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Interaction of Polycyclic Aromatic Hydrocarbon compounds in fish primary hepatocytes: From molecular mechanisms to genotoxic effects

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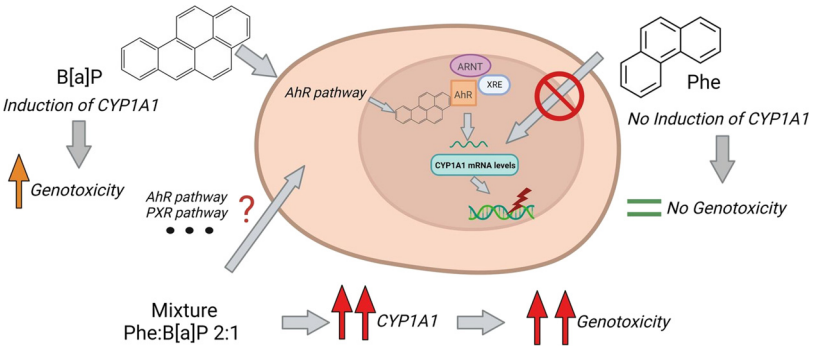
HIGHLIGHTS

- Individual PAH compounds induced CYP1A1 at individual rates: B[*a*]P ⋙ B[*b*]F > Phe.
- DNA damage was related to CYP1A1 activity with B[*a*]P > B[*b*]F > Phe.
- PAH mixtures increased CYP1A1 activity and genotoxicity relatively to equimolar levels of individual compounds.
- Environmental guidelines should consider mixture effects when addressing PAH toxicity.

GRAPHICAL ABSTRACT



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ABSTRACT

Polycyclic Aromatic Hydrocarbons (PAHs) are persistent pollutants normally found in the environment as complex mixtures. Although several individual PAHs are classified as mutagenic and carcinogenic pollutants, the interaction effects between compounds in a mixture may trigger different toxicological mechanisms and, consequently, yield different effects to organisms which are not accounted for in risk assessment guidelines. Given the ubiquity of PAHs, understanding the mechanistic features of their mixtures is a pressing research need. Therefore, the present work aimed to disclose the interaction effects of three PAHs with different carcinogenic potential and chemical structure, in primary hepatocyte cells of gilt-headed seabreams (*Sparus aurata*). Hepatocytes were exposed to Phenanthrene (Phe), Benzo[*a*]pyrene (B[*a*]P) and Benzo[*b*]fluoranthene (B[*b*]F) and their mixtures at different proportions and several cellular responses were analyzed: cellular viability, CYP1A1 activity (EROD assay) and protein expression level (Western blot); transcript (mRNA) levels of CYP1A1, EPXH1 and GST-3 (qRT-PCR); genotoxic effects (DNA strand breakage) by the Comet assay. Results show that B[*a*]P induced CYP1A1 gene and protein expression increasing its activity and, therefore, increasing the production of metabolites that trigger genotoxic DNA damage (%). Most importantly, mixtures containing Phe and B[*a*]P increased even further CYP1A1 mRNA levels and DNA damage (up to 70 %) which suggests that, although Phe is considered a non-carcinogenic PAH, it potentiates CYP1A1 synthesis induced by B

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[a]P, increasing its genotoxicity. These findings indicate that the upregulation of CYP1A1 by carcinogenic PAHs will not weaken even when in mixtures with non-carcinogenic PAHs. On contrary, non-carcinogenic PAHs may potentiate the genotoxic effect of carcinogenic PAH and therefore mixture composition should be taken in account when assessing PAH toxicity. In fact, our results point to the need of redefining Environmental Risk Assessment protocols for mixtures of carcinogenic pollutants.

1. Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are carbon and hydrogen compounds resulting from the fusion of 2 or more aromatic rings (Branco et al., 2021) during the incomplete combustion of organic matter, mainly due to fossil fuel combustion (Dat and Chang, 2017).

Many PAH compounds are procarcinogenic and promutagenic, thus requiring hepatic biotransformation to form reactive metabolites and exert toxicity (Branco et al., 2021; Silva Junior et al., 2021; Tarantini et al., 2011; Jarvis et al., 2013; Haritash and Kaushik, 2009). It is during phase I transformation, namely by cytochrome P450 monooxygenase system that those reactive metabolites are formed such as diol-epoxides which can covalently bind to the DNA to form adducts (Chen, 2020). Diol-epoxides are primarily detoxified by Epoxide Hydrolase (EPHX) to dihydrodiols, but these compounds are substrates to CYP1A1 which converts them to reactive dihydrodiol epoxides. EPHX cannot detoxify these metabolites and thus Phase II conjugation is necessary (El-Sherbeni and El-Kadi, 2014).

Phase II reactions involve the conjugation of PAH metabolites, with glutathione to produce water-soluble conjugates that are easily excreted by organisms (Sinaei and Zare, 2019). The enzymes involved in this conjugation are, for instance, the Glutathione-*S*-Transferases (Chen, 2020; Hayes et al., 2005). At a molecular level, PAHs induce the production of reactive oxygen species (ROS) affecting macromolecules such as lipids, proteins, and nucleic acids (Santana et al., 2018), which may culminate in inflammation and cell death (Santana et al., 2018).

Various epidemiological and toxicological studies (Sarigiannis et al., 2015; Armstrong et al., 2004) showed a correlation between exposure to PAHs and an augmented risk for cancer development. As such, several PAH compounds (e.g. Benzo[a]pyrene – B[a]P) are classified as carcinogens (Group 1) or potential carcinogens (Group 2) to humans by the International Agency for Research on Cancer (IARC) and they are also included in the Priority Substances List of the Marine Strategy Framework Directive (MSFD) and highlighted by U.S. Environmental Protection Agency (EPA) and the World Health Organization (WHO) as priority substances to be analyzed in various environmental matrices. However, these international directives fail to recognize that PAHs exist in the environment mainly as complex compound mixtures, a fact that may radically change their toxicology (Branco et al., 2021) and increase the uncertainty of risk assessment. Thus, the study of mixtures of PAHs represents a great challenge in the scientific community.

