 and 763 ng g^{-1} for TEL and PEL, respectively.

The artificial sediments were spiked in order to obtain the following nominal PAH concentrations: 250 ng g^{-1} (Phe-C1), 600 ng g^{-1} (Phe-C2), 250 ng g^{-1} (B[b]F-C1) and 800 ng g^{-1} (B[b]F-C2). However, the first experimental procedure, performed with SPMDs, indicated that, in order to account for volatile Phe losses during sediment mixing, the sediments hat to be spiked with three–fold the concentration of the PAH to obtain a final concentration within the expected range.

Appropriate amounts of Phe and B[b]F (obtained from Sigma, At Louis, MO, USA) stock solutions (in DMSO) were directly added to 2 L of wet sediment, which were allowed to equilibrate for 48h at 4 ºC after 15 min of mechanical mixing (Hickey and Roper, 1992; Costa et al., 1998; Martins et al., 2013). The control sediments were prepared similarly, albeit spiked with DMSO only. Samples of spiked sediment were collected and frozen at -20 ºC until PAH analysis.

2.2. SPMD construction

The SPMDs were constructed from low-density polyethylene (LPDE) lay-flat tubing (Brentwood Plastics, MO, USA), with 2.5 cm width and 70 µ thickness, cut into pieces of 2 cm in length and soaked with *n*-hexane for 24 h to remove impurities (Booij et al., 2006). After hand-drying of any residual solvent, 4.4 µL of triolein (Sigma, 95% purity) was added using a displacement syringe. The tubing was then heat-sealed at both ends. The amount of triolein per SPMD (4.08 mg) contributes for 4% of total SPMD weight and was selected corresponding to the estimated lipid content per clam. The lipid content in clams was analysed by Soxhlet extraction with *n*-hexane, as described in Ferreira and Vale, 2001.

2.3. Clam collection and acclimatization

Subadult (to avoid interferences of sex and maturation stage) *Ruditapes decussatus* (200 healthy clams) were collected from a coastal, unpolluted, shellfish bed (Ria Formosa, Southern Portugal) and

acclimatized in the laboratory for seven days before the experiments. The clams (26.2 ± 1.5 mm shell length, 2.83 ± 0.40 mm shell height and 1.23 ± 0.16 g whole soft-body) were acclimatized in tanks containing clean sand and filtered, aerated seawater with recirculation (temperature of 18 ± 1 ºC, salinity of 32 ± 1, pH 7.9 ± 0.2, total ammonia ≈0 mg L^{-1} and dissolved oxygen between 92 and 95%) and fed daily with cultured microalgae (*Isochrysis galbana*).

2.4. Experimental procedure

The two sets of laboratory 28-days bioassays (SPMD and clams), in duplicate, were prepared by placing 2 L of each of the five test sediments (control, Phe-C1, Phe-C2, B[b]F-C1 and B[b]F-C2) in 8 L-capacity tanks to which were added 5 L of filtered seawater. Sediments were allowed to settle for 48h before the beginning of the assays. Twelve SPMDs (the devices were suspended in water, above the sediment layer) and twenty randomly-selected clams were deployed to each test tank. Clams were feed daily with *I. galbana*. Each test tank was continuously aerated and 25% of water was changed weekly to ensure constancy of water parameters with minimum removal of contaminants. At the same time, water samples were taken for PAH analysis. Water parameters were found similar to the animals' acclimation conditions.

Six SPMD and ten clams (per replicate) were collected from each treatment at days 14 (T_{14}) and 28 (T_{28}). SPMD and clams (unexposed) collected at day 0 (T_0) were also collected for analyses. After removal of biofouling, the SPMDs were wrapped in aluminum foil, to prevent cross-contamination and photo-degradation of PAHs and stored at -20 ºC until analyses. Immediately after collection, clams were dissected to retrieve digestive gland and gills. Each organ was pooled to five samples per sampling time and stored at -20 ºC, for subsequent analysis.

2.5. Analytical methods

2.5.1. Phe and B[b] extraction

Sediment. Phenanthrene and B[b]F compounds were extracted from dry sediment samples, previously spiked with surrogate standards, by accelerated solvent extraction, using an ASE 200 model from Dionex (USA). The extracts were fraccionated with a silica/alumina glass chromatography column and concentrated under a gentle stream of N_2 prior to analysis (Martins et al., 2012).

Water. PAHs were extracted as described by Martinez et al. (2004), with some modifications by Martins et al. (2013). Briefly, surrogate standards were added to each water sample, previously mixed with 10% (v/v) methanol. The samples were percolated through a C18 speedisk previously

conditioned using a Baker vacuum system (J. T. Baker, The Netherlands). The compounds were then eluted with an ethyl acetate/dichloromethane mixture (1/1), subsequently evaporated and reconstituted in *n*-hexane.

SPMD. The SPMDs were extracted twice with 100 ml pentane for 24h with the addition of surrogate standards (Booij et al., 2006). The extracts were passed once over silica glass chromatography columns with pentane and concentrated under a gentle stream of N_2 prior to analysis.

Clam tissues. Surrogate standards were added to thawed digestive gland and gill samples and Phe and B[b]Fl were extracted by ASE, similarly to the procedures described for sediments, adapted to biological tissues by Martins et al. (2008).

2.5.2. Gas chromatography-mass spectrometry (GC-MS) analysis

All extracted samples, reference materials and procedural blanks were analyzed by GC-MS using a Thermo DSQ system in selected ion monitoring (SIM) mode as described in Martins et al. (2008). The identification of Phe and B[b]F was based on the comparison of their retention times and mass spectrum with appropriate individual standards. Both PAHs were quantified through the internal standard peak method, using deuterated standards containing phenanthrene-d_{10} and perylene-d_{12} (Supelco) following a 9-point calibration curve (Martins et al., 2008).

2.5.3. Quality control

Certified sediment reference material SRM1941b (NIST, USA) and reference mussel tissue SRM 2977 (NIST,USA) were analyzed by the same procedure, yielding recoveries of 80 - 120% for sediments and 73 - 112% for biological tissues for both Phe and B[b]F compounds. Analysis of spike water samples yielded recoveries between 99-102% for Phe and 86-88% for B[b]F. SPMD triolein samples showed recoveries between 92-109% for both compounds.

2.6. Bioaccumulation factors (BAF)

Two bioaccumulation factors were computed as the ratio between the PAH concentrations in both SPMDs and clam tissues relatively to PAH levels in either sediment or water (Baumard et al., 1999). The concentrations in the digestive gland and gills of clams are expressed as ng g^{-1} wet weight (ww), as ng g^{-1} for SPMDs, and concentrations in sediments and water given in ng g^{-1} dw and in ng L^{-1}, respectively.

2.7. Statistical analysis

The Shapiro–Wilks' and Levene's test were employed to check the normality of data and homogeneity of variances, respectively. Following invalidation of at least one of the assumptions for parametric analysis of variance, the non-parametric Mann-Whitney *U* test was applied for pairwise comparisons within tests and sampling times and the Spearman's rank-order correlation *R* statistic to address the link between PAH concentrations in SPMDs or clam tissues and concentrations in water. The significance level for all analyses was set at p=0.05. All statistics were performed with Statistica (Statsoft), following Zar (1996).

Table 3.1.. Nominal Phe and B[b]F concentrations (ng g^{-1} sediment) used for spiking the artificial sediment from SPMD (ng g^{-1}) and clams assays (ng g^{-1} ww) and Phe and B[b]F effective concentrations in the beginning (T_0) and in the end of the experiment (T_{28}) in both assays.

	Control				**Phe**				**B[b]F**			
	Phe		B[b]F		C1		C2		C1		C2	
Bioassay	SPMD	Clams	SPMD	Clams	SPMD	Clams	SPMD	Clams	SPMD	Clams	SPMD	Clams
Nominal	0	0	0	0	250	250	600	600	250	250	800	800
T_0	13.0±3.0	21.3±1.8	43.4±1.5	132.7±3.7	73.15±2.9	305.6±63.6	145.2±.37	856.8±78.8	297.7±1.0	436.9±24.2	753.9±124.9	1379.1±173.5
T_{28}	11.8±2.2	17.6±1.0	49.6±7.5	120.1±0.2	62.3±4.5	121.7±16.1	130.7±8.0	483.3±51.4	234.8±13.0	426.6±111.9	500.3±24.5	1223.0±80.5

3. Results

3.1. Phe and B[b]F concentrations in sediments

Table 3.1 presents the Phe and B[b]F concentrations obtained for each test sediment in the beginning (T_0) and end (T_{28}) of the SPMD and clam experiments. In general, the PAH concentrations in sediment were within the proposed levels, i.e., C1 between TEL and PEL and C2 above PEL, with the exception of Phe in the SPMD assays in which lower values of C1 and C2 were obtained, for the reason already stated above. The control sediment presented only trace concentrations of both compounds, in comparison with the spiked sediments. The PAH sediment concentrations decreased with time of exposure for both assays, albeit more significantly for Phe (yielding a 56-60 % loss).

3.2. SPMD experiment

Variation of Phe and B[b]F levels in water during the SPMD assays are presented in Fig. 3.1A and 3.1B, respectively. The water samples collected at T_0 were shy above the detection limit, for both compounds. Similar water levels of both PAHs were obtained for controls, without noticeable

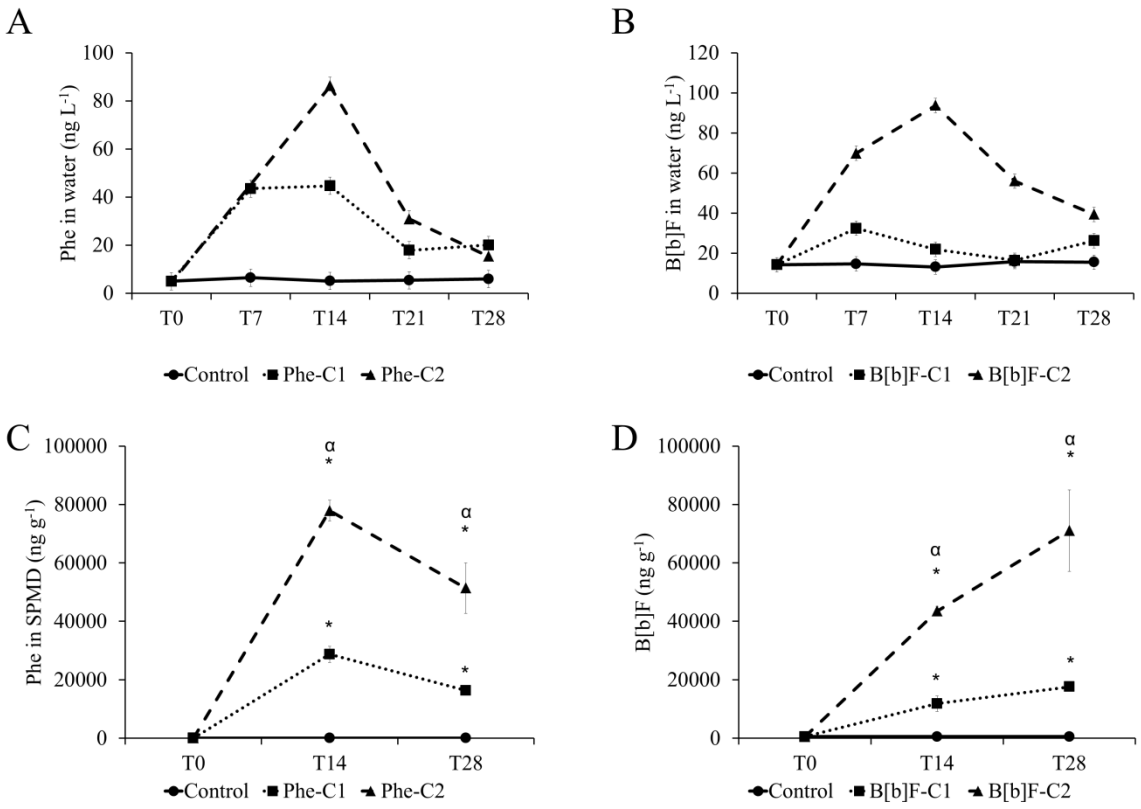

Fig. 3.1. Variation of phenathrene (Phe) and benzo[b]fluoranthene (B[b]F) levels in water (ng L^{-1}; A,B) and SPMD (ng g^{-1}; C,D) during 28 days of exposure to test sediments (control, Phe-C1, Phe-C2, B[b]F-C1 and B[b]F-C2). All data points represent the average ± SD (n = 6). * significantly different to control (Mann-Whitney U test, $p < 0.05$). $^{\alpha}$ significantly different to C1 (p Mann-Whitney U test, $p < 0.05$).

variation along the duration of the assay. Concentrations of Phe in water varied in time similarly between C1 and C2 tests, attaining higher levels following 14 days of assay. However, C2 presented higher levels of Phe in water at T_{14} than C1.Similarly, for the B[b]F-C2 test, B[b]F levels in water was higher at T_{14}, while for B[b]F-C1 test yielded concentrations consistently lower than C2, with scant variation along with the time-course.

The concentration of Phe in SPMDs was significantly higher (Mann-Whitney *U*, $p < 0.05$) in the C2 test comparatively to C1, however varied similarly with time of exposure, reaching higher concentrations at T_{14} (Fig. 3.1C). Concentration of B[b]F in SPMD was also significantly higher ($p < 0.05$) for the C2 test, even though, contrarily to the Phe test, increased in time reaching the maximum value at T_{28} (Fig. 3.1D).

3.3. Clam experiment

The variation of Phe and B[b]F concentrations in water during the 28-day bioassay with clams is presented in Figs. 3.2A and 3.2B, respectively. The Phe concentrations in water increased to maximum levels at T_{28} and were higher in the Phe-C2 test. In Phe-C1 test, water concentrations were consistently lower. The concentrations of B[b]F, generally increased with time of exposure and were higher in the C2 test, even though an unexpected decrease for C2 was observed at T_{14}. For C1 test, the variation of B[b]F in water varied only slightly with time. The control test presented water levels similar to T_0 for both PAHs and was always below the 9 ng L^{-1} recorded maximum.

The concentrations of Phe and B[b]F in the digestive gland of clams varied similarly, attaining higher levels at T_{14}, followed by a decrease at T_{28} (Fig. 3.2C and 3.2D, respectively). Also, the concentrations of both compounds were significantly higher (Mann-Whitney *U*, $p < 0.05$) in digestive gland of clams exposed to the highest concentrations (C2). The concentration of Phe (Fig. 3.2E) and B[b]F (Fig. 3.2F) in gills depicted a similar trend, however, the levels were significantly lower ($p < 0.05$) than in the digestive gland.

3.4. Accumulation of Phe and B[b]F by SPMDs and clams

The correlations between Phe and B[b]F concentrations in water and SPMD are presented in Fig. 3.3, combining all concentrations and sampling times. The most significant correlation was obtained in SPMDs (Fig. 3.3A and 3.3B) exposed to B[b]F ($R = 0.9$, $p < 0.05$). Exposure to Phe also yielded significant correlations between water and SPMD concentrations, however weaker ($R = 0.69$, $p < 0.05$). Phe concentrations in digestive gland and gills were better correlated to Phe concentrations in

water (Fig. 3.3C and 3.3D) than B[b]F. No significant correlation was observed between B[b]F in water and the concentration of the PAH in both tissues (Figs. 3E and 3F).

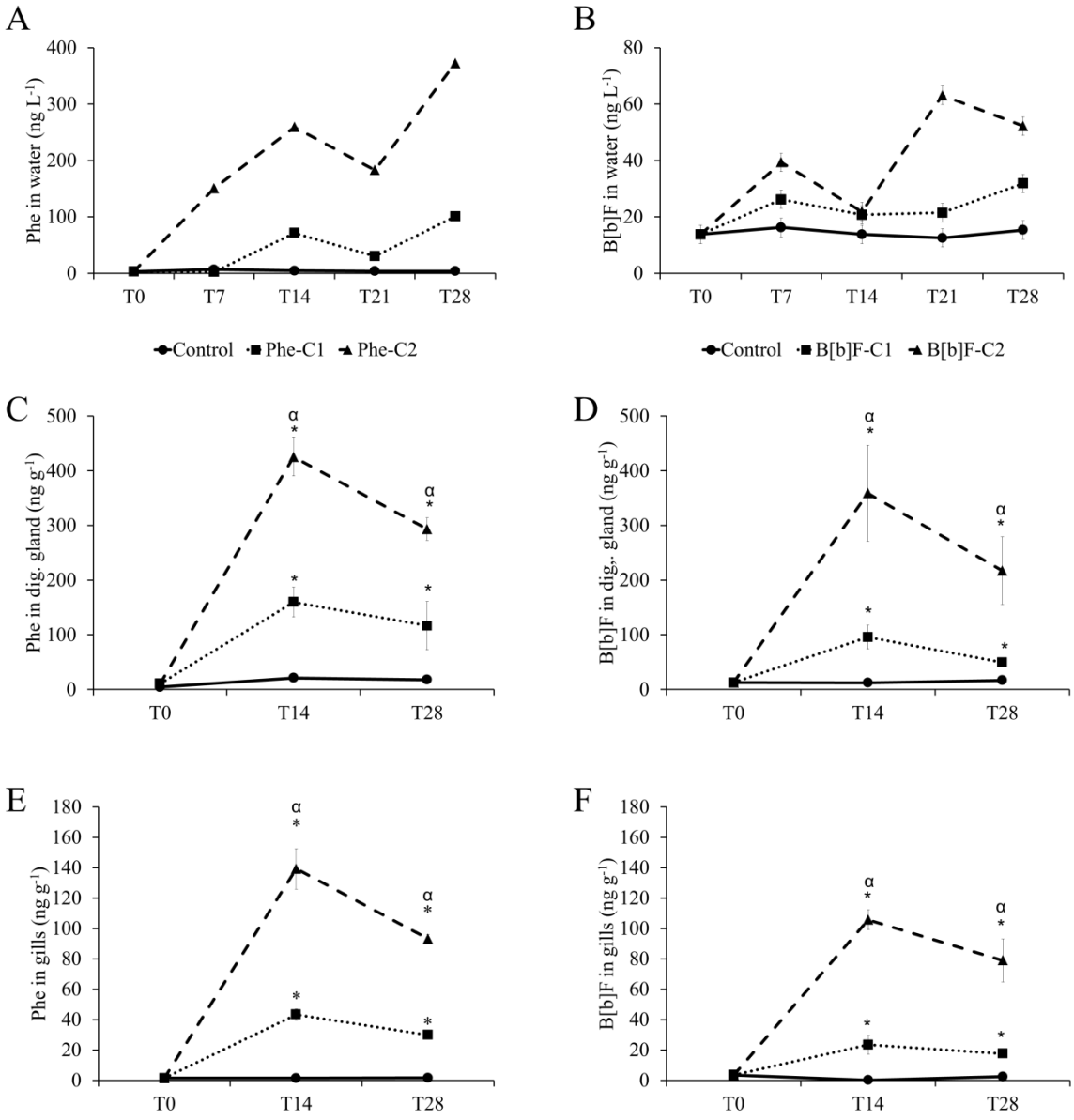

Fig. 3.2. Variation of A) phenanthrene (Phe) and B) benzo[b]fluoranthene (B[b]F) levels, respectively, in water (ng L^{-1}) and C, D) in digestive gland (ng g^{-1} ww) and E, F) in gills (ng g^{-1} ww) of clams during 28 days of exposure to test sediments (control, Phe-C1, Phe-C2, B[b]F-C1 and B[b]F-C2). All data points represent the average ± SD (n = 5). * indicates significant differences to control (Mann-Whitney *U* test, $p < 0.05$). α significantly different to C1 (Mann-Whitney *U* test, $p < 0.05$).

The BAFs calculated for SPMDs, digestive gland and gills of clams exposed to spiked sediments for 28 days are presented in Table 3.2. The estimated BAFs (relatively to both sediment and water) for SPMDs were higher than 1 for Phe and B[b]F exposures, however the highest values were found for Phe test. Nevertheless, the BAFs considering the PAH concentrations in water were always higher

than BAF-sediment. Significant differences ($p < 0.05$) between SPMD C1 and C2 tests were observed for both compounds. In contrast, no noticeable bioaccumulation was observed in the digestive gland and gills of clams, since BAF-sediments were always < 1. On the other hand, BAFs considering the concentrations in water showed that the digestive gland accumulated B[b]F at either concentration and Phe (C1) in sediments, whereas the BAF-water estimated for gills were > 1 for B[b]F-C2 test.

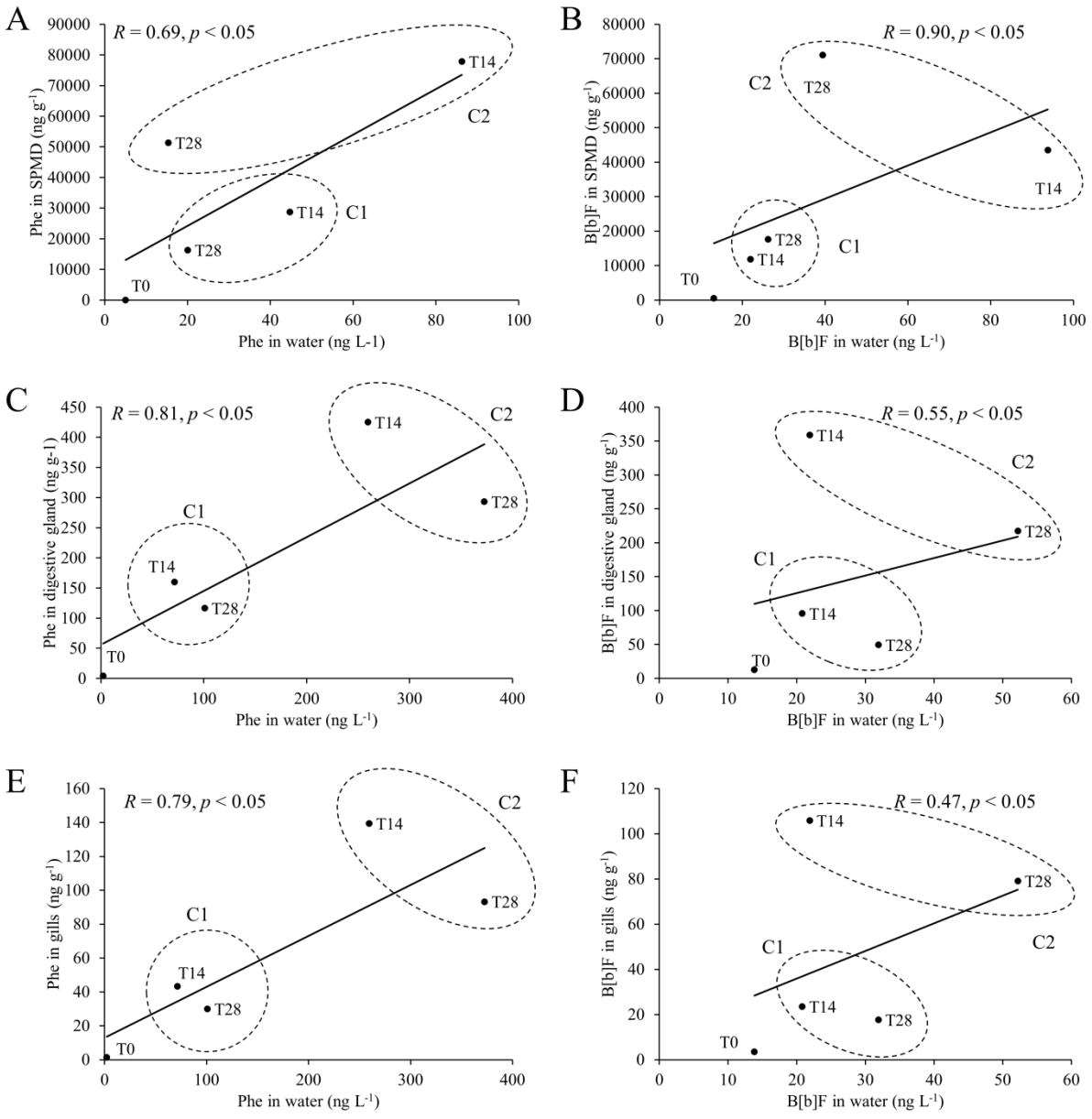

Fig. 3.3. Relations between Phe and B[b]F concentrations in water and SPMD (A, B) digestive gland (C, D) and gills of clams (E, F) exposed to test sediments (Phe-C1, Phe-C2, B[b]F-C1 and B[b]F-C2) during 0, 14 and 28 days. Spearman correlation coefficient and statistical significance are represented by *R* and *p*, respectively.

Table 3.2. Bioaccumulation factors (BAF) obtained for SPMDs, digestive gland and gills of clams exposed to test sediments (Phe-C1, Phe-C2, B[b]F-C1 and B[b]F-C2), for 28 days (T_{28}). Different letters indicate significant differences between C1 and C2 tests for the same compound (Mann-Whitney *U*, $p < 0.05$).

		Phe		B[b]F	
		Phe-C1	Phe-C2	B[b]F-C1	B[b]F-C2
BAF sediment	SPMD	223.01±15.0[a]	353.68±53.28[b]	59.112±4.75[a]	88.95±16.26[b]
	Digestive gland	0.38±0.15[a]	0.34±0.02[a]	0.11±0.01[a]	0.16±0.05[a]
	Gills	0.10±0.01[a]	0.11±0.01[a]	0.04±0.01[a]	0.06±0.01[b]
BAF water	SPMD	813.37±54.60[a]	3347.75±504.28[b]	670.46±53.92[a]	1805.36±353.78[b]
	Digestive gland	1.16±0.44[a]	0.79±0.06[a]	1.55±0.19[a]	4.16±1.19[b]
	Gills	0.30±0.03[a]	0.25±0.01[b]	0.55±0.08[a]	1.52±0.27[b]

4. Discussion

The present work demonstrated that both phenanthrene and benzo[b]fluoranthene were desorbed from the sediments to the water column rendering them bioavailable to SPMDs and clams. In fact, bioavailability could be verified by the accumulation results of the two compounds in the devices and both clam organs. However, while SPMDs and clam tissues presented a similar pattern for Phe exposures, this was not the case for B[b]F. In fact, phenanthrene accumulation does not seem to be integrative over time of exposure but rather better correlated with Phe concentrations in the water column (Fig. 3.3). On the other hand, while B[b]F accumulation in SPMDs increased over time and is well correlated with concentrations in water, bioaccumulation in the digestive glands and gills of clams demonstrated a pattern consistent with biotransformation and elimination processes in organisms that does not seem to occur during Phe exposure. The results also indicate significant differences between the processes that affect the portioning of the two PAHs, especially at and sediment/water interface; while demonstrating that the concentrations in water indeed reflect the most bioavailable phase, as corroborated by much higher BAFs normalized to water concentrations, when compared to BAFs estimated from sediment concentrations of the two PAHs (Table 3.2).

The adsorption and desorption of PAHs from aquatic sediments are complex processes that depended on the compound itself as well as on the nature of the sediments' fine particle/organic matter matrix and biological factors modulating turbation and biotransformation (Eggleton and Thomas, 2004). The findings indicate a more significant reduction in the levels of sediment Phe from T_0 to T_{28}. However, while Phe in sediments during the SPMD assays yielded a reduction ≈1.2-fold from T_0 to T_{28}, during the clam assays this reduction attained up to ≈2.5 fold. Conversely, B[b]F concentrations remained relatively unchanged throughout all assays, regardless of initial concentrations (Table 3.1). The

differences between Phe and B[b]F are likely related to their chemical properties, since the lower K_{ow} for Phe, and its related lower molecular weight, promoted its release from sediments, comparative to its 5-ring counterpart. It must be stressed that the artificial sediments, designed to mimic natural sediments, contain important levels of organic matter, which promotes trapping and storage of high hydrophobicity compounds like B[b]F (Chen et al., 1999). It must also be noted that the higher volatility and photo-oxidation of phenanthrene in comparison with 4 to 6-ring PAHs, such as B[b]F (Juhasz and Naidu, 2000) may contribute to explain the differential losses from sediments between the two PAHs. It is thus possible that the bioassay arrangement for Phe consists, in fact, not a two compartment model (i.e. sediment, water) but rather a three-compartment (sediment, water, atmosphere), where the continuous loss of Phe calls for a constant redefinition of steady-state conditions by continuously promoting the release of the toxicant from the sediment to the water column to compensate losses. Also, the differences between Phe concentrations in sediments by the end of the SPMD and clam assays may be partially explained by bioturbation since *R. decussatus*, although sedentary, is a burrowing organism, which may cause some resuspension and, moreover, shifts in the oxic/anoxic status of the sediment layer, promoting the release of PAHs sorbed to fine particles and organic matter (refer to Latimer et al., 1999). It must also be noted that the complexity of the reactions at the sediment/water interface interfering with PAH adsorption and release can be verified by the unexpected peak of Phe and B[b]F concentrations in water at T_{14}, more obvious in the assays with SPMDs (Fig. 3.1), which likely relates to unknown processes during the first stage of the assays, during which the sediments may still undergoing stabilization.

Unexpectedly, Phe concentrations in both SPMDs and clams are not linearly integrated in time. In fact, concentrations were better correlated with point concentrations in water whereas a continuous increment in time would be expected (Fig. 3.3). These findings are in accordance with the observations by Huckins et al., 1990, who stated that, under steady-state conditions, the concentrations of PAHs in both SPMDs and bivalves correlate with those in water. Under this point-of-view, it is plausible to assume that either SPMDs or clams are just as effective bioindicators of exposure to low molecular weight, low K_{ow} PAHs, like Phe. In contrast, the concentrations of B[b]F in water were more feebly correlated with the concentrations in clams, comparatively to SPMDs. This disparity may, at least in part, be explained by the clams' potential ability to biotransform and eliminate the compound. Conversely, B[b]F is more hydrophobic than Phe and holds higher affinity to triolein, which may contribute to constant accumulation of the compound, while delaying the reach for a steady-state condition (Baussant et al., 2001).

The BAFs were consistently higher in the clams' digestive glands, more obviously for B[b]F, comparatively to gills (Table 3.2). Nevertheless, the accumulation pattern for both PAHs between digestive glands and gills is essentially similar, indicating similar biological processes between the two

organs, albeit more pronounced in digestive glands. This information is in accordance with other studies with bivalves, reporting either accumulation or toxic effects exerted by PAHs in these organs (e.g. Darby et al., 1993; Fernández et al., 2010; Martins et al., 2013). Additionally, despite higher Phe bioavailability (via water), clams accumulated similar levels of Phe and B[b]F. These results may be attributed to the fact that filter-feeding organisms are capable of accumulating PAHs from water, either dissolved (especially those with lower K_{ow}, like Phe) or bound to suspended particles (Piccardo et al., 2001). Narbonne et al. (1999) concluded that assimilation from ingested materials may be more significant for PAHs with higher affinity for particulate matter, such as B[b]F, which may explain why the digestive gland is the main organ of bioaccumulation (and, likely, biotransformation), whereas the gills are primarily the main route of apical entry, conveying, rather than storing, dissolved substances to the remaining organs. The relative importance of these two uptake routes, i.e., through gill epithelia and ingestion, in filter-feeding organisms is thus controlled by the relative abundance of the xenobiotic between dissolved and particulate phases, respectively (Baussant et al., 2001). On contrary, SPMDs only sequester organic contaminants from the freely dissolved phase; however, this mechanism is mainly governed by a simple partitioning of the compound between membrane and water (Huckins et al., 1990).

5. Conclusions

The current study showed that SPMDs may accumulate higher net amounts of PAHs, even though caution is mandatory when comparing the accumulation of the xenobiotics between the devices and bivalves. It has been demonstrated that sediments contaminated with moderate, ecologically-relevant, concentrations of two distinct PAHs release significant amounts of both substances to the water column, rendering them bioavailable to organisms and prone for adequate detection by SPMDs. Still, while the pattern of bioaccumulation for the more hydrophilic Phe was similar between the devices and the clams, B[b]F bioaccumulation in clams yielded results that are more consistent with a continuous process of uptake and biotransformation/elimination that may be a significant confounding factor when considering these organisms as sentinels for the exposure to higher molecular weight, metabolizable, PAHs when accumulation is taken as the key end-point. Considering that these compounds are acknowledged as the most hazardous PAHs, most of which being known mutagens and carcinogens for wildlife (and potentially to humans), the present findings indicate the need to carefully balance ecologically-relevant approaches (e.g. using sentinel organisms) and cost-effective procedures (e.g. deploying semi-permeable devices), when it is aimed at developing biomonitoring programs for sediments contaminated with toxicants, for which the accumulation and detoxification mechanisms hold yet many challenges for ecotoxicologists.

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CHAPTER 4. COMPARATIVE DNA DAMAGE AND OXIDATIVE EFFECTS OF CARCINOGENIC AND NON-CARCINOGENIC SEDIMENT-BOUND PAHS IN THE GILLS OF A BIVALVE †

† Martins et al. (2013). *Aquat.Toxicol.* 142-143, 85- 95 (doi: 10.1016/j.aquatox.2013.07.019).

