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Comparative effects of sediments contaminated by carcinogenic and non-carcinogenic PAHs in *Dicentrarchus labrax*: a semi-quantitative histopathological approach

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Abstract

Polycyclic Aromatic Hydrocarbons (PAHs) are considered priority pollutants due to their high risk to environmental and human health. Due to their hydrophobic character, in aquatic environments, these substances tend to adsorb to the particulate fraction and accumulate in the sediments. Despite their division into carcinogenic, potentially carcinogenic and non-carcinogenic to humans, little is known about the differences between modes of action of carcinogenic and non-carcinogenic PAHs in aquatic organisms.

In order to understand the toxicity mechanisms of these two classes, laboratory assays were performed with juvenile basses (*Dicentrarchus labrax*) exposed to contaminated artificial sediments for 28 days. Sediment 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 analysed through an indice-based semi-quantitative histopathological approach in hepatic tissue, due to the role of liver in the accumulation and detoxification of xenobiotics.

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. Furthermore different toxicants caused different patterns of histopathological lesions and alterations. The results also show that histopathological condition indices of mixture-exposed individuals do not match the expected additive effects, suggesting 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 in organisms, in this case, a demersal fish. On the other hand, 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, seabasses, histology, liver, carcinogenic, non-carcinogenic, sediment contamination

Resumo

Os hidrocarbonetos aromáticos policíclicos (PAHs) são poluentes considerados prioritários devido ao seu elevado risco para a saúde ambiental e humana. Devido ao seu carácter hidrofóbico, estas substâncias tendem a adsorver à fracção particulada e a acumular-se no ambiente sedimentar. Apesar da divisão dos PAHs em cancerígenos, potencialmente cancerígenos e não-cancerígenos para humanos, pouco se sabe sobre a diferença entre o modo de acção destas substâncias em organismos aquáticos.

Com o intuito de compreender as diferenças entre os mecanismos de toxicidade entre duas classes de PAHs, realizaram-se ensaios laboratoriais com robalos juvenis (*Dicentrarchus labrax*) expostos durante 28 dias a sedimentos artificiais contaminados com diferentes concentrações ambientalmente relevantes de benzo[b]fluoranteno (PAH cancerígeno) e fenantreno (PAH não cancerígeno), isolados ou em mistura. Considerando o fígado como órgão-alvo, devido ao seu papel na acumulação e desintoxicação de xenobióticos, os efeitos da exposição aos PAHs foram analisados através de uma abordagem de índices histopatológicos semi-quantitativos.

No geral, foram detectadas alterações significativas no tecido hepático causadas por ambas as substâncias, isoladas ou em mistura, comparativamente ao teste controlo. Indivíduos expostos a benzo[b]fluoranteno, classificado como cancerígeno, apresentaram mais lesões hepáticas, em termos de severidade e disseminação, comparativamente à exposição a fenantreno. Para além disso, foram identificados padrões diferentes de lesões histopatológicas consoante o contaminante. Os resultados mostraram também que o índice de condição histopatológica em indivíduos expostos à mistura não corresponde aos efeitos cumulativos esperados, o que sugere uma possível interacção sinergística entre os contaminantes. Este trabalho permite concluir que concentrações de PAHs nos sedimentos, mesmo que consideradas baixas, embora ambientalmente relevantes, podem causar efeitos adversos nos organismos, neste caso um peixe de carácter demersal. Por outro lado, os resultados sugerem também que um PAH não cancerígeno pode ser responsável por efeitos tóxicos consideráveis, mesmo se presente em concentrações moderadas, possivelmente exponenciados pela co-exposição a PAHs cancerígenos. Desta forma torna-se premente requalificar os níveis de risco destas substâncias uma vez que os PAHs (tal como outros poluentes) estão normalmente presentes no meio ambiente incluídos em misturas complexas de substâncias.

Palavras-chave: Hidrocarbonetos Aromáticos Policíclicos, robalos, histologia, fígado, cancerígeno, não-cancerígeno, contaminação de sedimentos

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Abbreviation list

AHR – Aryl Hydrocarbon Receptor

ARNT – Aryl Hydrocarbon Nuclear Translocator

B[a]P – Benzo[a]pyrene

B[b]F – Benzo[b]fluoranthene

B[k]F - B[k]fluoranthene

CAT - Catalase

CYP – Cytochrome P450

DMSO – Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

EPA – Environmental Protection Agency

EQS – Environmental Quality Standards

EROD - ethoxyresorufin-*O*-deethylase

EU – European Union

FAO – Food and Agriculture Organization

FF – Sediment Fine Fraction

GPx – Glutathione peroxidase

GSH – Reduced glutathione

GST – Glutathione *S*-Transferase

IARC – International Agency for Research on Cancer

I_h – Histopathological condition indice

MFO – Mixed Function Oxygenase

MSFD – Marine Strategy Framework Directive

PAH – Polycyclic Aromatic Hydrocarbon

PCD – Programmed Cell Death

PEL – Probable Effects Level

Phe – Phenanthrene

RNA – Ribonucleic Acid

ROS – Reactive Oxygen Species

SOD – Superoxide Dismutase

TEL – Threshold Effects Level

TOM – Total Organic Matter

WFD – Water Framework Directive

XRE – Xenobiotic Response Element

1. **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. In an attempt to set water protection policies, 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), both pointing objectives for water protection in the future by setting quality standards and suggesting local policies to determine the degree of impacts and ensuring that clean waters remain unpolluted. The WFD states that EU Member States should not only apply Environmental Quality Standards (EQSs) for superficial waters, but also, if possible, for the biota or sediments in order to achieve good ecological and chemical status. The WFD lists various priority substances for which ESQs are set, one major group of those contaminants being Polycyclic Aromatic Hydrocarbons (PAHs). Also, the International Agency for Research on Cancer (IARC) classified PAHs as non-carcinogenic, potentially carcinogenic and carcinogenic to humans.

Polycyclic aromatic hydrocarbons have two or more fused benzene rings, often containing alkyl side groups, and they are present in the marine environment as a result of forest fires, volcanism and petroleum sweeps, combustion processes and petroleum–based products (reviewed by Meador et al., 1995). Low molecular-weight PAHs are usually defined as those possessing two or three aromatic rings while high-molecular PAHs possess four or more rings. PAHs are usually associated with sediments due to their high hydrophobicity and low solubility in water, whereas hydrophobicity increases with their molecular weight (Meador et al., 1995). In the aquatic environment, those contaminants are present as complex mixtures of aromatic and aliphatic compounds (see for instance Douben, 2003 for a review).

Owing to PAH partitioning between the different environmental compartments and due to different routes of uptake for organisms, differential accumulation is expected for different species (Meador et al., 1995). High hydrophobicity ensures that, in an organism with weak metabolic ability for these compounds, PAHs are accumulated in fatty tissues, which is hardly reversible (Eisler, 1987). Various aquatic organisms like mussels,clams, crabs, polychaetes and others, with slow rates of PAH metabolization and excretion, have shown adverse effects when exposed to PAHs (eg: Pisoni et al., 2004; Martins et al., 2013).

In fish, as for other vertebrates, the liver is the organ most commonly involved in the detoxification of PAHs, hence being targeted to determine the effects of exposure to PAHs. Biotransformation enzymes present in the livers of fish, convert lipid-soluble organic xenobiotics to water-soluble,

more excretable, metabolites. PAHs are easily metabolized by the phase I enzymes of the mixed function oxygenase system (MFO), to more hydrophilic products like phenols, dihydrodiols, quinones and epoxides, although most PAHs are only excreted after conjugation (adding a large polar group) by phase II enzymes such as GST-glutathione through conjugation with GSH-reduced glutathione (refer to van der Oost et al., 2003, for a review).

However, the biotransformation of metabolizable PAHs (termed bioactivation) may yield a metabolite that is more toxic than the parent compound, while producing reactive oxygen species (ROS) as by-products (reviewd by Altenburger et al., 2003). The different size and structure of various PAH compounds also influence the type and magnitude of toxicological effects such as narcotic effects, carcinogenicity, mutagenicity, and genotoxicity (Logan, 2007). In general, toxicity exerted by metabolites increases as molecular weight from parent PAH increases. In fact, metabolites from higher weight PAHs 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 (as reviewed by Douben, 2003).