Indeed, although the mode of action of some individual compounds, such as B[a]P, is well known, there is a knowledge gap relatively to the synergistic, additive or antagonistic effects resulting from a mixture and how these effects vary depending on the type and proportion of each PAH (Branco et al., 2021).

Therefore, the present study aims to disclose the toxic interaction effects of different PAHs, that are usually present in the aquatic environment at different proportions, using primary hepatocytes from an ecologically relevant species, the gilthead-seabream. Primary hepatocytes of wild organisms constitute a relevant model in ecotoxicology since they maintain biochemical pathways found in vivo and are generally more sensitive than immortalized cell lines (Figueiredo et al., 2021) and also complies with the 3R's principle. The expression and activity of enzymes involved in the toxic mechanism of action of the PAHs, and their relation to genotoxic effects were evaluated comparing individual compounds to mixtures with different proportions.

2. Materials and methods

2.1. Chemicals

Regents were all acquired from Sigma-Aldrich/ Merck and included: L-15 (Leibovit'z) medium; Antibiotic Antimycotic Solution (100×); Fetal Bovine Serum (FBS); Trypsin-EDTA (0.25 %); Benzo[a]pyrene (B[a]P; CAS number 50–32-8; ≥96 %); Phenanthrene (Phe; CAS number 85–01–8; ≥98 %); Benzo[b]fluoranthene (B[b]F; CAS number 205–99–2; ≥98 %).

2.2. Primary hepatocyte isolation and culture

Primary hepatocytes of *Sparus aurata* were isolated with the Pancreatin Digestion method as described by Figueiredo et al. (2021). Briefly, the liver was removed from the fish and cut into small fragments in cool sterile balanced salt solution. Preheated (37 °C) pancreatin digestive juice (126 mM NaCl, 4.28 mM KCl, 1.5 mM KH_2PO_4, 6.1 mM Na_2HPO_4, 0.54 mM Na_2-EDTA, 0.1 % pancreatin) was mixed with the liver fragments for 30 min and centrifuged at 100 ×*g* for 5 min, at 4 °C before purification with Histopaque (Sigma) and centrifuged again. The hepatocytes were collected from the supernatant and placed in Hank's Balanced Salt Solution (HBSS) medium.

The isolated hepatocytes showing >85 % of viability were seeded in plates with a complete culture medium (L-15 (Leibovit'z)) supplemented with 10 % heat-inactivated FBS, 1 % penicillin (10.000 U/mL)/ streptomycin (10 mg/mL) and amphotericin B (25 μg/mL) and maintained in an incubator at 18 °C. After 24 h, culture medium was changed, and cells were used for PAH exposure assays (Fig. S1 - Supplementary material). For each batch of assays were added two controls (cells and solvent).

2.3. Cell viability

Cells were plated in 96-well plates (5×10^4 cells/well) and after 24 h, several concentrations of Phe (0.1; 1; 10 μM), B[a]P (0.1, 1; 10 μM) and B[b]F (0.1, 1; 10 μM) and binary mixtures (total concentration: 0.1; 1; 10; 50 μM) with varying ratios of Phe:B[a]P (1:1; 1:2; 2:1) and B[a]P:B[b]F (1:1; 1:2; 2:1) were added to cell cultures during 24 h and 48 h. Cell viability was assessed by the MTS assay which evaluates the metabolic capability of viable cells to reduce MTS to formazan. The MTS assay kit purchased from abcam (ab197010) was used to the kit instructions. The formazan dye formed by viable cells was quantified by measuring the absorbance at 495 nm.

2.4. Total protein concentrations

The total protein concentration was determined in cell lysates, using the Bradford method (Bradford, 1976). Briefly, each sample was mixed with diluted (5×) Coomassie dye (BioRad) in 96-well plates and the absorbance was measured at 595 nm on a microplate reader. The protein concentrations were calculated from a calibration curve (1–12 μg of protein μL^{-1}) using BSA as standard.

2.5. CYP1A1 activity determined by EROD assay

EROD activity was measured as described by Ferreira et al. (Ferreira et al., 2014). Cells were seeded in a 24-well plate (3×10^5 cells/well) and left for 24 h before exposure to PAH. Exposures to Phe (0.1; 1;

10 μM), B[*a*]P (0.1, 1; 10 μM) and B[*b*]F (0.1, 1; 10 μM), lasted 24–48 h, after which the medium was removed and cells washed once with cold PBS followed by the addition of a reaction mixture (1 mg/mL BSA, 5 μM 7-ethoxyresorufin (7-ER), and 0.5 mM NADPH). Following incubation for 30 min at 37 ± 1 °C in the dark. Afterwards, fluorescence was measured at 560 nm excitation and 610 nm emission. A resorufin standard curve (0.78 ng/μL - 50 ng/μL) was used to calculate EROD activity. After EROD measurement, activity was normalized for total protein concentration (see Section 2.4).

2.6. CYP1A1 protein levels

Cells (1.5×10^6 cells/well) were exposed in 6-well plates for 24 h to: Phe, B[*a*]P or B[*b*]F (0.1, 10 and 50 μM); Phe:B[*a*]P and B[*a*]P:B[*b*]F mixtures (2:1; 1:1; 1:2 ratios for final concentrations of 0.1, 10 and 50 μM). Expression of CYP1A1 was evaluated by Western Blot in the soluble fraction (40 μg of protein) by SDS–PAGE on a 4–12 % Bis–Tris gel with MES running buffer under reducing conditions (140 V for 1 h). Electrophoresis was followed by dry transfer (20 v for 7 min) to a nitrocellulose membrane with the iBlot™ 2 Dry Blotting System (Invitrogen) and blocked for 1 h at RT with a 5 % milk solution. Primary antibody incubation proceeded overnight at 4 °C, followed by washing with PBS and incubation with the appropriate secondary antibody. Protein expression levels were normalized for total protein loading on the gel which was assessed by Ponceau S staining (Branco et al., 2014). The primary antibody was a rabbit anti-CYP1A1 polyclonal antibody (Biosense laboratories AS) and the secondary was a mouse anti-rabbit IgG-HRP antibody (sc-2357, Sta. Cruz).

