NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF ENVIRONMENTAL SCIENCES AND ENGINEERING

Molecular insight on the toxicity of mixtures of Polycyclic Aromatic Hydrocarbons to improve environmental guidelines

BEATRIZ ISABEL MANSO DE MATOS

Master in Biochemistry

DOCTORATE IN ENVIRONMENT AND SUSTAINABILITY

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“Courage is the root of change -
and change is what we're chemically designed to do"
Bonnie Garmus- Lessons in Chemistry

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are persistent environmental pollutants with toxic, mutagenic, and carcinogenic properties. They are usually present in the environment as a mixture of carcinogenic and non-carcinogenic compounds, that may induce interaction effects whose mechanisms remain poorly understood. This thesis investigates PAH toxicity using both *in vitro* and *in vivo* approaches, focusing on key toxicological endpoints, including gene and biochemical responses related with PAH detoxification mechanisms, oxidative stress and genotoxicity. By examining PAH interactions within complex mixtures, in different ratios, in cellular models and whole-organism studies, it provides insights into their mode of action and implications for environmental and human health risk assessment.

An optimized pancreatin digestion method enabled the isolation of fish hepatocytes for PAH toxicity assessment. Primary hepatocytes of *S. aurata* showed that, in general, exposure to mixtures of phenanthrene (Phe) and benzo[a]pyrene (B[a]P) significantly altered several biochemical responses with the Phe:B[a]P 2:1 mixture, leading to a 70% increase in DNA damage and enhanced CYP1A expression (5-fold). In juvenile *S. aurata* exposed for 42 days, PAHs triggered organ-specific effects, including oxidative stress in gills and liver, and a 7-fold increase in hepatic GST3 expression after Phe:B[a]P 2:1 exposure. MDS analysis further identified biological response patterns, providing insight into how fish cope with PAHs.

To assess interactions between PAHs in mixtures over Aryl Hydrocarbon Receptor (AhR) activation, *in vitro* experiments using HepG2 cells were conducted. Results showed that AhR activation, a major regulation pathway of CYP1A mediated metabolism, varied with mixture composition, exhibiting antagonistic interactions at lower concentrations, likely due to Phe competition with other PAH compounds.

Overall, these results highlight the complexity of PAHs mixture toxicity and its context-dependent effects. While individual PAHs triggered distinct biochemical responses, mixtures altered toxicity patterns. For instance, the 2:1 Phe:B[a]P mixture showed synergistic effects in fish models but antagonistic interactions in HepG2 cells. Previous studies in PAHs mixtures toxicity suggested that they might interact synergistically or antagonistically depending on the toxicity model. Furthermore, our findings suggest that different ratios and types of PAHs in mixture can activate not only CYP1A1 but another pathways, highlighting their unpredictable behaviour emphasizing the need for a more comprehensive risk assessment approach.

Keywords: PAHs, Mixtures, *in vitro*, *in vivo* , Genotoxicity

RESUMO

Os hidrocarbonetos aromáticos policíclicos (HAPs) são poluentes ambientais persistentes com propriedades tóxicas, mutagénicas e cancerígenas. Estão geralmente presentes no ambiente como uma mistura de compostos cancerígenos e não cancerígenos, que podem induzir efeitos de interação cujos mecanismos são ainda pouco compreendidos. Esta tese investiga a toxicidade dos HAPs utilizando abordagens *in vitro* e *in vivo*, com foco nos principais desfechos toxicológicos, incluindo respostas genéticas e bioquímicas relacionadas com os mecanismos de desintoxicação dos HAPs, stress oxidativo e genotoxicidade. Ao examinar as interações dos HAPs em misturas complexas, em diferentes proporções, em modelos celulares e estudos de organismos inteiros, são fornecidas informações sobre o seu modo de ação e implicações para a avaliação de riscos ambientais e para a saúde humana.

Um método otimizado de digestão com pancreatina permitiu o isolamento de hepatócitos de peixe para avaliação da toxicidade dos HAPs. Os hepatócitos primários de *S. aurata* mostraram que, em geral, a exposição a misturas de fenantreno (Phe) e benzo[a]pireno (B[a]P) alterou significativamente diversas respostas bioquímicas com a mistura Phe:B[a]P 2:1, levando a um aumento de 70% dos danos no ADN e a um aumento da expressão do CYP1A (5 vezes). Em juvenis de *S. aurata* expostos durante 42 dias, os HAP desencadearam efeitos específicos nos órgãos, incluindo stress oxidativo nas brânquias e no fígado, e um aumento de 7 vezes na expressão hepática de GST3 após exposição a Phe:B[a]P 2:1. A análise do MDS identificou ainda mais padrões de resposta biológica, fornecendo informações sobre a forma como os peixes lidam com os HAPs.

Para avaliar as interações entre os HAPs em misturas durante a ativação do recetor de hidrocarbonetos arílico (AhR), foram conduzidas experiências *in vitro* utilizando células HepG2. Os resultados mostraram que a ativação do AhR, uma importante via de regulação do metabolismo mediado pelo CYP1A, variou com a composição da mistura, exibindo interações antagónicas a concentrações mais baixas, provavelmente devido à competição do Phe com outros compostos de PAH.

No geral, estes resultados realçam a complexidade da toxicidade da mistura de HAPs e os seus efeitos dependentes do contexto. Enquanto os HAPs individuais desencadearam respostas bioquímicas distintas, as misturas alteraram os padrões de toxicidade. Por exemplo, a mistura 2:1 Phe:B[a]P apresentou efeitos sinérgicos em modelos de peixes, mas interações antagónicas em células HepG2. Estudos anteriores sobre a toxicidade de misturas de HAPs sugeriram que estes podem interagir sinergicamente ou antagonicamente, dependendo do modelo de toxicidade. Além disso, as nossas descobertas sugerem que diferentes proporções e tipos de HAPs na mistura podem ativar não só o CYP1A1, mas outras vias, destacando o seu comportamento imprevisível e enfatizando a necessidade de uma abordagem de avaliação de risco mais abrangente.

Palavras-chave: HAPs, Misturas, *in vitro*, *in vivo*, Genotoxicidade

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ACRONYMS

A549. Lung cancer cell line

AhR. Aryl hydrocarbon receptor

AOPs. Adverse outcomes pathway

ARNT. AhR nuclear translocator

B[a]A. Benzo[a]anthracene

B[a]P. Benzo[a]pyrene

B[b]F. Bezno[b]fluoranthene

BPDE. 7,8-diol-9,10-epoxide

BSA. Bovine Serum Albumin

CA. Concentration Addition

CAR. Constitutive adrostane receptor

CAT. Catalase

CYPs. Cytochrome P450 enzymes

DMEM. Dulbecco's modified eagle medium

DMSO. Dimethyl sulfoxide

DTNB. 5,5′-dithiobis-(2-nitrobenzoic acid

ECHA. European Chemicals Agency (ECHA)

EDCs. Endocrine-disrupting chemicals

EDTA. Ethylenediaminetetraacetic acid

ENAs. Erythrocytic nuclear abnormalities

EPA. Environmental Protection Agency

EPXH. Epoxide Hydrolase

EQSD. Environmental Quality Standards Directive

EROD. Ethoxyresorufin-O-deethylase

EU. European Union

F-12. Ham's F12 medium

FBS. Fetal Bovine Serum

FLT. Fluoranthene

GAPDH. Glyceraldehyde-3-phosphate dehydrogenase

GC-MS. Gas chromatography–mass spectrometry

GPx. Glutathione Peroxidase

GR. Glutathione Reductase

GSH. Glutathione

GSSG. Oxidized Glutathione

GST. Glutathione-S-transferase

HBSS. Hanks' Balanced Salt Solution

HeLa. Cervical cancer cell line

HEPES. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HepG2. Hepatoblastoma cell line

HRP. Horseradish peroxidase

HT-29. Coloretal adenocarcinoma line

IA. Independent Action

IARC. International Agency for Research on Cancer

IATA. Integrated Approaches to Testing and Assessment

ISO. International Organization for Standardization

LDH. Lactate dehydrogenase

LPO. Lipid peroxidation

MDR. Model Deviation Ratio

MEI. Mixture Effect Index

MOA. Mode-of-action

MPs. Microplastics

MSFD. Marine Strategy Framework Directive

MTS. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

MTT. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide

NADPH. Nicotinamide adenine dinucleotide phosphate

NAMs. New approach methodologies

NMDS. Non-Metric Multidimensional Scaling

NPs. Nanoplastics

NQO1. NADPH quinone dehydrogenase

Nrf-2. Transcription factor

OECD. Organisation for Economic Co-operation and Develpment

PAHs. Polycyclic aromatic hydrocarbons

PCBs. Polychlorinated biphenyls

PCDDs. Polychlorinated dibenzo-p-dioxins

PCDFs. Polychlorinated dibenzofurans

PCPs. Personal Care Products

PFAS. Per and polyfluoroalkyl substances

Phe. Phenanthrene

POPs. Persistent Organic Pollutants

PXR. Pregnane X receptor

QSAR. Quantitative Structure Activity Relationship

ROS. Reactive oxigen species

RTL-W1. Rainbow trout liver cell line

SDGs. Sustainable Development Goals

SDS. Sodium dodecyl sulphate

SOD. Superoxide dismutase

TBARS. Thiobarbituric acid-reactive substance

TCA. Trichloroacetic acid

TEF. Toxic Equivalent Factors

TEQ. Total toxic equivalent

TP53. Tumour protein 53

TU. Toxic Unit

UGT1A. UDP glucuronosyltransferase 1 family, polypeptide A cluster

WFD. Water Framework Directive

WHO. World Health Organization

XREs. Xenobiotic responsive elements

ZFL. Zebrafish hepatocytes line

1

Introduction

1.1 General Overview

1.1.1 Aquatic Ecosystems

Aquatic ecosystems are vital components of our planet's ecological and biological networks, encompassing a wide range of habitats that support immense biodiversity and essential ecological functions[1]. These ecosystems provide critical resources and services that are essential to human life, such as food, climate regulation, and recreational opportunities, but they also play a pivotal role in maintaining the health and stability of the global environment[2,3]. Marine ecosystems exhibit remarkable diversity and can be classified into several types based on their unique characteristics and functions.

Oceans absorb around 90% of anthropogenic CO_2[4], helping mitigate global climate change by channelling excess CO_2 into deep-sea sediments[5].

In recent decades, pollution in aquatic ecosystems has garnered significant attention and is now a major societal challenge, posing serious health risks to living organisms.[6] Population growth, rapid industrialization, increasing urbanization, and careless use of natural resources have all negatively impacted water quality. Identifying contamination sources and estimating their effects is complex due to the diversity of contaminant types and concentrations, as well as the specificities of the ecosystems affected.[7]

Among all environmental compartments, aquatic environments are particularly vulnerable, as they often serve as the final reservoir for numerous contaminants[7]. Therefore, effective management and conservation strategies are crucial to preserving their ecological integrity and ensuring a sustainable future for both nature and humanity.

1.1.2 Anthropogenic Sources of Pollution

Anthropogenic activities are major contributors to aquatic pollution through various sources as recognized by the United Nations Convention on the Law of the Sea (UNCLOS) 1982[8,9]:

- **From Land**: Human activities on land are responsible for 80% of marine pollution. The major input of toxic substances into marine waters comes from pipelines discharging

pollutants of all kinds. Additionally, rivers transport toxic substances from entire catchment areas to the sea, contributing significantly to aquatic pollution.

- **From Air**: The atmosphere plays a crucial role in aquatic pollution, as global toxic inputs to the sea are due to atmospheric transport and discharges. Pollutants from industrial emissions and other airborne sources settle into waters, exacerbating contamination.
- **From Water:** Oily discharges are released daily through ballast and bilge waters due to the illegal dumping of waste from ships. Furthermore, toxic substances are often accidentally lost from ships. Other continuous sources of pollutants include dredging materials, sewage sludge, fly ash, and oil-based sludge, all of which contribute to the degradation of aquatic environments[10].

Pollution may occur continuously, such as through sewage discharge in cities or industrial waste released into water bodies. It can also occur intermittently, occurring at either irregular or regular intervals. Additionally, pollution can be temporary or accidental, as seen in cases of unexpected environmental disasters[6]. Collectively, these pollution sources degrade water quality, disrupt ecosystems, and pose severe health risks to both aquatic organisms and humans.

For instance , Macau, one of the most densely populated regions worldwide, was identified as a critical polluted area due to significant levels of pollutants such as mercury, benzo[a]anthracene (B[a]A), fluoranthene (FLT), and benzo[b]fluoranthene (B[b]F) originating from vehicle emissions, hazardous materials, and municipal waste[11].

Another dramatic example of anthropogenic pollution was the Minamata Disaster that occurred in Japan during the mid-20th century[12]. The disaster was linked to the Chisso Corporation, a chemical company that began operations in Minamata in 1908, which released industrial wastewater containing methylmercury, into Minamata Bay and the Shiranui Sea. The methylmercury in the wastewater accumulated in the marine environment, contaminating fish and shellfish, the primary food source for the local population. Over time, the methylmercury bioaccumulated in the food chain, reaching toxic levels to the local population who consumed the seafood.

A notable example occurred in November 2002, with the Prestige disaster[13]. This environmental catastrophe took place off the coast of Galicia, Spain, when the oil tanker

Prestige sank while, carrying about 77,000 tons of heavy fuel oil. The spill released was highly toxic, contaminating beaches and coastal waters. The spill had devastating effects on marine life, including fish, birds, and other wildlife. Cleanup efforts took years to restore the affected areas.

These incidents represent just a small sample of anthropogenic pollution, highlighting the diverse sources and their significant impacts on various ecosystems, underscoring the need for ongoing monitoring and effective regulatory measures.

1.1.3 Environmental pollutants and general biological effects

Water pollutants can be categorized in different categories: Toxic Metals, Pharmaceuticals and Personal Care Products, Microplastics and Nanoplastics, Nanoparticles, Agrochemicals, Nutrients, Radioactive Substances and Persistent Organic Pollutants. Figure 1.1 depicts the different chemical pollutants that end up in the aquatic environment.

These pollutants possess diverse physicochemical properties, including solubility, biodegradability and volatility which significantly influence their behaviour in aquatic environments. These characteristics determine how pollutants disperse, persist, and accumulate in water bodies. Consequently, contaminants interact differently with ecosystems, posing varied and potentially severe risks to aquatic life, other organisms, and even human health through the contamination of drinking water and seafood. Understanding these properties is crucial for assessing environmental impacts and developing effective mitigation strategies.

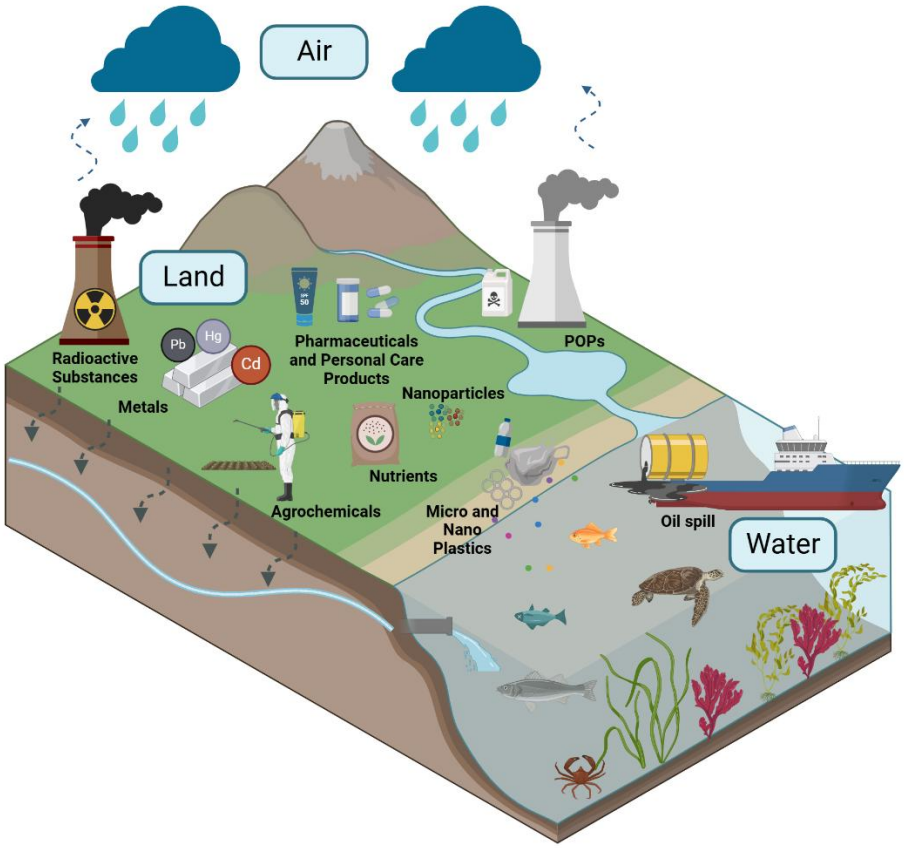


Figure 1.1 - Categories of pollutants that enter to the aquatic environment due to anthropogenic activities. Created in BioRender.com

1.1.3.1 Toxic Metals

Metals and metalloids constitute the majority of the chemical elements in the periodic table. Although many are biologically essential, others have no known biological function and are generally regarded as highly toxic. These include metals such as cadmium (Cd), lead (Pb), mercury (Hg) and the metalloid arsenic (As)[14] .

Although human activity does not create or destroy these elements, it can significantly impact their biogeochemical cycle, changing the chemical speciation, increasing movement across ecosystems, bioavailability and toxicity. These toxic metallic elements are released daily into water from various natural and anthropogenic sources, including industrial, agricultural, and domestic activities (e.g., garbage, detergents)[15], as well as mining[16]. In the aquatic environment, toxic metals are commonly measured in μg/L or mg/L in water, and in mg/kg in sediments and biota. In many regions worldwide, the average concentrations of toxic metals

in surface water bodies exceed the maximum allowed values for drinking water[15]. Examples of this values establish by WHO in drinking water are: 10 μg/L for Lead, 3 μg/L for Cadmium and 6 μg/L for Mercury[17]. Metals are particularly toxic to aquatic organisms, with documented genotoxic[18], neurotoxic[19], and endocrine-disrupting effects[20]. These metals can cause DNA damage, disrupt normal neurological functions, and interfere with hormone systems, leading to reproductive and developmental issues in aquatic life. Additionally, metals can exist in different chemical forms (metal speciation)[21] which can greatly influence the metal's behaviour, bioavailability and mobility in the environment[22]. Various methods have been developed to remove toxic metals from water, but conventional treatments often have significant drawbacks, such as incomplete removal, high energy requirements, and the production of toxic sludge. As a result, approximately 40% of the planet's lakes and rivers have been polluted by toxic metals[23]. Since metals are non-degradable, at best, treatments transfer them to another environmental compartment [24].

1.1.3.2 Pharmaceuticals and Personal Care Products (PCPs)

With the rapid development of medicine and the increasing consumption of various personal care products (PCPs), large quantities of pharmaceuticals and PCPs are being released into the environment. Major sources of these pollutants include domestic sewage[25], agricultural waste[26], industrial discharges[27], and improper disposal of unused medications[28]. Common pharmaceuticals found in the environment include anti-inflammatory drugs, antibiotics, antiretroviral drugs, analgesics, antidepressants, and hormones, while PCPs encompass products like fragrances, disinfectants, and cosmetics[26,27,29,30].

These substances are widely used globally, contributing to their pervasive presence in the environment[27,29]. After ingestion or application, many of these pharmaceuticals and PCPs are not fully absorbed or metabolized by humans and farm animals. Consequently, they are excreted in urine and faeces as either active substances or metabolites. These excreted compounds frequently enter wastewater systems, where traditional wastewater treatment plants may not efficiently remove them[31]. As a result, concentrations of these substances can range from ng/L to mg/L[32] in surface waters, depending on the compound and environmental context.

While some countries have begun to set regulatory limits for certain pharmaceuticals and PCPs in environmental matrices, there is still a significant lack of comprehensive global legislation. The established limits often focus on specific high-risk substances, but many

emerging contaminants remain unregulated[33]. In aquatic environments, these pollutants are known to have various toxic effects on organisms, including genotoxic[34], neurotoxic[35], and endocrine-disrupting properties[36]. These effects can lead to adverse outcomes such as reproductive disorders, behavioural changes, and developmental abnormalities in aquatic species[37].

The main challenges in addressing the presence of pharmaceuticals and PCPs in the environment include the vast number of these substances, their diverse chemical properties, and the complexity of their interactions within ecosystems[38]. Moreover, the lack of efficacy of existing wastewater treatment technologies, coupled with the lack of comprehensive environmental monitoring and regulation, further exacerbates the issue. As a result, there is a growing need for advanced treatment methods, stricter regulations, and more extensive research to better understand and mitigate the environmental impact of these contaminants[26].

1.1.3.3 Microplastics and Nanoplastics

Plastics have been widely used for their practicality and versatility since the last century. They can be released into the aquatic environment through various pathways, including improper disposal of plastic waste[39], runoff from landfills[40], industrial discharges[41], and wastewater effluents[42]. Once in the environment, plastics suffer degradation or breakdown due to ultraviolet radiation, mechanical abrasion, or biological mechanisms, resulting in smaller particles of micro (0.1–1000 mm) and nanoscale size (<100 nm)[43–45]. Microplastics (MPs) are also intentionally introduced into the environment through manufactured products, such as microbeads in personal care products, while nanoplastics (NPs) are extensively used in electronics, paints, and drug delivery systems[43].

These plastic particles accumulate and persist in aquatic habitats for years. Harmonizing microplastic sampling protocols[40,46] and addressing technological challenges are crucial for accurately assessing their impact on marine life and their role as pollutants in aquatic environments. MPs and NPs are considered emerging pollutants since they are relatively recent and their fate, interactions and effects on organisms are poorly understood[41]. However, it is well-established that they negatively impact aquatic organisms' by affecting growth, development, behaviour, reproduction, and survival[44], posing a significant environmental threat. Furthermore, these plastic particles can exacerbate contaminant transport, underscoring the need for a better understanding of their mobility, aggregation behaviour, and potential for enhanced risk prediction[45].

1.1.3.4 Nanoparticles

Nanoparticles are materials with dimensions measured in nanometres, typically between 1 and 100 nm. Due to their small size, nanoparticles exhibit unique physical and chemical properties, making them valuable in various industries, including medicine, electronics, and environmental remediation[47,48]. Their widespread use has led to increased release into the environment, where they can interact with biological systems in ways that differ from larger particles[49]. Nanoparticles enter aquatic environments through various sources, including industrial processes[50], medical applications[51] and consumer products (cosmetics, textiles, sunscreens)[52]. The concentration of nanoparticles in surface waters is typically estimated to range from ng/L to mg/L, depending on the specific type of nanoparticles and the characteristics of the environment[53]. Nanoparticles are known to have toxic effects on aquatic organisms, including genotoxicity[47], oxidative stress[54] and neurotoxicity[55]. The main challenges in addressing nanoparticle pollution include the difficulty in detecting and quantifying them in environmental samples, due to their physical transformation[49], understanding their long-term ecological effects, and establishing effective regulatory frameworks to manage their risks[52]

1.1.3.5 Agrochemicals

With the increasing world population, agricultural systems face growing pressure to meet food production demands[56]. Consequently, larger quantities of pesticides, including herbicides, fungicides, nematicides, and fertilizers, are being used to control pest populations and increase crop yields.[57]. These agrochemicals often enter freshwater and marine ecosystems through agricultural runoff and industrial discharge[58]. As a result, the concentration of these compounds in aquatic environments is rising, with reported levels ranging from ng/L to μg/L, depending on the specific compound and the proximity to agricultural activities[59]. These agrochemicals can cause significant harm to aquatic ecosystems by disrupting food chains and degrading water quality, ultimately affecting both biodiversity and human health[60]. Pesticides are known to exhibit a range of toxic effects on aquatic organisms, including genotoxic[60], neurotoxic[57], and endocrine-disrupting[58] properties. These effects can lead to behavioural changes, reproductive impairments, and increased mortality in aquatic species[60]. The main

challenges in addressing agrochemical pollution in aquatic environments include the diversity of chemical properties among different pesticides, their varying persistence in the environment, and the difficulty in monitoring and regulating their presence[56]. The environmental fate of these compounds can vary widely, with some being highly persistent and prone to bioaccumulation, while others degrade rapidly into potentially harmful byproducts [61]

1.1.3.6 Nutrients

Increased concentrations of nutrients, particularly nitrogen (N) and phosphorous (P) resulting from anthropogenic activities have emerged as a significant global environmental concern impacting water quality[5]. The main sources of nutrient pollution include agricultural runoff (from fertilizers and manure)[62], wastewater discharge[63], industrial effluents[64], and atmospheric deposition[65]. While these nutrients are vital for plant growth, their excessive presence in water bodies can give rise to various ecological and health issues[63]. An aquatic environment is considered eutrophic when phosphorus concentrations range from 30 to 100 μg/L and nitrogen concentrations range from 1 to 2 mg/L, depending on the proximity to pollution sources and the specific conditions of the water body[66]. Eutrophication a process primarily driven by nutrient overload - causes increased suspended particles due to macroalgal blooms, reduced water clarity, and destruction of benthic habitats from shaded vegetation. It also leads to bottom-water hypoxia, CO_2 production from organic matter decomposition, and enhanced water acidification. Additionally, eutrophication alters biogeochemical processes, resulting in sediment anoxia, accumulation of harmful hydrogen sulphide, and disrupted nutrient cycling[62,64,67]. In the long term, eutrophication may lead to ecological changes[68]. The main challenges in addressing nutrient pollution include the diffuse nature of nutrient sources, the complexity of nutrient cycles in aquatic ecosystems, and the limitations of existing treatment technologies. Biological treatment processes, which are commonly used in wastewater treatment, often fail to reduce nutrient levels to meet disposal standards. Therefore, further research is essential to develop more effective and sustainable treatment technologies[62,69].

1.1.3.7 Radioactive Substances

Radioactive substances or radionuclides are naturally present in seawater or sediments since the beginning of the universe[70], the majority of which are short lived radionuclides that decay within a few days. However, there are some cases in which radionuclides were introduced in the ocean by technical / anthropogenic processes becoming pollutants due to the long decay periods. These include radionuclides such as Potassium (^{40}K), Uranium (^{238}U and ^{235}U), Thorium (^{232}Th), Iodine (^{131}I), Cesium (^{134}Cs and ^{137}Cs,) and Radium (^{226}Ra))[71–73] . Ionizing radiation from radioactive particles can cause severe biological effects, including DNA damage and necrosis in tissues of aquatic organisms and long-term effects in humans[74–76]. Effective detection and decontamination methods are essential for managing radioactive contamination. However, further research is needed to fully understand the long-term ecological impacts and pathways of radioactive particles in the environment[74]. Additionally, raising global safety awareness on the handling and disposal of hazardous radioactive materials and, establishing radioactive waste management programs are essential steps toward mitigating risks [71].

1.1.3.8 Persistent Organic Pollutants

Persistent Organic Pollutants (POPs) are industrial chemicals introduced into aquatic ecosystems primarily through industrial processes, waste disposal, oil spills, and fossil fuel combustion. Notable examples of POPs include polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), Per and polyfluoroalkyl substances (PFAS), and polycyclic aromatic hydrocarbons (PAHs)[77,78]. These chemicals are characterized by their resistance to biological, chemical, and photolytic degradation, allowing them to persist in the environment for years or even decades and enabling their transport over thousands of miles from their sources[79–81].The persistence and mobility of POPs make tracing their occurrence, distribution, and fate in the environment particularly challenging. POPs can exist in various phases, such as gas, dissolved in water, or attached to airborne particles or solid particulate matter (SPM). They can also be exchanged among different environmental compartments, further complicating their monitoring[80]. POPs are highly toxic and pose significant health risks, including development of certain cancers[82], birth defects[83], immune and reproductive system dysfunctions[84], increased susceptibility to cardiovascular disease[85], and diabetes[86]. Given their potential to cause severe health issues,

monitoring and managing POPs in aquatic environments is critical[78]. Quantification of POPs is analytically challenging because they usually occur in water at very low concentrations (ρg/L range), requiring highly sensitive analytical methods. Additionally, POPs tend to accumulate in sediments, soils, and biota, where they become even harder to detect due to the presence of interfering compounds. Sample preparation is necessary to isolate POPs from these matrices, but this process is time-consuming and complex[87]. Advanced analytical techniques, on-site detection methods, and biomonitoring are among the approaches being explored to improve the detection and mitigation of these persistent pollutants. The Stockholm Convention is an international agreement aimed at reducing and eliminating the production, use, and release of persistent organic pollutants (POPs) adopted in 2001 and came into force in 2004 under the auspices of the United Nations Environment Programme (UNEP). Some measures of Stockholm Convention are: Identification and Listing of POPs, National Implementation Plans, Provisions for Specific POPs, Monitoring and Reporting and Review and Amendment. The latest significant update to the Stockholm Convention occurred at the 11th Conference of the Parties (COP-11) held in May 2023. Additionally, the global monitoring plan for POPs is continuously updated to include newly listed chemicals and to streamline data harmonization and usage procedures[88]

A summary of the main characteristics of each type of pollutant is presented in Table 1.1.

Table 1.1 - Main pollutants in the aquatic environment and their effects on aquatic animals.

Pollutants	Source	Effects	Examples
Toxic Metals	Domestic activities Agricultural waste Industrial discharges Mining	Genotoxic Neurotoxic Endocrine-disrupting	Copper (Cu) Cadmium (Cd) Zinc (Zn) Lead (Pb) Mercury (Hg)
Pharmaceuticals and PCPs	Domestic sewage Agricultural waste Industrial discharges Improper disposal of unused medications	Genotoxic Neurotoxic Endocrine-disrupting	Anti-inflammatory drugs Antibiotics Analgesics Hormones Fragrances Disinfectants
Micro and Nanoplastics	Microbeads in personal care products Electronics Paints Drug delivery systems	Growth Development Behaviour Reproduction Survival	Plastic pellets Plastic fibres
Nanoparticles	Industrial processes Medical applications Consumer products (cosmetics, textiles, sunscreens)	Genotoxicity Oxidative stress Neurotoxicity	Carbon nanotube Liposome Gold nanoparticle Quantum Dot
Agrochemicals	Agricultural runoff Industrial discharge	Genotoxic Neurotoxic Endocrine-disrupting	Insecticides Herbicides Fungicides Fertilizers
Nutrients	Agricultural runoff Wastewater discharge Industrial effluents	Reduced water clarity Macroalgal bloom Bottom-water hypoxia Water acidification	Nitrogen (N) Phosphorous (P)
Radioactive Substances	Technical and anthropogenic processes	DNA damage Necrosis in tissues	Potassium (40K) Uranium (238U and 235U) Cesium (134Cs and 137Cs,) Radium (226Ra))
Persistent Organic Pollutants	Industrial processes Waste disposal Oil spills Fossil fuel combustion	Cancers Birth defects Immune and reproductive system dysfunctions	Polychlorinated biphenyls (PCBs) Polychlorinated dibenzo-p-dioxins (PCDDs) Polycyclic aromatic hydrocarbons (PAHs)

1.1.4 Bioaccumulation and Biomagnification

Having discussed the various categories of aquatic pollutants, it is essential to understand the processes through which these contaminants interact with aquatic organisms. Specifically, the concepts of bioaccumulation and biomagnification are critical in elucidating how pollutants can concentrate and amplify through the food web, leading to significant ecological and health impacts.

Aquatic ecosystems are particularly suitable for such assessments as they serve as the final receptors of contaminants released into the environment. Bioconcentration refers to the uptake and retention of chemical substances by organisms directly from water [89–91]. Absorption can also occur from other sources such as soil and sediment or through the ingestion of contaminated food[92,93].

Bioaccumulation refers to the gradual accumulation of substances, such as pesticides or other chemicals which are moderately hydrophobic and poorly metabolized or excreted by organisms. [94] It is a kinetic process that compares the internal concentration of the compound at a given moment in time with its concentration at a later time. This process occurs when the absorption rate of a compound exceeds its elimination rate, meaning that the substance accumulates faster than it is excreted. Bioaccumulation can occur in both aquatic and terrestrial environments[95,96]. Furthermore, the scarcity of empirical data on bioaccumulation compared to other parameters underscores the need for further research in this area, especially given the challenges of poor data quality and inadequate procedural documentation[97]. Additionally, bioaccumulation models have limited applicability across all chemical classes, highlighting the complexity of assessing bioaccumulative potential. Accumulation levels depend on factors such as size, dietary habits, and reproductive stage, as well as environmental variables like pH, dissolved oxygen, and water temperature[93].

On the other hand, biomagnification describes the phenomenon wherein pollutants become increasingly concentrated as they move up the food chain, since the chemical concentration in an organism exceeds the concentration of its food when the major exposure route occurs from the organism's diet [10,91,92] . For instance, predatory fish species at higher trophic levels are particularly prone to biomagnification of metals, serving as important vectors for the transfer of these contaminants to humans through diet[98]. Biomagnification factors (BMFs) represent the ratio of the concentration of a substance in an organism to the concentration of the substance in its food[99]. Numerous studies have highlighted the significant influence of physicochemical properties, environmental conditions, and food chains on the

bioaccumulation of chemicals and persistent organic pollutants across various species[100]. Bioaccumulation and biomagnification have profound ecological and health implications due to the accumulation of toxic substances in organisms and their increased concentration at higher trophic levels. Ecologically, these processes can cause severely impact wildlife populations and ecosystems. Persistent pollutants can cause reproductive, neurological and immune system deficiencies in several species, ultimately leading to population decline and disruption of ecosystem dynamics[19]. From a human health perspective, the biomagnification of contaminants in food chains poses direct risks. As an example humans consuming seafood contaminated with toxic metals can suffer from various health issues, including developmental problems, cancer, and immune system suppression[101]. The presence of pharmaceuticals in aquatic ecosystems also highlights the risk of chronic exposure to low concentrations of these chemicals, potentially leading to long-term health effects[30]. A comprehensive understanding of these processes is essential for mitigating the adverse effects of environmental pollution on ecosystems and human well-being. Furthermore, implementing robust laws and regulations is essential for the conservation of water sources. Regular monitoring of industrial effluents and pollutant discharges is necessary, along with strict enforcement of environmental protection measures. Effective legislation in both developed and developing economies plays a vital role in safeguarding aquatic environments.

1.2 Environmental Guidelines and Strategies

As seen in the previous section, the presence of aquatic pollutants has become an increasing concern over the past few decades. In response, various guidelines have been established to regulate and control the increase of these chemicals in this ecosystem. These guidelines provide a framework for monitoring, assessing, and managing the risks associated with pollutants in aquatic environments, helping to mitigate their impact on marine ecosystems and human health. In this chapter, these guidelines will be analysed in detail.

1.2.1 European Union's Water Framework Directive (WFD)

The Water Framework Directive (WFD), established in 2000, is a comprehensive piece of legislation aimed at protecting and enhancing the quality of water resources across the European Union (EU). Its primary goal is to achieve "good status" for all EU waters—rivers, lakes, groundwater, and coastal waters—by a set deadline[102].

Directive 2013/39/EU is an amendment to the WFD that specifically updates the list of priority substances which pose significant risks to or via the aquatic environment. This includes a wide range of chemicals such as pesticides, industrial chemicals, and pharmaceuticals.

In recent years, the European Commission has introduced updates through implementing decisions to maintain and refine these standards. For example, the 1st Watch List was established in 2015, and subsequent updates were made in 2018 and 2020 to include new substances for monitoring. The latest update in 2022 involved reviewing and adding substances like plant protection products, antibiotics, and pharmaceuticals to ensure comprehensive risk assessment and high-quality monitoring data collection[103,104]. These continuous updates are crucial for maintaining the effectiveness of water quality management and adapting to emerging pollutants that pose risks to aquatic environments and human health.

To update these guidelines, member states must follow the implementation rules:

i) Member states are required to monitor the concentrations of priority substances and report their findings. This data helps assess compliance with the EQS and the effectiveness of pollution control measures[102,105].

ii) The directive highlights the need to develop and implement advanced water treatment technologies to address recalcitrant pollutants that are not effectively removed by conventional treatment processes. Techniques such as advanced

oxidation processes (AOPs) are promoted for their ability to degrade persistent compounds[102,106,107].

iii) Member states must develop and implement measures to reduce the concentrations of priority substances in water bodies. This includes regulatory controls on industrial discharges, agricultural practices, and urban runoff[102,108].

iv) Public access to information and involvement in the decision-making process are required to ensure transparency and public engagement in water quality management[102].

The Environmental Quality Standards Directive (EQSD), initially enacted as Directive 2008/105/EC and later amended by Directive 2013/39/EU, is a critical part of the European Union's water policy framework. Its primary goal is to protect surface water bodies from pollution by hazardous substances by setting concentration limits for specific pollutants in water, sediment, or biota. These standards are designed to safeguard aquatic life and human health. The directive establishes and periodically updates EQS for various substances to ensure effective control and protection of water quality.[106,109]

1.2.2 European Union's Marine Strategy Framework Directive (MSFD)

The European Union's Marine Strategy Framework Directive (MSFD)[110] is a comprehensive environmental policy framework aimed at protecting the marine environment across Europe. Adopted in 2008, the directive seeks to achieve "Good Environmental Status" (GES) of the EU's marine waters by 2020 and to protect the resource base upon which marine-related economic and social activities depend[111]. This directive promotes an ecosystem-based approach to the management of human activities that impact the marine environment, ensuring the sustainable use of marine resources. The GES is defined by a set of 11 descriptions[112] that cover aspects such as biodiversity, fish populations, eutrophication, sea floor integrity, and contaminants.

The MSFD requires Member States to cooperate at the regional level to develop and implement marine strategies that are coordinated across marine regions and sub-regions, recognizing that marine ecosystems transcend national boundaries. The directive emphasizes the importance of public participation in the development of marine strategies and requires Member States to inform and consult the public. Additionally, Member States are required to

report to the European Commission on the implementation of their marine strategies. The directive also interacts with other EU legislation, such as the Water Framework Directive and the Habitats Directive, which together aim to achieve integrated management of the EU's aquatic environments.

1.2.3 Zero Pollution Action Plan

The zero-pollution vision for 2050 is part of the European Green Deal and aims to reduce air, water, and soil pollution to levels that are no longer harmful to health and natural ecosystems, respecting the planet's capacity to cope and creating a toxic-free environment. This vision is supported by key targets for 2030 to accelerate pollution reduction at the source. These targets include:

- Improve air quality to reduce premature deaths caused by air pollution by 55%.
- Enhance water quality by reducing waste, plastic litter at sea by 50%, and microplastics released into the environment by 30%.
- Improve soil quality by reducing nutrient losses and chemical pesticide use by 50%.
- Reduce the share of EU ecosystems where air pollution threatens biodiversity by 25%.
- Decrease the proportion of people chronically disturbed by transport noise by 30%.
- Significantly reduce waste generation and cut residual municipal waste by 50%.

The Action Plan aims to support the EU's leadership in green, digital, and economic sectors while creating a healthier and more socially equitable Europe and planet. It provides a strategic framework to integrate pollution prevention into all relevant EU policies, enhance the implementation of existing EU legislation, and identify potential gaps[113].

1.2.4 World Health Organization (WHO)

The World Health Organization (WHO) provides comprehensive guidelines for the control of pollutants and chemical compounds to protect public health and the environment. These guidelines cover various aspects such as: Air Quality Guidelines, Guidelines for Drinking-water Quality, Chemical Safety Guidelines, Guidelines on Occupational Exposure, Guidelines for Indoor Air Quality and Pesticide Evaluation Scheme. These guidelines serve as international benchmarks for controlling pollutants and chemical compounds, aiming to

protect human health and the environment. They provide a foundation for national and local authorities to develop and implement effective environmental and public health policies[114].

1.2.5 The 2030 Agenda for Sustainable Development

The Sustainable Development Goals (SDGs), established by the United Nations in 2015, aim to address global challenges and promote prosperity while protecting the planet. These goals are intended to be achieved by 2030, and their progress is continually assessed.

This thesis is closely aligned with Sustainable Development Goals (SDGs) 3 and 14. SDG 3, which aims to ensure good health and well-being for all, is directly relevant as pollutants in aquatic systems can have serious repercussions for both human health and the health of aquatic organisms. By investigating the sources, distribution, and effects of various pollutants, this thesis supports efforts to mitigate health risks associated with environmental contamination. At the same time, SDG 14, which seeks to conserve and sustainably use the oceans, seas, and marine resources, is critical because pollution poses significant threats to marine ecosystems and biodiversity. This way, addressing these pollution challenges is essential for achieving a healthier planet and safeguarding the well-being of current and future generations.

1.2.6 OECD Test Guidelines for chemicals

OECD guidelines[115], developed by the Organisation for Economic Co-operation and Development (OECD), first published in 1981. They are internationally recognized standards used in the testing of chemicals to ensure their safety for human health and the environment. These guidelines are especially relevant for regulatory purposes, such as evaluating the safety of industrial chemicals, pesticides, pharmaceuticals, and other substances. These guidelines are split into 5 sections: Section 1: Physical Chemical Properties, Section 2: Effects on Biotic Systems, Section 3: Environmental Fate and Behaviour, Section 4: Health Effect, and Section 5: Other Test Guidelines. These guidelines are used in various sectors, including pharmaceuticals, agriculture (pesticides), industrial chemicals, and cosmetics. Guidelines are under constant review, with guidelines being periodically updated, new guidelines being adopted, and guidelines being withdrawn. They are essential in ensuring that products reaching the market are safe and meet international regulatory standards.