In order to provide information on the potential exposure to toxicants, biomarkers are commonly employed in ecotoxicological studies. A biomarker is defined as a change in a sub-individual biological response (ranging from molecular through cellular and physiological responses to behavioral changes), which can be related to exposure to, or toxic effects of, environmental chemicals (Martín-Díaz et al., 2004). The utility and applicability of histological biomarkers has been surveyed for a wide range of marine organisms and, in a majority of those studies, fish are used as model organisms. In an attempt to determine the degree of histopathological alterations in fish, several authors developed indices that lead to a better understanding of histological findings after contaminant exposure (see, for instance: DelValls et al., 1998; Oliva et al., 2009). Histo-cytological responses are relatively easy to determine by expert researchers, and causal relationships have indeed been established between fish histopathological lesions and levels of pollution in the marine environment (Au, 2004). Fish liver histopathology studies are considered a useful tool to be incorporated in monitoring programmes assessing the health of ecosystems and the effect of contaminants (Lang et al., 2006). The growing number of studies on histopathological biomarkers is linked to the notion that they reflect fish health more realistically than biochemical biomarkers and can thus be better extrapolated to community- and ecosystem-level effects of toxicity (Au, 2004). Also, this category of biomarkers allows easier examination of specific organs (for example gills, kidneys, liver) responsible for vital functions such as respiration, accumulation and biotransformation of xenobiotics in fish, for which alterations may serve as warning signs of adverse effects of animal health (Stentiford et al., 2003). Individual histopathological indices are gaining particular interest, especially weighted indices. These indices are based on the premise that

distinct histological changes may not share the same impact (biological significance) to the animal. In this case, the empirical value of a given histopathological alteration is based on two factors: the extension of a pathological change and the pathological importance of the alteration or "weight" (Bernet et al., 1999; Costa et al., 2011).

Fish are often chosen as models in PAH bioassay-based ecotoxicology studies for several reasons, such as: being particularly vulnerable receptors to PAH contamination; their habitats may be close to human settlements and thus close to potential sources of contaminations (which is of particular relevance for coastal ecosystems); and also ecological importance as well as recreational and commercial value (Logan, 2007). Studies dealing with PAH exposure in fish vary widely in their approach, mainly because there are different possible exposure routes and apical organs of entry, such as gills for their role in gas exchange and salt uptake and excretion; gut from food, sediment or detritus ingestion, and even direct absorption through the integument (see, for instance: Fragoso et al., 2006; Gonçalves et al., 2008; Kopecka-Pilarczyk and Correia, 2009; Sanchez et al., 2009). Also, bioassays may be performed in laboratory or in situ (field). While the latter may yield more ecologically-relevant outcomes, the former holds the considerable advantage of eliminating much effect from noise variables on biomarker responses, including histopathological traits, even when complex toxicant matrices are involved, such as contaminated sediments (Costa et al., 2012).

The European sea bass (*Dicentrarchus labrax* Linnaeus, 1758, Perciformes: Moronidae) is an eurythermic and euryhaline coastal demersal species that often inhabits estuaries and other confined waters subject to strong anthropogenic stressors. It is found in waters all around Europe, from eastern Atlantic Ocean to the Mediterranean Sea and Black Sea, including Portuguese waters. The species also holds high economic importance for fisheries and aquaculture, as well as high ecological value (being a top-chain predator). The species is known to be sensitive to PAH and able to metabolize many of these compounds (Gravato and Santos, 2002; Ferreira et al., 2010). However, studies with sea bass and other species often deal with biochemical responses only and, furthermore, with exposures to single substances at high concentrations that surpass ecologically-realistic concentrations in waters and natural sediments. In addition, there is, to date, no information regarding the comparison between the effects of PAHs deemed non- or potentially carcinogenic on fish or even the majority of organisms and, especially, their combination, even though PAHs in the environment occur in mixtures. In fact, the most common efforts to determine sediment toxicity focus on the measurement of acute responses, whereas sediment-associated contaminants usually results in sub- lethal, chronic effects (Martín-Díaz et al., 2004).

2. Objectives

In order to compare the effects and responses of a sediment-bound carcinogenic and non-carcinogenic PAHs in a benthic fish, animals were exposed to phenanthrene (Phe), a low molecular PAH classified as a non-carcinogenic to humans and 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, 2013) and included the list of priority substances (WFD).

Phenanthrene is commonly used as a model substrate for studies involving the metabolism of low molecular PAHs, since it is composed of three benzene rings. Although neither considered mutagenic nor carcinogenic, its toxicity has been demonstrated in aquatic organisms (USEPA, 1990). On the other hand, benzo[b]fluoranthene is a five-ring PAH and knowledge about its toxicological effects is currently scarce. Nevertheless, a similar PAH, benzo[a]pyrene (also 5-ring), is probably the best known model PAH, and its toxicity, as well as carcinogenicity, is widely acknowledged in ecotoxicological research (Varanasi et al., 1986; Akcha et al., 2000; Gravato and Guilhermino, 2009).

Specifically, the main objectives of this thesis may be summarized as follows:

- To identify histological lesions and alterations in the liver of *Dicentrarchus labrax* exposed to ecologically-relevant concentrations of sediment-bound phenanthrene and benzo[b] fluoranthene;
- Estimate individual weighted indices through a semi-quantitative histopathological approach;
- Distinguish the effects in the hepatic parenchyma caused by exposure to a carcinogenic and a non-carcinogenic PAH, whether isolated or in mixture.
- To seek for time- and dose-dependent relationships, i.e., between time of exposure and sediment PAH concentrations and histopathological changes in the liver of the test species.

3. Material and Methods

3.1. Sediment spiking

The artificial sediment was prepared by mixing sandy and muddy estuarine sediments (1:3) in order to obtain a final sediment holding 5-10% total organic matter (TOM) and 50% fine size particles, i.e.< 67 μm (FF), which stands for the average composition of estuarine sediments from potentially polluted areas in Portugal (see for instance Carreira, 2013). For the purpose, sediment samples were collected from the Mira estuary, an area considered devoid of direct input of hazardous substances and one of the least impacted coastal areas in Portugal (Vasconcelos et al., 2007). Final sediment FF was determined by hydraulic sieving after digestion with H_2O_2 and disaggregation with pyrophosphate, yielding the value of 46.2%, relative to sediment dry weight. Total organic matter was inferred from carbon loss-on-ignition by combustion at 450 ± 50 ºC. The final sediment TOM was recorded to be 6%.

Sediments were spiked with two different concentrations of phenanthrene (Phe) and benzo[b]fluoranthene (B[b]F), hereforth termed "low" (C1) and "high" (C2). In order to achieve ecological relevance, the choice of the concentrations was based on the thresholds determined by (MacDonald et al., 1996) as numerical sediment quality guidelines (SQGs). Accordingly, the toxicant SQGs may attain the concentrations below which adverse effects only rarely occur (TEL - Threshold Effects Level) or surpass the concentration above which adverse effects frequently occur (PEL - Probable Effects Level). In accordance, the concentrations referred to as "low" were targeted between TEL and PEL, whereas "high" as directly above PEL. Due to the lack of a guideline available for benzo[b]fluoranthene, the guideline used referred to benzo[a]pyrene, considering the chemical similarity between the two compounds. The TEL and PEL values are, respectively, 86.7 ng g^{-1} and 544 ng g^{-1} for phenanthrene and 88.8 ng g^{-1} and 763 ng g^{-1} for benzo[a]pyrene (McDonald et al., 1996).

Sediments were spiked according to the method described by Hickey and Roper (1992) and Martins et al (2013). The contaminants (dissolved in Dimethyl sulfoxide - DMSO) were directly added to the artificial sediment expected nominal concentrations of 250 ng g^{-1} (C1) and 600 ng g^{-1} (C2) for phenanthrene and 250 ng g^{-1} (C1) and 800 ng g^{-1} (C2) for benzo[b]fluorenthene (Table 3.1), for isolated exposures. For the mixture assays, different combinations of both PAHs were used. As such, M1 comprised the lowest concentrations of both PAHs, while M2 contained the highest (C2). Assays comprising different concentration levels of each PAH are regarded as M3 (Phe-C2 + B[b]F-C1) and M4 (Phe-C2 + B[b]F-C1) (Table 3.1). The sediments were allowed to

equilibrate for 48h at 4ºC after 15 min of mechanical mixing. The control sediments were prepared in a similar way and spiked only with DMSO.