2.7. Expression of PAH metabolism-related genes

Cells were seeded in 6-well plate at a density of 1.5×10^6 cells/well. After exposure for 24 h to PAHs (10 μM of Phe, B[*a*]P or B[*b*]F; 10 μM of Phe:B[*a*]P and B[a] P:B[*b*]F mixtures in compound ratios of 2:1; 1:1; 1:2), RNA was extracted from each sample using the NZY Total RNA Isolation Kit (NZYTech, Portugal), following the manufacturer's instructions. Following cell lysis with a specific lysis buffer and β-mercaptoethanol, samples were homogenized by filtration (NZY spin columns), mixed with ethanol 70 % and then purified by desalting and DNase I digestion with several washing steps prior to the final RNA elution with RNase free water. Samples were stored at −80 °C until analysis. The RNA quantification was performed using a NanoDrop 2000 (Thermo Scientific) and purity was assessed by looking at the 260/280 nm and 260/230 nm ratios. For cDNA synthesis the NZY First-Strand cDNA Synthesis Kit (NZYTech, Portugal) was used, following the manufacturer's instructions. Briefly, 0.1 μg of template RNA was mixed with an enzyme and oligo mix and incubated for 10 min at 25 °C followed by 30 min at 50 °C. After inactivation for 1 min at 85 °C and cooling on ice, 1 μL of *E. coli* RNase was added to each sample and incubated for 20 min at 37 °C. Samples were stored at −20 °C until qPCR.

Quantitative analysis of CYP1A1, Epoxyhydrolase (EPHX1) and Glutathione-S-Transferases (GST-3), was done with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), using GAPDH as the housekeeping gene. Primers were designed with Primer-blast using Reference Sequences from GeneBank (Table 1). Primer efficiency was tested with a dilution in series (1:10–1:1000000) of the cDNA template (Table 1) and ranged from 95 % to 108 %. A total of 100 ng of template cDNA was amplified using the NZY ROX qPCR Green Master Mix (NZYTech) with forward and reverse primers (400 nM) and molecular grade water for a total reaction volume of 10 μl. A total of 40 cycles of amplification (15 s at 95 °C followed by 1 min at 60 °C) were performed after incubation at 50 °C for 2 min and 95 °C for 10 min. The qRT-PCR was performed on an Applied Biosystems QuantStudio™ 7 Flex real-time PCR system (Applied Biosystems, Foster City, CA, USA).

2.8. Comet assay

In a 6-well plate, 1.5×10^6 cells/well primary hepatocyte cells were exposed for 24 h and 48 h to Phe and B[*a*]P (0.1- 50uM) individually and in mixtures with 1:1, 1:2 and 2:1 (Phe:B[*a*]P) compound ratio. The standard alkaline Comet assay was performed as previously described (Martins et al., 2018). Briefly, PBS cell suspensions were diluted in liquid low melting-point agarose (1 % w/v) (LMPA; Sigma) and placed on slides pre-coated with normal melting-point agarose (1 % w/v). After solidification the slides were dipped for 1 h at 4 °C in lysis solution (2.64 % NaCl, 3.72 % EDTA and 5 mM Tris, 10 % (v/v) DMSO and 1 % (v/v) Triton-X 100) and subsequently incubated (40 min) with cold electrophoresis solution (pH 13) followed by 30 min at 25 V. After neutralization (15 min) with 0.1 M Tris–HCl buffer (pH 7.5), slides were stained with ethidium bromide (20 μg/mL) and examined with a DMLB microscope using an epifluorescence light source (EL6000 with N2.1 filter from Leica Microsystems). Around 100 random comets were analyzed per slide with software CometScore (TriTek, VA, USA) (Martins et al., 2018; Martins et al., 2013).

2.9. Statistical analysis

Differences between groups were evaluated with a One-way ANOVA followed by Fisher's post-hoc test using Statistica7®. For non-parametric data the Mann-Whitney test was applied. For all assays, a minimum of 3 independent experiments was performed and Figures represent the mean ± standard error of no less than 3 independent experiments. Also, two control groups were used (negative control with L-15 complete medium and solvent control with L-15 complete medium containing 0.5 % DMSO). Differences between groups were classified as significant at $p < 0.05$ and very significant at $p < 0.01$.

3. Results

3.1. Cell viability results

Cell viability of primary hepatocytes of *S. aurata* was evaluated following 24 and 48 h of exposure relatively to the solvent control (DMSO)by the MTS assay (Fig. 1). The viability of negative control cells and solvent control cells (DMSO) was not significantly different ($p > 0.05$). Exposure to

Table 1
Primers used in the quantification of CYP1A1, EPXH1 and GST-3 mRNAs. GAPDH was used as the control gene. Primers were design with Primer-Blast from the following reference sequences for *S. auratus* retrieved from Gen Bank.