1.2.7 ECHA-REACH Regulation

ECHA-REACH regulation[116] is associated with the European Chemicals Agency (ECHA) and the REACH regulation, which stands for Registration, Evaluation, Authorisation, and Restriction of Chemicals. This is a comprehensive regulatory, framework implemented by the European Union (EU) in 2007, to ensure the safe use of chemicals within the EU and to protect human health and the environment[117].

The primary goals of REACH are as follows:

1) Compile a suite of physicochemical, toxicological, and ecotoxicological data for each substance.

2) Establish safe usage parameters by conducting chemical safety assessments (CSAs).

3) Allow for regulatory evaluation of substances to determine potential hazards based on the compiled data.

4) Prevent the use of substances of very high concern (SVHCs) without approval of the European Chemicals Agency (ECHA).

5) Restrict the use of chemicals for which no safe usage parameters can be established.

The implementation of REACH will enhance and broaden the scope of applied toxicology and exposure assessment by driving advancements and innovation in sampling techniques, toxicology testing, exposure modelling, alternative testing methods, and risk assessment practices.

1.3 Mixtures of enviornmental contaminants

While European Union and World Health Organization guidelines and directives have been effective in reducing levels of individual pollutants and improving global environmental quality, they have significant limitations in fully addressing the complexities associated with mixtures of pollutants[118]. The cumulative and synergistic effects of multiple pollutants, which can interact in ways that amplify or attenuate their individual impacts, are not adequately captured by current regulatory frameworks that focus predominantly on single substances[119].

The need for more integrated and comprehensive approaches to risk assessment and management is fundamental. Traditional risk assessments have often focused on evaluating pollutants individually, which may limit consideration of the potential for combined exposures to contribute to increased health risks or unanticipated environmental impacts. Furthermore, the implementation of these guidelines varies significantly between different regions and is strongly influenced by the commitment and capacity of national and local governments.

Likewise, although the World Health Organization guidelines are globally influential and serve as important references, their implementation largely depends on the adoption and integration of these recommendations by national governments into their own regulatory frameworks. Particularly, developing countries may face significant challenges in adopting these guidelines due to limited resources and infrastructure, which may hinder their effective implementation.

To ensure comprehensive protection of human health and the environment, future efforts should prioritize strengthening the assessment and management of pollutant mixtures. This includes the development and adoption of more sophisticated risk assessment models that can consider interactions between multiple pollutants, as well as the implementation of policies that promote coordinated action across different regulatory frameworks. By addressing these challenges, we can move towards a more holistic and effective approach to environmental protection and public health.

1.3.1 Approaches to assess effects of mixtures

As previously mentioned, several entities have made significant efforts to control the release of chemicals into the environment through various guidelines and regulations. In this

chapter, the complex issue of mixtures of pollutants will be deepened, examining their combined effects and interactions on marine ecosystems.

Studying chemical mixtures is crucial for understanding the full scope of their impact on environmental health. Unlike individual chemicals, compounds in mixtures can interact often leading to unpredictable and potentially more severe health effects. Several models and approaches have emerged to identify interactions between different chemical compounds. The aim of being able to predict the interactions between two chemicals has been a research topic for more than a century, and the two major concepts underlying all valid assessments of joint chemical effects were framed already in the first part of the twentieth century by Loewe and Muischnek (1926)[120] and Bliss[121] (1939).

1.3.1.1 Concentration Addition (CA) and Independent Action (IA) models

The concept of Concentration Addition (CA) developed by Loewe and Muischnek[120,121] has been re-invented several times. It assumes that all chemicals in a mixture act on the same biological target site, contributing to the overall effect in proportion to its concentration and potency. The total effect is the sum of the effects of each chemical as if they were acting independently but additively on the same target.

The Independent Action (IA) model, developed by Bliss[121,122], is another approach used in toxicology to predict the combined effects of chemical mixtures. This model is based on the principle that the components of a mixture act independently from each other, affecting different biological targets or mechanisms. Therefore, the total effect of the mixture is a function of the individual effects of each chemical, if each effect occurs independently of the others. The IA model uses a probabilistic approach to predict the combined effects. It calculates the probability of observing a response due to at least one of the chemicals in the mixture. The combined effect is determined by the complement of the product of the probabilities of no effect from each chemical.

Nowadays these models are very important in the field of toxicology and are widely used in risk assessment. They provide a baseline for understanding additive effects. When the actual observed effects of a chemical mixture are different from those predicted by these models, it suggests the presence of synergistic or antagonistic interactions.

The word “synergos” from the Greek means “working together”[123]. In other words, synergism is a coordinated or correlated action of two or more physiological structures, agents or processes such that the combined action is greater than the sum of each acting separately. For

example, if a mixture of pesticides results in greater toxicity than predicted by additive models, it indicates synergistic interaction. On the opposite side, antagonism is a phenomenon wherein two or more agents in combination have an overall effect that is less than the sum of their individual effects[123]. For instance, if the presence of one chemical reduces the toxicity of another, it indicates antagonistic interaction.

1.3.1.2 Model Deviation Ratio (MDR)

The Model Deviation Ratio (MDR) proposed by Belden et al[124] is a metric used to quantify these deviations. MDR compares the observed effect of a chemical mixture to the effect predicted by an additive model (either CA or IA). Final score should be interpreted as the following:

0.5 < MDR < 2: Indicates perfect additivity, meaning the observed effect matches the predicted effect.

MDR > 2: Indicates synergism, where the observed effect is greater than predicted.

MDR < 0.5: Indicates antagonism, where the observed effect is less than predicted[121,124].

By interpreting MDR values, researchers and regulators can identify and characterize synergistic and antagonistic interactions within chemical mixtures, leading to improved risk assessments and better protective measures for human health and the environment.

1.3.1.3 Toxic Unit (TU) approach

Toxic Unit (TU) approach is a concept first proposed by Sprague and Ramsay[125] in 1965, with juvenile salmon as experimental model. It was later developed by Höss et al.[126] in 2011, with nematodes as model organism, to evaluate the toxicity of complex mixtures specially in aquatic environments. It is a standardized measure used to express the toxicity of a chemical within a mixture relative to a specific biological endpoint. It allows for the comparison and summation of the toxic effects of various chemicals in a mixture, facilitating the assessment of their combined impact. For example, the TU for an individual chemical in a mixture is calculated by dividing the concentration of that chemical by its concentration that causes a specific effect (e.g., LC_{50}, the concentration lethal to 50% of the test organisms). The TUs for all the individual chemicals in the test mixture are then summed and the following values can be interpreted:

$\sum$TU = 1: Indicates that the mixture has reached the toxic threshold, producing an effect equivalent to the individual LC_{50} concentrations of the components.

∑TU > 1: Indicates a higher than expected toxicity, suggesting potential synergistic effects.

∑TU < 1: Indicates lower than expected toxicity, suggesting potential antagonistic effects[127,128]

This approach allows the standardization of the toxicity of different chemicals to a common scale, enabling researchers and regulators to evaluate the overall risk posed by mixtures, ensuring effective environmental management and protection of aquatic ecosystems.

1.3.1.4 Toxic Equivalent Factors (TEF) concept

Another method, the Toxic Equivalent Factors (TEF) concept, was developed during the mid-1980s by researchers such as Barnes et al.[129]and Safe et al.[130] and has been reviewed several times by the WHO, with the latest update in 2022[131]. Toxicological and mechanistic research have shown that the adverse health effects associated with exposure to chlorinated dioxins and dioxin-like compounds are primarily mediated through the aryl hydrocarbon receptor (AhR). This understanding led to the development of the Toxic Equivalency Factor (TEF) concept. TEFs express the toxicity of various chemicals relative to their potency to produce an aryl hydrocarbon receptor (AhR)-mediated biological or toxicological effect compared with the most potent congener, allowing for a standardized assessment of their combined toxic effects. The reference compound is typically the most toxic member of a chemical group, such as 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) for dioxins[132,133]. The total toxic equivalent (TEQ) is calculated by multiplying the concentration of each chemical by its TEF and summing these products. For example, in a mixture containing different dioxins, furans, or PCBs, each compound's concentration is multiplied by its respective TEF to obtain its contribution to the overall toxicity. These contributions are then summed to determine the total TEQ. TEFs are applied in environmental monitoring programs to assess contamination levels of dioxins, PCBs, PAHs, and related compounds in soil, water, and biota, ensuring that these levels are within safe limits. This standardized approach provides a clear and consistent method for evaluating and managing the risks associated with complex chemical exposures. In 2022 the WHO updated the TEFs based on new scientific data and methodologies that have emerged since the last revaluation in 2005. The process included an extensive review of congener-specific data, updating the relative potency database, and employing advanced statistical methods such as Bayesian meta-regression to derive "Best-Estimate" TEFs[131]. The

new TEFs application to dioxin-like chemical concentrations indicated that the TEQs are generally lower compared to those calculated using the 2005 TEFs. This suggests a potential reduction in the estimated health risk from these compounds. These updated TEFs are crucial for regulatory agencies and environmental health practitioners. They provide a more accurate tool for assessing the risk posed by exposure to mixtures, ensuring that safety standards reflect the most current scientific understanding.

1.3.2 Limitations and challenges in assessing mixture effects

Even though the previously mentioned models are very important in the evaluation of mixtures, they still have some limitations. In general, almost all models (CA, TUs, and TEFs) assume that the effects of chemicals in the mixture act according to the additive model and have similar modes of action. On the other hand, the model IA considers that all chemicals act independently, excluding the fact that some may follow the same pathways and have the same mechanism of action.

Furthermore, the TU model depends on the availability of other data such as the calculation of EC_{50} for all components of the mixture. TEF is based on AhR-mediated effects, which can sometimes overlook other pathways that are relevant for some compounds in mixtures.

Therefore, while these models provide valuable frameworks for assessing the toxicity of chemical mixtures, their limitations stress the need for complementary approaches and continuous refinement. Accurate risk assessment requires considering potential interactions, variability in data, and environmental factors to ensure that protective measures are effective and based on the best available science.

1.4 Polycyclic Aromatic Hydrocarbons (PAHs)

As mentioned before, aquatic environments are increasingly contaminated by a diverse range of pollutants from multiple sources, resulting in a complex "cocktail" of chemicals. These pollutants span several categories, each with distinctive physical and chemical properties, leading to different toxic effects that are unpredictable and can be exacerbated in aquatic ecosystems. Even within the same group of pollutants, such as PAHs, PCBs and dioxins, or heavy metals, individual chemicals can vary significantly in their molecular structure and toxicological impact, contributing to a multifaceted threat to aquatic life.

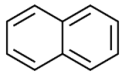
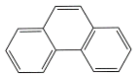
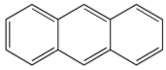
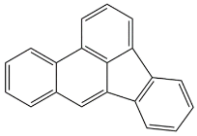
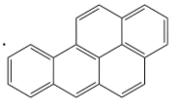
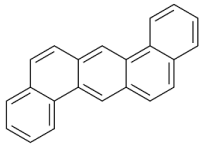
Among these, polycyclic aromatic hydrocarbons (PAHs) are particularly concerning as persistent organic pollutants due to their widespread presence in environmental mixtures and their harmful effects to aquatic organisms.

This section will explore the characteristics of PAHs, their pathways into aquatic ecosystems, and the risks they pose, providing a comprehensive overview of these hazardous pollutants.

1.4.1 Sources and Properties

PAHs are compounds composed of carbon and hydrogen, formed by the fusion of two or more aromatic rings (AR)[134]. Examples of PAHs are represented in Table 1.2: naphthalene (2 ARs), phenanthrene (3 ARs), anthracene (3ARs), benzo[b]fluoranthene, benzo[a]pyrene and Dibenz[*a*,*h*]anthracene (5 ARs). PAHs originate from various sources, including industrial activities (such as the petrochemical industry, petroleum refining)[134,135], transportation (use and combustion of gasoline, diesel, and other fossil fuels), domestic activities (wood and coal combustion)[136], domestic activities (smoking, cooking, incense burning)[134,137] and natural events (volcanic eruptions, forest fires, and tree exudates)[138,139]. These compounds are ubiquitously present in the environment and typically appear as colourless, white, or pale yellow solids[140]. They are hydrophobic and lipophilic, have high melting and boiling points, and low vapour pressure[141].

Table 1.2 - Physical properties[142] and IARC classification of PAHs (Group 1 – Carcinogenic to humans, Group 2A – Probably carcinogenic to humans, Group 2B – Possibly carcinogenic to humans and Group 3 – Probably not carcinogenic to humans).

PAH	Molecular weight	Melting point °C	Boiling Point °C	Vapor pressure kPa	Solubility in water (mg/L)	IARC Classification	Structure
Naphthalene	128.18	80.2	218	$1.1x10^{-2}$	3.93	2B	
Phenanthrene	178.24	96-101	339-340	$2.3x10^{-5}$	1.2	3	
Anthracene	178.24	216-219	340	$36x10^{-6}$	0.076	3	
Benzo[b]fluoranthene	252.32	167-168	481	$6.7x10^{-8}$	0.0012	2B	
Benzo[a]pyrene	252.32	177-179	493-496	$7.3x10^{-10}$	0.0023	1	
Dibenz[*a*,*h*]anthracene	278.35	266-270	524	$1.3x10^{-11}$	0.0005	2A	

PAHs are distributed in the atmosphere in various phases, including gas and particulate matter. Their distribution is influenced by several factors such as sources of emissions, atmospheric conditions, and physical-chemical properties of the PAHs themselves, namely on the molecular weight of the PAH compounds. Lower molecular weight PAHs are typically found in the gas phase, while higher molecular weight PAHs are predominantly found in the particulate phase[143]. PAHs are often associated with fine particulate matter (PM2.5), studies have shown that a significant portion of PAHs are bound to particles less than 2.5 μm in diameter, allowing them to penetrate deep into the human respiratory system and indicating their potential for long-range transport and deep lung deposition.[144].

Precipitation is a primary pathway for the deposition of PAHs from the atmosphere onto the soil surface, making soil one of the largest sinks for atmospheric PAHs[145]. Over time,

PAHs can accumulate in soils and street dust. These PAHs can then be transported into aquatic environments through runoff, especially during rainstorms and stormwater flow[146,147].

Due to their low solubility in water, which decreases with increasing molecular weight, PAHs are not easily degraded and tend to persist, often adsorbed onto fine particles and organic matter, becoming trapped in the complex geochemical matrix of aquatic sediments[148,149]. Sediments with higher organic matter and fine particle content, such as estuarine sediments, are particularly important reservoirs for PAHs[147,150]. Consequently, organisms in contact with the bottom sediments or those in the water column can be directly or indirectly affected, as PAHs can be adsorbed onto resuspended particulate matter or dissolved in water[151]. These pollutants can be taken up by aquatic organisms and gradually transferred through food chains, posing a threat to the ecosystem (Figure 1.2).

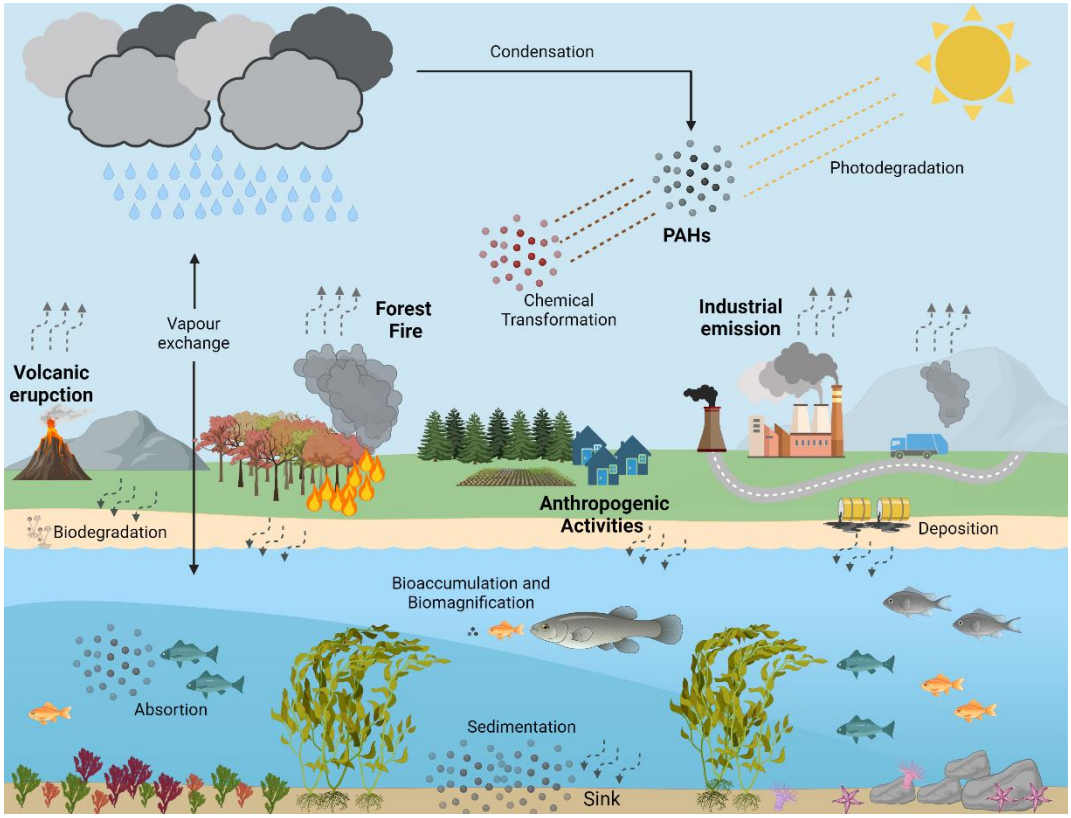


Figure 1.2 Biogeochemical cycle of PAHs. The main sources of PAHs include volcanic eruptions, forest fires, industrial emissions, and various anthropogenic activities. PAHs are released into the atmosphere as gases or bound to particulate matter, where they undergo photodegradation and chemical transformations. Through wet and dry deposition, PAHs are transferred to land and water surfaces. They enter aquatic systems via runoff, vapor exchange, or atmospheric deposition. In aquatic environments, PAHs can bioaccumulate and biomagnify in organisms or bind to fine particles in sediments, forming long-term sinks. In soils and sediments, microbial degradation can break down PAHs, although they may persist for long periods. Created in BioRender.com

To better understand PAHs in aquatic environments, below are some case studies that highlight the occurrence, sources, and ecological consequences of PAH pollution in various marine and freshwater ecosystems.

One example of PAH contamination was a case study in Chalk River Systems, France that investigated PAH levels in surface water ($[PAH]_{total}$ 73.5 - 728 ng/L), suspended particulate matter (SPM) ($[PAH]_{total}$ 749.6 - 2463 μg/Kg), and sediments upstream and downstream of wastewater treatment plant (WWTP) ([PAH]total 690.7 - 3625.6 μg/Kg), in the Loue and Doubs rivers. PAH levels were significantly higher in SPM and sediments compared to surface water, indicating that sediments are crucial for assessing overall PAH contamination. Biota was also impacted by WWTP effluents, showing the necessity of monitoring sediment and SPM for a comprehensive understanding of PAH pollution[152].

Another case study concerned long-term contaminated riverbank sediments of the Mahoning River (Northeast Ohio, USA). The study detected extremely high PAH concentrations ($[PAH]_{total}$ 94,000 - 560,000 μg/Kg), indicating both pyrolytic and petrogenic sources, making this aquatic ecosystem one of the most polluted in the world. The ecological risk assessment showed significant risks to aquatic organisms, underscoring the need for remediation strategies in long-term contaminated ecosystems[153].

There are also examples of PAH contamination in Portugal, such as in the Sado Estuary and Ria de Aveiro. Ribeiro *et al*.[154] found that sediments in these regions show significant contamination with PAHs, reaching concentrations up to 7,350 ng/g. This contamination stems from industrial, agricultural, and urban discharges, affecting both sediments and biota. High levels of other persistent organic pollutants, such as polychlorinated biphenyls (PCBs), further exacerbate the ecological risk in these aquatic environments[106]. Moreover, Rocha *et al*.[155] investigated PAH levels in water and surface sediments of the Douro River estuary and nearby Atlantic seacoast. The results showed ubiquitous distribution of PAHs, with total concentrations averaging 55 ng/L in water and 52 μg/g dry weight in sediments. The area is heavily polluted by PAHs from various sources, posing potential mutagenic and carcinogenic risks to humans and aquatic life.

Several individual PAHs, such as B[a]P, are classified as effective or potential carcinogens to humans by the International Agency for Research on Cancer (IARC, group 1)[156], as numerous epidemiological and toxicological studies have shown a correlation between exposure to PAHs and an increased risk of developing cancer[136,143]. Additionally, PAHs are included in the Priority Substances List of the Marine Strategy Framework Directive (MSFD)[110]

and Water Framework Directive (WFD)[102] and are highlighted by the U.S. Environmental Protection Agency (EPA)[157] and the World Health Organization (WHO)[114] as priority substances for analysis in various environmental matrices.

The widespread presence and ecological risks of PAH contamination in aquatic ecosystems highlight the importance of studying these pollutants, particularly considering that they always occur in the environment as complex mixtures. Such mixtures can have unpredictable toxic effects on aquatic life, making it even more crucial to effectively monitor and manage these pollutants. They emphasize the importance of using a combination of monitoring matrices (water, sediment, biota) to capture the full extent of contamination and highlight the need for continued management efforts, review of environmental guidelines and remediation methods to protect aquatic environments from risks posed by PAH pollution.

1.4.2 PAH metabolism and toxic effects

At a molecular level, PAHs can induce effects in cellular macromolecules, such as lipids, proteins, and nucleic acids[158]. Acute exposure to these compounds generates oxidative stress by increasing the levels of reactive oxygen species (ROS), leading to inflammation and cell death[159]. Moreover, PAHs have been extensively studied for their carcinogenic and mutagenic effects due to their metabolites (e.g., diol epoxides) that can covalently bind to DNA and form an altered base, known as a DNA adduct[149,158,160]. DNA adducts can also arise from endogenous processes, including normal metabolism, oxidative stress, and chronic inflammation[158]. As a result of the carbon and hydrogen structure, PAH, are poorly reactive, requiring metabolic activation to exert toxicity[161]. In this sense, the inducibility of metabolic activation and detoxification enzymes plays a crucial role in the metabolism of PAHs. Metabolic enzyme induction occurs when a molecule or compound alters the activity of these enzymes, thereby influencing the metabolism of foreign compounds. Moreover, the balance between activation and detoxification activities is critical in determining the toxic effects of reactive intermediates and metabolites produced during xenobiotic metabolism[162]. PAHs are procarcinogenic and promutagenic, meaning parent compounds are less toxic than the metabolites formed after hepatic biotransformation. In other words, they become more toxic after metabolism, leading to the formation of DNA adducts[160]. This biotransformation is facilitated by various enzymes, typically classified into two types: phase I and phase II enzymes[158].

The Phase I cytochrome P450 monooxygenase system is a large family of heme proteins that introduce polar groups into xenobiotic molecules through oxidative, reductive, or hydrolytic processes. Cytochrome P450 is responsible for forming quinones and diol-epoxides, which can react with DNA to form bulky adducts[161,163]. These metabolites can cause nucleotide oxidation and other potentially irreparable changes. The activity and amount of cytochrome P450 enzymes (CYPs) are important indicators for assessing environmental disturbances caused by chemical contamination. For example, the ethoxyresorufin-O-deethylase (EROD) activity, catalysed by CYP1A enzymes, is a widely used biomarker for contamination by planar halogenated and polycyclic aromatic hydrocarbons[158].

An important feature of PAHs is their ability to induce their own metabolism by binding to the aryl hydrocarbon receptor (AhR). AhR is a ligand-activated transcription factor. Upon binding to ligands, AhR activates the promoter regions of its target genes, leading to the upregulation of several xenobiotic-metabolizing enzymes, such as CYP1A1 and CYP1A2[161].

Between phase I oxidation reactions and phase II conjugation reactions, the enzyme epoxide hydrolase is generally found. This enzyme plays a crucial role in the metabolic activation of xenobiotics such as drugs and PAHs. Epoxide hydrolase metabolizes foreign compounds containing an epoxide residue, such as those generated from CYP450-catalyzed oxidative activation[164]. It converts epoxide residues into two hydroxyl residues through dihydroxylation, forming dihydrodiol products. Additionally, epoxide hydrolases catalyse the trans addition of water to alkene and arene oxides formed by P450 bioactivation. While epoxide hydrolases are often considered activation enzymes, they are also sometimes referred to as detoxification enzymes based on their functional properties[162].

Phase II reactions involve the conjugation of xenobiotics or their phase I metabolites with polar endogenous constituents such as glucuronic acid, sulphate, glutathione, or amino acids to produce water-soluble conjugates that are easily excreted by organisms[162]. The enzymes involved in phase II are called conjugation enzymes. Glutathione S-transferase (GST) belongs to a superfamily of dimeric water-soluble enzymes responsible for various functions, including intracellular transport, eicosanoid biosynthesis, and critically, the conjugation of glutathione (GSH) to xenobiotics in phase II reactions, by lowering the pKa of the thiol group in GSH's Cys residue[158,164].

For example, B[a]P is a PAH containing a bay region, a structural feature often associated with carcinogenicity and is metabolized by CYP1A1/1B1 enzymes to form 7,8-epoxide. This 7,8-epoxide is further metabolized by epoxide hydrolase to form 7,8-diol, and

then again by CYP1A1/1B1 to form 7,8-diol-9,10-epoxide (BPDE), the ultimate carcinogenic metabolite of B[a]P[165] (Figure 1.3 a.) BPDE reacts with the N2 position of guanine in DNA to form a DNA adduct, a critical event in the initiation of carcinogenesis[160]. Several studies have linked DNA adduct formation to modifications in specific genes implicated in animal and human cancers. DNA adduct formation is sequence-specific and can cause mutations in the tumour suppressor gene p53[166,167]. The p53 gene acts as a brake on cellular replication, causing cell cycle arrest to prevent the propagation of errors and thus prevent tumour development. When DNA adducts form on the gene encoding p53, they result in mutations and malfunction of the gene, allowing cells to propagate with less regulation[168].

In comparison, phenanthrene is the simplest PAH containing a bay region but Phe itself is not generally considered carcinogenic. The metabolism of Phe to diol epoxides follows pathways like those of B[a]P. It starts with formation of Phe-3,4-epoxide by CYP1A1/B1 followed by epoxide hydrolase activity to form Phe-3,4-diol that is again metabolized by CYP1A1/B1 to form Phe-3,4-diol-1,2epoxide (Figure 1.3 b.) The majority of B[a]P metabolites are produced through the bay region diol epoxide pathway. In contrast, most Phe metabolites are generated via the reverse diol epoxide pathway, which is associated with significantly weaker mutagenicity and carcinogenicity[169]. This explains the higher carcinogenic potential of B[a]P, attributed to its metabolism through the bay region diol epoxide pathway that forms stable DNA adducts, compared to Phe.

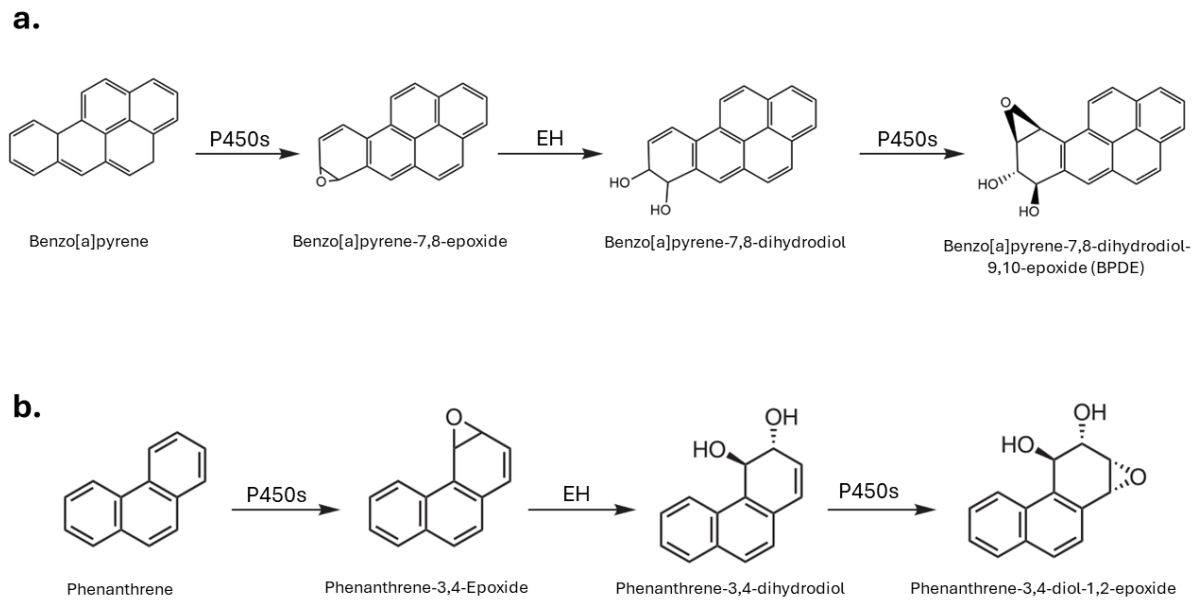


Figure 1.3 - Major pathway of metabolic activation of PAHs **a**. Benzo[a]pyrene and **b**. Phenanthrene

Besides the carcinogenic effects of PAHs, their metabolism produces reactive oxygen species (ROS), which can lead to oxidative stress, oxidative DNA damage, and GSH depletion, which is the most important non-enzymatic defence against ROS. GSH protects sensitive protein thiols from overoxidation through glutathionylation, serves as a cofactor for glutathione peroxidases (GPx) in the degradation of H_2O_2[162] and glutaredoxins in thiol redox cycles[170], and is used in the conjugation with ROS-generating electrophiles in conjugation reactions.

Superoxide dismutase (SOD) and catalase (CAT) form the first enzymatic line of antioxidant defense[162]. SOD converts superoxide radicals ($O_2^{\bullet-}$) into H_2O_2, which is then converted to water by CAT. By reducing the levels of $O_2^{\bullet-}$ and H_2O_2, SOD and CAT play a crucial role in preventing the formation of hydroxyl radicals (HO•). Hydroxyl radicals are highly unstable and reactive, capable of oxidizing many biomolecules, including RNA, DNA, proteins, and lipids, leading to toxicity[158,162]. Hence, although living organisms possess robust antioxidant defences, excessive ROS production and impairment of these defences can cause oxidative damage to biomolecules. Particularly, lipid peroxidation (LPO) is a series of chain reactions triggered by radicals like hydroxyl radicals, leading to the loss of membrane integrity and secondary damage to DNA and proteins through the formation of adducts with toxic aldehydes produced from lipid peroxides[171,172]

1.4.3 PAH as compound mixtures

The main issue when studying the effects of PAHs is that they occur in the environment not as single compounds, but always in complex mixtures[159]. However, the traditional approach to regulatory risk assessment focuses primarily on individual chemicals rather than their mixtures. Although environmental guidelines place limits on the total concentration of PAHs in a certain environment, they do not address the composition of these mixtures[173–175]. As an example, in the European Union, the maximum concentration allowed of PAHs in drinking water is 0.1 μg/L[176], while for the WHO) the recommended concentration for PAHs in drinking water is up to 0.2 μg/L[17]. This represents a significant gap in our understanding and management of PAH pollution.

Although there is considerable knowledge about the mode of action of some individual PAHs, such as B[a]P[177], the combined effects of complex PAH mixtures present greater challenges. The toxic effects and thresholds induced by individual PAHs are not necessarily the same when PAHs are present simultaneously in a mixture. Previous research has shown that environmental PAH mixtures can have synergistic, additive, or antagonistic effects, leading to unpredictably higher or lower toxicity than expected from individual PAHs[178].

For example, some PAHs can inhibit metabolic pathways that detoxify other PAHs, thereby increasing the overall toxicity of the mixture. Conversely, other interactions, such as those with organic matter[179], or even plastics[180], can reduce the bioavailability of certain PAHs, diminishing their toxic potential[177]. This variability complicates the assessment of environmental risk and the establishment of effective remediation strategies.

Therefore, it is important to change the paradigm for formulating environmental guidelines so that they do not only focus on the total concentration of PAHs but also address the profile of the mixtures, including the number and types of PAHs present[181]. For certain profiles, the acceptable concentration limits may need to be higher or lower. A more complete approach would consider the specific interactions and combined effects of PAH mixtures, providing a more accurate assessment of their ecological and health risks.

Additionally, research should focus on understanding the behaviour of PAH mixtures in various environmental contexts. This includes studying their degradation pathways, bioaccumulation in the food web, and long-term ecological impacts. Advanced analytical methods and models can help predict the behaviour of these mixtures, supporting the development of comprehensive guidelines that reflect the true nature of PAH pollution. By

incorporating the complexity of PAH mixtures into environmental regulations, we can improve the protection of aquatic ecosystems and human health.

1.4.4 Most used methodologies/biomarkers to study PAHs toxicity in aquatic organisms

Polycyclic aromatic hydrocarbons exhibit distinct mode-of-action, requiring specific biomarkers and methodologies to assess their toxicity in aquatic organisms.

A biomarker, is a measurable indicator of a biological state or condition, typically reflecting a biological response to a chemical or environmental stressor. In the context of toxicology and environmental science, biomarkers serve as early-warning signals of contamination that can be measured in biological samples such as tissues, cells, or body fluids. These indicators are crucial because they can reveal the presence of environmental impacts before they manifest at higher biological levels, such as in populations or ecosystems[158,182,183]. Based on their specific roles in assessing biological responses, biomarkers are categorized into three main types: biomarkers of exposure, biomarkers of effect, and biomarkers of susceptibility:

- **Biomarkers of exposure:** Are biological indicators that reflects the presence and extent of exposure to a chemical or environmental stressor. These biomarkers are considered important tools in toxicology because they provide an estimate of the levels at which chemical substances are absorbed by an organism. This is achieved by quantifying the toxic substances, their metabolites or adducts in biological samples, such as urine, blood and bile, which are considered reliable matrices for assessing exposure[184,185].

Several types of biomarkers of exposure are commonly used to assess the exposure to PAHs:

Metabolomics: Direct measurement of PAH metabolites in bile[186]

DNA Adducts: Formation of PAH-DNA adducts, which directly indicates genotoxic exposure to PAHs[187].

- **Biomarkers of effect:** Are measurable biological responses that indicates an organism's physiological or biochemical changes resulting from exposure to a chemical or environmental stressor. These biomarkers reflect the impact of the exposure, such as

cellular damage, altered enzyme activities, or changes in gene expression, which may lead to adverse health outcomes[184,185].

Examples of biomarkers of effect that are commonly used to assess the effects to PAHs are:

Oxidative Stress Biomarkers:

Lipid Peroxidation (LPO): Indicates oxidative damage to lipids as a result of PAH exposure[158].

Antioxidant Enzyme Activities: Includes enzymes like Glutathione S-transferase (GST), Glutathione Peroxidase (GPx), Catalase (CAT), reflecting the organism's response to oxidative stress[158].

Genotoxicity Biomarkers:

Comet Assay: Assesses DNA strand breaks.[188]

Micronucleus and Erythrocytic Nuclear Abnormalities assays: Detect chromosomal damage[189].

Histopathological Changes: Examination of tissues (e.g., liver, gills) for pathological alterations due to PAH exposure[190].

Biochemical pathway analysis:

Metabolomics: Reveal changes in broader metabolic pathways that indicate how the organism's biochemistry is responding to the exposure[186].

Proteomics and Transcriptomics: Uncover changes in protein and gene expression profiles[191,192].

Behavioural Changes: Observes alterations in behaviour in organisms, such as swimming patterns or feeding, as a consequence of PAH toxicity[193].

- **Biomarkers of susceptibility:** Are indicators that assess the variability in how individuals or organisms respond to environmental stressors, based on their intrinsic characteristics. These biomarkers encompass genetic factors, such as polymorphisms in certain enzymes or molecules, that can influence how the body interacts with a chemical substance. Additionally, they include other biological factors related to an individual's nutritional

status, health condition, lifestyle, and life stage, all of which can affect their susceptibility to chemical exposure. While some enzymes and molecules may exhibit genetic polymorphisms that play a crucial role in determining the extent and type of response to exposure, biomarkers of susceptibility generally reflect the systemic origin rather than being specific to a particular organ or tissue[184,185].

Genetic Polymorphisms: Assess the variations in genes involved in PAH metabolism (e.g., CYP1A1) can predispose certain organisms to increased susceptibility[194].

The use of biomarkers as tools to assess PAH exposure in aquatic environments offers a powerful and multifaceted approach to understanding the impacts of these persistent contaminants. Biomarkers of exposure, such as EROD activity and PAH metabolites in bile, provide direct and reliable indicators of the presence and levels of PAHs within organisms. Biomarkers of effect, including oxidative stress markers, DNA damage assessments, and histopathological changes, reveal the biological consequences of PAH exposure, helping to elucidate the pathways of toxicity and potential long-term impacts on aquatic species. Lastly, biomarkers of susceptibility highlight the importance of individual and species-specific factors that may influence the response to PAH exposure, offering insights into the variability of effects across different organisms. Together, these biomarkers create a comprehensive toolkit that enhances our ability to monitor, understand, and mitigate the ecological risks posed by PAHs in aquatic environments (Figure 1.4).

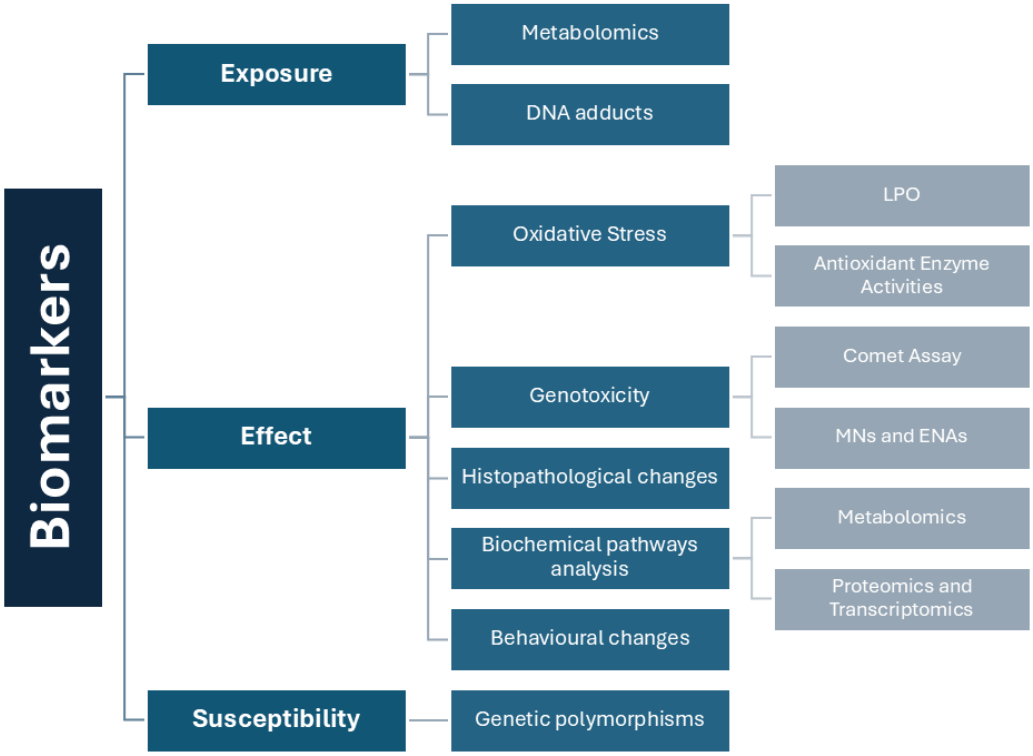


Figure 1.4 - Schematic representation of the most commonly used biomarkers for studying PAH toxicity in aquatic organisms.

1.5 Aquatic Toxicology: Model Organisms and Bioassays

Following the discussion on PAHs, their sources, and their effects in the previous section, herein we will address the use of model organisms and bioassays—specifically *in vitro*, *in vivo*, *in situ*, and *in silico* approaches—to study the impacts of PAH mixtures. We will explore how these tools play a crucial role in the field of environmental toxicology, providing the basis for ecological risk assessment and guiding environmental management strategies.

1.5.1 Model organisms

Ecotoxicology is the study of the effects of toxic chemicals across various biological and ecological scales, from populations to the ecosystem. For ecosystems already impacted by pollution, ecotoxicological studies in varied spatial and temporal scales can inform the best course of action to restore ecosystem functions. Practical ecotoxicology often operates within the Ecological Risk Assessment (ERA) framework[195,196]. Ecotoxicology has emerged as the applied science addressing the central issues of contaminants in the biosphere, making it vital for environmental health and sustainability. [197].

Model organisms, found among prokaryotes, protists, fungi, plants, and animals, are non-human species extensively studied to understand biological phenomena across various scales, from unicellular organisms to entire ecosystems [198]. They serve as proxies for examining the movement, transformation, and impact of contaminants, providing insights into toxicity mechanisms. The ultimate goal is to extrapolate findings to more complex organisms, including humans, and predict pollution effects across ecological niches and environmental contexts, guiding effective prevention and remediation efforts [199].