3.2. Bioassays

The laboratory assay was prepared according to Costa et al. (2009) and Martins et al (2013). Briefly: 2 L of freshly-collected sediments were placed in 15 L-capacity white polyvinyl tanks with blunt edges to which 10 L of clean, 0.45 µm-filtered water was added. Sediments were then allowed to settle for 48h before the beginning of the assay. The tanks were supplied with continuous aerations. A weekly 25% water change was done to maintain constancy of parameters with minimal removal of suspended particles and contaminants. Photoperiod was set at 16:8 h light:dark. Water parameters were monitored weekly and were observed to be: pH = 7.9 ± 0.2, salinity = 32 ± 1g L^{-1}, temperature = 18 ± 1 °C, dissolved O_2 ranged between 92 and 95% and total ammonia was maintained within 2-4 mg L^{-1}. Fish were fed daily with M2 grade commercial fish pellets (Aquasoja, Portugal). The full experimental procedure was divided into nine experimental treatments: Control, Phe-C1, Phe-C2, B[b]F-C1, B[b]F-C2, plus the combination assays, termed M1, M2, M3 and M4 (see Table 3.1). Two hundred hatchery-brood *Dicentrarchus labrax* juveniles (standard length = 85.2 ± 8.5 mm; total wet weight = 9.90 ± 2.31) were divided into the different treatments. Assays were performed in duplicate with each tank containing 10 individuals.

Table 3.1. Nominal PAH concentrations (ng g^{-1}) used for spiking artificial sediments of isolated (Phe-C1, Phe-C2, B[b]F-C1, B[b]F–C2) and combined assays (M1, M2, M3, M4).

	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

3.3. Sample preparation for histological analyses

Five animals were collected at days 0 (T_0), 14 (T_{14}) and 28 (T_{28}) of each experiment, euthanized by cervical sectioning and dissected immediately. Liver samples were prepared for histological analyses following Martoja and Martoja (1967). Fresh liver samples were immersed in Bouin-Hollande's fixative (20 mL 37% v/v formaldehyde, 5 mL of 100% v/v acetic acid and picric acid added until saturation). Fixation was done at 4ºC for ≈ 48h.

Sample dehydration was performed in a progressive series of ethanol followed by an intermediate impregnation with xylene and embedded in paraffin. Sections (5 µm thick) were cut (using a Jung RM2035 model rotary microtome) and at least 8 sections per slide were obtained. A clearing bath was performed using xylene after staining.

The sections were stained with haematoxylin in order to stain basophilic structures like nucleic acids such as cell nucleus and ribosomes. Counterstain was made with alcoholic eosin. The slides were allowed to dry and were prepared for optical microscope analysis with a mounting media (DPX mountant). Microscopic analysis was conducted with a DMLB model microscope equipped with a DFC480 digital camera (Leica Microsystems).

3.4. Histopathological condition indices

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. 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). The weight of the alterations is classified into three important factors: 1 - minimal pathological importance, the lesion is easily reversible; 2 - moderate pathological importance, the lesion is reversible in most cases if the stressor is neutralized; and 3 - marked pathological importance, the lesion is generally irreversible, leading to partial or total loss of the organ function. Every alteration is assessed using a score ranging from 0 to 6, depending on the degree and extent of the alteration. The score can attain the values of 0 where a lesion is infrequent up until 6, in case of a severe occurrence. Intermediate values were also considered. The histopathological condition indices were estimated according to the formula proposed by Costa et al. (2013):

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

Where I_h is the histopathological condition for the individual *h*; w_j 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). For each individual, the respective pathological changes were classified into three reaction patterns: circulatory disturbances, regressive and progressive alterations. 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 weights for each histopathological trait were defined by Bernet et al (1999) and

Costa et al. (2009, 2011). Table 3.2 summarizes the histopathological biomarkers surveyed and their respective weights. A blind review was performed at the end of analyses in 25% of the samples to confirm accuracy of observations, the error being ≈ 12%.

Table 3.2. Histopathological alterations (biomarkers) observed in liver of *D. Labrax* and their respective condition weight (*w*)

Reaction pattern	Alteration	w
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. (2011)

[c] Weights according to Costa et al. (2013)

3.5. Statistical analyses

Failing to meet least one of the assumptions to perform parametric analysis of variance, namely the homogeneity of variances (tested through the Levene's test), led to the employment of non-parametric analysis, namely Mann-Whitney *U* test to determine pairwise differences between the mean I_h values for different treatments. Cluster analyses based on the 1-Pearson correlation r statistic was used to investigate links between the weight × score values for the different histopathological traits. Discriminant analysis was employed to determine the relative significance of each reaction pattern in the distinction between assays, according to concentration and sampling time. Also, simple comparison was made with mixtures and isolated contaminants according to the concentration of each contaminant. For example, the treatment involving the mixture of highest concentration of contaminants was compared with the treatments with the isolated contaminants at highest concentration and so on. A significant level of $\alpha = 0.05$ was set for all analyses. Statistics were performed using Statistica (StatSoft Inc).

4. Results

4.1. Liver histopathology

Fish collected at the beginning of the experiment (T_0) presented a hepatic architecture consistent with that of normal juvenile teleosts with normal hepatocytes presenting a fairly polyedric shape with a translucent-clear cytoplasm and a spherical nucleus with conspicuous nucleoli (see, e.g., Hibyia, 1982). The parenchyma contained blood vessels, carrying few blood cells (mostly erythrocytes), that branch into sinusoids surrounded by hepatic cells (Fig. 4.1A). Also, these livers displayed little or no signs of inflammatory response, with defence cell infiltration being rare or absent, usually reduced to few macrophages (probably Kupffer cells) intruding the parenchyma, typically near blood vessels. Similarly, progressive and regressive alterations were infrequent. Control fish collected at both sampling times displayed high resemblances to T_0 fish.

Overall, fish exposed to either contaminant, be it isolated or in mixture, presented signs of hepatic alterations relatively to control animals. Alterations in fish subjected to longer exposures (28 days) presented greater severity and dissemination than those sampled at T_{14}. Likewise, livers of fish exposed to the contaminant mixture also sustained more damage-related lesions (such as haemorrhage and necrosis) than isolated exposures, in both exposure times (T_{14} and T_{28}).

Amongst the alterations most often observed in livers of fish exposed to PAHs, circulatory disturbances and regressive changes were some of the most conspicuous. Hyperaemia was often observed in livers of fish exposed for 28 days, although no sample presented signs of dilation and blood flow changes on all its blood vessels (usually, this alteration was observed in the most affected livers, in the majority of blood vessels). Intrusion of erythrocytes into the parenchyma (haemorrhage) from ruptured vessels (Fig 4.1C) was often observed in hyperaemic vessels (Fig 4.1B) and was infrequent in animals exposed to low concentrations of either toxicants or their mixtures. Macrophages were also commonly detected in all samples, with higher prevalence in the most damaged livers and, again, fish subjected to mixtures or longer exposures presenting greater Kupffer cell infiltration as well as macrophage agglomerates (Fig. 4.1D). Sporadic melano-macrophage intrusion was detected on animals from every bioassay, even in controls, albeit with higher prevalence in fish exposed to mixtures, occasionally forming dense centres (Fig. 4.1D, inset). Higher number of macrophage agglomerates was also found in livers that sustained higher damage, especially in or nearby necrotic tissue (Fig. 4.2A). Melanomacrophages were chiefly identified by the presence of vesicles containing melanin-like pigments. Melanomacrophages containing ceroid/lipofuscin-like pigments (also termed chromolipoid pigments) were rare.

Necrotic foci, considered as a regressive alteration, were observed in all assays, but its dissemination and occurrence is clearly distinct from control and T_0 fish to animals exposed to PAHs, the latter presenting more diffuse necrotic tissue areas, as opposed to small, occasional foci. Necrotic foci in livers of fish exposed to isolated contaminants was observed chiefly at higher exposure times (T_{28}) while livers of mixture assays presented a similar degree of necrotic tissue dissemination regardless of exposure time (Fig 4.2A). Necrotic tissue usually presented nuclear pleomorphisms, such as pyknosis or hypertrophy. Also, higher evidence of apoptosis, i.e., programmed cell death (PCD), was found in these samples and was identified by agglomerates of apoptotic bodies and early stages of PCD (Fig. 4.2B).