Abstract

Polycyclic aromatic hydrocarbons (PAHs) regarded as carcinogenic and non-carcinogenic to humans are ubiquitous hydrophobic pollutants that tend to be trapped in aquatic sediments. As a consequence of their acknowledged toxicity and pro-mutagenic or even carcinogenic potential, PAHs are deemed prioritary in biomonitoring programmes. Still, the differences between the toxicity of carcinogenic and non-carcinogenic PAHs are poorly known especially, when aquatic organisms are exposed to ecologically-relevant concentrations of these compounds in sediments. Laboratory bioassays with sediments spiked with phenanthrene (Phe) and benzo[b]fluoranthene (B[b]F), non-carcinogenic and carcinogenic PAH, respectively, were conducted and the effects of exposure (related to DNA damage and oxidative stress) were analysed in the gills of a burrowing clam, *Ruditapes decussatus* (Bivalvia, Veneridae). To ensure eco-logical relevance, two contaminant concentrations (termed "low" and "high") were selected in accordance with available PAH sediment quality guidelines. The results showed that, even in "low" concentrations, both compounds caused a likely genotoxic effect in the gills, which is in accordance with the link between PAHs in water. Glutathione S-transferase activity and glutathione biosynthesis appear to be associated with limited lipid peroxidation even though they were insufficient to prevent higher and faster genotoxicity induced by exposure to the carcinogenic B[b]F, comparative to Phe. Overall the findings indicate that low concentrations of sediment-bound PAHs, carcinogenic or not, may be rendered significantly bioavailable to benthic filter-feeders as to induce genotoxicity, revealing that even PAHs considered non-carcinogenic to humans detain a latent, albeit significant, pro-mutagenic hazard to bivalve molluscs.

Keywords

Oxidative stress; Comet assay; Phenanthrene; Benzo[b]fluoranthene; Sediment contamination; *Ruditapes decussatus*

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) constitute a ubiquitous class of environmental chemical pollutants many of which are known to exert pro-mutagenic effects (Swartz et al., 1990; Canova et al., 1998; Pérez-Cadahía et al., 2004). In this context the International Agency for Research on Cancer (IARC) has classified PAHs as non-carcinogenic, potentially carcinogenic and carcinogenic to humans, hence their allocation within priority substances in biomonitoring programmes (see Wogan et al., 2004). In accordance, the European Water Framework (updated through de Directive 2008/105/EC), which provides the legislative framework for the protection of quality of inland and coastal waters in the European Union, includes eight PAHs in the list of Priority Substances: naphthalene, anthracene, fluoranthene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene and benzo[g,h,i]perylene.

Both carcinogenic and non-carcinogenic PAHs are present in the aquatic environment as a result of oil spills, ship traffic, pyrolytic processes plus domestic and industrial wastewater discharges. Due to their high hydrophobicity, they tend to be trapped and stored in aquatic sediments, depending on the geochemical properties of the sediment, of which fine particle proportion and organic matter load are paramount (Narbonne et al., 1999). Sediments are thus critical reservoirs of these pollutants that may directly or indirectly affect organisms in contact with bottom floors or those in the water column exposed to PAHs adsorbed to re-suspended particulate matter or dissolved in water.

Polycyclic aromatic hydrocarbons are a particular class of pollutants in the sense that most of their toxicity is derived from the cell's own detoxification mechanisms. Their elimination process begins with the addition of an electrophilic group to the molecule to increase its solubility in water, a reaction that is termed "activation" and is especially catalysed by microsomal CYP monooxygenases, also termed mixed function oxygenases (MFOs). The mechanisms of PAH detoxification are reasoned to be similar in vertebrates and invertebrates (including bivalves).

However, it is generally believed that the cellular responses in molluscs are reduced when compared to fish and mammals, even though it is long known that PAHs induce the MFO system and increase CYP expression in bivalves (Canova et al., 1998). However, PAH activation renders highly reactive and toxic PAH metabolites (such as PAH quinones and diol epoxides), many of which are able to form adducts with DNA. Also, the process of activation generates reactive oxygen species (ROS) which, depending on the radical, may cause direct nucleobase oxidation (see, e.g. Cavalieri and Rogan, 1995; Penning et al., 1996; Ohnishi and Kawanishi, 2002, for details on PAH activation and radical formation). As such, CYP-activatable PAHs may contribute to increase genotoxicity and pro-mutagenesis since misrepaired DNA damage may be propagated and initiate tumourigenic mutations,

especially if occurring in the sequence of oncogenes of tumour suppressorgenes, hence the acknowledged link between genotoxicity and oncogenesis (see Wogan et al., 2004). Thus, it is long reckoned that the role of CYP monooxygenases, in the metabolic activation of PAHs, is related to the carcinogenicity of these chemicals (Conney, 1982). However, the distinction between "carcinogenic" and "non-carcinogenic" PAHs is mostly based on information retrieved from vertebrate model animal testing (especially mammals) and applies essentially to human risk. Still, the differences between the modes of action of the two groups of PAHs are poorly understood.

Due to their association with the benthos, combined with their high ecological and economic importance, bivalve molluscs are extensively employed as sentinel organisms in areas affected by contaminated sediments. Considerable bioaccumulation of PAHs has, in fact, been detected in these organisms collected from impacted areas (e.g. Coughlan et al., 2002; Wetzel and Vleet, 2004; Martins et al., 2012). Also, genetic damage as a consequence of exposure to the known carcinogenic PAH benzo[a]pyrene (B[a]P)has already been demonstrated in bivalves, either resulting from the formation of DNA adducts (Venier and Canova, 1996) or from the generation of ROS (Mitchelmore et al., 1998; Giannapas et al.,2012). In fact, most fundamental studies on PAH toxicity to aquatic organisms, as well as to mammals, report exposure to this model toxicant (e.g. Livingstone, 1998, 2001, Canova, 1998 for a case study in molluscs). However, as for other organisms, little information is available about the toxic mechanisms of other carcinogenic (or non-carcinogenic) PAHs (and their comparison) in bivalves. In addition, most fundamental studies neither focus on exposure to contaminated sediments nor consider exposure to ecologically-relevant PAH concentrations, which render the gap between laboratory mechanism-oriented studies and field-based approaches critical. Establishing cause-effect relationships is difficult due to the many confounding factors biasing *in situ* research.

Ruditapes decussatus (=*Venerupis decussata*) is a high-value commercial clam in Southern Europe that burrows in sandy-muddy sediments of coastal ecosystems, especially in enclosed areas such as estuaries and coastal lagoons (see, e.g., Bebianno et al.,2004; Carreira et al., 2013; Costa et al., 2013, and references therein), which are prone to be impacted by multiple anthropogenic pressures. The species has been proposed as a substitute for mussels in biomonitoring studies in areas where these are scarce or absent (Bebianno et al., 2004). Still, in spite of the relevance of the species to the field of environmental toxicology, fundamental information relating the genotoxic and oxidative stress effects and responses to PAH exposure (especially sediment-based) are in essence lacking, as for most molluscs.

The main goal of the present study is to compare the effects and responses of sediment-bound "carcinogenic" and "non-carcinogenic" PAHs in a bivalve species exposed to ecologically-relevant concentrations in sediments with special focus on genotoxicity and oxidative effects, due to their

acknowledged link to pro-mutagenesis and potential tumourigenesis. For the purpose, two priority PAHs usually present in contaminated aquatic sediments were selected: phenanthrene (Phe) and benzo[b]fluoranthene (B[b]F), for which toxicological data are much scarcer than for the well-known model PAH, B[a]P. Phenanthrene, with three fused benzene (aromatic) rings, although regarded neither as mutagenic nor as carcinogenic (thus being allocated in IARC's group 3), has been shown to cause toxic effects, such as ROS production and reduction of immune function, to marine organisms (Yin et al., 2007; Hannam et al., 2010). The five-ring PAH, benzo[b]fluoranthene, in its turn, has been defined as a possible or probable human carcinogen (IARC, group 2B), being regarded as a high-risk environmental pollutant.

2. Materials and methods

2.1. Sediment spiking

Artificial sediment was prepared by mixing freshly-collected muddy and sandy sediment batches collected from the Mira estuary, one of the least impacted coastal areas in Portugal (e.g. Vasconcelos et al., 2007). The choice of sediment proportion (3/1; muddy/ sandy) aimed at obtaining a final artificial sediment with5–10% total organic matter (TOM) and ≈50% fine fraction (FF), which is compatible with the characteristics of sediments of impacted estuaries inhabited by the species (Carreira et al., 2013).Final sediment TOM (6%) and FF (46.2%) were determined by combustion at 450 ± 50◦C and by hydraulic sieving after digestion with H_2O_2 and disaggregation with pyrophosphate, respectively.

Sediments (wet) were spiked with two concentrations (here-after termed "low" and "high") of each individual PAH (Phe and B[b]F), taking into consideration their differential toxicity thresh-olds. For the purpose, the selection of "low" (C1) and "high" (C2)nominal PAH concentrations was based on available sediment quality guidelines for the toxicants, namely the Threshold Effects Level (TEL) and the Probable Effects Level (PEL) retrieved from MacDonald et al. (1996). In accordance, C1 was targeted between TEL and PEL whereas C2 was intended to attain a value immediately above PEL. In the absence of a specific guideline for B[b]F, the guidelines of B[a]P were considered, accounting for chemical similarities between the two carcinogens. The TEL and PEL sediment quality guidelines for Phe and B[a]P were 86.7 and 88.8 ng $g^{-1}$and 544 and 763 ng g^{-1}, respectively (refer to Macdonald et al., op. cit).The sediment spiking procedure was based on Hickey and Roper (1992) and Costa et al. (1998). In brief, aliquots of the stock solutions of Phe (2500 g mL^{-1} in DMSO) and B[b]F (1020 g mL^{-1} in DMSO) were directly added to the sediments in order to achieve the four nominal concentrations: 250 ng g^{-1}(Phe-C1), 600 ng g^{-1}(Phe-C2), 250 ng g^{-1}(B[b]F-C1) and 800 ng g^{-1}(B[b]F-

C2), which was followed by 15 min of mechanic mixing. To account for the volatile Phe losses during the sediment spiking, the stock solution was prepared so that nominal sediment concentrations were three times higher than intended. Sediments were allowed to equilibrate for 48 h at 4 ºC before the beginning of the assays. The control sediment was prepared in a similar manner and spiked only with DMSO. Both PAHs were obtained from Sigma (St Louis, MO, USA).

2.2. Experimental design

Approximately 200 healthy clams (*Ruditapes decussatus*) were collected from Ria Formosa (South Portugal), from a high oceanic influence shellfish bed, previously described as unimpacted by pollutants (Barreira et al., 2007; Cravo et al., 2012), and acclimatised to laboratory conditions for one week before the experiment, in tanks containing clean sand and filtered and constantly aerated recirculated seawater.

The experimental assay consisted of a semi-static arrangement of 8 L-capacity tanks in which 2 L of each sediment (control, Phe-C1, Phe-C2, B[b]F-C1 and B[b]F-C2) and 5 L of filtered seawater were allocated. The bioassays had the duration of 28 days and were per-formed in duplicate for each experimental treatment. Sediments were allowed to settle for 48 h before the beginning of assays. The seawater in the experimental tanks was continuously aerated and 25% of the water was changed weekly (avoiding accessory resuspension) in order to ensure constancy of water parameters with minimum removal of contaminants. Water samples were taken for PAH analyses at days 0, 14 and 28. The physico-chemical parameters of water were also monitored weekly and were found similar to rearing (temperature = 18 ± 1 ºC, salinity = 32 ± 1, pH = 7.9 ± 0.2, total ammonia = 2-4 mg $L^{-1}$and the dissolved oxygen between 92 and 95% of air saturation).

Twelve randomly-selected clams (26.2 ± 1.5 mm shell length, 2.83 ± 0.40 mm shell height and 1.23 ± 0.16 g whole soft-bodyweight) were placed in each tank. Clams were fed daily with microalgae specifically cultured for the experiment (*Isochysis galbana*). Sampling was performed at days 14 (T_{14}) and 28 (T_{28}) for all experimental conditions, with six clams per replicate being collected and immediately processed for biomarker analyses. Upon collection, all individuals were measured for total length and weight. The condition index (CI) for each clam was calculated according to Lawrence and Scott (1982) by dividing the wet weight of soft tissue by the total wet weight of the clam (shell included).The gills of each clam were excised and divided for the Comet assay (processed immediately) or stored at −80 ºC for subsequent bio-chemical analyses (lipid peroxidation, glutathione and glutathione *S*-transferase activity). The choice of gills as target organ relates to their role in apical entry of toxicants, which implies increased sensitivity while withstanding the organism's first line of defence towards chemical challenge, especially in a filter-feeder like *R.decussatus*.

2.3. Phe and B[b]F analyses in sediments

Sediment PAHs were quantified by gas chromatography-mass spectrometry techniques (GC-MS) using a Thermo DSQ system in selected ion monitoring (SIM) mode, as described by Martins et al. (2012). In brief: sediment PAHs were extracted using a Dionex ASE 200 accelerated solvent extraction apparatus with an ace-tone/hexane mixture (v/v), fractionated with silica/alumina (g/g) glass column and concentrated to 0.5 mL under a gentle stream of N_2. Concentrations of PAHs were measured through the internal standard peak method following a 9-point calibration curve for each compound (Martins et al., 2008). Certified sediment reference material SRM1941b (NIST, USA) was analysed through the same method and the analysis yielded values within the certified range (recovery rates varied between 80 and 120% for both compounds).

2.4. Phe and B[b]F analyses in water

Water PAHs were analysed as described by Martinez et al. (2004) with some modifications. Briefly: to avoid adsorption of PAHs upon glassware, 10% (v/v) methanol was added to 1 L of each water sample. The solution was mixed thoroughly and then spiked with a surrogate standard (from Supelco). The C18 Speedisk was conditioned with 10 mL of ethyl acetate followed by adding10 mL methanol and 10 mL distilled water, at a rate of 10 mL min^{-1},using a Baker vacuum system (J.T. Baker, The Netherlands). The water sample was percolated through the speedisks at a flow rate of 20 mL min^{-1}. The speedisk was dried under vacuum for10 min and an elution was performed with a 5 × 5 mL ethyl acetate/dichloromethane mixture (1/1). The extract was evaporated under nitrogen and reconstituted in *n*-hexane. Both Phe and B[b]F were quantified by GC–MS (Martins et al., 2008). The limits of detection (LODs) calculated at a signal-to-noise ratio of 3 were 0.1 and 3 ng L^{-1} for Phe and B[b]F, respectively.

2.5. Biochemical biomarker analyses

2.5.1. Tissue handling and preparation

Gill samples were homogenized in cold phosphate-buffered saline (PBS), pH 7.4, with 0.7% NaCl and centrifuged at 7000 × g for 10 min. The clear homogenates were then divided in aliquots, one of which was used to determine total protein content according to the method of Bradford (1976), using bovine serum albumin as standard, in order to normalize all biochemical biomarker data. The activity of the phase II enzyme GST was determined from the same aliquot while GSH-related biomarkers and lipid peroxidation (LPO) were determined from sample aliquots de proteinised with5% (m/v) 5-sulfosalicylic acid and centrifuged to remove debris and precipitated protein.

2.5.2. Lipid peroxidation

Lipid peroxidation was determined using the thiobarbituric acid-reactive species (TBARS) protocol originally developed by Uchiyama and Mihara (1978) adapted to a microplate reader by Costa et al. (2011). Briefly: 1% (m/v) thiobarbituric acid solution was added to the deproteinated and acidified supernatant and incubated in a boiling water bath for fifteen minutes to conjugate thiobarbituric acid with lipid peroxides (in heat and low pH), forming a reddish pigment. The absorbance was measured at 530 nm using a Benchmark model microplate reader (Bio-Rad). Malondialdehyde bis(dimethylacetal), from Merck, was used as the standard to build an eight-point calibration curve. The results are expressed as pmol TBARS per mg of protein.

2.5.3. Glutathione determination

Glutathione (total, reduced and oxidized) was measured in the clear homogenates with a commercial kit (from Cayman), based on the enzymatic recycling method (using glutathione reductase), following the manufacturer's instructions. The assay allows the quantification of total glutathione (GSHt) and glutathione disulphide (GSSG) by derivatiing GSH in samples with 2-vinilpyridine (Sigma–Aldrich). Total GSH and GSSG were calculated from a standard calibration curve. Activity was determined spectrophotometrically using the aforesaid microplate reader (absorbance 412 nm). The results were expressed as nmol per mg of protein. The GSH/GSSG ratio was calculated as GSH/(GSSG/2).

2.5.4. Glutathione *S*-transferase activity

Glutathione *S*-transferase activity was measured using a commercial kit (from Sigma-Aldrich), using chloro-2,4-dinitrobenzene(CDNB) as substrate, following manufacturer's instructions. Activity was determined spectrophotometrically (using the aforementioned microplate reader) by measuring the increase in absorbance at 340 nm during 5 min at 1 min intervals. Activity was estimated as nmol conjugated CDNB/min/mg protein.

2.6. Assessment of total DNA strand breakage

The alkaline Comet assay was applied in clam gills mainly based on the protocol described by Costa et al. (2008), adapted from Singh et al. (1988). In brief: individual gill samples were excised and then chopped in 100 µL cold Kenny's salt solution (KSS: 0.4 M NaCl, 9 mM KCl, 0.7 mM K_2HPO_4, 2 mM $NaHCO_3$). After a low-power centrifugation to precipitate cellular debris (1500 rpm, 4 ºC for 2 min), 10 µL of the supernatant was diluted in 180 µL of liquid (35-40 ºC) 1% (w/v) low-melting-point agarose (LMPA, Sigma) prepared with KSS. The cells suspended in LMPA were then pipet-ted (2 ×

75 µL) onto glass microscopy slides pre-coated with dry1% (w/v) normal melting-point agarose prepared in TAE buffer. Afterwards, slides were immersed for 1 h (in the dark), at 4 ºC, in lysis solution (2.64% NaCl w/v, 3.72% EDTA w/v and 5 mM TRIS) to which 10% (v/v) DMSO and 1% (v/v) Triton-X 100 were added just before use. Slides were then placed in cold (4 ºC) electrophoresis solution (pH 13) for 40 min to promote DNA unwinding and enhanced expression of alkali-labile sites. Electrophoresis was run at 25 V, for 30 min, at 4 ºC, in the dark. Finally, the slides were neutralized in 0.1 N Tris–HCl buffer (pH 7.5) for 15 min. The employment of dark/dim light environment and cold solutions aimed at reducing accessory DNA damage and gel lifting from slides. Slides were stained with ethidium bromide (0.02 mg mL^{-1}) for 5 min and examined with a DMLB microscope adapted for epifluorescence with an EL6000 light source with mercury short-arc reflector lamps and a N2.1 filter (Leica Microsystems). Approximately one-hundred random comets were analysed per slide using CometScore (TriTek, VA, USA). The percentage of DNA in the tail was employed as a direct measure of DNA strand breakage (Lee and Steinert, 2003). Results are expressed as average percentage of DNA in tail, per individual.

2.7. Statistical analysis

After data failing to comply with parametric ANOVA assumptions, the non-parametric Mann–Whitney *U* test was applied for pairwise comparisons between experimental conditions and sampling times. Multivariate statistics include principal component (correlation-based) and discriminant analysis to address possible relationships between all accounted variables and the relative importance of each to differentiate bioassays, respectively. A significance level $\alpha = 0.05$ was set for all analyses. Statistics follow Zar (1996) and Sheskin (2000). All analyses were computed with Statistica (Statsoft, OK, USA).

3. Results

3.1. PAH concentrations

The Phe and B[b]F concentrations in sediment and water samples collected from each bioassay at both sampling times are presented in Table 4.1. Sediment spike contaminations were within the proposed ranges: “low” concentrations (C1) between TEL and PEL values and “high” concentrations (C2) higher than PEL values. The control sediment presented very low concentrations of both compounds (comparative to the TEL threshold). The sediment concentrations of PAHs in sediments decreased with time of expo-sure for all experiments, however, the highest declines between T_0 (beginning) and T_{28} (end of the assay) were observed for Phe. The water samples collected at T_0 were devoid (i.e.

below detection limit) of both compounds and in the control assay were always below the 9 ng L^{-1} maximum. The Phe concentrations in water were generally higher at T_{28} and in the Phe-C2 assay. The concentrations of B[b]F were highest at T_{28} and for B[b]F-C2, while similar at T_{14} between B[b]F-C1 and C2 assays.

Table 4.1. - Nominal Phe and B[b]F concentrations (ng g^{-1}) used for spiking the artificial sediment, and Phe and B[b]F effective concentrations in the beginning (T_0) and end of the experiment (T_{28}). Phe and B[b]F water concentrations measures in bioassays (control, Phe-C1, Phe-C2, B[b]F-C1 and B[b]F-C2) at sampling time (T_0, T_{14} and T_{28}).

		Control		**Phe**		**B[b]F**	
		Phe	B[b]F	C1	C2	C1	C2
Sediment (ng g^{-1})	Nominal	0	0	250	600	250	800
	T0	21.3±1.8	132.7±3.7	305.6±63.6	856.8±78.8	436.9±24.2	1379.1±173.5
	T28	17.6±1.0	120.1±0.2	121.7±16.1	483.3±51.4	426.6±111.9	1223.0±80.5
Water (ng L^{-1})	T0	<0.1	<3.0	<0.1	<3.0	<0.1	<3.0
	T14	4.5±0.1	7.3±3.1	71.4±0.1	259.4±3.6	20.8±3.1	21.9±3.3
	T28	6.7±0.1	8.8±3.0	100.8±0.1	372.6±3.2	31.9±3.3	52.2±3.2

3.2. Physiological parameters

Clam shell length (L_s) and total wet weight (ww_t) were significantly correlated (Spearman $R = 0.89$, $p < 0.05$). No significant differences (Mann-Whitney U, $p > 0.05$) were found between bioassays and sampling times regarding either measure. The condition index observed in clams exposed to control and spiked sediments ranged between 0.18 and 0.21 (Fig. 4.1A). No significant differences were found between sampling times and experimental treatments (Mann-Whitney U, $p > 0.05$).

3.3. Biochemical biomarkers

Globally, LPO tended to decrease with time in animals exposed to either compound, when compared to the control assay, the exception being the gills of clams exposed to B[b]F. Lipid per-oxidation was significantly higher (Mann-Whitney U, $p < 0.05$) in clams from control and B[b]F-C1 assays (Fig. 4.1B) after 14 days of exposure while Phe (C1 and C2) and B[b]F-C2 assays presented similar values. At T_{28}, an increase in LPO was registered for all treatments, compared to T_{14}, especially in Phe exposure (C1 andC2) (Mann-Whitney U, $p < 0.05$). Still, the pattern was similar to that observed at T_{14}. No clear dose-response effect was observed for either Phe or B[b]F bioassays.

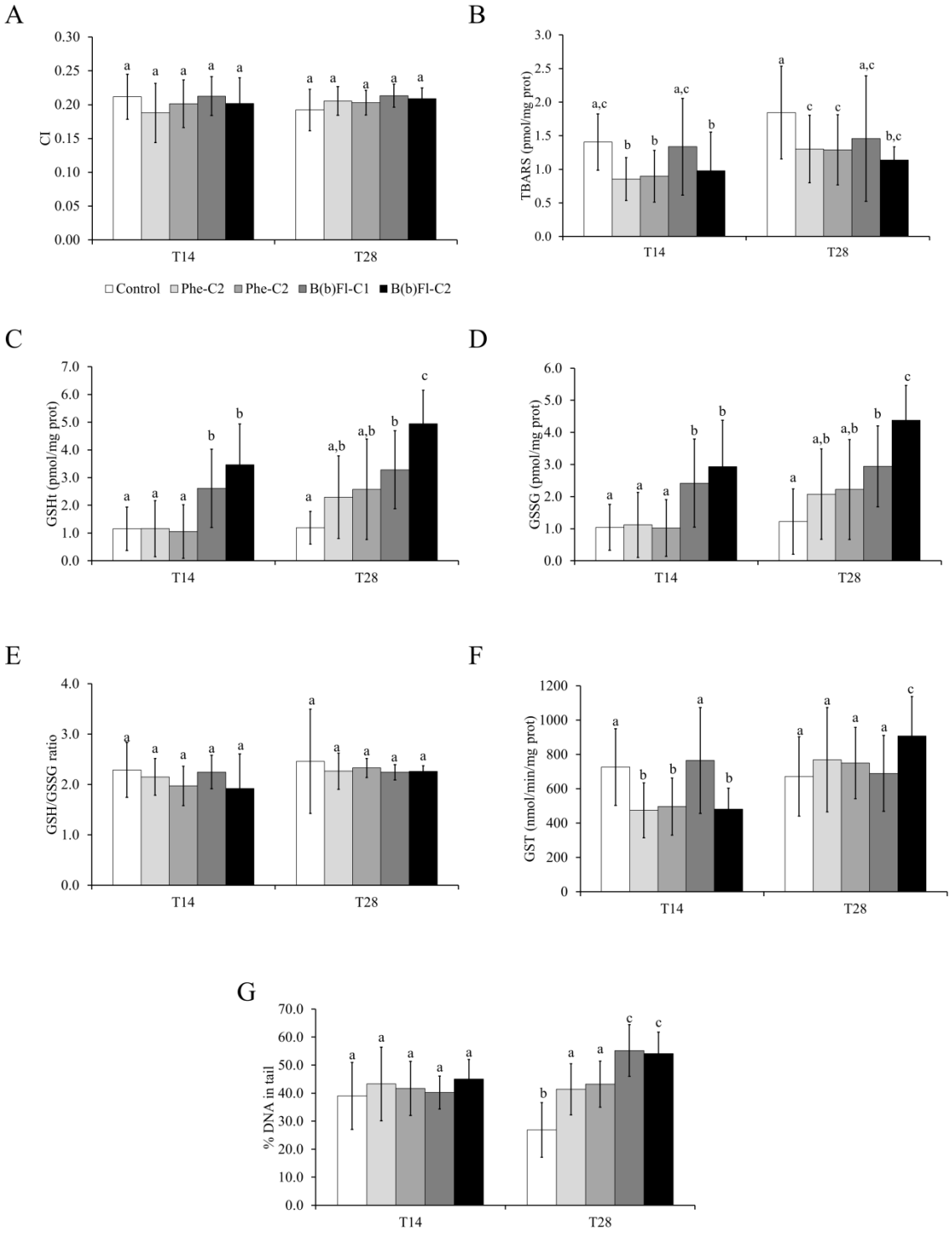

Fig. 4.1. Average biomarker responses in gills of clams exposed to tested sediments (control, Phe-C1, Phe-C2, B[b]F-C1 and B[b]F-C2), at day 14 (T_{14}) and day 28 (T_{28}). A) Condition index (CI). B) Lipid peroxides (given by TBARS). C) Total Glutathione (GSH_t). D) Glutathione disulfide (GSSG). E) GSH/GSSG ratio. F) Glutathione *S*-transferase activity (GST). G) DNA strand breakage (Comet assay; % DNA in tail). Error bars indicate standard deviation. Different letters indicate significant differences (Mann-Whitney *U*, $p < 0.05$).

A similar pattern was observed between the total (GSH_t) and the oxidized (GSSG) forms of glutathione (Fig. 4.1C and 4.1D, respectively). The gills of clams exposed to both concentrations of B[b]F presented significantly higher GSH and GSSG concentrations (Mann-Whitney *U*, $p < 0.05$) than those in Phe assays (C1 and C2), at T_{14}. However, at T_{28}, GSH and GSSG concentrations increased in all experiments reaching significantly higher concentrations than earlier in the B[b]F-C2 assay. Control and Phe experiments were similar at T_{14}, without significant differences at T_{28} (Mann-Whitney *U*, $p > 0.05$). The GSH/GSSG ratio (Fig. 4.1E) did not vary with assay or sampling time (Mann-Whitney *U*, $p > 0.05$) and ranged between ≈ 2 and 2.5. A similar pattern between GST activity and LPO was observed at T_{14} (Fig. 4.1F) with highest values being attained in the control and B[b]F-C1 bioassays. However, at T_{28}, clam gills from the Phe (both concentrations) and B[b]F-C2 bioassays showed a significant increase in the enzyme activity compared to T_{14} animals from the same experimental conditions (Mann-Whitney *U*, $p < 0.05$), especially the latter, where the highest values were scored. However, this increase in GST activity was observed to be transitory compared to controls since only B[b]F-C2 yielded significant differences to controls at this sampling time.

3.4. Total DNA strand breakage

The average percentage of DNA in the nucleoid's tail as a measure of total DNA-strand breakage (DNA-SB) is presented in Fig. 4.1G. No significant differences were observed for any experiment at T_{14}.However, at T_{28}, B[b]F assays (C1 and C2) yielded significantly higher percentage of DNA in tail (Mann-Whitney *U*, $p < 0.05$) com-pared to all other assays, reaching 2-fold values of control animals. These differences were mostly due to the significant increments of cells with up to 60% of DNA in tail and the decrease of cell with20% of DNA in tail observed in B[b]F assays (Fig. 4.2). Moreover, DNA damage decreased with sampling time in control assays (Fig. 4.1G) with a significant decrease on the number of cells with 60-100% of DNA in tail. In its turn, Phe induced significant DNA damage over controls at T_{28} as well. However, the percentage of DNA in tail was lower than for B[b]F exposed animals in both concentrations.

3.5. Statistical integration of data

Principal component analysis combining all individual biomarker responses plus assay conditions gave two models: model 1, for the Phe bioassays (Fig. 4.3A) and model 2 for the assays with B[b]F (Fig. 4.3B). Model 1 yielded three main factors that, combined, explained about 71.5% of the total variance. Total and reduced glutathione were consistently correlated and were the most significant contributors to factor 1 (0.58 and 0.57, respectively). The most important contributors to factor 2 were LPO (0.69), GST (0.46) and GSH/GSSG ratio (0.38) while in factor 3 the most important variables were CI (0.55) and TSB (0.69).

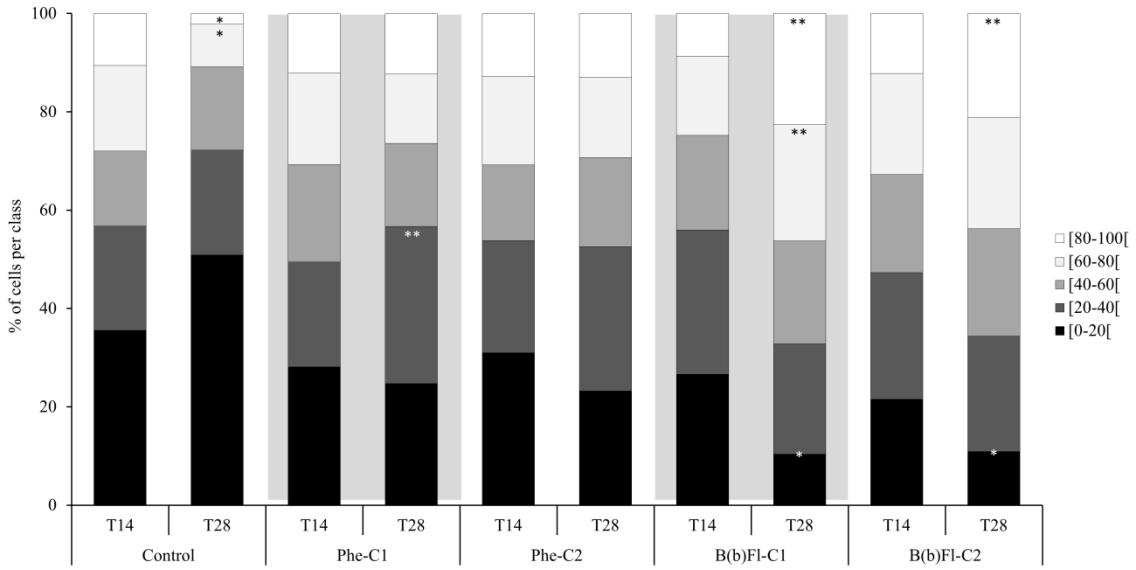

Fig. 4.2. Frequency distribution of cells per DNA strand-breakage class: [0-20[, [20-40[, [40-60[, [60-80[and [80-100] percentage of DNA in tail. * and ** indicate significant differences between sampling times (T_{14} and T_{28}) within each experimental treatment (Mann-Whitney *U*, $p < 0.05$).

No clear link was observed between TSB and the other biomarker responses and its overall significance in the model was low. Time of exposure (T) showed significance in the model and was positively correlated with LPO and GST whereas sediment and water contamination (S_c and W_c, respectively) failed to achieve any clear trend with other biomarker responses or even overall significance in the model. Model 2 retrieved three main factors that, combined, explain about 74.9% of the total variance. GSH (0.60) and GSSG (0.60) were the main contributors to factor 1, followed by CI (0.57), LPO (0.48), the GSH/GSSG ratio (0.61) to factor 2, and TSB (0.48), LPO (0.54) and GST (0.59) to factor 3.