Traditionally, the term "model organism" has been applied to species such as mice and Drosophila that facilitate experimental laboratory research due to their small size and short generation times[200]. However, with the advent of genome sequencing projects, this definition has broadened to include a wider variety of species. The most widely acknowledged inventory of these organisms includes those officially recognized by the US National Institutes of Health (NIH) in 1999 as model organisms for biomedical research, listing thirteen species including the mouse (*Mus musculus*), rat (*Rattus norvegicus*), zebrafish (*Danio rerio*), fruit fly (*Drosophila melanogaster*), nematode (*Caenorhabditis elegans*), baker's yeast (*Saccharomyces cerevisiae*), and thale cress (*Arabidopsis thaliana*)[200]. Nonetheless, several other models are frequently used,

obeying to criteria such as ecological relevance, economical relevance, genetic traceability, and specific experimental requirements.

Model organisms offer a variety of well-recognized experimental and pragmatic advantages. They are typically easy to breed and maintain in large numbers under laboratory conditions. Research involving model organisms often benefits from the standardization of the organism and the accumulation of extensive knowledge and resources on it[199].

Economics has significantly influenced the selection of organisms for study, prioritizing agriculturally important species, such as rice, and those linked to human health, like the malarial parasite Plasmodium[200]. For instance, the zebrafish has been designated as the standard experimental fish by the Organization for Economic Cooperation and Development (OECD) and the International Organization for Standardization (ISO) due to its well-defined genetic background and numerous advantages[197].

1.5.2 *in vitro* bioassays

in vitro assays are experimental procedures conducted in controlled laboratory environments, outside of a living organism[201]. These assays play a crucial role in biomedical, environmental and toxicological research by providing detailed mechanistic insights at the cellular and molecular levels[202]. The primary advantage of *in vitro* assays lies in the highly controlled environment, enabling precise manipulation of experimental conditions and enhancing result reproducibility [203]. They are also ethically favourable, as they reduce the need for animal testing, aligning with the 3Rs (Replacement, Reduction, and Refinement) ethical principle[204]. This guiding principle of research in the fields of toxicology and other biomedical sciences, emphasizes the replacement of animal models with alternative methods, the reduction of the number of animals used, and the refinement of techniques to minimize pain and distress. By implementing *in vitro* assays, researchers can adhere to these ethical guidelines, promoting humane and responsible scientific practices[201].

However, despite their numerous benefits, *in vitro* assays have limitations. They lack the complexity of whole organisms, which can affect the predictive accuracy of the results, as they do not fully replicate the interactions between different cell types, tissues, and organ systems. Additionally, certain biological phenomena, such as immune responses and behaviour, cannot be studied *in vitro*. Also, they do not provide information on the

toxicokinetic behaviour of xenobiotics, since they cannot mimic the physiologically relevant functions of human or animal organs and tissues[205].

Despite these challenges, *in vitro* assays remain indispensable tools in scientific research, providing foundational data that informs and directs further *in vivo* and clinical studies[206,207]. Two primary types of *in vitro* models are commonly used: immortalized cell lines and primary cells.

Immortalized cell lines are derived from a single cell type that has undergone genetic modifications allowing them to proliferate indefinitely[208]. Ideally, these cells are phenotypically and genetically similar to the cells of the original tissue. Cell lines are widely used in toxicology due to i) their ease of use and possibility to culture and maintain over long periods[209] ii) providing a consistent and uniform population of cells, which is crucial for reproducibility in experiments iii) availability from biobanks that certify origin and characteristics. Some examples are HepG2, HepaRG, HT-29, HeLa, A549. As a limitation, due to genetic modifications, these cell lines may not accurately represent the physiological state of normal cells, leading to potential discrepancies in toxicological responses as for example altered metabolism.

Primary cells are directly isolated from living tissues and cultured for experiments. Some examples of techniques for the isolation of primary cells are: enzymatic digestion[210], perfusion[211], Magnetic-Activated Cell Sorting (MACS)[212]. Primary cells maintain more of the physiological characteristics of the original tissue compared to immortalized cell lines[213]. Thus, these cells possess natural metabolic pathways, making them more suitable for studying the biotransformation and bioactivation. However, primary cells have a finite lifespan which can limit their use for long-term studies. Also, there can be significant variability between batches of primary cells due to differences in donor characteristics and isolation techniques, potentially affecting experimental outcomes. Isolating and culturing primary cells is also more labour-intensive and expensive compared to immortalized cell lines.

Both immortalized cell lines and primary cells have their strengths and limitations. Immortalized cell lines offer ease of use and consistency, making them suitable for high-throughput screening. In contrast, primary cells provide greater physiological relevance and are better suited for detailed mechanistic studies, but used living organisms, although much less than in vivo experiments. The choice between these models should be guided by the specific objectives of the research, balancing the need for physiological relevance with practical considerations of experimental design.

Another *in vitro* approach is co-culture systems, where two or more different cell types are grown together in the same environment. This method is designed to study cell-cell interactions, better replicate the in vivo environment, and explore complex biological processes that are not observable in monocultures (where only one cell type is cultured)[214,215]. Co-cultures are particularly useful for investigating the mechanisms of action of xenobiotics and identifying their potential targets, serving as a bridge between monoculture techniques and animal models. These systems offer realistic insights into cell-cell interactions mediated by secreted factors, which play a crucial role in various metabolic functions such as energy balance, muscle atrophy, obesity, and related co-morbidities[216].

Co-culture systems are generally categorized into two main types: direct and indirect methods.

Direct Co-Culture: In this approach, also known as mixed cultures[217], different cell types are cultured in direct physical contact, allowing for close cell-cell interactions. This setup facilitates communication through gap junctions and the exchange of signals via membrane-bound proteins[216,218].

Indirect Co-Culture: Here, cells are cultured near each other but are separated by a permeable membrane (e.g., in transwell systems). This arrangement allows the exchange of soluble factors such as cytokines and growth factors, enabling interaction without direct cell-cell contact[216,218].

Also, co-cultures can be implemented in both 2D and 3D systems. In 2D co-culture systems, cells are grown on flat surfaces, typically in a monolayer on culture plates. This is the more traditional and widely used approach due to its simplicity and ease of imaging and analysis. In another hand, 3D co-culture systems allow cells to grow in a three-dimensional environment, closely mimicking the architecture and microenvironment of tissues *in vivo*. Cells can be cultured in 3D matrices, scaffolds, or as spheroids/organoids[219,220].

Co-culture systems are powerful tools for studying cell interactions and modelling complex biological systems, offering a more physiologically relevant environment compared to monocultures. However they present some disadvantages like increased complexity and costs, reproducibility issues, technical challenges, standardization, and potential unintended interactions[215,216,218,219].

The main endpoints evaluated in *in vitro* bioassays can be broadly categorized into several areas, each providing crucial insights into different aspects of cellular and molecular responses to xenobiotics. Some of them are: Cytotoxicity (EC_{50}, LC_{50}), Genotoxicity,

Mutagenicity, Toxicokinetic studies and Cellular and Functional Responses (Protein/ Gene Expression)[203].

1.5.3 *in vivo* bioassays

in vivo bioassays are experimental procedures used to evaluate the biological effects of substances by testing them in living organisms. These assays help determine the toxicokinetics and biological activity, namely organ-directed toxicity, of compounds by observing their impact on whole organisms under controlled conditions. Unlike *in vitro* bioassays, which are performed on isolated cells or tissues, *in vivo* bioassays consider the organism as a whole and provide a more comprehensive understanding of the relation between the mechanism of action and the mode of action, making the results more applicable environmental and health risk assessments[221,222]. Some common endpoints include mortality rates, growth, reproduction, behavioural changes, and specific physiological or biochemical responses[223,224].

Regarding exposure time there are two major types of bioassays, acute and chronic, which can further be subdivided into sub-acute and sub-chronic respectively. Acute bioassays are designed to assess the immediate effects of a substance on living organisms over a short period, typically ranging from a few hours to a few days. The primary focus is on determining the lethal concentration (LC_{50}) or effective concentration (EC_{50}) that causes 50% of the test population to exhibit a specific adverse effect, such as mortality or immobility. These bioassays are crucial for identifying the potential short-term toxicity of chemicals, pollutants, or pharmaceuticals[225]. Chronic bioassays, on the other hand, evaluate the long-term effects of a substance over an extended period, which can range from several weeks to the entire lifespan of the test organisms. These assays help in understanding the cumulative and prolonged exposure effects, like genotoxic effects providing insights into the potential risks and impacts on populations and ecosystems[225]. The OECD[226,227] provides internationally recognized guidelines for the testing of chemicals, including both acute and chronic bioassays. These guidelines ensure that testing is conducted in a standardized and scientifically sound manner, facilitating the comparison of results across different studies and regulatory bodies.

Overall, while *in vivo* biomodels are indispensable for capturing complex biological interactions, they present some disadvantages, including ethical concerns, high costs, time-consuming procedures, and challenges with variability and reproducibility, underscoring the need for continued refinement and integration of alternative methods to enhance their relevance and applicability.

1.5.4 *in situ* bioassays

in situ bioassay is a type of biological assay conducted in the natural environment of the organism being studied rather than in a laboratory setting. The term "*in situ*" is Latin for "in the original place," indicating that the bioassay occurs in the natural habitat where the organism normally lives. These assays help determine the real-world impact of contaminants on the ecosystem, providing more accurate and environmentally relevant data than laboratory tests. Various biological responses are measured, such as growth, reproduction, behaviour, physiological changes, or biochemical markers. Since the assay is conducted in the natural environment, the results reflect actual ecological interactions and conditions. It provides data on the real impact of pollutants, taking into account factors like weather, the presence of other species, and complex environmental interactions[228,229].

The natural environment, unlike a controlled laboratory setting, presents numerous challenges that must be carefully considered in *in situ* bioassays. External influences, such as vandalism, loss of cages or equipment, uncontrollable covariates, and the potential spread of diseases or pests, can significantly impact the integrity of the study. Additionally, confounding factors such as abiotic variables—temperature, pH, salinity, and other compounds—are difficult to control during exposure and can influence the study's outcomes. As a result, these experiments often exhibit greater variability in results and are more logistically challenging and time-consuming, particularly when conducted in remote or adverse environments[230–232].

1.5.5 *in silico* models

An *in silico* model for toxicological assessment refers to the use of computer-based simulations and computational techniques to predict the toxicological properties of substances[233]. These models are designed to evaluate the potential adverse effects of chemicals, drugs, or other compounds on biological systems. These models aim to complement *in vitro* and *in vivo* toxicity testing to potentially minimize the need for animal testing, reduce the cost and time of toxicity testing, and improve toxicity prediction and safety assessment. These models are much less expensive in terms of time and money and are very useful in scenarios where it is impossible to carry out *in vivo* or *in vitro* tests or for example in emergency situations where rapid responses are required[234]. There are several methodologies that are used in *in silico* methods such as Quantitative Structure-Activity Relationship (QSAR) Models, Read-across and Molecular Docking.

QSAR models are computational methods that predict the biological activity or toxicity of chemical compounds based on their chemical structure. The fundamental premise is that similar chemical structures will exhibit similar biological activities[235]. It is used to predict various toxicological endpoints such as mutagenicity and carcinogenicity.

Read-Across is a technique used to predict the toxicity of a chemical by comparing it to structurally similar chemicals (analogues) with known toxicological data. It uses existing data to make predictions, which can save time and resources and is useful when experimental data for the target chemical is scarce[233,236].

Molecular docking is a computational technique that predicts how a chemical (ligand) interacts with a biological target (usually a protein). It estimates the binding affinity and the preferred orientation of the ligand within the binding site of the target. It provides detailed insights into the molecular interactions[235,237]

In conclusion, each model offers distinct advantages and unique limitations, making them suitable for different types of studies and research questions. When used together, these models complement one another, providing a holistic understanding of environmental toxicology. Table 1.3 presents a summary of their key characteristics.

Table 1.3 - Comparison between *in vitro*, *in vivo*, *in situ* and *in silico* bioassays.

Parameter	***in vitro***	***in vivo***	***in situ***	***in silico***
Objective	Mechanistic insights	Whole organism responses	Real-world ecological impacts	Predictive modelling
Biological model	Cell lines, primary cells	Fish, mussels and other aquatic organisms	Aquatic organisms in natural habitats	Computational models
Duration	Short-term (hours to days)	Short to long-term (days to months)	Long-term (months to years)	Not applicable (virtual)
Advantages	Cost-effective High throughput	Ecologically relevant	Real-world relevance, ecological integration	Rapid Large datasets Low cost
Limitations	Limited to cellular responses Aseptic conditions	Ethical concerns, resource-intensive Logistically complex	Logistically complex, variable conditions Complex data	Requires validation, dependent on existing data

1.5.6 Integration of different approaches

The integration of *in vitro*, *in vivo*, *in situ*, and *in silico* bioassays offers a comprehensive approach to understanding the effects of polycyclic aromatic hydrocarbon (PAH) mixtures on aquatic organisms.

In Figure 1.5 a scheme about the connections between the 4 types of bioassays is presented.

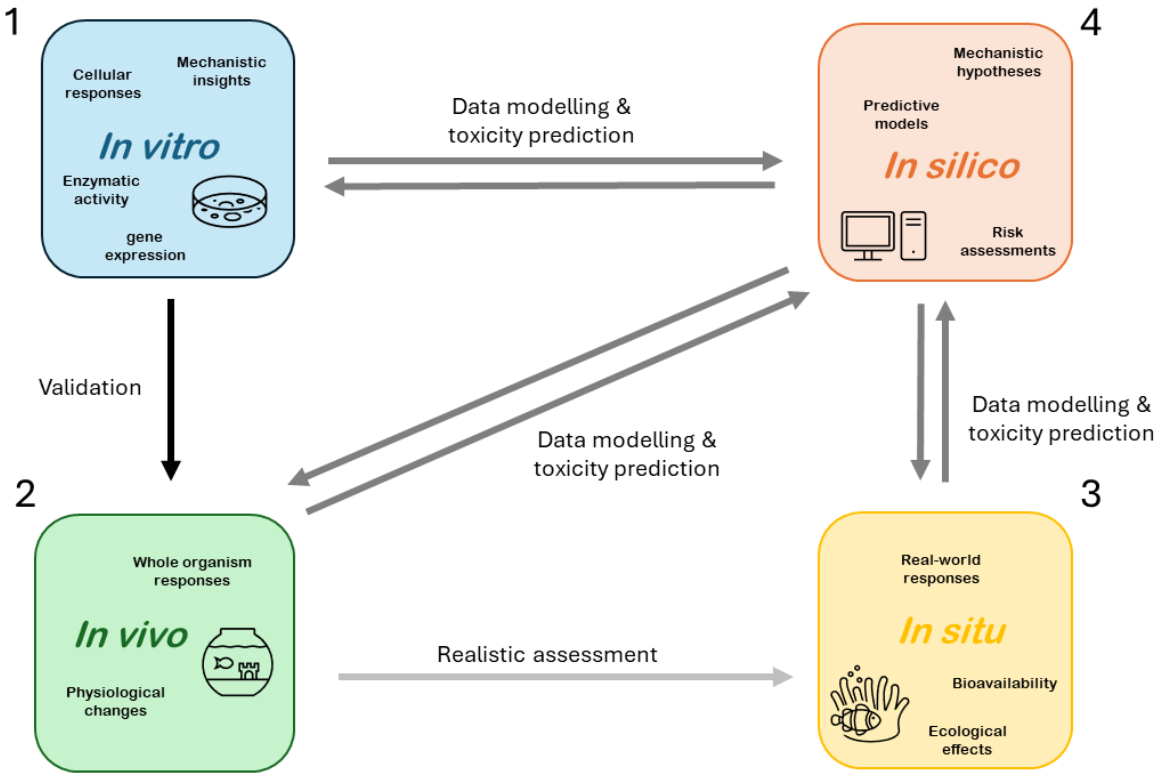


Figure 1.5 - Connections between *in vitro*, *in vivo*, *in situ* and *in silico* bioassays.

Then, *in vitro* bioassays (1) provide initial mechanistic insights by examining cellular and molecular responses to PAHs, such as gene expression changes and enzyme activity, under controlled conditions. These findings help identify potential biomarkers and pathways affected by PAH exposure (arrow 1 to 2). I*n vivo* bioassays (2) extend these insights to whole organisms, such as fish, allowing researchers to observe physiological, behavioural, reproductive effects and toxicokinetics over short and long-term exposures as well as some biochemical analysis in specific organs of the fish. This organism-level data is crucial for validating the relevance of *in vitro* (1) results and for understanding the systemic impacts of PAHs. *In situ* bioassays (3) further contextualize these findings by assessing the real-world ecological effects of PAHs in natural environments. By exposing organisms to contaminated

sediments or water bodies, *in situ* (3) studies account for environmental variables such as bioavailability and interactions with other pollutants, providing a realistic assessment (arrow 2 to 3) of ecological risks. Finally, *in silico* bioassays (4), through predictive modelling and quantitative structure-activity relationships (QSAR), integrate data from *in vitro* (1), *in vivo* (2), and *in situ* (3) studies to simulate potential outcomes and risks associated with PAH exposure. These models help predict toxicity, guide experimental designs, and inform risk assessments, particularly when empirical data is limited (arrows 1 to 4 and 4 to 1, 2 to 4 and 4 to 2 and 3 to 4 and 4 to 3). Together, these interconnected bioassay approaches enable a thorough evaluation of PAH toxicity, from cellular mechanisms to ecological impacts, enhancing our ability to assess and mitigate environmental risks.

1.5.7 New Approach Methodologies (NAMs)

in vivo toxicity data are available for only a limited number of substances, which poses a significant challenge given the rapidly increasing number of chemicals in commerce[238]. Consequently, it is anticipated that most chemicals will be evaluated using New Approach Methodologies (NAMs) rather than relying solely on traditional *in vivo* toxicity models. This shift is also important for assessing chemical mixtures, where the complexity of dose-response relationships is far greater than for individual chemicals[239].

Therefore, NAMs represent a significant advancement in toxicology, moving beyond traditional animal-based testing towards more sophisticated, human-relevant, and ethical approaches[240,241]. These methodologies include alternative or complementary techniques that enhance the predictive power of hazard assessments, adhering to the principles of the 3Rs[239]. NAMs employ high-throughput screening (HTS)[242] methods, omics-based technologies[243], 3D cell co-cultures[244], organoids[245], and micro-physiological systems (MPS)[246], among others to predict the biological effects of chemical exposures.

In addition to serving as alternatives to animal testing, NAMs can be integrated with traditional *in vivo* methods, *in vitro* studies, and clinical data. This integration is facilitated by frameworks such as Integrated Approaches to Testing and Assessment (IATA) and Adverse Outcome Pathways (AOPs).

IATA are structured frameworks that combine multiple sources of information and methodologies to evaluate the safety and risks associated with chemical substances. IATA is not a single method or test but rather an overarching strategy that integrates various data

types and approaches to provide a comprehensive assessment of a substance's potential hazards[247]. IATA is used in various regulatory and research contexts, including the assessment of industrial chemicals, pharmaceuticals, cosmetics, and environmental pollutants. The flexibility of IATA makes it applicable to a wide range of chemical safety assessments, from human health to environmental impacts[248].

AOPs are conceptual frameworks that describe the sequence of events leading from a molecular-level interaction between a chemical and a biological system to an adverse health outcome at the individual or population level. AOPs help to organize and connect different levels of biological organization, from molecular changes to cellular, tissue, organ, and whole-organism responses, ultimately linking these events to population-level impacts[249,250].

IATA use the framework of AOPs as a basis to systematically link molecular-level events with adverse health outcomes, enabling a more structured and predictive assessment of chemical risks that can integrate multiple data sources and reduce the reliance on animal testing[247].

Given their success in human research, these methodologies hold great potential for application in aquatic toxicology as well. By adopting NAMs in aquatic environments, researchers could gain deeper insights into the effects of pollutants on aquatic organisms and ecosystems, improving the accuracy of environmental risk assessments and advancing the protection of aquatic life.

1.6 Case Studies on the Environmental Toxicology of PAH Mixtures

In this section several case studies are presented to illustrate the application of various models and approaches in investigating the complex dynamics of PAH mixture toxicology. These examples underscore the significance of selecting the appropriate model system for specific research questions. Additionally, integrating insights from multiple models contributes to a more comprehensive understanding of the behaviour and toxicity of PAHs in aquatic ecosystems. For this purpose, a literature review was conducted using Pubmed to assess the number of research articles focused on the effects of PAH mixtures in the aquatic environment across different types of bioassays. The search targeted studies published between **2014 and 2024**, employing specific queries tailored to each bioassay type. Relevant keywords and Boolean operators were used (e.g., "PAHs mixtures" AND ("in vitro" OR "cell culture" OR "cell line" OR "fish cell")). Filters were applied to ensure that only research articles within the specified date range were included. The search yielded a total of 585 articles for *in vitro* studies, 93 for *in vivo* studies, 72 for *in situ* studies, and 66 for *in silico* studies.

Although the search primarily targeted mixtures of PAHs, some of the obtained articles had a broader or somewhat different focus (e.g., mixtures of PAHs with other contaminants). Nevertheless, these articles were included in the final count because this pattern was consistent across all searches. This approach ensures that the results reflect the overall research trend in the field of study (the application of different models to study PAH mixtures).

Thus, the data reveal a decreasing trend in the number of studies as the complexity of the bioassays increases (*in vitro* > *in vivo* > *in situ*), with a relatively low number of *in situ* studies compared to *in vivo* and *in vitro* research. Through this chapter, possible explanations for these patterns will be explored.

1.6.1 *in vitro* studies

Despite the recent development of fish cell lines like ZFL and RTL-W1, these models are still less commonly used compared to human cell lines. As a result, studies specifically focused on PAH mixtures in fish cells are relatively scarce.

Therefore, to provide a more comprehensive analysis, the search for *in vitro* articles was broadened to include studies involving various cell types, not limited to fish cells and

only PAH mixtures. The human cell line HepG2 has been fundamental in investigating the interaction mechanisms of PAHs at environmentally relevant concentrations and mixtures. Furthermore, other models, such as A549 alveolar cells and 3D bronchial epithelial cultures, have also been employed to explore the toxicological impacts of PAHs on different tissue types.

Immortalized rainbow trout cell lines are among the most extensively used models for assessing the environmental risks of chemical contaminants. Studies by Stadnicka-Michalak et al. (2018)[251] and Langan et al. (2018)[252] employed three distinct rainbow trout cell lines (RTL-W1 (hepatocytes), RTgill-W1 (epithelial cells from gills) , and RTgutGC (epithelial cells from intestine)) to investigate the biotransformation processes and activation of the CYP1A enzyme pathway in response to B[a]P. Similarly, Ferreira et al. (2014)[253] explored the effects of B[a]P on primary seabass cells, focusing on CYP1A transcription and (EROD) activity as markers of biotransformation. While this study is limited to single PAH compounds and do not encompass PAH mixtures, its inclusion in this discussion highlights the recent advancements in the application of *in vitro* fish cell models for understanding the molecular and biochemical impacts of B[a]P exposure.

Yazdani et al. (2018)[254] took a step closer to addressing the effects of PAH mixtures by exposing primary rainbow trout hepatocytes to four individual PAHs, naphthalene, fluoranthene, pyrene, and benzo[a]pyrene, for 24 hours. Although not strictly a mixture study, the aim was to compare the cytotoxicity, effects on glutathione (GSH) levels, and DNA damage across the different PAH treatments. The study found that all PAHs, except B[a]P, significantly reduced GSH levels, and all tested compounds exhibited genotoxicity in the hepatocytes. In terms of cytotoxicity, only pyrene and naphthalene resulted in reduced cell viability. These findings underscore the importance of understanding the effects of individual PAHs as a foundation for studying the more complex interactions that occur in PAH mixtures.

In 2024, Pannetier et al.[255] investigated the effects of water-accommodated fractions (WAFs) from two oil sources—Arabian light crude oil (LO) and refined oil from the Erika spill (HO)—both containing polycyclic aromatic hydrocarbons (PAHs), on RTL-W1 cells. Several toxicological endpoints were assessed, including cytotoxicity, EROD activity, DNA damage (using micronucleus and comet assays), and reactive oxygen species (ROS) production. Cells were exposed to varying dilutions of WAF for 24 hours. The results showed that LO WAF exposure led to a significant increase in EROD activity, ROS production, and DNA damage, as indicated by the comet and micronucleus assays. In contrast, HO WAF exhibited limited

toxicity, primarily inducing EROD activity. While this study focuses on PAH-containing mixtures, it approaches them in a broader context, highlighting differences in the overall PAH content between the two oils rather than comparing individual PAHs to mixtures. Nonetheless, the study provides valuable insights into the toxicological effects of complex matrices, emphasizing the relevance of mixtures in environmental toxicology research.

Branco et al. (2021)[178] assessed the toxicity of PAH mixtures in HepG2. So, the cells were exposed to Phe and B[b]F and their mixtures with varying compounds ratios. Results showed that Phe exhibited higher cytotoxicity compared to B[b]F, largely due to greater ROS production. Also, B[b]F increased GSH levels significantly and increased protein-S-glutathionylation levels, a posttranslational modification which protect sensitive thiols in proteins from oxidative damage. This GSH upregulation was linked to Nrf2 signalling activation and increased mRNA levels of GCLC, GCLM, and GS, the three enzymes involved in GSH synthesis. On the contrary, this was not observed in Phe exposure. Notably, all mixtures of Phe and B[b]F were more cytotoxic than the individual compounds, with the mixture 1:1 Phe:B[b]F showing the highest cytotoxicity and ROS production. However, GSH levels were not significantly increased, even in the B[b]F-enriched mixture. These findings highlight GSH as a key modulator of PAH toxicity and reveal the complex toxicological behaviour of PAH mixtures, even with just two compounds in different ratios.

In another study with HepG2 cells, Peng et al. (2015)[256] used mixtures with different PAHs, mixtures of heavy metals and mixtures with PAHs and heavy metals, and assessed the formation of micronucleus and the induction of AhR. Results showed that PAH mixtures containing naphthalene, phenanthrene and pyrene did not significantly increase micronucleus formation, however the mixtures containing B[a]P increased the occurrence of micronucleus. The same pattern was observed for AhR induction. In the mixture of PAHs and heavy metals, the mixture containing B[a]P and cadmium was the one that induced the highest levels of micronucleus. These results suggested that an additive effect may exist between PAHs and heavy metal/loids in a compound and concentration-dependent manner. This study examines not only the interactions between different PAHs but also their combined effects with other environmental contaminants, specifically heavy metals. This is a highly relevant study because it reflects real-world exposure scenarios, where organisms are often exposed to complex mixtures of different pollutants.

In 2019 Chang et al.[257] used a primary human 3D bronchial epithelial culture to assess different mechanisms of toxicity of B[a]P and dibenzo[def,p]chrysene (DBC) and their mixture

(CTE). Dosing was chosen based on relative potency in B[a]P equivalents (B[a]Peq) for DBC (~50 B[a]Peq) and CTE (0.4 B[a]Peq). They assessed the gene expression after 48h of exposure. Results showed that the compounds acted oppositely, whereas B[a]P and CTE upregulated CYP1A1 and ALDH3A1, DBC downregulated CYP1A1 and CYP1B1. Also, they found that other processes were regulated in common by all PAH treatments, B[a]P, DBC and CTE.

In 2016 Genies et al.[258] exposed A549 alveolar cells to a binary mixture of 12 different PAHs with B[a]P or a complex mixture of all of them to assess cell viability, EROD activity, the induction of genes coding for metabolization enzymes as well as diol epoxide adduct formation. Results showed that, compared to pure B[a]P, both types of PAH mixtures resulted in decreased CYP450 activity, as measured by the EROD test, and showed a similar trend in the formation of DNA adducts. The complex mixtures were more potent than B[a]P at the same concentration in inducing genes coding for CYP.

Castel et al. (2023) [259] studied the effects of PAHs (individual and in mixtures) that are present in settled dust. They used adherent human adenocarcinoma gastric cells to assess genotoxic effects through the micronucleus and comet assays. They also used the concept of Toxic Equivalent Factors (TEF) based on the hypothesis of additive effects to estimate Genotoxic Equivalent Factors (GEF). Results showed that B[b]F and B[a]P had a synergistic effect on DNA damage. Also, GEFs calculated for PAH individual were lower than GEFs for PAHs in mixtures; thus, mixtures induce greater DNA/chromosomal damage than expected.

The previous studies used different human immortalized cell lines to explore the effects of PAH mixtures, comparing them to individual compounds. As previously said, this approach is essential for uncovering complex interactions like synergism or antagonism, offering deeper insights into how combined exposures can differ from single-compound effects.

In conclusion, *in vitro* studies on PAH mixtures employ several different approaches, reflecting the complexity of analysing the effects of these contaminants. Traditionally, most research on PAH mixtures has been conducted using human cell lines, mainly due to their relevance in human health risk assessments. These studies generally focus on cytotoxicity, genotoxicity, and metabolic activation pathways, providing information on how individual and combined PAHs affect human cells.

However, in recent years there has been increasing interest in using fish cells, which is particularly significant given the ecological context of PAH contamination.

These studies are essential for predicting how PAHs interact with aquatic organisms at the cellular level, helping to bridge the gap between human toxicology and environmental toxicology.

1.6.2 *in vivo* studies

This section will go through detailed case studies to evaluate PAH mixtures focusing on animal models and methodologies, highlighting key findings and their implications for environmental health research.

In 2014 Vignet et al.[260] exposed zebrafish embryos and larvae during 4 days to a natural sediment spiked with 3 individual PAHs (Pyrene, Phenanthrene and Benzo[a]pyrene). The study quantified metabolites in larvae exposed to PAHs, confirming contamination and active metabolism, particularly for pyrene and benzo[a]pyrene. Results showed growth disruption appearing at five months and a trend towards lower reproductive ability. Adults from PAH-exposed embryos exhibited lethargic and anxiety-like behaviours, which were also observed in offspring at the larval stage. These effects could negatively impact fish performance and their contribution to population recruitment.

Martins et al (2015)[189] studied the interactions of two PAHs, carcinogenic B[b]F and non-carcinogenic phenanthrene, in seabass over a 28-day bioassay with spiked sediments. Genotoxicity was observed as DNA strand breaks in fish, though no clear dose or time response was detected, likely due to low exposure levels and shifts in PAH bioavailability. Clastogenic/aneugenic lesions were seen only in fish exposed to B[b]F, while combination assays revealed a synergistic effect at the chromosome level. The study highlighted that even low-to-moderate concentrations of PAH mixtures in sediment pose a significant genotoxic risk, showing that non-carcinogenic PAHs can still induce mutagenesis in marine organisms. This underscores the potential for long-term DNA damage from low-level PAH exposure, including mixtures with non-carcinogenic compounds, in aquatic ecosystems.

in vivo assays can employ various approaches to study the effects of PAH exposure on aquatic organisms. Despite differences in their duration, the two studies mentioned above both used spiked sediments as the contamination matrix. These studies underscore the significance of sediments in PAH exposure, as PAHs, due to their hydrophobic nature, tend to adsorb onto suspended particles and accumulate in sediments, particularly those rich in

fine particles and organic matter. As a result, sediments can act as reservoirs for pro-mutagenic substances like PAHs, posing long-term risks to benthic organisms and the broader ecosystem.

Pannetier et al. (2024)[261] exposed Japanese medaka in two life stages, larvae and juveniles to two water-accommodated fractions (WAFs) from Arabian light crude oil (LO) and refined oil from Erika (HO) for 48h. They examined EROD activity, DNA damage, photomotor response, and sensitivity to nervous necrosis virus (NNV) infection. Their findings showed that both oils significantly induced EROD activity, caused DNA damage, and led to developmental anomalies, though no behavioural changes were observed. In larvae, NNV infection resulted in high mortality and a marked reduction in response to light stimulation. Co-exposure to WAFs and NNV further increased mortality, indicating that WAFs may impair the fish's defence mechanisms. Toxic effects of WAFs in juveniles were only evident following NNV challenge, with a higher sensitivity to HO WAFs compared to LO WAFs.

Another approach to studying the effects of PAH mixtures in aquatic environments is using crude oil, which contains a complex mix of PAHs. As highlighted in the *in vitro* bioassay section, this study is highly relevant because it is simulated real-world contamination scenarios. However, it only focusses on differentiating between heavy oil (HO) and light oil (LO) without comparing the effects of individual PAHs within the mixtures, in addition it is possible that these mixtures contain other hydrocarbons than just PAHs.

Nakken et al. (2024)[262] aimed to identify PAH metabolites and their metabolic pathways in Atlantic haddock (*Melanogrammus aeglefinus*) by injecting the fish with either individual PAHs or complex oil mixtures. The fish were euthanized three days post-injection, and bile extracts were analysed to profile PAH metabolites. This approach led to the development of an interactive library containing 33 metabolites derived from eight PAHs, providing new insights into the detoxification pathways and previously undiscovered metabolites in fish exposed to crude oil.

In 2014 Larcher et al[263] studied the chronic (14 months) dietary exposure of zebrafish to PAH mixtures from a polluted site in the Seine estuary (PY), Erika fuel (HO) and Arabian light crude oil (LO). Results showed a decrease in survival after 3 months of exposure for the fish fed with HO and LO. Also, all PAH fractions caused preneoplastic and neoplastic disorders in zebrafish. The most severe carcinogenic effects were induced by dietary exposure to HO compared to exposure to LO or PY after 9 to 10 months of exposure. In contrast, earliest carcinogenic effects were detected as soon as 3 months after exposure to LO, including the lowest concentration, or to PY.

In another study, Meier et al. (2020)[264] evaluated DNA damage in juvenile haddock exposed to low doses (0.3–0.7 mg PAH/kg fish/day) of petrogenic and pyrogenic PAHs through ingestion over two months, followed by a two-month recovery period. The PAHs came from various sources: (1) crude oil dominated by 2-ring PAHs, (2) crude oil with 3-ring PAHs, and (3) a heavy pyrogenic PAH mixture of 4/5/6-ring PAHs. Additionally, a group was injected with 12 individual 4/5/6-ring PAHs. The study assessed DNA adducts, liver PAH load, bile metabolites, CYP1A gene/protein expression, GST activity, lipid peroxidation, skeletal deformities, and liver histopathology. Elevated DNA adduct levels were found in the liver three days after single-compound PAH exposure and following ingestion of 2- and 3-ring PAH extracts, persisting across all exposed groups even after the recovery period. Fish exposed to oil or heavy PAHs exhibited high DNA adduct levels in the intestines, unlike those in the oil-produced water or control groups. This suggests that the intestinal epithelium is a primary target for PAH toxicity during oral exposure and likely serves as the first line of interaction with these contaminants. The presence of DNA adducts in the intestinal tissue indicates that PAHs can cause damage at the point of entry, before they are metabolized or distributed to other organs.

These three studies introduce important variation in the exposure route, since in the first study, the fish were injected directly with individual PAHs or complex oil mixtures. In the second study, the oil mixtures were administered via ingestion, and in the last one have both methods, ingestion and injection. The use of injection as an exposure method contrasts with the more common routes, such as ingestion or water exposure, offering a direct and controlled way to assess PAH metabolism and toxicity with high precision. While previous studies focused only on the effects of heavy oil (HO) or light oil (LO), these studies compare different oils with individual PAHs, making it easier to determine the formation of metabolites and evaluate potential interactions between PAHs in mixtures providing a more detailed understanding of the risks associated with mixed exposures.

Albarano et al. (2023)[265] exposed the crustacean *Artemia franciscana* to binary, tertiary, and quaternary mixtures of polycyclic aromatic hydrocarbons (PAHs) for 48 hours. They quantified synergistic and antagonistic interactions based on individual inhibitory effects measured as LC_{50} values and assessed gene expression. The results indicated that most PAH combinations produced additive or synergistic effects, except for the naphthalene and phenanthrene mixture, which showed no significant interaction. In contrast, the combination of naphthalene and benzo(k)fluoranthene exhibited an antagonistic effect. Additionally, real-

time qPCR analysis revealed that all PAH mixtures affected the expression levels of five key genes involved in Artemia's stress response (hsp 26, hsp 60, hsp70, COXI and COXIII).

Barranco et al. (2017)[266] studied the effects of mixtures of five PAHs benzo[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[k]fluoranthene, and chrysene on zebrafish embryos. Gene expression related to the aryl hydrocarbon receptor pathway was analysed. After 24 h of exposure, PAHs and their mixture did not cause significant levels of mortality or malformations. Also, all PAHs, except for benzo[a]anthracene, acted as potent inducers of the cyp1a gene. Benzo[k]fluoranthene was the major inducer; the effect caused by the mixture was two times higher than that caused by any of the compounds tested individually and greater than an additive upregulation. Furthermore, the great induction of cyp1a by the mixture was due to the presence of BkF, since another experiment was carried out in which a mixture without BkF was tested which did not increase cyp1a induction.

In 2022 Eriksson et al.[267] exposed rainbow trout alevins to retene, fluoranthene and their mixture for 1, 3, 7 and 14 days. They assessed morphological alterations after each exposure period and conducted a microarray analysis on heart tissue. The results indicated that retene and the mixture, but not fluoranthene, significantly reduced growth by day 14 compared to the control group. Additionally, exposure to the mixture led to a significant increase in the BSD (blue sac disease) index from day 3 onwards. Pathway analysis revealed that the mixture overexpressed pathways associated with growth, amino acid and xenobiotic metabolism, and oxidative stress responses. Alevins exposed to the individual PAHs showed altered receptor signalling pathways: retene downregulated genes involved in G-protein signalling, while fluoranthene upregulated genes related to GABA signalling. Moreover, both retene and fluoranthene exposure affected the expression of genes encoding proteins related to calcium and potassium ion channels, suggesting potential impacts on heart structure and function.

Li et al. (2020)[268] exposed Manila clam (*Ruditapes philippinarum)* to different concentrations of (0.05, 0.5, and 5 μg/L) a quaternary mixture (B[a]P:B[b]F:B[a]A:Chr) with a 1:1:1:1 proportion. The exposure lasted for 21 days, followed by a 15-day depuration period to assess bioaccumulation and oxidative damage, including DNA damage, lipid peroxidation (LPO), protein carbonylation (PC), and oxidative stress biomarkers. PAHs accumulated in the gill, digestive gland, adductor muscle, and soft tissue in the following descending order: chrysene > benzo[a]anthracene > benzo[a]pyrene > benzo[b]fluoranthene. All PAH mixtures,

except at the lowest concentration, significantly induced oxidative damage. The correlation analysis indicated that aryl hydrocarbon hydroxylase, glutathione S-transferase, superoxide dismutase, DNA strand breaks, PC, and LPO in both the gill and digestive gland could serve as potential early indicators of exposure to PAH mixtures.

The previous four studies examined PAH contamination directly in water, using four different species to evaluate the effects of PAH mixtures and observed various interactions between the PAHs within the mixtures. Water as the exposure medium closely replicates real-world environmental contamination, where organisms are consistently exposed to dissolved PAHs, providing valuable insights into how these contaminants behave in natural aquatic ecosystems.

Overall, there are fewer *in vivo* studies dealing with PAH mixture exposure than *in vitro* studies, as previously noted. This discrepancy can be attributed to ethical considerations, particularly the objective of adhering to the principle of the 3Rs which emphasizes minimizing the use of live animals in research, as well as greater logistics compared to *in vitro* tests.

In terms of species diversity, studies cover a variety of aquatic organisms, including freshwater and saltwater species such as fish, mussels and crustaceans. This variety highlights the importance of considering different biological and ecological contexts when evaluating the toxicological effects of PAHs, making findings more broadly applicable to freshwater and marine ecosystems. Still, zebrafish, (embryos, larvae and adults), are the most used *in vivo* model, likely due to their small size, ease of maintenance and well-established protocols. Furthermore, it is important to note that saltwater species require more complex infrastructure for their maintenance, making them less frequently used.

As mentioned previously, these studies take various approaches to contamination sources (water, sediment, or food), providing valuable data on different exposure pathways and their effects. Exposure periods also varied widely, from 24 hours to 14 months. This range is appropriate for PAH studies as short-term exposure allows investigation of detoxification mechanisms (such as CYP1A activation) and metabolite formation, while long-term exposure is crucial for assessing cumulative damage, including DNA damage and chromosomal.

1.6.3 *in situ* studies

In the following section, we will present *in situ* studies that investigated the effects of PAH exposure in natural environments.

Martins et al. (2015)[231] conducted *ex situ* and *in situ* bioassays during 28 days in the Portuguese Sado estuary. Juvenile soles (*Solea solea*) were exposed to different moderately contaminated (heavy metals and PAHs) sediments. A qualitative histopathological screening yielded scant lesions to gills, albeit alterations such as epithelial hyperplasia being evident and more frequent in fish exposed *ex situ*.

In 2015, Lacroix et al.[269] evaluated the ecotoxicological status of the Brest harbour (Bay of Brest, Brittany, France) using native and caged mussels (*Mytilus spp*.) to assess the effects of chronic contamination. PAH concentrations were measured, and biomarkers related to biotransformation, antioxidant defence, metabolism, and oxidative damage were analysed. The results revealed moderate PAH contamination in the digestive glands of both native and caged mussels at the exposed site. Biomarker modulations in native mussels suggested ongoing chemical stress. Overall, the findings indicated that mussels chronically exposed to contamination had developed metabolic adaptations.