Fat degeneration (potentially leading toward lipidosis in the most severe cases) was the most common hepatocytic alteration found. In this progressive change, hepatocytes tend to lose their polyedric shape due to hepatocellular lipid vacuolation, increasing their size and revealing nuclei and cytoplasm compressed against the plasmalemma (Fig. 4.2C). Microvesicular fat degeneration (potentially leading to steatosis) (Fig. 4.2D), identified by the intracellular accumulation of multiple small lipid vesicles, was restrained to small foci, usually in livers where lipidosis was already present in a moderate to low dissemination degree. Exposure to isolated compounds presented more signs of this microvesicular alteration, where fat degeneration (also referred to as lipid vacuolation) was substantially high.

No significant evidence of bile duct structural alterations was found, although fish exposed to PAH mixtures (at higher concentrations of either contaminant) presented vacuolization of bile duct epithelial cells, revealing some degree of alterations to bile tubules (Fig. 4.2A). The most infrequent histopathological trait was fibrosis, and only on highly affected livers.

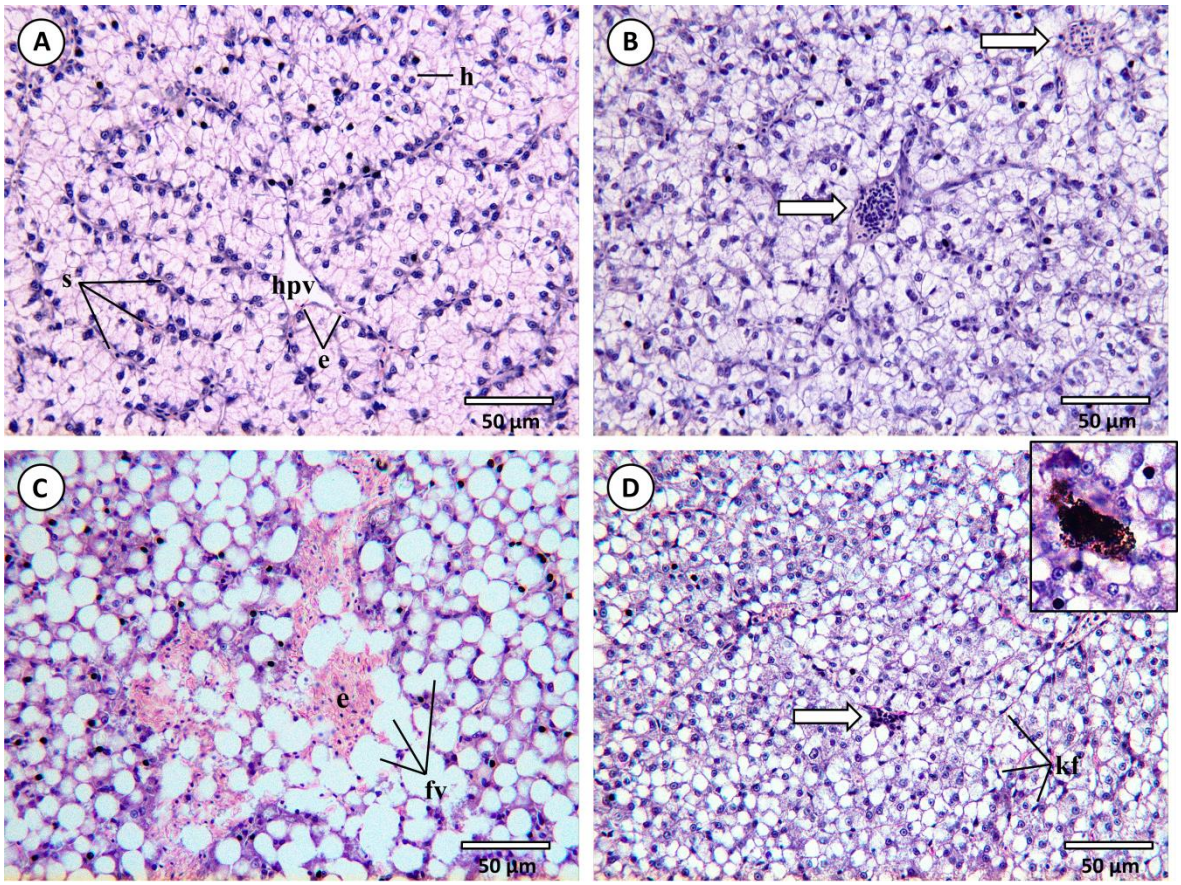

Fig. 4.1. Common histopathological lesions and alterations observed in the livers of *D. Labrax* (H&E). (A) Overall aspect of the morphology of a normal juvenile liver from a control individual, exhibiting sinusoids (s) that diffuse from a branch of the hepatic portal vein (hpv) containing few blood 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) Swollen blood vessels, with erythrocytes and defence cell accumulation, indicating hyperaemia (arrows) in a fish exposed to high concentration of Phe after 14 days. (C) 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). (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, with kupffer cells invading the parenchyma (kf). Inset: melanomacrophage aggregate containing mostly melanin-like pigments.

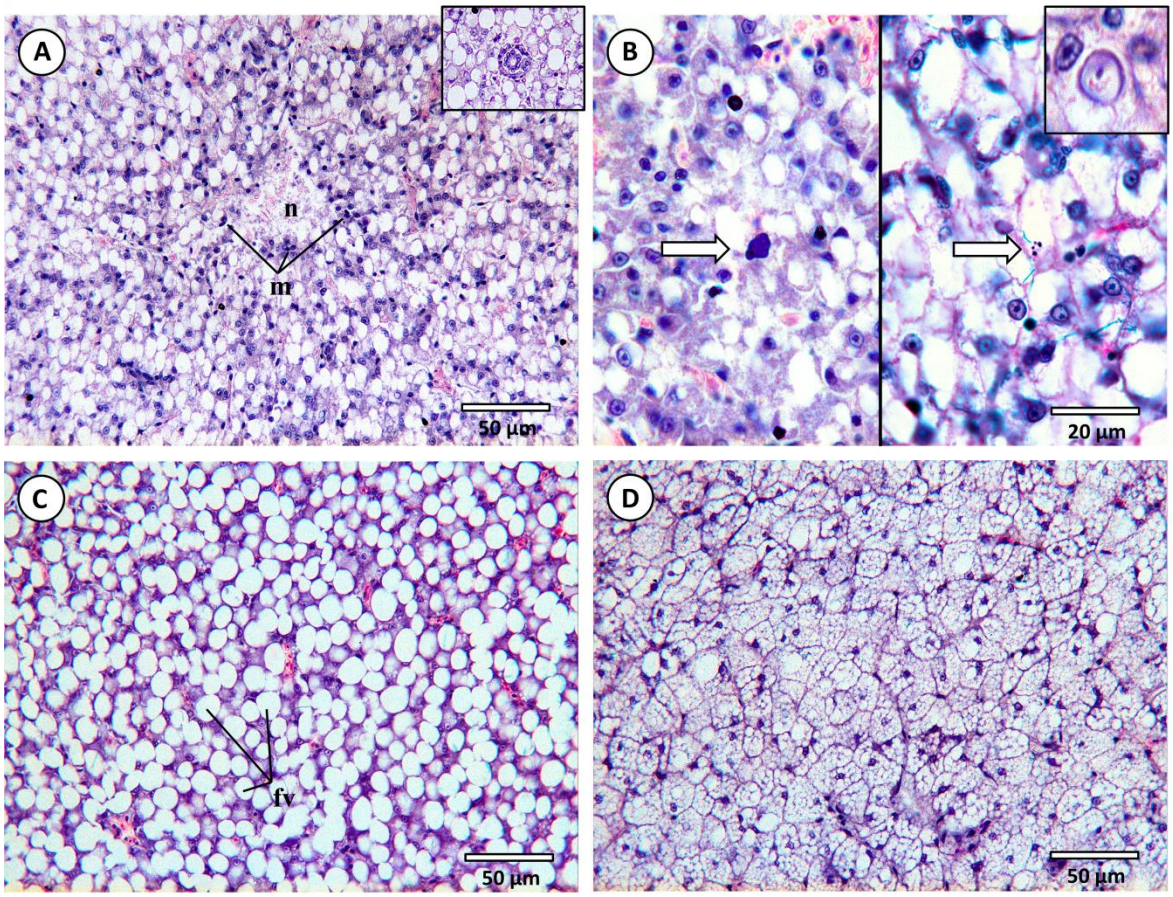

Fig. 4.2. Common histopathological lesions and alterations observed in the livers of *D. Labrax* – continuation (H&E). (A) Necrotic foci (n) with macrophage defence cell intrusions (m) from a fish exposed to mixture treaments at T_{28}. Inset: detail of a bile duct with fat vacuolation. (B) Left panel indicates early stage of apoptosis from an animal exposed to the "high" concentration of B[b]F after 28 days. Right panel: apoptotic bodies as a result of programmed cell death (PCD) (arrows). Inset: detail of a nuclear pleomorphism. (C) Fat vacuoles (fv) leading towards severe lipidosis, common in fish exposed to either PAHs, in this case, to the lowest concentration of Phe after 28 days. (D) Diffuse microvesicular fat vacuolation (steatosis) caused by excessive lipid accumulation in hepatocytes in a fish from the previously mentioned assay.