Gene	Primer	Sequence	NCBI Reference Sequence	Primer Efficiency (%)
CYP1A1	Forward	5′-CCTTCCTGGAGGCCTTTATCC-3′	XM_030415642.1	95
	Reverse	5’-GTCTTTTGACGAGCAGTGAGG-3′		
EPXH1	Forward	5′-CCAGCCCCACTCTAAGATACCAG-3′	XM_030405605.1	107
	Reverse	5′-CGGGCCATCGTCGTGAAAG-3′		
GST-3	Forward	5′-TTGCCCTTCACTCCAGATCC-3′	XM_030441496.1	108
	Reverse	5′-CCCGGACAGCACCTTTTCG-3′		
GAPDH	Forward	5′-ATCACTGTTTTCCACGAGAGGG-3′	XM_030394814.1	108
	Reverse	5′-TCAACAACGTACTGGGCACC-3′		

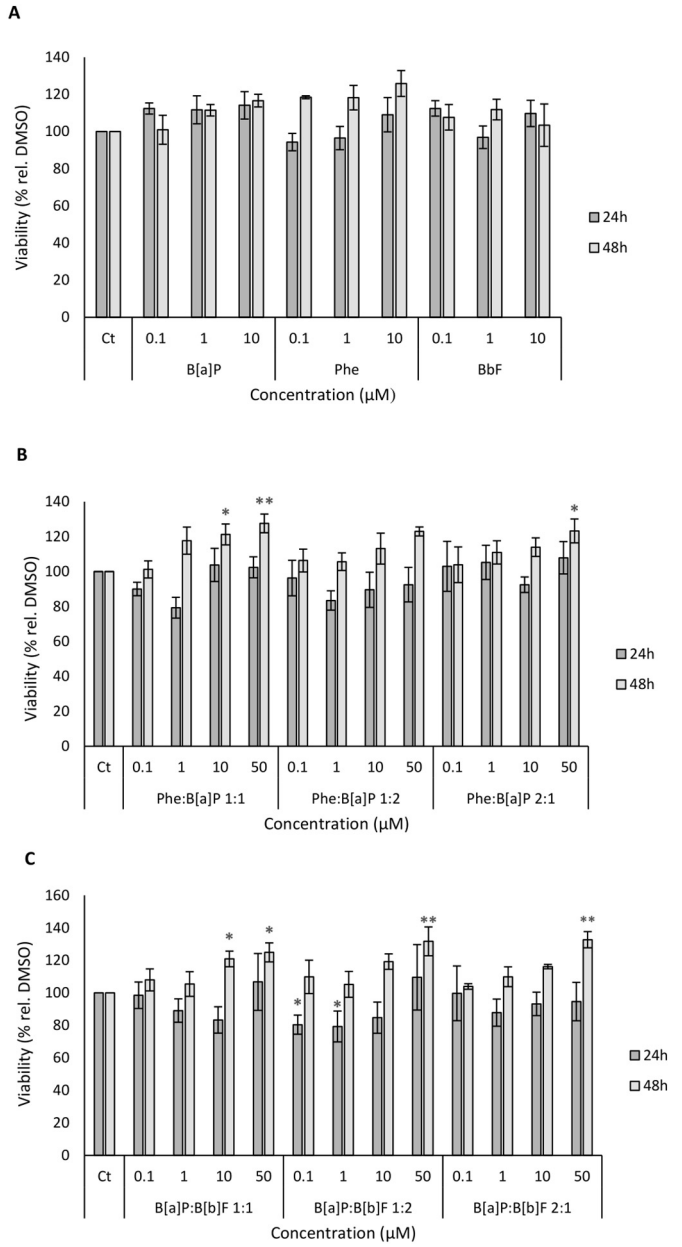

Fig. 1. Cell viability (% rel. DMSO) performed by MTS assay, indicating the percentage of metabolically active cells. Cells were exposed to B[*a*]P, Phe, B[*b*]F (A) (0.1, 1 and 10 μM) or their mixtures (B, C) (total PAH = 0.1, 1, 10 and 50 μM). Data (mean ± S.E.) from 3 to 6 independent experiments. Ct represents Control Group. *significantly different from control ($p < 0.05$); ** very significantly different from control ($p < 0.01$).

the individual compounds did not cause a significant ($p > 0.05$) alteration in cell viability after 24 h. The mixture Phe:B[*a*]P increased cell viability after 48 h at the ratio of 1:1 10 and 50 μM, and also at 2:1 50 μM, as seen in Fig. 1B ($p < 0.05$). Concerning mixture, B[*a*]P:B[*b*]F co-exposure (1:2 ratio) at 0.1 and 1 μM were the only mixture decreasing cell viability significantly ($p < 0.05$) after 24 h.

3.2. EROD assay

The results for the EROD assay (Fig. 2) showed a significant increase of activity after 24 ($p < 0.01$) and 48 ($p < 0.05$) hours of exposure to B[*a*]P 0.1 μM and a decrease of the activity with B[*a*]P 10 μM ($p < 0.05$). Phenanthrene exposure demonstrated a decrease at CYP1A1 activity after

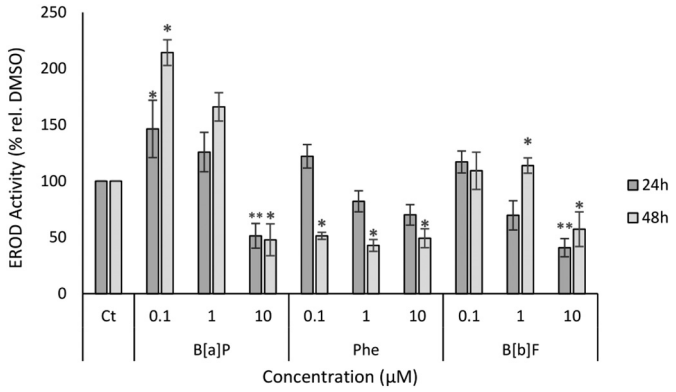

Fig. 2. EROD activity (% rel. DMSO) of primary hepatocytes of *S. aurata* after 24 and 48 h of exposure. Cells were exposed to B[*a*]P, Phe, B[*b*]F (total PAH = 0.1, 1 and 10 μM). Data (mean ± S.E.) from least 3 independent experiments. Ct - Control. *significant difference versus control ($p < 0.05$); ** very significant difference versus control ($p < 0.01$).