Maloney et al. (2023)[270] performed an *in situ* bioassay for 2 years across 8-11 sites in the Milwakee Estuary (WI. USA) to evaluate the mixture contamination. To do so, caged fathead minnows (*Pimephales promelas)* were exposed for 96h on each site-specific. After that, chemicals were grouped based on structure/mode of action, bioactivity and pharmacological activity. Analyses identified 14 mixtures and 16 chemicals that significantly contributed to cumulative effects (such as PAHs).

Sandrini-Neto et al. (2016)[271] investigated the effects of repeated diesel spills on the bivalve *Anomalocardia brasiliana*, the gastropod *Neritina virginea,* and the polychaete *Laeonereis culveri* by monitoring oxidative stress biomarkers *in situ* in a subtropical estuary in Paranaguá Bay, southern Brazil. The study compared oil spill effects using different diesel dosages in a factorial experiment with asymmetrical controls. The hypotheses tested included: (i) the overall impact of oil spills, (ii) the influence of diesel dosage and exposure frequency, and (iii) the effect of time since the last spill. PAH concentrations were measured from sediment samples taken a day after the final spill. Results showed PAH contamination, with frequent low-dosage exposures inducing stronger antioxidant responses than infrequent high-dosage ones. The bivalve *A. brasiliana* and the polychaete *L. culveri* proved to be more effective sentinel species due to their greater sensitivity to oil and broader geographical range.

As previously discussed, conducting *in situ* bioassays is more labour-intensive and complex due to the challenges posed by environmental conditions, which limit control over experimental variables, increase costs, and result in more complex and variable data for

analysis. Like *in vivo* studies, there has been a decreasing trend in the number of studies using aquatic organisms to assess PAH mixtures, likely due to the increased complexity and logistical demands.

However, the importance of *in situ* bioassays in understanding the real-world impacts of contamination by multiple pollutants is unquestionable. Typically conducted in areas with existing contamination, *in situ* studies take a more comprehensive approach by identifying and quantifying various contaminants rather than focusing only on a single pollutant, such as PAHs. They also highlight the effectiveness of using sentinel species to monitor ecosystem health, revealing how different contamination patterns and mixture effects influence biological responses.

1.6.4 *in silico* studies

This section will explore the computational models and simulations used to assess the effects of PAH mixtures, emphasizing the methodologies and endpoints analysed in these *in silico* studies.

Gaskill and Bruce (2016)[272] combined *in silico* methods (QSAR) to modulate the toxic effects of PAHs based on *in vivo* experiments. Clone-9 rat liver cells were used to analyse cell proliferation, viability and genotoxicity of 15 PAHs in single doses and binary mixtures. Tests revealed that many mixtures have non-additive toxicity, presenting varying effects depending on the composition of the mixture. Also, the variation in endpoint responses underscores the complexity of PAH mixture toxicity and the importance of considering multiple endpoints in risk assessment. Different biological processes may be affected differently by PAH mixtures, leading to endpoint-specific interaction patterns. Many of these mixtures showed antagonism, similarly to other published studies. QSARs were then developed using a gene function approximation algorithm to predict the toxic activity of both individual PAHs and binary mixtures. QSAR algorithms for mixtures were adjusted to consider observed interactions and mixture composition (proportions) to evaluate the accuracy of QSARs on mixtures. Based on the results, the authors concluded that toxic addition is unlikely and therefore environmental PAH mixtures do not exhibit additive responses when complex interactions between components occur.

Bak et al (2019)[273] implemented a study regarding the contamination of fish in Kesennuma Bay, Japan, by heavy oil containing PAHs. Six specific PAHs were detected at

high levels in fish tissues and the effects of these PAHs on the AhR signalling pathway were then evaluated. Initially, PAH concentrations and the activities of cytochrome P4501A enzymes (EROD and MROD assay) were measured. Subsequently, AhR-mediated responses were investigated with an *in vitro* reporter gene assay system where red seabream (Pagrus major) AhR1 (rsAHR1) or rsAhR2 expression plasmids were transiently transfected into COS-7 cells. This assay showed transactivation depended on isoform, PAH type, and dose. Concentrations effective to induce 20% of the maximal benzo[a]pyrene (REC20-B[a]P) response were determined for each PAH. The data were then modulated using *in silico* models that suggested that the activation power of PAHs could be predicted from an rsAHR2 model. These toxicity estimations can be incorporated into risk assessments for compounds without toxicity data, allowing for accurate predictions and a better understanding of the relationship between composition and toxicity.

Cabral et al. (2022)[274] used an *in silico* approach to assess the potential proinflammatory effects of various carcinogenic PAHs by evaluating their binding affinity to the human toll-like receptor 4 (TLR4). Using Autodock Vina, they identified the best docking poses and binding affinities for each PAH. The results showed that out of the 14 PAHs studied, indeno[1,2,3-cd] pyrene, benzo[ghi]perylene, and benzo[a]pyrene had the highest binding affinities, with values of −10, −9, and −8.9 kcal/mol, respectively. Molecular dynamics simulations and RMSF analysis further confirmed that TLR4 maintained a stable conformation despite ligand interactions.

Chen et al. (2021)[163] used a QSAR approach, combined with docking methods, QM/MM, and *ab initio* calculations, to investigate the physicochemical and electronic properties affecting PAH metabolism and PAH-CYP1A1 interactions. They aimed to identify key structural properties influencing binding ability, metabolic clearance, and mutagenicity. The results revealed that van der Waals interactions, influenced by PAH molecular weight and hydrophobicity, were crucial for binding to CYP1A1. The interaction between PAHs and the heme group, specifically the distance between iron and PAH carbons (Fe_Cmin) and heme van der Waals forces, impacted PAH metabolic clearance. Additionally, the electronic property (ESP negative variance) played a significant role in mutagenicity by affecting the formation of epoxide metabolites.

Despite significant technological advancements, *in silico* models for studying the effects of PAH mixtures are still in the development phase, with relatively few studies focusing on this area. One of the main challenges limiting the broader application of *in silico* bioassays in

PAH mixture research is the difficulty in obtaining essential input data. Many of the existing studies rely on data from *in vivo* or *in vitro* experiments, which, as previously discussed, come with their own limitations. Additionally, much of the toxicity research is primarily focused on human toxicology, with less emphasis on environmental toxicology. This gap constrains the development of *in silico* models capable of effectively predicting the effects of PAH mixtures in aquatic ecosystems and other natural environments, highlighting the need for more specific environmental data to improve the accuracy and applicability of these models.

1.7 Future Perspectives and Conclusions

As the study of PAH mixtures progresses, several emerging trends in pollution assessment are expected to shape future research. New biomodels and technologies are being developed to enhance our understanding of the complex interactions within PAH mixtures. For example, the use of advanced fish cell lines such as ZFL and RTL-W1 is anticipated to grow, providing more relevant data for aquatic environments. Additionally, the development of more sophisticated *in silico* models leveraging artificial intelligence and machine learning will enable researchers to predict the behaviour and impact of PAH mixtures more accurately, offering a powerful tool for environmental risk assessment. Also, the integration of omics technologies, including genomics, proteomics, metabolomics, and transcriptomics, represents a significant advancement in pollution assessment. These technologies allow for a comprehensive analysis of the molecular responses of organisms exposed to PAH mixtures. By examining changes in the genetic, protein, and metabolic levels, researchers can gain deeper insights into the mechanisms of toxicity and identify potential biomarkers for exposure and effects. This holistic approach will improve the sensitivity and specificity of bioassays, facilitating early detection of environmental contaminants and a better understanding of their impacts on health and ecosystems.

Furthermore, the findings from studies on PAH mixtures carry significant policy implications. As our understanding of the interactions and combined effects of PAHs improves, it will be essential to update regulatory frameworks to address the complexities of environmental pollution. Policymakers should consider adopting guidelines that account for the cumulative and synergistic effects and composition of chemical mixtures, rather than evaluating pollutants individually. This shift would ensure more comprehensive environmental protection and stronger public health safeguards. Thus, future research should prioritize the following directions:

Enhanced Monitoring Programs: Implementing advanced monitoring programs that utilize new biomodels and omics technologies to track PAH contamination in various environments.

Standardization of Methods: Developing standardized methodologies for *in vitro*, *in vivo*, *in situ* and *in silico* bioassays to ensure consistency and comparability of results across studies.

Long-term Studies: Conducting long-term studies to better understand the chronic effects of PAH mixtures on ecosystems and human health.

Interdisciplinary Collaboration: Fostering collaboration among toxicologists, chemists, environmental scientists, and policymakers to address the multifaceted challenges of PAH pollution.

Public Engagement and Education: Engaging and educating the public about the risks associated with PAH mixtures and the importance of regulatory measures to mitigate these risks.

AI disclosure

AI tools were used in the development of this thesis to support various stages of the research process. Their use is detailed as follows:

Literature Search: As a starting point, Chat GPT 4.0 (www.chatgpt.com), Perplexity (www.perplexity.ai) and Consensus (www.consensus.app) tools were used to search and identify relevant academic literature

Code Generation: ChatGPT 4.0 was used to generate code for use in the R software (statistical data processing)

Language Refinement: As the author is not a native English speaker, ChatGPT 4.0 was used to refine academic language (checking grammatical structure, punctuation and vocabulary)

All AI-generated content was critically reviewed and adapted to ensure accuracy, relevance, and academic integrity.

2 Objectives and research approach

2.1 Objectives

The main objective of this thesis is to understand the toxicological mechanisms of environmentally relevant polycyclic aromatic hydrocarbon (PAH) mixtures, with a particular emphasis on mutagenesis and carcinogenesis-related effects on marine fish species.

The central hypothesis is that the relative proportions of individual PAHs within a mixture, while maintaining a constant total PAH concentration, modulate the mixture's overall toxicological potency and mode of action, particularly with respect to mutagenic and carcinogenic endpoints in marine fish.

To test this hypothesis, the study combines *in vitro* assays using fish primary hepatocytes to investigate mechanistic aspects, followed by *in vivo* validation using an ecologically relevant fish species, *Sparus aurata*. The final phase involves the use of HepG2 human liver cells to further investigate the molecular mechanisms. The research focuses on key molecular pathways, such as AhR activation and CYP-mediated bioactivation, and employs a combination of molecular, biochemical techniques to provide a comprehensive understanding of the toxicity mechanisms associated with complex PAH mixtures.

The specific objectives are:

i) To develop a reliable and reproducible protocol for the isolation and culture of primary hepatocytes from marine fish species in line with the 3R (Replacement, Reduction, Refinement) principles of animal experimentation.

ii) To evaluate the cytotoxic and genotoxic effects of PAH mixtures in fish primary hepatocytes, focusing on cell viability, DNA damage, and CYP1A1 expression at the mRNA and protein levels.

iii) To validate, *in vivo*, the mechanisms identified in the fish-based cellular models using *Sparus aurata* as a test species, integrating molecular biomarkers.

iv) To investigate the AhR and CYP-mediated bioactivation mechanisms yielded by the PAH mixtures.

This thesis aligns with the goals outlined in the UN's 2030 Agenda, specifically focusing on ensuring good health and well-being for all (Goal 3) and increasing knowledge to conserve and sustainably use oceans, seas, and marine resources (Goal 14).

2.2 Research Approach

PAHs are widespread environmental contaminants, particularly in aquatic environments, where they commonly occur as complex mixtures. Traditionally, the toxic effects of substances are assessed by studying each compound individually; however, in aquatic ecosystems, PAHs are rarely isolated, and their presence in mixtures poses a significant challenge for toxicity evaluation due to their unpredictable interactions, such as additive, antagonistic, or synergistic effects.

To better understand the contribution of individual components and their interactions within PAH mixtures, this study employs a design in which the total concentration of PAHs remains constant, while the relative proportions of individual PAHs are systematically varied. This strategy allows for the assessment of how changes in mixture composition influence toxicological outcomes, such as cytotoxicity, genotoxicity, and metabolic activation. By analysing mixtures with differing ratios, mimicking environmentally relevant scenarios, this research seeks to uncover whether specific PAHs act as key drivers of toxicity, or if effects arise from complex interactions within the mixture.

To achieve this, different biological models are employed, each contributing complementary insights into the mechanistic and systemic effects of PAH mixtures.

The first phase of the study focused on *in vitro* experiments. Initially, a protocol was established and optimized for the extraction of primary hepatocytes from sea bream (*Sparus aurata*), validating this cellular model for toxicity assays involving PAHs. The selected PAHs for the study included benzo[a]pyrene (B[a]P, a well-known carcinogenic compound with five rings), phenanthrene (Phe, a non-carcinogenic compound with three rings), and benzo[b]fluoranthene (B[b]F, which has a structure similar to B[a]P). These compounds were tested individually and in mixtures at varying ratios (1:1, 1:2, and 2:1), with exposure durations of 24 and 48 hours. The analysis of toxicological endpoints included the assessment of genotoxicity, investigation of molecular pathways associated with CYP-mediated bioactivation, and examination of cellular processes such as oxidative stress and DNA repair.

The second phase of the study involved the *in vivo* validation of the results obtained from the *in vitro* assays. A bioassay was conducted using *Sparus aurata*, exposing the fish to PAH mixtures selected based on the *in vitro* findings. This phase included the analysis of molecular biomarkers to confirm and expand upon the discoveries made in the cellular model.

In the third phase, the study investigated the molecular details related with the observed effects using HepG2 human liver cells, particularly the AhR pathway role in the metabolism of PAHs mixtures and on evaluating the interactions among the PAH compounds in mixtures, (additive, antagonistic, or synergistic).

The study culminated in an integrative analysis that combined *in vitro* and *in vivo* data for a general understanding of toxicity mechanisms. This comprehensive research approach aimed to elucidate the molecular mechanisms underlying the toxicity of PAH mixtures but also to provide valuable insights for environmental management and the protection of human health and aquatic ecosystems, namely regarding the Environmental Risk Assessment of PAH mixtures, leading to recommendations for future environmental guidelines, such as those outlined by the Marine Strategy Framework Directive (MSFD).

2.3 Outline of the Thesis

The present PhD dissertation comprises seven chapters (Figure 2.1). Chapter 1 provides a general introduction, including a detailed literature review that covers an overview of aquatic pollutants, their sources, and their biological effects. It also discusses the main current guidelines and strategies for addressing aquatic pollution, as well as approaches for dealing with mixtures of environmental contaminants. Additionally, it offers an overview of PAHs, including their chemistry, sources, and biological effects, the various model organisms and bioassays used to study PAH effects, and some relevant case studies. chapter 2 outlines the aims, research approach, and structure of the thesis. The first chapter presenting original data (chapter 3) introduces a protocol for the extraction of primary cells from *Sparus aurata*. chapter 4 investigates the interaction of PAH mixtures in these primary cells, while chapter 5 aims to validate the *in vitro* results *in vivo* using *S. aurata*. chapter 6 uses the HepG2 cell line to extrapolate previous findings to a human model, validating the results within a human cellular context and comparing the effects observed in fish and human cells. Furthermore, predictive models were developed to analyse interactions among PAHs in complex mixtures, exploring additive, antagonistic, and synergistic effects. Finally, chapter 7 synthesizes the key

findings, critically examines research limitations, and proposes future directions, integrating the thesis's core elements and their broader implications. Chapters 3, 4 and 5 are published manuscripts, while. chapter 6 is in preparation for submission. A summary of the thesis structure and research approach is provided in Figure 2.1.

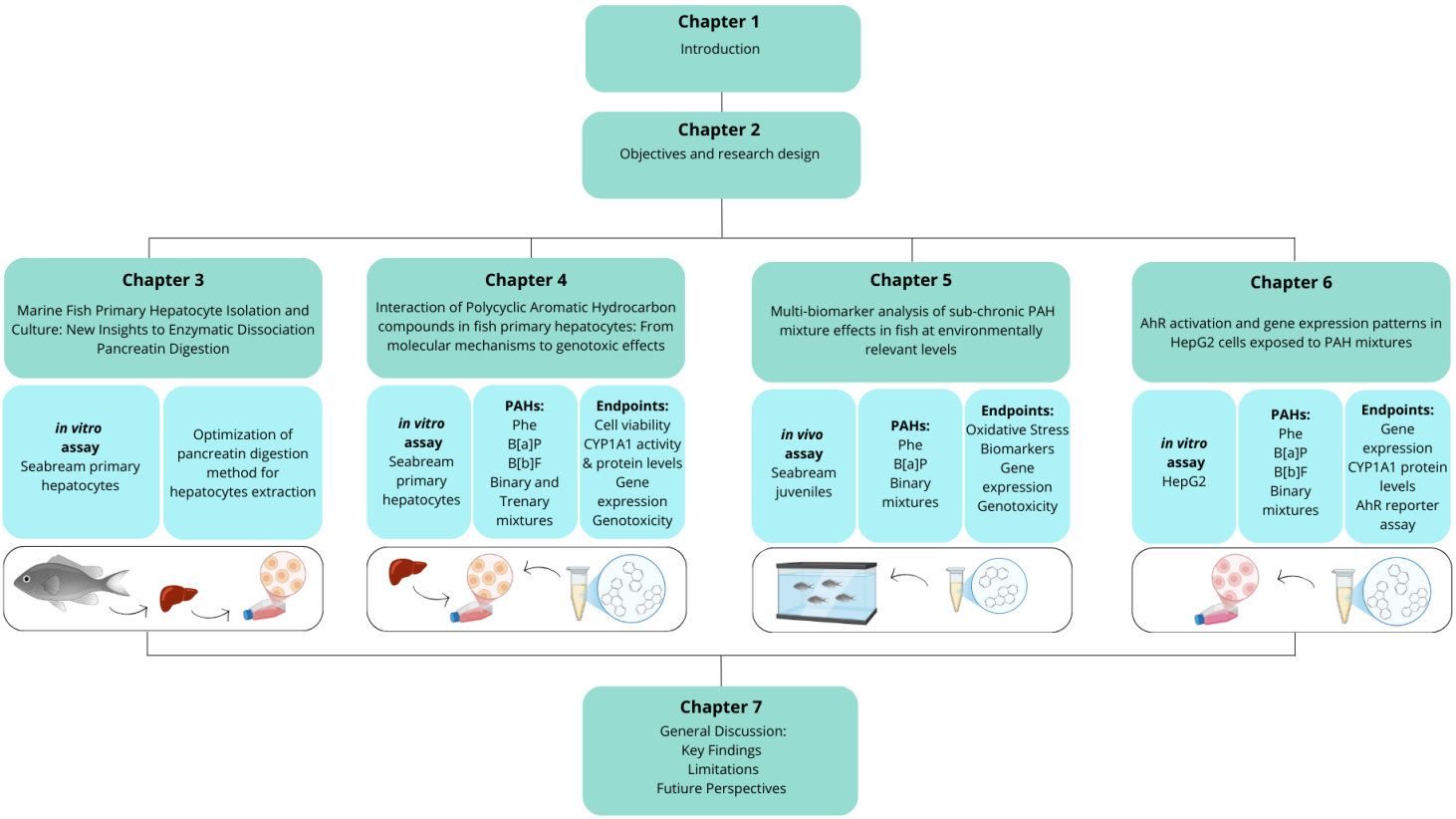


Figure 2.1 - Thesis structure and Research Approach

2.4 Thesis Outputs

The work developed during this PhD has resulted in the following scientific contributions, including published articles, submitted manuscripts, and presentations in scientific meetings.

Published articles

- Figueiredo, Neusa; Matos, Beatriz; Diniz, Mário; Branco, Vasco; Martins, Marta. "Marine Fish Primary Hepatocyte Isolation and Culture: New Insights to Enzymatic Dissociation Pancreatin Digestion". International Journal of Environmental Research and Public Health 18 4 (2021): 1380. Doi: 10.3390/ijerph18041380

- Bramatti, Isabella*; Matos, Beatriz*; Figueiredo, Neusa; Pousão-Ferreira, Pedro; Branco, Vasco; Martins, Marta. "Interaction of Polycyclic Aromatic Hydrocarbon compounds in fish primary hepatocytes: From molecular mechanisms to genotoxic effects". Science of The Total Environment (2022): 158783. Doi:10.1016/j.scitotenv.2022.158783

* These authors contributed equally to this work.

- Matos, Beatriz; Bramatti, Isabella; Santos, Carlos David; Branco, Vasco; Martins, Marta. "Multi-biomarker analysis of sub-chronic PAH mixture effects in fish at environmentally relevant levels". Aquatic Toxicology (2025): 107350. Doi:10.1016/j.aquatox.2025.107350

To be submitted

- Matos, Beatriz; Branco, Vasco; Martins, Marta. "AhR activation and gene expression patterns in HepG2 cells exposed to PAH mixtures"

Presentations in scientific meetings

- Matos, Beatriz; Diniz, Mário; Branco, Vasco; Martins, Marta. "The unpredictable response of in vivo and in vitro biological models to mixtures of Polycyclic Aromatic Hydrocarbons"- IX International Symposium on Marine Sciences (2024) - Oral communication

- Matos, Beatriz; Diniz, Mário; Branco, Vasco; Martins, Marta. Genotoxic Effects of PAHs Mixtures in Primary Hepatocytes of the Gilt-Headed Seabream (*Sparus aurata*) - 31st SETAC Europe (2021) - Oral communication

- Matos, Beatriz; Diniz, Mário; Branco, Vasco; Martins, Marta. "An overview of the effects of Polycyclic Aromatic Hydrocarbons (PAHs) mixtures in both *in vivo* and *in vitro* models" - SETAC Europe 34th Annual Meeting (2024) - Poster presentation

- Matos, Beatriz; Diniz, Mário; Branco, Vasco; Martins Marta. "Induction of CYP1A1 by Benzo[a]pyrene and Phenanthrene in zebrafish hepatocytes". SETAC Europe 33rdAnnual Meeting (2023) - Poster presentation

- Matos, Beatriz; Bramatti, Isabella; Diniz, Mário; Branco, Vasco; Martins, Marta. "Cytotoxic effects of PAHs mixtures in immortalized cells of Zebrafish (*Danio rerio*)". SETAC Europe 32nd Annual Meeting (2022) - Poster presentation

3

MARINE FISH PRIMARY HEPATOCYTES ISOLATION AND CULTURE: NEW INSIGHTS TO ENZYMATIC DISSOCIATION PANCREATIN DIGESTION

This chapter is a published manuscript:

Figueiredo, Neusa; Matos, Beatriz; Diniz, Mário; Branco, Vasco; Martins, Marta. "Marine Fish Primary Hepatocyte Isolation and Culture: New Insights to Enzymatic Dissociation Pancreatin Digestion". International Journal of Environmental Research and Public Health 18 4 (2021): 1380. Doi: 10.3390/ijerph18041380

Abstract

Primary cell cultures from wild organisms have been gaining relevance in ecotoxicology as they are considered more sensitive than immortalized cell lines and retain the biochemical pathways found *in vivo*. In this study, the efficacy of two methods for primary hepatocyte cell isolation was compared using liver from two marine fish (*Sparus aurata* and *Psetta maxima*): (i) two-step collagenase perfusion and (ii) pancreatin digestion with modifications. Cell cultures were incubated in L-15 medium at 17 ± 1 ◦C and monitored for up to six days for cell viability and function using the trypan blue exclusion test, MTT test, lactate dehydrogenase (LDH) activity, and ethoxyresorufin O-deethylase (EROD) activity after Benzo[a]Pyrene exposure. The results showed significant differences between the number of viable cells ($p < 0.05$), the highest number being obtained for the pancreatin digestion method (average=4.5 ± 1.9 ×107 cells). Moreover, the hepatocytes showed solid adherence to the culture plate and the rounded shape, changing into a triangular/polygonal shape. The cell viability and function obtained by pancreatin digestion were maintained for five days, and the EROD induction after exposure to the B[a]P showed that cells were metabolically active. This study shows that the optimized pancreatin digestion method is a valid, cost-effective, and simple alternative to the standard perfusion method for the isolation of primary hepatocytes from fish and is suitable for ecotoxicological studies involving marine pollutants, such as PAHs.

Keywords: primary hepatocytes; *in vitro* assays; *Sparus aurata*

3.1 Introduction

Aquatic ecosystems, from coastline to open ocean, are widely impacted by a countless number of xenobiotics (e.g., heavy metals, persistent organic pollutants (POP), pharmaceuticals (e.g., triclosan), endocrine-disrupting chemicals (EDCs), and plastic wastes and their related chemicals (e.g., BPA, phthalates))[173,189,275–282]. The presence of these xenobiotics in both water and sediments eventually results in ecotoxicological effects in aquatic organisms, from algae to fish [173,189,277]. For instance, exposure to polycyclic aromatic hydrocarbons (PAHs), such as Benzo[a]Pyrene (B[a]P), is known to produce carcinogenic effects following bioactivation by cytochrome P450 (CYP) and epoxide hydrolases[278].

Traditionally, in aquatic toxicology, the toxic effect of xenobiotics is mostly evaluated using *in vivo* models (e.g., algae, such as *Pseudokirchneriella subcapitata*; invertebrates, such as chironomids or daphnia; and fish, such as rainbow trout, fathead minnow, stickleback, medaka, or zebrafish)[283]. Besides being highly costly and time-consuming, this approach requires a substantial number of animals[284,285], which creates pressure for the replacement, reducing, and refinement (3Rs principle) of *in vivo* tests, particularly when the research goal is focused on understanding toxicity mechanisms. In this context, in vitro cell cultures offer advantages to the *in vivo* approach, as cells in culture retain the basic characteristics of the *in vivo* conditions[286,287] while allowing the precise control of physicochemical conditions (pH, temperature, osmotic pressure, and O2 and CO2 tension) and physiological conditions (such as hormone and nutrient concentrations) of the experiment, reducing the variability of results[287]. Furthermore, the costs and time expended are greatly diminished, as the maintenance of cell cultures is far less demanding than conducting assays with live fish.

The main limitation to the *in vitro* approach, especially when using immortalized cell lines, is the genetic and phenotypic instability that may introduce variability from one passage to another and the lack of environmental relevance, which compromises the effective extrapolation to whole organisms[288]. However, this can be partially overcome by the usage of freshly isolated primary cultures from species with environmental relevance. These cultures are a mixture of several cell types obtained from a piece of minced or enzyme dispersed tissue, thus retaining the characteristics of their source tissue. In particular, in research using hepatocytes, the maintenance of many *in vivo* characteristics is the most important factor. For instance, Segner and Cravedi[289] found that primary hepatocytes express stable levels of phase

I and phase II enzymes, such as CYP1A1, an important factor for studies of metabolically activated contaminants[253,290–292].

The critical point of using primary hepatocyte cultures is to obtain a viable culture that will survive and be functional long enough for toxicological assessment. The low viability and cell membrane damage associated with simple mechanical or enzymatic digestion methods have discouraged their usage. At the expense of these methods, the collagenase perfusion method became the classic and routinely used method for the isolation of primary hepatocytes by producing a higher number of hepatocytes, which are able to maintain vitality and function for a prolonged period[293]. Two-step collagenase perfusion was originally described by Berry and Friend[293] for the rat liver, but Birnbaum et al.[294] adapted it for the fish liver as well[210]. The authors Baksi and Frazier[287] and Pesonen and Andersson[292] revised the properties and usage of fish primary cell cultures and, even now, a considerable number of studies are still reporting the application of these cultures as a good alternative for the replacement of aquatic organisms [14,19,25–29][253,286,295–299]. However, most of these studies used freshwater species models, and there is a lack of information regarding the isolation and culture conditions for marine fish hepatocytes. Moreover, perfusion requires skillful handling of procedures, namely in the cannulation step, which may undermine the success of the culture.

Therefore, this study aims to optimize an effective and simple procedure for the isolation and maintenance of viable primary hepatocytes from marine fish, which can be used for ecotoxicological assessment of marine pollutants. To achieve this goal, two marine species with ecological and economic relevance, *Sparus aurata* and *Psetta maxima*, were used to isolate primary hepatocytes by two different methods: two-step collagenase perfusion and pancreatin digestion. The viability and functionality of the isolated hepatocytes were assessed under different culture conditions, including 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT), lactate dehydrogenase (LDH), and ethoxyresorufin O-deethylase (EROD) assays.

3.2 Materials and Methods

3.2.1 Fish Acclimatization

The turbot (*Psetta maxima*, Linnaeus, 1758) and gilt-headed seabream (*Sparus aurata*, Linnaeus, 1758) weighing 46.8–75.1 g and 186.7–271.3 g, respectively, were acquired from a local aquaculture farm (Aquinova-Mira and Setúbal, Portugal, respectively). *P. maxima* is a bottom-dwelling marine fish, and *S. aurata* is the most economically important pelagic marine sparid fish species cultured along the Mediterranean coast and has been used in ecotoxicological studies[173,189,300–303]. The fish were transported to the MARLab laboratories at the Department of Environmental Sciences and Engineering (FCT NOVA) and maintained in controlled conditions (17 ± 1 ◦C, 34‰ salinity, with a regular photoperiod set at 12 h light/12 h dark). During this time, the fish were fed with standard commercial chow (as used by the fish supplier) twice a day.

3.2.2 Primary Hepatocyte Cell Isolation

All animals were randomly collected from the tanks, evaluated regarding the general health status, measured for total weight, and irreversibly anesthetized with an aqueous solution of 2-phenoxyethanol (0.25 ppm)[304]. After disinfection with 70% alcohol, a longitudinal incision from the anus to the gills was performed to expose the liver. Internal organs were observed for their general condition, and fish with excessively fat liver or alterations in tissue consistency were excluded from the procedure. The primary hepatocytes were isolated using the two-step collagenase perfusion and pancreatin digestion methods, according to Ferreira et al. [253] and Yanhong et al.[210], respectively, with modifications (Figure 3.1 and Figure 3.2) as subsequently described. In total, 15 fish were used for both procedures.

3.2.2.1 Two-Step Collagenase Perfusion

A cannula (24G) was inserted in the portal vein, and the liver was first perfused for 20 min with solution A (176 mM NaCl, 4.82 mM KCl, 0.44 mM KH2PO4, 3.6 mM NaHCO3, 0.35 mM Na2HPO4.2H2O,10mM HEPES, 5mM Na2-EDTA, pH7.6) at a flow rate of 10 mL min^{-1}.

The second perfusion step was performed for 15 min with solution B (solution A without Na2EDTA, with 2.5 mM CaCl2 and 0.02 mg mL−1 collagenase IV) at the same flowrate (10 mL min^{-1}). Following the two perfusions, the liver was removed and transferred to a chilled dish containing solution C (solution B without collagenase IV and with 1% BSA) for 30 min and mechanically disrupted by using sterile stainless-steel scissors. The liver fragments were then filtered twice through a stainless-steel 200 and 64 μm mesh, and the cells were collected by low-speed centrifugation (100× g, 5 min, 4 ◦C). The cell pellet was washed three times in cooled non supplemented Hank's balanced salt solution (HBSS, Lonza) and then resuspended in HBSS medium supplemented with 5% fetal bovine serum (FBS), penicillin (10 U mL^{-1}), streptomycin (10 μg mL^{-1}), and amphotericin (0.025 μg mL^{-1}). The cell yield was counted in a Neubauer chamber and their viability was examined by the trypan blue exclusion assay. The percentage of viable cells was calculated as (A − B) / A × 100, with A being the average of total cells and B the average of nonviable cells. After opening the abdomen, the liver was: (A) perfused *in situ*, digested with collagenase, and disrupted with stainless-steel forceps; (B) cut into small pieces, clipped into fragments, and digested with pancreatin digestive juice. The digested fragments were filtered through stainless-steel meshes (200 μm and 60 μm), and the cells were collected by low-speed centrifugation (100× g).

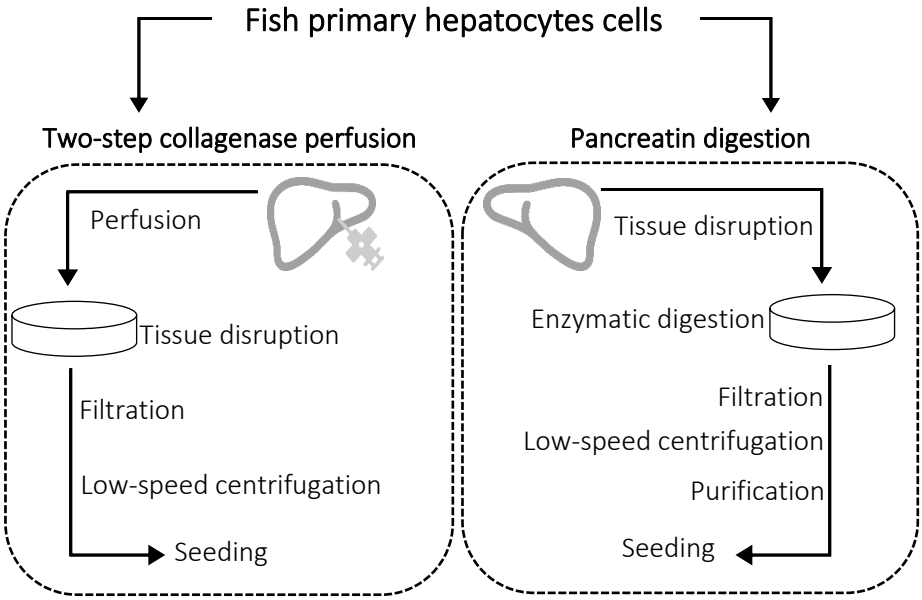


Figure 3.1 - Primary fish hepatocytes isolation methods applied: (A) two-step collagenase perfusion and (B) pancreatin digestion.

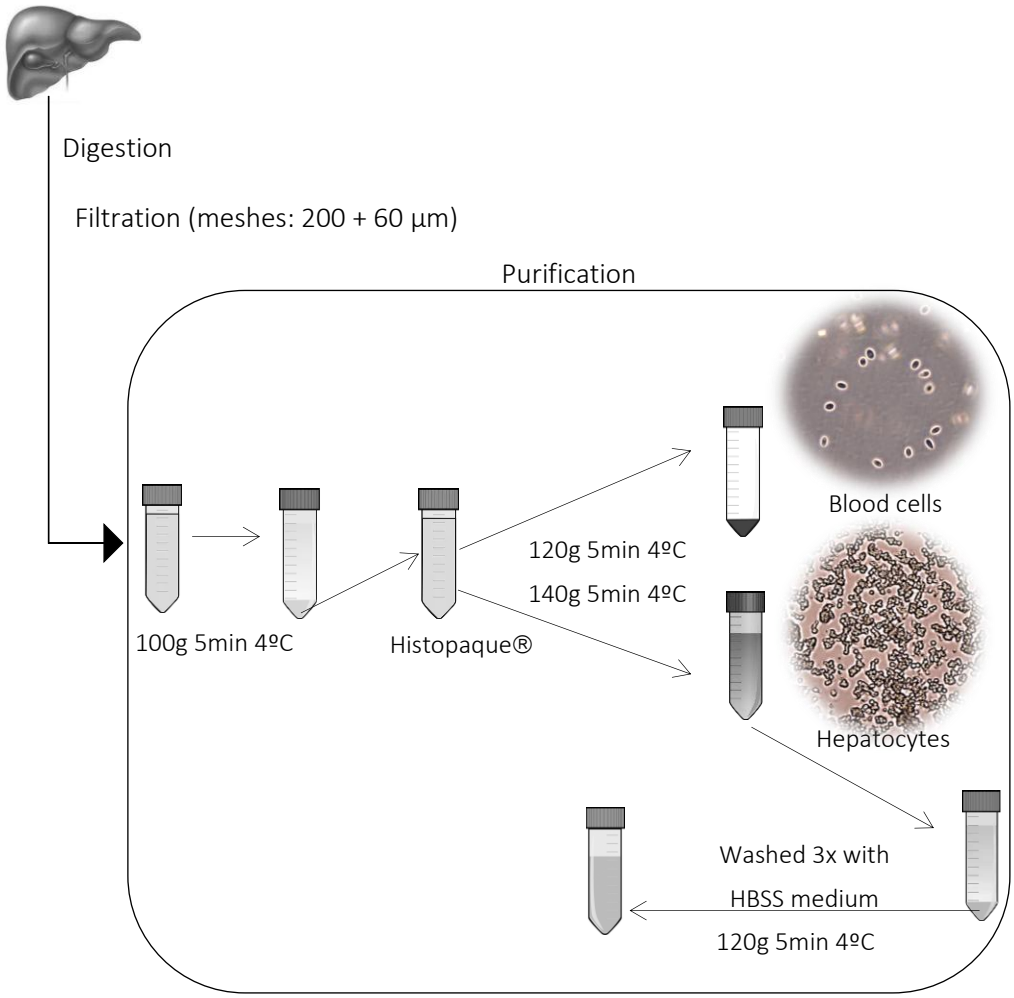


Figure 3.2 - Pancreatin digestion method by Yanhong et al.[24] with modifications to increase culture purity

3.2.2.2 Pancreatin Digestion

The liver was removed from the abdomen, weighed, and placed in a cool dish containing sterile dissection balanced salt solution (DBSS) (126 mM NaCl, 4.82 mM KCl, 1.5 mM KH2PO4,6.1 mM Na2HPO4,2 1.9 mM HEPES, pH7.4). The liver was then cut into 1.0–2.0 mm3 pieces, washed three times with cool sterile DBSS, and clipped into fragments. The preheated (37 ◦C) pancreatin digestive juice (126 mM NaCl, 4.82 mM KCl, 1.5 mM KH2PO4, 6.1 mM Na2HPO4, 0.54 mM Na2-EDTA, 0.1% pancreatin) was added to the dish to digest the fragments for 30 min. During the digestion, the fragments were blown gently with a sterile Pasteur pipette. The digested fragments were then filtered through a stainless-steel mesh (200

μm and 60 μm, respectively) and collected by low-speed centrifugation (100× g, 5 min, and at 4 ◦C). The pellet was purified with Histopaque® (Sigma)[305] (120× g, 5min 4◦C and 140×g, 5min 4◦C). The hepatocytes were collected from the supernatant, washed three times with non-supplemented HBSS, and then resuspended in HBSS medium supplemented with 5% FBS, penicillin (10 U mL^{-1}), streptomycin (10 μg mL^{-1},) and amphotericin (0.025 μg mL^{-1}). The cell yield was counted in a Neubauer chamber, and viability was examined by the trypan blue exclusion assay. The percentage of viable cells was calculated as described in Section 3.2.2.1.

3.2.3 Seeding Fish Primary Hepatocytes

To establish marine fish primary hepatocyte cultures, the isolated hepatocytes showing ≥85% viability[306] were cultured in a complete cell medium consisting of L-15 medium, supplemented with 5% FBS, penicillin (10 U mL^{-1}), streptomycin (10 μg mL^{-1}), and amphotericin (0.025 μg mL^{-1}), in 96-well microplates (10^4 cells/well), 24-well microplates (10^5 cells/well), 6-well microplates (10^6 cells/well), and 35 mm cell culture dish (10^6 cells/well), with a distinct growth surface coating. The growth surface of each type of plate was previously coated with 0.01% poly-L-lysine solution (Sigma-Aldrich) and Biocoat Collagen I (Corning). In parallel, plates without treatment were used as controls. The cell cultures were allowed to attach for 24 h (hereafter defined as T0 cells) and maintained in an incubator at 17 ± 1 ◦C (the fish optimal growth temperatures). The medium was changed every 2 days.

3.2.4 Monitoring of Fish Primary Hepatocyte Cultures: Morphology, Viability, and Functionality

This assessment was performed after 24 (T24), 48 (T48), 72 (T72), 96 (T96), and 144 h (T144) (6 days) of cell attachment (T0), depending on the endpoint.

3.2.4.1 Cell Morphology

Cultured hepatocytes in 35 mm dish cultures were monitored daily under inverted light microscopy (Olympus) (10× and 20× objectives) to observe the morphological changes over time (i.e., from single rounded cells to aggregate polyhedral morphology typical of hepatocytes)[307].

3.2.4.2 Cell Attachment and Viability

Cell attachment and viability were assessed at T24. Briefly, after the removal of culture media from culture plates (0.01% poly-L lysine, collagen I, and without coating), the primary hepatocytes were washed with sterile 1× PBS and detached with trypsin (0.25% trypsin–EDTA, Sigma). Cell detachment was observed under the microscope and an aliquot (50 μL) of the cell suspension was taken, and cells were counted in a Neubauer chamber by the trypan blue exclusion assay. Cell viability was expressed relative to the T0 cell number.

3.2.4.3 Cell Viability by MTT

The MTT assay was performed using cell cultures at T0, T24, T48, T72, T96, and T144 and according to the methodology described by Mosmann and modified by Carvalho et al. [38,39]. Briefly, the MTT solution (at a final concentration of 400 μg mL^{-1} per well) was added to 96-well plates prepared previously with 10^4 cells/well followed by incubation at 16 or 18 ◦C (depending on fish species) for 4h. Following the incubation period, the medium with the MTT was removed and the formazan crystals dissolved using a 150 μL/well 4:1 mixture of dimethyl sulfoxide (DMSO)/glycine buffer (50 mM glycine, 50 mM sodium chloride/NaOH, pH 10.5) by shaking for 20 min at 200 rpm. The absorbance was measured at 550 nm on a microplate reader. Cell viability was expressed as the percentage of T0 cells.

3.2.4.4 Detection of LDH in the Supernatant

Lactate dehydrogenase (LDH) release was used as an indicator of hepatocyte mortality. This assay was performed using the culture medium collected at T0, T24, T48, T72, T96, and T114. After the collection of the medium from 24-well plates previously cultured with 105 cells/well, the medium was centrifuged at 4 ◦C for 10 min (12,000 g) to remove floating cells. The LDH activity was measured using the Pierce LDH cytotoxicity assay kit (Thermo Scientific, USA), according to the manufacturer's instructions. The absorbance of each sample was read at 490 nm and 680 nm using a microplate reader. A positive LDH control (from the kit) and negative control (L-15 medium without cells) controls were run in parallel.