Table 4.2. Results from the discriminant analysis between Phe and B[b]F bioassays. The best model was assessed according to the lowest Wilks' λ statistic and forward stepwise addition of variables. Variable significance within each model was determined using *F* tests ($p < 0.05$).

				Variables									
		Model		CI		LPO		GSSG		GST		%DNA in tail	
Case		Wilks' λ	*p*	Wilks' λ	*p* to remove	Wilks' λ	*p* to remove	Wilks' λ	*p* to remove	Wilks' λ	*p* to remove	Wilks' λ	*p* to remove
C1	T_{14}	0.635	0.189	0.998	n.s.	0.948	n.s.	0.929	n.s.	0.850	n.s.	0.940	n.s.
	T_{28}	0.363	0.005	0.937	n.s.	0.916	n.s.	0.826	n.s.	0.709	0.026	0.524	0.002
C2	T_{14}	0.581	0.202	0.974	n.s.	0.972	n.s.	0.697	0.041	0.998	n.s.	1.000	n.s.
	T_{28}	0.426	0.032	0.947	n.s.	0.993	n.s.	0.785	n.s.	0.952	n.s.	0.861	n.s.

Total DNA strand breakage (DNA-SB) and GST were consistently correlated and were linked to time of exposure (T) and water concentrations (W_c). Sediment PAH concentration had some relation to these biomarker responses in spite of its relatively low significance in the model. The condition index was negatively correlated to GSH/GSSG ratio and LPO despite its uncertain position relative to all other variables. The most significant discriminant analysis models (Table 4.2), concern T_{28} for both concentrations of toxicants (C1 and C2). For the "low" concentrations (C1) the variables that contributed most to differentiate Phe and B[b]F bioassays at T_{28} were GST and the percentage of DNA in tail (Wilks' λ = 0.71, $p < 0.05$ and Wilks' λ = 0.52, $p < 0.01$, respectively). However, for higher concentrations (C2), GSSG was the variable that contributed the most to differentiate the two PAH compounds at T_{14} (Wilks' λ = 0.70, $p < 0.05$).

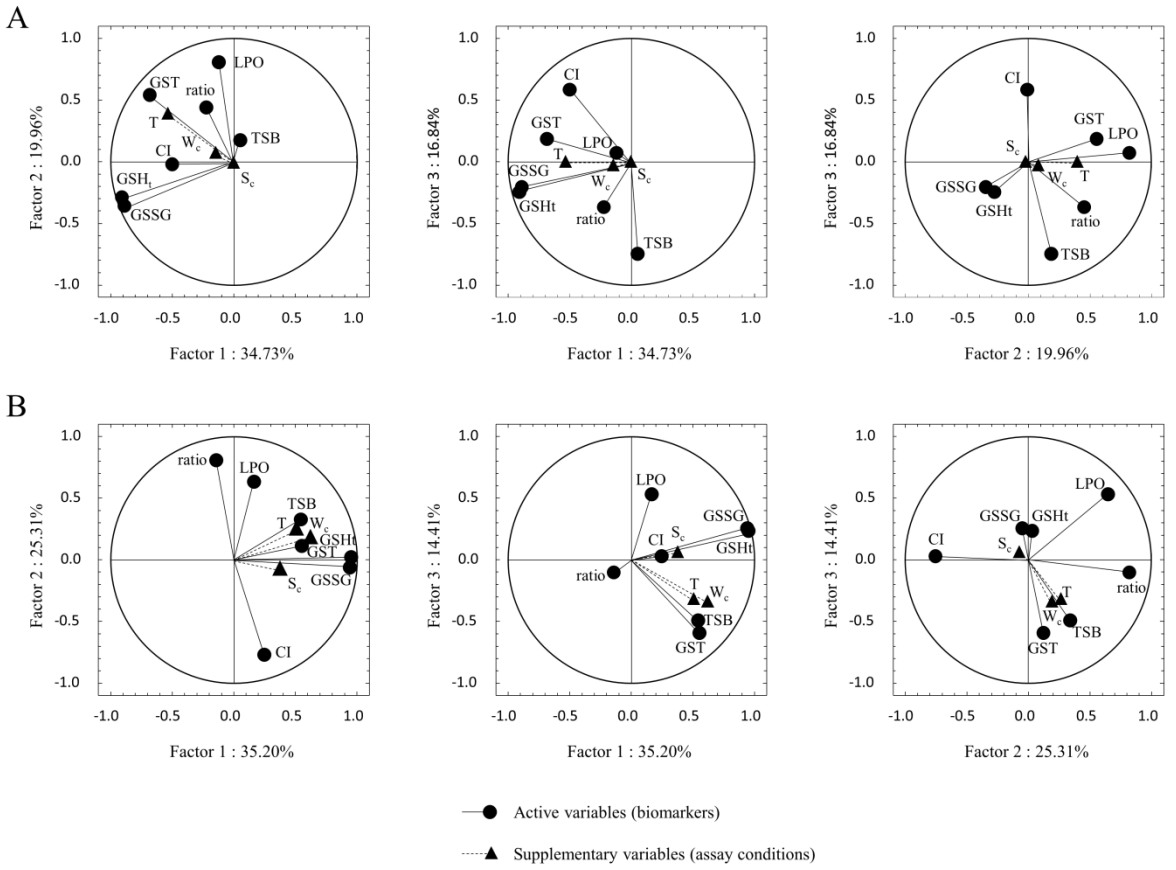

Fig. 4.3. Principal component analysis results for all biomarker data and respective factor contributions for explained variables (condition index, CI; lipid peroxidation, LPO; total glutathione, GSHt; glutathione disulfide, GSSG; GSH/GSSG ratio, ratio; glutathione *S*-transferase activity, GST; total DNA strand breakage, TSB), plus the assay conditions (time, T; sediment contamination, S_c; water contamination, W_c) as supplementary variables. A) Model 1 - including data from Phe bioassay. The total cumulative variance explained by the three factors is 71.5%. B) Model 2 - including data from B[b]F bioassay. The total cumulative variance explained by the three factors is 74.9%.

4. Discussion

The present findings suggest differences between the two PAHs, concerning not only their release from the sediment but also, most importantly, to their mode of action *in vivo*. The differences between Phe and B[b]F concentrations in water point to the different solubility of these aromatic compounds, which should contribute to modulate bioavailability, since *R. decussatus* is essentially a filter-feeding bivalve. In fact, the solubility of aromatic compounds decreases as the octanol–water partitioning coefficient (K_{ow}) increases (Means et al., 1980). Also, molecular weights and molecular areas, which are descriptors related to the dimension of molecules, are inversely correlated with desorption rates. Because of this, PAHs with high K_{ow} values and higher molecular weight such as benzo[a]pyrene (B[a]P) and B[b]F are preferentially adsorbed onto or associated with particulate matter, while PAHs with low K_{ow} values and low molecular weight, such as Phe, are more water soluble (Narbonne et al., 1999), which is in good agreement with the much higher desorption of Phe from the sediments to water, compared to B[b]F (Table 4.1). These different physico-chemical properties explain the higher enrichment of Phe observed in water phase compared to B[b]F. In fact, Narbonne et al. (1999) have demonstrated that more than 50% of the Phe concentrations in any given steady-state sediment was transferred to the water phase, while only less than 10% of B[a]P (that shares physicochemical properties with B[b]F) was available for dissolution in water. Thus, from these chemical properties, which corroborate the current findings, it may be inferred the importance of sediments in the trapping and storage of potential carcinogens such as many PAHs, especially those with higher molecular weight. It must also be stressed that PAH levels in the water were similar to those found in moderately to highly contaminated estuaries (Fernandes et al., 1997; Guo et al., 2007). Also, the responses (GST activity and GSH induction) and effects (DNA damage and lipid peroxidation) of organisms relate better to concentration in water (Fig. 4.3A and 4.3B), i.e., to the toxicants released from the sediments, than to the concentrations of PAHs in the sediments themselves, which further dictates the need to understand the phenomena modulating bioavailability.

It is generally acknowledged that PAHs, as many other toxicants, trigger toxicological effects by causing oxidative stress, either by eliciting direct damage or depletion of the anti-oxidant defences of the cells, such as GSH (see, e.g., Winston and Di Giulio, 1991;Frenzilli et al., 2001; Livingstone, 2001). Also, gills, as the main entry organ of waterborne toxicants, either dissolved or bound to particulate matter (especially in filter-feeders as bivalves), are more prone to experience oxidative stress than other tissues which should imply that antioxidant mechanisms have to be more rapid and efficient in this tissue than in others (Regoli and Principato,1995). One of primary targets of oxidizing agents are membrane lipids, where lipid peroxidation (LPO) is caused. Lipid peroxidation is initiated by ROS that attack polyunsaturated fatty acids in membranes (e.g. Livingstone, 2001). The present findings, however, suggest reduction, rather than increase, in gill LPO, as a consequence of exposure

to either PAH, compared to control animals. Interestingly, the formation of oxidized glutathione (GSSG) was compensated for by increased GSH biosynthesis of increased GSSG recycling, which results in more or less constant reduced/oxidized glutathione ratios throughout the assays (Fig. 4.1). Given the aim of surveying ecologically relevant, moderate, exposures to PAHs, the results indicate an over-compensatory anti-oxidant response that ultimately led to decreased LPO, i.e., low biochemical oxidative damage to gill tissue. This helps understanding why the condition index showed no significant alterations. In fact, other authors reported increased LPO in bivalve haemolymph with depletion of GSH, although following exposure to higher Phe concentrations (200 µg L^{-1}), than those employed here (Hannam et al., 2010). Conversely, and in accordance with the present findings, decreased LPO has been recorded in mussel gills, and is a probable consequence of elevated levels of antioxidant enzymes and GSH (Cheung et al., 2001). Altogether, the findings suggest a compensatory hormetic response in clam gills triggered by exposure to relatively low concentrations of both PAHs (see Mattson, 2008, for a definition of terms). Nevertheless, both total and oxidized glutathione were significantly higher in B[b]F-exposed clams, as early as after 14 days, unlike in Phe bioassays, which indicates higher oxidative challenge.

Besides its role in conjugating electrophilic toxicants or their by-products (as some PAH metabolites), a process that is catalysed by glutathione *S*-transferases, glutathione (a tripeptide thiol) is involved in the scavenging free radicals which offers a first protective step against oxyradicals, thereby preventing lipid peroxidation (Fernández et al., 2012). Glutathione is predominantly present in cells in its active reduced form (GSH) and turns to its oxidized form (GSSG) in the presence of oxidative agents, like H_2O_2. Oxidation of GSH is catalysed by glutathione peroxidase (see Lushchak, 2011, for a review). Thus, the glutathione molecule's dual role as radical scavenger and conjugating agent for organic xenobiotic metabolites may fit the mechanisms underlying exposure to metabolisable PAHs (like B[b]F), which are, e.g., detoxified by phase I enzymes that yield highly reactive, promutagenic/carcinogenic hydrophilic metabolites and oxidative radicals as by-products. Nevertheless, while the process is well described in vertebrates, the subject is not yet well understood in other animals such as molluscs. Still, the activation of organic xenobiotics is long known to occur in bivalves, where it may result from the activity of microsomal enzymes such as CYP-like monooxygenases or cytosolic enzymes (refer to Stegeman, 1985; Díaz-Méndez et al., 1998). In addition, GSH induction in bivalves has already been linked to the detoxification of organic xenobiotics like PAHs and PCBs (e.g. Osman et al., 2007), either by radical scavenging or metabolite conjugation. Results on such induction include exposure to benzo[a]pyrene, a known carcinogen with similar structure and properties as B[b]F (Cheung et al., 2001).

The GSSG values registered in clam gills exposed to Phe (at both concentrations) were similar to those of the control assays indicating that no significant oxidative stress occurred during the first 14 days of

exposure. In contrast, the elevated levels of GSSG registered in the B[b]F assays could mean that higher production of reactive oxygen species (ROS) occurs as a result of exposure to this PAH. However, as noted, the production of GSH appears to offset the formation of reactive oxygen species. Still, GSSG recycling back to reduced glutathione may have also been enhanced by increased activity of enzymes like glutathione reductase, not surveyed here. Nevertheless, after 28 days of exposure, GSSG values were also elevated in gills of Phe-exposed clams, which may indicate time-dependent oxidative stress caused by Phe, albeit delayed compared to its "carcinogenic" counterpart. It must be highlighted at this point, that glutathione (total and reduced) was more important to explain variation between experiments than LPO, as inferred from PCA analysis, which indicates that anti-oxidant responses may be more effective biomarkers when low-moderate exposures are prone to deliver null oxidative effects like LPO and GHS/GSSG ratios.

Glutathione *S*-transferases (GSTs) play an important role in the conjugation (phase II of detoxification) of various electrophilic compounds derived from PAH activation (e.g. the formation of the highly reactive and genotoxic PAH epoxides) with GSH (Kaaya et al., 1999). These enzymes also play a role in protecting against oxidative stress by catalysing selenium-independent glutathione peroxidase activity (Prohaska, 1980). In fact, no significant increase in GST activity, compared to controls, was observed during the first 14 days of exposure to Phe and B[b]F, which is in line with LPO results, indicating reduced oxidative stress. Additionally, the results indicate efficient defences towards ROS (via GSH scavenging) being triggered by B[b]F at T_{14}. Conversely, at T_{28}, increased GST activity was registered for both Phe and B[b]F assays relative to T_{14}, although, in the case of Phe, the activity was similar to that of the control bioassay, indicating a transient situation. Increased GST activity has long been documented to occur in laboratory exposures of bivalves to various organic chemicals (see Livingstone,1991) and, recently, it has been also suggested that GST is one of the most active anti-oxidant enzymes in the mussel *Mytilus edulis* (Vidal-Liñan et al., 2010). There is evidence that GST induction may modulate PAH, e.g. Phe, bioaccumulation in aquatic animals (Yin et al., 2007). The gills of the scallop *Chlamys farreri* can resist low concentrations of the carcinogenic PAH benzo[k]fluoranthene, e.g. by increasing the activity of anti-oxidant enzymes, GST included (Pan et al., 2006). Although little studied in invertebrates, GST gene expression depends on the Keap1/Nrf2 pathway, according to which Keap1 (the specific repressor of the Nrf2 transcription factor) releases Nrf2 in the presence of oxidative agents/electrophilic toxicants, which may then migrate to nucleus and bind to the ARE (anti-oxidant response element) of target genes, promoting transcription. As such, increased ROS production as a consequence of B[b]F activation (during exposure to the highest concentration) likely contributed to the increase in GST activity at T_{28}. Conversely, effective ROS scavenging by baseline defences (as glutathione) may account for the lack of a clearer dose- and time-dependent increase of GST activity.

In spite defence mechanisms (such as GSH) which appear to have provided protection towards biochemical damage (namely, LPO), exposure to Phe- and B[b]F-spiked sediments elicited DNA damage in the gills, at later stages of exposure and more consequent for animals exposed to the carcinogenic PAH, likely indicating a significant genotoxic effect. Still, although a clear dose-response was absent in either case, individual total DNA strand breakage (TSB) was better correlated to other biomarker data in B[b]F- than in Phe-exposed clams, and, furthermore, consistently linked to the concentration of the PAH in water (Fig. 4.3B), which was likely the main route of exposure.

Genotoxic damage has been detected and measured in marine invertebrates following chronic exposure to sediments contaminated with various toxicants (Coughland et al., 2002; Hartlet al., 2004; Frenzilli et al., 2009), including in the gills of mussels exposed *in situ* during dredging activities, where a strong correlation between DNA damage and sediment B[b]F was found (Martins et al., 2012). Polycyclic aromatic hydrocarbons are not direct carcinogens. Rather, they may be metabolized to genotoxic metabolites. One route of activation common among carcinogenic PAHs concerns the sequential action of CYP (cytochrome P450) monooxygenases and microsomal epoxide hydrolase, which results in the formation of a bay-region, highly genotoxic, diol epoxide. This mechanism, which is present in molluscs, is believed to be of greater importance in vertebrates (Canova et al., 1998). Nevertheless, research is still needed to disclose the mechanism of PAH activation in invertebrate species. The PAH diol epoxides have been identified as the ultimate carcinogenic metabolites which can produce bulky DNA adducts, therefore weakening the DNA chain, promoting instability and breakage (Livingstone et al., 1990; Venier and Canova, 1996). Another activation pathway involves one-electron oxidation step to yield quinones and semi-quinone radicals, which cause the formation of DNA adducts and production of ROS via redox cycling (Livingstone et al., 2003). These may themselves be genotoxic by causing nucleobase oxidation, although not all forms of ROS are directly genotoxic (such as the superoxide anion, unlike its genotoxic breakdown product OH^{-}). Still, whereas nucleobase oxidation may be repairable, usually involving base-excision (Cadet et al., 2010), reverting DNA lesions like adducts is trickier, leading to potential propagation of mutations and, therefore, increasing the risk of tumourigenesis, hence the hazard inherent to PAHs like B[b]F and similar compounds.

Although DNA damage occurred as a consequence of exposure to both PAHs, higher DNA-SB was registered in the gills of clams exposed to B[b]F, with significant increments in the frequencies of nucleoids with up to 60% of DNA in tail (Fig. 4.2). Furthermore, the results from discriminant analysis showed that %DNA in tail was the variable that contributed the most to differentiate between Phe and B[b]F bioassays in exposures to the lowest (C1) PAH concentrations. These findings indicate that Phe and B[b]F have different genotoxic and, most likely, carcinogenic potentials towards clam gills, concerning the time of exposure needed to elicit damage and the mechanisms of action upon the

DNA molecule. Comparative carcinogenicity studies with mice demonstrated that bay-region dihydrodiol epoxides of Phe exhibit no significant tumorigenic activity *in vivo* (Buening et al., 1979), due to the relatively high polarity of the Phe metabolites compared to those of larger PAHs such as B[a]P and B[b]F, which are considered to be effective carcinogens to humans by the IARC. As compared to larger PAHs, Phe metabolites may be associated with reduced DNA adduct formation, wider intracellular distribution or facilitated excretion of metabolites (Wood et al., 1979). In addition, Phe is metabolized by microsomal CYP enzymes at much lower rates than, for instance, B[a]P, yielding many distinct metabolites, which altogether likely accounts for the differences between these contaminants' carcinogenic potential (see Pangrekar et al., 2003, and references therein). Thus, it is possible that Phe exposures caused genetic damage in gills of clams mainly as a consequence of ROS formation, whereas B[b]F induced both oxidative and non-oxidative damage. It should be noticed that the formation of PAH-DNA adducts has been demonstrated in molluscs, following *in-* and *ex-situ* exposures (Canova et al., 1998; Xu et al., 1999). Inclusively, the induction of bulky B[a]P metabolite-DNA adducts in mussels has also been demonstrated during laboratory exposures to the PAH (Canova et al., 1998; Akcha et al., 2000). Regarding the specific case of B[b]F (a non-alternant PAH, meaning that it possesses other rings in addition to six-carbon), in contrast to other PAHs like B[a]P (alternant PAH), there is evidence to suggest that the biotransformation of its bay-region dihydrodiol epoxide does occur to change the phenolic dihydrodiol to 5,9,10-trihydroxy-11,12-epoxy-9,10,12-tetrahydrobenzo[b]fluoranthene which has been linked to the specific genotoxic activity of B[b]F in mouse skin (Weyand et al., 1993). It is clear, though, that further research is still need to fully understand the effects and their mechanisms when aquatic invertebrates are exposed to PAHs whose classification of "carcinogenic" or "non-carcinogenic" applies chiefly to humans and needs yet to be demonstrated in molluscs.

5. Concluding remarks

The present study showed that, even in concentrations that may be considered "low" and ecologically relevant, both PAHs were released from sediments, either in the dissolved form or bound to particulate matter, and elicited DNA lesions in the gills of clams, likely through genotoxic effects. Still, higher genotoxicity was promoted by the exposure to a "carcinogenic" PAH. On the other hand, the biochemical changes caused by Phe, considered a "non-carcinogenic" PAH to humans, occurred posteriorly, which is indicative of the differential toxicity between the two compounds. Seemingly, these alterations occurred in organisms that were still able to cope with oxidative stress and retained a condition index similar to unexposed animals, thus rendering genotoxicity as the keystone adverse effect. Given the critical hazard of DNA damage to clams and that only a fraction of the contaminants was made readily available to clams via water (whose toxicant concentrations better related to

toxicological alterations), the current findings call for a redefinition of risk thresholds associated to toxicants in steady-state sediments and mandate caution when determining which responses may be the most informative of potential deleterious effects to individuals and populations.

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CHAPTER 5. COMPARING THE GENOTOXICITY OF A CARCINOGENIC AND A NON-CARCINOGENIC PAH, SINGLY AND IN BINARY COMBINATION, ON PERIPHERAL BLOOD CELLS OF THE EUROPEAN SEA BASS[†]

† Martins et al. (submitted).

Abstract

Research on the toxicological mechanisms of PAHs deemed carcinogenic and non-carcinogenic has mostly been developed for individual compounds even though, in the environment, PAHs invariably occur in mixtures. The present work aimed at understanding the interaction effects of two model PAHs, the carcinogenic benzo[b]fluoranthene (B[b]F) and the non-carcinogenic phenanthrene (Phe) to a marine fish (the bream *Dicentrarchus labrax*). The study endeavoured an ecologically-relevant scenario with respect to concentrations and contaminant matrix, sediments, which are the main reservoirs of these substances in the environment, due to their hydrophobic nature. For the purpose, 28-day laboratorial bioassays with spiked sediments (with individual and combined PAHs at equitoxic concentrations) were conducted. Genotoxicity was determined in peripheral blood through the "Comet" assay and by scoring erythrocytic nuclear abnormalities (ENA). The results showed that exposure to either PAHs induced similar levels of DNA damage, although without a clear dose- or time-response, likely due to the low concentrations of exposure. However, clastogenic/aneugenic lesions were only observed in fish exposed to B[b]F-spiked sediments. Conversely, the combination assays revealed a supra-additive effect especially at chromosome level, linked to concentrations of PAHs in water. A decrease in DNA-strand breakage was observed over time during all assays, which indicated the ability of fish to cope with this DNA lesion. Overall, the findings indicate that low-moderate concentrations of sediment-bound mixed PAHs may significantly increase the hazard of mutagenesis even when the individual concentrations indicate low risk, especially considering that chromosome-level damage is unlikely to be repaired, leading to the fixation of DNA lesions upon prolonged exposures.

Keywords

Comet assay, Chromosomal clastogenesis, Phenanthrene, Benzo[b]fluoranthene, Mixtures, Oxidative-stress.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) constitute one of the priority classes of environmental genotoxic compounds. These organic compounds have long been acknowledged to cause DNA damage to humans and wildlife. Moreover, genotoxic effects exerted by some PAHs have been linked to chromosomal clastogenesis, teratogenesis and oncogenesis, which greatly contributes to confer to these substances their top-ranking amongst the pollutants of concern (see Miller and Ramos, 2001; Gangar et al., 2010; Yang et al., 2010 for a review). Studies with experimental animals as well as epidemiological investigations, performed from the 1970s onwards, PAHs became classified as non-carcinogenic, potentially carcinogenic and carcinogenic to humans, by the International Agency for Research on Cancer (IARC).

Interestingly, the bulk of the genotoxic effects caused by PAHs results mostly from the action of the metabolites rather than the parent compounds. In fact, the genotoxic effects of the PAHs begin with the cell´s own detoxification mechanisms through PAH activation by CYP monoxygenase (also termed mixed function oxidases), which yields highly reactive intermediates such as PAH quinones and diol epoxides. These intermediates can directly interact with DNA to form bulky adducts and cause other lesions to the DNA molecule that may turn into fixed mutations if not adequately repair and if at all repairable (Baird et al., 2005). In addition, PAH activation generates reactive oxygen species (ROS) that, on their turn, may cause direct nucleobase oxidation (Penning et al., 1996; Ohnishi and Kawanishi, 2002). In either cases, mutations occurring at specific sites mat trigger oncogenes or suppress the expression of tumour suppressor genes, potentially leading the cell to become anaplastic or neoplastic (Sarasin, 2003).

Still, in spite of similar chemical properties, the mechanisms by which carcinogenic and non-carcinogenic PAHs are genotoxic and pro-mutagenic may differ. In addition, when combined in mixtures (as commonly found in the environment), their effects may be synergistic, antagonistic, or additive, remain largely unknown. As such, the current state-of-art, which considers only the hazard of individual compounds, may grossly underestimate the risks of PAH-induced genotoxicity on human wildlife health.

There are a number of techniques designed to assess the genotoxic damage exerted by a wide range of substances, from PAHs to metals, aiming at distinct, albeit potentially linked endpoints. These endpoints include determining chromosomal clastogenesis by analysing nuclear aberrations (such as through the micronucleus test (MN) in mammalian cells); detecting formation of DNA-adducts or measuring the DNA strand breakage (DNA-SB). For the purpose, the extension of the MN assay to non-mammalian organisms for which erythrocytes are nucleated led to the development of the

erythrocytic nuclear abnormality (ENA) test (Çavaş and Ergene-Gözükara, 2005; Bolognesi and Hayashi, 2011). On its turn, the single-cell gel electrophoresis (SCGE) or "Comet" assay (Singh et al., 1988), rapidly became one of the most widespread protocols to analyse DNA-SB and is nowadays applied to a variety of model and non-model organisms (see for instance van der Oost et al., 2003). However, the measured endpoints of these different techniques displayed distinct types of DNA damage and different consequences to the cell. While MN and ENA tests detect irreparable DNA damage, namely clastogenic lesions, the SCGE identifies DNA chain breaks (single and/or double-strand) or lesions that can be converted into strand breaks under the strongly alkaline conditions of the electrophoresis. Many of these lesions can be repaired through the complex nuclear enzymatic machinery that mediates processes such as mismatch repair plus nucleotide and base excision repair (for single-strand lesions) and homologous recombination (for double-strand breakage) (e.g. Collins, 2009). Conversely, clastogenesis refers essentially to chromosomes fragmentation or loss of whole chromosome during faulty mitosis. These events are reflected in abnormal shapes of nuclei, such as micronuclei or nuclear buds, due to failure of incorporating chromosome fragments or displaced chromatids into the genome of the daughter-cell, compromising cell viability (see Heddle et al., 1991 and Fenech et al., 2011, for a review). Due to the differences between cytokinesis-blocked cultures cells and cells obtained from peripheral fluids or from solid tissues, chromosomal clastogenesis is often assessed, in studies involving whole-animals, as the sum of all nuclear abnormalities, since true MN are infrequent in fully differentiated cells. For such reason, determining ENA is one of the most common endpoints when surveying whole-blood in fish (see for instance Bolognesi and Hayashi, 2011). Due to the differences between the two biomarkers of DNA damage, i.e. at chromosome- and chain-level, several genotoxicity studies have been employing both ENA and Comet assays to the same cell populations (Andrade et al., 2004, Costa et al., 2008, 2011). Furthermore, their relationship and complementarity was reported by different authors for numerous organisms, even if higher sensitivity of the Comet assay has been reported (Costa et al., 2008).

Both carcinogenic and non-carcinogenic PAHs are ubiquitous in the aquatic environment as a result of discharges from industrial and urban sources, oil spills and atmospheric deposition (Meador et al., 1995). Due to their hydrophobic properties, PAHs tend to be adsorbed to suspended particles and to be trapped in sediments, especially if holding high percentages of fine particles and organic matter (Chen and White, 2004). Consequently, sediment may pose as a reservoir of pro-mutagenic substances such as PAHs (Canova et al., 1998). Sediment-bound genotoxicants and pro-mutagens can be transferred to the water column (e.g. via resuspension), affecting fish by through several toxicological pathways (Myers et al., 1991; Pinkney et al., 2004; Costa t al., 2008). Several *in vitro* bioassays have been developed to assess the genotoxic potential of sediment-bound genotoxicants. For example, fish cell lines have been successfully employed to assess the effects of sediment extracts (e.g. Kammann et al., 2004; Yang et al., 2010). On the other hand, *in* and *ex situ* bioassays deploying fish as target

organisms have been used to assess sediment-derived genotoxic effects. Peripheral blood is a convenient target and tends to reflect the global health status of the individual. Moreover, fish red blood cells are nucleated and therefore suitable for MN, ENA or SCGE assays (for instance Costa et al., 2008, 2011). Nevertheless, most of these studies neither deal with the mechanistic of toxicant interactions nor focuses on the differences between distinct PAHs, which properties ultimately modulate the adsorption or release from such an intricate matrix as aquatic sediments.

The present work aims at filling in the gaps the genotoxic potential of PAHs regarded as "carcinogenic" and" non-carcinogenic" to fish under realistic exposure scenarios, specifically, at ecologically-relevant concentrations, bound to sediments and considering interaction effects. Analysis was complemented with the determination of lipid peroxidation in blood plasma as a potential biomarker of oxidative stress. For the purpose, the "carcinogenic" benzo[b]fluoranthene and the "non-carcinogenic" phenanthrene were considered as model PAHs. Both PAHs are included in the list of priority substances and are usually present on contaminated sediments (e.g. Martins et al., 2012). Benzo[b]fluoranthene (B[b]F) consists of five fused aromatic rings and is considered a probable human carcinogen (thus belonging to IARC, group 2B). Phenanthrene (Phe), on its turn, is a three ring- PAH, neither regarded as a mutagen nor a carcinogen to humans (IARC, group 3). Still, toxic effects induced by Phe, like ROS production and impairment of immune functions, have been demonstrated in marine organisms (Yin et al., 2007; Hannam et al., 2010).

The European sea bass (*Dicentrarchus labrax* Linnaeus, 1758, Perciformes: Moronidae) was chosen as target organism for gathering paramount characteristics that render the species as an appealing surrogate for piscine wildlife. The species is a eurythermic coastal demersal species that often inhabits estuaries and other confined waters subjected to strong anthropogenic stressors. It is found in waters all around Europe, from the eastern Atlantic Ocean to the Mediterranean Sea and Black Sea. The species also holds high economic importance for fisheries and aquaculture, as well as high ecological value (being a top-chain predator). Furthermore, the species is known to be sensitive to PAHs and able to metabolize many of these compounds (Gravato and Santos, 2002; Ferreira et al., 2010), which greatly contributes to its potential as a sentinel.

2. Material and Methods

2.1. Animals

Juvenile hatchery-brood seabass, belonging to the same cohort (85.2 ± 8.5 mm standard length; 9.90 ± 2.31 total wet weight) were obtained from Maresa (Spain) and acclimatized in clean, aerated seawater

for 10 days prior to experimentation (water temperature was set at 19±0.2 ºC and photoperiod at 12/12h light/dark). Fish were fed once a day with commercial fish pellets (Aquasoja, Portugal).

Table 5.1. Target concentrations of Phe and B[b]F (ng g^{-1}) used for spiking the artificial sediments, and the respective Phe and B[b]F concentrations in water (ng L^{-1}) at 14 (T_{14}) and 28 (T_{28}) days of exposure to the nine experimental treatments.

			Bioassay								
			Control	**P1**	**P2**	**B1**	**B2**	**M1**	**M2**	**M3**	**M4**
Sediments	Target	Phe	0	86.7<C1<544	C2>544	0	0	86.7<C1<544	C2>544	C2>544	86.7<C1<544
(ng g^{-1})	concentrations	B[b]F	0	0	0	88.8<C1<763	C2>763	88.8<C1<763	C2>763	88.8<C1<763	C2>763
Water (ng L^{-1})	T_{14}	Phe	<2.8	45.7±1.2	201.6±1.5	6.7±0.9	7.3±0.8	62.4±1.2	348.7±2.0	392.1±2.2	69.9±2.3
		B[b]F	<13.8	16.5±1.1	<13.8	52.1±1.4	29.5±1.1	<13.8	22.1±1.0	16.3±1.0	40.6±2.1
	T_{28}	Phe	<2.8	23.12±1.3	62.7±1.0	2.7±0.9	<2.8	36.4±1.2	8.5±1.0	9.3±0.9	<2.8
		B[b]F	<13.8	13.3±1.3	<13.8	15.3±1.0	22.2±1.2	<13.8	<13.8	<13.8	22.8±1.2

2.2. Sediment preparation

Artificial sediment (containing 6% total organic matter, 42.2 % fine fraction), obtained by mixing clean sandy and muddy sediments from an uncontaminated estuary, the Mira, SW Portugal (see Martins et al., 2013 for specifics), was spiked with two different concentrations (here forth termed C1 and C2) of Phe and B[b]F, either isolated or combined, resulting in nine sediment assays (Table 5.1): control, Phe-C1, Phe-C2, B[b]F-C1, B[b]F-C2, and four mixtures (M1 to M4). The target PAH concentrations were selected according to available toxicity thresholds guidelines for coastal sediments, namely the Threshold Effects Level (TEL) and the Probable Effects Level (PEL), retrieved from MacDonald et al. (1996). In accordance, C1 concentration was targeted between TEL and PEL values, whereas C2 was intended to be above PEL. In absence of a specific guideline for B[b]F, the guidelines for benzo[a]pyrene were considered, due to the physicochemical similarities between the two carcinogens.