48 h of exposure to 0.1, 1 and 10 μM. Furthermore, B[*b*]F increased the activity after 48 h of exposure to 1 μM but at 10 μM was observed a decrease in CYP1A1 activity after 24 and 48 h.

3.3. CYP1A1 protein levels

Fig. 3 shows that all the concentrations tested of B[*a*]P (0.1, 10 and 50 μM) demonstrated significant differences ($p < 0.05$), increasing the expression of protein levels of CYP1A1, in a not dose-dependent manner. The exposure to the other PAH compounds demonstrated an increase in the expression of only Phe 10 μM and B[*b*]F 0.1 μM. The mixture Phe:B[*a*]P increased the expression of CYP1A1 in all ratios ($p < 0.05$), with the exception of Phe:B[*a*]P 1:2 10 μM; comparing all ratios and concentrations the increase was higher when exposed to Phe:B[*a*]P 2:1 at a final concentration of 50 μM. B[*a*]P:B[*b*]F ratios 1:2 and 2:1 demonstrate a significant increase ($p < 0.05$) at CYP1A1 expression at all concentrations, especially at 2:1.

3.4. Expression of PAH metabolism-related genes

Results of gene expression of metabolism-related enzymes, CYP1A1 and GST-3, showed significant differences after 24 h. The mRNA level of CYP1A1 (Fig. 4A) obtained for the mixture Phe:B[*a*]P exposure was significantly ($p < 0.05$) increased (up-to 6 fold) relatively to control group, by all compound ratios (1:1, 1:2 and 2:1), but more notoriously for the 2:1 mixture. Remaining treatments did not up-regulate CYP1A1b gene transcription significantly. GST-3 mRNA levels were unchanged by all treatments (Fig. 4B). EPXH1 mRNA levels were not significantly impacted by any treatment, nonetheless, it is possible to observe an increase at the exposure to Phe:B[*a*]P 2:1 10 μM (Fig. 4C). The mixture B[*a*]P:B[*b*]F did not demonstrate significant alterations at the mRNA levels of CYP1A1, GST-3 and EPXH1 in any of the exposed concentrations after 24 h.

3.5. Comet assay

DNA damage results are expressed through %DNA in tail and showed an increase in a concentration dependent manner for B[*a*]P with more % of DNA in tail for highest concentrations (Fig. 5 A). B[*b*]F exposure demonstrated similar results to B[*a*]P exposure, increasing the % of DNA in tail in a dose dependent manner at 48 h.

For the mixture Phe:B[*a*]P, highest values of % DNA in tail were observed, particularly after 48 h ($p < 0.01$). Interestingly, this increase was more pronounced when mixtures were enriched in Phe (2:1 ratio) with an increase of approximately 73 % (Fig. 5B). As seen in Fig. 5C, the mixture B[*a*]P:B[*b*]F increase the % of DNA damage in all ratios after