3.2.4.5 Ethoxyresorufin O-Deethylase (EROD) Activity

EROD (CYP1A) activity was measured according to the method adopted for the 24-well plate described by Ferreira et al.[19]. Briefly, after 24 h and 48 h of exposure to 0.1 μM, 0.5 μM, and 1 μMB[a]P, the medium was removed, and the cells washed once with cold PBS. EROD activity was measured by adding a EROD reaction mixture containing 1 mg/mL BSA, 5 μM7-ethoxyresorufin (7-ER), and 0.5 mM NADPH and incubated for 30 min in the dark at 37±1◦C. Fluorescence was measured at 560nm excitation and 610nm emission. EROD activity was calculated based on the resorufin standard curve obtained for each independent experiment. After EROD measurement, total protein concentration was determined in the same well by the Bradford method with BSA as a standard.

3.2.5 Data Analysis

Statistical significance of results was evaluated with the software Statistica 8.0 Statsoft, 2007 (Tulsa, USA) by applying the nonparametric Mann–Whitney U test to compare two independent variables or the parametric t-test for independent variables, depending on the observation of parametric test assumptions, i.e., normality of distribution (Shapiro–Wilk's test) and homogeneity of variances (Levene's test). All data were expressed as means ± SE of more than three independent experiments, and statistical differences were established as significant at $p < 0.05$ and very significant $p < 0.01$.

3.3 Results

3.3.1 Cell Yield and Viability

The cell yield obtained from *S. aurata* liver ranged from 1.04 × 10^7 to 2.47 × 10^7 for the two-step collagenase perfusion method and 3.3 × 10^7 to 7.31 × 10^7 for the pancreatin digestion method (Table 3.1), and the cell viability ranged from 70.7 to 72.8% for the two-step collagenase perfusion method and 95.1% to 100% for the pancreatin digestion method (Table 3.1). The cell yield obtained for *P. maxima* liver by pancreatin digestion ranged from 1.08 × 10^7 to 3.44 × 10^7 (Table 3.1). The small size of livers (average = 0.47 ± 0.19 g) made it difficult to perform the two-step collagenase perfusion method in this species.

Table 3.1 - Total number of primary hepatocyte cells (range and average) isolated from the liver of *S. aurata* and *P. maxima* fish obtained by different cell isolation methods, two-step collagenase perfusion and pancreatin digestion, and their respective cell viability (%).

Method	Fish Species	Weight (g)		[1] Number of Cells (x 10^7)	% of Viability
		Fish	Liver		
Two-step perfusion	*Sparus aurata*	234 ± 43.3	4.80 ± 1.66	1.04–2.47 x =1.91 ± 0.77 [a]	70.7-72.8
Pancreatin digestion	*Sparus aurata*	236 ± 41.2	4.47 ± 1.37	3.3–7.31 x =4.5 ±1.9 [b]	95.1 - 100
	Psetta maxima	62.5 ± 8.57	0.47 ± 0.19	1.08–3.44 x =1.97 ± 0.76 [c]	100

[1]Range of cell yields and the respective average of 3 a, 4 b, and 8 c independent assays.

The recommended work viability is ≥85%[36]. For pancreatin digestion, the viability was ≥95%, which is in good agreement with other results (>98.4%)[24]. This high viability is related to the lower concentration of pancreatin used, which should cause fewer injuries to isolated hepatocytes[24]. The viability achieved with two-step collagenase perfusion was below 85% (max 73%), which is likely related to the long-time involved in the cannulation procedure and also needs accurate technical skills. Based on the viability and cell yield results, we proceeded with the pancreatin digestion method in *S. aurata* in subsequent experiments.

Cell Culture Purity

The presence of blood cells and tissue debris was observed in the first extractions with the pancreatin digestion method. To improve the culture purity, we applied some modifications to the original pancreatin digestion method described by Yanhong et al.[24]. As mentioned in Section 2.2.2, these modifications included the usage of two meshes (200 and 60 μm) during the filtration process and two centrifugation steps with Histopaque® (Figure 3.2). The usage of these two meshes improved the removal of tissue debris, and the two subsequent centrifugations (120× g and 140× g, 5 min at 4 ◦C) with Histopaque® improved the removal of blood cells from the cell culture (Figure 3.2). The presence of blood cells in the resulting culture is a critical point. In the two-step perfusion method, the perfusion step aims to clean up the liver from these cells. However, several authors have suggested the low-speed centrifugation with Percoll gradient as an alternative to guarantee a pure culture of hepatocytes[24,40,41]. Taking this into account, we combined enzymatic-based dissociation with the Percoll gradient purification, using Histopaque® to obtain a high-purity culture. Histopaque® is a solution of polysucrose and sodium diatrizoate, adjusted to a density of 1.077 g/mL, which is commonly used in isolation and purification of blood cells[35,42]. This proved to be an adequate substitute for the perfusion method, greatly simplifying procedures. In most cases, after this purification process, the hepatocyte culture purity was 100%, as determined by microscopic observation. The three final washing steps further improved the removal of dead cells.

3.3.2 Cell Culture Condition: Morphology, Viability and Function

The marine fish primary hepatocyte cultures obtained by the optimized pancreatin digestion method are presented in Figure 3.3. The culture shows ≥85% viability and 100% hepatocyte purity. The monitoring data at T0 to T144 showed that the cells were attached to a plate coated with the poly-L lysine and collagenase I coated-plate.

Immediately upon isolation, hepatocytes were seen as single separated cells, which were showing a rounded shape (Figure 3.3A). Twenty-four hours after isolation, and after 24h to allow cell attachment, this initial rounded shape was changed into a triangular/polygons shape, some hepatocytes began to flatten, and the cell–cell contact was evident (Figure 3.3B). After 48 h in culture, cells were clustering into small groups (Figure 3.3C), and larger clusters were observed after 72 h, with the subsequent loss of the initial rounded appearance as the boundaries between the cells increased (Figure 3.3D).

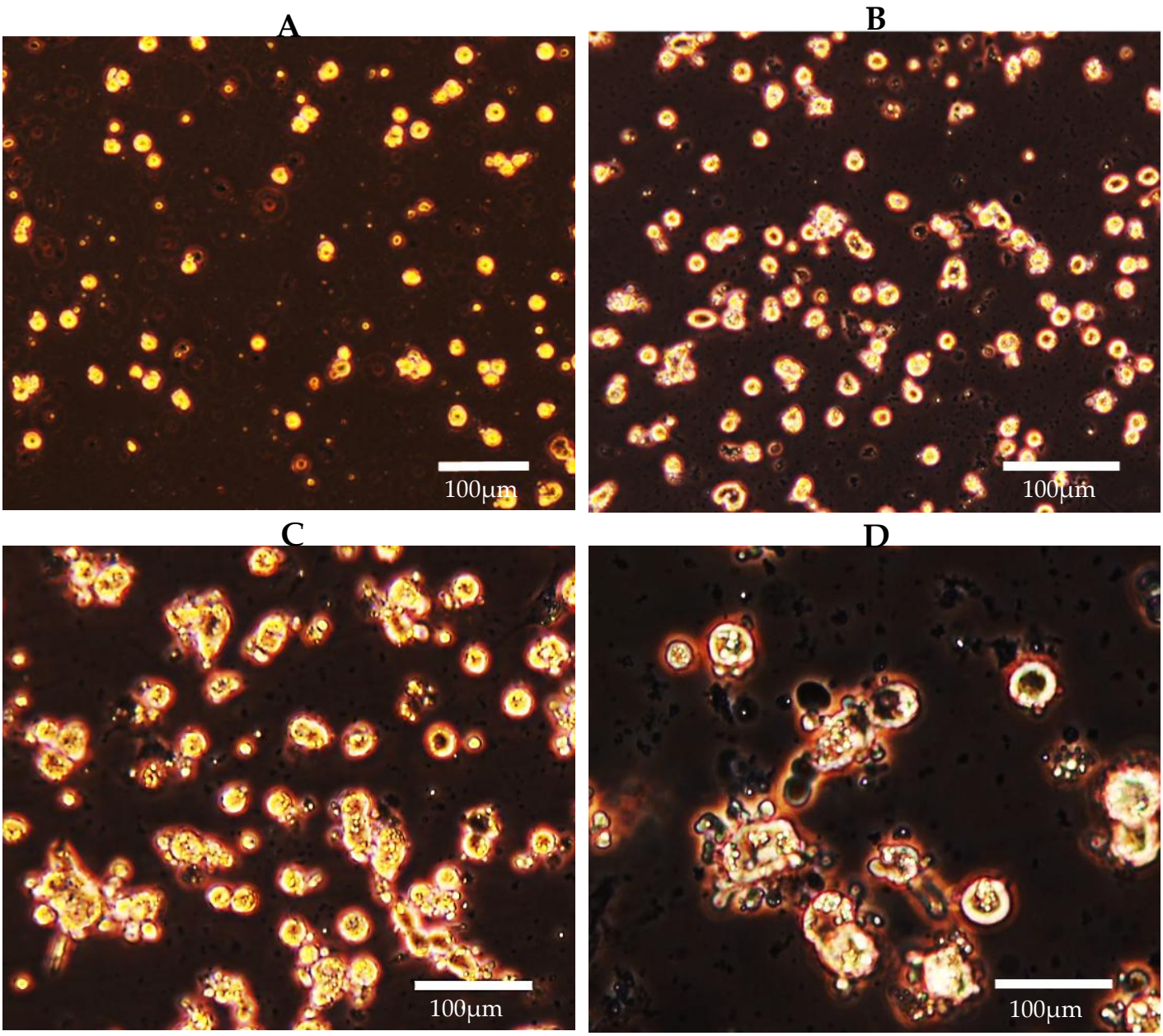


Figure 3.3 - Culture of *S. aurata* primary hepatocytes isolated by pancreatin digestion with modifications (A) and after incubation in poly-L lysine-coated plates up to 24 (B), 48 (C), and 72 h (D). The observations were made under inverted light microscopy (Olympus) with a 10× objective (A,B) and a 20× objective (C,D).

Primary hepatocytes' viability was assessed by the trypan blue exclusion and MTT assays. The trypan blue exclusion was performed at T24, and the results showed cell viability of 94.8 ± 14.1% for the collagen I plate and 98.1 ± 11.5 for the poly-L lysine plate (Table 3.2). The MTT viability test was performed at T24, T48, and T72. Comparing the results for plates coated with collagen I, the viability decreased slightly, reaching a minimum of the viability of 78.7 ± 0.47% at T72 (Table 3.2). Interestingly, no significant differences in cell viability were

obtained when comparing the manual poly-L-lysine coating and commercial collagen I coating plates (Table 3.2).

Table 3.2 - The viability of primary hepatocytes isolated by pancreatin digestion after T24, T48, and T72 in culture with different plates, no-treatment, and coated with the poly-L-lysine solution (Sigma) and collagen I (Sigma). Cell viability was evaluated by trypan blue assay (TBA) and MTT. All the data are expressed as % of T0 and are expressed as the mean ± SD of more than 3 independent isolations. Significant differences from the T0 was observed for the plates coated; * $p < 0.05$.

Viability Tests	**Plate Coating**	
	Plate Coated w/ Poly-L Lysine	**Plate Coated w/ Collagen I**
TBA (24h)	98.1 ± 11.5	94.6 ± 14.1
LDH (24h)	97.5 ± 3.4	95.5 ± 4.9
	MTT	
24h	84.1 ± 8.13*	91.8 ± 2.73
48h	82.7 ± 4.157*	89.0 ± 2.93*
72h	82.7 ± 8.15*	78.7 ± 0.47*

The assessment of LDH release was performed in cell culture media and collected after T24, T48, and T144. Analysis of the culture supernatants showed that LDH secretion increased gradually over time, with a peak at T144 (Figure 3.4).

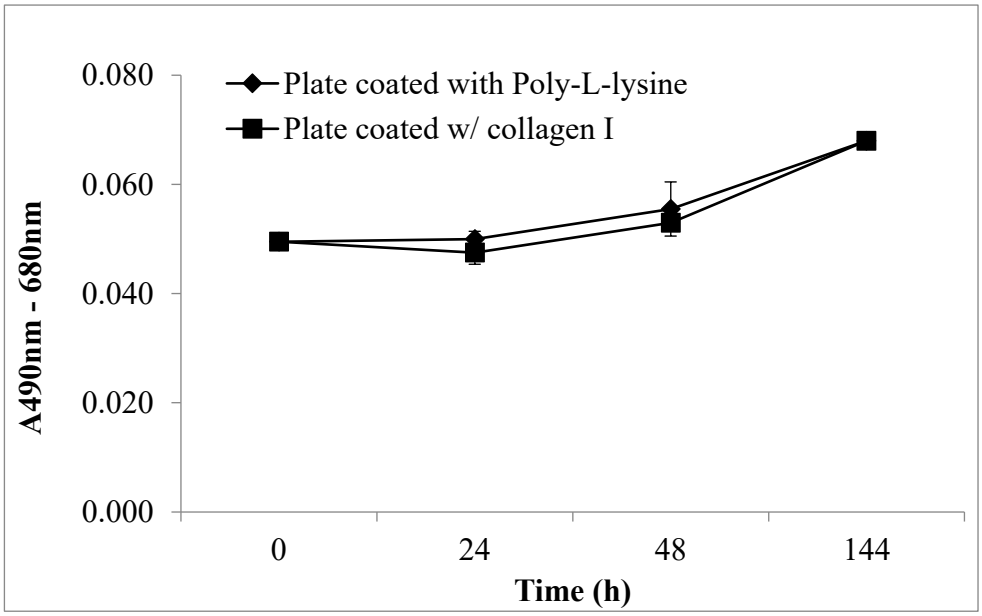


Figure 3.4 - Lactate dehydrogenase (LDH) released by *S. aurata* primary hepatocytes cultured in a 24well plate coated with poly-L-lysine and collagen I. After the overnight incubation of 105 cells/well, the L-15 medium was collected at 24, 48, and 144 h to assess LDH activity. A significant difference was observed between the control and T144; ** $p < 0.01$.

To assess cellular function, namely xenobiotic phase I metabolism, primary hepatocytes of *S. aurata* cultured on the poly-L-lysine plates were exposed to 0.1, 0.5, and 1 μM of B[a]P to assess CYPA1 activity via EROD-based assay (Figure 3.5). The results showed a significant increase of EROD activity at T24 and T48, relative to control cells ($p < 0.05$).

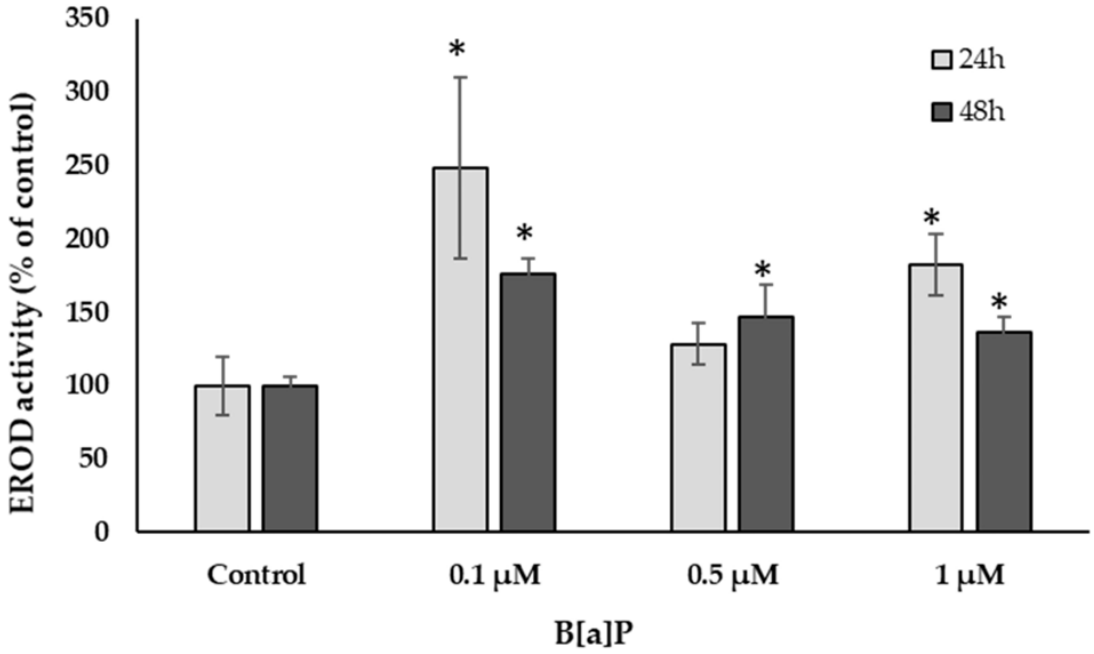


Figure 3.5 - EROD activity of *S. aurata* primary hepatocytes exposed to different concentrations of Benzo(a)pyrene (B[a]P) for 24 and 48 h. Ethoxyresorufin O-Deethylase (EROD) activity is expressed relative to control cells (DMSO). Values are mean ± SE of 4 to 5 independent extractions. A significant difference was observed between the control and T24 and T48; * $p < 0.05$.

3.4 Discussion

The use of primary cells carries several challenges, including (1) the process of cell isolation, (2) the maintenance of viable and functional cells in culture, and (3) reproducibility[308,309]. Among these, the most critical challenge is to have an optimized isolation

method adjusted to the demands of the investigation. Among the available methodologies, two methods were used to obtain primary hepatocytes culture from marine fish: the two step collagenase perfusion and a common enzymatic-based dissociation by a pancreatic enzyme. Two-step collagenase perfusion combines the perfusion technique with the dissociation action of collagenase and has been successfully used to isolate primary hepatocytes for toxicological evaluation of aquatic xenobiotics purposes[253,286,310–318].

Although enzymatic dissociation methods are not so commonly used, the pancreatin digestion method described in the present research (Figure 3.1 and Figure 3.2) proved to be effective to isolate marine fish primary hepatocytes in terms of (1) its applicability for small/medium/large fish, (2) low cost and simple procedure, and (3) high cell yield and viability achieved (Table 3.1). In comparison with the two-step collagenase perfusion method, the pancreatin method proved to be very versatile, being suitable for extracting cells from smaller livers where it is difficult or impossible to apply the liver cannulation procedure, required for the two-step collagenase perfusion method. Enzymatic digestion is in general a better alternative when the model in the study is small, such as the case of zebrafish[319].

Besides these advantages, pancreatin digestion also proved to be more effective in obtaining a significantly higher number of viable cells in comparison with those obtained by the two-step collagenase perfusion method (Table 3.1). Using healthy *S. aurata* livers of a similar weight ($p > 0.05$), the number of viable cells obtained by pancreatin digestion was upto four-fold higher than by collagenase perfusion. Additionally, the percentage of viable cells extracted using pancreatin digestion was 30% higher than with the two-step collagenase perfusion method. This is extremely important since obtaining a high number of viable cells is the first critical point of using primary cells. After isolation, primary cells must remain viable and functional long enough to allow successive experiments. Thus, the viability of the isolated primary hepatocytes was monitored over two to six days in culture by several tests (trypan blue exclusion test, MTT assay, and LDH release). All these assays showed a similar result (Table 3.2); however, MTT and LDH data had a smaller standard error, suggesting that these tests may be a more reproducible method than trypan blue exclusion by eliminating error associated with cell counting. In general, until 72 h in culture (three days after the isolation), the viability of primary hepatocytes plated in coated plates was on average >80% (Table 3.2). This suggests that when seeded in coated plates, either with the commercial collagen or manually with poly-L lysine, after the initial attachment, the majority of cells remain viable and stable. Furthermore, the results of the LDH release test, used to assess cell death, showed

that the cells remain viable and functional for six days. Indeed, a significant increase in cell death ($p < 0.01$) was only observable after six days in culture (Figure 3.4). This result agrees with other studies, which showed a viability >85% after the first five days in culture and a significant decline for longer periods[286].

Freshwater species (such as *Oncorhynchus mykiss*, *Cyprinus carpio*, *Oreochromis niloticus Pelteobagrus fulvidraco*, and *Ctenopharyngodon idellus*) are the most used fish models to assess the mode of action of environmental pollutants[286,310,311,313,316,320–324]. However, in the context of marine environmental toxicology, marine fish primary hepatocytes are an interesting approach when assessing the effects of xenobiotics, such as polycyclic aromatic hydrocarbons(PAHs), since it increases the ecological and economic relevance to the studies. Among PAHs, B[a]P is a five-ring PAH that can be found in aquatic environments[280] and is a potent CYP1A1 inducer[253,278,300,325]. The CYP1A mediated activation of B[a]P produces DNA reactive intermediates, such as B[a]P-7,8-diol-9,10-epoxide, which can form stable DNA adducts[278,325] .

Several studies have already demonstrated the toxicological response of *S. aurata* towards B[a]P exposure [300–303], including the induction of EROD activity in fish both for *in vivo* and *in vitro* models[253,278,300,325,326]. To verify the metabolic activity of primary hepatocytes obtained from *S. aurata* liver by the pancreatin digestion method, the EROD based assay was used after cell exposure to B[a]P. In fact, after 24 and 48 h of exposure to three different concentrations of B[a]P (0.1, 0.5, and 1 μM), EROD activity increased significantly relative to control cells, showing that *S. aurata* primary cells were active and able to metabolize this xenobiotic by liver cytochrome P450 dependent-monooxygenases.

A nondependent dose–response and higher EROD activity being obtained at the lowest B[a]P concentration tested was also observed. This pattern has already been verified by Wessel et al. [326], who reported similar results for sole hepatocytes exposed to 0.1, 0.5, 1, 5, and 25 μM B[a]P concentrations. In contrast, Ferreira et al.[253], who used primary hepatocytes isolated from *Dicentrarchus labrax,* reported a dose–response result for EROD activity with a peak at a 1 μM B[a]P concentration (maximum studied concentration). However, the CYP1A1 response of fish to contaminants is known to be species specific[326]. Comparing the two exposure times, EROD activity was higher after 24 h of exposure than 48 h. This observation is in line with previous studies that reported a peak of EROD activity after 24 h of exposure of *S. aurata* treated with 20 mg/Kg of intraperitoneal B[a]P injections [300].

3.5 Conclusions

This study presented an optimized methodology for the isolation of marine fish primary hepatocytes, which can be maintained viable and functional for up to five days, an essential feature for using primary cultures in ecotoxicity testing. This optimized protocol for pancreatin digestion, besides being a good alternative to the commonly used perfusion method, proved to be a suitable method to be used in future ecotoxicological studies involving marine pollutants, such as PAHs. This is particularly relevant in the present-day context, where compound mixtures in the aquatic environment are abundant, therefore demanding fast and thorough analysis of toxicity mechanisms, using alternatives to *in vivo* models as stated in the 3Rs principle.

Author Contributions: Conceptualization, N.F. and M.M.; formal analysis, N.F., B.M., V.B., and M.M.; funding acquisition, M.M.; investigation, N.F. and B.M.; methodology, N.F., B.M., and M.D.; supervision, V.B. and M.M.; writing—original draft preparation, N.F.; writing—review and editing, N.F., B.M., V.B., M.D., and M.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board 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 are authors of this manuscript (M.M. and M.D.). Housing of fish took place in certified

fish facilities approved by the apropriate Portuguese authority, Direção Geral da Alimentação e Veterinária (ref: 0421/000/000/2013).

Informed Consent Statement: Not applicable. Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

4

INTERACTION OF POLYCYCLIC AROMATIC HYDROCARBON COMPOUNDS IN FISH PRIMARY HEPATOCYTES: FROM MOLECULAR MECHANISMS TO GENOTOXIC EFFECTS

This chapter is a published manuscript:

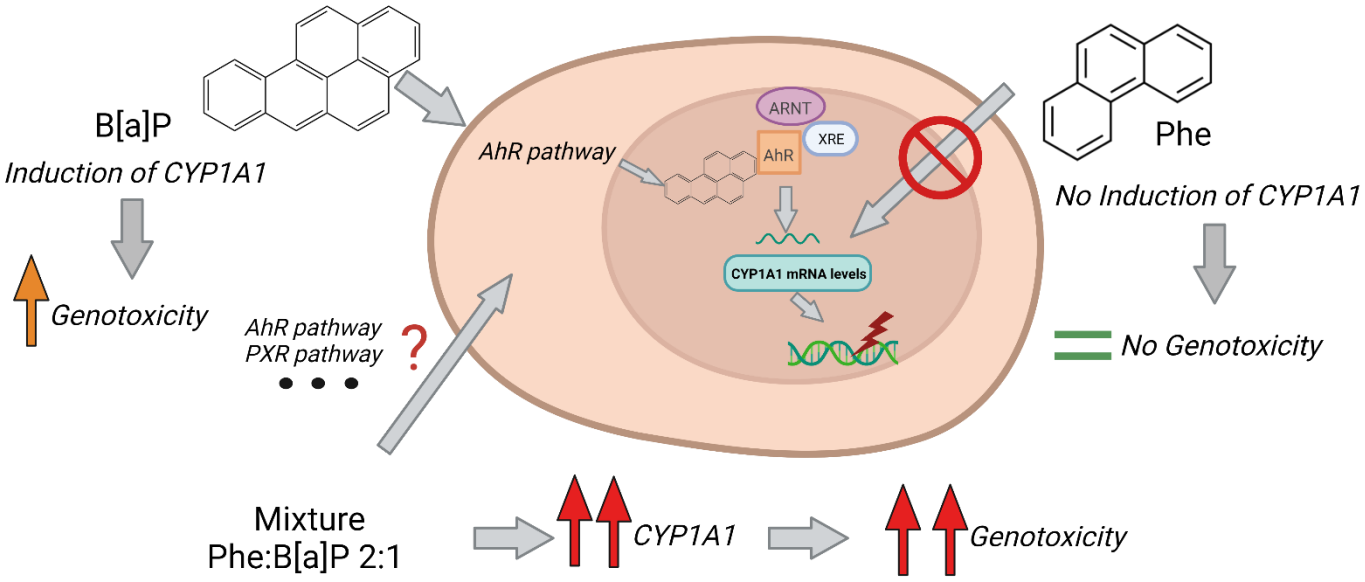
Bramatti, Isabella*; Matos, Beatriz*; Figueiredo, Neusa; Pousão-Ferreira, Pedro; Branco, Vasco; Martins, Marta. "Interaction of Polycyclic Aromatic Hydrocarbon compounds in fish primary hepatocytes: From molecular mechanisms to genotoxic effects". Science of The Total Environment (2022): 158783. Doi:10.1016/j.scitotenv.2022.158783

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 analysed: 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[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.

Keywords: CYP1A1, DNA strand breakage, Phenanthrene, Benzo[a]pyrene, Benzo[b]fluoranthene

Graphical Abstract



4.1 Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are carbon and hydrogen compounds resulting from the fusion of 2 or more aromatic rings[178] during the incomplete combustion of organic matter, mainly due to fossil fuel combustion[327]. Many PAH compounds are procarcinogenic and promutagenic, thus requiring hepatic biotransformation to form reactive metabolites and exert toxicity [178,328–331]. It is during phase I transformation, namely by cytochrome P450 monooxygenase system that those reactive metabolites are formed such as diolepoxides which can covalently bind to the DNA to form adducts[162]. 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 [332]. Phase II reactions involve the conjugation of PAH metabolites, with glutathione to produce water-soluble conjugates that are easily excreted by organisms [333]. The enzymes involved in this conjugation are, for instance, the Glutathione-S-Transferases [162,334]. At a molecular level, PAHs induce the production of reactive oxygen species (ROS) affecting macromolecules such as lipids, proteins, and nucleic acids [158], which may culminate in inflammation and cell death [158].

Various epidemiological and toxicological studies [335,336] 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 [178]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 [178]. 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 [337] 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.

4.2 Materials and Methods

4.2.1 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 %).

4.2.2 Primary hepatocyte isolation and culture

Primary hepatocytes of *Sparus aurata* were isolated with the Pancreatin Digestion method as described by Figueiredo et al (2021) [337]. 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 KH2PO4, 6.1mM Na2HPO4, 0.54 mM Na2-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).

4.2.3 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.

4.2.4 Total protein concentrations

The total protein concentration was determined in cell lysates, using the Bradford method (Bradford, 1976)[338]. 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.

4.2.5 CYP1A1 activity determined by EROD assay

EROD activity was measured as described by Ferreira et al. [253]. 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 4.2.4).

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 [339]. 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).

4.2.7 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 µLofE. 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-ΔΔCT method [340] using GAPDH as the housekeeping gene. Primers were designed with Primer-blast using Reference Sequences from GeneBank (Table 4.1). Primer efficiency was tested with a dilution in series (1:10–1:1000000) of the cDNA template (Table 4.1) 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).

Table 4.1 - 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. aurata* 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	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′		

4.2.8 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- 50µM) 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)[278]. 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) [148,278].

4.2.9 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 $p < 0.01$.

4.3 Results

4.3.1 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 (Figure 4.1) The viability of negative control cells and solvent control cells (DMSO) was not significantly different ($p > 0.05$). Exposure to 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 Figure 4.1B ($p < 0.05$). 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.

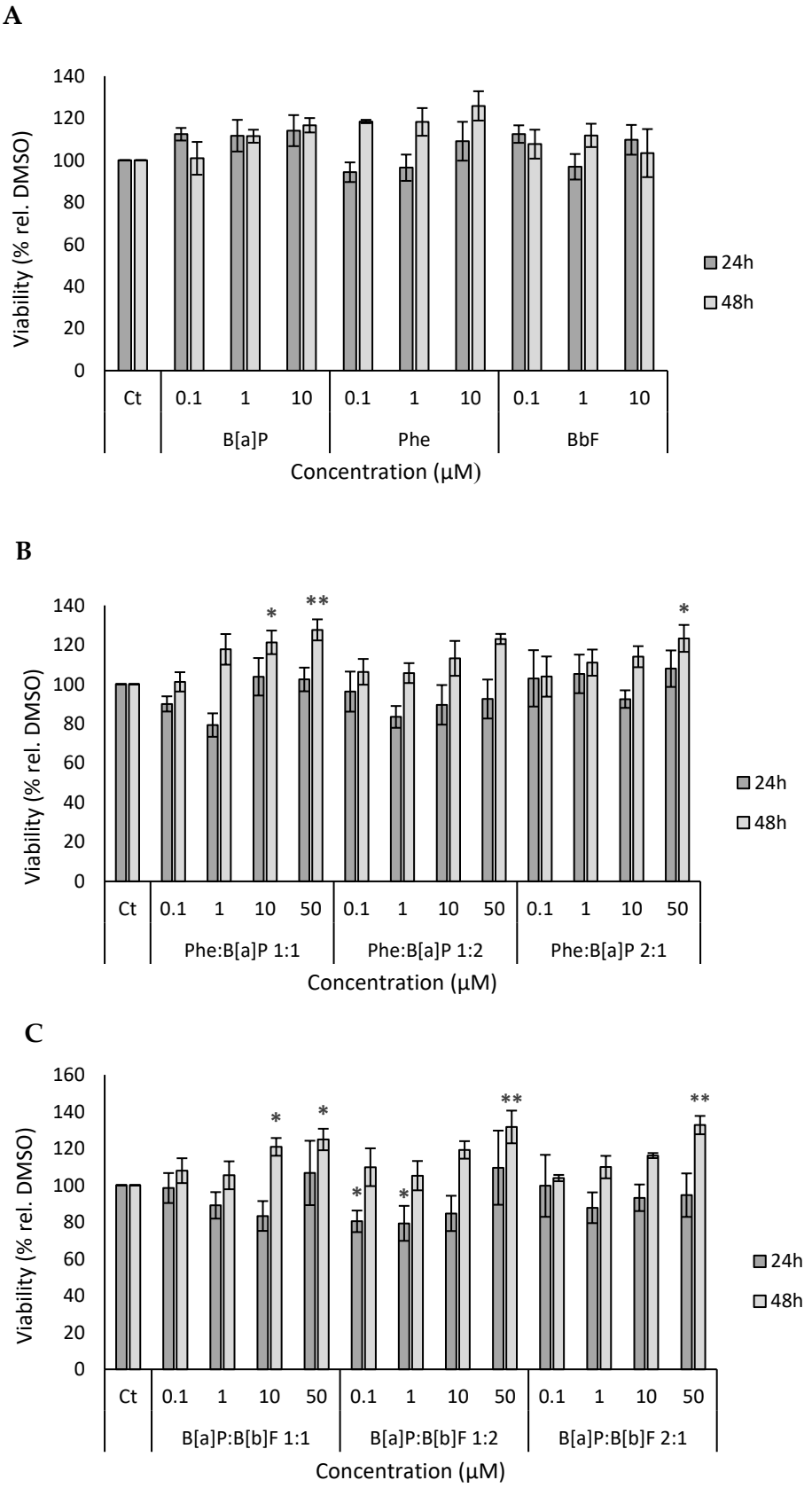


Figure 4.1 - Cell viability (% rel. DMSO) performed by MTS assay, indicating the percentage of metabolically active cells. Cells were exposed to B[a]P, Phe, B[b]F (A) (0.1, 1 and 10 µM) or their mixtures (B, C) (total PAH = 0.1, 1, 10 and 50 µM). Data (mean ± S.E.) from 3 to 6 independent experiments. Ct represents Control Group. *significantly different from control ($p < 0.05$); ** very significantly different from control ($p < 0.01$).

4.3.2 EROD assay

The results for the EROD assay (Figure 4.2) showed a significant increase of activity after 24 ($p < 0.01$) and 48 ($p < 0.05$) hours of exposure to B[a]P 0.1μM and a decrease of the activity with B[a]P 10 μM($p < 0.05$). Phenanthrene exposure demonstrated a decrease at CYP1A1 activity after 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.

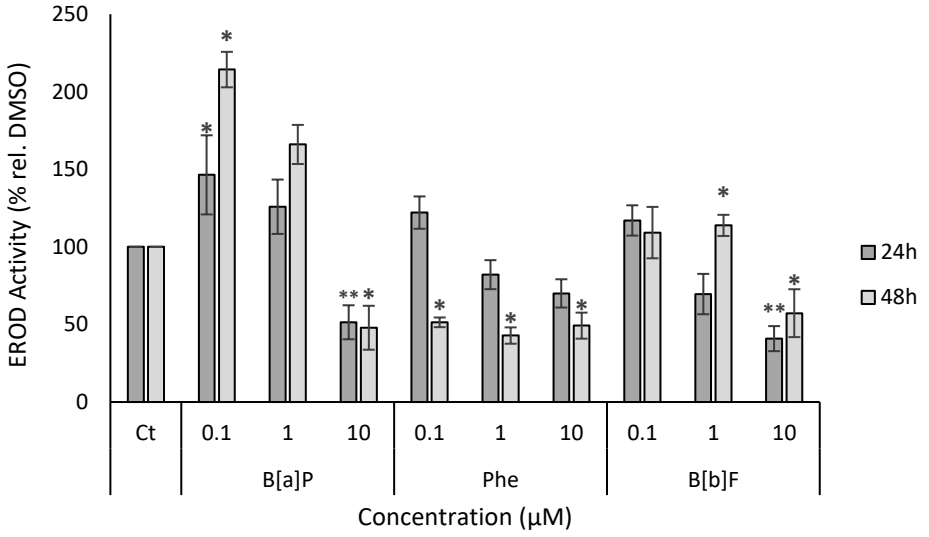


Figure 4.2 - EROD activity (% rel. DMSO) of primary hepatocytes of *S. aurata* after 24 and 48 h of exposure. Cells were exposed to B[a]P, Phe, B[b]F (total PAH = 0.1, 1 and 10 μM). Data (mean ± S.E.) from least 3 independent experiments. Ct - Control. *significant difference versus control ($p < 0.05$); ** very significant difference versus control ($p < 0.01$).

4.3.3 CYP1A1 protein levels

Figure 4.3 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.

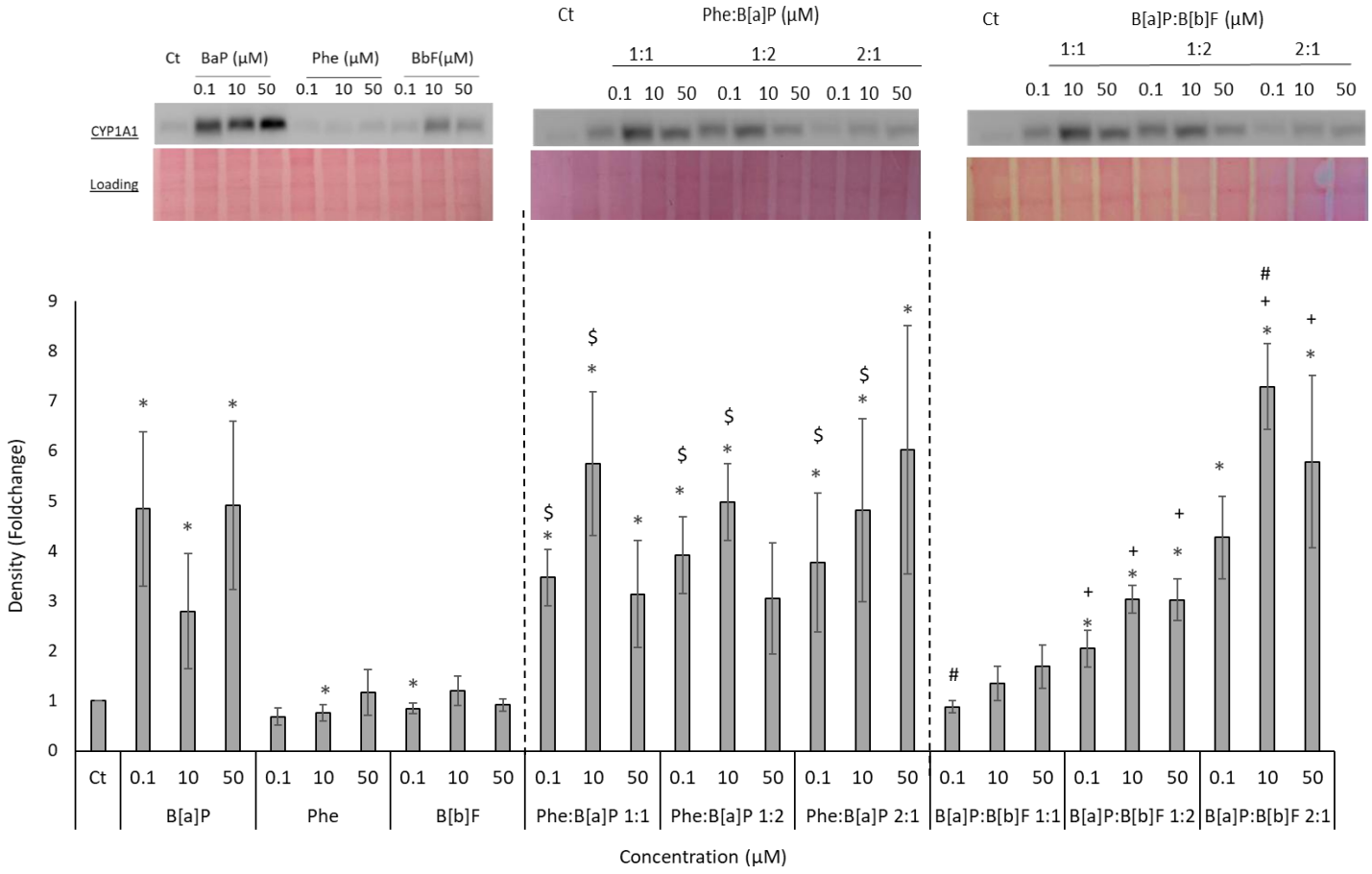


Figure 4.3 - Expression of CYP1A1 by Western blot after 24 h of exposure in primary hepatocytes of *S. aurata* exposed to B[a]P, Phe, B[b]F and mixtures (tPAH = 0.1, 10 and 50 µM). Ct - Control. Data (mean ± S.E) from 3 to 6 independent experiments. *significant difference vs. control ($p < 0.05$). #significant 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 ($p < 0.05$).

4.3.4 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 (Figure 4.4A) 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 (Figure 4.4B). 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 (Figure 4.4C). 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.