The sediment spiking procedure was based on Hickey and Roper (1992), Costa et al. (1998) and Martins et al. (2013). In brief: 2 L of sediment was spiked with appropriate aliquots of stock solutions of Phe (2500 µg mL^{-1} in DMSO) and B[b]F (1020 µg mL^{-1} in DMSO), in order to achieve the target concentrations of each sediment test. After 15 min of mechanical mixing, each sediment test was allowed to equilibrate for 48h at 4 ºC before the beginning of the bioassays. The control sediment was prepared similarly spiked with DMSO only. Both PAHs were obtained from Sigma (St Louis, MO, USA).

2.3. Bioassays

The 28-days bioassays were performed with two replicates per sediment test, making a total of 18 assays. Two liters of each sediment were allocated in 15L capacity tanks with blunt edges, to which was added 12L of filtered seawater. After 24h to allow sediments settling, ten randomly-selected fish were placed in each tank. To ensure the constancy of the water parameters (salinity = 31±1, pH = 7.8±0.2 and total ammonia = 1.5-2 mg L^{-1}), 25% of the total water volume was changed weekly. Water temperature, oxygen saturation, photoperiod and feed were set to mimic rearing conditions. Ten fish per assay (5 per replicate) were sampled at days 14 (T_{14}) and 28 (T_{28}) of exposure. Blood samples were immediately collected, from each individual, from the caudal with a syringe previously washed with EDTA (0.1 M) to prevent clotting. Fish collected at day 0 (T_0) consisted of 10 animals retrieved directly from rearing. Water samples were also collected at T_{14} and T_{28} for PAH analyses. The bioassay procedures followed the norms mandated by the Directive 2010/63/EU of the European Parliament and of the Council for Laboratory Animal Welfare. In accordance, facilities, researchers and research were credited and cleared by the Portuguese General Directorate for Veterinary.

2.4. PAH analyses in water

Phenanthrene and B[b]F levels were determined in water samples according to Martinez et al (2004) with modifications (Martins et al., 2013). In brief: water samples were spiked with a surrogate standard solution (from Supelco), vigorously mixed and percolated through speedisks previously conditioned with ethylacetate and methanol, using a Baker vacuum system (J.T. Baker, The Netherlands). PAHs were eluted from the speedisk with an ethylacetate/dicloromethane (v/v) mixture, under vacuum and the extracts were evaporated under nitrogen and reconstituted in *n*-hexane. Phe and B[b]F were quantified by gas chromatography-mass spectrometry (GC-MS) (Thermo DSQ) in selected-ion monitoring (SIM) mode and their identification was performed through the internal standard peak method (Martins et al., 2008).

2.5. Assessment of DNA damage

DNA damage was determined in fish peripheral blood by scoring erythrocytic nuclear abnormalities (ENA) and by the single-cell gel electrophoresis (SCGE or "Comet") assay, based on the protocol described by Costa et al. (2007, 2008). Immediately after collection, blood aliquots were either smeared on glass microscopy slides (followed by air-drying) for ENA analysis or diluted (1/100) in cold PBS (phosphate-buffered saline) for the Comet assay.

ENA analysis. After fixation in methanol for 15 min, the blood smears were stained with 0.1 g L^{-1} acridine orange (Sigma) for 30 min and mounted with DPX, as described by Costa and Costa (2007). At least 1000 mature, intact, erythrocytes were scored per individual. The criteria of the scoring of cells with nuclear abnormalities were based on Fenech et al. (2003), Costa and Costa (2007) and Bolognesi and Hayashi (2011).

Comet assay. Cell suspensions (20 uL) was diluted in 180 uL of melted (35-40 ºC) 1% (w/v) low-melting point agarose (LMPA, Sigma) prepared with PBS. Aliquots of the cell suspension (2×75 uL) were placed in glass microscopy slides previously coated with 1% (w/v) normal melting-point agarose (NMPA, Sigma) prepared in TAE buffer. After agarose solidification (15 min, 4 ºC, in dark), slides were dipped for 1 h in cold (4 ºC, in dark) lysis solution (2.64% NaCl w/v, 3.72% EDTA w/v and 5 mM TRIS) to which 10% (v/v) DMSO and 1% (v/v) Triton-X 100 were added just before use. DNA unwinding and enhanced expression of alkali-labile sites was promoted by immersing slides for 40 min, in electrophoresis buffer (pH 13). Electrophoresis was run during 30 min, at 25 V. Afterwards, the slides were neutralized in 0.1 N Tris-HCl buffer (pH 7.5) for 15 min. Approximately one-hundred random comets were analysed per slide using the CometScore (TriTek, VA, USA) software, after staining with ethidium bromide for 5 min. The percentage of DNA in tails was employed as metric.

A DMLB microscope adapted for epifluorescence with an EL6000 light source with mercury short-arc reflector lamps was used for ENA and Comet analyses, equipped with an I3 and an N2.1 filter (Leica Microsystems) for acridine-orange and ethidium bromide staining, respectively. Data from the ENA and Comet assay were expressed per individual as fold changes/variation coefficients (VC_{ENA}, VC_{TSB}) relativeto T_0 fish, as calibrator group, in order to normalize all biomarker data (Costa et al., 2011).

2.6. Plasma lipid peroxides analysis

Lipid peroxides were determined in plasma samples through the thiobarbituric acid reactive species (TBARS) assay described by Uchiyama and Mihara (1978) and adapted to a microplate reader by Costa et al. (2011). In brief, after blood centrifugation at 10 000 × g, for 10 min, 5 µL of plasma were diluted in 45 µL PBS (pH 7.5, 0.7% NaCl) to which was added 100 µL of ice-cold trichloroacetic acid (20% m/v). Following a 15 min incubation (4 ºC), samples were centrifuged for 15 min, at 2 200 × g and 100 µL of the supernatant was collected and added 100 µL of thiobarbituric acid (1%, m/v). Samples were then incubated for 15 min on boiling water. The absorbance of the reddish pigment was measured at 530 nm with a Benchmark model microplate reader (Bio-Rad). Quantification was performed through an eight-point calibration curve using malondialdehyde (MDA) from Merck, as standard. Plasma lipid peroxides are expressed as variation coefficient (VC_{TBARS}) relative to T_0 fish (Costa et al., 2011).

2.7. Statistical analysis

After the invalidation of the assumptions of normal distribution of data and homogeneity of variances, non-parametric statistic were employed, namely the Mann-Whitney *U* test for pairwise comparison between assays. Correlation based principal component analysis (PCA) was employed to rank the variables that contributed the most to explain the variation of responses within Phe and B[b]F be, singly or in binary mixtures, and evaluate potential links between variables. All statistics were computed using Statistica (Statsoft).

3. Results

3.1. Phe and B[b]F concentrations in water

The PAH concentrations in water (Table 5.1) obtained for isolated and mixed PAH bioassays presented similar variation in time, i.e., the higher levels were registered at T_{14}. Moreover, the assays prepared with C2 sediment concentrations yielded higher concentrations in water relative to C1

sediments. However, the Phe concentrations in water were always higher than B[b]F. In the control assay the levels were close to the detection limit of both PAHs.

3.2. ENA

Nuclear buds, lobed nuclei or a combination of both alterations (Fig. 5.1) were the most common erythrocytic nuclear abnormalities. The variation coefficients of the percentage of ENA (VC_{ENA}) for isolated and combined Phe and B[b]F bioassays are presented in Fig. 5.2. During the single PAH assay, exposures to B[b]F-spiked sediments (B1 and B2) yielded a significantly higher (Mann-Whitney *U*, $p < 0.5$) percentage of ENA-bearing cells than Phe and control assays at both 14 and 28 days of exposure (Fig. 5.2A). Also, both concentrations of B[b]F in sediments induced a similar proportion of cells exhibiting ENA in fish peripheral red blood cells, without evidence of variation with time. On the other hand, isolated exposure to the Phe (P1 and P2) and control (C) sediments did not cause a significant increase in ENA-bearing cells relative to T_0 with the exception of P1at T_{14} and P2 at T_{28}. Regarding the combined assay (Fig. 5.2B), all tests exhibited significantly higher percentage of mature erythrocytes with nuclear abnormalities to control (Mann-Whitney *U*, $p < 0.5$). In general, all tests elicited highest VC_{ENA} at day 14 (T_{14}). The mixtures containing higher B[b]F concentrations (M2 and M4) yielded greater clastogenic/aneugenic effects at T_{14}, however, at T_{28}, VC_{ENA} decreased significantly (Mann-Whitney *U*, $p < 0.5$).

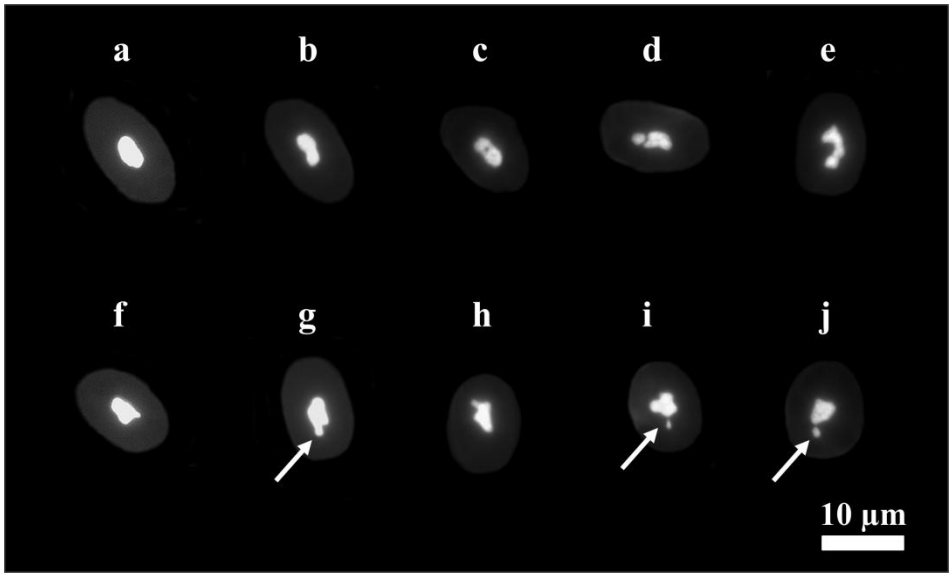

Fig. 5.1. Mature blood cells exhibiting erythrocytic nuclear abnormalities in *D. labrax* exposed to spiked sediments. (a) normal mature erythrocyte; (b-e) common nuclear abnormalities in mature erythrocytes: (b) lobed nucleus, (c) fragmenting nucleus, (d) fully fragmented nucleus (binucleated cell); (e) cell exhibiting combination of multiple abnormalities; (f-j) different stages of micronucleus formation: (f-h) lobed nucleus with nuclear bud (arrow), (i) lobed nucleus with nucleoplasmic bridge (arrow), (j) micronucleus (arrow).

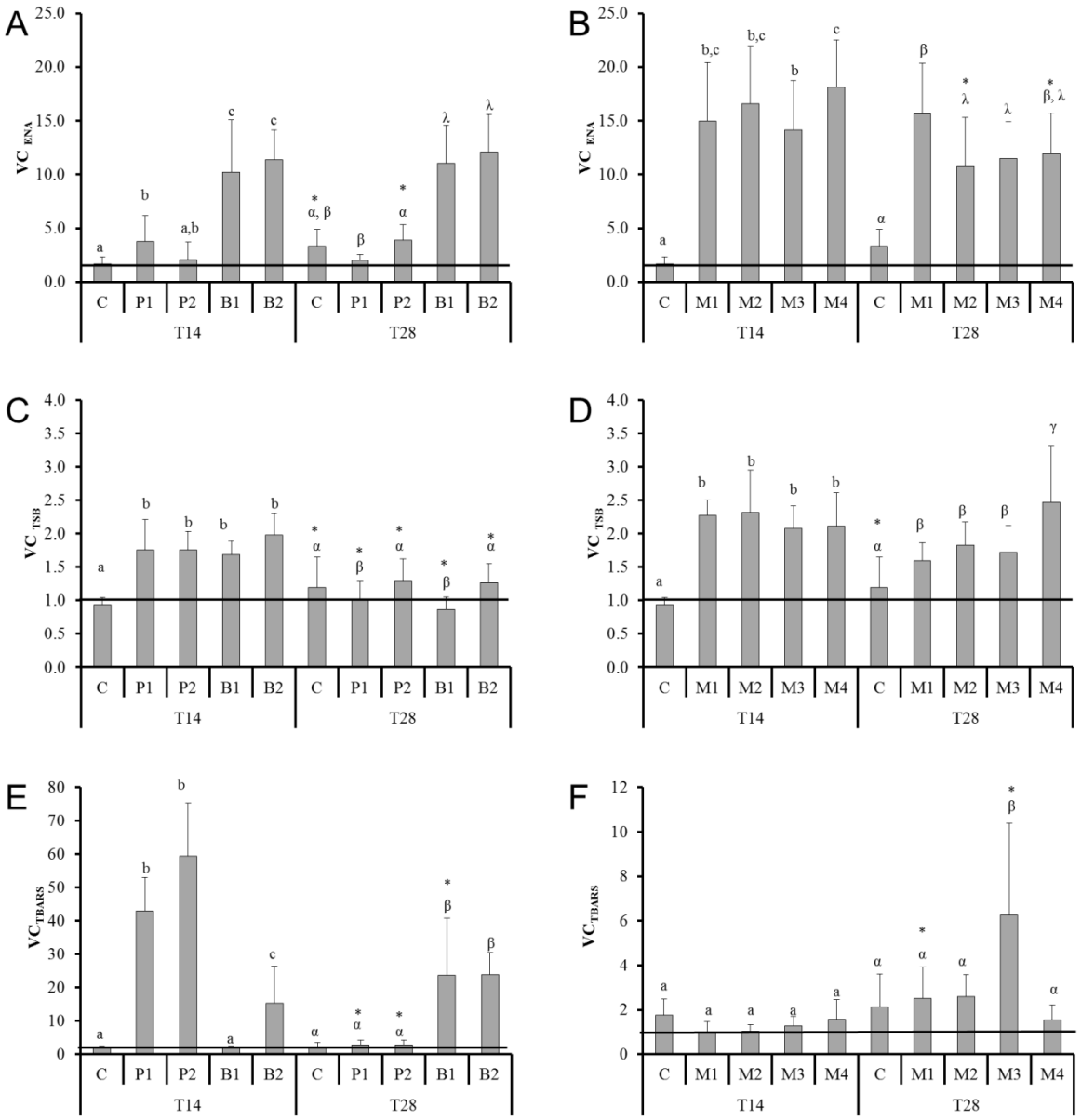

Fig. 5.2. Variation coefficients (VC) relative to T_0 animals exposed to singly and binary combination Phe and B[b]F contaminated sediments: A and B) VC of the percentage of mature red blood cells exhibiting erythrocytic nuclear abnormalities (VC_{ENA}) in fish exposed to isolated or combined PAHs, respectively; C and D) VC of the total DNA strand breakage (VC_{TSB}) in blood; E) and F) VC of lipid peroxides (VC_{TBARS}) in blood plasma. Different letters (latin and greek) indicate significant differences (Mann-Whitney *U*, $p < 0.05$) within fish collected during the same sampling time, i.e., T_{14} or T_{28}; respectively; * mean significant differences (Mann-Whitney *U*, $p < 0.05$) between sampling times (T_{14} versus T_{28}). Error bars indicate the standard deviation. C-control assay; P1 and P2 - Phe concentration 1 and 2 assay, respectively; B1 and B2 - B[b]F concentration 1 and 2 assay, respectively.

3.3. Comet assay

The comet examples are presented in Fig. 5.3. Exposure to sediments spiked with the single compounds yielded a significant increase (Mann-Whitney *U*, $p < 0.5$) in total DNA-SB, at $T_{14,}$ relative

to controls, which was not observed for animals collected at T_{28} (Fig. 5.2C). However, no clear compound- or dose-response effect was observed for either PAH. At T_{28}, no significant differences in variation was observed between control or Phe and B[b]F sediment exposures. In contrast, exposure to sediments spiked with mixtures of the two PAHs yielded significant differences to controls at both sampling times (Fig. 5.2D). However, while at T_{14} no significant differences were observed between treatments, at T_{28}, the highest increase in DNA-SB relative to T_0 animals occurred in fish exposed to mixture M4 (≈ 2.5 fold), significantly distinct from the remaining treatments and controls.

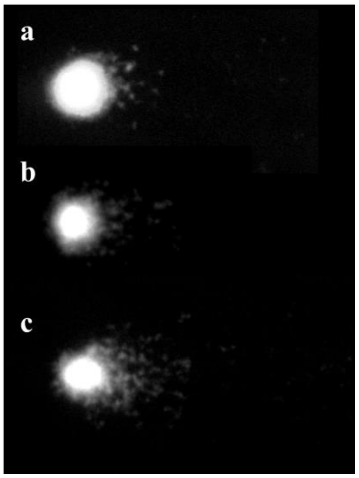

Fig. 5.3. Comet examples from tested fish: ≈ 0% (a), ≈ 20% (b) and ≈ 60% (c) DNA-SB.

3.4. Lipid peroxidation

Exposure to phenanthrene-spiked sediments (P1 and P2) yielded the highest increase in plasma lipid peroxides (determined as TBARS), up to ≈70 fold, which was not observed at T_{28}, where only fish exposed to either concentrations of B[b]F promoted significant differences compared to control (Fig. 5.2E). The bioassays with PAH mixtures failed to reveal significant differences to controls, with the exception of mixture M3 at T_{28}, i.e., the combination between the highest concentrations of Phe and lowest of B[b]F (Fig. 5.2F).

3.5. Statistical integration of data

Principal component analysis (PCA) integrating the variation coefficients for ENA, TSB and TBARS plus assay conditions (time, Phe and B[b]F concentrations in water) obtained for the assays with C1 (Fig. 5.4A) and C2 (Fig. 5.4B) concentrations of either isolated PAH or with the combined PAHs (Fig. 5.4C) produced two main factors that explained 77.8, 81.3 and 80.4% of the total variance, respectively. Concentrations of PAHs in water were included instead of concentrations in sediments

for being potentially the most indicative of bioavailability. The most important contributors in isolated exposures to C1 sediment concentrations were VC_{TBARS} (relative contribution = 0.36 in factor 1) and VC_{TSB} (0.61 in factor 2). However, VC_{TBARS} and VC_{ENA} were consistently linked with Phe and B[b]F concentrations in water, respectively., unlike VC_{TSB} and time of exposure, which yielded, inclusively, opposite trends. Similar results were obtained for the model encompassing C2 concentrations of either PAH, albeit a more conspicuous link between Phe concentrations in water, VC_{TBARS} and VC_{TSB}. However, the highest relative contribution for factor 2 was obtained by VC_{ENA} (0.70).

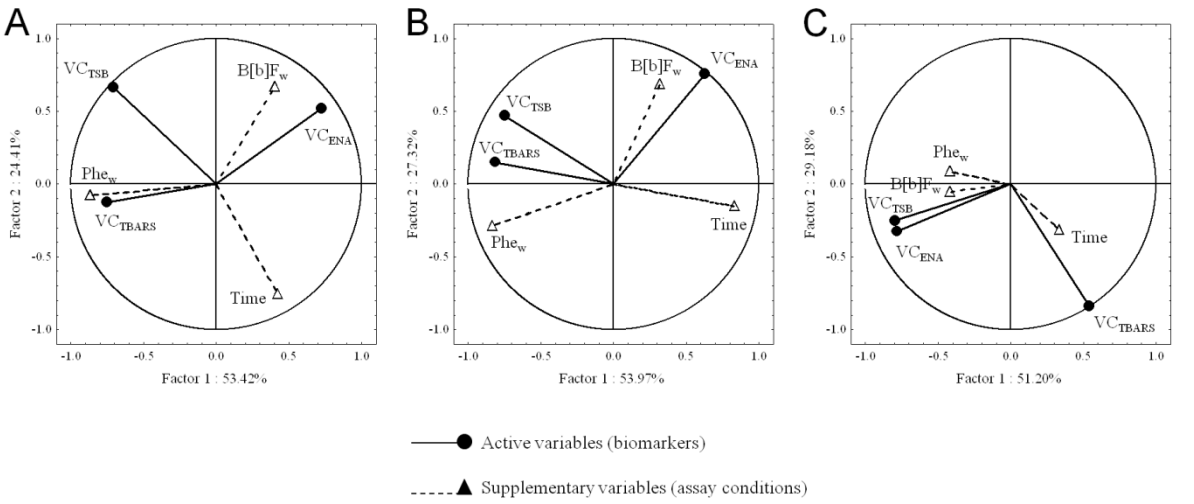

Fig. 5.4. Plot of the principal component analysis (PCA) results for VC_{ENA}, VC_{TSB} and VC_{TBARS} (variation coefficients of erythrocytic nuclear abnormalities, total DNA strand breakage and plasma lipid peroxides, respectively) plus the assay conditions, time, Phe and B[b]F concentrations in water (indicated as Time, Phe_w and $B[b]F_w$, respectively). A) C1-Phe and B[b]F isolated sediment assays (factor 1 eigenvalue=1,60 ; factor 2 eigenvalue=2,33); B) C2-Phe and B[b]F isolated sediment assay (factor 1 eigenvalue= 1,62 ; factor 2 eigenvalue=2,44); C) Phe and B[b]F combined sediment assay (factor 1 eigenvalue= 1.54; factor 2 eigenvalue=2.41). Only the most significant PAC factors are shown (factor 1 and 2). The percentages for each PCA factor indicate the % total explained variance.

Distinct results were retrieved from the model including PAH mixtures. In this model, PAH concentrations in water lost relative significance in the overall model, whereas VC_{TSB} and VC_{ENA} attained now the highest relative contributions in factor 1 (0.41 and 0.40, respectively). Additionally, these two variables were seemingly more correlated while depicting a clear link between PAH concentration in water. Conversely, VC_{TBARS}, albeit a significant variable in the model (relative contribution = 0.80 for factor 2) failed to exhibit a clear trend with the remaining variables, excluding time of exposure.

4. Discussion

The present work demonstrated that sediment-bound PAHs with distinct toxicological and chemical characteristics may yield significant genotoxic effects at ecologically-relevant concentrations. However, the two PAHs, when isolated, cause distinct patterns of DNA lesions, with respect to intensity or type of damage, being significantly modulated by the duration of exposure. On the other hand, the findings from the combination assay revealed a supra-additive enhancement of damage to the genetic material. Nonetheless, this increase, when compared to exposure to the isolated compounds, diluted the effects of each individual compound. It must also be noted that the most significant genotoxic effects were related to chromosomal clastogenic/aneugenic effects as revealed by the erythrocytic nuclear abnormalities assay, which attained up to a ≈ 20-fold increase whereas for DNA-SB only an approximate 3-fold increase in damage was reached relatively to controls (in either case, during the mixture assay). Also, there were no significant differences between DNA strand breaks elicited by either individual PAH, regardless of concentration. Additionally, such genotoxic effects were higher at earlier stages of exposure. This confirms that clastogenic/aneugenic events are indeed the most significant effects caused by exposure to the moderate concentrations of the substances and indicates some ability to cope with DNA-SB along the duration of exposure. Since whole-chromosome damage is unlikely to be repaired, this may indicate higher risk of occurring mutagenesis through the fixation of DNA lesions and, consequently, the increase in the probability of anaplasms/neoplasms being formed upon more prolonged exposure. As such, the present findings disclose that low-moderate concentrations of sediment-bound PAHs may very significantly increase the risk of the incidence of mutagenesis and hinder the interpretation of fundamental genotoxicity biomarker data by diluting time- and dose-response effects.

It is well known that PAHs are strong genotoxic agent, even though their DNA-damaging effects are caused by metabolites and not by the parent compounds *per se* (Xue and Warshawsky, 2005). Their genotoxic action follows PAH activation by the microsomal MFO system, rendering highly reactive, more hydrophilic metabolites and reactive oxygen species (ROS) as by-products. These metabolites potentially attack the DNA molecule, especially by forming bulky adducts (Pavanello et al., 2008), while nucleobase oxidation by action of ROS may also occur (Penning et al., 1996). The carcinogenic potential of PAHs results from the fixation of unrepaired DNA damage, leading to mutation in pro-oncogenes and tumor suppressor genes. On the other hand, accumulation of ROS and excessive DNA damage may lead to cell death, promoting inflammation, for which there is a known link to tumourigenesis by increasing cell proliferation (Sarasin, 2003). Three pathways of PAH activation have been described (Jin and Penning, 2007), involving the formation of: i) radical cations (P450-peroxidase derived), ii) diol-epoxides (P450-epoxide hydrolase derived) and iii) redox active *0*-quinones [Aldo-keto reductases (AKR) derived].

Bioactivation of PAHs and substances with similar properties (toxicants or not) is mainly controlled by a transcriptional feedback loop termed the aryl hydrocarbon receptor (AHR) pathway. Ligands like PAHs bind to available, cytosolic, AHR [(bound to two heat-shock 90Kda proteins (HSP90)]. The complex then enters the nucleus (loosing then the HSP90 chaperones) and forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT). It is this heterodimer that bind to the xenobiotic response element (XRE) of specific genes (such as CYP1A), enabling transcription. As such, more ligands imply induction of CYP MFOs, potentially increasing the ability of the cell to metabolize the substance. As such, elevated biosynthesis of active CYP enzymes associated to CYP-metabolizable PAHs (i.e. substrate) may increase the production of genotoxic PAH-metabolites and therefore the potential risk of elicited neoplastic disease (refer to Nebert et al., 2004, for a review).

However, the metabolization of PAHs varies with each PAH physicochemical properties. For example, a study that investigated the induction of CYP1A1 and CYP1A2 enzyme activity in human cell lines demonstrated that molecules with aligned rings are less able to induce CYP1A, whereas molecules with clustered rings, like 5-ring PAHs, can more easily bind to AHR receptor and be transported into the nucleus. Moreover, the bay-region of the PAH molecule is considered to be an important factor to promote CYP1A induction, since it confers higher affinity to the AHR active binding site (Skupinska et al., 2007). The different PAHs also yield distinct metabolites, with respect to their genotoxic potential. It is generally acknowledged that PAH that results in diol epoxides (such as B[a]P and other bay-region, higher molecular weight PAHs) are stronger pro-mutagens and carcinogens (Wogan et al., 2004).

Phenanthrene is a known CYP substrate and represents the simplest angular PAH forming a bay-region (Buening et al, 1979; Wood et al., 1979). However, when compared to the corresponding bay-region of diol-epoxides from 5-ring PAHs, such as B[b]F or BaP (considered carcinogenic PAH), the bay-region diol-epoxides of phenanthrene exhibit low or null tumorigenic activity in mice, possibly due to poor ability to form DNA adducts (Wood et al., 1979). This may aid explaining the low clastogenic/aneugenic effecs caused by sediment-bound Phe, in contrast with the relatively high frequencies of erythrocytes exhibiting ENA in fish exposed to B[b]F (Fig. 5.2A). In fact, the increase in the frequency of erythrocytes with nuclear abnormalities has already been associated with higher mutation rates and clastogenicity (Michelmore and Chipman, 1998; Baršienė et al., 2006). Moreover, a link between carcinogenic PAH metabolites, DNA adducts and micronuclei has already been established, at least in humans (Pavanello et al. 2008). It must be noticed that, although far less studied than its model counterpart (B[a]P), B[b]F holds many similar properties to bay-region high molecular weight PAHs, hence its classification as a carcinogen to wildlife and potential carcinogen to humans. Still, this compound has already been found to form hepatic PAH-DNA adducts in freshwater fish, (Erickson and Balk, 1999), which indicates the ability to trigger DNA lesions.

The differences in the patterns of DNA damage observed between the two PAHs during the present study are corroborated by the PCA analysis (Fig. 5.4A and 5.4B), which revealed Phe as a more significant inducer of DNA-SB, while exposures to B[b]F was consistently correlated with the formation of ENAs, following exposure to ecologically-relevant concentrations of the sediment-bound PAHs that should, at least theoretically, be regarded as equitoxic. Nonetheless, the findings did not indicate the existence of significant dose-response effects for either case, likely due to the relatively low concentrations tested. Still, the results suggest distinct genotoxic mechanisms underneath exposure to PAHs that are reckoned to hold linearly opposite effects as environmental carcinogens. These differential mechanism are most likely related to the ability to induce genotoxic metabolites able to form bulky adducts with DNA and organisms' ability to cope with DNA lesions and oxidative stress, the latter of which is a critical consequence of exposure to this class of pollutants.

It is already known that Phe metabolism causes DNA strand breakage by action of ROS (e.g. Sun et al., 2006; Yin et al., 2007). In fact, the production of hydroxyl radicals, one of the most potent nucleobase oxidating agents (see Cadet et al., 2010, for a review), has already been found to be induced by phenanthrene in *Carassius auratus* (Sun et al., 2006). Therefore, production of oxidative radicals may explain, at least in part, the more significant increase in DNA-SB. It is possible that exposure to Phe, isolated, promoted DNA-SB through oxidative stress-related processes whereas B[b]F acted as a clastogenic agent, mainly through the formation of PAH-DNA adducts, while causing DNA-SB as well, via adducts and direct chain oxidation, originating fragments and relaxed DNA loops measurable through the Comet assay (Frenzilli et al., 2009). Also, mis- or unrepaired DNA-SB faulty mitosis may occur, originating chromosome and chromatid fragments and aneugenic events (see Fenech et al., 2011). By their turn, the variation of blood plasma lipid peroxides yielded inconclusive results for the majority of treatments, with the exception for exposure to Phe for 14 days, which is accordant with the notion that this PAH may exert toxic effects primarily by inducing oxidative radicals, which is accordant with the PCA findings (Fig. 5.4A). The absence of a clear relationship between lipid peroxidation and the remaining treatments (including mixtures) likely indicates that i) plasma TBARS may not be an entirely efficient indicator of oxidative stress and ii) oxidative stress is modulated by complex mechanisms that involve a complex battery of enzymatic and non-enzymatic defences that may result, inclusively, in hormetic responses (see Costa et al., 2011). It must also be emphasized that besides no obvious dose-response effects were observed regarding either biomarker for both substances, no time-dependent effects were noted for ENA, whereas strand breakage was reduced at the end of the experiments. These findings indicate that adaptation and DNA repair significantly modulated DNA-SB as measured by the standard alkaline Comet assay. On the other hand, chromosome-level damage is unlikely to be repaired, thus demonstrating the higher mutagenic risk of B[b]F compared to Phe.

Pathways of DNA repair, such as base and nucleotide excision repair (BER and NER, respectively) have been identified in fish (Kienzler et al., 2013). While BER is the major mechanism for repairing oxidative DNA base lesions and single strand breaks, NER is the main repair system for DNA cross-links and bulky adducts generated from exposure to compounds such as PAHs. However, previous experiments with fish exposed to genotoxicant highlighted the persistence of bulky DNA adducts and slower NER than mammals (Bailey et al., 1996). Such weaker NER capability makes fish prone to accumulate highly mutagenic DNA adducts, which may contribute to explain the relatively high incidence of clastogenic/aneugenic events in erythrocytes of fish exposed to B[b]F and to the mixture of both compounds.

In contrast to the single PAH assays, when Phe and B[b]F were combined, a supra-additive genotoxic effect was observed at both nucleobase and whole-chromosome levels. As a consequence, the concentrations of both PAHs in water (which should constitute the most bioavailable fraction) were hitherto linked with DNA-SB and ENA results (Fig. 5.4C), albeit without a clear dose- or time-dependent trend. In fact, studies indicated that lower concentrations of PAHs (e.g. below 0.5 g/ml) may cause genotoxicity in an additive or slightly less than additive trend (White, 2002). Also, in the current work, the combination of two distinct PAHs, both at low, realistic, concentrations likely contributed to the dilution of dose- and time-dependent responses from the mixture assays. Still, another probable explanation for the modulation of genotoxic effects caused by the combined PAHs is the agonistic action of PAHs toward the AHR pathways. It must be highlighted that B[b]F has a bay-region and higher length/width ratio that, comparatively, provides higher affinity for AHR than Phe. The higher affinity of B[b]F towards AHR renders this PAH more efficient to promote CYP induction through positive transcriptional control. As a consequence, increased CYP transcription may raise Phe activation and the subsequent production of Phe metabolites and ROS. Moreover, it must be noted that Phe and B[b]F hold differential stereoselectivity for distinct CYP isoenzymes, with consequences for the production of metabolites with different reactivity towards the DNA molecule (see Pangrekar et al., 2003). As such, the combination of the two toxicants may induce the activity of a wider array of CYP MFOs, therefore increasing the range of PAH metabolites types for either compound (especially those with higher ability to form adducts), which could have contributed to increase the genotoxicity of Phe in presence of a strong CYP inducer such as B[b]F.