4.2. Hepatic histopathological condition indices

With the exception of individuals exposed to lower concentrations of benzo[b]fluoranthene at T_{14}, all tests caused an increase in the global hepatic histopathological indice I_h compared to T_0 and control fish (Fig 4.3). T_0 and control fish presented no significant differences. The livers of fish exposed to isolated contaminants yielded distinct I_h between exposures to higher and lower concentrations, with animals subjected to the higher concentrations of either toxicant presenting higher I_h. This difference is visible at both sampling times T_{14} and T_{28}, however, statistical differences were only identified on fish exposed to benzo[b]fluoranthene at T_{14} and Phenanthrene at T_{28} (Mann-Whitney U, $p<0.05$). No clear differences were detected in livers of animals exposed to mixture treatments, with both sampling times presenting similar global I_h for all treatments,

regardless of concentrations. Also, fish subjected to these treatments revealed a resemblance to those exposed to higher concentrations of isolated contaminants at T_{28}.

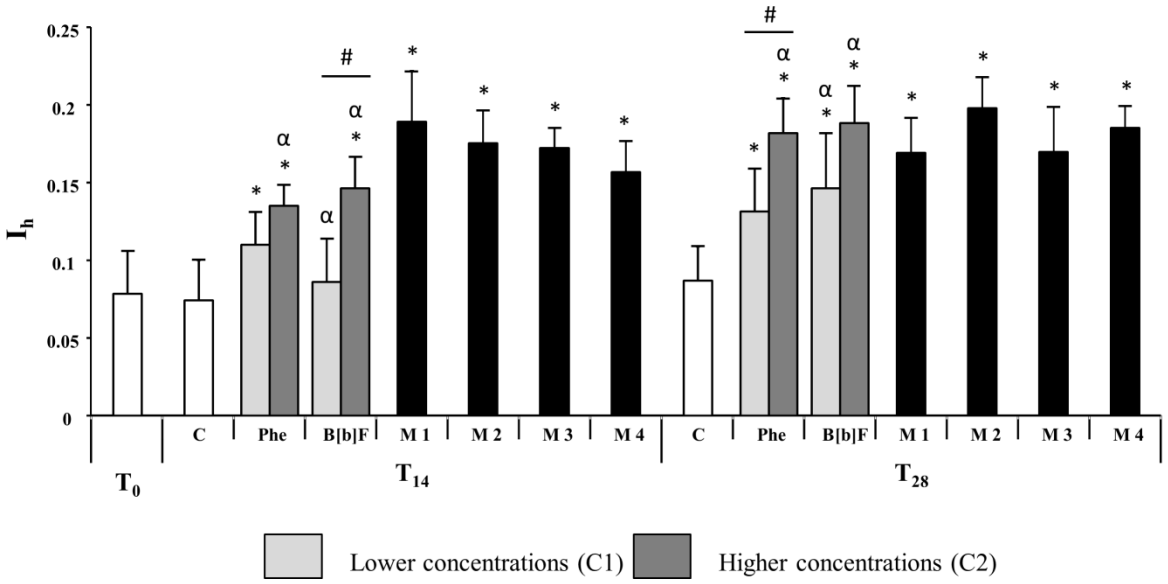

Fig. 4.3. Comparison of the average global hepatic histopathological indice (I_h) between fish exposed to isolated and mixture contaminated sediments at sampling times T_0, T_{14} and T_{28}; * means significant differences between contaminated and control assays, $p < 0.05$ (Mann-Whitney U test). α 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.

All indices for each reaction pattern presented a similar variation to that of I_h (Fig. 4.4). Accordingly, mixtures and higher concentrations of isolated assays commonly held higher scores of histopathological alterations for each reaction pattern, when comparing to control and T_0 fish. Inflammatory response (I_1) presented statistical differences between fish exposed to both concentrations of B[b]F for both sampling times (Fig. 4.4A). Regressive changes (I_2), however, revealed significant differences between sampling times of higher concentrations on isolated assays for both contaminants (Fig. 4.4B). Average histopathological indice score regarding progressive changes (I_3) also exhibited no clear statistical variations between higher and lower concentrations, however, sampling times for B[b]F assays in both concentrations displayed relevant statistical disparities (Fig.4.4C).

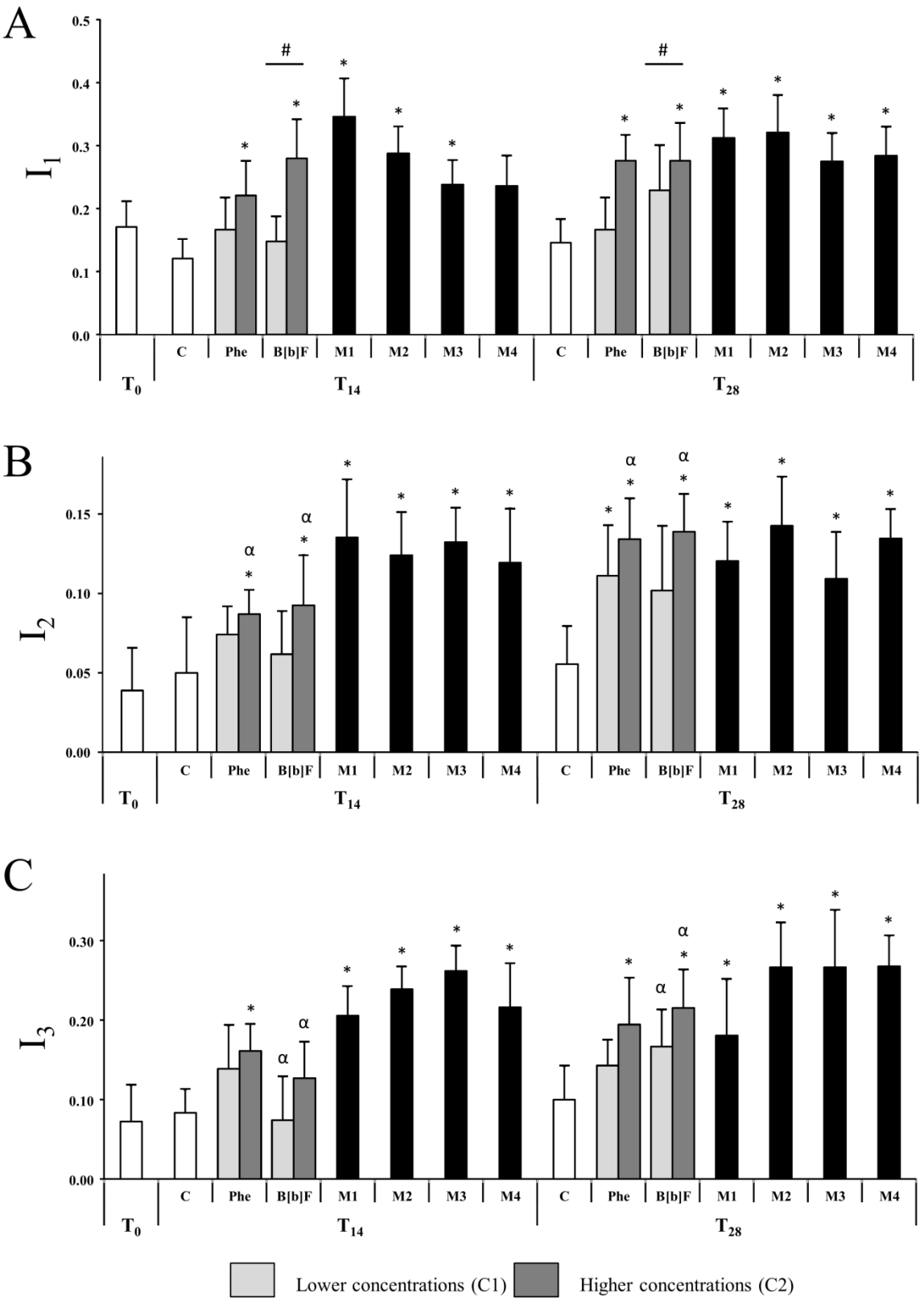

Fig. 4.4. Comparison of the average hepatic histopathological indice for each reaction pattern between fish exposed to isolated and mixture contaminated sediments at sampling times T_0, T_{14} and T_{28}; (A) Circulatory disturbances/Inflammatory response (I_1); (B) Regressive alterations (I_2); (C) Progressive alterations (I_3); * 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.