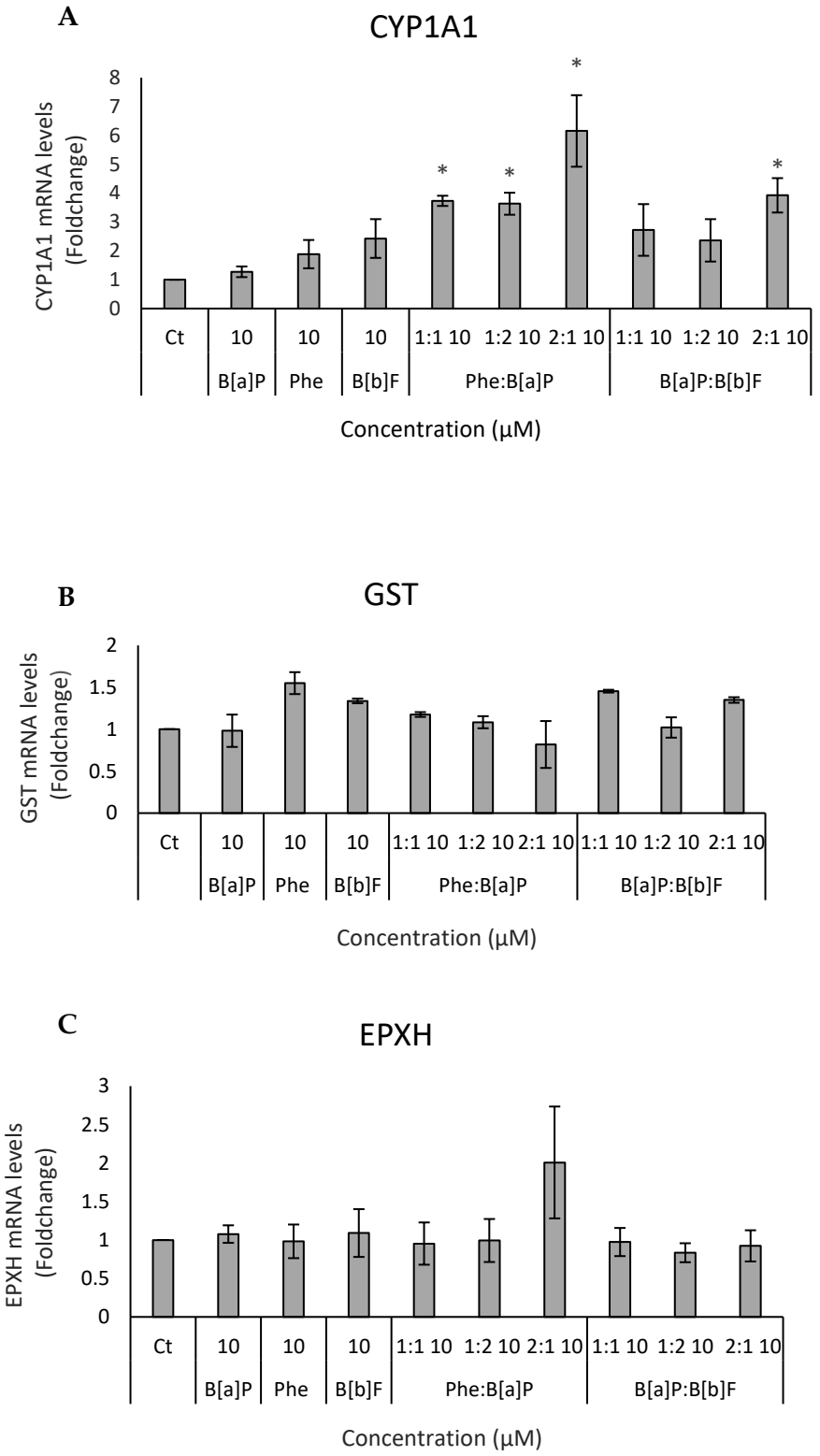


Figure 4.4 - mRNA levels for CYP1A1 (A), GST-3 (B) and EPXH1 (C) after 24 h of exposure to B[a]P, Phe, B[b]F or PAH mixtures (tPAH = 10 μM). Data (mean ± S.E) from at least 3 independent experiments. Ct - Control. *significant difference vs. control ($p < 0.05$).

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4.3.5 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 (Figure 4.5A). 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 % (Figure 4.5B). As seen in Figure 4.5C, the mixture B[a]P:B[b]F increase the % of DNA damage in all ratios after 48 h with the exception of 1:1 0.1 μM. ($p < 0.05$), and that increase appears to be dose dependent.

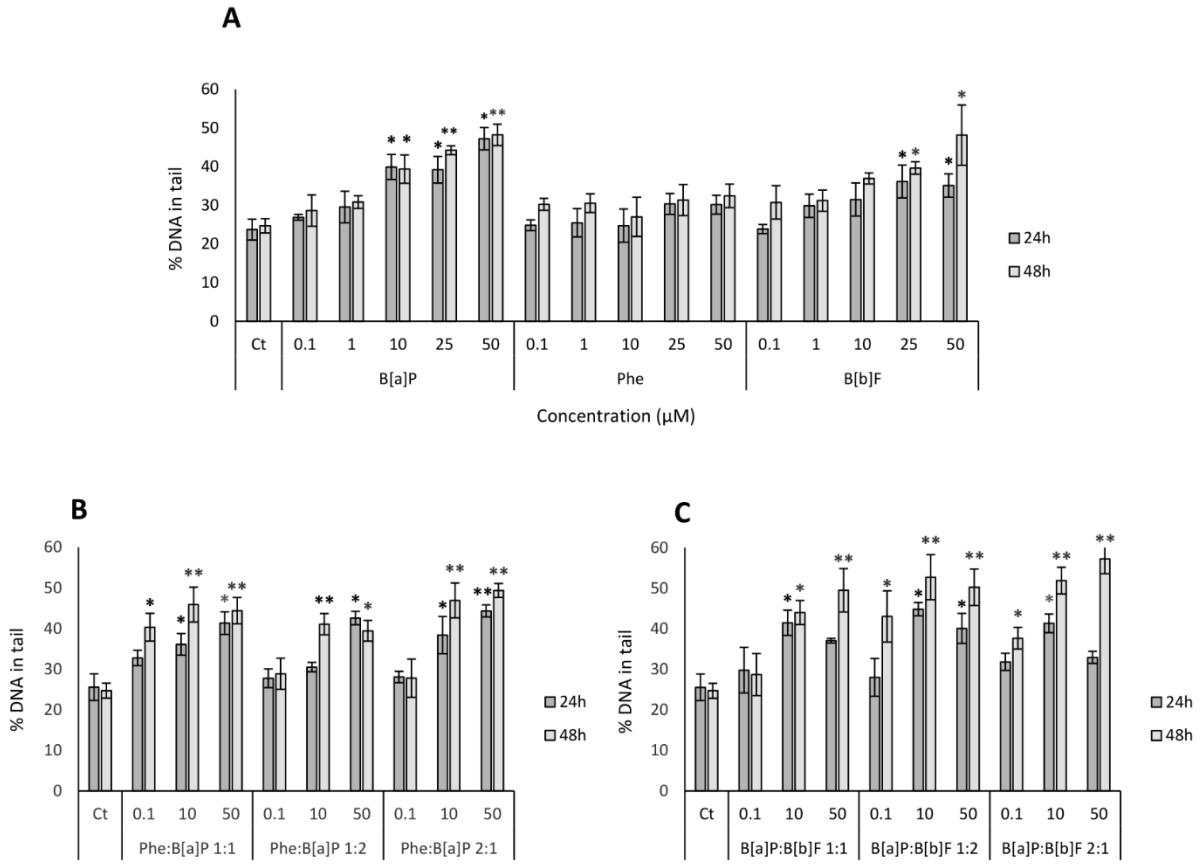

Figure 4.5 - Percentage (%) of DNA in tail of primary hepatocytes of *S. aurata* after 24 and 48 h of exposure. Cells were exposed to B[a]P, Phe, B[b]F (A) or their mixtures (B, C) (total PAH = 0.1, 10 and 50 μM). Data (mean ± S.E) of 3 to 6 independent experiments. Ct - Control Group. *significant difference vs. control ($p < 0.05$); ** very significant difference vs. control ($p < 0.01$).

4.4 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 (Figure 4.1). 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 [178]. 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). (Figure 4.2). In line with our results, the work by Kienzler et al. (2012) [341] 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 [173]. 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 (Figure 4.3). 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 [342]. Interestingly, we did not observe an increase in CYP1A1 mRNA levels, contrary to reported elsewhere [343–345]. Activation of B[a]P generates reactive epoxides which can form adducts with DNA [346] which explains the high degree of DNA strand breakage observed (Figure 4.5A). B[b]F can also bind to AhR, and reportedly has a stronger agonist activity than B[a]P [161,347], but here no significant alteration in CYP1A1 protein expression and mRNA levels was observed (Figure 4.3), which also translated in lower levels of DNA damage (Figure 4.5A).

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 (Figure 4.5A) and with previous reports on Phe's non-carcinogenic nature [348]. This repression of CYP1A1 could be a feature of lower molecular weight PAH since fluoranthene is known to behave similarly [349]. 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 (Figure 4.3), especially at the 2:1 ratio (50 μM) with results being even higher than when cells were exposed to B[a]P individual. 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 [348], while B[a]P is a well-known carcinogen and a strong inducer of CYP1A1 through AhR binding [350]. 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 [348] and induce oxidative stress therefore promoting DNA strand breakage [189]. The mechanism is probably related with the poor induction of Nrf-2 signalling by Phe albeit it generates ROS production [178]. Some studies show a higher cytotoxicity for Phe than other PAHs [351] and in zebrafish (*Danio rerio*) larvae it caused a similar toxic response when in comparison to B[a]P [351]. However, others [352] 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 [353–355]. 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 [161,343,356] which may potentiate synergistic effects between PAHs [353,357]. 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 (Figure 4.3) but did not alter the mRNA levels of CYP1A1. This result differs from other studies, such as Martins et al. (2015)[173], 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 (Figure 4.5C). 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 (Figure 4.4C). 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 [358]. Since GSTs are involved in Phase II metabolism of PAHs, acting in the cellular detoxification and excretion [359], 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 [345].

4.5 Conclusions

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. Supplementary data to this article can be found online at https://doi. org/10.1016/j.scitotenv.2022.158783.

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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5

MULTI-BIOMARKER ANALYSIS OF SUB-CHRONIC PAH MIXTURE EFFECTS IN FISH AT ENVIRONMENTALLY RELEVANT LEVELS

This chapter is a published manuscript:

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Abstract

Polycyclic Aromatic Hydrocarbons (PAHs) are persistent pollutants in aquatic ecosystems, occurring as complex mixtures with unpredictable toxicity. Although PAHs are procarcinogenic, their harmful effects require metabolic activation, leading to reactive metabolites and reactive oxygen species (ROS) that can cause DNA damage.

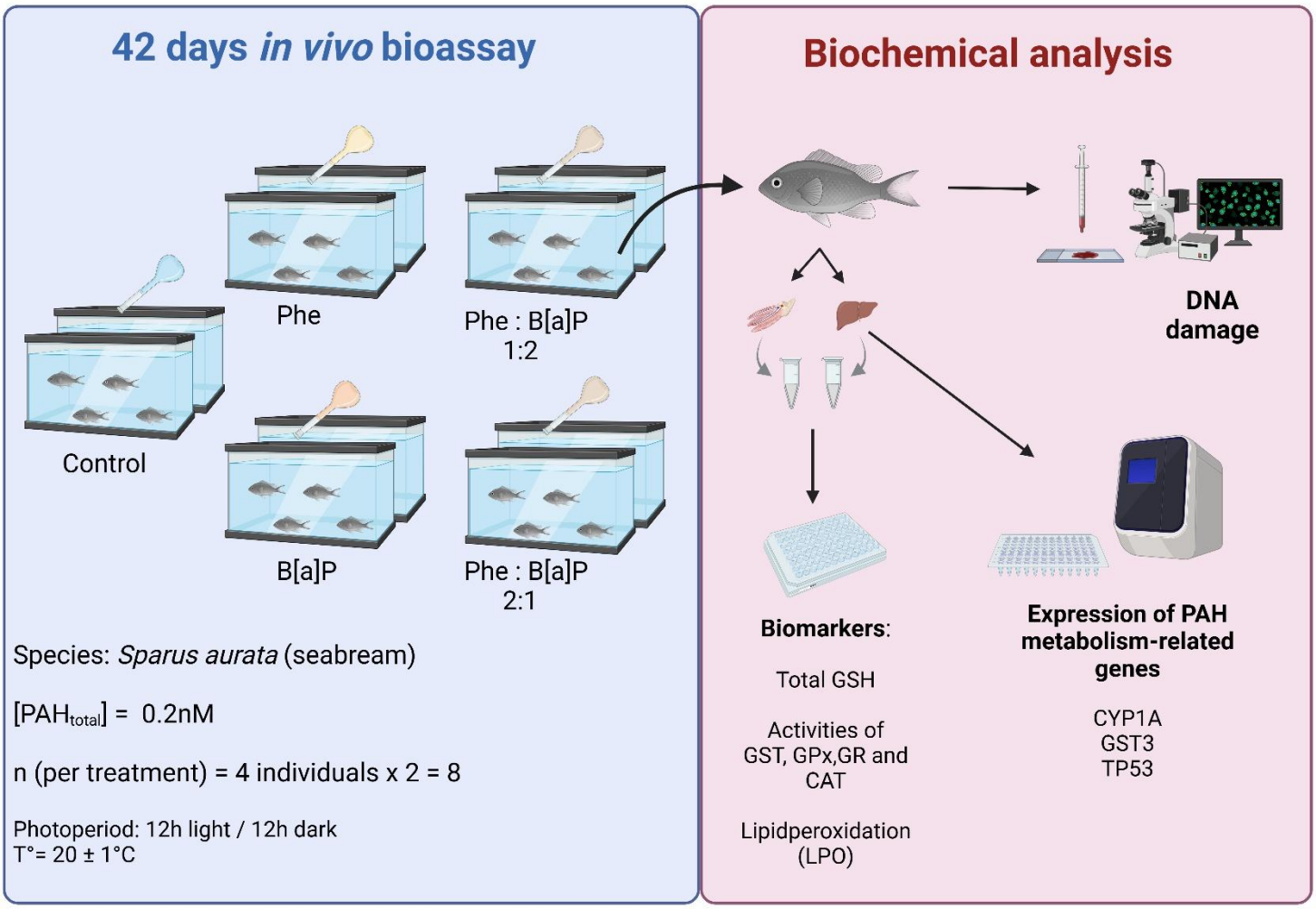
This study assessed the toxic effects of individual PAHs (Phenanthrene and Benzo[a]pyrene) and their mixtures (1:2 and 2:1 ratios) on juvenile seabream (*Sparus aurata)* after 42 days of exposure at 0.2nmol.L^{-1} . Biomarkers related to oxidative stress, detoxification, and lipid peroxidation were analysed in the liver and gills (e.g., glutathione (GSH), glutathione peroxidase (GPx), glutathione-S-transferase (GST), catalase (CAT), lipoperoxidation (LPO). Liver gene expression (Cytochromes P450 (CYP1A), GST3, tumour protein p53 (TP53) and blood cell DNA damage were also studied. Correlation analyses and Non-Metric Multidimensional Scaling (NMDS) were used to relate treatments and biomarkers.

Results suggested differences in organ responses, with the gills generally showing the most significant changes in GSH levels, GST activity, and LPO compared to the control group. DNA repair mechanisms appeared to prevent significant genotoxicity as assessed by the comet assay. However, erythrocytic nuclear anomalies (ENAs) were notably higher in fish exposed to Phe, B[a]P, and the 2:1 B[a]P:Phe mixture compared to the control group. Interestingly, the 2:1 Phe:B[a]P mixture appeared to have an enhanced effect, showing a marked upregulation of GST3 mRNA (up to 7-fold), possibly influenced by the higher proportion of Phe. MDS analysis proved to be a valuable tool in identifying patterns among biological responses, offering insight into how fish cope with PAH exposure and helping to uncover the unpredictable effects of chemical mixtures.

This study highlights the need for further research into the interactions of PAH mixtures, employing multi-analysis approach and underscores the importance of revising environmental guidelines to account for their effects.

Keywords: Phenanthrene; Benzo[a]pyrene; genotoxicity; *Sparus aurata*; environmental guidelines

Graphical Abstract



5.1 Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are pollutants containing 2 or more fused benzene rings that are ubiquitously present in the environment at concentration between 0.061 – 45 ng L^{-1} (Σ28 PAHs) across the world[360], as a result of incomplete combustion reactions of anthropogenic (e.g. fossil fuel burning) or natural (e.g. forest fires) origin [361]. These compounds are persistent organic pollutants (POPs)[362], due to their resistance to degradation and, consequently, a long environmental half-life enabling accumulation in sediments. This facilitates uptake by aquatic organisms and the gradual transfer along the food chain, posing a threat to the ecosystem[363]. At the molecular level, several PAHs are considered procarcinogenic since they originate reactive metabolites following hepatic biotransformation [160]. Metabolites can also originate from endogenous processes (oxidative stress and chronic inflammation), but the most common pathway is phase I transformation by the cytochrome P450 monooxygenase system, namely CYP1A, regulated via activation of the Aryl Hydrocarbon Receptor (AhR). Some of these metabolites are responsible for the carcinogenic and mutagenic effects by biding covalently to DNA forming adducts [364]. As such, several PAHs (e.g. Benzo[a]pyrene, hereafter B[a]P) are classified as effective or potential carcinogens to humans by the International Agency for Research on Cancer (IARC) since various epidemiological and toxicological studies showed a correlation between exposure to PAHs and an increased risk of developing cancer [365] and genotoxicity is a hallmark of exposure to many of these compounds (Silva Junior et al. 2021). After Phase I functionalization, metabolites undergo Phase II reactions, which involve the GST-mediated conjugation with glutathione to produce water-soluble conjugates that are easily excreted by organisms [160]. During metabolism, PAHs have the potential to stimulate the production of reactive oxygen species (ROS), damaging macromolecules such as lipids, proteins, and nucleic acids [158].

Studying the effects of PAHs in aquatic ecosystems poses a significant challenge since they are commonly found in the aquatic environment as complex mixtures, rather than as isolated compounds [178,366]. The conventional approach to regulatory risk assessment typically focuses on individual chemicals, neglecting the complexities of chemical mixtures[367]. Although

extensive knowledge exists regarding the mode of action of specific PAHs, such as B[a]P, the combined effects of PAH mixtures present a distinct toxicological challenge for organisms and a complex research topic for the scientific community.

Recent studies indicate that PAH mixtures enhance toxic effects in fish beyond single compound exposure, disrupting development, causing carcinogenic and oxidative stress-related impacts, and interfering with metabolic processes [368]. Research on Japanese medaka (*Oryzias latipes*) [369], European sea bass (*Dicentrarchus labrax*) [173], and zebrafish (*Danio rerio*) [263] has shown that PAH mixtures delay hatching, cause developmental abnormalities, disrupt swimming activity, and induce DNA damage, with toxicity levels varying by PAH composition. In medaka and sea bass, PAH mixtures lead to metabolic and liver disruptions, with mixtures sometimes generating supra-additive effects that amplify mutagenic risks. Exposure to binary PAH mixtures in zebrafish embryos increased toxicity and expression of AhR-regulated genes (ahr2 and cyp1a), along with genes critical for cardiovascular development and function. AhR knock-down significantly reduced cardiovascular toxicity caused by 6H-BPO and its mixture with B[a]P, highlighting the AhR's central role in these effects [370]. An *in vitro* study with seabream hepatocytes exposed to mixtures of B[a]P and Phenanthrene (Phe) demonstrated supra-additive effects over CYP1A transcription and DNA damage, particularly in mixtures with higher proportion of Phe relatively to B[a]P [366]. Additionally, another study with HepG2 cell line revealed that all PAH mixtures were more cytotoxic than individual PAHs, with the 1:1 mixture of Phe and benzo[b]fluoranthene (B[b]F) showing the highest cytotoxicity and reactive oxygen species (ROS) production [178].

These complex interactions between PAH in a mixture with different proportions of each individual PAH, highlight the need for a more comprehensive risk assessment approach that considers the combined toxicity of PAH compounds. Therefore, the objective of this research is to evaluate and validate the interactions between Phe (group 3 - not classifiable as carcinogenic to humans, IARC) [365] and B[a]P (group 1 – carcinogenic to humans, IARC) [365], in juvenile gilthead seabream (*Sparus aurata*), after a 42-day sub-chronic exposure. Previous studies [173,279,371] in fish with exposures of 14 to 28 days have shown that the effects of PAH, from CYP1A activation to DNA damage, can appear in the first 3-4 days and persist for up to 28

days. Taking this into consideration, we opted for a longer period of exposure to assess whether organisms can adapt and recover, and evaluate which biomarkers best translate toxicity. Consequently, the exposure was conducted using low concentrations of PAHs, yet maintaining ecological relevance [173,190]. Afterwards, several biochemical responses related with oxidative stress, PAH metabolism-related genes and genotoxicity were analysed in fish liver and gills.

5.2 Materials and Methods

5.2.1 Chemicals

Reagents were all acquired from Sigma-Aldrich/ Merck and include: Benzo[a]pyrene (B[a]P; CAS number 50–32-8; ≥96 %); Phenanthrene (Phe; CAS number 85–01–8; ≥98 %); Ethylenediaminetetraacetic acid (EDTA); 5,5'-dithio-bis-[2-nitrobrenzoic acid] (DTNB); 2,4-Dinitrochlorobenzene (CDNB); Glutathione Reductase (GR); Reduced Glutathione (GSH); Oxidized Glutathione (GSSG); Ponceau S; Dimethyl sulfoxide (DMSO); Bovine Serum Albumin (BSA); Nicotinamide adenine dinucleotide phosphate (NADPH); Formaldehyde; Purpald. Coomassie dye was acquired from Biorad.

5.2.2 *in vivo* bioassay

The juvenile seabream (*Sparus aurata*) (N=40; mean weight: 11.19 ± 2.70 g; mean total length 8.89 ± 0.74 cm) were obtained from the Olhão Fish Farming Pilot Station (EPPO, IPMA, Portugal) and acclimated for one week in a closed-circuit system at NOVA FCT with filtered seawater. The fish were subjected to a photoperiod of 12 h light:12 h darkness, and the water was kept at a temperature of 20 ± 1 ◦C and a pH of 7.4 ± 0.2, with continuous aeration, enough to keep the dissolved oxygen above 6.0 mg/L. Fish were feed daily at *ad libitum* with commercial formula (Sparos). Ten aquaria were filled with 12.0 L of seawater and the water conditions (temperature, pH and dissolved oxygen) were the same as the acclimation period. After the acclimatization period, 40 fish were randomly distributed by each aquarium with 4 fish per aquarium. Fish were exposed for 42 days to two PAHs, B[a]P and Phe, singly and combined Phe:B[a]P at different proportions (Phe:B[a]P, 1:2 and 2:1) in a final total PAH concentration of 0.2 nmol.L $^{-1}$, corresponding to 35.64 ng.L^{-1} for Phe and 50.46 ng.L^{-1} for B[a]P . Thus, besides a control group (Ct), the four experimental treatments were: Phe at 0.2 nmol.L $^{-1}$, B[a]P at 0.2 nmol.L $^{-1}$, Phe:B[a]P 1:2 (Phe 0.067 nmol.L $^{-1}$:B[a]P 0.134 nmol.L $^{-1}$) and Phe:B[a]P 2:1 (Phe 0.134 nmol.L $^{-1}$:B[a]P 0.067 nmol.L $^{-1}$) (Fig. 1). The final concentration of PAHs was based on previous works [173,190], which focused on environmental relevant concentrations. The DMSO was used to solubilize the stock solutions of PAHs and for intermediate PAHs solutions

in order to have 0.002% of this solvent in the test aquaria. During the exposure period 70% of the water volume was renewed every 48 h, for the maintenance of water parameters, and the aquariums were re-contaminated according to each specific exposure condition. Fish were feed at the same way as acclimation period.

After the exposure period, blood samples were immediately collected from the caudal artery of each fish with a syringe previously washed with heparin (50 U) to prevent clotting. Afterwards, all animals were measured for total weight and length and euthanized by cervical sectioning and dissected immediately according to the norms mandated by Directive 2010/63/EU of the European Parliament and the Council for Laboratory Animal Welfare. Immediately following collection, organs such as the liver and the gills were stored at -80 °C for further analysis.

5.2.3 Biochemical analysis

Seabream liver and gills were homogenized in ice-cold conditions, in a proportion of 1 mg of tissue to 5 μL of homogenization buffer containing phosphate-buffered (pH 7.4) saline solution and 1 tablet of protease inhibitor cocktail (Roche) per 10 mL. Tissue homogenates were centrifuged at 10,000 g (15 min at 4 ◦C) and the supernatant frozen at -80 °C until further analyses. All biochemical analyses were performed at least in duplicate.

5.2.3.1 Total protein concentration

The total protein concentration in cell lysates was assessed using the Bradford method [338]. Briefly, each sample was combined with a diluted (5×) Coomassie dye (BioRad) in 96-well plates, and the absorbance was recorded at 595 nm using a microplate reader. Protein concentrations were determined from a calibration curve (1–16 μg of protein per μL) with BSA as the standard.

5.2.3.2 Evaluation of total glutathione

Total glutathione (GSH) levels were determined using the enzymatic recycling method outlined by Rahman et al., (2006)[372], as follows: 5 μL of each sample were incubated with 5 mmol.L^{-1} DTNB, 50 mmol.L^{-1} NADPH, and 50 nmol.L^{-1} glutathione reductase (100 U mL^{-1} GR) in 0.1 M potassium phosphate buffer with 5 mmol.L^{-1} EDTA (pH 7.5), and the formation of 5'-thio-2-nitrobenzoic acid (TNB) was monitored at 412 nm for 2 minutes. Calibration curves

with a GSH standard (range: 0.5–50 μmol.L-1) were employed for quantification. Results are expressed in relation to the total protein concentration (nmol of GSH/mg protein).

5.2.3.3 Determination of GPx activity

The activity of cytosolic glutathione peroxidase (GPx1) was assessed using the method described by Esworthy et al. [373]. Briefly, 30 μg of protein from the homogenates were combined with 2 mmol.L^{-1} NADPH, 10 mmol.L^{-1} glutathione, 1.125 mmol.L^{-1} sodium azide, and 100 U mL-1 of glutathione reductase. Hydrogen peroxide (5 mmol.L^{-1}) served as the substrate. The reduction in absorbance at 340 nm reflecting the rate of NADPH consumption, was monitored for 5 minutes to calculate GPx1 activity. Results are expressed in relation to the total protein concentration (μmol of NADPH/min/mg protein).

5.2.3.4 Determination of GR activity

The activity of glutathione reductase was determined in homogenates following Mannervik's method (1999)[374]. In 96-well microplates, 30 μg of protein were combined with 0.2 mmol.L^{-1} phosphate buffer (pH 7.0), 2 mmol.L^{-1} NADPH, and 20 mmol.L^{-1} GSSG. The consumption of NADPH was monitored at 340 nm for 5 min, at 30 °C. Results are expressed in relation to the total protein concentration (μmol of NADPH/min/mg protein).

5.2.3.5 Determination of GST activity

Glutathione S-transferase activity was measured with a commercial kit (from Sigma-Aldrich), using chloro-2,4-dinitrobenzene (CDNB) as substrate, according to manufacturer's instructions. Activity was determined by measuring the increase in absorbance at 340 nm during 5 min. Results are expressed in relation to the total protein concentration (μmol of CDNB/min/mg protein).

5.2.3.6 Determination of catalase activity

Catalase activity was determined as described by Johansson & Håkan Borg (1988)[375] following adaptation to 96-well microplates. Briefly, 20 μL of each sample or standard, 100 μL of assay buffer and 30 μL of methanol were added to each well of a 96-well microplate. Then, the reaction was initiated by adding 20 μL of hydrogen peroxide (30%) to all the wells and the microplate was incubated for 20 min on a shaker. In this reaction, catalase catalyses the

oxidation of methanol to formaldehyde while reducing H_2O_2. Afterwards, 30 μL of potassium hydroxide and 30 μL of Purpald in HCl were added into each well and incubated for 10 min in a shaker at room temperature. Afterwards, 10 μL of potassium periodate in KOH was added to each well and allowed to incubate for 5 min. Then, the absorbance was read at 540 nm in a microplate reader. Formaldehyde concentration of the samples was determined based on a calibration curve using a formaldehyde standard (0 to 75 $\mu mol.L^{-1}$). Results are expressed in relation to the total protein concentration (nmol of formaldehyde/min/mg protein).

5.2.3.7 Determination of lipid peroxidation

Lipid peroxidation was determined with the TBARS assay as described by Uchiyama and Mihara (1978)[376]. In a microtube, 5 μL of each sample were added to 45 μL of monobasic sodium phosphate buffer. Next, 12.5 μL of sodium dodecyl sulphate (SDS), 93.5 μL of trichloroacetic acid (TCA) and 93.5 μL of thiobarbituric acid were added. Then, 50.5 μL of MilliQ- water was added into each microtube and mixed for 30s in a vortex and incubated for 10 min at 100 °C. Afterwards, the reaction was stopped in ice, and 62.5 μL of MilliQ water was added to each microtube. Then, microtubes were centrifuged at 5000 g for 5 min. Duplicates of 150 μL of the supernatant of each reaction were added into a 96-well microplate and absorbance was read at 530 nm. A calibration curve using MDA standards (0 – 1 nmol mL^{-1}) was used to determine the peroxides concentration. Results are expressed in relation to the total protein concentration (nmol of MDA/mg protein).

5.2.4 Expression of PAH metabolism related genes

RNA was extracted from each sample (30 mg of liver tissue) 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 CYP1A, tumour suppressor protein (TP53) and Glutathione-S-Transferases (GST-3), was done with the 2-$\Delta\Delta$CT method (Livak and Schmittgen, 2001)[340], using GAPDH as the housekeeping gene. Primers were designed with Primer-blast using Reference Sequences from Gen Bank (Table 5.1). Primer efficiency was tested with a serial dilution of the (1:10–1:1000000) of the cDNA template 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 nmol.L-1) and molecular grade water for a final 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 an initial incubation at 50 °C for 2 min and 95 °C for 10 min. No template and no reverse transcription controls were included for all genes and no amplification was observed. The qRT-PCR was performed on an Applied Biosystems QuantStudio™ 7 Flex real-time PCR system (Applied Biosystems, Foster City, CA, USA).

5.2.5 Assessment of DNA damage

DNA damage in fish peripheral blood was assessed by scoring erythrocytic nuclear abnormalities (ENA) and by the Comet assay, following the procedures described by Costa et al. (2008) [188]. Upon collection, blood samples were either smeared on glass microscopy slides (with subsequent air-drying) for ENA analysis or diluted (1:100) in cold phosphate-buffered saline (PBS) for the Comet assay. For ENA preparations, slide samples were fixed with methanol during 15 min and then blood smears were stained with 0.1 g L21 acridine orange for 30 min and mounted with DPX [188]. A minimum of 1000 mature, intact erythrocytes were scored for each individual. The scoring criteria for cells with nuclear abnormalities were established following the guidelines of Bolognesi and Hayashi (2011) [377], Costa et al. (2008) [188], and Fenech et al. (2003) [378].

The standard alkaline comet assay was conducted following the procedure described by Martin et al. (2018) [278]. In brief, PBS cell suspensions were mixed with liquid low melting-point agarose (LMPA) and applied to slides pre-coated with normal melting-point agarose.

Once solidified, the slides were immersed for 1 hour at 4 °C in a lysis solution and subsequently incubated for 40 min with cold electrophoresis solution (pH 13), followed by 30 min at 25 V. After neutralization (15 minutes) with Tris–HCl buffer (pH 7.5), slides were stained with ethidium bromide (20 μg mL^{-1}) and examined using a DMLB microscope equipped with an epifluorescence light source (EL6000 with N2.1 filter from Leica Microsystems). Approximately 100 random comets were analysed per slide using CometScore software (TriTek, VA, USA) (Martins et al. (2013), Martins et al. (2016) [189,279].

5.2.6 Statistical analysis

Differences in biomarker levels, gene expression and DNA damage between the different treatments and the control group were evaluated with a Nested-ANOVA, where the aquarium was set as nesting factor. The Nested-ANOVA was followed by Tukey's HSD test to allow pairwise comparisons among the treatment groups. Assumptions of normality and homogeneity of variances were checked prior to analysis, using the Shapiro-Wilk test and Levene's test, respectively. Differences between groups were classified as significant at $p < 0.05$. These statistical analyses were conducted in R [379], while graphical representations were created using GraphPad Prism.

A Non-Metric Multidimensional Scaling (NMDS) analysis was performed to examine the effects of treatment on the joint responses of biochemical markers using functions from the vegan R package [380]. We standardized biomarker data with the decostand function, built a Bray-Curtis dissimilarity matrix with the vegdist function, performed the NMDS with the metaMDS function and overlayed biomarker vectors fitted with the function envfit. The NMDS represented in a reduced-dimensional space the degree of similarity in the responses of individual fish, allowing for the visualization of possible clusters of individuals exposed to the same treatment and their correlation with biomarker trends. To focus on how mixtures relate to or differ from individual compounds, we excluded the control group from this analysis. A PERMANOVA was performed to evaluate the statistical differences between treatments using the adonis2 function (also from vegan package), followed by a pairwise comparison of treatments using the function pairwise.adonis of the pairwise Adonis R

package [380]. To complement the NMDS, a correlation analysis was conducted to assess relationships between biomarkers, further highlighting their interactions.

5.3 Results

5.3.1 Biochemical Results

After 42 days for exposure, total glutathione showed significant higher levels than control for B[a]P in liver (Figure 5.1 **c**., Table 5.2 in the Supplementary Information) and for Phe, B[a]P and the 1:2 mixture in gills (Table 5.3). For GST activity the results were significantly higher for Phe, B[a]P and 2:1 mixture in gills (Table 5.3) but no differences were found for liver (Figure 5.1**d**.), relatively to control animals. For lipid peroxidation, measured as MDA content, statistically significant lower levels were found for B[a]P and both mixtures for gills (Table 5.3), while no differences were found for liver samples (Figure 5.1. **f.**), compared to controls. Treatment showed no significant differences from control for GPx, GR and CAT activities (Figure 5.1 **a**, **b** and **e**) in the liver and gills of fish exposed for 42 days.

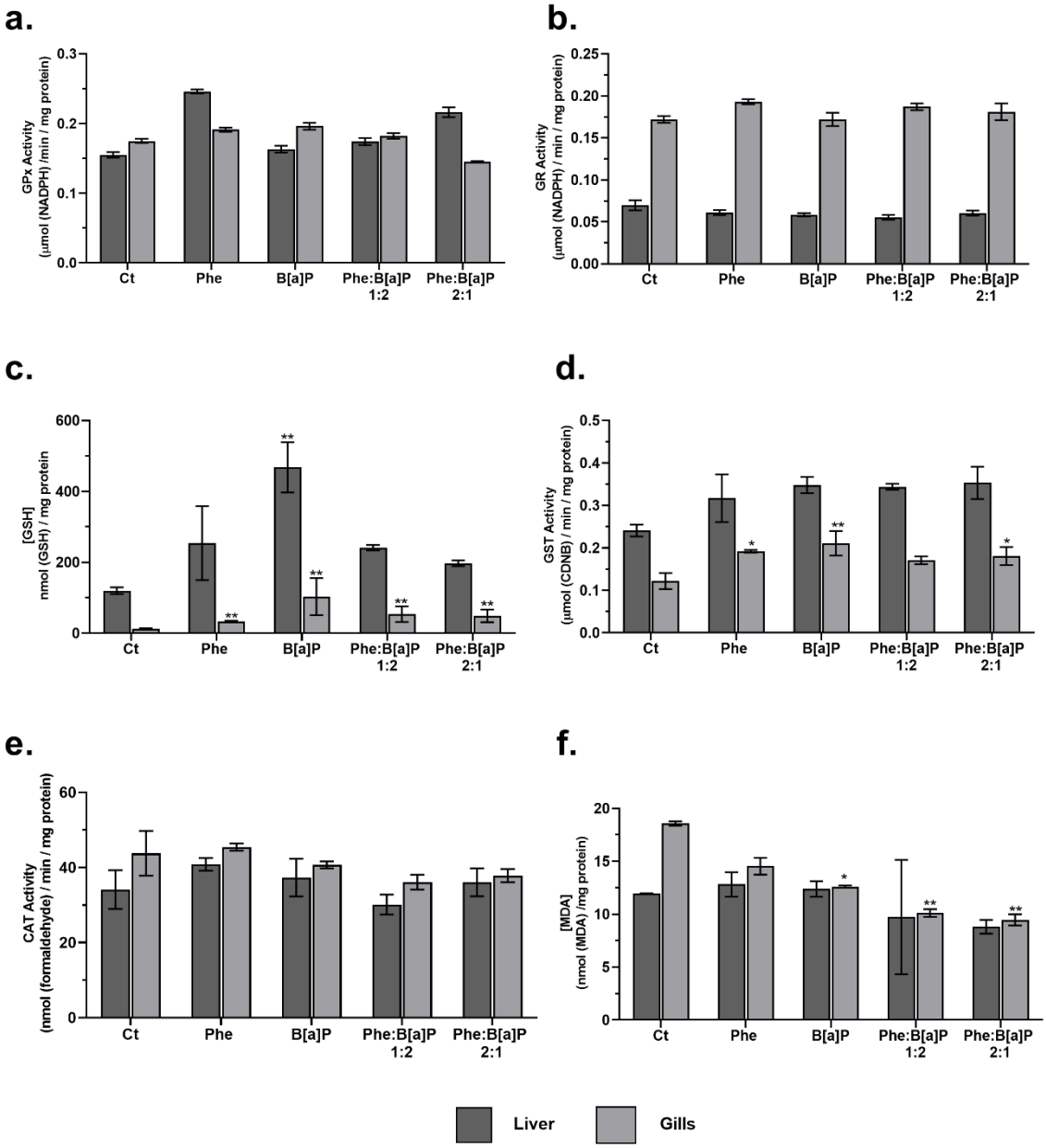


Figure 5.1- Biomarker analyses in liver and gills of juvenile liver of seabream exposed to 0.2 nM of Phe, B[a]P and the mixtures Phe: B[a]P (1:2) and Phe:B[a]P (2:1) for 42 days compared to a control group. a. GPx activity (µmol (NADPH)/min/mg protein), b. GR activity (µmol (NADPH)/min/mg protein), c. Total glutathione content, [GSH] (nmol (GSH)/ mg protein), d. GST activity (µmol (CDNB)/min/mg protein), e. CAT activity (nmol (formaldehyde)/min/mg protein) and f. Lipid peroxidation represented as MDA content (nmol (MDA)/mg protein. Data (mean ± S.E.) from 8 individuals per treatment. Ct represents control group. Error bars represent model standard errors. Statistical differences between treatments and the control group are represented as * $p < 0.05$ and **: $p < 0.01$

5.3.1.1 Non-Metric Multidimensional scaling (NMDS)

Despite PERMANOVA analysis for liver samples showed no significant differences in biomarkers for the treatment groups, the NMDS bidimensional representation showed trends of point segregation of treatment groups that are worth to be interpreted (Figure 5.2**a**.). The treatments B[a]P and the Phe:B[a]P mixture at a 1:2 ratio tended to be positioned in the positive region of axis 1, corresponding to higher levels of GSH, while Phe and the Phe:B[a]P mixture at a 2:1 ratio tended to be located on the negative side of axis 1, corresponding to higher activity of GPx (Figure 5.2**a**) LPO and CAT represented trends along axis 2, but this axis had little effect on the segregation of data points (Figure 5.2 **a**.).

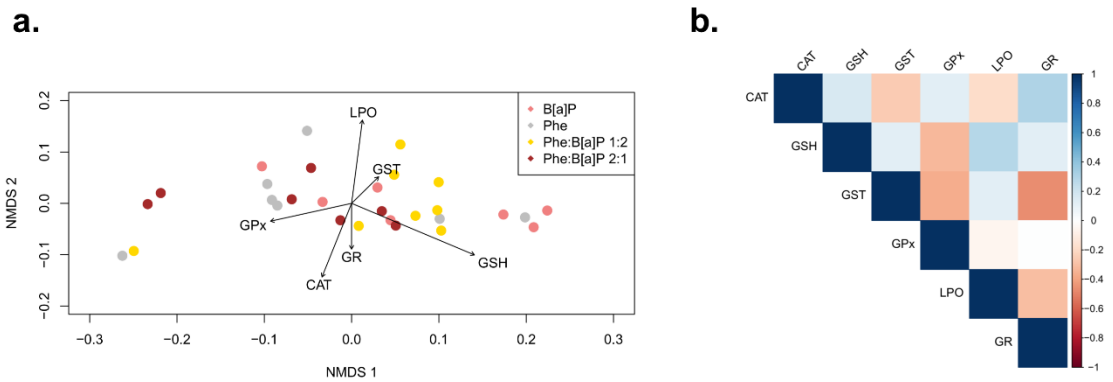


Figure 5.2 - a. NMDS results showing segregation of individual fish, coloured by treatment, regarding the joint response to biomarker activity in liver. PERMANOVA analysis showed no statistically significant differences between treatments. **b.** Correlation plot of all biomarkers in liver, represented by a colour gradient where the colour intensity indicates the strength and direction of correlations.

The correlation analysis of biochemical responses in liver tissue revealed a balanced distribution of positive and negative associations among the biomarkers, with approximately 50% of the plot showing positive correlations and 50% showing negative correlations (Figure 5.2 **b**., Table 5.4). GST and GR exhibited a negative significant correlation (Figure 5.2 **b.**, Table 5.4). GST also showed a weaker negative significant correlation with GPx (Figure 5.2**b.**, Table 5.4). In general, other biomarkers showed correlations close to zero, indicating a lack of association in their responses to PAH exposure. These findings reflect both notable relationships and the absence of others, underscoring the complex but selective biochemical response in liver tissue.

Unlike the liver, in gills, showed a significant effect of treatment on biomarkers activity (PERMANOVA, Table 5.5). Specifically, there were significant differences between B[a]P vs Phe and Phe vs Phe:B[a]P 1:2 mixture (pairwise comparisons Table 5.5). The NMDS analysis also showed a clearer separation of treatment groups based on biochemical responses. There is an almost full segregation between B[a]P and Phe along the axis 1, with B[a]P assuming mostly positive values and Phe only negative values (Figure 5.3 **a.**). Both mixtures assumed an intermediate position in this axis (Figure 5.3 **a**.), although Phe:B[a]P 1:2 and Phe segregated considerably, this being consistent with the PERMANOVA results. GSH content was aligned to some extent with axis 1, meaning that B[a]P tended to show higher GSH levels and Phe lower levels. (Figure 5.3 **a**.).