5. Concluding remarks

Sediments are the most important reservoir of carcinogenic PAHs in the aquatic milieu. Depending on their geochemical properties and oxic/anoxic shifts, PAHs may be returned to pore waters and to the water column, becoming more bioavailable to fish. The current study showed that concentrations of

individual PAHs, in sediments, judged to pose low risk increase the hazard of occurring severe genotoxic effects at the whole-chromosome level, even if and when animals seemingly have not exceeded their abilities to cope with DNA strand breakage and oxidative stress. Moreover, if such effects could indeed be expected for a higher molecular weight PAH, considered to be carcinogenic to wildlife (B[b]F), its combination with a non-carcinogenic PAH, in the case, phenanthrene, clearly increased risk of occurring unrepairable DNA damage, therefore augmenting the possibilities of occurring mutagenesis. Considering that PAHs are present in the environment as mixtures the concept of carcinogenic risk for these substances should be redefined even in scenarios where low concentrations, routes of exposure and realistic toxicant vehicles (as aquatic sediments) impair the recognition of clear-cut dose-effect relationships.

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CHAPTER 6. EFFECTS OF CARCINOGENIC *VERSUS* NON-CARCINOGENIC AHR-ACTIVE PAHS AND THEIR MIXTURES: LESSONS FROM ECOLOGICAL RELEVANCE†

† Martins et al. (submitted).

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are priority environmental mutagens and carcinogens that occur in the environment as mixtures rather than the individual compounds for which guidelines are issued. The present work aimed at the interaction pathways between carcinogenic and non-carcinogenic PAHs in the marine fish *Dicentrarchus labrax*. Laboratory assays under ecologically-relevant parameters were conducted for 28 days with sediments spiked with low-moderate concentrations (250-800 ng g^{-1}) of two model PAHs, phenanthrene (non-carcinogenic) and benzo[b]fluoranthene (carcinogenic to fish). Both PAHs induced hepatic histopathological changes that indicate metabolic failure and inflammation, especially in animals exposed to mixtures. Phenanthrene elicited biochemical changes better related to oxidative stress (lipid peroxidation, glutathione and glutathione *S*-transferase activity) and CYP function, whereas B[b]F disrupted metabolic responses and defences to toxicological challenge. Conversely, mixed PAHs yielded lesions and responses that, altogether, are compatible with the AHR dependent pathway (the basis of PAH mutagenicity), generating a supra-additive effect. Nonetheless, the low, ecologically-relevant, concentrations of PAHs diluted dose and time-response relations. Overall, although seemingly predicting the risk of individual PAHs, environmental guidelines may not apply to mixtures by underestimating adverse effects, which calls for a redefinition of standards when determining the true risk of toxicants under realistic scenarios.

Keywords

Aryl hydrocarbon receptor; oxidative stress; histopathology; Phenanthrene; Benzo[b]fluoranthene; *Dicentrarchus labrax.*

1. Introduction

Organic xenobiotics are a potential threat to humans and the environment, especially the polycyclic aromatic hydrocarbons (PAHs) which are known to have immunotoxic, teratogenic, clastogenic and carcinogenic properties (Miller and Ramos, 2001; Gangar et al., 2010; Yang et al., 2010). Owing to their toxicity and ubiquity in the environment, PAHs are regarded as priority pollutants, some of which being classified as carcinogenic to humans (IARC, 2012). As such, many PAHs, especially those of higher molecular weight, generally considered as holding higher mutagenic risk, are included in the list of priority substances of the European Water Framework, U.S. Environmental Protection Agency (USEPA) and the World Health Organization (WHO).

Polycyclic aromatic hydrocarbons are commonly present in the environment as mixtures. The individual compounds may hold very distinct mechanisms of toxicity, regardless of chemical similarities among the class (Mayer and Reichenberg, 2006). However, environmental quality guidelines have been invariably issued to single compounds (Altenburger and Greco, 2009). Moreover, ecological realism is often neglected in studies dealing with the toxic effects of PAHs, whether it concerns interaction effects, realistic target subjects, concentrations or toxicant vehicle. Altogether, the standard approaches may lead to a misinterpretation of risk under realistic scenarios. For instance, the tendency of both non-carcinogenic and carcinogenic PAH to induce (or suppress) the CYP activity may directly influence metabolic activation of other potentially carcinogenic PAHs (Jarvis et al., 2014).

The carcinogenicity of PAHs has been attributed to the role of CYP-mixed-function oxygenases (MFOs) in the metabolic activation of the compounds, yielding highly reactive intermediates, such as quinones and diol-epoxides while generating reactive oxygen species (ROS) (Conney, 1982, Stegeman and Lech, 1991; Ohnishi and Kawanishi, 2002). These metabolites can damage DNA, e.g. through formation of bulky adducts, along with oxidation by ROS, generating fixed mutations if not repaired (Cavalieri and Rogan, 1995; Baird et al., 2005). Moreover, PAHs may mediate the induction of CYP MFOs through the aryl hydrocarbon receptor (AHR) pathway, a ligand-activated transcription factor found in vertebrate species from fish to humans (Schmidt and Bradfield, 1996). Hence, AHR agonists trigger a positive feedback loops, potentially increasing the ability of cells to metabolise PAHs, and thus the production of genotoxic metabolites (Nerbert et al., 2004). Despite the higher affinity of five-ring PAHs to AHR, the differences between toxicity mechanisms of carcinogenic and non-carcinogenic PAHs are largely unknown, as are the interaction effects between individual compounds.

Both carcinogenic and non-carcinogenic PAHs are present in the aquatic environment as a result of petrogenic and pirolytic sources (Meador et al., 1995). Due to their hydrophobicity, PAHs tend to be

sorbed to fine particles and organic matter, thus becoming easily trapped in the complex geochemical matrix that characterizes aquatic sediments (Narbonne et al., 1999). Therefore, sediments with higher organic matter and fine particle contents, like estuarine sediments, are important reservoirs for PAHs, whose bioavailability depends on multiple factors that break the sediment' steady state, such as oxic/anoxic shifts during disturbance (Eggleton and Thomas, 2004). Moreover, sediment quality guidelines are poorly representative of the real environmental risk, since they are drawn for a single compounds and do not integrate the effects of complex sediment contaminant mixtures (Chapman, 1990). This, when linked to ecologically-relevant low-moderate concentrations of PAHs in sediments, hinders the interpretation of adverse effects to biota.

The aim of the present work was to assess the mechanisms of toxicity of carcinogenic and non-carcinogenic PAHs in a fish, the European sea bass (*Dicentrarchus labrax* L. (Perciformes: Moronidae), as a surrogate for representative estuarine vertebrates, which are known to hold a well-developed MFO system (Uno et al., 2012, for a review). Specifically, it was aimed at comparing metabolic effects and responses of two model PAHs, isolated and combined, taking sediments as the toxicant vehicle and under ecologically-relevant concentrations. For the purpose, two priority PAHs, common in aquatic sediments were considered: the "carcinogenic" benzo[b]fluoranthene (B[b]F) and the "non-carcinogenic" phenanthrene (Phe). The former, a high molecular weight PAH, composed of five rings, is classified as possible carcinogenic to humans (IARC, group 2B), and its genotoxicity and carcinogenicity have been demonstrated in clams (Martins et al., 2013) and rats and mice skin (Weyand et al., 1993), respectively. On its turn, phenanthrene, a lower molecular weight PAH, with three-benzenoid rings, although regarded as non-carcinogenic to humans (IARC, group 3), has been shown to cause genotoxicity (Oliveira et al., 2007; Martins et al., 2013) and oxidative stress (Yin et al., 2007; Oliveira et al., 2008; Hannam et al., 2010) in aquatic organisms.

2. Materials and methods

2.1. Exposure of Dicentrarchus labrax to sediment-bound PAHs

Artificial sediment (6% total organic matter, 42.2 % fine fraction) was spiked with two different concentrations (termed C1 and C2) of Phe and B[b]F, singly or combined, resulting in nine sediment assays (Table 6.1): control, Phe-C1, Phe-C2, B[b]F-C1, B[b]F-C2, M1, M2, M3, M4. The PAH target concentrations were selected taking into account their toxicity thresholds guideline, namely the Threshold Effects Level (TEL) and the Probable Effects Level (PEL), retrieved from MacDonald et al. (1996). In accordance, C1 concentration was targeted between TEL and PEL values, whereas C2 was

Table 6.1. Target Phe and B[b]F concentrations (ng g^{-1}) used for spiking the artificial sediment, and Phe and B[b]F effective concentrations in the beginning (T_0) and end (T_{28}) of the nine bioassays. Phe and B[b]F water concentrations (ng L^{-1}) measures in all bioassays at T_{14} and T_{28}.

			Bioassay								
			Control	**Phe-C1**	**Phe-C2**	**B[b]F-C1**	**B[b]F-C2**	**M1**	**M2**	**M3**	**M4**
Sediments (ng g^{-1})	Target concentrations	Phe	0	86.7<C1<544	C2>544	0	0	86.7<C1<544	C2>544	C2>544	86.7<C1<544
		B[b]F	0	0	0	88.8<C1<763	C2>763	88.8<C1<763	C2>763	88.8<C1<763	C2>763
	T_0	Phe	12.3±1.6	383.8±27.4	917.2±53.5			382.3±1.5	850.1±17.7	889.8±137.4	326.4±1.3
		B[b]F	28.4±1.2			267.3±24.5	981.9±39.2	328.6±32.6	1266.6±83.6	289.0±54.7	1111.9±15.2
	T_{28}	Phe	11.9±1.0	189.2±23.0	528.9±17.0			216.3±5.6	485.6±24.9	517.4±42.1	229.2±33.1
		B[b]F	28.3±1.3			265.1±43.6	964.5±42.8	254.7±7.1	816.0±44.0	281.5±30.8	920.6±22.4
Water (ng L^{-1})	T_{14}	Phe	<2.8	45.7±1.2	201.6±1.5	6.7±0.9	7.3±0.8	62.4±1.2	348.7±2.0	392.1±2.2	69.9±2.3
		B[b]F	<13.8	16.5±1.1	<13.8	52.1±1.4	29.5±1.1	<13.8	22.1±1.0	16.3±1.0	40.6±2.1
	T_{28}	Phe	<2.8	23.12±1.3	62.7±1.0	2.7±0.9	<2.8	36.4±1.2	8.5±1.0	9.3±0.9	<2.8
		B[b]F	<13.8	13.3±1.3	<13.8	15.3±1.0	22.2±1.2	<13.8	<13.8	<13.8	22.8±1.2

intended to be above PEL (Table 6.1). In the absence of guideline available for B[b]F, the guideline for benzo[a]pyrene was considered, allowing for the physicochemical similarities between the two carcinogens. The TEL and PEL sediment quality guidelines for Phe and B[a]P were 86.7 and 88.8 ng $g^{-1}$and 544 and 763 ng g^{-1}, respectively (refer to MacDonald et al., op. cit.).

To each sediment test, 2L of sediment were spiked with stock solutions of Phe (2500 g mL^{-1} in DMSO) and B[b]F (1020 g mL^{-1} in DMSO), mechanically mixed during 15 min, placed in a 15-L capacity tank and allowed to equilibrate for 48h, at 4ºC (Hickey and Roper, 1992; Costa et al., 1998; Martins et al., 2013). Control tests were prepared similarly, although spiked with DMSO only. The bioassays were performed in duplicate and were prepared by adding 12 L of filtered seawater to each sediment tank (two tanks per sediment test). After 24h to allow sediment settling, two hundred hatchery-brood *Dicentrarchus labrax* (juveniles), obtained commercially (MARESA, Spain), all from the same cohort (85.2 ± 8.5 mm standard length; 9.90 ± 2.31 total wet weight) were randomly distributed, with 10 animals being allocated per tank. The 28-day bioassays were performed with constant aeration, 25% of the total water volume being changed weekly. Water parameters (monitored daily) were within the rearing conditions (temperature = 19.0±0.2 ºC, salinity = 31±1, pH = 7.8±0.2, dissolved oxygen between 90-94%, and total ammonia = 1.5-2 mg L^{-1}). Commercial fish pellets (Aquasoja, Portugal) were provided to fish once a day. Photoperiod was set at 12 h light: 12 h dark. After 14 (T_{14}) and 28 (T_{28}) days of exposure, five fish per replicate were collected from each tank. All animals were measured for total weight (ww_t) and standard length (L_s). The liver of each fish was excised and divided for biochemical (stored at -80 ºC) and histological analyses. Water and sediment samples were also collected (only water at T_{14}) and stored at -20 ºC for PAH analyses.

2.2. PAH analyses

Sediment samples were oven-dried (37 ºC) and extracted by using an accelerated solvent extraction apparatus, ASE 200 (Dionex, USA) with an acetone/hexane (v/v) mixture followed by fractionation with a silica/alumina (g/g) glass column (Martins et al., 2008). Water samples were percolated through speedisks (J.T. Baker) previously conditioned with ethylacetate and methanol, using a Baker vacuum system (J.T. Baker, The Netherlands) and PAHs were eluted from the speedisk with an ethylacetate/dicloromethane (v/v) mixture, under vacuum, as described by Martins et al. (2013).

Identification and quantification of PAHs was performed on a Thermo DSQ gas chromatography-mass spectrometry (GC-MS) system in selected-ion monitoring (SIM) mode (Martins et al., 2008). The identification of Phe and B[b]F was performed through the internal standard peak method, using deuterated standards containing phenanhrene-d_{10} and perylene-d_{12} (Supelco, Sigma), following a 9-point calibration curve. Quality control was assessed using sediment reference material, SRM1941b

(NIST, USA) and spiked water analyses yielding recoveries between 80-120% and 86-102%, respectively.

2.3. Histological analyses

Liver samples from each fish were processed for histological analyses according to Martoja and Martoja-Pierson (1967). In brief: samples were fixed in a Bouin-Hollande's solution for 48 h (at room temperature). Afterwards, samples were dehydrated in a progressive series of ethanol and intermediately infiltrated in xylene and embedded in paraffin. Sections of 5 µm thickness were stained with haematoxylin and counterstained with alcohol eosin (H&E stain). The slides (prepared in duplicate for each liver sample) were allowed to dry and were mounted with DPX resinous medium (from BDH, Poole, England) for further optical microscope analysis. DMLB model microscope equipped with a DFC480 digital camera (Leica Microsystems) was used for microscopy observations. A blind review of slides was performed at the end of analyses in 25% of the samples to confirm accuracy of observations, the average error between reviews being ≈ 12% or less.

Table 6.2. Histopathological alterations (biomarkers) observed in the liver of *D. Labrax* and their respective condition weights.

Reaction pattern	Histological alterations	Weight
1. Circulatory disturbances/Inflammatory response	Haemorrhage	1[a]
	Hyperaemia	1[b]
	Macrophage infiltration	2[a]
2. Regressive	Hepatocyte necrosis	3[a]
	Bile duct atrophy	2[a]
	Nuclear pleomorphisms	2[a]
	Apoptosis	2
3. Progressive	Fat vacuolation/lipidosis	1[b]
	Microvesicular fat vacuolation/steatosis	1
	Fibrosis	2[c]

[a] Weights according to Bernet et al., 1999.
[b] Weights according to Costa et al., 2009b.
[c] Weights according to Costa et al., 2013.

2.4. Histopathological condition indices

Hepatic histopathological alterations were divided into three reaction patterns (Table 6.2), namely circulatory disturbances, regressive alterations (implying reduction/loss of function) and progressive changes (meaning increased/altered function) for a semi-quantitative approach, as developed by Bernet et al. (1999), with modifications. The method is based on the product between the biological significance of each histopathological alteration (weight) and its degree of dissemination (score). The weight of alteration ranges between 1 (reversible, low severity) and 3 (irreversible, high severity) and the score ranged from 0 (absent) to 6 (diffuse). The global histopathological condition indice (I_h) was estimated according to the formula proposed by Costa et al. (2013):

$$I_h = \frac{\sum_1^j w_j a_{jth}}{\sum_1^j M_j} \quad [1]$$

Where w_j is the weight of the j^{th} histopathological alteration; a_{jh} the score attributed to the *h*th individual for the *j*th alteration and M_j is the maximum attributable value for the j^{th} alteration, i.e., weight × maximum score. The equation's denominator normalizes I_h to a value between 0 and 1, thus allowing inter-study comparisons (Costa et al., 2013).

2.5. Biochemical analyses

Liver samples were homogenized in 400 µL of ice-cold buffer (pH 7.4), containing 0.1 M sodium phosphate, 0.15 M KCl, 1mM EDTA, 1 mM dithiothreitol (DTT) and 10% (v/v) glycerol and centrifuged at 12 000 × g for 20 min, at 4 ºC. The supernatant was collected and centrifuged at 100 000 × g for 60 min. Then, the post-mitochondrial supernatant (PMS) was collected and divided in aliquots for biochemical analysis (GST, GSH and LPO) and stored at -80 ºC. The microsomal (MS) pellet was resuspended in buffer pH 7.4 (0.15 M KCl, 1mM EDTA, 1 mM DTT and 20% glycerol) and stored at -80 ºC for subsequent analyses of ethoxyresorufin-O-deethylase (EROD) activity and cytochrome P450 1A (CYP1A.) The post-mitochondrial supernatant and the microssomal total protein were determined by the Bradford (1976) method.

Glutathione *S*-transferase activity was determined using a commercial kit (from Sigma-Aldrich) following the conjugation of reduced glutathione with chloro-2,4-dinitrobenzene (CDNB) at 340 nm, using a Benchmark model microplate reader (Bio-Rad), according to manufacturer's instructions. Liver GST activity was estimated as nmol conjugated CDNB/min/mg post-microssomal protein.

Glutathione was determined in PMS sample previously deproteinized with 5% (m/v) 5-sulfosalicylic acid solution. Glutathione was measured using a commercial kit (from Sigma-Aldrich), following manufacturer's instructions. The assay allows the quantification of total GSH (GSHt) and glutathione disulphide (GSSG) by derivatizing GSH in samples with 2-vinilpyridine (Sigma-Aldrich). Activity was determined spectrophotometrically using the aforesaid microplate reader at 412 nm. The results are expressed as nmol/mg post-microssomal protein. The GSHt/GSSG ratio was calculated as GSHt/(GSSG/2).

The peroxidative damage of lipids (LPO) was determined by the thiobarbituric acid-reactive species (TBARS) method (Uchiyama and Mihara, 1978, adapted to microplate reader by Costa et al., 2011). The PMS fraction was deproteinized (as previously described) and after centrifugation the supernatant was incubated at 100 ºC, for 15 min, with thiobarbituric acid (TBA). The absorbance was measured at 530 nm using the aforementioned microplate reader. Malondialdehyde bis(dimethylacetal), from Merck, was used as standard. LPO is expressed as nmol TBARS/mg post-microssomal protein.

Ethoxyresorufin-*O*-deethylase activity (EROD) was measured in MS fraction, according to Gagné and Blaise (1993). Briefly, microssomal suspension (10 µl) was incubated (20 min, at 37 ºC) in fluorescence-compliant microplate wells by the addition of 150 µl of EROD reaction mix (1.0 mg/ml BSA, 5 µM ethoxyresorufin, 0.5 mM NADPH). The enzymatic reaction was stopped by adding 100 µl of 2 M glycine (pH 10.3-10.4). Resorufin fluorescence was analyzed at 560 nm excitation and 610 nm emission wavelengths with an Infinite 200 microplate reader (Tecan, Männedorf, Switzerland). Resorufin (Sigma) was used as standard. The results are given in ng resorufin/mg microssomal protein.

Cytochrome P450 1A relative induction was determined by enzyme-linked immunosorbent assay (ELISA) in the MS fraction, according to the protocol of Nilsen et al. (1998). Rabbit anti-fish CYP1A polyclonal antibody (Biosense Laboratories) was employed as the primary antibody and the alkaline phosphatase conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich) was used as the secondary antibody. The absorbance was read at 405 nm with the aforementioned microplate reader. Due to the absence of a commercial CYP protein for quantification, CYP1A induction was determined semi-quantitatively and the results are given in absorbance (Abs)/mg microssomal protein.

2.6. Statistical analysis

After data failing to comply with the homogeneity of variances and/or the normality assumptions for parametric analyses of variance, the non-parametric Mann-Whitney *U* test was applied for pairwise comparisons between sediment assays and sampling times. Cluster analyses based on the 1-Pearson

correlation *r* statistic were used to determine links between biomarker responses. Discriminant analyses were applied to determine the relative significance of each biomarker in the distinction between experiments. The significance level for all analyses was set at $p = 0.05$. Statistics were obtained using Statistica (Statsoft, OK, USA).

3. Results

3.1. Phe and B[b] concentrations in sediments and water samples

Spiked sediment contaminants (T_0) were within the proposed ranges: C1 between TEL and PEL and C2 higher than PEL (Table 6.1). The control sediment displayed low Phe and B[b]F concentrations in comparison with TEL values. The concentrations of both PAHs decreased between the beginning (T_0) and the end (T_{28}) of both isolated and combined assays, however the higher reductions (1.8 to 2 times) were observed for Phe. The Phe and B[b]F concentrations in water samples varied similarly with time (Table 6.1), i.e., higher concentrations at T_{14}. The highest levels were registered in water samples from assays prepared with highest concentrations (C2) in sediments. Also, Phe levels in water were always higher than B[b]F for both isolated and combined assays. The water samples collected in control tanks presented concentrations close to detection limit for both PAHs.

3.2. Histopathological analyses

Control fish presented a hepatic architecture consistent with that of a normal juvenile teleost (Fig. 6.1A). Exposure to either PAH, isolated or combined, caused more severe and diffuse hepatic, especially at T_{28}. Overall, circulatory disturbances and regressive changes were the most obvious alterations. Hyperaemia was often observed in livers of fish exposed for 28 days. Haemorrhage was often observed, although infrequent in animals exposed to low concentrations of either toxicants or their mixtures. Macrophages were more commonly detected in fish exposed to mixtures and for a longer time. Necrotic foci were present in fish subjected to all assays, chiefly at T_{14} for combined exposures and at T_{28} for any experimental condition, a similar patter being observed for apoptosis. Fatty degeneration was the most common alteration, leading towards lipidosis in mixture assays, at T_{28} (Fig. 6.1B). Microvesicular fatty degeneration (potentially leading to steatosis), identified by intracellular accumulation of small lipid vesicles, was restrained to small foci, usually in livers where lipidosis was already present in a moderate low degree of dissemination (Fig. 6.1C). This hepatic alteration was presented in liver of fish exposed to isolated compounds at T_{28} (for futher details see also Santos et al. 2013, annex 2).

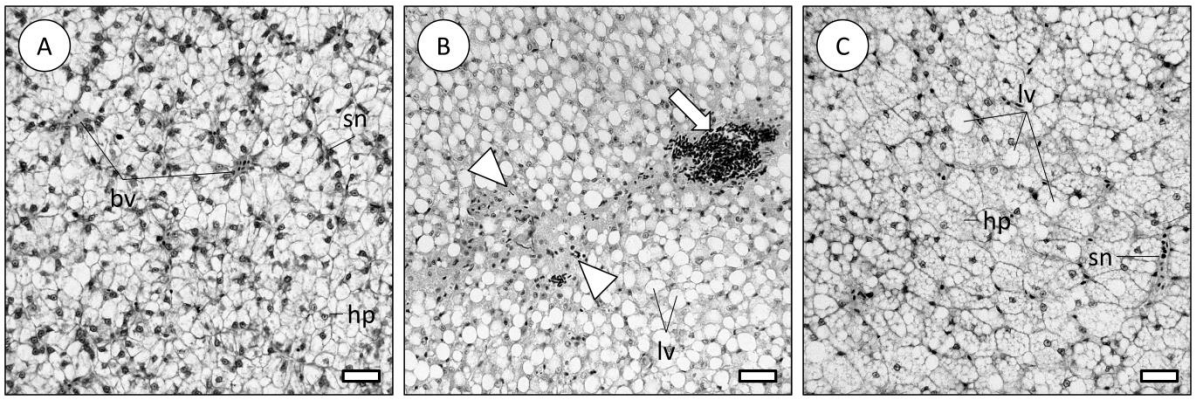

Fig. 6.1. Representative micrographs of liver sections (Bouin's, H&E). A) Section of a control bass collected at T_{14}, revealing normal structure of the hepatic parenchyma crossed by sinusoids (sn) and larger blood vessels (bv), namely venules, with roughly polyedric hepatocytes (hp) bearing a concentric nuclei with conspicuous nucleoli. B) Diffuse fatty degeneration progressing into full lipidosis in a fish exposed to mixture M2 for 28 days. Note the large lipid vacuoles in hepatocytes (lv). Defence cells (mostly macrophages) are infiltrating the hepatic parenchyma (arrow) adjacently to a necrotic foci where localized haemorrhage and infiltrating leukocytes are clearly visible (arrowhead). Altogether, the findings indicate an inflammatory response to injury. C) Microvesicular fatty degeneration developing to diffuse steatosis in the a fish exposed to the highest concentration of B[b]F for 28 days. Note the many small lipid vesicles within the typically enlarged hepatocytes (hp). Lipid vacuoles (lv) are also present. There is no significant sign of inflammation–related alterations in the sections, as observed through normal sinusoids (sn), devoid of hyperaemia–related swelling and absence of infiltrating defence cells.

3.3. Biomarker analyses

All biomarker responses, with the exception of GSHt yielded significant differences to controls, regardless of substance, time of exposure or concentrations of singly PAH in sediment (Fig. 6.2). However, the only notorious dose- and time-effect relationships were obtained for I_h, for which, inclusively, higher values were retrieved from the livers of B[b]F-exposed animals (Fig. 6.2H). The GSH/GSSG ratio, EROD activity and CYP1A induction were elevated in animals collected at T_{14}, decreasing at T_{28} and, in the case of the latter two, unexpectedly higher in Phe-exposed fish, in a dose-response manner. With the exception of the GSH/GSSG ratio and LPO, biomarker responses tended to be clearly elevated in fish exposed to either mixture or sampling time, relatively to controls. (Fig.6.3). Although the responses were higher than those elicited by exposure to the single compounds, no clear dose- or time-response effects were detectable among the multiple biomarkers responses as well. The most significant increases relatively to controls were observed for GST, GSH and GSSG, followed by I_h. Liver EROD activity from fish exposed to M2 and M4 assays was higher than control animals, at T_{14}, while at T_{28}, only fish subjected to M3 assay yielded increased activity. Similar results were retrieved from CYP1A induction, which was elevated in animals collected at T_{14}, especially those exposed to mixtures M1 and M2.

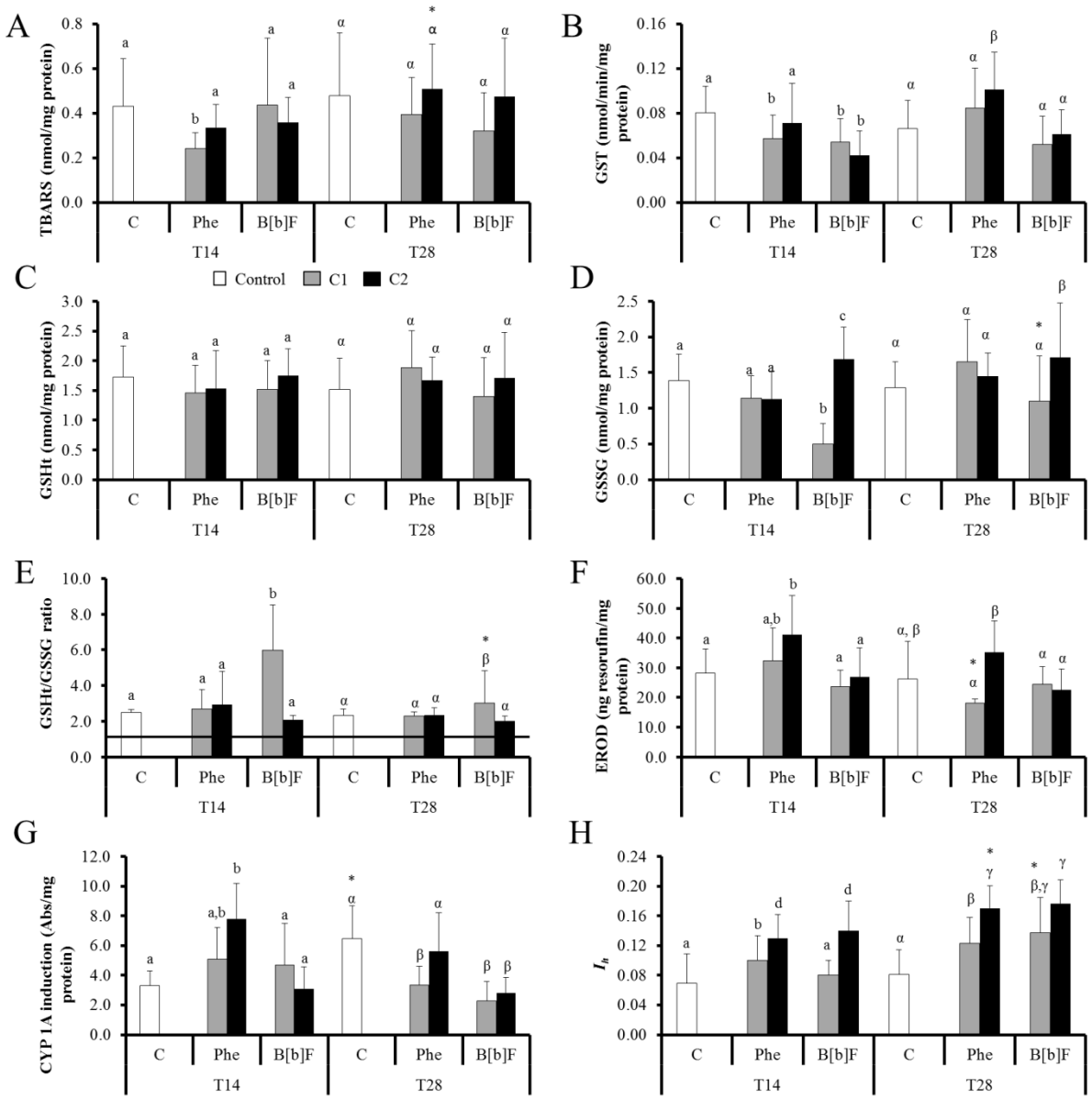

Fig. 6.2. Average biomarker responses in liver of fish exposed to isolated concentrations of Phe and B[b]F in sediments (control:C; Phe-C1: P1, Phe-C2: P2, B[b]F-C1: B1, B[b]F-C2: B2). A) Lipid peroxides (given by TBARS). B) Glutathione S-transferase activity (GST). C) Total Glutathione (GSHt). D) Glutathione disulfide (GSSG). E) GSH/GSSG ratio. F) Ethoxyresorufin-*O*-deethylase activity (EROD). H) Cytochrome P450 induction (CYP 1A). I) Hepatic histopathological condition indice (I_h). Different letters, lattin and greek, indicate significant differences (Mann-Whitney *U*, $p<0.05$) within fish collected during the same sampling time, i.e., T_{14} or T_{28}; respectively.* mean significant differences (Mann-Whitney *U*, $p < 0.05$) comparing sampling times. Error bars indicate standard deviation.

3.4. Comparison between isolated and combined Phe and B[b]F exposure

In Fig. 6.4 are presented the biomarker fold change (FC) for combined bioassays over exposure to the respective isolated compounds. In general, the biomarker fold change was higher in fish exposed to mixtures than isolated assays. The highest fold changes were obtained for GST activity and GSH, ranging between 2 and 6 (Figs. 6.4C and 6.4D), however without a clear dose- and time-response.

Conversely, the GSHt/GSSG ratio presented a negative fold change for both PAHs. Fish exposed to mixtures containing lowest concentrations of Phe presented higher I_h and LPO fold changes, at T_{14}, in comparison to fish exposed to higher concentrations of Phe (Fig. 6.4A). Mixtures containing lower concentrations of B[b]F yielded higher I_h comparatively to the isolated compounds at similar concentrations (Fig. 6.4B). EROD and CYP1A induction presented similar pattern for Phe (Fig. 6.4E). Mixtures containing higher concentrations of B[b]F yielded higher CYP1A induction when contrasted to isolated exposure (Fig. 6.4F), while EROD was similar between low and high B[b]F exposures.