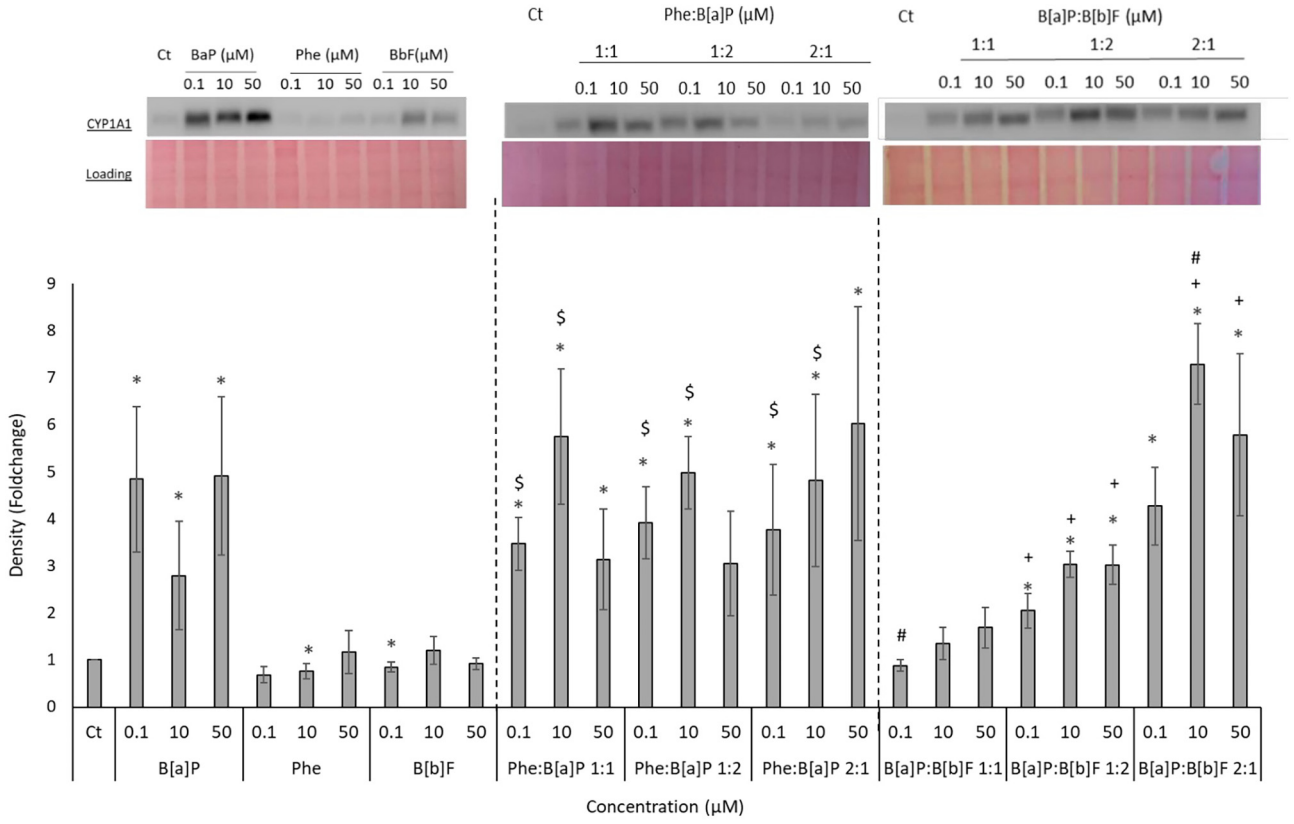

Fig. 3. Expression of CYP1A1 by Western blot after 24 h of exposure in primary hepatocytes of *S. aurata* exposed to B[*a*]P, Phe, B[*b*]F and mixtures (tPAH = 0.1, 10 and 50 μM). Ct - Control. Data (mean ± S.E) from 3 to 6 independent experiments. *significant difference vs. control ($p < 0.05$). #significant difference vs. the respective concentration of B[*a*]P ($p < 0.05$). $ significant difference vs. the respective concentration of Phe ($p < 0.05$). + significant difference vs. the respective concentration of B[*b*]F ($p < 0.05$).

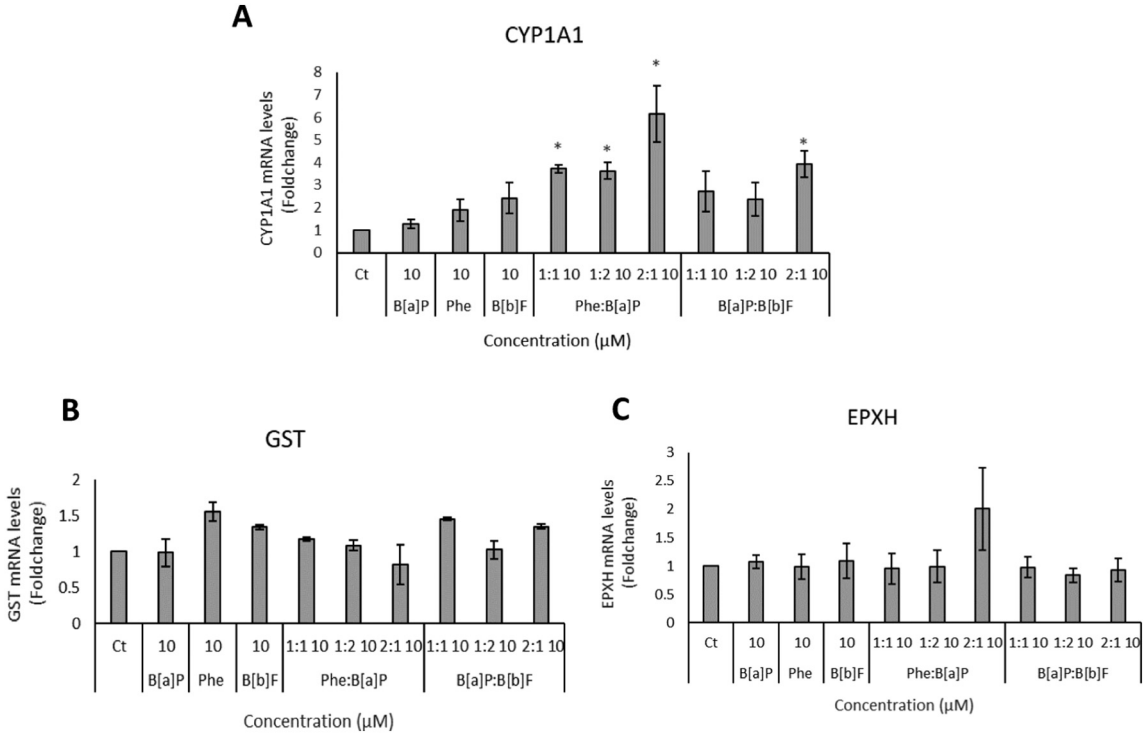

Fig. 4. mRNA levels for CYP1A1 (A), GST-3 (B) and EPXH1 (C) after 24 h of exposure to B[*a*]P, Phe, B[*b*]F or PAH mixtures (tPAH = 10 μM). Data (mean ± S.E) from at least 3 independent experiments. Ct - Control. *significant difference vs. control ($p < 0.05$).