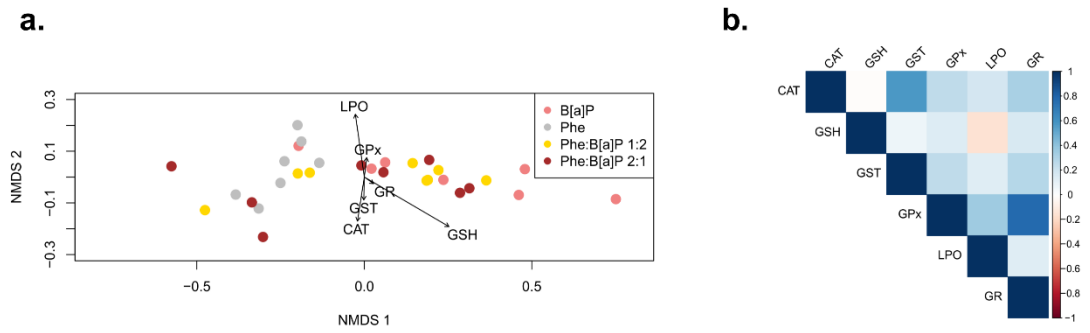


Figure 5.3 - NMDS results showing segregation of individual fish, coloured by treatment, regarding the joint response to biomarker activity in gills. PERMANOVA analysis showed significant differences between treatments (p = 0.017). Specifically, Phe (grey cluster) was significantly different from B[a]P and Phe:B[a]P 1:2 mixture (p= 0.001 and p=0.03 respectively, Table 5.5, Appendices.). **b.** Correlation plot of all biomarkers in liver, represented by a colour gradient where the colour intensity indicates the strength and direction of correlations.

The correlation analysis of biochemical responses in gill samples (Figure 5.3 **b**. and Table 5.6) indicated generally stronger positive associations compared to correlations of biomarkers obtain for liver tissue. The highest positive significant correlation was observed between GPx and GR, with a correlation value greater than 0.7 (Figure 5.3 **b.** and Table 5.6). The second strongest positive significant correlation was between CAT and GST (0.578). These findings suggest a more coordinated positive response, with almost all biomarkers working at the same time, in gill tissue, with limited antagonistic interactions.

5.3.2 Expression of PAH metabolism and genotoxicity - related genes

Changes in expression were found for several PAH metabolism-related genes following exposure to the different treatments (Figure 5.4). Interestingly, a 7-fold increase in GST3 expression was detected in fish exposed to the Phe:B[a]P 2:1 mixture. TP53 mRNA levels were significantly upregulated 4-fold in fish exposed to Phe.

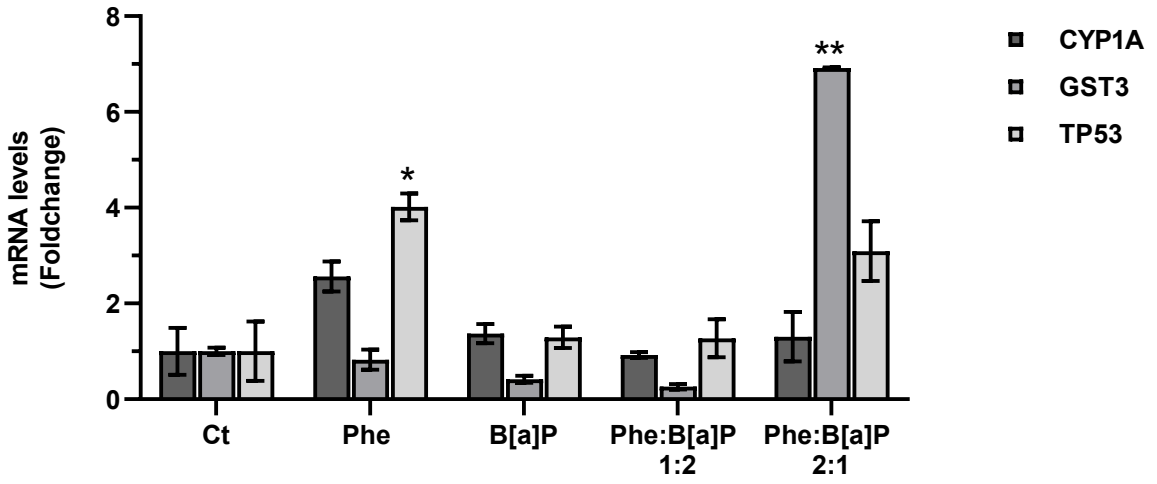


Figure 5.4 - mRNA levels for CYP1A, GST3 and TP53 of juvenile liver of *S. aurata* exposed to 0.2 nM of Phe, B[a]P and the mixtures Phe: B[a]P (1:2) and Phe:B[a]P (2:1) for 42 days. Error bars represent model standard errors. Statistical differences between treatments and the control group are represented as * $p < 0.05$ and **: $p < 0.01$.

5.3.3 DNA damage

Results from the Comet assay showed no significant differences between treatments for the percentage (%) of DNA in tail, indicating the absence of DNA strand-brakes (Figure 5.5**a.**), for the PAH concentrations tested. However, the percentage of total ENAs was significantly higher for Phe, B[a]P and the mixture Phe : B[a]P 1:2 than in the control group (Figure 5.5**b**., see Figure 5.5, Appendices)

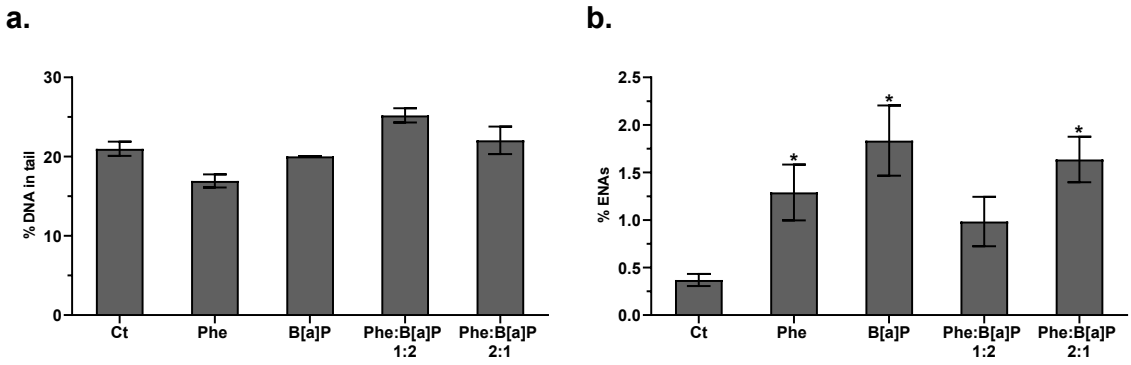


Figure 5.5 - DNA damage in blood cells of juvenile seabream exposed to 0.2 nM of Phe, B[a]P and the mixtures Phe: B[a]P (1:2) and Phe:B[a]P (2:1) for 42 days and a control group (Ct) represented as: **a.** Percentage (%) of DNA in tail **b.** % of Total Erythrocyte Nuclear Abnormalities (ENAs). Error bars represent model standard errors. Statistical differences between treatments and the control group are represented as * $p < 0.05$

5.4 Discussion

In the present study, an *in vivo* sub-chronic 42-days exposure was implemented, involving two distinct PAHs usually present in the aquatic environment as complex mixtures, each one being known for having different carcinogenic potentials, Phe and B[a]P. These PAHs were administered through water, to juvenile seabream, both individually and as a mixture with different proportions of each PAH at a final concentration of 0.2 nmol.L^{-1}, corresponding to 36.64 ng.L^{-1} for Phe and 50.46 ng L^{-1} for B[a]P.

Across all exposure conditions, after 42 days of exposure, no significant effects were observed in the activities of key antioxidant enzymes, GPx, GR, and CAT (Figure 5.1 **a**, **b**, and **e**), which are typically involved in detoxification processes and oxidative stress defence. These findings are consistent with Machado et. al. (2014) [381] that observed that GR activity was not altered both in liver and gills after the exposure of Guaru fish (*Poecilia vivipara)* to Phe at 10, 20 and 200 mg.L^{-1}. In the same way Bastolla et. al (2023) [382] verified a lack of significative differences in GPx and CAT activities after the exposure of *Crassostrea* oysters to Crysene (a PAH with 4 aromatic rings) at 10 μg/L. Also, Danion et. al (2014) [383] found that the activities of antioxidant defence enzymes, including GPx, SOD, and CAT, measured in the liver, were not significantly different in seabass (*Dicentrarchus labrax)* exposed to PAHs at 835±53 ng.L^{-1} in the water-soluble fraction of Arabian crude oil, for 21 days.

However, GSH levels (Figure 5.1 c) increased significantly in fish liver exposed to B[a]P singly, and in the gills, significant increases were observed across all treatments compared to the control group, after 42 days of exposure. Unlike electrophilic xenobiotics such as mercury [178], parent PAH compounds are chemically stable and do not directly interact with GSH. However, Phase I metabolism via CYP1A1 generates reactive metabolites which are actively detoxified by GST-mediated conjugation with GSH. Interestingly, the mixtures with B[a]P resulted in a comparatively smaller increase in GSH than exposures to singly B[a]P, which may suggest a reduced detoxification capacity under mixed exposure conditions and potentially increased toxicity. Similar findings were reported by Branco et. al (2021) [178] involving Phe and B[b]F (a PAH structurally similar to B[a]P and classified as possibly carcinogenic to humans, group 2B by IARC [365]), where lower GSH levels were observed in mixtures compared to B[b]F. Nonetheless, the metabolism of PAHs generates reactive oxygen species (ROS), which can induce oxidative stress. To counteract this, the Nrf2 (Nuclear Factor Erythroid 2-related Factor 2) pathway can be activated. In response to ROS, Nrf2 dissociates from its repressor Keap1, translocates to the nucleus, and binds to the ARE (Antioxidant Response Element), promoting the expression of antioxidant genes such as glutathione peroxidase, glutathione reductase, and other enzymes involved in glutathione cycle [173]. Consequently, GSH levels rise as part of the antioxidant response, enhancing the detoxification of ROS and reactive PAH intermediates. These findings are also consistent with Zeng et. al. (2023) [384] that observed an increase in GSH levels in the liver of large yellow croaker after B[a]P exposure (4, 8, 16 μg/L)

GST activity was increased in the gills, although no changes were observed in the liver (Figure 5.1 d). This differential response between the liver and gills could suggest that the gills, as the primary site of direct exposure to waterborne pollutants, may mount a stronger detoxification response via GST activity. Though these findings are unexpected, they have also been observed in other studies with liver or gills [384–387].

The observed changes to enzymatic activities are further supported by PCR analysis of key detoxification-related genes, providing insights into the underlying transcriptional regulation triggered by PAH exposure. This molecular data complements the biochemical

findings, offering a more detailed understanding of the organism's adaptive responses to prolonged PAH exposure.

In contrast with GST activity results (Figure 5.1 **d.**), gene expression results, revealed a very significant ($p<0.01$) increase in GST3 mRNA levels in the liver of fish exposed to the 2 B[a]P : 1 Phe mixture (Figure 5.4). The lack of synchronization between mRNA expression levels and enzymatic activities can be explained by the fact that antioxidant enzymes such as GST are encoded by multiple gene subtypes [344] [388]. GST3, belonging to the pi class of GSTs, is often a predominant isoform in fish species, playing a crucial role in xenobiotic metabolism [389]**.** The marked response of GST3 to the PAH mixture aligns with previous studies demonstrating the sensitivity of GST pi to environmental pollutants in various marine species (fish and clams) [390]**.** [391] found an increase in GSTpi mRNA expression after exposing zebrafish to different PAHs and mixtures, specifically B[a]P individual and a mixture of B[a]P and fluoranthene. Yao et. al. (2017) [392] observed an increase of GSTs pi class in the digestive glands of the scallop *Chlamys farreri* exposed to B[a]P and Chrysene. In addition to GST3, another isoform of particular interest in fish species is GST rho. [393–395]. While our study focused on GST3, future investigations could benefit from examining GST rho expression in response to PAH exposure, potentially revealing species-specific detoxification mechanisms. The inclusion of multiple GST isoforms in toxicological studies could provide a more comprehensive understanding of how fish respond to and metabolize PAHs at the molecular level. Additionally, spatial and temporal variations in mRNA, it is natural decay, along with the local availability of resources for protein biosynthesis, significantly influence the relationship between protein levels and their corresponding transcripts [396]. Moreover, discrepancies between mRNA levels and enzymatic activity may arise due to the time intervals required for gene transcription, translation, and protein modification processes. The intriguing result of GST3 expression (Figure 5.4) for the 2:1 mixture aligns with previous findings *in vitro* [366], suggesting synergistic effects, underscoring the unpredictable nature of mixtures. It appears that, as observed previously, Phe, despite being classified as a non-carcinogenic PAH, may interact synergistically with B[a]P.

Additionally, lipid peroxidation (LPO), a key marker of oxidative effects, showed a slight decrease in the gills for all treatments, except in fish treated with Phe (Figure 5.1 **f**) . Similar reductions in LPO levels have been observed in other species, such as planaria exposed to PAHs by Simão et. al (2022) [397] , in bivalves by Piazza et. al (2016) [398] and Martins et al (2013) [279]. This decrease in LPO likely reflects an over-compensatory antioxidant response, with increased antioxidant defences progressively reducing oxidative damage throughout the exposure period, even before the 42-day mark. Interestingly, in the liver, LPO levels remained like control animals. In contrast, a study by Rossner et al. (2020) [399] suggests that B[a]P may inhibit lipid peroxidation by acting as an agonist of the AhR. While B[a]P is known to activate the AhR, leading to the induction of cytochrome P450 enzymes (CYP1A) and the subsequent formation of reactive metabolites, AhR activation may also inhibit the arachidonic acid (AA) pathway. This inhibition could reduce isoprostane formation, a biomarker of lipid peroxidation, thereby contributing to the observed decrease in LPO.

Results from the comet assay (Figure 5.5) showed no significant increase in DNA strand breaks across treatments, suggesting that PAH exposure did not lead to immediate DNA damage after 42-days of exposure. In addition, the upregulation of TP53, particularly in response to Phe may have contributed to the prevention of strand breaks by activating DNA repair pathways during the sub-chronic exposure. TP53, a key tumour suppressor, plays an essential role in maintaining genomic stability by responding to DNA damage and triggering repair mechanisms [400]. These findings are consistent with those of Larcher et. al. (2014) [263], who reported no increase in genotoxicity, assessed by the comet assay, after exposing zebrafish to PAH mixtures in diets spiked with pyrolytic (PY), petrogenic light oil (LO), or petrogenic heavy oil (HO). Conversely, Dupuy et. al. (2014)[401] observed an initial increase in DNA damage after 29 days of exposure to a PAH mixture, followed by a return to basal levels, suggesting the activation of DNA repair processes.

Despite the absence of significant DNA strand breaks in the comet assay, attributed to the activation of DNA repair mechanisms, a significant increase in ENAs was observed in the Phe, B[a]P, and Phe:B[a]P 2:1 mixture treatments, indicating that chromosomal damage still occurred. This increase in ENAs was expected, as PAHs are known genotoxic compounds that

can produce DNA reactive intermediates during metabolism [160,189]. These intermediates can lead to persistent chromosomal alterations that are often irreversible, even when immediate DNA strand breaks are mitigated by repair mechanisms. Thus, the complementary results from the comet assay and ENA quantification highlight different aspects of chronic genotoxic stress: while the comet assay captures immediate DNA strand breaks, ENAs provide insight into the more lasting chromosomal damage expected from PAH exposure.

In addition to NMDS, a correlation analysis was performed to identify direct associations between individual biomarkers. The correlation analysis complemented NMDS by providing insights into the strength and direction of relationships between specific biomarkers, such as the positive or negative correlations between antioxidant enzymes and oxidative stress markers. Together, NMDS and correlation analysis provided a comprehensive view of both sample-level patterns and biomarker-specific interactions, allowing us to explore global trends and specific relationships that contribute to our understanding of biochemical responses to PAH exposure.

The PERMANOVA results shows relevant differences in liver and gill in the responses of biomarkers to treatments. While no significant differences in treatments were found in liver, there were significant differences between Phe and B[a]P, and Phe and Phe:B[a]P 1:2 in gills. Despite the lack of statistical significance for liver analysis, it is interesting to note that liver and gills had some consistent trends in the NMDS results. In both the liver and gills, B[a]P and the Phe occupied opposite positions along the NMDS axis 1. This suggests that these treatments induce distinct biochemical changes. However, a key difference emerged with the Phe:B[a]P 1:2 mixture: while in the liver it tended to be distributed towards higher values of axis 1, thus closer to the B[a]P points; in gills it assumed a more intermediate location between Phe and B[a]P points. This suggests a more variable response, possibly resulting from the unpredictable nature of the mixtures' effects.

There was also same consistency for both organs in the direction of biomarker vectors. The most relevant of such relationships was the alignment of GSH with axis 1, as the treatments were mostly segregated along this axis. From that relationship we may conclude

that B[a]P tended to show higher levels of GSH in both organs, implying that this treatment likely induces a detoxification response involving glutathione conjugation.

It is also worth to note that Phe points in the liver were positioned nearer to GPx, indicating that this treatment may more prominently activate oxidative stress defence mechanisms.

While the NMDS plot results showed similarities in both organs, the correlation plot revealed distinct differences. In the liver, correlation analysis uncovered key relationships, including a positive correlation between GST and GSH, and negative significant correlations between GST and both GPx and GR. This indicates that GSH play a role as subtract to GST activity enhancing detoxification processes, particularly under treatments like B[a]P and the 1:2 mixture. These findings indicate that B[a]P and mixtures with a higher proportion of B[a]P favour glutathione-based detoxification via conjugation and most likely Nrf-2 activation. On the other hand, Phe and Phe-enriched mixtures promote oxidative stress management via enzymes like GPx, which consumes available GSH, and because Phe is not a strong Nrf-2 inducer [178], its levels are not replenished properly. This illustrates a treatment-specific modulation of antioxidant and detoxification pathways, with mixtures displaying a blend of these responses depending on their composition.

From the correlation plot, it was observed significantly positive correlations (GPx – GR, CAT – GST, LPO - GPx) in the gills indicating that both detoxification and oxidative stress mechanisms are being activated simultaneously in response to PAH exposure. This supports the idea that both detoxification and oxidative stress responses are being coordinated, depending on the treatment. These findings align well with the functional differences between the gills and the liver. The gills, as a primary site of direct chemical exposure, exhibit a higher susceptibility due to their extensive surface area [383,402,403], which may display more complex and overlapping responses to pollutants compared to the liver.

To disclose such complex interactions, between compounds, biochemical responses and different organs, a multi-biomarker comprehensive approach proved to be an invaluable tool. In contrast to a single-biomarker approach, which often falls short in identifying sources of toxicological risk, MDS combined with correlation analysis allowed for the integration of

multiple biomarkers, revealing subtle trends and interactions that might otherwise be overlooked. Together, MDS provides a spatial representation of treatment effects, while correlation plots highlight specific relationships among biomarkers, offering an overall view of the detoxification and oxidative stress responses across different tissues.

5.5 Conclusion

The complexity of asses the toxicity responses of pollutants that are usually present in the environmental as a complex mixture of different molecules, such as Polycyclic Aromatic Hydrocarbons, constitute a challenge, especially when ecologically relevant concentrations are considered. However, multi-biomarkers analyses showed to be a strong and valuable tool to disclose the toxicity pathways in fish. Although several enzymatic activities did not exhibit significant changes following sub chronic exposure to PAHs, distinct biological responses at transcriptomic level and genotoxicity were observed and were dependent on both the treatment and the organ involved. In general, B[a]P and the B[a]P-enriched mixture were closely linked to the GSH system, whereas Phe was more associated with GPx and the antioxidant system. Also, mixtures resulted in wider spread of points in the multivariate analysis, which testifies the variability in toxicological responses and the difficulty in predicting toxicological outcomes for such type of exposure.

Furthermore, genotoxicity assessments showed that while at low concentrations, the organs can deal with DNA damage by activating repair mechanisms, there was a marked rise in erythrocyte nuclear abnormalities (ENAs), at 42 days of exposure.

In conclusion, the findings emphasize the need to update existing guidelines to require the evaluation of PAH mixtures not only based on total concentration but also considering the specific components and their proportions. Additionally, incorporating a multi-biomarker approach is critical for capturing the full spectrum of biological responses to toxicants and accurately assessing environmental risks, especially when dealing with complex chemical mixture.

CRediT authorship contribution statement

Institutional statement

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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5.6 Appendices

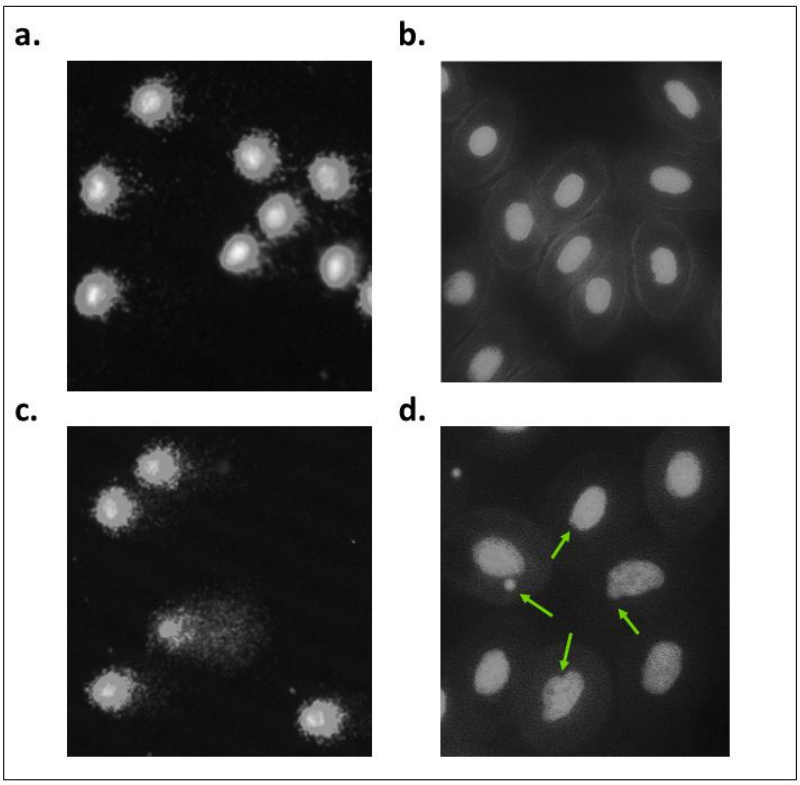


Figure 5.6 - Mature blood cells of seabream exposed to 0.2 nM of Phe, B[a]P and the mixtures Phe: B[a]P (1:2) and Phe:B[a]P (2:1) for 42 days. a. Comet assay from control group b. Erythrocytic nuclear abnormalities from control group. c. Comet assay from Phe:B[a]P 2:1 treatment b. Erythrocytic nuclear abnormalities from Phe:B[a]P 2:1 treatment.

Table 5.1 - Primers used in the quantification of CYP1A, TP53 and GST-3 mRNAs. GAPDH was used as the control gene. Primers were designed with Primer-Blast from the following reference sequences for *S. aurata* retrieved from Gen Bank.

Gene	Primer	Sequence	NCBI Reference Sequence
CYP1A	Forward	5′-CCTTCCTGGAGGCCTTTATCC-3′	XM_030415642.1
	Reverse	5′-GTCTTTTGACGAGCAGTGAGG-3′	
TP53	Forward	5′-GCGGACAACCCAAAGATCCC-3′	XM_030431663.1
	Reverse	5′-ACGGTCGGTATGGTCGAGAT-3′	
GST	Forward	5′-TTGCCCTTCACTCCAGATCC-3′	XM_030441496.1
	Reverse	5′-CCCGGACAGCACCTTTTCG-3′	
GAPDH	Forward	5′-ATCACTGTTTTCCACGAGAGGG-3′	XM_030394814.1
	Reverse	5′-TCAACAACGTACTGGGCACC-3′	

Table 5.2 - Pairwise comparisons between groups using Tukey's test based on biochemical analysis on liver samples.

Liver					
GPx					
Comparison	**estimate**	**SE**	**df**	**t.ratio**	**p.value**
B[a]P - Control	0.0079	0.0357	27	0.2210	0.9994
B[a]P - Phe:B[a]P 1:2	-0.0110	0.0351	27	-0.3130	0.9978
B[a]P - Phe:B[a]P 2:1	-0.0525	0.0366	27	-1.4360	0.6109
B[a]P - Phe	-0.0830	0.0393	27	-2.1140	0.2433
Control - Phe:B[a]P 1:2	-0.0189	0.0357	27	-0.5290	0.9836
Control - Phe:B[a]P 2:1	-0.0604	0.0371	27	-1.6270	0.4938
Control - Phe	-0.0909	0.0398	27	-2.2850	0.1806
Phe:B[a]P 1:2 - Phe:B[a]P 2:1	-0.0415	0.0366	27	-1.1350	0.7867
Phe:B[a]P 1:2 - Phe	-0.0720	0.0393	27	-1.8350	0.3755
Phe:B[a]P 2:1 - Phe	-0.0305	0.0406	27	-0.7530	0.9417
GR					
Comparison	**estimate**	**SE**	**df**	**t.ratio**	**p.value**
B[a]P - Control	-0.1617	0.1580	27	-1.0250	0.8418
B[a]P - Phe:B[a]P 1:2	0.0540	0.1550	27	0.3480	0.9967
B[a]P - Phe:B[a]P 2:1	-0.0621	0.1620	27	-0.3840	0.9951
B[a]P - Phe	-0.0632	0.1740	27	-0.3640	0.9960
Control - Phe:B[a]P 1:2	0.2157	0.1580	27	1.3670	0.6533
Control - Phe:B[a]P 2:1	0.0996	0.1640	27	0.6070	0.9727
Control - Phe	0.0985	0.1760	27	0.5600	0.9797
Phe:B[a]P 1:2 - Phe:B[a]P 2:1	-0.1161	0.1620	27	-0.7180	0.9505
Phe:B[a]P 1:2 - Phe	-0.1172	0.1740	27	-0.6750	0.9602
Phe:B[a]P 2:1 - Phe	-0.0011	0.1790	27	-0.0060	1.0000
GSH					
Comparison	**estimate**	**SE**	**df**	**t.ratio**	**p.value**
B[a]P - Control	1.5764	0.3560	27	4.4280	**0.0012**
B[a]P - Phe:B[a]P 1:2	0.5655	0.3500	27	1.6150	0.5012
B[a]P - Phe:B[a]P 2:1	0.7657	0.3640	27	2.1010	0.2489
B[a]P - Phe	0.6144	0.3920	27	1.5690	0.5288
Control - Phe:B[a]P 1:2	-1.0109	0.3560	27	-2.8400	0.0597
Control - Phe:B[a]P 2:1	-0.8107	0.3700	27	-2.1910	0.2135
Control - Phe	-0.9620	0.3970	27	-2.4250	0.1392
Phe:B[a]P 1:2 - Phe:B[a]P 2:1	0.2002	0.3640	27	0.5490	0.9811
Phe:B[a]P 1:2 - Phe	0.0489	0.3920	27	0.1250	0.9999
Phe:B[a]P 2:1 - Phe	-0.1513	0.4040	27	-0.3740	0.9956
GST					
Comparison	**estimate**	**SE**	**df**	**t.ratio**	**p.value**
B[a]P - Control	0.1063	0.0474	27	2.2430	0.1950
B[a]P - Phe:B[a]P 1:2	0.0030	0.0467	27	0.0640	1.0000
B[a]P - Phe:B[a]P 2:1	-0.0058	0.0486	27	-0.1210	0.9999
B[a]P - Phe	0.0305	0.0522	27	0.5850	0.9762
Control - Phe:B[a]P 1:2	-0.1033	0.0474	27	-2.1790	0.2178
Control - Phe:B[a]P 2:1	-0.1122	0.0493	27	-2.2760	0.1835
Control - Phe	-0.0758	0.0529	27	-1.4350	0.6112
Phe:B[a]P 1:2 - Phe:B[a]P 2:1	-0.0088	0.0486	27	-0.1830	0.9997
Phe:B[a]P 1:2 - Phe	0.0275	0.0522	27	0.5270	0.9838
Phe:B[a]P 2:1 - Phe	0.0363	0.0539	27	0.6750	0.9601
CAT					
Comparison	**estimate**	**SE**	**df**	**t.ratio**	**p.value**
B[a]P - Control	4.5010	5.3700	27	0.8390	0.9161
B[a]P - Phe:B[a]P 1:2	7.1880	5.2800	27	1.3610	0.6565
B[a]P - Phe:B[a]P 2:1	0.7460	5.5000	27	0.1360	0.9999
B[a]P - Phe	-4.0910	5.9000	27	-0.6930	0.9563
Control - Phe:B[a]P 1:2	2.6870	5.3700	27	0.5010	0.9866
Control - Phe:B[a]P 2:1	-3.7550	5.5800	27	-0.6730	0.9606
Control - Phe	-8.5910	5.9800	27	-1.4360	0.6105
Phe:B[a]P 1:2 - Phe:B[a]P 2:1	-6.4420	5.5000	27	-1.1720	0.7667
Phe:B[a]P 1:2 - Phe	-11.2780	5.9000	27	-1.9110	0.3360
Phe:B[a]P 2:1 - Phe	-4.8360	6.1000	27	-0.7930	0.9303
LPO					
Comparison	**estimate**	**SE**	**df**	**t.ratio**	**p.value**
B[a]P - Control	0.4548	3.3300	27	0.1370	0.9999
B[a]P - Phe:B[a]P 1:2	2.6514	3.2700	27	0.8100	0.9251
B[a]P - Phe:B[a]P 2:1	2.6008	3.4100	27	0.7640	0.9388
B[a]P - Phe	0.1764	3.6600	27	0.0480	1.0000
Control - Phe:B[a]P 1:2	2.1965	3.3300	27	0.6600	0.9631
Control - Phe:B[a]P 2:1	2.1459	3.4600	27	0.6210	0.9705
Control - Phe	-0.2785	3.7100	27	-0.0750	1.0000
Phe:B[a]P 1:2 - Phe:B[a]P 2:1	-0.0506	3.4100	27	-0.0150	1.0000
Phe:B[a]P 1:2 - Phe	-2.4750	3.6600	27	-0.6770	0.9598
Phe:B[a]P 2:1 - Phe	-2.4244	3.7800	27	-0.6420	0.9667

Table 5.3 - Pairwise comparisons between groups using Tukey's test based on biochemical analysis on gill samples

Gills					
GPx					
Comparison	**estimate**	**SE**	**df**	**t.ratio**	**p.value**
B[a]P - Control	0.02018	0.0346	28	0.5840	0.9764
B[a]P - Phe:B[a]P 1:2	0.01339	0.0346	28	0.3870	0.9950
B[a]P - Phe:B[a]P 2:1	0.0502	0.0346	28	1.4520	0.6007
B[a]P - Phe	0.00482	0.0359	28	0.1340	0.9999
Control - Phe:B[a]P 1:2	-0.0068	0.0332	28	-0.2050	0.9996
Control - Phe:B[a]P 2:1	0.03002	0.0332	28	0.9040	0.8931
Control - Phe	-0.01536	0.0346	28	-0.4440	0.9915
Phe:B[a]P 1:2 - Phe:B[a]P 2:1	0.03681	0.0332	28	1.1080	0.8008
Phe:B[a]P 1:2 - Phe	-0.00856	0.0346	28	-0.2480	0.9991
Phe:B[a]P 2:1 - Phe	-0.04538	0.0346	28	-1.3120	0.6859
GR					
Comparison	**estimate**	**SE**	**df**	**t.ratio**	**p.value**
B[a]P - Control	0.000223	0.0371	28	0.0060	1.0000
B[a]P - Phe:B[a]P 1:2	0.014763	0.0371	28	-0.3980	0.9944
B[a]P - Phe:B[a]P 2:1	0.008774	0.0371	28	-0.2370	0.9993
B[a]P - Phe	0.020444	0.0385	28	-0.5310	0.9833
Control - Phe:B[a]P 1:2	0.014986	0.0356	28	-0.4210	0.9931
Control - Phe:B[a]P 2:1	0.008997	0.0356	28	-0.2520	0.9990
Control - Phe	0.020667	0.0371	28	-0.5570	0.9801
Phe:B[a]P 1:2 - Phe:B[a]P 2:1	0.005989	0.0356	28	0.1680	0.9998
Phe:B[a]P 1:2 - Phe	0.005681	0.0371	28	-0.1530	0.9999
Phe:B[a]P 2:1 - Phe	-0.01167	0.0371	28	-0.3150	0.9977
GSH					
Comparison	**estimate**	**SE**	**df**	**t.ratio**	**p.value**
B[a]P - Control	2.2610	0.3410	28	6.6310	**<.0001**
B[a]P - Phe:B[a]P 1:2	0.6010	0.3410	28	1.7640	0.4137
B[a]P - Phe:B[a]P 2:1	0.7430	0.3410	28	2.1780	0.2174
B[a]P - Phe	0.9700	0.3540	28	2.7420	0.0727
Control - Phe:B[a]P 1:2	-1.6600	0.3280	28	-5.0660	**0.0002**
Control - Phe:B[a]P 2:1	-1.5180	0.3280	28	-4.6350	**0.0007**
Control - Phe	-1.2910	0.3410	28	-3.7850	**0.0062**
Phe:B[a]P 1:2 - Phe:B[a]P 2:1	0.1410	0.3280	28	0.4310	0.9924
Phe:B[a]P 1:2 - Phe	0.3690	0.3410	28	1.0810	0.8144
Phe:B[a]P 2:1 - Phe	0.2280	0.3410	28	0.6670	0.9618
GST					
Comparison	**estimate**	**SE**	**df**	**t.ratio**	**p.value**
B[a]P - Control	0.0891	0.0198	28	4.4970	**0.0010**
B[a]P - Phe:B[a]P 1:2	0.0404	0.0198	28	2.0370	0.2754
B[a]P - Phe:B[a]P 2:1	0.0302	0.0198	28	1.5230	0.5570
B[a]P - Phe	0.0188	0.0206	28	0.9150	0.8889
Control - Phe:B[a]P 1:2	-0.0488	0.0190	28	-2.560	0.1058
Control - Phe:B[a]P 2:1	-0.0589	0.0190	28	-3.0950	**0.0331**
Control - Phe	-0.0703	0.0198	28	-3.5470	**0.0112**
Phe:B[a]P 1:2 - Phe:B[a]P 2:1	-0.0102	0.0190	28	-0.5350	0.9829
Phe:B[a]P 1:2 - Phe	-0.0216	0.0198	28	-1.0880	0.8113
Phe:B[a]P 2:1 - Phe	-0.0114	0.0198	28	-0.5740	0.9779
CAT					
Comparison	**estimate**	**SE**	**df**	**t.ratio**	**p.value**
B[a]P - Control	-0.0768	0.1280	28	-0.5990	0.9741
B[a]P - Phe:B[a]P 1:2	0.1167	0.1280	28	0.9100	0.8906
B[a]P - Phe:B[a]P 2:1	0.0710	0.1280	28	0.5540	0.9805
B[a]P - Phe	-0.0522	0.1330	28	-0.3930	0.9947
Control - Phe:B[a]P 1:2	0.1935	0.1230	28	1.5710	0.5275
Control - Phe:B[a]P 2:1	0.1478	0.1230	28	1.2000	0.7514
Control - Phe	0.0246	0.1280	28	0.1920	0.9997
Phe:B[a]P 1:2 - Phe:B[a]P 2:1	-0.0457	0.1230	28	-0.3710	0.9957
Phe:B[a]P 1:2 - Phe	-0.1689	0.1280	28	-1.3180	0.6828
Phe:B[a]P 2:1 - Phe	-0.1233	0.1280	28	-0.9610	0.8700
LPO					
Comparison	**estimate**	**SE**	**df**	**t.ratio**	**p.value**
B[a]P - Control	-5.9590	1.7000	28	-3.513	**0.0122**
B[a]P - Phe:B[a]P 1:2	2.5010	1.7000	28	1.474	0.5869
B[a]P - Phe:B[a]P 2:1	3.1420	1.7000	28	1.852	0.3654
B[a]P - Phe	-1.9340	1.7600	28	-1.098	0.8058
Control - Phe:B[a]P 1:2	8.4600	1.6300	28	5.191	**0.0002**
Control - Phe:B[a]P 2:1	9.1010	1.6300	28	5.584	**0.0001**
Control - Phe	4.0260	1.7000	28	2.373	0.1526
Phe:B[a]P 1:2 - Phe:B[a]P 2:1	0.6410	1.6300	28	0.393	0.9947
Phe:B[a]P 1:2 - Phe	-4.4350	1.7000	28	-2.614	0.0948
Phe:B[a]P 2:1 - Phe	-5.0760	1.7000	28	-2.992	**0.0419**

Table 5.4 - Pearson correlation matrix showing the relationships between the biomarkers analysed in liver. Significant correlations are indicated as follows: ** for $p < 0.01$ and * for $p < 0.05$

	CAT	GSH	GST	GPx	LPO	GR
CAT	1	0.166321	-0.25545	0.120685	-0.18333	0.302207
GSH	0.166321	1	0.120922	-0.32492	0.284522	0.120464
GST	-0.25545	0.120922	1	-0.36114 *	0.12066	-0.4689 **
GPx	0.120685	-0.32492	-0.36114 *	1	-0.05446	0.009771
LPO	-0.18333	0.284522	0.12066	-0.05446	1	-0.306
GR	0.302207	0.120464	-0.4689 **	0.009771	-0.306	1

Table 5.5 - Results of a PERMANOVA performed on the MDS ordination of gill samples

Gills					
Comparison	**Df**	**SumsOfSqs**	**F.Model**	**R2**	**p.value**
Phe vs B[a]P	1	0.222709	13.69042	0.5329	**0.001**
Phe vs Phe:B[a]P 1:2	1	0.072151	5.416679	0.294118	**0.03**
Phe vs Phe:B[a]P 2:1	1	0.03786	2.111109	0.139706	0.164
B[a]P vs Phe:B[a]P 1:2	1	0.048716	2.056432	0.136582	0.193
B[a]P vs Phe:B[a]P 2:1	1	0.088034	3.110411	0.193068	0.086
Phe:B[a]P 1:2 vs Phe:B[a]P 2:1	1	0.007717	0.312346	0.021824	0.624

Table 5.6 - Pearson correlation matrix showing the relationships between the biomarkers analysed in gills. Significant correlations are indicated as follows: *** for $p < 0.001$ and * for $p < 0.05$

Gills					
Comparison	**Df**	**SumsOfSqs**	**F.Model**	**R2**	**p.value**
Phe vs B[a]P	1	0.222709	13.69042	0.5329	**0.001**
Phe vs Phe:B[a]P 1:2	1	0.072151	5.416679	0.294118	**0.03**
Phe vs Phe:B[a]P 2:1	1	0.03786	2.111109	0.139706	0.164
B[a]P vs Phe:B[a]P 1:2	1	0.048716	2.056432	0.136582	0.193
B[a]P vs Phe:B[a]P 2:1	1	0.088034	3.110411	0.193068	0.086
Phe:B[a]P 1:2 vs Phe:B[a]P 2:1	1	0.007717	0.312346	0.021824	0.624

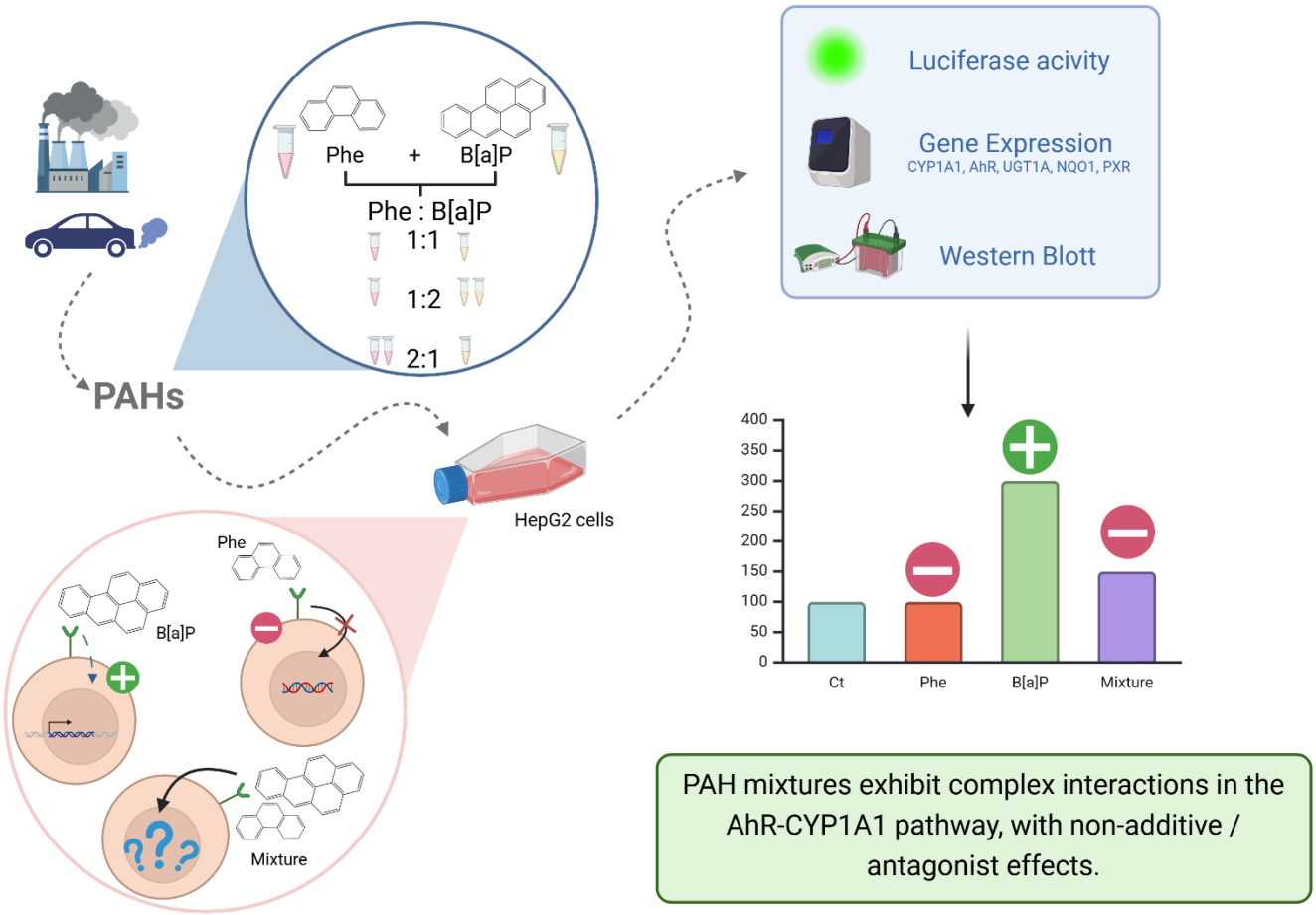
6

AhR activation and gene expression patterns in HepG2 cells exposed to PAH mixtures

This Chapter is a manuscript in preparation for submission:

Matos, Beatriz; Branco, Vasco; Martins, Marta. "AhR activation and gene expression patterns in HepG2 cells exposed to PAH mixtures"

Graphical abstract



Abstract

Polycyclic aromatic hydrocarbons (PAHs) are a group of chemical compounds composed of fused aromatic rings. They are commonly found in the environment as a result of incomplete combustion of organic materials, such as fossil fuels, wood, and tobacco. These compounds are widespread environmental pollutants and are known for their persistence in the environment in complex mixtures. PAHs have been linked to various adverse health effects, including carcinogenic, mutagenic, and endocrine-disrupting properties, primarily through their interaction with the aryl hydrocarbon receptor (AhR). The aim of this study, is to investigate the behaviour of PAH mixtures in human hepatocyte cells (HepG2), focusing on how different proportions of binary PAH mixtures affect AhR signalling and related downstream molecular events. The PAHs were tested in HepG2 as mixtures with final concentrations equivalent to the individual compounds, using varying ratios (1:1, 1:2, 2:1) to examine potential additive, synergistic, or antagonistic interactions. AhR activation was assessed through a luciferase reporter assay, while quantitative PCR was used to evaluate the expression of PAH metabolism-related genes (CYP1A1, AhR, UGT1A, NQO1 and PXR). Additionally, CYP1A1 protein levels were evaluated by western blot as a marker of AhR activation. Statistical analysis via t-tests was performed to determine the nature of the interactions within the mixtures. This integrated approach provides a detailed understanding of how PAH mixtures influence AhR-mediated responses, offering insights into their complex behaviour and potential environmental and health impacts.