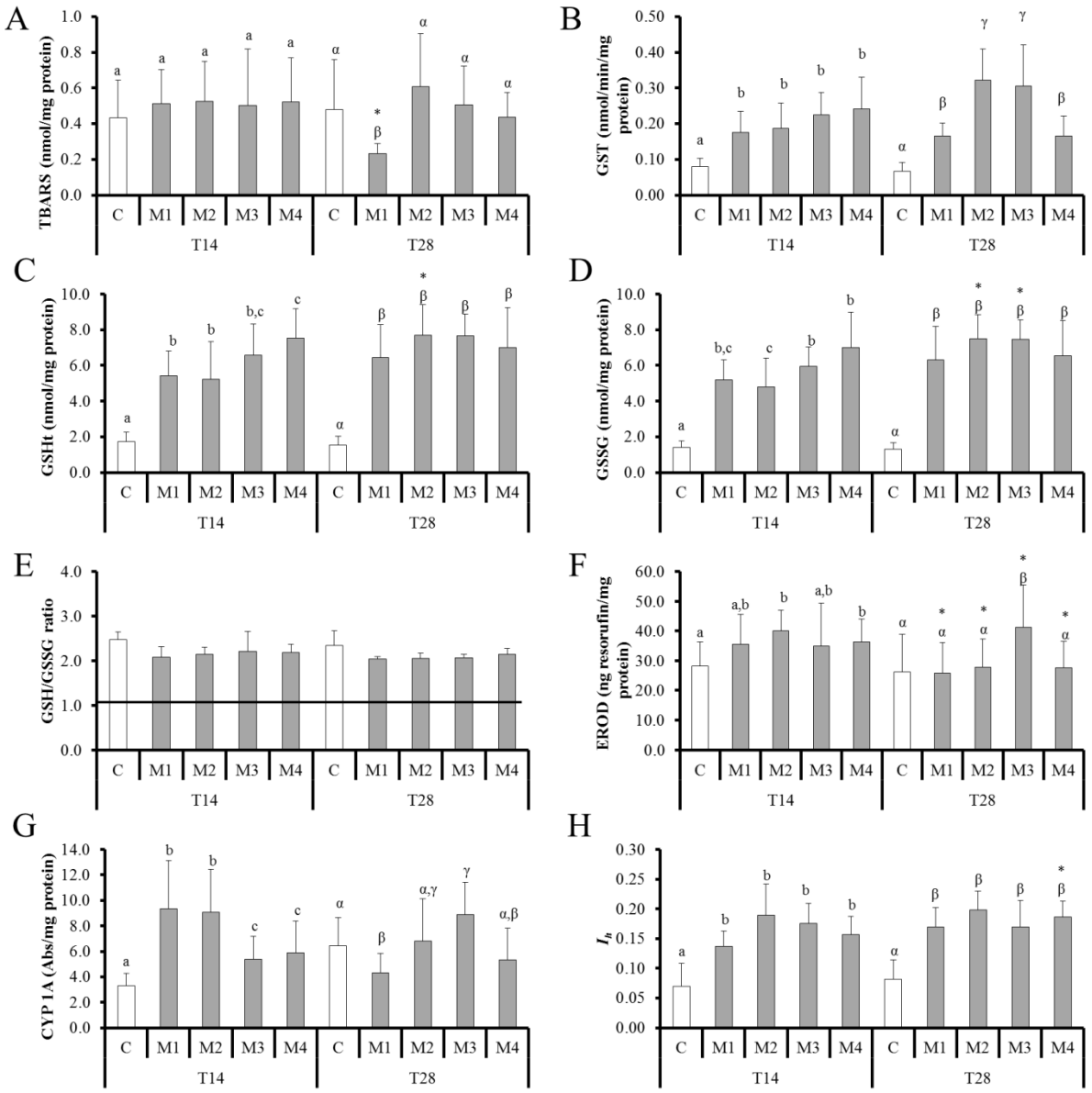

Fig. 6.3. Average biomarker responses in liver of fish exposed to combined concentrations of Phe and B[b]F in sediments (control:C; M1, M2, M3, M4). A) Lipid peroxides (given by TBARS). B) Glutathione S-transferase activity (GST). C) Total Glutathione (GSHt). D) Glutathione disulfide (GSSG). E) GSH/GSSG ratio. F) Ethoxyresorufin-*O*-deethylase activity (EROD). H) Cytochrome P450 induction (CYP 1A). I) Hepatic histopathological condition indice (I_h). Different letters, latin and greek, indicate significant differences (Mann-Whitney *U*, $p<0.05$) within fish collected during the same sampling time, i.e., T_{14} or T_{28}; respectively. * mean significant differences (Mann-Whitney *U*, $p < 0.05$) comparing sampling times. Error bars indicate standard deviation.

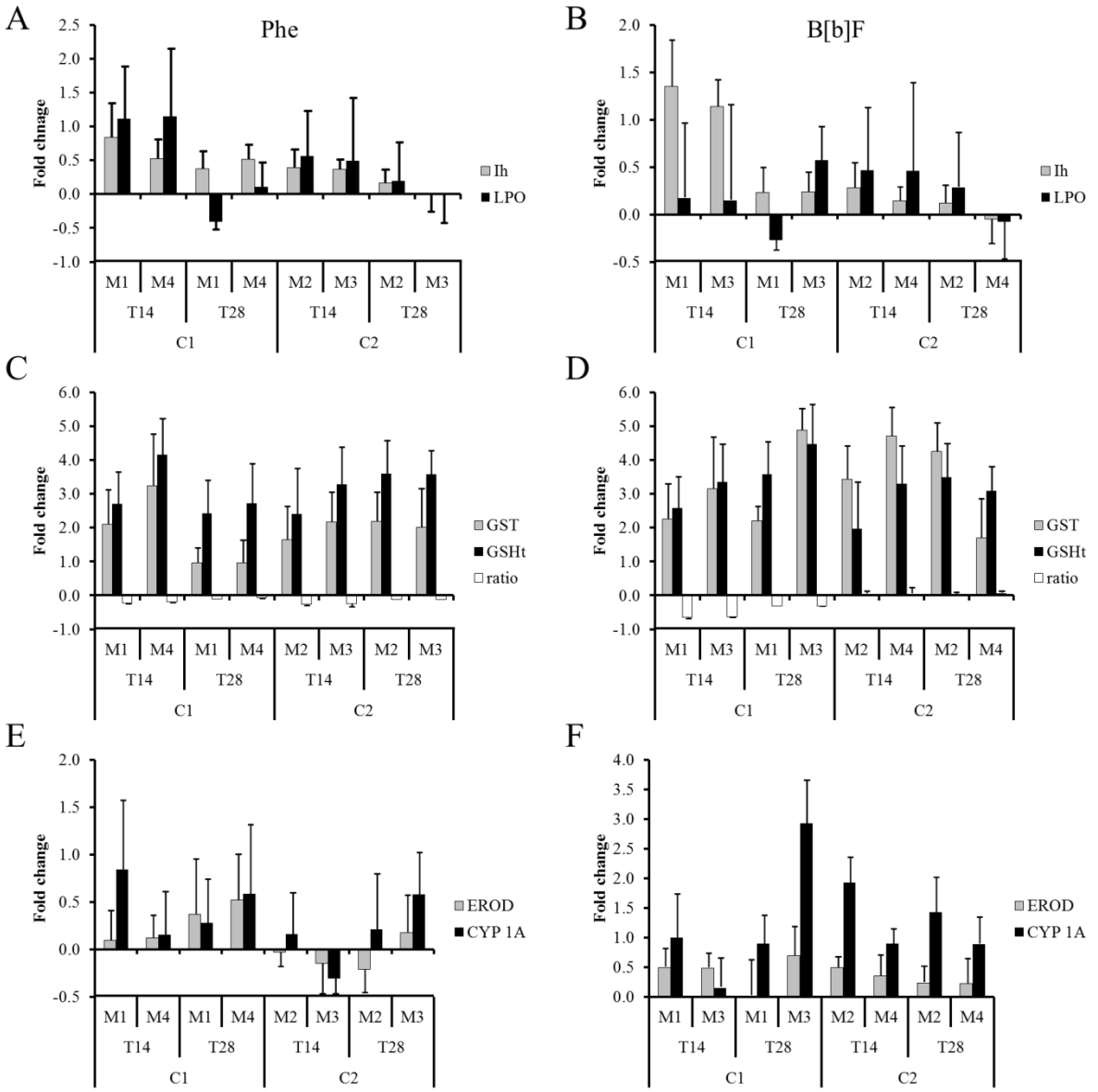

Fig. 6.4. Average biomarker responses expressed as fold changes obtained for the combination assays (M) over exposure to the respective isolated compound. A) Hepatic histopathological condition indice (I_h) and lipid peroxides (LPO). B) Glutathione S-transferase activity (GST), total Glutathione (GSHt) and GSH/GSSG ratio. C) Ethoxyresorufin-O-deethylase activity (EROD) and cytochrome P450 induction (CYP 1A).

Cluster analyses for each assay showed distinct biomarkers correlations pattern. Regarding isolated Phe assays, two groups of biomarkers are distinct (Fig. 6.5A). The first group comprises the most correlated biomarkers, GST and LPO, which are linked to I_h and GSH/GSSG ratio. The second cluster includes EROD and CYP1A induction and, to a lesser extent, the GSH/GSSG ratio. In B[b]F exposure the strongest correlation was obtained between LPO and CYP1A induction, which, together, are linked to GSH/GSSG ratio, forming a distinct cluster (Fig. 6.5B). The I_h indice was the most distant from the other biomarkers. For combined exposures, GST activity, LPO, GSH and GSH/GSSG, were clustered apart from EROD, CYP 1A induction and I_h (Fig. 6.5C). From the discriminant analysis

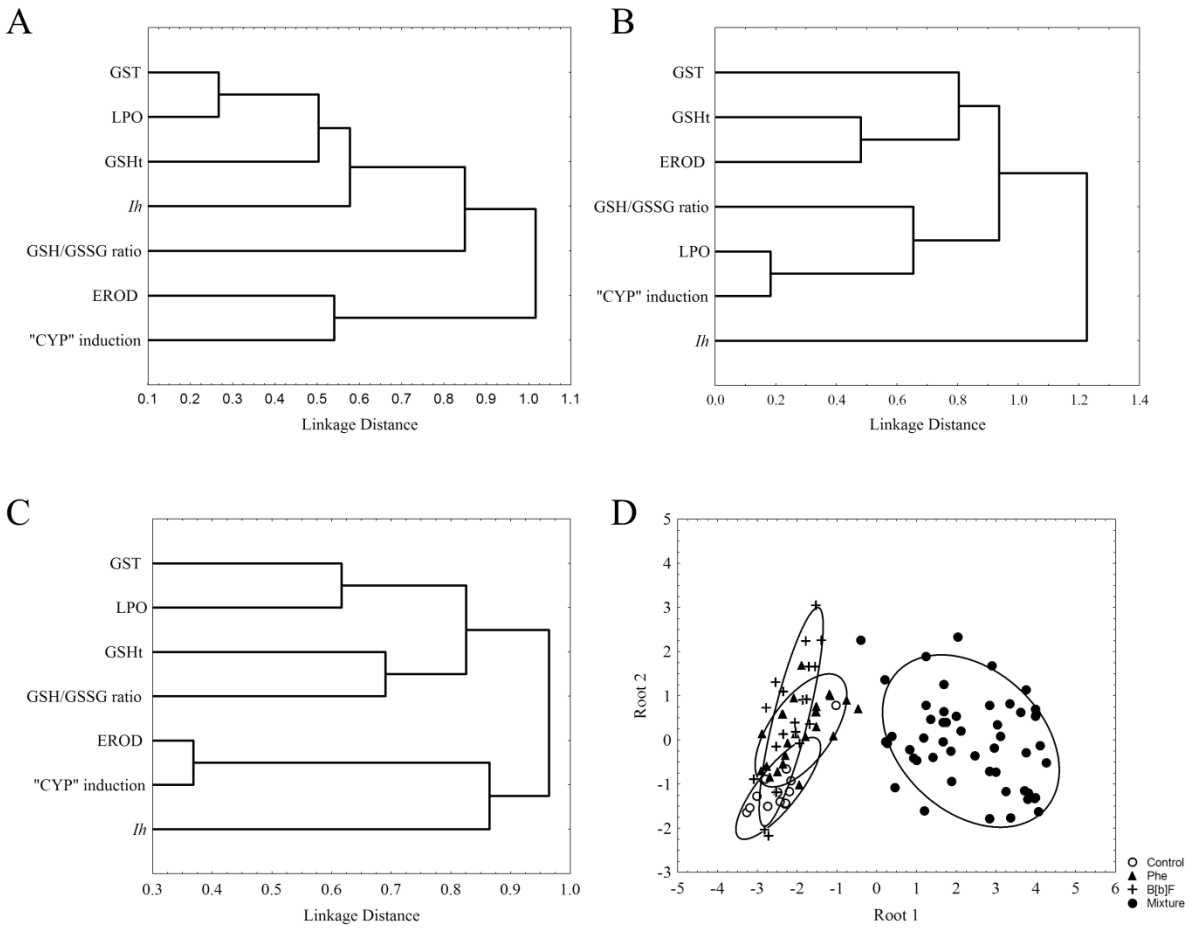

Fig. 6.5. Cluster analysis for all biomarkers analyzed in liver of fish exposed to A) isolated concentrations of Phe, B) isolated concentrations of B[b]F and C) combined concentrations of Phe and B[b]F (M), in sediments. Distances are based on the 1-Pearson correlation statistic *r* between condition indices. D) Discriminant analysis scatterplots. The model combined all variables fish exposed to test sediments.

it is noteworthy that biomarker responses obtained for combined assays are clearly detached from the isolated assays (Fig. 6.5D), being GST activity, GSHt and I_h that contribute the most to differentiate all assays (Table 6.3).

Table 6.3. Discriminant analysis results taking all assays and sampling time as grouping variables (factors). The best model was assessed according to the lowest Wilks' λ statistic (Wilks' λ = 0.12; $p < 0.01$). Variable significance within the model was determined using *F* tests ($p < 0.05$).

Variables	**Partial λ**	***p*-level**
GST	0.924	0.05
GSHt	0.522	0.00
LPO	0.952	0.18
EROD	0.956	0.21
CYP induction	0.929	0.06
I_h	0.720	0.00

4. Discussion

Environmental quality guidelines invariably dismiss the fact that pollution caused by a single toxicant seldom occurs, with the potential exception of spills and other accidents that, in most cases, likely allow a better recovery of ecosystems than prolonged, diffuse, contamination. Mixtures of toxicants, furthermore low concentrations may trigger unforeseen biological effects and responses, hindering risk assessment. To this are added the particular constraints posed by complex sediment matrices such as sediments, whose geochemical properties ultimately dictate the labile ratio between toxicant storage and bioavailability. The present findings show that a combination of sediment-bound PAHs with distinct toxicological properties elicited significant pathological and metabolic changes in fish, albeit unforeseen by exposure to either isolated compound. Moreover, the tested concentrations should indicate reduced risk to biota, according to acknowledged sediment quality guidelines.

The current work integrated a series of biomarker responses that relate to PAH bioactivation and production of highly hazardous metabolites and ROS as by-products. In particular, high molecular weight PAHs (like B[b]F) are generally acknowledged to hold more affinity to CYP MFOs, which contributes to its relatively higher toxicity when compared to low molecular weight PAHs such as the 3-ring Phe. Nonetheless, the present circumstances of assessment, which more closely resemble those in the natural environment, showed that the different effects and responses form an intricate web of alterations, diluting specificity of biomarkers and, notably, dose-effect relationships. Moreover, whereas exposure to Phe and B[b]F (isolated) induced histopathological alterations seemingly unlinked to biochemical changes, the combination assays yielded lesions and responses that, altogether, are compatible with exposure to AHR-compatible organic substances. This pattern of alterations clearly shows a supra-additive interaction effect between the two toxicants, albeit without a clear dose-response relation. Overall, the present findings suggest: i) the sediment-bound PAHs were rendered bioavailable to fish; ii) exposure to either isolated compound elicited hepatic histopathological lesions and alterations, albeit the low, ecologically-relevant concentrations diluted time- and dose-response effects; iii) exposure to Phe (termed non-carcinogenic) elicited biochemical changes better related to oxidative stress and CYP induction whereas B[b]F (carcinogenic) disrupted metabolic responses and defences to toxicological challenge; iv) the combination of both PAHs triggered metabolic functions that relate to enhanced histopathological lesions and AHR-dependent metabolic pathways.

Sediments are major reservoirs of toxicants, especially for hydrophobic toxicants like PAHs and other organic pollutants. These substances may be rendered bioavailable to aquatic organisms via ingestion of sediment particles and pore water during feeding or via gills through the solubilized fraction of the substances in water column. Sediment disturbance, through oxic/anoxic shifts that unbalance the

steady-state of the sediments is one of the most important factors favouring PAH release from sediments (see Eggleton and Thomas, 2004, for a review). Also, low- and high-molecular weight possess different desorption rates from sediments that, being slower in the latter case, contribute to the concerns about high molecular weight PAH contamination of sediments over extended periods of time (Narbonne et al., 1999). Under this scope, the present work showed that, regardless of the specific cause, sediment-bound PAHs may become bioavailable to demersal fish and elicit toxic effects, even when the concentrations in the sediments would imply reduced risk. The results are in agreement with previous laboratory bioassays performed under similar circumstances with bivalves and fish exposed to natural sediments contaminated by mixed substances, including low concentrations of low to high molecular weight PAHs (e.g. Costa et al., 2008; Martins et al., 2013). As such, the present findings further contribute to the important notion that sediment toxicant characterization, on itself, provides a fairly incomplete notion of risk.

Low molecular weight PAHs like Phe (more hydrophilic) are acknowledged to pose higher risk of acute toxicity to aquatic organisms (hence the higher water solubility and lower PEL than B[b]F) whereas higher molecular weight PAHs hold higher risk to cause neoplasia-related chronic effects. As such, distinct toxicological mechanisms of toxicity should be expected between the two classes of substances. Nonetheless, the mechanisms by which Phe is toxic, especially to fish, are not well understood. However, there is experimental evidence for induction of oxidative stress in fish, with emphasis on the production of the highly reactive hydroxyl radical (e.g. Sun et al., 2006). Although both Phe and B[b]F have a bay-region, Phe has been found to be a much weaker AHR agonist than B[b]F by five orders of magnitude, in fish (Barron et al., 2004). Nonetheless, the present findings revealed that Phe was the only compound that, isolated, significantly induced CYP and (as expected) EROD activity (Fig. 6.2). In fact, both CYP induction and EROD activity yielded little significance as explanatory variables between treatments (Table 6.3). Considering that CYP transcription is AHR-mediated, this pathway seems to account poorly for the differences of *modus operandi* between the two compounds, under the present circumstances. Quite surprisingly, when performing correlation analyses to draw potential mechanisms (Fig. 6.5), exposure to Phe (isolated) yielded a pattern of responses and effects more consistent with phases I (CYP and EROD) and II (GST and GSH) of detoxification.

Although the cellular responses were low following exposure to Phe-spiked sediments, correlation analysis (Fig. 6.5A) revealed two distinct clusters of measured endpoints, each directly relatable to two transcriptional pathways: one encompassing CYP induction and EROD that relate to the AHR-XRE pathway and the other including GST activity and glutathione, both of which relate to the Nrf2-(N2-related factor) -ARE (antioxidant response element) pathways. In accordance, lipid peroxidation (even though it failed to produce significant alterations compared to controls) is clustered within. In

fact, the Nrf2 pathways is directly mediated by ROS and even some lipid peroxides, which favour the release of the Nrf2 transcription factor from the complex with Keap1, rendering it free to migrate to the nucleus where it may bind to the ARE for the promoter region for GST and GSH genes (e.g. Milder et al., 2010). Interestingly, I_h is clustered amongst anti-oxidant endpoint which indicates a link between histological lesions and oxidative stress, regardless of the reduced levels of the previous. Conversely, exposure to B[b]F alone yielded no immediately obvious pattern of response and effect related to toxicological challenge, furthermore relating to a known AHR agonist (Fig. 6.5B). Furthermore, histopathological alterations were not linked to any of the surveyed biochemical endpoints. In fact, histopathological alterations caused by exposure to either compound were very unspecific (such and inflammation-related and lipid degeneration). Altogether, it appears that exposure to Phe caused oxidative stress without, however, overwhelming the liver's natural ability to cope with oxidative challenge through its biochemical resources, although unsufficient to avoid some extent of histological damage. As such, the pattern observed in Fig. 6.5A likely mirrors the baseline, unchanged metabolic pathways for the surveyed endpoints. On the other hand, B[b]F caused metabolic disturbance, leading to more pronounced histological damage. The toxicological pathways of exposure to this PAH, under the present circumstances, cannot be fully disclosed. Overall, either "low" or "high" concentrations of either PAH in sediment were still too low as to cause obvious dose-response effects. In addition, it must be highlighted that other, non-surveyed, mechanisms of defence and response (such as other antioxidant pathways and conjugating agents and enzymes) to toxicological challenge may have contributed to maintain the oxidative status of the liver tissue. As such, the present work not only confirms the importance of surveying multiple endpoints but also stress that determining exposure to low, realistic, concentrations of toxicants through traditional biomarker approaches mandates caution when interpreting the results.

Exposure to the combined toxicants, conversely, yielded more conclusive results and a distinct pattern of responses and effects from the other experimental treatments (Fig. 6.5D). All endpoints were responsive to exposure, except GSH/GSSG ratio. Nonetheless, the constancy of the ratio, relative to controls, may be explained by increased GSSG being compensated by glutathione biosynthesis (Fig. 6.3). Still, as previous, dose- and time-dependent effects were illusive. Nonetheless, when contrasting mixture assays to the respective exposure to isolated compounds (Fig. 6.4), indicate additive, or even synergistic effects of mixtures. It is the case of lipid peroxidation (Fig. 6.4A) and the I_h (Fig. 6.4B) of animals exposed to mixtures containing lowest doses of Phe and B[b]F, respectively, when contrasted to the effects of the isolated compound at similar concentrations. In comparison, exposure to mixture M2 (both compounds in highest concentrations) did not, as could be expected, elicit the highest responses, regardless if compared to controls or exposure to the isolated compounds. In addition, the results do not indicate significant antagonistic effects between the two PAHs. These findings are accordant with previous studies with binary mixtures of PAHs *in vitro*, reporting scarce antagonist

effects in favour of agonist and potentially synergic responses and effects, depending on concentration of the compounds and endpoint (Staal et al., 2007; Tarantini et al., 2011). As such, the current results, obtained *in vivo* at low concentrations of the pollutants tend to mirror those *in vitro* in the sense that predicting toxicity from PAH mixtures may lead to results of difficult interpretation. Still, the present findings clearly show that ecologically-relevant scenarios pose additional constraints by most notoriously diluting dose-response relationships. In fact, even *in vitro* studies with environmental mixtures of PAHs reported that, as the current work, the effects of mixtures are far more complex than additivation (e.g. Tarantini et al., 2009).

One of the most unexpected findings relates to CYP1A-related responses. Previous research reported positive links between the expression of CYPs and the carcinogenic potential of PAHs when comparing exposure to isolated PAHs (e.g. Staal et al., 2006). Contrarily, discriminant analysis revealed that both CYP1A induction and EROD activity failed to become distinctive endpoints between the multiple treatments, concerning exposure to the isolated or mixed compounds (Table 6.3). Moreover, these CYP1A and EROD showed a trend to decrease over time of exposure following a peak at T_{14} that, interestingly, was higher in animals exposed to Phe, a less potent AHR agonist than B[b]F. Although the decrease in CYP MFO activity of protein contents with time of exposure has been described to occur in fish exposed to PAHs, isolated or mixed, carcinogenic and non-carcinogenic (e.g. Costa et al., 2009a; Bravo et al., 2011), the underlying mechanism is not yet understood. There is also indication *in vitro* that the interactions between high molecular weight, carcinogenic PAHs, do modulate the AHR pathway, production of specifics metabolites and, therefore, carcinogenicity (e.g. Spink et al., 2008). However, data on carcinogenic/non-carcinogenic PAH interactions are scarce, especially at the rim of realistic exposures. It must be noticed, though, that the mechanisms of the AHR pathway are complex and, furthermore, interlinked with other molecular pathways, such as the Keap1/Nrf2, since bioactivation of PAHs generates ROS, which may trigger Nrf2 release and subsequent activation. The metabolites of bioactivatable PAHs and oxidative by-products may then be conjugated, naturally or catalysed by GSTs, to glutathione, forming covalent bond with the sulfhydryl groups of cysteine resides of this peptide. This mechanism thus takes part in a form of negative feed-back loop of both the AHR and Nrf2 pathways. On the other hand, there are multiple CYP enzyme isoforms, for which PAHs and other substances with similar properties preferentially bind to. Among these, CYP1A, CYP1B and CYP2E MFOs (and respective subfamilies), namely monooxygenases and hydrolases, appear to be the most important in xenobiotic metabolization, through the oxidation of the parent compounds, increasing their electrophilic disposition and, as a consequence, their solubility in water and reactivity (see Ioannides and Lewis, 2004, for a review). This aspect also determines the chemical characteristics of metabolites and by-products (especially ROS) produced through the bioactivation process. It must be highlighted that it is precisely the chemical nature of the metabolites that determined its reactivity towards other organic molecules, especially DNA, since the formation of

adducts/apurinic sites is an acknowledged factor in the mutagenic hazards of PAHs and, therefore, in their relative risk to induce cancer (see for instance Xue and Warshawsky, 2005). In fact, high molecular weight PAHs (the best known example being B[a]P) tend to yield PAH diol epoxides, which are secondary metabolites derived from oxidation of primary hydroxides, again via CYP MFOs, namely epoxide hydrolases. Diol-epoxides are acknowledged as the most potent PAH-derived mutagens (including for B[b]F), unlike Phe-hydroxides (Weyand et al., 1993). The type of DNA damage also determines its proneness for repair, for instance by base (BER) or nucleotide excision repair (NER), the latter or which involved in the removal of adducts (Kienzler et al., 2013). Altogether, the complexity of these mechanisms adds many confounding factors to the understanding the specific pathways of toxicity and response to challenge, especially when dealing with reduced concentrations of mixed PAHs (as occurs in the aquatic milieu) and limited durations of exposure.

It must also be noticed that the toxic effects of PAHs are not restricted to the interlinking between AHR and Nrf2 pathways and most certainly not to the restricted endpoints hereby measured such as CYP1A induction and the activity of cytosolic GST. Besides the many CYP isoforms mentioned above, there are multiple isoforms of GSTs, including mitochondrial GSTs (mitochondria specific, like GSTk or there migrated from the cytoplasm like GSTα) which are known to take part in phase II detoxification processes and being able, depending on isoform, to be regulated by Keap1/Nfr2 as well (see Raza et al., 2002, and Raza, 2012). Nonetheless, this issue clearly needs further research concerning PAHs and other pollutants. Also, PAH toxicity depends on the full antioxidant capacity of cells, which is also linked to energetic demands and ATP hydrolysis and biosynthesis, which affects basal metabolism (including gene expression) and specific responses to toxicological challenge, like DNA repair. This way, addressing complex toxicological scenarios clearly demands surveying multiple endpoints, albeit without full guarantee that most will yield clear-cut results, as in the present study. Related to these aspects, the results are indicative of general metabolic failure, which is corroborated by the hepatic histopathological assessment. In fact, one of the most significant histopathological alterations was lipid degeneration, which is very unspecific and recognized as a potential indicator of carbohydrate metabolism/energy production disorders when fish are exposed to toxicants, even though it may occur naturally as a result of age and feeding regime (see Costa et al., 2009b,2011, and references therein). This alteration was more obvious in B[b]F and mixture-exposed animals indicates metabolic unbalancing as a major consequence of exposure, even if the occurrence of more severe alterations, such as necrotic foci (accompanied by inflammation) or even apoptosis, although elevated, was more modest. Still, even such an unspecific trait like lipidosis has been link to elevated oxidative stress and risk of acquiring neoplasia-related disease in experimental animals (see Sánchez-Pérez et al., 2005, and Costa et al., 2011). On their turn, both oxidative stress and DNA damage are linked to cell death, inflammation and neoplasia/preneoplasia (e.g. Cadet et al., 2010; Reuter et al., 2010, and Martins and Costa, 2014). Overall, the extent of histopathological alterations

indicates similar conditions of exposure, in the natural environment, would induce very significant chronic effects to organisms, even considering that the fish surveyed in the present study are contaminant-naïve, therefore, little adapted to contaminated natural sediments. Nonetheless, the test species is an ecologically-relevant species that inhabits estuaries and other confined coastal waters, from which it may be inferred that the tested mixtures of PAHs cause significant toxic effects even if the individual concentrations would predict low or null risk. It must be stressed, though, that the duration of the assays performed in the present study was certainly too short to allow the development of full neoplasms. Still, the histopathological findings here observed tended, unlike most biochemical responses and effects, to become more severe in diffuse with time and duration of exposure and, moreover, were significantly more prominent in fish exposed to the mixtures of toxicants, without evident for amelioration or adaptation (unlike, for instance, CYP-related responses). This information indicates that: i) the animals endured significant tissue-level alterations when exposed to the mixed toxicants; ii) histological observations, regardless of low or absent specificity, yielded more obvious effects that better relate to the actual health status of the animals in an intricate scenario of exposure that hindered the interpretation of more standardized and specific biochemical biomarkers.

Carcinogenesis is a complex biological process that cannot be simply derived from the simple toxicity testing approaches upon which most toxicant guidelines are derived from. In fact, risk assessment for PAH mixtures should be a holistic approach that begins with the realistic vehicle for the xenobiotics (in this case, the sediments), through the assessment of multi-level effects and responses up to the detection of full neoplasms. The present work also highlighted how little is still understood about the mechanisms of toxicity for carcinogenic and non-carcinogenic PAHs and their mixtures under ecologically-relevant circumstances. Finally, it has been shown that sediment quality guidelines, although effectively predicting risk of individual PAHs, should not apply to mixtures, thus mandating caution when determining the environmental status of a given ecosystem taking these artificial levels as definite thresholds, under the serious risk, as hereby demonstrated, of grossly underestimating risk.

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CHAPTER 7. POLYCYCLIC AROMATIC HYDROCARBON METABOLITE FINGERPRINTING IN THE BILE OF FISH EXPOSED TO SEDIMENT-BOUND SINGLE AND COMBINED COMPOUNDS – PRELIMINARY RESULTS†

† Martins et al. (submitted).

Abstract

The toxicity of PAHs is set upon their metabolites and not on their parent compounds. The wide diversity of PAHs, in spite of similar basic chemical properties deems differential affinities towards phase I enzymes (especially CYPs) and, consequently, deems a wide variety of metabolites with different toxicological effects and hazards, among which the most acknowledged relate to DNA damage. Still, little is known about the metabolites of many PAHs and how these are produced under realistic circumstances of exposure, which includes ecologically-relevant organisms, concentrations and mixtures of PAHs. The present work attempted to survey the patterns of metabolites in the bile of sea basses exposed (via sediments) to two distinct PAHs, phenanthrene (Phe) and benzo[b]fluoranthene (B[b]F), considered non-carcinogenic and carcinogenic to fish respectively; singly or in mixture through a battery of laboratorial 28-day bioassays. Although the interpretation of the findings was hindered by the lack of standards, especially for B[b]F, the results indicate distinct patterns between exposure to the isolated and mixed compounds. Whereas Phe metabolites were mostly hydroxy-Phe, B[b]F metabolites were more diverse, albeit yet unidentified. Also, co-exposure appears to have an agonist effect towards Phe bioactivation, the opposite effects being observed for B[b]F. Altogether, this study indicates the importance of understanding the patterns of PAH metabolites in order to acquire a realistic measure of risk and that these patterns are more complex than could be judged *a priori*.

Keywords

Phenanthrene; Benzo[b]fluoranthene; PAH bioactivation; Hydroxy-PAHs; GC-MS; Interaction effects

1. Introduction

The key factor in polycyclic aromatic hydrocarbon (PAHs) toxicity is the formation of highly reactive metabolites. By other words, the parent compounds are not the toxic agents *per se* but their metabolites, whose production follows a process commonly designated by bioactivation. The metabolic activation of PAHs is mainly governed by phase I cytochrome P450 (CYP) mixed function oxidases (MFOs), and in some cases followed by the action of secondary enzymes such as microsomal epoxide hydrolases, that transform hydrophobic PAHs into more electrophilic, more easy to eliminate, but more reactive metabolites (see for instance Stegeman and Hahn, 1994). These metabolites may form adducts with DNA, or be conjugated by phase II enzymes such as glutathione-*S*-transferase, UDP-glucuronyltransferase and sulphotransferases (Omiecinski et al., 2011), rendering the metabolites inactive and facilitating their elimination. In addition reactive oxigen species (ROS) are produced during PAH bioactivation, which, besides general metabolic disruption and cellular damage, may cause direct nucleobase oxidation (Penning et al., 1996; Ohnishi and Kawanishi, 2002). As such, PAH-induced genotoxicity may increase the probability of occurring misrepaired and unrepaired damage, leading to fixed mutations and carcinogenicity (Sarasin, 2003). In fact, many PAHs are already classified as effective or potential carcinogens to humans by the International Agency for the Research on Cancer (IARC, 2012). In spite of similar chemical properties among PAHs, the nature and reactivity of the PAH metabolites are primarily a function of the structure of the parent compounds. Also, PAHs hold different affinities to CYP isozyme active sites, which further contribute to the production of different metabolites. Altogether, the molecular weight, the stereochemistry and even the position of the epoxide group in the bay-region of the PAH intermediate may determine the affinity of a given metabolite to bind to DNA and form bulky adducts (Xue and Warshawsky, 2005). Diol-epoxides are acknowledged as the most potent mutagenic PAH metabolites (see for instance, Wogan et al., 2004). However, the bioactivation mechanisms and the respective metabolites of most PAHs are still unknown and, furthermore, little is known about the interaction effects between PAHs even though these toxicants are present in the environment as complex mixtures. In fact, when addressing PAH mutagenic effects, ecological realism is often neglected, with respect to concentrations, model organism and toxicant vehicle.