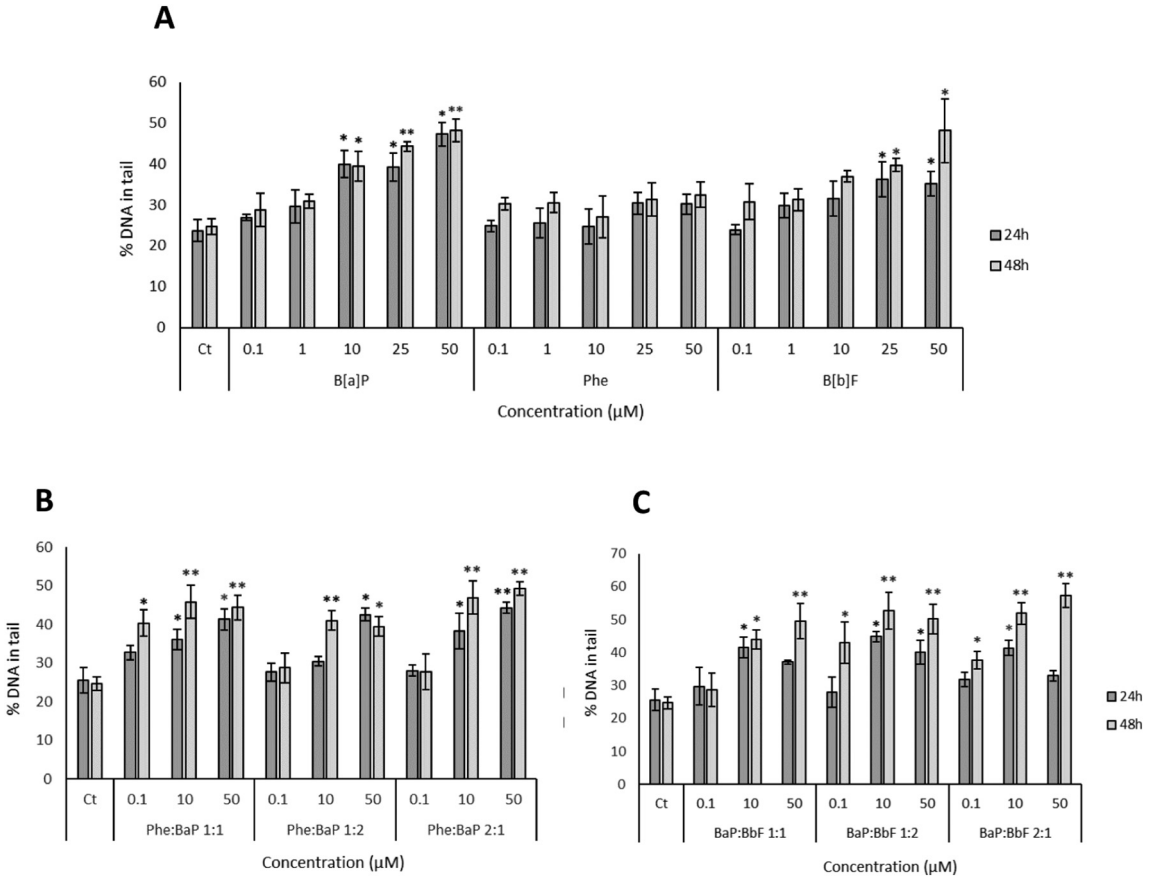

Fig. 5. Percentage (%) of DNA in tail of primary hepatocytes of *S. aurata* after 24 and 48 h of exposure. Cells were exposed to B[*a*]P, Phe, B[*b*]F (A) or their mixtures (B, C) (total PAH = 0.1, 10 and 50 μM). Data (mean ± S.E) of 3 to 6 independent experiments. Ct - Control Group. *significant difference vs. control ($p < 0.05$); ** very significant difference vs. control ($p < 0.01$).

48 h with the exception of 1:1 0.1 μM. ($p < 0.05$), and that increase appears to be dose dependent.

4. Discussion

Environmental guidelines concerning PAHs focus on individual compounds, such as B[*a*]P, driven by the extensive knowledge on its mechanism of toxicity, genotoxicity and carcinogenicity. However, in a mixture, even if in low concentrations, PAHs may trigger unpredicted biological effects. Indeed, this study showed that when PAHs are mixed, the effects at molecular level changed, contributing for the increase of genotoxic effects.

Overall, exposure to PAH compounds (0–50 μM range) both individually and in mixture had little impact over primary hepatocyte viability (Fig. 1). However, when cells were exposed to B[*a*]P:B[*b*]F there was a slight decrease in the cellular viability of primary hepatocytes at 24 h. This is in line with previous observations in HepG2 showing that at concentrations ranging from 1 to 100 μM, both Phe and B[*b*]F had little impact over cellular viability (Branco et al., 2021). Nonetheless, this does not mean that these compounds do not exert toxic effects at these environmentally relevant concentrations.

The EROD activity in the gilt-head seabream primary hepatocytes was considerably increased by B[*a*]P and slightly by B[*b*]F exposures especially at an earlier time-point (24 h). (Fig. 2). In line with our results, the work by Kienzler et al. (2012) also reported a time-dependent alteration of EROD induction after B[*a*]P exposure, where EROD activity was higher at earlier times of exposure (8 h) and, then, after 16 and 24 h, the EROD activity decreased.

EROD activity is a common biomarker to evaluate PAH biotransformation since it reflects induction of CYP1A1. Indeed, PAHs are largely inert compounds that require metabolic activation to be reactive. Primary activation occurs through the CYP-family of P450 monooxygenases which are regulated via the AhR pathway, a ligand-activated transcription factor found in vertebrates (Martins et al., 2015). Since these PAHs are AhR agonists it was expected that increasing five-ring PAHs concentrations (B[*a*]P and B[*b*]F) would enhance, the transcription of CYP-family P450 monooxygenases and, consequently, the EROD activity. Accordingly, we could also observe an increase in the protein expression of CYP1A1 during exposure of B[*a*]P (Fig. 3). B[*a*]P is a model AhR agonist, and acts by binding to this receptor, that when translocated to the nucleus dimerizes with AhR nuclear translocator (ARNT) and binds on XREs (xenobiotic-responsive elements) in the promoter of AhR target genes. These include phase I enzymes such as CYP1A1 and CYP1A12, and phase II enzymes, such as GST (Vogel et al., 2020). Interestingly, we did not observe an increase in CYP1A1 mRNA levels, contrary to reported elsewhere (Goedtke et al., 2021; Kang et al., 2022; Soltani et al., 2019). Activation of B[*a*]P generates reactive epoxides which can form adducts with DNA (Ewa and Danuta, 2017) which explains the high degree of DNA strand breakage observed (Fig. 5A). B[*b*]F can also bind to AhR, and reportedly has a stronger agonist activity than B[*a*]P (Goedtke et al., 2020; Vakharia et al., 2001), but here no significant alteration in CYP1A1 protein expression and mRNA levels was observed (Fig. 3), which also translated in lower levels of DNA damage (Fig. 5A).