Through discriminant analysis (Table 4.1) it was observed that there were no reaction patterns that significantly contributed to differentiate between the isolated contaminant assays, when testing for concentration of exposure. On the other hand, discriminant analysis revealed that regressive (I_2) and progressive changes (I_3) contributed the most to differentiate between sampling times. Also, comparing mixture treatments, particularly M1×M3 and M1×M4, revealed circulatory disturbances/inflammatory response (I_1) to be the most significant reaction pattern contributing to the differentiation between tests, principally at sampling time T_{14} (Table 4.2). Inflammatory response/circulatory disturbances (I_1) was the most significant reaction pattern contributing to differentiate between isolated and mixture assays at lower concentrations (Table 4.3). 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 (Table 4.3). The model that best described the differences between mixture and isolated contaminants was observed mainly at T_{14} (Wilk's $\lambda = 0.17$, $p \approx 0$).

Table 4.1. Discriminant analysis results taking sampling time and concentration of exposure as grouping variables (factors) for isolated assays. Lowest Wilk's λ statistic was employed to assess best model. F-tests determined the most significant variables ($\alpha = 0.05$). 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								
Sampling time: $T_{14} \times T_{28}$	P1	0.69	0.17	0.75	0.30	**1.00**	**0.03**	0.74	0.34
	P2	0.44	0.01 *	0.45	0.68	**0.83**	**0.00**	0.58	0.06
	B1	0.57	0.05	0.66	0.17	0.57	0.91	0.77	0.05
	B2	0.36	0.01	0.38	0.88	**0.63**	**0.02**	**0.71**	**0.01**
Phe-C1 × B[b]F-C1	T14	0.80	0.33	0.86	0.31	0.83	0.52	0.96	0.10
	T28	0.73	0.31	0.94	0.10	0.81	0.29	0.80	0.35
Phe-C2 × B[b]F-C2	T14	0.78	0.34	0.91	0.17	0.78	0.99	0.85	0.28
	T28	0.95	0.89	0.96	0.71	0.98	0.61	0.99	0.47
Phe-C1 × Phe-C2	T14	0.82	0.36	0.92	0.18	0.85	0.49	0.86	0.39
	T28	0.52	0.06	**0.77**	**0.04**	0.52	0.91	0.53	0.55
B[b]F-C1 × B[b]F-C2	T14	0.47	0.03 *	**0.80**	**0.01**	0.50	0.42	0.47	0.96
	T28	0.71	0.24	0.71	0.86	0.81	0.22	0.85	0.15

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

Table 4.2. Discriminant analysis results taking sampling time and concentration of exposure as grouping variables (factors) for mixture assays. 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								
Sampling time: $T_{14} \times T_{28}$	M1	0.93	0.78	0.95	0.55	0.93	0.96	0.96	0.51
	M2	0.86	0.48	0.91	0.37	0.90	0.44	0.93	0.28
	M3	0.81	0.42	0.90	0.25	0.92	0.21	0.82	0.69
	M4	0.76	0.21	0.84	0.22	0.85	0.18	0.79	0.46
M 1 × M 2	T14	0.76	0.21	0.89	0.12	0.79	0.46	0.86	0.17
	T28	0.74	0.22	0.76	0.54	0.78	0.38	0.93	0.08
M 1 × M 3	T14	0.51	0.03 *	**0.76**	**0.03**	0.59	0.20	0.55	0.38
	T28	0.76	0.26	0.79	0.44	0.78	0.59	0.93	0.10
M 1 × M 4	T14	0.66	0.10	**0.97**	**0.02**	0.66	0.88	0.69	0.48
	T28	0.67	0.11	0.11	0.38	0.72	0.31	**0.92**	**0.03**
M 2 × M 3	T14	0.73	0.24	0.90	0.11	0.82	0.23	0.79	0.32
	T28	0.83	0.38	0.88	0.35	0.92	0.20	0.83	0.86
M 3 × M 4	T14	0.86	0.60	0.86	0.91	0.89	0.52	0.97	0.24
	T28	0.88	0.52	0.90	0.63	1.00	0.15	0.89	0.67

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

Table 4.3. 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

Cluster analysis based on correlation statistic 1-Pearson r indicated the degree of correlation between the observed individual histopathological changes (Fig. 4.5). The analysis indicated a high correlation of hepatocyte necrosis with macrophage infiltration and a high link with these and fat vacuolation. These alterations, together with haemorrhage and hyperaemia formed a distinct cluster. Nuclear pleomorphisms indicated a feeble correlation degree with steatosis. By their turn, fibrosis, bile duct atrophy and apoptosis, although showing some link between them, comprised a distinct cluster to that of preceding alterations.

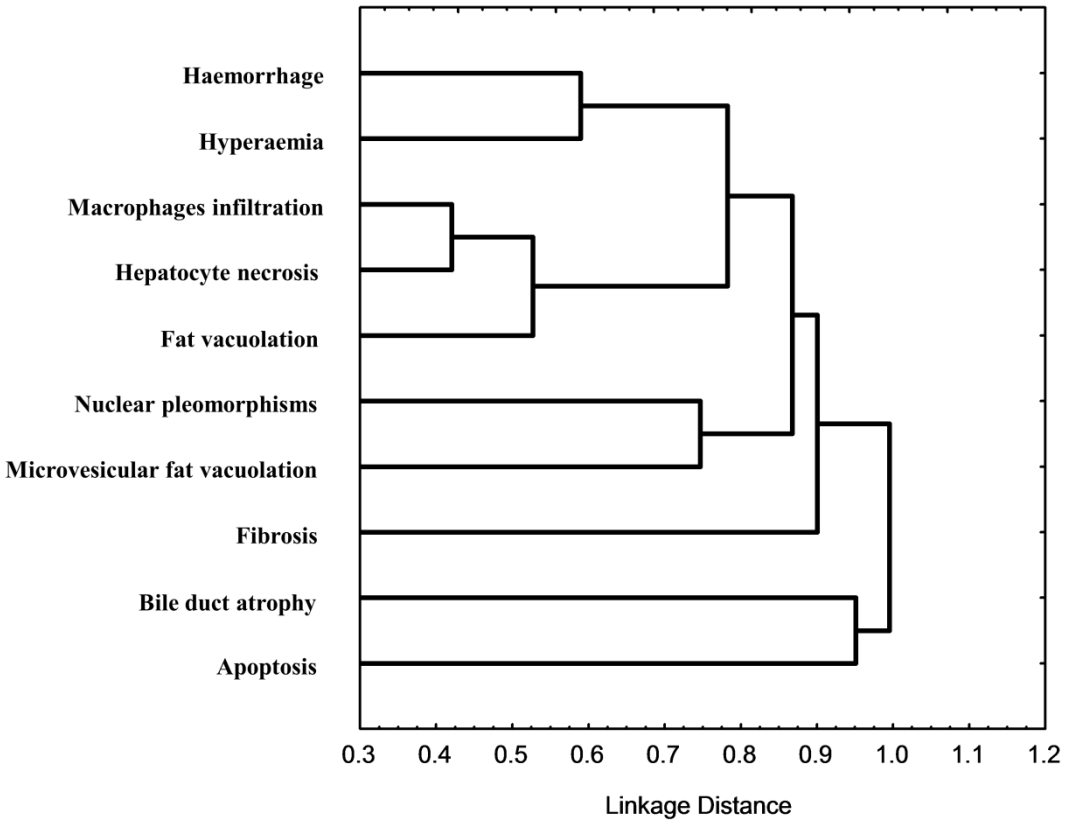

Fig. 4.5. Cluster analysis for all histopathological biomarkers observed. Distances are based on the 1-Pearson correlation statistic r between condition indices. Amalgamation was based on unweighted pair-group averages.