The present work aims at comparing the profiles of PAH metabolites present in bile of fish exposed to two distinct PAHs, the “non-carcinogenic” PAH (IARC, group 3), phenanthrene (three-ringed PAH) and the “carcinogenic” PAH (group 2B), benzo[b]fluoranthene (five-ringed PAH) under ecologically-relevant circumstances of exposure. Specifically, it was aimed at obtaining a metabolite fingerprint when the exposure was isolated or combined and contrast it to PAH bioaccumulation in fish species.

2. Materials and methods

2.1. Experimental design

In order to achieve ecological relevance, sediments were chosen as the toxicant vehicle, due to their recognised reservoir of hydrophobic toxicants. Two low-moderate, equitoxic, concentrations of either substances were selected according to available toxicity thresholds guidelines (MacDonald et al., 1996). The sea bass *Dicentrarchus labrax* was chosen as the ecologically-relevant organism being suitable model of estuarine fish, which are likely subjects of sediments-bound pollution by PAHs and known to possess a well-developed MFO system (Uno et al., 2012, for a review).

Two liters of sediment were spiked with appropriate aliquots of stock solutions of Phe and B[b]F (in DMSO), in order to achieve the target concentrations of 86.7 and 544 ng g^{-1} for Phe and 88.8 and 763 ng g^{-1} for B[b]F, as described in Martins et al. 2013, making a total of nine test sediments: control (only DMSO), Phe-C1, Phe-C2, B[b]F-C1, B[b]F-C2, M1 (Phe-C1 plus B[b]F-C1), M2 (Phe-C2 plus B[b]F-C2), M3 (Phe-C2 plus B[b]F-C1) and M4 (Phe-C1 plus B[b]F-C2) (Table 7.1). Spiked sediments were placed in 15 L capacity tanks to which were added 12 L of filtered seawater.

Ten randomly-selected juvenile hatchery-brood sea basses, all belonging to the same cohort (85.2 ± 8.5 mm standard length; 9.90 ± 2.31 total wet weight) were placed in each tank as described in Martins et al., 2014 and Chapter 5. The assays were performed in duplicate. After 14 and 28 days of exposure, 10 fish (5 per replicate) were collected and the liver and gallbladder were harvested for the determination of PAH bioaccumulation and PAH metabolites, respectively.

2.2. Phe and B[b]F analysis in water

Water samples were spiked with surrogate standard solution (from Supelco), percolated through speedisks using a vaccum system, followed by elution with and ethyl/dicloromethane (v/v) mixture, according to Martinez et al (2004) with modifications (Martins et al., 2013). Phenanthrene and B[b]F quantification was performed by chromatography-mass spectrometry (GC-MS) system (Thermo DSG) in selected-ion monitoring (SIM) mode and their identification was achieved through the internal standard peak method (Martins et al., 2008). The recoveries obtained for surrogate standards were 74% and 87% for Phe and B[b]F respectively. Analysis of spike water samples yielded recoveries between 99-102% for Phe and 86-88% for B[b]F.

Table 7.1.. Target concentrations of Phe and B[b]F (ng g^{-1}) used for spiking the artificial sediments, and the respective Phe and B[b]F concentrations in water (ng L^{-1}) at 14 (T_{14}) and 28 (T_{28}) days of exposure to the nine experimental treatments.

			Bioassay								
			Control	**Phe-C1**	**Phe-C2**	**B[b]F-C1**	**B[b]F-C2**	**M1**	**M2**	**M3**	**M4**
Sediments (ng g^{-1})	Target concentrations	Phe	0	86.7<C1<544	C2>544	0	0	86.7<C1<544	C2>544	C2>544	86.7<C1<544
		B[b]F	0	0	0	88.8<C1<763	C2>763	88.8<C1<763	C2>763	88.8<C1<763	C2>763
	T_0	Phe	12.3±1.6	383.8±27.4	917.2±53.5			382.3±1.5	850.1±17.7	889.8±137.4	326.4±1.3
		B[b]F	28.4±1.2			267.3±24.5	981.9±39.2	328.6±32.6	1266.6±83.6	289.0±54.7	1111.9±15.2
	T_{28}	Phe	11.9±1.0	189.2±23.0	528.9±17.0			216.3±5.6	485.6±24.9	517.4±42.1	229.2±33.1
		B[b]F	28.3±1.3			265.1±43.6	964.5±42.8	254.7±7.1	816.0±44.0	281.5±30.8	920.6±22.4
Water (ng L^{-1})	T_{14}	Phe	<2.8	45.7±1.2	201.6±1.5	6.7±0.9	7.3±0.8	62.4±1.2	348.7±2.0	392.1±2.2	69.9±2.3
		B[b]F	<13.8	16.5±1.1	<13.8	52.1±1.4	29.5±1.1	<13.8	22.1±1.0	16.3±1.0	40.6±2.1
	T_{28}	Phe	<2.8	23.12±1.3	62.7±1.0	2.7±0.9	<2.8	36.4±1.2	8.5±1.0	9.3±0.9	<2.8
		B[b]F	<13.8	13.3±1.3	<13.8	15.3±1.0	22.2±1.2	<13.8	<13.8	<13.8	22.8±1.2

2.3. Phe and B[b]F analysis in liver

Phenanthrene and B[b]F were determined in liver of fish as described in Martins et al., 2008. In brief: in order to ensure enough biomass to survey PAHs, liver samples were pooled into three replicates per test and were spiked with surrogate standards (from Supelco). PAHs were then extracted by ASE (accelerated solvent extraction). The extracts were concentrated, fractionated with silica/alumina (g/g) glass column and re-concentrated to 0.5 ml under gentle stream of N_2 prior to analysis. The PAH quantification was performed on a Thermo DSQ gas chromatography-mass spectrometry (GC-MS) system in selected ion monitoring (SIM) mode. Standard reference material SRM 2977 (NIST, USA) was analysed to validate the procedure and the obtained PAH levels were found within certified range.

2.4. Analysis of PAH metabolites in fish bile

The analysis of PAH metabolites in bile was performed mainly as described by Jonsson et al., 2003, with some modifications. Prior to the analysis, individual bile samples of each duplicate were pooled. Ten microliters of the pooled samples were treated with 1 ml of β-glucuronidase (3000 units dissolved in 0.4 M acetate buffer, pH 5.0). The internal standard, 1-hydroxypyrene (10 µL) and the antioxidant butyl-hydroxytoluene (BHT) were added, and the mixture was incubated at 40 ºC for 2 hours. Hydrolysed metabolites were then extracted four times with 500 µl of the mixture of ethylacetate (with 10% methanol). The combined extracts were dried with anhydrous sodium sulphate and subsequently evaporated to dryness under a gentle nitrogen stream. Derivatisation of the samples was carried out by adding 50 µl of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), for 20 min at room temperature (Grova et al., 2005). Finally, trimethylol propane (50 µl) was added as a GC-MS performance standard prior to injection.

The determination of PAH metabolites was performed on a Hewlett Packard 6890 GC equipped with a CP Sil-8 capillary column (length 50 m, diameter 0.25 mm, film thickness 0.25 µm), and a Hewlett Packard 5973 mass-selective detector (MSD). The split/splitless injector was maintained at 300 ºC and the injection volume was 1 µl. Helium was used as carrier. The temperature program was: 60 ºC (then holding 0.5 min), increased by 15 ºC min^{-1} to 200 ºC (hold 0 min), then followed by another increase of 6 ºC min^{-1} up to 330 ºC (hold 15 min). Selective ion monitoring (SIM) mode was employed as routine to achieve higher sensitivity. A full scan run was performed on most samples to confirm the SIM analysis results and for screening for any additional other PAH metabolites. Phe metabolites were identified by comparing retention times and mass spectra with those of reference standards (Table 7.2). However, due to the limited number of Phe standards available and the absence of B[b]F metabolite standards, identification was chiefly based on interpretation of the mass spectra, which are characterised by the presence of the molecular ion and specific fragment ions. The quantification of

individual metabolites was based on their GC-MS response relative to that of an internal standard. The recovery of internal standard was 80 ± 59 %.

Table 7.2. List of screened analytes and internal standards.

Compounds	GC retention time (min)[4]	Quantification ion (*m/z*)	Major fragment ion (*m/z*)
9-mono hydroxyphenathrene[1,3]	17.9	266	251, 235, 165
1-mono-hydroxyphenanthrenes[3]	18.28	266	251, 235, 165
2-mono-hydroxyphenanthrenes[3]	18.67	266	251, 235, 165
3-mono-hydroxyphenanthrenes[3]	18.15	266	251, 235, 165
4-mono-hydroxyphenanthrenes[3]	17.31	266	251, 235, 165
1,2-dihydroxy dihydrophenanthrene[3]	19.8	356	266, 253
3,4-dihydroxy dihydrophenanthrene[3]	16.95	356	266, 253
9,10-dihydroxy dihydrophenanthrene[3]	16.62	356	341,266
1-hydroxypyrene[1]	22.95	290	275
monohydroxy B[b]F[2]		340	325
dihydroxy dihydro B[b]F[2]		430	413
quinones B[b]F[2]		282	254

[1] Internal Standard.
[2] Based on benzo[a]pyrene metabolites reported in Takahashi et al. (1979).
[3] Johnson et al. (2003); Krahn et al. (1992); Yu et al., (1995).
[4] Retention times are only indicative

3. Results and Discussion

The bioaccumulation of PAHs by aquatic organisms is highly dependent of the physico-chemical properties of these xenobiotics (Meador et al., 1995). The findings indicated distinct liver bioaccumulation patterns between Phe and B[b]F exposure, whether isolated or combined. Also, in spite of the constraints related to the complexity of the matrix (bile) and the quantification of B[b]F metabolites and many Phe metabolites (due to the absence of standards), the results revealed that the bioactivation and elimination mechanisms of *D. labrax* yielded distinct patterns of PAH metabolites between isolated and combined-PAH assays.

In general, B[b]F bioaccumulation in liver was lower than Phe by an order of magnitude (Fig. 7.1). In comparison to controls, fish exposed to Phe yielded elevated Phe concentrations in liver, at T_{14}, in comparison with T_0 followed by a plateau stage until the end of the assay (Fig. 7.1A). Fish exposed to higher concentrations of Phe in sediments also bioaccumulated the highest amounts of the PAH. In contrast, no obvious B[b]F bioaccumulation occurred (Fig. 7.1B). As previously, Phe bioaccumulated in the liver of fish exposed to combined PAHs in a dose-response manner (Fig. 7.1C). However, B[b]F bioaccumulation in the liver was more obvious during the mixture assays even if in a much lower scale than its non-carcinogenic counterpart and yielding a less obvious dose-response (Fig. 7.1D). Altogether, there are two main issues that likely contributed to the major differences between Phe and B[b]F bioaccumulation: bioavailability and detoxification. In fact, the lower molecular weight Phe is

less hydrophobic than B[b]F, which most likely had two immediate consequences: it favoured its desorption of sediments, leading to increased Phe concentrations in water, the most bioavailable fraction (Table 7.1), and favours its transport through biological barriers, therefore increasing uptake.

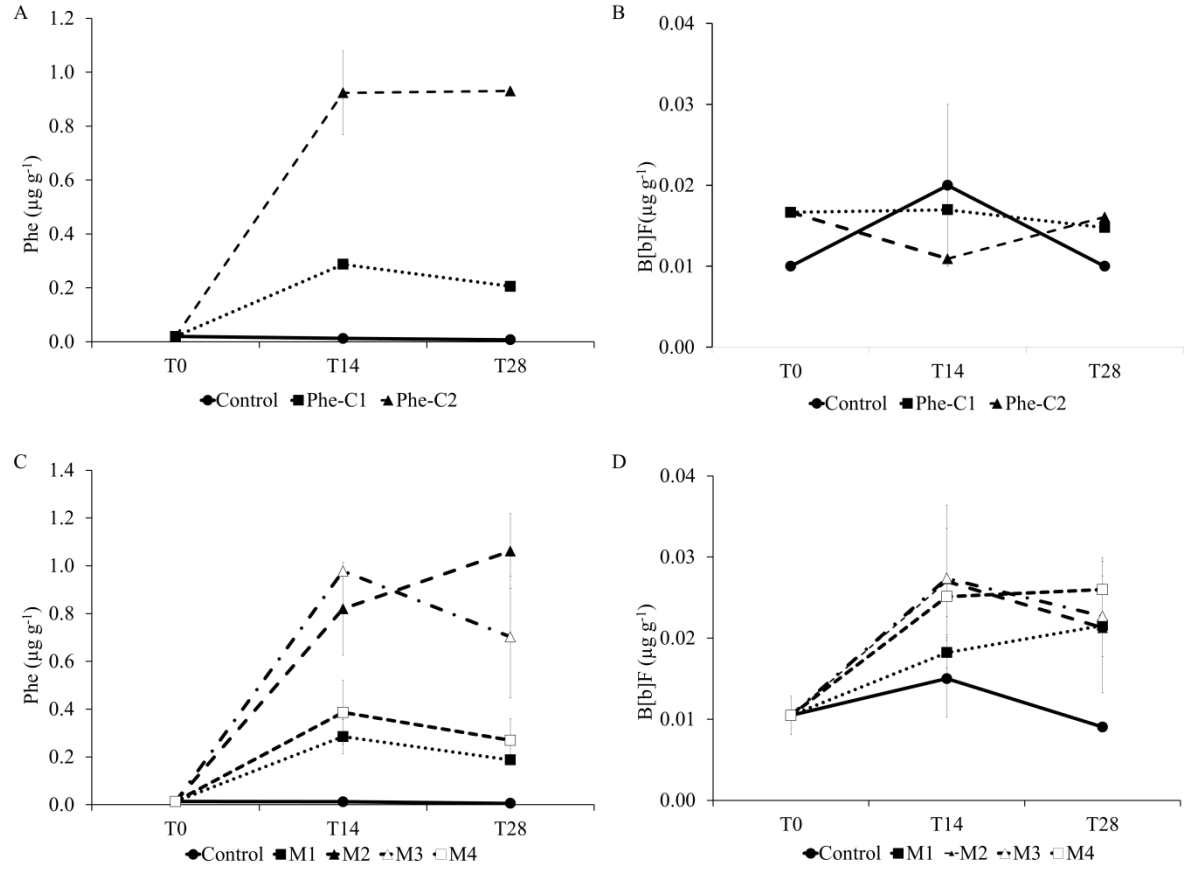

Fig. 7.1. Average concentrations (µg g^{-1} ww) of Phe and B[b]F compounds in liver of *D. labrax* exposed to isolated and combined PAHs.

Apart from the bioavailability, the differences between bioaccumulation of Phe and B[b]F may be attributed to the higher elimination rate of the latter. In fact, Thakker et al. (1985) observed that bullhead liver microssomes metabolised Phe to a lesser extent than less hydrophilic, higher molecular weight, PAHs, suggesting that the latter compounds are better substrates for bullhead hepatic microsomal enzymes.

The detoxification of PAHs by hepatic phase I enzymes may yield the formation of several metabolites, such as, hydrodiols, epoxides, phenols, quinones, dihydrodiols, dihydrodiol epoxides, tetrahydrotriols and tetrahydrotetrols, which may be concentrated in the bile of fish prior to elimination through the digestive tract. However, unlike for Phe, in absence of specific standards, B[b]F metabolites could only be pinpointed by screening a few known ions (Fig. 7.2 and Table 7.2). Still, overall differences between the patterns of potential metabolites were found in fish bile even

though, in face of the many constraints, caution is required when interpreting the results. Overall, the findings suggest relatively higher amounts of Phe metabolites in bile (Fig. 7.3), which is contradictory with the notion that the animals metabolised B[b]F more efficiently. Nonetheless, the results also

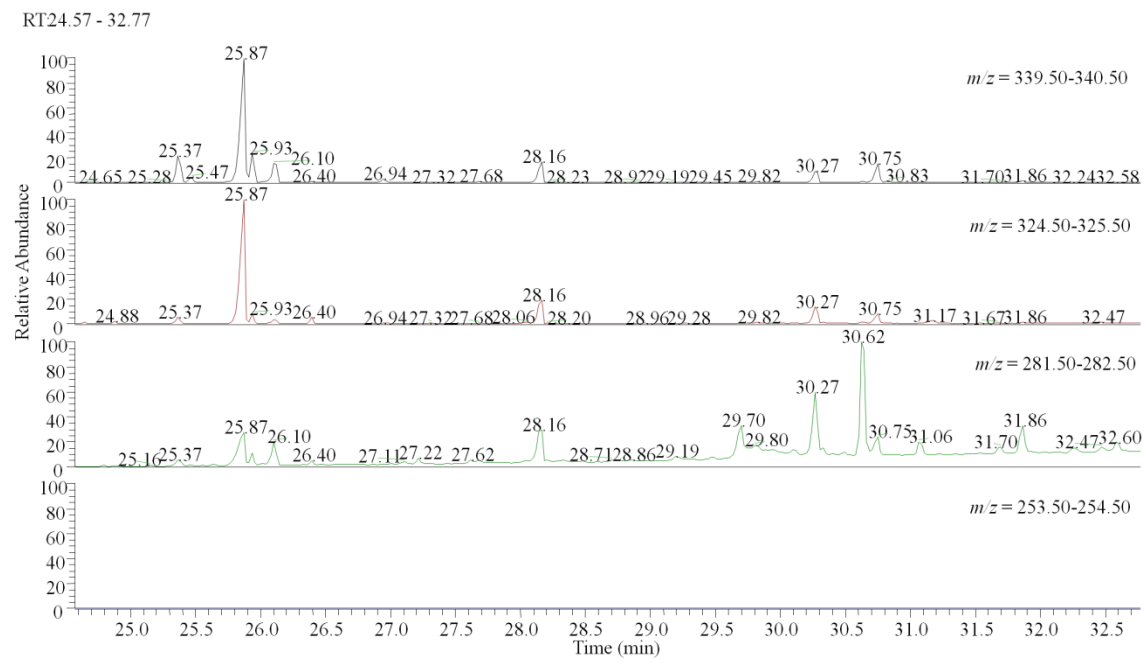

Fig. 7.2. Ion chromatograms corresponding to B[b]F selected ions (*m/z* 340, *m/z* 282, *m/z* 254) obtained from bile of fish collected from B[b]F isolated bioassay.

indicate that exposure to B[b]F, under the present circumstances at least, may yield a more complex pattern of metabolites than Phe, with respect to number of potential candidates and their relative proportion. As such, it is possible that B[b]F bioactivation results in a wider array of metabolites that is translated in overall higher relative amounts of total metabolites. Nonetheless, it must be highlighted that this is a preliminary result and further research is still needed, with respect to identification and quantification to fully disclose B[b]F metabolization.

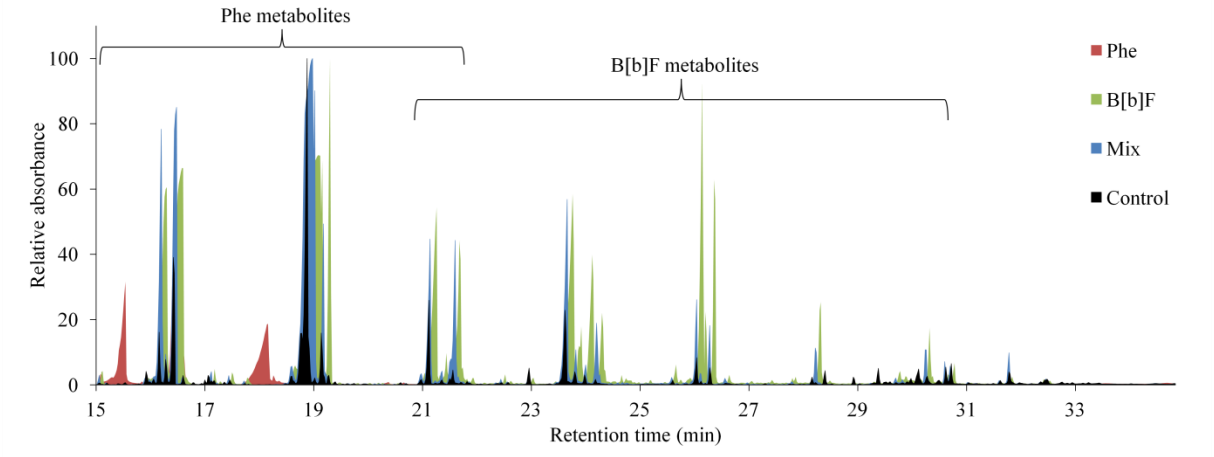

Fig. 7.3. GC-MS chromatograms of Phe and B[b]F metabolites present in bile of *D. labrax* exposed to tested sediments.

One of the most important differences between the patterns of metabolites was observed between exposure to isolated and combined PAHs even though, among the latter, no clear differences could at this stage be identified. Co-exposure seemingly increased Phe metabolite amounts while yielding the opposite effects for B[b]F (Fig. 7.3), which suggests agonist and antagonist interaction effects, respectively, of mixtures on the metabolism of the two PAHs. These potential antagonist effects on the bioactivation of B[b]F aid explaining some unexpectedly low biomarker responses (Aryl Hydrocarbon Receptor (AHR)-pathway related), and/or dilute dose-effect relationships retrieved from animals subjected to same assays (Martins et al., 2014b; Chapter 6).

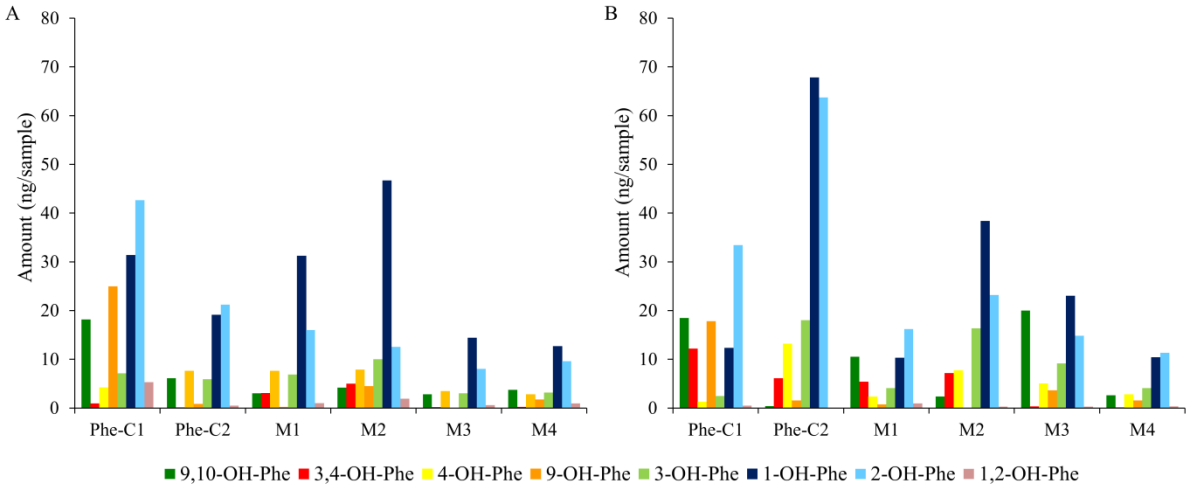

Fig. 7.4. Amount of Phe metabolites in bile of D. labrax exposed to the spiked-sediments assays during 14 (A) and 28 days (B).

Overall, the most significant metabolites able to be directly matched by co-chromatography were 1- and 2-hydroxyphenanthrenes in bile of fish exposed to Phe singly or combined (Fig. 7.4). Still, no clear pattern with time of exposure was observed for the metabolites, being the exception the increment registered for 1- and 2- hydroxyphenanthrene at T_{28}, Phe-C2 assay. Although it is long known that the most significant Phe metabolites are hydroxyls, which should yield reduced genotoxic effects when compared to epoxides produced from higher molecular weight PAHs (Wood et al., 1979), these results do not fully explain the genotoxic effects observed in fish peripheral blood cells (Martins et al., 2014a; Chapter 5). Overall, the present findings suggest that the pattern of PAH metabolites is intricate, whether interaction effects are involved or not, although clearly more so in the first case. It must also be highlighted that most of the literature on the subject is focused on the reactivity of PAHs towards DNA, due to the acknowledge relation between genotoxicity and mutagenesis and even carcinogenesis. However, even hydroxyls pose significant toxicological hazards, for instance, due to their high affinity to proteins (on the account of reacting with carboxyl groups, rendering stable esters), forming adducts that disrupt folding on proteins such as haemoglobin (Ragin et al., 2008). Thus, as previous findings indicate, Phe elicits more metabolic than genotoxic

effects, compared to B[b]F, whereas the mixture of both is likely involved in both. Considering the importance of adequate protein folding in basal cellular metabolism and on all upstream processes, including enzyme activity and gene transcription, it may be reasoned that traditional biomarker responses, such as EROD and GST activities and CYP induction (via AHR pathway), among others may be compromised. As such, there may be a gross underestimation of the toxic effects of non-carcinogenic PAHs like Phe and, moreover, of mixtures that tend to increment its metabolization through processes still little (or not at all) understood. Also, common biomarkers such as determining total PAH metabolites in fish bile may indicate exposure (Beyer et al., 2010) but not true toxicological risk. Therefore, the current work demonstrated how little is known about PAH metabolites and their production, and the importance of acquiring such knowledge to gather a realistic, and holistic, notion of hazard imposed by these priority pollutants.

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Martins, M., Santos, J.M., Diniz, M.S., Ferreira, A.M., Costa, M.H., Costa, P.M. (2014b, submitted). Effects of carcinogenic versus non-carcinogenic AHR-active PAHs and their mixtures: lessons from ecological relevance.

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CHAPTER 8. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Concluding remarks

Addressing the effects and responses of organisms to toxicological insult under ecologically-relevant circumstances remains one of the most challenging approaches within the field of Environmental Toxicology. Notwithstanding the need to produce realistic data, therefore apt for integration in ERA strategies and environmental management, there is relatively scarce research that attempts to integrate multiple biotic and abiotic factors within experimental designs. The multiplicity of these factors generates bias and renders difficult the interpretation of data. However, it is clear that the drawing of environmental quality guidelines, toxicity thresholds and identifying substances (or mixtures of substances) cannot disregard realism. In accordance, the present work demonstrated that, under ecologically-relevant circumstances indicating low risk to the biota, both carcinogenic and non-carcinogenic PAHs elicited toxic effects and responses in invertebrate and vertebrate species, being genotoxicity one of the keystone adverse effects. However, the results indicate distinct modes of action between the toxicants and, moreover, complex interaction effects between the substances, that surpass linear additive effects and responses, when binary mixtures of carcinogenic and non-carcinogenic PAHs were tested. Overall, the present work highlighted the need of understanding how toxicants become pollutants in the natural milieu and calls for further research on PAH mixtures and a redefinition of environmental standards in order to allocate potential effects of co-exposure since toxicants seldom, if ever, act isolated onto the biota.

Acknowledging that aquatic sediments may act as reservoirs of organic toxicants may lead to underestimating risk, since steady-state and disturbed sediments yield different abilities to release pollutants back to the biota. In fact, the first results (Chapter 2), obtained from *in situ* bioassays with mussels to address the effects of dredging operations within a harbour, indicated that the remobilization of toxicants from low-moderately contaminated sediments occurred and was able to induce significant effects in *M. edulis*. As a consequence, it became clear that sediment disturbance very significantly increases risk by favouring bioavailability of contaminants, including PAHs, thus contradicting environmental guidelines and norms for contaminated sediments, which predicted low or null risk. This finding, on its own, holds critical implications for environmental management of impacted marine ecosystems. The risk hitherto determined was associated to increased bioaccumulation, in *M. edulis* (moreover a commercial species), of the most hazardous PAH classes, namely 5/6-ring PAHs (especially B[b]F), deemed carcinogenic to aquatic organisms and humans. Also, increased bioaccumulation was correlated to elevated DNA strand-breakage. Also, even low levels of bioaccumulation of 3-ring PAHs (like Phe), in mussels, were correlated with oxidative stress. These results indicated the importance of surveying distinct classes of PAHs and their mixtures, as they occur in the environment, and disclosed that guidelines may not realistically reflect risk when complex toxicant matrices are involved, especially when dealing with toxicant mixtures whose

bioavailability is changed by the disruption of the sediments' steady-state.

Following these first findings, a fully laboratorial bioassay procedure (to reduce the effects of confounding factors) was undertaken, taking the two most representative PAHs in sediments classified as non-carcinogenic (Phe) and carcinogenic (B[b]F) as model substances. However, since the main goal was to establish a bridge between mechanistic studies and environmental monitoring, several assumptions were integrated into the experimental design to assure realism, specifically, selecting ecologically-relevant concentrations, a realistic vehicle (sediment), ecologically-relevant organisms and mixtures of PAHs. The development of bioassays under these specific circumstances constituted an important novelty in the field. Overall, the application of this holistic approach revealed that even concentrations that may be considered "low" and PAHs judged to pose low risk, like the non-carcinogenic Phe, are able to induce adverse effects in organisms. However, the low concentrations of exposure, as well as potential confounding factors from the vehicle (sediments) and organisms seemingly diluted the specificity of some biomarkers and, notably, dose-effect relationships in both clams (Chapter 4) and fish (Chapter 5 and 6). It must be noticed that the choice of target species is far from idle. With the prejudice of differences between the toxicological mechanisms *in vivo* of vertebrates and invertebrates (hereby demonstrated, at least in part), test species needed selection according to the type of bioassay and testing conditions: whereas mussels appeared adequate for deployment in cage-based experiments (for being hard-substrate bivalves), clams are seemingly adequate for testing in and *ex situ* when stable sediments are involved. Additionally, following the results obtained from bivalves, testing a fish of high ecological relevance, the sea bream, as a surrogate for bottom dwelling estuarine teleosts, steers the research towards animal models resembling higher-order vertebrates for which metabolic pathways are far better understood. The results indicated the adequacy of the species for testing or biomonitoring under complex toxicological scenarios where realism should be safeguarded. On the other hand, the deployment of substitutes for organisms in monitoring procedures, such as SPMDs, is a promising tool albeit untested in many scenarios and still in need of contrasting to living species in order to validate its purpose.

It should be noted that toxicological studies with aquatic organisms involving contaminants in water or in sediments are typically constrained by variables such as xenobiotic bioavailability and interactions, even though these issues are seldom addressed, with particular respect to PAHs. In fact, the desorption rates of Phe from sediments are higher than those for B[b]F due to his higher solubility in water. However, it is possible that the laboratory assays with Phe consisted in not just a three compartment model (i.e. sediment, water, organism) but rather a four-compartment (sediment, water, organism, atmosphere). As such, Phe losses to the atmosphere may continuously promote the release of this xenobiotic from the sediment to the water column, which may contribute to explain why the sediments' steady-state was not achieved during the exposure time, as first-hand demonstrated by the

SMPD assay (Chapter 3). The bioaccumulation results in clams (filter-feeders) showed the ability of these organisms to accumulate PAHs from either the dissolved phase or bound to suspended particles, unlike SPMDs, in the latter case. As such, bioaccumulation in clams was generally more consistent with a continuous process of uptake, biotransformation and elimination of metabolizable PAHs that may hinder risk assessment if PAH concentration in tissues is taken as main, or sole, endpoint. Still, it must be emphasized that little is known about the significance and metabolization mechanisms of PAHs by bivalves, when compared to fish and other vertebrates. Under this point-of-view, SPMDs may be of particular use to predict bioavailability, particularly of more easily excretable low molecular weight PAHs like Phe, albeit the need to ensure that equilibrium between water and the device is achieved.

Research comparing *in situ* and *ex situ* bioassays with living organisms has several inherent constraints, mainly related with sediment disturbance during handling, which should favour toxicant desorption. However, *in situ* bioassays performed during dredging operations (Chapter 2) and *ex situ* (i.e. laboratorial) assays with bivalves (Chapters 3 and 4) seem to provide comparable results, particularly when dredging is performed in a confined area like a harbour. In fact, similar results were obtained among the two types of assays in relation to bioavailability of PAHs plus genotoxic and oxidative stress effects, regardless of bivalve species. The *ex situ* bioassay results also demonstrated that genotoxicity was the main adverse effect elicited by the exposure to sediment-bound Phe and B[b]F, in both clams and sea bass, albeit higher for the latter compound (Chapter 5). Conversely, both fish and bivalves seem to have the ability to cope with the oxidative challenge elicited by exposure to low-moderate concentrations of either PAH in sediments, although unsufficient to avoid histological damage in fish and, besides DNA lesions. In fact, the present findings aid demystifying the notion that bivalves and other invertebrates are unable to efficiently metabolize PAHs, which should have been translated in reduced toxicological effects. However, it is clear that PAH detoxification pathways, especially concerning CYP equivalents, in molluscs and invertebrates in general is in need of further research. Furthermore, performing bioassays with PAH mixtures with bivalves as model organisms would be an important future endeavour to disclose the interaction mechanisms between the substances in these highly important invertebrates, whose abilities to bioactivate PAHs have often been underestimated.