Exposure to the 3-ring PAH, Phe, demonstrated to be a poor inducer of EROD activity, decreasing its activity at all tested concentrations. Furthermore, it did not alter the expressions of protein levels or mRNA levels of CYP1A1. This lack of CYP1A1 induction is in line with the lack of genotoxic effects (Fig. 5A) and with previous reports on Phe's non-carcinogenic nature (Billiard et al., 2006). This repression of CYP1A1 could be a feature of lower molecular weight PAH since fluoranthene is known to behave similarly (Willett et al., 1998). The Phe:B[*a*]P mixture demonstrated the most

interesting and intricate results. It increased CYP1A1 protein expression in all compounds ratios and concentrations when compared to control group (Fig. 3), especially at the 2:1 ratio (50 μM) with results being even higher than when cells were exposed to B[*a*]P alone. Likewise, mRNA levels were increased at all Phe:B[*a*]P ratios but, also mostly at the 2:1 compound ratio. This is a very interesting and unforeseen result since Phe, as mentioned above, is considered a non-carcinogenic compound and a non-inducer of CYP1A1 (Billiard et al., 2006), while B[*a*]P is a well-known carcinogen and a strong inducer of CYP1A1 through AhR binding (Franco et al., 2018). This suggests that an effect due to Phe activity, presumably AhR-independent, should be involved.

Previous studies reported that Phe has the ability to interfere in CYP1A1 (Billiard et al., 2006) and induce oxidative stress therefore promoting DNA strand breakage (Martins, 2015). The mechanism is probably related with the poor induction of Nrf-2 signaling by Phe albeit it generates ROS production (Branco et al., 2021). Some studies show a higher cytotoxicity for Phe than other PAHs (Piasecka et al., 2011) and in zebrafish (*Danio rerio*) larvae it caused a similar toxic response when in comparison to B[*a*]P (Piasecka et al., 2011). However, others (Søfteland et al., 2014) suggest a possibility that Phe toxicity in fish can be induced via the AhR, in line with some studies with other low-molecular-weights PAHs (Zhou et al., 2020; Sakshi and Haritash, 2020; Incardona et al., 2005). Additionally, in humans Phe can bind to other receptors, such as CAR (constitutive androstane receptor), a receptor that can influence the expression of CYP family enzymes. In fish, PXR (pregnane x receptor) is the homologous receptor to CAR (Goedtke et al., 2021; Goedtke et al., 2020; Yang et al., 2019) which may potentiate synergistic effects between PAHs (Zhou et al., 2020; Kim et al., 2018). Although this mechanism requires further elucidation, it is clear that the environmental guidelines need to be reviewed since these do not properly account for the particularities of PAH mixtures.

The mixture B[*a*]P:B[*b*]F increased the expression of CYP1A1 at the ratios of 1:1 and 2:1 at all concentrations (Fig. 3) but did not alter the mRNA levels of CYP1A1. This result differs from other studies, such as Martins et al. (2015), where it was observed that mixtures containing B[*b*]F presented a higher CYP1A1 induction than isolated exposure. Since both B[*a*]P and B[*b*]F are potent AhR inhibitors, activation and gene transcription could have occurred earlier than 24 h of exposure and, therefore only the increase CYP1A1 protein levels is observed. In fact, B[*a*]P: B[*b*]F mixture led to a time-dependent increase in DNA damage especially with the ratio 2:1 (Fig. 5C).

Since PAHs are activated into epoxide intermediates by CYP1A1 and further converted to more reactive diol-epoxides by epoxide hydrolase (EPXH), we also examined the mRNA levels of EPXH1. There were no significant alterations caused by the exposures but it is possible to detect a slight increase with the mixture Phe:B[*a*]P 2:1 10 μM (Fig. 4C). Most importantly, we observed no statically significant alterations in GST-3 levels with any treatment, in agreement with studies reporting a low induction of GST activity by PAHs (Pushparajah et al., 2008). Since GSTs are involved in Phase II metabolism of PAHs, acting in the cellular detoxification and excretion (Bebianno and Barreira, 2009), the lack of changes to its activity and expression demonstrates that PAH metabolites conjugation to glutathione and subsequent detoxification is not up-regulated.

Hence, when there is no induction of GST but there is a simultaneous induction of CYP1A1 activity, there is a higher probability of DNA damage due to accumulation of reactive PAH metabolites (Soltani et al., 2019).

5. Conclusion

Our study demonstrated that a mixture between PAHs with and without carcinogenic potential may lead to higher CYP1A1 induction and consequently to increased genotoxicity in fish primary hepatocytes. This is a very relevant result since Polycyclic Aromatic Hydrocarbons exist mainly as mixtures in the environment. Because different PAHs have distinct mechanisms of action and toxicity (e.g. carcinogenic vs. non-carcinogenic), the interaction effects of their mixtures may be very different from the observed for the individual compounds. Notwithstanding, environmental quality guidelines almost exclusively address PAH contamination and toxicity from a single compound point of view. This can significantly underestimate risk and stresses the need to properly evaluate the effects of PAH mixtures in more realist scenarios such as chronic in vivo assays or in situ experiments. To further reinforce the need to address PAH toxicity from a mixture toxicology point of view, it should be mentioned that herein we examined the effects of a mixture of two compounds but, in nature, mixtures may involve a much larger number of compounds, making effects even harder to predict.

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CRediT authorship contribution statement

Isabella Bramatti: writing – original draft, formal analysis, investigation; **Beatriz Matos:** writing – original draft, formal analysis, investigation; **Neusa Figueiredo:** formal analysis; **Pedro Pousão-Ferreira:** resources; **Vasco Branco:** Conceptualization, methodology, validation, resources, writing – review and editing; **Marta Martins:** Conceptualization, methodology, validation, supervision, resources, writing – review and editing, Project administration and funding acquisition.

All authors have read and agreed to the published version of the manuscript.

Institutional statement

Ethical review and approval were waived for this study, due to the absence of experiments with live animals. Only primary cells were collected from fish using humane procedures performed by FELASA category C certified researchers, which is author of this manuscript (M.M.). Housing of fish took place in certified fish facilities approved by the appropriate Portuguese authority, Direção Geral da Alimentação e Veterinária (ref: 0421/000/000/2013).

Informed consent statement

Not applicable.

Data availability

The data presented in this study are available on request from the corresponding author.

Declaration of competing interest

The authors declare no conflict of interest.

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