5. 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 exposed fish. However, basses exposed to the combination of Phe (considered non-carcinogenic to humans) and B[b]F (carcinogenic) endured the overall highest level of histopathological alterations, unlike for the isolated PAH assays, without a clear dose- or time-dependent pattern. Nevertheless, in any case, the histopathological alterations are consistent with chronic hepatic disease (rather than acute), given the overall moderate degree of severity and dissemination of changes to the hepatic parenchyma. On the other hand, progressive changes were the most significant contributors to differentiate between the hepatic condition indices of animals exposed to mixtures from those exposed to the isolated toxicants (refer to Fig. 4.2 and Table 4.3).
It must be noted that the present study surveyed phenanthrene and benzo[b]fluoranthene concentrations between the range of two sediment quality guidelines (TEL and PEL), meaning within 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 histopathological alterations observed in the liver of animals exposed to the spiked sediments. However, the mixed PAHs caused distinctively higher levels of histopathological alterations and at earlier stages of exposure, which reveals that interactions between contaminants are not contemplated in the SQGs while antagonistic, synergistic or additive effects likely exist in sediments contaminated by mixtures of toxicants, especially organic, whose high hydrophobicity render sediments as the most important trap in aquatic environments.

The occurrence of liver lesions and alterations in fish exposed to both isolated contaminants augmented with exposure time, with I_h revealing a clear increase from animals collected at T_{14} to T_{28} animals. However, B[b]F induced only marginally higher histopathological alterations compared to its termed “non-carcinogenic”, lower molecular weight counterpart (Phe). There is yet a need for more studies towards benzo[b]fluoranthene and its effects. However, a similar and extensively studied high-risk PAH may be used for comparison, benzo[a]pyrene (B[a]P), which is a potential carcinogen for humans and a well-known carcinogen for wildlife, including fish (also a five-ring PAH). Chemical differences such as the number of benzene rings or differences in its metabolic pathway in vivo may aid explaining why B[a]P is more hepatotoxic than Phe. In fact, B[a]P is more prone to metabolic activation by CYP mixed-function oxygenases (MFO) than Phe, meaning faster elimination from tissues, albeit generating reactive oxygen species (ROS) and highly genotoxic metabolites (such as diol epoxides) which are highly reactive and known to covalent binding to DNA, leading to the formation of bulky adducts (Akcha et al., 2000; Pisoni et al., 2004). On the other hand, although not considered carcinogenic, phenanthrene is also known

for its toxicity to aquatic organisms and for inducing oxidative stress by the production of ROS as well (Correia et al., 2007; Yin et al., 2007; Aimová and Poljaková, 2010). Some PAHs (such as B[a]P) may promote the transcription of specific genes involved in bioactivation, since PAHs may bind to the aryl hydrocarbon receptor (AHR), which, in turn, binds to the aryl hydrocarbon nuclear translocator (ARNT) that can be transferred to the cell nucleus, where on its turn, it binds to the specific xenobiotic response element (XRE) of the gene, promoting transcription (Bucheli and Fent, 1995). B[a]P is known to express AHR-mediated effects, representing higher enzymatic activity and thus increased production of toxic metabolites, as opposed to Phe, which is a weak AHR agonist (Mu et al., 2012). Nevertheless, the differences between the toxicological pathways underneath exposure to these two PAHs are not well understood, especially regarding CYP-mediated activation and relative potency as toxic agents.

It is well known that polycyclic aromatic hydrocarbons in fish are metabolized ("activated") during Phase I by the cytochrome P450 (CYP1A) monooxygenases system, followed by Phase II enzymes, which are involved in the elimination or inactivation of toxic or hazardous metabolites. For instance, glutathione S-transferases (GST), conjugate glutathione (GSH) with highly toxic activated metabolites and also some forms of ROS (Pretti et al., 2001). The genotoxicity of activated PAHs in aquatic animals was already confirmed, including fish (see for instance, Gravato and Santos, 2002; Holth et al., 2009; Pacheco and Santos, 1997; Taylor et al., 2011) as well as mutagenicity (Pacheco and Santos, 1997) and potential carcinogenicity (Varanasi et al., 1986). In accordance, the global histopathological indice for each contaminant showed that, as expected, B[b]F exposure may lead to higher hepatic damage, when comparing to phenanthrene, confirming its higher toxicity. It is likely that B[b]F induces higher levels of hepatic histopathological alterations through a combination of factors resulting from its activation, from direct action of ROS to DNA damage, however, previous research attempting to link known PAH effects, such as DNA lesions, to specific histological traits is essentially absent.

The activation of PAHs triggers a series of metabolic defence mechanisms that may affect the integrity of tissues and organs, depending on exposure concentrations and duration, thus contributing to mask the toxicopathic effects of exposure. In fact, phase II defence systems such as the enzymes GST, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) can be induced even by moderate increases in intracellular oxidative radicals, as a compensatory response (Xu et al., 2009). However, severe oxidative stress will either suppress or saturate the activities of anti-oxidative enzymes and lead to increasing oxidative damage (Sun et al., 2006). This can explain higher I_h at T_{28}, when comparing assays with either isolated contaminant. Also, comparing T_{14} and T_{28} animals exposed to phenanthrene revealed an obvious, albeit unexpected, increase in regressive alterations (Table 4.1), since phenanthrene is presumably less toxic than

B[b]F, as generally acknowledged for lower molecular weight PAHs. It must be noticed that, under severe toxicological challenge, hepatocytes are damaged by the production of ROS and other metabolites and may be unable to recover from these lesions to normal hepatic function (Yin et al., 2007). However, Sun et al (2006) showed that after some depuration time, fish exposed to waterborne phenanthrene (thus expectedly more bioavailable) would present levels of antioxidant enzyme activities similar to control level, returning cellular antioxidant defence to normal physiological conditions, even following acute exposures to this toxicant. Should this indicate reduced toxicity of Phe comparative to B[b]F, this information contradicts the reduced histopathological alterations induced by fish exposed to the latter after 14 (but not 28) days of exposure for the lowest concentration exposure (C1) (Fig. 4.3).

As previously stated, metabolites resulting from the activation of higher molecular weight PAHs are presumably more toxic. However, the high risk of B[a]P and other high molecular weight PAHs contradicts, at least partly, the reduced I_h obtained for the lowest concentration of B[b]F at T_{14}, which was not significantly raised over controls. The global I_h from B[b]F-C1 at 14 days of exposure suggests that this contaminant at lower concentrations is metabolized by fish without harsh consequences to their hepatic health. On the other hand, benzo[b]fluoranthene assays with higher concentration (B[b]F-C2) revealed noticeable effects on progressive and regressive changes, where an increase of both reaction patterns is striking (see Table 4.1). The present results suggest the existence of cumulative effects of exposure to B[b]F (i.e. derived from ROS and activated metabolites), particularly at higher concentrations and more prolonged exposures, thus accordant with time- and dose-related effects. Altogether, it may be inferred that fish, even PAH-naïve, may be able to respond to B[b]F at lower concentrations, likely for being able to metabolize this compound within the ability to cope with ROS and other by-products of activation, resulting in reduced histopathological alterations. However, the effects of elevated phenanthrene-induced alterations at lower concentrations remain elusive.

Fish exposed to the mixture treatments presented a global histopathological indice relatively higher than fish exposed to the two isolated contaminants, which likely reveals some sort of additive effect between the two toxicants, which is in accordance with the works of Basu et al (2001) and Fleming and Di Giulio (2011) when testing for mixtures of PAHs. However, the degree of global histopathological alterations was unexpectedly lower than that sum of effects elicited by the two substances and, moreover, dose- and-time-dependent effects were unclear. Previous studies on fish exposure to natural PAH-contaminated sediments, containing complex PAH mixtures, revealed histological alterations in livers and other organs, such as gills, however, revealing complex interaction scenarios that greatly diluted the effects of concentration and time of exposure (Costa et al., 2009; Kerambrun et al., 2012). It must be, at point, noted that interaction effects may be

classified as synergistic (meaning a different effect rather than simply the addition of isolated effects), antagonistic (when the mixture effect is less than the isolated effects) or agonistic/additive (where the mixture effects are lower than the sum of the individual effects) (Gonçalves et al., 2008). However, the lack of clear dose- and time-effect relationships indicates other factors modulating the cumulative toxicity of the two toxicants, which could include dose-dependent inhibition of toxicity caused by phase I enzyme impairment. In fact, Taylor et al (2012) noted that the activity of cytochrome CYP1A could be inhibited at higher concentrations of some inducers, which leads to hypothesize that the PAH mixture had an antagonistic effect.