Yet another challenging subject, analytically and in terms of data interpretation, the analyses of PAH metabolites in fish bile revealed the presence of a wide range of potential B[b]F metabolites, which suggests a higher number of candidates to form DNA-adducts in comparison with Phe metabolites (Chapter 6). However, the presence of hydroxyl-phenanthrenes may have also contributed to the formation of some adducts which may explain the unforeseen genotoxicity of these low-molecular PAH. Furthermore, the findings from the combined PAH assays suggest, to some extent, that co-

exposure could have favoured Phe metabolism, thus increasing the formation of hazardous Phe metabolites. This task confirmed the potential value of analysing PAH metabolites as biomarkers of exposure, however, it also made clear that such study is technically challenging and in great need of further research for instance, regarding the identification of specific metabolites or sets of metabolites for which no standards are available. However, it has been shown that exposure to combined PAHs modulates the pattern of metabolites which likely results in different genotoxic, mutagenic and even carcinogenic hazards comparatively to exposure to the isolated compounds. As such, in spite of its present technical handicaps, the current study was a ground-breaking initiative that showed that the application of mass spectrometry to identify distinct (potentially unknown) PAH metabolites may provide a better notion of risk than the common fixed-wavelength fluorescence analyses that are extensively used in monitoring programs.

One of the most important achievements of this work relates to the analysis of multiple effects and responses that point towards the various processes involved in PAH bioactivation and toxicity. Despite the fact than the combination of ecologically-relevant factors, particularly the low concentrations of the two PAHs, yielded complex patterns of effects and responses to exposure, surveying a battery of biomarkers in fish liver contributed to understand some of the aspects of the toxicological pathways of Phe and B[b]F and their combination. For instance, the results showed that, whereas exposure to isolated Phe yielded biochemical changes better related to oxidative stress, B[b]F disrupted metabolic responses (Chapter 6) and caused higher clastogenic/aneugenic effects, which, altogether represent the most severe DNA damage (Chapter 5). On the other hand, mixtures of PAHs yielded effects and responses compatible with the AHR dependent pathway, generating additive, if not synergistic, effect, which is accordant with elevated DNA damage (Chapter 5). Also, the histopathological assessment disclosed a variety of unspecific hepatic alterations related to inflammations and metabolic imbalance. However, exposure to B[b]F and, especially, the mixture of PAHs yielded the most diffuse and severe alterations, with particular respect to hepatocellular degenerative alterations, some of which may lead to pre-neoplasms (Chapter 6). Overall, even though the full disclosure of the toxicological mechanisms underlying exposure to either substances and their combination needs further research, especially at the molecular level, it was shown that the analysis of multiple biomarkers can provide an overall pattern of multi-level responses and effects that is consistent with toxicological challenge, even when the individual responses yield unclear results. In fact, one of the most important achievements of the present work was the creation of background knowledge that aids steering future research on the mechanisms of PAH-induced carcinogenesis, as well as contributing to the re-evaluation of the meaning of environmental quality norms.

In face of the present findings, it may be inferred that guidelines for single PAHs (and most likely for other substances as well) may be poorly significant in the context of environmental risk assessment by

underestimating toxicity thresholds modulating toxicant interaction effects, at least. Also, by analysing ecologically-relevant species it is possible to gather more objective information on the true risk underneath toxicological challenge. It must be highlighted that most, if not all, mechanistic studies consider acknowledged model species, from zebrafish to mice, which may hinder realism when extrapolating the findings to natural environments. Also, predicting risk without considering the different properties of PAHs and the differential proportions of specific PAHs, or classes of PAHs, within complex mixtures, may provide a biased overview of the mutagenic potential of contaminated sediments or any other realistic matrix. In fact, environmental risk assessment for toxicant mixtures, especially those whose mode-of-action is intricate at a subcellular level, like PAHs and other organic xenobiotics, should be a holistic approach focusing at multiple levels of biological organization. This is likely the most adequate perspective to search for expeditious biomarkers or sets of biomarkers apt for risk assessment *in situ*. However, it is clear that mechanisms need to be validated in the field in order to establish conclusive cause-effect relationships. For instance, the detection and quantification of specific genotoxic/mutagenic metabolites in fish bile, or perhaps the determination of specific forms of DNA adducts can be pertinent, even though this subject needs yet much research. Finally, it must be highlighted that the present study contributed to draw a bridge between the environment and the mode-of-action of environmental carcinogens, showing that the interaction effects between these substances may lead to results inconsistent with either theory or environmental guidelines, which, altogether, calls for a reinterpretation of risk analysis paradigms when addressing toxicologically-driven cancer as one of the 21st century's global epidemics.

Future perspectives

The present research consisted of a ground-breaking study, in design and results, on the interactions of sediment-bound PAHs. As such, the findings also revealed gaps and constraints related to the toxicological pathways of these substances. Although there are many issues to be investigated regarding PAH interaction effects in marine organisms, the following should be addressed in a more immediate term:

i) Research is need to enlighten the interaction effects of PAHs in invertebrate organisms, for which available information on the subject is essentially absent, concerning both mechanism and effect, especially considering realistic scenarios of exposure. This would imply, for instance, the molecular characterization of molluscan CYPs, or equivalents, gene sequencing and determining their activity.

ii) Even in fish, the molecular mechanisms underlying exposure to mixed PAHs need further research in order to disclose, for instance, alterations in the transcriptional patterns of AHR-mediated genes,

such as CYPs and to understand potential antagonistic/agonistic interactions. For the purpose, the deployment of state-of-art molecular tools, especially high-throughput techniques such as RNASeq would permit inferring on complex transcriptional patterns to steer research towards more specific pathways.

iii) Related to the previous, more mechanistics-oriented research on complex PAH interactions should be performed with other biological models, especially *in vitro* (e.g. with commercial fish or human cells liver or even primary cultures of fish hepatocytes), to further eliminate confounding factors. Still, it would be critical to validate the findings *in vivo* under ecologically-relevant scenarios, including on wild organisms. However, further research should focus on more complex mixtures of PAHs than binary combinations.

iv) Isolation and chemical characterization of PAH metabolites produced during combined exposures needs enhancement, including the optimization of extraction and identification techniques. This would permit determining specific metabolites that could be subjected to isolation and toxicological testing themselves to infer, for instance, on its genotoxic potential, thus leading to future applications of metabolite determination as biomarkers of exposure to carcinogenic mixtures of PAHs.

v) To develop environmental quality guidelines for mixtures containing distinct proportions of PAHs of different classes. This is probably one of the most challenging future endeavours, since it would imply the combination of all aforementioned topics and their integration through computational models and/or Best Expert Judgement approaches. Nonetheless, this approach is paramount to bring about the knowledge and promote its direct application within environmental monitoring, with particular emphasis on mutagenic and carcinogenic mixtures of PAHs.

ANNEXES

Annex 1. Outputs of Thesis

The following articles have been produced on the course of this thesis:

1. **Martins, M.**, Costa, P.M., Raimundo, J., Vale, C., Ferreira, A.M., Costa, M.H. (2012). Impact of remobilized contaminants in *Mytilus edulis* during dredging operations in a harbour area: bioaccumulation and biomarker responses. *Ecotoxicology and Environmental Safety*. 85: 96–103. [Chapter 2]

2. **Martins, M.**, Costa, P.M., Ferreira, A.M., Costa, M.H. (2013). Comparative DNA damage and oxidative effects of carcinogenic and non-carcinogenic sediment-bound PAHs in the gills of a bivalve. *Aquatic Toxicology*. 142-143: 85-95. [Chapter 4]

3. Santos, J.M., Costa, P.M., **Martins, M.** Costa, M.H. (2013). Comparative effects of sediments contaminated by carcinogenic and non-carcinogenic PAHs in *Dicentrarchus labrax*: a semi-quantitative histopathological approach. In: Borrego, C., Miranda, A.I., Arroja, L., Fidélis, T., Castro, E.A., Gomes, A.P. (Eds.). Actas da 10ª Conferência Nacional do Ambiente. Departamento de Ambiente da Universidade de Aveiro, Portugal. vol. I, pp. 51-56. [Annex 2]

4. **Martins, M.**, Costa, P.M. (2015). The Comet assay in Ecological Risk Assessment of marine pollutants: applications, assets and handicaps of surveying genotoxicity in non-model organisms. (doi:10.1093/mutage/geu037). [Chapter 1]

5. **Martins, M.**, Costa, M.H., Ferreira, A.M., Costa, P.M. (submitted). Comparing the genotoxic potential of carcinogenic and non-carcinogenic sediment-bound PAHs in fish peripheral blood: isolated versus interaction effects. [Chapter 5]

6. **Martins, M.**, Costa, M.H., Ferreira, A.M., Costa, P.M. (submitted). Differential uptake and accumulation sediment-bound phenanthrene and benzo[b]fluoranthene: a comparison between semi-permeable membrane devices and filter-feeding organisms. [Chapter 3]

7. **Martins, M.**, Santos, J.M., Diniz, M.S., Ferreira, A.M., Costa, M.H., Costa, P.M. (submitted). Effects of carcinogenic versus non-carcinogenic AHR-active PAHs and their mixtures: lessons from ecological relevance. [Chapter 6]

8. **Martins, M.**, Ronald van Bommel, Kees Booij, Ana M. Ferreira, Maria H. Costa, Pedro M. Costa. (submitted). Polycyclic aromatic hydrocarbon metabolite fingerprinting in the bile of fish exposed to sediment-bound single and combined compounds – preliminary results. [Chapter 7]

Results presented in scientific meetings:

1. **Martins M.**, Costa P. M., Raimundo J., Vale C., Ferreira A. M., Costa M. H., 2010. Impact of remobilized contaminants in *Mytilus edulis* during dredging operations in a harbour area: bioaccumulation and biomarker responses. ECSA 47 Symposium, Figueira da Foz, Portugal, 14-19 de Setembro 2010.

2. **Martins M.**, Ferreira, A.M., Costa, M.H., Costa, P.M., 2013. Comparing the genotoxic potential of carcinogenic ad non-carcinogenic sediment-bound PAHs in fish peripheral blood. ICOETox – 2 nd International Conference on Occupational & Environmental Toxicology, Porto, Portugal, 16-17 de Setembro de 2013.

3. Santos, J.M., Costa, P.M., **Martins, M.** Costa, M.H., 2013. Efeitos comparativos da exposição a sedimentos contaminados com PAHs cancerígenos e não cancerígenos em *Dicentrarchus labrax*: estudos histopatológicos. 10ª Conferência Nacional do Ambiente, Aveiro, Portugal, Novembro de 2013. Oral communication.

4. Pereira, S., **Martins, M.**, Costa, M.H., Costa, PM., 2014. Analysis of the interaction of polycyclic aromatic compounds in a model organism: integration of genotoxic and histopathological effects. IMMR - International Meeting on Marine Research, Peniche, Portugal, 10-11 de Julho 2014.

Awards:

Best presentation (Poster) in ECSA 47 Symposium, Figueira da Foz 2010, with the scientific work untitled "Impact of remobilized contaminants in *Mytilus edulis* during dredging operations in a harbour area: bioaccumulation and biomarker responses."

Annex 2. Comparative effects of sediments contaminated by carcinogenic and non-carcinogenic PAHs in Dicentrarchus labrax: a semi-quantitative histopathological approach[1]

J.M.Santos, P.M. Costa, M. Martins, M.H. Costa

Abstract

Polycyclic Aromatic Hydrocarbons (PAHs) are considered priority pollutants due to their high risk to the environment and to their carcinogenic potential to humans. Considering their hydrophobic properties, these substances tend to be trapped and stored in aquatic sediments. In order to understand the toxicity between carcinogenic and non-carcinogenic PAHs, laboratory assays were performed with juvenile seabasses (*Dicentrarchus labrax*) exposed to contaminated artificial sediments for 28 days. Sediments were spiked with environmentally-relevant concentrations of benzo[b]fluoranthene (a carcinogenic PAH) and phenanthrene (non-carcinogenic), either isolated or in mixture. Exposure effects were assessed in the liver, through an indice-based semi-quantitative histopathological approach. Overall, significant alterations in the hepatic tissue were detected relatively to control tests, either for isolated or mixture assays, despite the low levels of exposure. Individuals exposed to benzo[b]fluoranthene presented higher severity and number of hepatic lesions compared to phenanthrene. Mixture results suggest a possible synergistic interaction effect between the contaminants. This work allows the conclusion that, albeit considered low, environmentally-relevant concentrations of PAHs in sediments may cause adverse effects to demersal fish, however, mixed contaminants dilute dose– and time–dependent effects, compromising risk assessment. The results also suggest that a non-carcinogenic PAH may be responsible for considerable toxic effects, even in moderate concentrations. Altogether, requalifying risk assessment for these substances becomes of the upmost importance since PAHs (as other pollutants) are usually present in the environment in complex mixtures.

Keywords

Polycyclic Aromatic Hydrocarbons, sediments, histopathology, seabass, liver

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Introduction

The rising worldwide concern for water pollution and its effects confirms that this may be one of the biggest environmental issues in today's world. The European Union (EU) adopted a legislative tool entitled Water Framework Directive (WFD, updated through the Directive 2008/105/EC), later followed by the Marine Strategy Framework Directive (MSFD, Directive 2008/56/EC). The directives link with the Priority Substances list, in which are allocated Polycyclic Aromatic Hydrocarbons (PAHs), classified by the International Agency for Research on Cancer (IARC) as non-carcinogenic, potentially carcinogenic and carcinogenic to humans.

PAHs are usually associated with sediments due to their high hydrophobicity (reviewed by Meador et al., 1995). The ultimate fate of those PAHs that are trapped in aquatic sediments is believed to be biotransformation and biodegradation by aquatic organisms. In fish, as for other vertebrates, the liver is the organ most commonly involved in the detoxification of PAHs, and their metabolization may yield a metabolite that is more toxic than the parent compound. The PAHs metabolites are known to be highly genotoxic and carcinogenic, since some of which (e.g. PAH epoxides) bind covalently to DNA or RNA, forming bulky adducts that are not, if at all, easily repaired.

In order to compare the histophathological effects of a sediment-bound carcinogenic and non-carcinogenic PAHs in a benthic fish, animals were exposed to phenanthrene (Phe), a low molecular PAH not classified as a carcinogenic to humans and to benzo[b]fluoranthene (B[b]F), a high molecular PAH, considered as possibly carcinogenic to humans, but estimated as carcinogenic for fish and other wildlife (IARC, 1983).

Material and methods

Artificial sediments (6% total organic matter, 46.2% fine fraction) were spiked with two different concentrations ("low" and "high") of phenanthrene (Phe) and benzo[b]fluoranthene (B[b]F), isolated or combined (Table 1). In order to achieve ecological relevance, the choice of the concentrations was based on sediment quality guidelines (SQGs) for the toxicants, namely the Threshold Effects Level (TEL) and the Probable Effects Level (PEL) (MacDonald et al., 1996). In accordance, the concentrations referred to as "low" (C1) were targeted between TEL and PEL, whereas "high" (C2) as directly above PEL. Due to the lack of a guideline available for benzo[b]fluoranthene, the guideline used referred to benzo[a]pyrene, due to the chemical similarity between the two compounds. The nominal sediment concentrations are presented in table 1.

Table 1 – Nominal PAH concentrations (ng g^{-1}) used for artificial sediments of isolated and combined assays.

	Test assays	Control	Phe-C1	Phe-C2	B[b]F-C1	B[b]F-C2	M1	M2	M3	M4
Nominal concentrations	Phe	0	250	600	0	0	250	600	600	250
(ng g-1)	B[b]F	0	0	0	250	800	250	800	250	800

The laboratory assay was prepared according to Martins et al. (2013). Two hundred hatchery-brood sea bass juveniles (*Dicentrarchus labrax*) (standard length = 85.2 ± 8.5 mm; total wet weight = 9.90 ± 2.31) were divided by the different treatments. Assays were performed in duplicate, with each tank containing 10 individuals.

Animals were collected at days 0 (T_0), 14 (T_{14}) and 28 (T_{28}) of the experiment, euthanized by cervical sectioning and dissected immediately. Liver samples were prepared for histological analyses following Martoja and Martoja (1967), sectioned at 5µm, stained with haematoxylin and counterstained with alcoholic eosin (H&E stain).

Hepatic histopathological alterations were surveyed through a semi-quantitative approach, based on the weighted histopathological condition indices proposed by Bernet et al. (1999), with slight modifications (Costa et al., 2013). In brief: the individual hepatic histopathological condition indice (I_h) was estimated according to the concepts of the differential biological significance of each surveyed alteration (weight) and a numerical attribute that reflects the degree of dissemination of the alteration within the surveyed organ (score).

For each individual, the respective pathological changes were classified into three reaction patterns: circulatory disturbances (I_1), regressive changes (I_2) and progressive alterations (I_3). Circulatory disturbances result from a pathological condition of blood and tissue fluid flow, although fluid content alterations in tissues related to inflammatory processes are also considered in this case. Regressive changes are processes which terminate in a function reduction or loss of an organ while progressive changes lead to an increased activity or function alteration of cells or tissues.

The failure to meet least one assumption to perform parametric analysis of variance (normality of data and homogeneity of variances), the non-parametric Mann-Whitney *U* test was employed to determine pairwise differences. Discriminant analysis was used to assess the relative significance of each reaction pattern in the distinction between assays. Statistics were performed using Statistica (StatSoft Inc).

Results

Fish collected at the beginning of the experiment (T_0) presented a hepatic architecture consistent with that of normal juvenile teleosts with hepatocytes presenting a fairly polyedric shape with a clear cytoplasm and a spherical nucleus with conspicuous nucleoli (Figure 1A). Lesions and alterations were infrequent. The liver of control fish collected at T_{14} and T_{28} times displayed high resemblances to T_0 fish.

Overall, fish exposed to either PAH, isolated or in mixture, presented higher hepatic alterations relatively to control animals. Longer exposure (28 days) caused greater severity and dissemination. Likewise, livers of fish exposed to the contaminant mixture also sustained more damage-related lesions (such as haemorrhage and necrosis), at both T_{14} and T_{28}. Focal haemorrhage was often observed in hyperaemic livers (Figure 1B), especially in fish exposed to higher concentrations of both contaminants and in mixture assays. The latter also presented more signs of necrosis commonly accompanied by macrophage intrusion (Figure 1C), which was common in all assays, albeit with higher prevalence in fish exposed to mixtures, occasionally forming dense centres (Figure 1D). With the exception of exposure to lower concentrations of B[b]F at T_{14}, all tests caused an increase in the global hepatic histopathological indice I_h compared to T_0 and control fish (Figure 1). The livers of fish exposed to higher concentrations (C2) of either PAH, at both sampling times, presented higher I_h than fish exposed to the isolated compounds. No clear differences were detected in livers of animals exposed to mixture treatments.

Overall aspect of the morphology of a normal juvenile liver from a control individual, exhibiting sinusoids (s) that diffuse from a branch of the hepatic portal vein (hpv) containing few blood cells (e). The blood vessels are surrounded by well-defined hepatocytes (h) with polyedric shape and a translucent-clear cytoplasm with a spherical nucleus. (B) Haemorrhage in a fish of a mixture assay regarding highest concentrations of both PAHs at T_{28}, characterized by blood cells (e) invading liver parenchyma possibly caused by an extensive fat vacuolation, potentially leading to lipidosis (fv). Inset: Detail of a swollen blood vessel, with erythrocytes and defence cell accumulation. (C) Necrotic foci (n) with macrophage defence cell intrusions (m) common in fish exposed to mixture treatments. Inset: Detail of a bile duct with fat vacuolation. (D) Macrophage aggregate (arrow) on a necrotic tissue identified by their high affinity towards haematoxylin (basophilic) in an individual exposed to the lowest concentration assay of B[b]F for 28 days. Inset: melanomacrophage aggregate containing mostly melanin-like pigments.

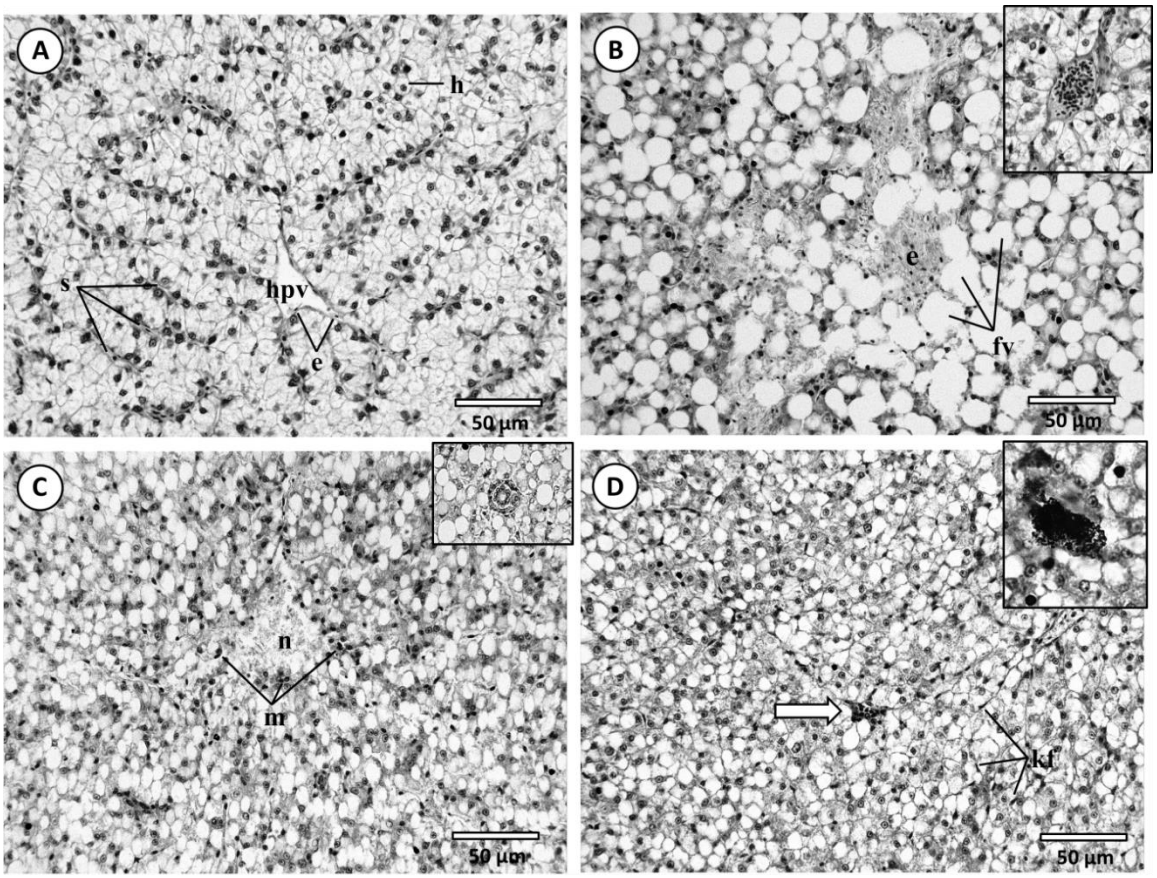

Figure 1 – Histopathological lesions and alterations observed in the livers of *D. labrax* (H&E). (A) Discriminant analysis revealed that inflammatory response/circulatory disturbances (I1) was the most significant reaction pattern contributing to differentiate between isolated and mixture assays at lower concentrations (M1). On the other hand regressive changes (I_2) contributed the most to differentiate between higher concentration mixture (M2) and higher concentration (C2) isolated assays. Also, different concentration mixtures (M3 and M4) and corresponding concentrations of isolated contaminants (C1 and C2) displayed differences in progressive alterations (I_3) (Table 2).

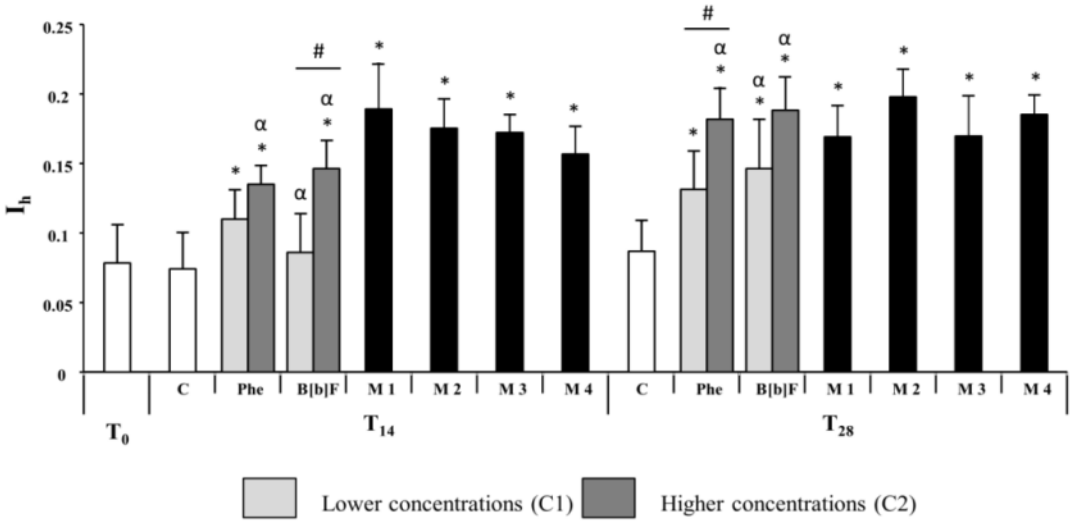

Figure 2 - Comparison of the average global hepatic histopathological indice (I_h) between fish exposed to isolated and mixture contaminated sediments at sampling times T_0, T_{14} and T_{28}; * means significant differences between contaminated and control assays, $p < 0.05$ (Mann-Whitney U test). α means significant differences between T_{14} and T_{28} assays, $p < 0.05$ (Mann-Whitney U test). # means significant differences between C1 and C2 concentrations in isolated assays, $p < 0.05$ (Mann-Whitney U test). Error bars indicate 95% confidence intervals.

Table 2 - Discriminant analysis results when comparing between mixture and isolated assays with the corresponding concentration as grouping variable factor. Lowest Wilk's λ statistic was employed to assess best model. *F*-tests determined the most significant variables (α = 0.05). The models' dependent variable is the hepatic histopathological condition indice (I_h) obtained for each individual.

		Variables							
		Model		I1		I2		I3	
		Wilk's λ	p to remove	Wilk's λ	p to remove	Wilk's λ	p to remove	Wilk's λ	p to remove
Factors to descriminate	Case								
M 1 × Phe-C1	T14	0.37	0.00 *	**0.64**	**0.00**	0.38	0.42	0.47	0.05
	T28	0.35	0.01 *	**0.93**	**0.00**	0.39	0.27	0.38	0.33
M 1 × B[b]F-C1	T14	0.26	0.00 *	**0.46**	**0.00**	0.29	0.22	**0.37**	**0.03**
	T28	0.76	0.33	0.95	0.11	0.76	0.76	0.79	0.53
M 3 × Phe-C2	T14	0.17	0.00 *	0.19	0.28	**0.44**	**0.00**	**0.55**	**0.00**
	T28	0.76	0.27	0.76	0.94	0.85	0.22	0.91	0.13
M 3 × B[b]F-C1	T14	0.24	0.00 *	0.29	0.14	0.24	0.92	**0.46**	**0.01**
	T28	0.47	0.16	0.74	0.37	0.71	0.60	**0.93**	**0.05**
M 4 × Phe-C1	T14	0.54	0.02 *	0.67	0.08	0.68	0.07	0.60	0.23
	T28	0.37	0.00 *	0.41	0.29	0.41	0.28	**0.54**	**0.02**
M 4 × B[b]F-C2	T14	0.53	0.05	0.64	0.15	0.54	0.83	**0.90**	**0.02**
	T28	0.85	0.46	0.85	0.76	0.85	0.74	0.99	0.13
M 2 × Phe-C2	T14	0.35	0.00 *	0.35	0.73	**0.54**	**0.01**	**0.71**	**0.00**
	T28	0.73	0.21	0.82	0.22	0.75	0.64	0.92	0.08
M 2 × B[b]F-C2	T14	0.29	0.00 *	0.34	0.18	**0.41**	**0.04**	**0.87**	**0.00**
	T28	0.81	0.38	0.89	0.25	0.81	0.79	0.94	0.16

*best model to assess discrimination between factors
Bold figures indicate significant variables within the model

Discussion

The present work revealed that sediments contaminated by ecologically-relevant concentrations of the two PAHs, either isolated or combined, caused significant histopathological alterations in the livers of fish, consistent with chronic hepatic disease (rather than acute). It must be noted that the present study surveyed PAH concentrations between the boundaries of low and high risk to exert deleterious effects to the biota. Under this point of view, the current findings are in accordance with the expected moderate levels of hepatic histopathological alterations. Isolated PAH assays revealed a clear increase from animals collected at T_{14} to T_{28} animals, although B[b]F induced only marginally higher histopathological alterations to its termed "non-carcinogenic", lower molecular weight counterpart (Phe). Higher number of benzene rings or differences in its metabolic pathway *in vivo* may explain B[b]F higher toxicity. Due to the lack of knowledge regarding B[b]P and its effects, a similar and extensively studied high-risk PAH may be used for comparison – Benzo[a]pyrene (B[a]P). B[a]P is more prone to metabolic activation by CYP mixed-function oxygenases (MFO) than Phe, meaning faster elimination from tissues, generating reactive oxygen species (ROS), which may lead to severe oxidative stress. Also, B[a]P is known to induce AHR-mediated gene expression (e.g. CYP1A), thus increasing the production of toxic metabolites (Akcha et al., 2000). Reduced I_h from B[b]F-C1 tests at

T_{14} suggests that even PAH-naïve fish may be able to respond to low B[b]F concentrations, likely being able to metabolize this compound within the ability to cope with ROS and other by-products of activation. However, high I_h in fish exposed to B[b]F at T_{28} (both concentrations) suggest cumulative effects, while the causes of elevated phenanthrene-induced alterations at lower concentrations remain elusive.

The combined exposures caused distinctively higher and earlier levels of histopathological alterations. However, the degree of global histopathological alterations was unexpectedly lower than the sum of effects elicited by the two substances and, moreover, dose- and time-dependent effects were unclear. This suggests an interaction between the contaminants, possibly linked to effects on the PAH metabolization pathway.

The lack of a temporal trend following combined exposure may be due to the "low" concentrations used in this work, compared to the high, often subacute, exposures usually employed in most PAH studies. Also, PAH concentration may be held constant in the water column, gradually being released from sediments, reaching a steady-state condition, and permitting some sort of adaptative in fish at latter stages of exposure, while fish collected at T_{14} were still enduring the initial phase of homeostatic and metabolic disturbance, (see Steinberg et al., 2008).

Interestingly, contrasting the effects of mixtures and isolated contaminants with their concentrations showed specific differences in reaction patterns for each mixture (Table 2). Fish exposed to the mixture comprising the lowest concentrations (M1) presented more signs of inflammation. Mixtures comprising combination of "high" and "low" concentrations of PAHs (M3 and M4) displayed an increase in progressive changes while M2, as the mixture comprising the highest concentrations of both PAHs, revealed and increase in regressive changes, considered of greater severity (such as necrosis). Also, fish exposed to this mixture endured more alterations at T14 than fish subjected to isolated PAH assays, which means that PAH mixtures may elicit alterations faster than the isolated contaminants.

Conclusions

The current findings confirmed that sediments contaminated with PAHs, even in "low" and environmentally-relevant concentrations, are able of inducing hepatic lesions and alterations in a demersal fish, consistent with sub-lethal toxicopathological effects. Individuals exposed to phenanthrene presented lower liver histopathological alterations than benzo[b]fluoranthene especially at T28, thus contributing to confirm a positive relation between the number of benzene rings and

toxicity. Also, mixture PAHs may induce higher damage, albeit without a clear dose- or time-dependent pattern, which may suggest interactions between the two contaminants, a critical issue that yet remains elusive, even though PAHs are usually present in the environment in complex mixtures.
This work shows that the SQGs hereby considered, were consistent with the overall moderate level of hepatic lesions, since these thresholds allocated exposure between the levels of "low" and "high" potential to cause adverse effects to organisms. Nevertheless, it must be pointed out that SQGs provide an empirical measure of risk that may not necessary integrate mixtures, which calls for further understanding on the effects of combined toxicants and their influence on establishing effective thresholds for risk assessment strategies.

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