Interaction effects between different contaminants have already been suggested, even in human toxicological studies, as well as the possible synergistic or antagonistic effects of CYP1A1 activity in mixed exposures (Tarantini et al., 2011). In fish, synergistic effects are mainly identified by the analysis of ethoxyresorufin-*O*-deethylase EROD activity, an indicator of phase I enzymes biotransformation. Basu et al (2001) observed a synergistic effect in mixtures of PAHs, where numerous PAHs could increase suprea-additively the mutagenicity of benzo[a]pyrene. However, combination bioassays with CYP1A-activatable PAHs may yield an underestimation of effects, probably due to an inhibitory action of other PAHs (Willett et al., 2001; Wills et al., 2010). However, synergistic effects may also be responsible for the present findings, for some PAHs may disturb the metabolization pathway of others. Altogether, it is clear that further research is needed to fully disclose toxicokinetics of PAHs and their interactions.

Several reasons may explain the lack of a temporal trend following combined exposure such as the short exposure time in the experiments, as pointed out by Taylor et al (2012). Also, it must be stressed out that the concentrations hereby tested were relatively “low”, in order to achieve ecological relevance, which may have resulted in less obvious responses compared to the high, often subacute, exposures typically employed in most baseline studies with PAHs (e.g. Danion et al., 2011; Kerambrun et al., 2012). Another explanation may come from PAH bioavailability to pelagic/benthopelagic fish species (as the sea bass) which is essentially linked to compounds present in the water column. Assuming that PAHs were being dissolved in a steady-state condition, matching the elimination rate of fish, it is possible that dissolved PAH concentration was maintained throughout the experiment thus balancing bioavailability to fish. In this case, sediments may act as a reservoir, where PAHs will be dissolved until they are bioavailable even at lower concentrations, maintaining concentrations balanced between sediments, water and fish, which aids explaining, at least in part, why fish exhibited an adaptative response that limited cumulative alterations. As such, exposure to low concentrations of the PAHs, as in the present study, induces defences that attenuate negative effects, limiting hepatic alterations to a steady-state that diluted dose- and time-dependent effects. It is also possible that the duration of this assay only

contemplates the initial phase of homeostatic and metabolic disturbance, where various defence mechanisms are activated to deal with that disturbance. According to Steinberg et al. (2008), after that initial phase, with a longer exposure time, effects may come to decrease, since the presence of metabolites may induce, not only the activation of more specific defensive mechanisms, but also repair mechanisms. This means that the fish undergo an adaptive compensatory response following that initial disruption. Also, the term "adaptive response" implies that similar defence mechanisms are activated regardless concentration, which may explain no clear differences in reaction patterns between different mixtures.

In the environment, contaminants usually occur in mixtures, generally at low levels of individual components, and an analysis of single chemicals is likely to be misleading when comparing with mixtures (Altenburger et al., 2003). Mixtures are rarely addressed in mechanistic toxicological studies. In particular, studying PAH mixtures may unravel complicated mechanisms associated to distinct PAH detoxification pathways that may be hard either to identify or compare with the effects of isolated toxicants. For instance, in human cells, it was observed that B[k]F (another 5-ring PAH) inhibits metabolization of B[a]P, probably through competition to bind to monooxygenase active sites, whereas, B[b]F and B[k]F did not seem to compete for metabolization (Tarantini et al., 2011). In fact, the same authors state that the differential affinity towards CYP1A MFO's active sites and the competition between the contaminants for these sites as an important factor in the formation of reactive metabolites. In a study with rainbow trout (*Onchrorhynchus mykiss*) involving acute toxicity tests, Bols et al. (1999), showed that fluoranthene (a 4-ring PAH) is a non-inducer EROD activity. In fact, in this same study, fluoranthene showed little or no evidence of differences between controls regarding the activity of this CYP enzyme often surveyed as biomarker of exposure, and, when in mixture with B[a]P, EROD activities were actually significantly lower than in fish treated only with B[a]P. Another rainbow trout study with different PAH mixture exposures presented an additive effect on EROD activity (Basu et al., 2001) . Synergistic effects following exposure to different classes of contaminants, such as PAH/metal mixtures have also been reported. For instance, a subacute combination of B[a]P and cadmium in soles (*Solea senegalensis*) revealed inhibited responses related to liver regeneration and apoptosis (Costa et al., 2010). Also, studies with killifish (*Fundulus heteroclitus*) embryos exposed to different waterborne PAH mixtures suggest that sites with PAH mixtures generally induce CYP1A activity, such as those containing B[a]P and B[k]F. However, environmental PAH mixtures may also contain compounds that can act as CYP1A inhibitors, like fluoranthene, which means that additive models currently used to estimate PAH toxicity may over or underestimate that same toxicity in PAH mixtures (Wassenberg and Di Giulio, 2004). In the present work, histopathological effects in mixtures yielded higher I_h values than in fish subjected to isolated assays, but the sum of effects is lower than expected, indicating a limited agonistic effect.

Interestingly, contrasting the effects of mixtures and isolated contaminants with their respective concentrations showed specific differences to reaction patterns for each mixture (see Table 4.3). Fish exposed to the mixture comprising the lowest concentrations of either PAH (M1) presented signs of greater inflammatory response, when compared to fish exposed to isolated contaminants (Table 4.1). Inflammatory response-related alterations (such as infiltration and hyperaemia) were highly correlated (see clusters), being this reaction pattern related to non-specific immunological responses. Inflammatory response is often associated with other reaction patterns and is the reaction pattern observed that is considered to have the less damaging potential to the organ. On the other hand, mixtures comprising combination of "high" and "low" concentrations of PAHs (M3 and M4) display an increase in progressive changes when comparing to isolated assays of each respective contaminant. Progressive changes regard alteration of hepatocyte functions, mainly fat vacuolation. This alteration, together with other progressive changes not observed in this work (like eosinophilic bodies), has also been observed in livers of juvenile soles (*Solea senegalensis*) exposed to PAH-contaminated sediments (Costa et al., 2009). It is possible that fat vacuolation, alongside other progressive alterations may be a little or null-specificity biomarker, albeit able to progress into severe cirrhosis (Koehler, 2004). This is supported by a high correlation between fat vacuolation and hepatocyte necrosis observed (Fig. 4.3). The mixture comprising the highest concentrations of both PAHs (M2) revealed an increase of lesions considered of greater severity, when comparing to the respective isolated assays (Table 4.3). The results also suggest that histopathological alterations are evident sooner, where fish exposed to this mixture endured more alterations at T_{14} than fish subjected to isolated PAH assays, which means that PAH mixtures may elicit alterations faster than the isolated contaminants.

6. 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 benthopelagic fish, consistent with sub-lethal toxicopathological effects. Also, this work showed that semi-quantitative indices based on the relative weights of lesions and quantitative data proved to be a useful and practical tool to assess fish health when exposed to xenobiotics. The data revealed that the histopathological alterations are consistent with chronic hepatic disease (rather than acute), given the overall moderate degree of severity and dissemination of changes to the hepatic parenchyma. Knowing that PAH concentrations used in this work were within the boundaries of low and high risk to exert deleterious effect to the biota, the current findings are in accordance with the expected moderate levels of histopathological alterations observed in the liver of animals exposed to the spiked sediments.

Individuals exposed to phenanthrene (considered non-carcinogenic to humans) presented lower liver histopathological alterations than benzo[b]fluoranthene (carcinogenic) especially at T_{28}, thus contributing to confirm a positive relation between the number of benzene rings and toxicity. Also, mixture treatments caused more hepatic damage, without a clear dose- or time-dependent pattern, which may suggest interactions between the two contaminants. However, there is still a lack of knowledge concerning these interactions and their connection to hepatic histopathological traits. Complementary histochemical biomarker analysis would be crucial in increasing knowledge of metabolic pathways and possible interactions because PAHs are usually present in the environment in complex mixtures and never isolated. Furthermore, additional histopathological analysis on a different organ, such as gills, due to their role as a main entry route for toxic agents, or kidneys as well and their high susceptibility to adverse environmental conditions, could prove useful in identifying the organs and mechanisms regarding apical entry and excretion of xenobiotics and their metabolites, respectively.

Finally, it has been shown 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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