This study demonstrates that varying PAH mixture ratios provides insights into component interactions and their effects on the AhR-CYP1A1 pathway. While B[a]P induced the strongest AhR and CYP1A1 activity, mixtures showed antagonistic interactions at lower concentrations, likely due to competition with Phe. These findings highlight the complexity of PAH mixture toxicity, where receptor activation does not always correlate with downstream effects. The results emphasize the importance of considering both individual PAH properties and their interactions for understanding their full toxicological impact and informing environmental risk assessments.

Keywords: PAHs; HepG2; AhR; CYP1A1; Luciferase

6.1 Introduction

PAHs, primarily from fossil fuel combustion, are persistent pollutants found in air, water, and soil[404]. In the atmosphere, they attach to particulate matter, contributing to air pollution and respiratory issues. In water and soil, their low solubility leads to accumulation in sediments, causing bioaccumulation in aquatic life and disrupting ecosystems[405]. Their toxicity and carcinogenicity pose serious risks to biodiversity and human health, making their environmental presence a significant concern[406].

The main risk regarding PAHs stem from their well-documented genotoxic, mutagenic, and carcinogenic properties. The International Agency for Research on Cancer (IARC)[156] classifies several PAHs, such as B[a]P, as either probable or confirmed human carcinogens. These classifications are based on extensive epidemiological and toxicological evidence linking PAH exposure to an increased risk of cancer. Furthermore, the toxicological relevance of PAHs has earned them inclusion in the Priority Substances List of the Marine Strategy Framework Directive (MSFD)[112] and recognition by both the U.S. Environmental Protection Agency (EPA)[407] and the World Health Organization (WHO)[408] as significant pollutants of concern.

PAHs are categorized as procarcinogens and promutagens, necessitating metabolic activation to manifest their carcinogenic properties. This metabolic activation is predominantly facilitated by cytochrome P450 (CYP) enzymes, leading to the generation of reactive intermediates such as diol-epoxides and quinones[160]. These intermediates interact with cellular macromolecules, forming bulky DNA adducts, promoting nucleotide oxidation, and inducing other types of DNA damage, some of which may be irreversible[409]. The efficient detoxification of these reactive metabolites hinges on Phase II conjugation processes mediated by Glutathione S-Transferases (GST), utilizing glutathione (GSH) as a substrate. However, PAH metabolism also produces reactive oxygen species (ROS), which exacerbate oxidative stress, enhance oxidative DNA damage, and contribute to GSH depletion[158,178]. The toxicity exerted by PAHs is contingent upon both the structure of the parent compound and the nature of the metabolites produced, a factor largely influenced by the specific CYP isoform involved in their metabolism. High molecular weight PAHs, especially those containing a reactive "Bay region," are more potent mutagens. This structural feature facilitates binding with DNA and

interactions with both aryl hydrocarbon receptor (AhR) and CYP enzymes, amplifying their biological activity[346].

AhR is a key player in PAH-mediated toxicity. Upon binding to PAHs, AhR is activated, translocates to the nucleus, and regulates the expression of target genes, including those encoding enzymes involved in xenobiotic metabolism[161]. To study AhR activation by PAHs, luciferase reporter assays provide a sensitive and efficient tool. In this assay, genetically modified cells are used, with a luciferase gene linked to AhR-responsive elements. PAH binding leads to transcriptional activation of luciferase, producing a quantifiable luminescent signal that correlates directly with the level of AhR activation. This rapid and sensitive approach is particularly valuable for evaluating the toxicological impact of PAH exposure and understanding the potential health risks associated with environmental contamination[410].

A significant challenge in assessing PAH toxicity lies in their environmental occurrence as complex mixtures rather than as isolated compounds[411]. While there is extensive knowledge of the mode of action for certain individual PAHs, the toxicity of PAH mixtures remains poorly understood, as interactions between different components can lead to non-additive effects[412]. Studies have indicated that synergistic or supra-additive interactions may occur within these mixtures, complicating the prediction of their overall toxicological impact[413]. Moreover, current environmental guidelines often fail to adequately account for these complex mixture effects, underestimating the risks posed by PAH exposure to aquatic organisms and ecosystems.

The HepG2 cell line, derived from a human hepatocellular carcinoma, is a widely used *in vitro* model for studying PAH toxicity and AhR activation. These cells maintain several key liver functions, such as plasma protein production and inducible expression of CYP enzymes, making them an ideal model for investigating liver function, drug metabolism, and xenobiotic toxicity[414].

The specific objective of this study is to assess whether changes in the proportions of individual compounds within PAH mixtures influence the detoxification mechanisms of response and to determine whether a mixture demonstrates synergism, additivity, or antagonism.

Existing tools for evaluating mixture effects, such as GraphPad Prism, Combenefit, SynergyFinder, and Mixtox, rely on comprehensive concentration-response datasets and are often ill-suited for analysing the effects of mixtures based solely on varying component proportions. To address this limitation, we propose a statistical test to classify PAH mixtures.

6.2 Materials and Methods

6.2.1 Cell Culture

HepG2 human hepatoma cells were generously provided by Dr. Maria João Silva (National Health Institute Dr. Ricardo Jorge). The cells were cultured in a complete medium consisting of a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and F-12, supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin (10,000 U/ml)/streptomycin (10,000 μg/ml). Cells were maintained in a humidified incubator at 37°C with 5% CO2. The culture medium was replaced every 2–3 days, and cells were sub-cultured at 70–80% confluence.

6.2.2 PAHs exposure

All treatments were prepared within a concentration range of 5–100 μM for individual compounds (Phenanthrene (Phe), Benzo[a]pyrene (B[a]P) and Benzo[b]fluoranthene (B[b]F). Binary mixtures were tested using ratios of 1:1, 1:2, and 2:1. These ratios indicate that the final concentration of each mixture matches the concentration of the individual components. For instance, if the target concentration is 5 μM, then for individual treatments, [Phe] = [B[a]P] = 5 μM and for the mixture [Phe]+[B[a]P] = 5 μM. In certain endpoints, an additional treatment of the 1:1 mixture was included to double the concentration of each individual compound. As an example, if the concentrations were set such that [Phe] = [B[a]P] = 5 μM individually, then resulted in a combined concentration of [Phe] + [B[a]P] = 10 μM in the mixture.

6.2.3 Expression of PAH metabolism-related genes

Cells were seeded in 6-well plates at a density of 1.5×10^6 cells per well. After a 24-hour exposure to different PAHs treatments (Section 6.2.2), RNA was extracted from each sample using the NZY Total RNA Isolation Kit (NZYTech, Portugal), following the manufacturer's protocol. After cell lysis using a specific lysis buffer and β-mercaptoethanol, samples were homogenized by filtration (NZY spin columns), mixed with 70% ethanol, and then purified through desalting and DNase I digestion, with several washing steps before the final elution of RNA with RNase-free water. Samples were stored at −80°C until analysis. RNA

quantification was carried out using a NanoDrop 2000 (Thermo Scientific), and purity was assessed by evaluating the 260/280 nm and 260/230 nm absorbance 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 minutes at 25°C, followed by 30 minutes at 50°C. Inactivation was performed for 1 minute at 85°C, and the samples were cooled on ice. Then, 1 µL of E. coli RNase was added to each sample and incubated for 20 minutes at 37°C. Samples were stored at −20°C until further qPCR analysis.

Quantitative analysis of cytochrome P450 family 1 subfamily A member 1 (CYP1A1), UDP glucuronosyltransferase family 1 member A (UGT1A), NAD(P)H quinone dehydrogenase 1 (NQO1), Aryl Hydrocarbon Receptor (AhR) and pregnane X receptor (PXR) expression was conducted using the 2^-ΔΔCT method (Livak and Schmittgen, 2001)[340], with GAPDH serving as the housekeeping gene. Primers were designed with Primer-blast using Reference Sequences from GeneBank. 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 final 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 an initial 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).

6.2.4 CYP1A1 protein levels

Cells (1.5×10^6 per well) were seeded in 6-well plates and exposed for 24 hours to different treatments (Section 6.2.2). After treatment, cells were lysed using a lysis buffer containing EDTA, Triton and protease inhibitors to extract proteins for further analysis.

CYP1A1 expression was assessed by Western blot analysis in the soluble protein fraction (40 µg of protein), using SDS-PAGE on a 4–12% Bis–Tris gel with MES running buffer under reducing conditions (140 V for 1 hour). Proteins were then transferred onto a nitrocellulose membrane by dry transfer (20 V for 7 minutes) using the iBlot™ 2 Dry Blotting System (Invitrogen), followed by blocking for 1 hour at room temperature with a 5% milk solution. The membrane was incubated with the primary antibody overnight at 4°C, followed by PBS washes and incubation with the appropriate secondary antibody. Protein expression

levels were normalized to total protein loading on the gel, which was assessed by Ponceau S staining (Branco et al., 2014)[178]. The primary antibody used was a goat anti-CYP1A1 polyclonal antibody (126887, Abcam), while the secondary antibody was a rabbit anti-goat IgG-HRP antibody (Sc-2768 Santa Cruz).

6.2.5 Reporter AhR assay

Cells (1×10^5 per well) were seeded in 24-well plates 24 hours prior to transfection. For the transfection, a mixture was prepared in opti-MEM such that 1 μL of the DNA mixture added per well contained 0.5 μg of luciferase reporter plasmid and 0.1 μg of β-galactosidase plasmid. The transfection mixture also included the appropriate amount of Lipofectamine reagent, as recommended by the manufacturer, to achieve optimal DNA-Lipofectamine complex formation. After 24 hours, cells were treated with PAHs (section 6.2.2) and incubated for 24h at 37°C. Following treatment, the medium was aspirated, and cells were gently washed with 500 μL of phosphate-buffered saline (PBS). Cell lysis was performed by adding a passive lysis buffer to each well. The plate was then frozen at -80°C to facilitate mechanical lysis. After freezing, the plate was thawed at room temperature, ensuring thorough lysis of the cells. Luciferase activity was measured by transferring 10 μL of cell lysate from each well into a luminescence microplate. To each sample, 70 μL of luciferase substrate solution was added, and luminescence was recorded using a microplate reader (SPECTROstar® Omega, BMG Labtech). For normalization, β-galactosidase activity was measured using a commercially available β-galactosidase assay kit. A 25 μL aliquot of the lysate was transferred to a separate plate, and the kit's reagents were added according to the manufacturer's instructions. Luminescence was then measured as for luciferase. Luciferase activity was normalized to β-galactosidase activity to account for variations in transfection efficiency. Normalized luciferase activity was plotted against the concentrations of the test compounds to determine fold induction or inhibition of AhR transcriptional activity compared to the controls.

6.2.6 Total protein determination

Total protein concentration in cell lysates was determined using the Bradford method (Bradford, 1976)[338]. In summary, each sample was mixed with a 5× diluted Coomassie dye (BioRad) in 96-well plates, and the absorbance was measured at 595 nm using a microplate

reader. Protein concentrations were calculated from a calibration curve (ranging from 1 to 16 μg/μL) using BSA as the standard.

6.2.7 Statistical analysis

Differences between groups were evaluated using a One-way ANOVA followed by Tukey's post-hoc test when the data met normality assumptions, using R software for all statistical analyses. For non-parametric data, the Kruskal-Wallis rank sum test was applied, followed by the Wilcoxon rank-sum test with Bonferroni correction for multiple comparisons when significant differences were observed. All assays were based on a minimum of three independent experiments, and the results are represented as mean ± standard error. Additionally, a control groups was used: a solvent control with HepG2 complete medium containing 0.5% DMSO. Differences between groups were considered significant at $p < 0.05$.

6.2.8 Mathematical approach for evaluating PAH mixtures

6.2.8.1 Mixtures Interaction

Evaluating the combined effects of multiple compounds is fundamental for understanding mixture interactions. Two of the most widely used theoretical frameworks for this purpose are Concentration Addition (CA) introduced by Loewe and Muischnek (1928)[120] and Independent Action (IA), proposed by Bliss (1939)[122]. Concentration Addition is used for mixtures of chemicals that share similar modes of action, where the predicted effect of the mixture is determined by adding the doses of the individual components that contribute to the same response level. This approach is suitable for compounds that act on the same biological target, assuming their toxicity or biological effect is proportional to their combined concentrations[415]. In contrast, Independent Action applies to mixtures of chemicals that operate via different mechanisms of action. In this model, the overall effect of the mixture is considered independent for each compound, implying that each component contributes to the final effect without influencing the activity of the others[121].

To quantitatively assess whether a mixture exhibits synergism, additivity, or antagonism, a ratio is used, which is defined as the observed effect of the mixture divided by the expected effect. Herein, this ratio is referred to as the Mixture Effect Index (MEI), although

it may be known by different names in other contexts[124,416]. The expected effect was calculated using the CA model, which was chosen over the IA model due to the structural and chemical similarities among the PAHs studied. These concepts form the basis for understanding how individual chemicals interact within a mixture and influence the overall toxicological or biological outcome.

To calculate the theoretical effect of each mixture based on CA model, weighted averages of observed individual effects were used, reflecting the specific ratio of each compound in the mixture. For example, in the Phe:B[a]P 1:1 mixture, the expected effect was calculated as:

$$Expected\ Effect\ (1{:}1) = \frac{1}{2}\ Effect\ Phe + \frac{1}{2}\ Effect\ B[a]P$$

This approach assumes that the contribution of each compound to the overall effect is proportional to its presence in the mixture. In the 1:1 mixture, each component contributes equally (50%) to the expected outcome, which is why their observed effects are averaged with equal weighting.

On the other hand, for the Phe:B[a]P 1:2 mixture, the expected effect was:

$$Expected\ Effect\ (1{:}2) = \frac{1}{3}\ Effect\ Phe + \frac{2}{3}\ Effect\ B[a]P$$

In this case, the weighting reflects the specific ratio of the components in the mixture, meaning that B[a]P, which is present in higher proportion, contributes more heavily to the expected effect. This weighted averaging approach was consistently applied to all mixture ratios to determine the theoretical effects.

Using the calculated theoretical value, the Mixture Effect Index (MEI) can be determined to assess the nature of the interactions within the mixtures. This approach allows us to quantitatively classify the interactions as synergistic, antagonistic, or additive, depending on the comparison between observed and expected effects. A MEI equal to 1 indicates additivity, suggesting that the observed effect aligns with the predicted effect according to the selected model. An MEI greater than 1 suggests synergism, meaning the

combined effect of the compounds exceeds the expected effect. Conversely, an MEI less than 1 implies antagonism, where the mixture's effect is less than expected.

Once the MEI has been determined, it is essential to evaluate the accuracy of this prediction. Therefore, the MEI was calculated for each replicate of the assay, followed by determining the mean, standard deviation, and standard error for each condition. Subsequently, a one-sample t-test was performed with a theoretical value of 1 (the threshold for additivity) to determine whether the observed MEI was statistically different from 1 ($p < 0.05$). A statistically significant difference from 1 indicates that the obtained MEI reliably represents a deviation from additivity, thereby providing confidence in the classification of the interaction as synergistic or antagonistic. After determining the MEI and evaluating its accuracy, the data can be visualized by plotting the MEI values in a bar plot. In this plot, the x-axis represents different mixture conditions, while the y-axis crosses at 1, representing the threshold for additivity. Bars represent the mean MEI values, including standard error, and asterisks (*) indicate statistical significance from the theoretical value of 1 ($p < 0.05$). This visual representation effectively demonstrates the nature of each mixture, highlighting whether the interaction is synergistic, antagonistic, or additive (Figure 6.1). In this example it is only possible to classify the mixture 1:2 as synergistic because it is significantly ($p<0.05$) different than 1.

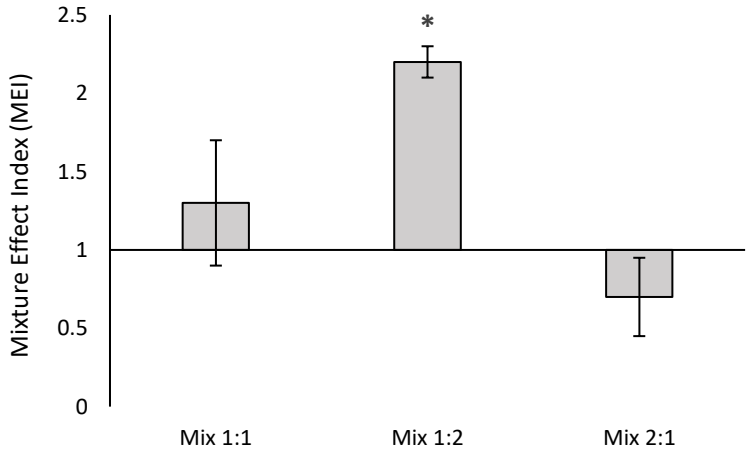


Figure 6.1 - Example of graphical representation of MEI (Mixture Effect Index)

6.3 Results

6.3.1 Expression of PAH metabolism-related genes

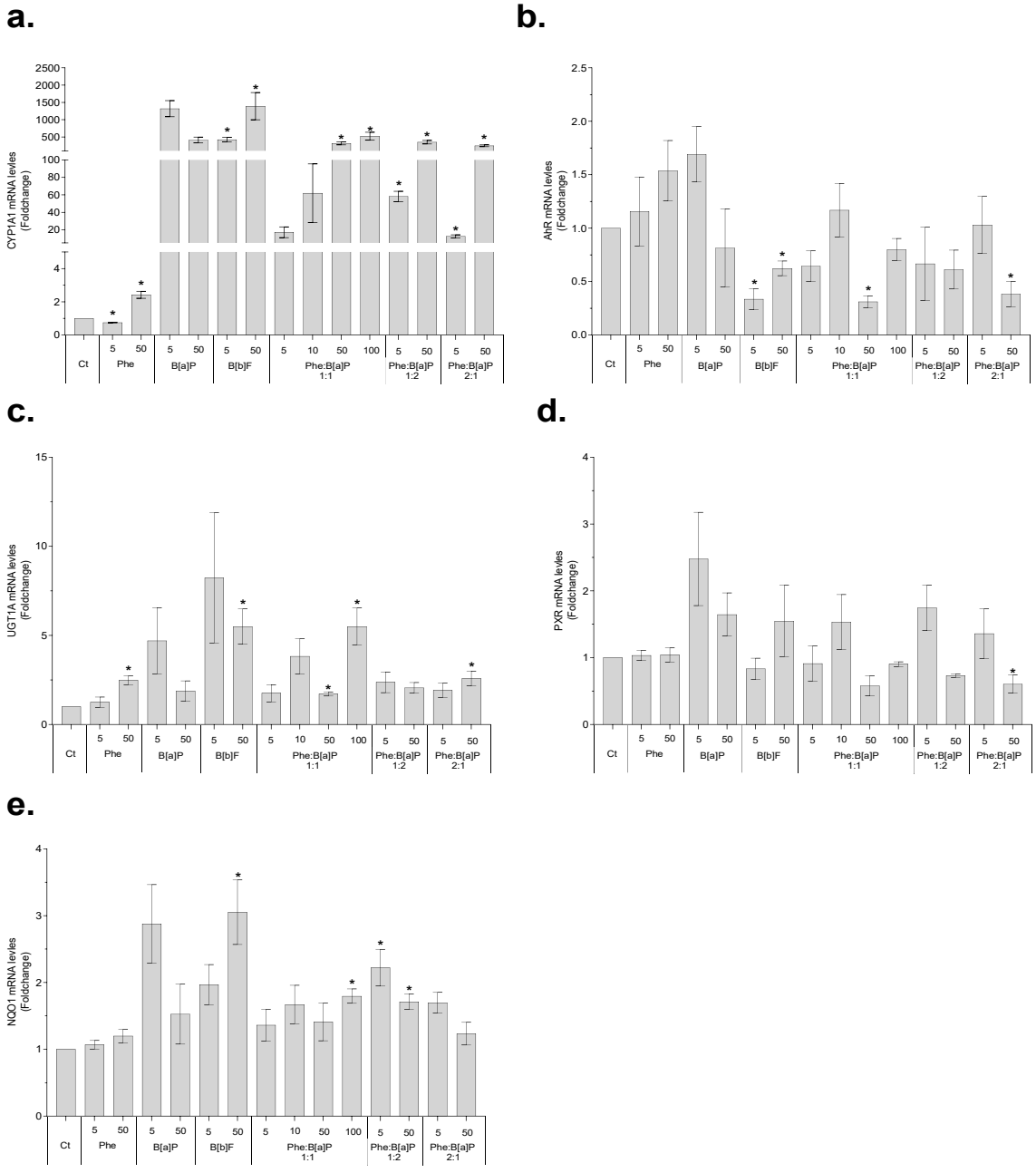


Figure 6.2 - mRNA expression levels for a. CYP1A1, b. AhR, c. UGT1A, d. PXR, and e. NQO1 in HepG2 cells exposed to 5, 10, 50, and 100 μM of Phe, B[a]P, B[b]F, and their mixtures (tPAH = 5, 10, 50, and 100 μM) after 24 hours. Data are presented as mean ± S.E. from four independent replicates. * $p < 0.05$ compared to control.

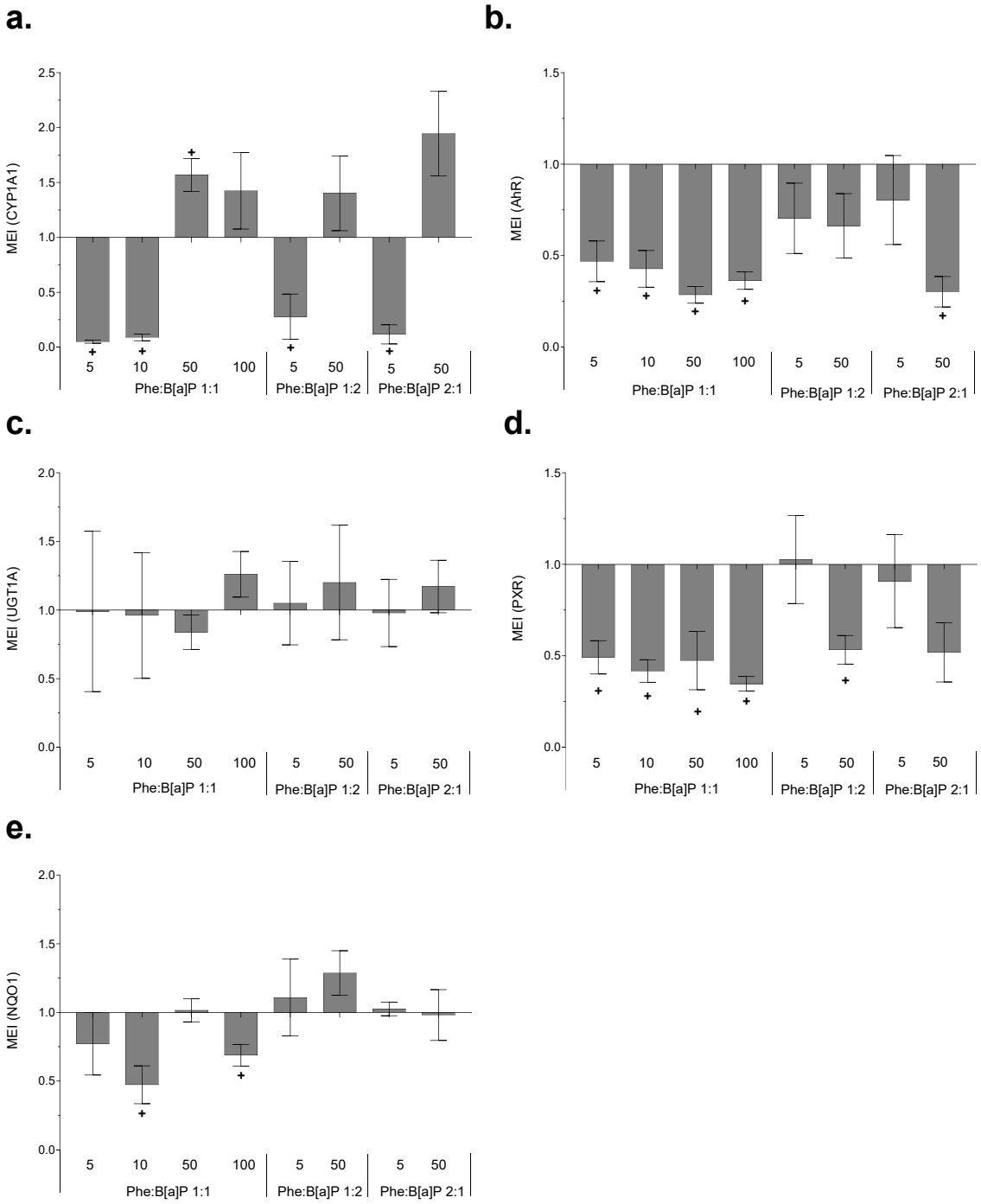


Figure 6.3 - Mixture Effect Index (MEI) for the Phe:B[a]P mixture related to PCR results for each gene: a. CYP1A1, b. AhR, c. UGT1A, d. PXR, and e. NQO1 + significant differences compared to theoretical value = 1

The analysis of mRNA expression revealed statistically significant ($p<0.05$) upregulation of CYP1A1 levels (Figure 6.2**a.**) following exposure to most treatments, with fold changes exceeding 15-fold compared to the control group, except for the Phe treatment. The

most pronounced effects were observed with B[a]P at 5 μM and B[b]F at 50 μM, which induced fold changes greater than 1000-fold.

Regarding the AhR gene (Figure 6.2 **b.**), significant decreases related to control in mRNA levels were observed for B[b]F at 5 μM and 50 μM, as well as for the 1:1 Phe:B[a]P mixture at 50 μM and the 2:1 Phe:B[a]P mixture at 50 μM.

For the UGT1A gene (Figure 6.2 **c.**), significant upregulation was noted at 50 μM for Phe, B[b]F, the 1:1 Phe:B[a]P and the 2:1 Phe:B[a]P mixtures. Furthermore, the 1:1 Phe:B[a]P mixture at 100 μM also exhibited significant mRNA induction.

In the case of PXR (Figure 6.2 **d.**), only the 2:1 Phe:B[a]P mixture at 50 μM showed a significant decrease in mRNA levels. Although not statistically significant, the treatments B[a]P, B[b]F 50 μM, and the mixtures Phe:B[a]P (1:1 10 μM, 1:2 5 μM, and 2:1 5 μM) showed fold-change values higher than those observed in the control.

Finally, NQO1 gene expression (Figure 6.2 **e.**) showed moderate upregulation, with fold changes ranging from 1.5 to 3 across various treatments including B[a]P, B[b]F, and select mixtures (1:1 100 μM and 1:2) . However, statistically significant increases were specifically observed for the 50 μM B[b]F exposure, the 1:1 Phe:B[a]P mixture at 100 μM, and both concentrations of the 1:2 Phe:B[a]P mixture.

Results from the calculation of the Mixture Effect Index (MEI) for CYP1A1 (Figure 6.3 **a.**) showed that at lower concentration levels (5 μM and 10 μM), all mixtures (1:1, 1:2, and 2:1) exhibited antagonistic behaviour. At higher concentrations all mixtures were additive, or synergistic in the case of the mixture Phe:B[a]P 1:1.

For AhR and PXR genes (Figure 6.3 **b.** and **d.** respectively), nearly all mixtures demonstrated antagonistic behaviour, with the exception of the 1:2 mixture (5 and 50μM) and 2:1 mixture at 5 μM for AhR and the 1:2 mixture (5μM) and 2:1 mixture (5 and 50μM) for PXR.

In the case of the UGT1A gene (Figure 6.3 **c.**), any mixture was significantly different than 1, making it impossible to draw definitive conclusions about whether they exhibit synergistic or antagonistic behaviour. In the absence of these interactions, their effects are assumed to be additive. For the NQO1 gene (Figure 6.3 **e.**), the only mixtures that could be clearly considered antagonistic were the 1:1 mixtures at 10 and 100 μM.

6.3.2 CYP1A1 protein levels

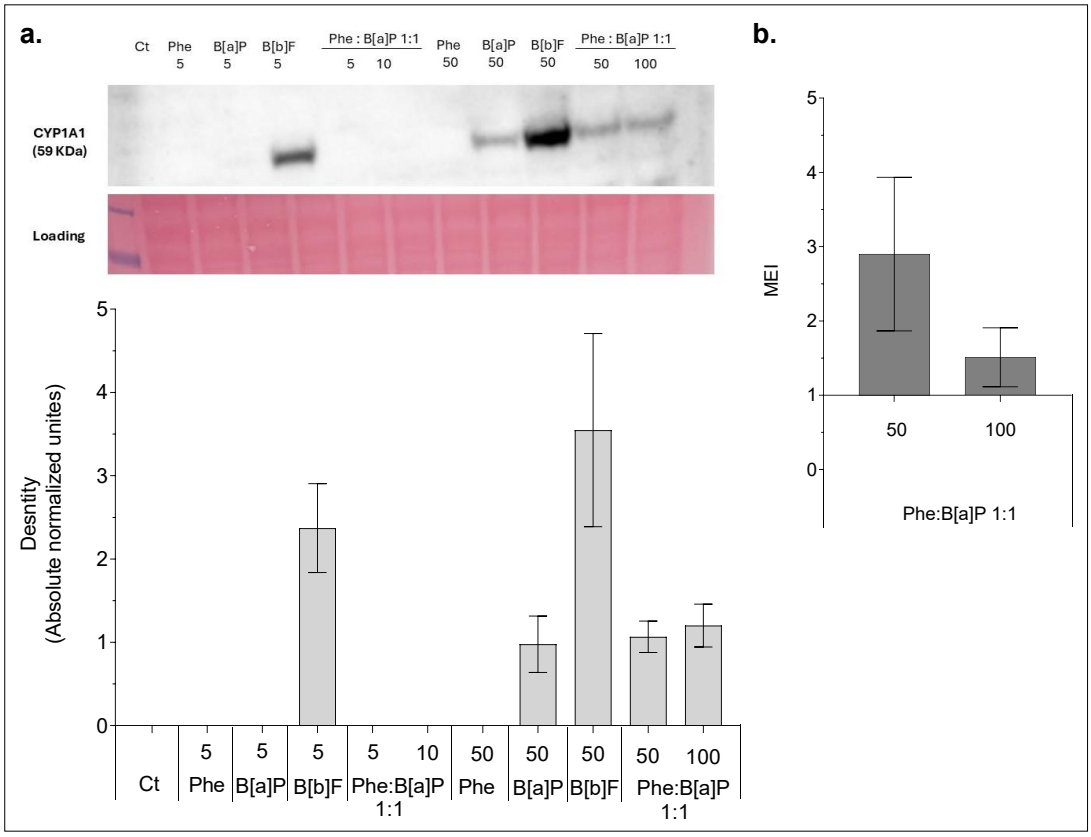


Figure 6.4 - CYP1A1 Expression (Density) in HepG2 cells after 24 h of exposure to B[a]P, Phe, B[b]F, and their mixtures at total PAH concentrations (tPAH) of 5, 10, 50, and 100 μM. Ct represents the control group. Data are presented as mean ± S.E. from four independent experiments. **a.** Grey bars represent the CYP1A1 expression levels. **b**. Dark bars represent the Mixture Effect Index (MEI) for the Phe:B[a]P 1:1 mixtures.

No detectable CYP1A1 expression was found in the following treatments: control, Phe (5 and 50 μM), B[a]P 5 μM, Mix 1:1 (5 μM and 10 μM) (Figure 6.4 **a.**). The highest expression levels were obtained for B[b]F at 5 and 50μM, being 2.3±0.5 and 3.5±1.1, respectively (Figure 6.4 **a.**). The results from the calculation of the MEI indicated that none of the mixtures showed a significant deviation from an index value of 1 (Figure 6.4 **b.**).

6.3.3 AhR reporter assay

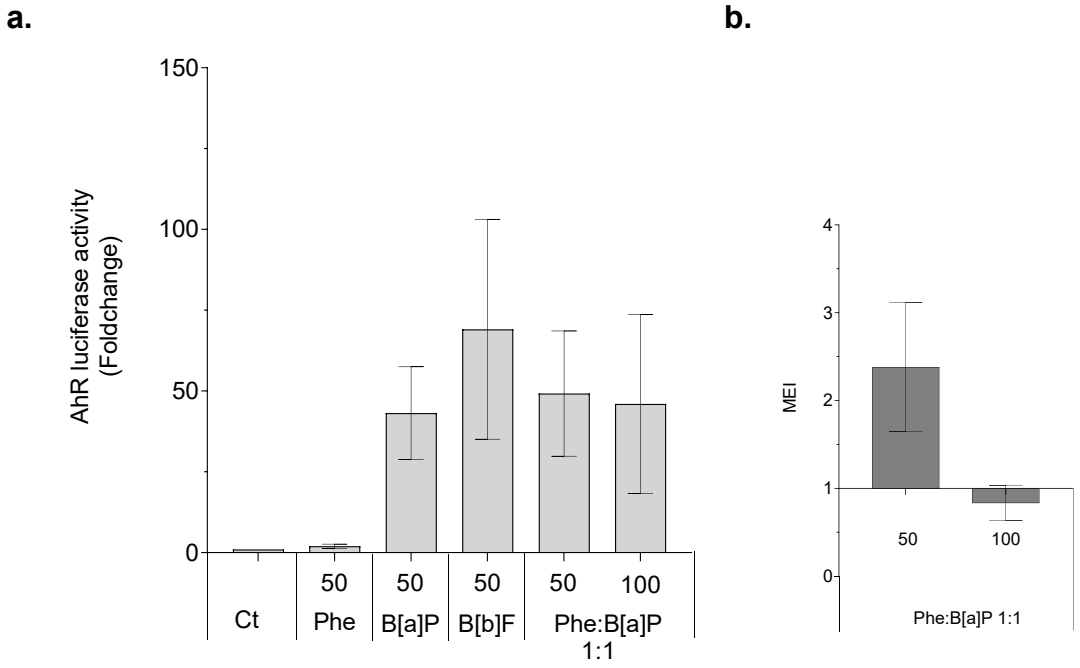


Figure 6.5 - AhR luciferase activity in HepG2 cells after 24 hours of exposure to B[a]P, Phe, B[b]F, and their mixtures at total PAH concentrations (tPAH) of 50 and 100 μM. Ct represents the control group. Data are presented as mean ± S.E. from four independent experiments. **a.** Bars represent the CYP1A1 expression levels; **b.** Bars represent the Mixture Effect Index (MEI) for the Phe:B[a]P 1:1 mixtures.

The AhR luciferase reporter assay (Figure 6.5 **a.**) revealed substantial induction of AhR activity across most treatments, with the exception of Phe. B[b]F demonstrated the most potent AhR activation, with a remarkable 69-fold increase (±33) in luciferase activity. The Phe: B[a]P mixture presented similar results to B[a]P exposures. Despite these notable fold changes, it's important to note that the differences did not reach statistical significance when compared to the control. The MEI calculation indicated that none of the mixtures deviated significantly from an index value of 1, suggesting predominantly additive effects in the mixture tested (Figure 6.5 **b.**).

6.4 Discussion

The present study evaluates PAH metabolism through the expression of related genes, CYP1A1 protein levels and AhR activity (luciferase reporter assay) in a human immortalized liver cell line, specifically focusing on the effects of different PAH mixture with different proportions of individual compounds. Therefore, an exposure experiment was conducted in the HepG2 cell line using PAHs individually and in mixtures.

Subsequently, the mixtures were modulated to assess their nature, whether additive, antagonistic, or synergistic. The Mixture Effect Index (MEI) was employed to modulate mixtures since conventional methods frequently overlook the effects of differing proportions of mixture components. By focusing on component ratios, this approach provided nuanced insights into mixture interactions. by calculating the ratio of observed to expected effects. The statistical robustness of this approach was ensured through t-tests comparing MEI values to a theoretical value of 1, indicating additivity.

While this method is practical, it is based on assumptions, such as compound similarity in the CA model, which may not fully account for the variability in individual PAH mechanisms. Additionally, the reliance on single concentrations may hide nuanced interactions detectable across broader dose-response studies. Despite these limitations, this approach provides a reliable framework for analysing complex mixtures.

The exposure to Benzo[a]pyrene elicited an activation of the AhR-CYP1A1 pathway in HepG2 cells. At 50 μM, CYP1A1 mRNA levels increased 500-fold (Figure 6.2 **a**.), leading to a corresponding increase in CYP1A1 protein expression (Figure 6.4). In contrast, at 5 μM, the mRNA increase was even more pronounced (1500-fold) (Figure 6.2 **a.**), yet no corresponding protein was detected. This suggests that while higher mRNA induction occurs at 5 μM, it does not translate into protein expression as observed at 50 μM. Additionally, the AhR reporter assay showed a 53-fold activation at 50 μM (Figure 6.5), confirming functional receptor translocation. However, despite this strong activation, AhR mRNA levels remained relatively unchanged (1.5-fold) (Figure 6.2 **b**.), suggesting post-transcriptional regulation[417]. The disconnect between gene activation and protein expression at this lower concentration suggests potential translational repression or enhanced protein degradation, mechanisms that were not evident at 50 μM. Kumar et al. (2022)[418] observed in human astrocytic cell lines significant changes in the mRNA expression of CYP enzymes at 1 μM B[a]P exposure, but no corresponding increase in CYP1A1 protein levels, suggesting a disconnect between gene

activation and protein expression. This aligns with the concept that mRNA translation requires exceeding a certain threshold to lead to an increase in protein levels [419]. This distinction is particularly relevant when considering environmental exposures, which are typically much lower than 5 μM. The fact that no detectable protein expression occurs at this concentration raises important questions about the functional relevance of mRNA induction under environmentally relevant exposure levels.

These results align with B[a]P's role as a benchmark compound in PAH toxicity studies. A five-ring PAH, B[a]P strongly interacts with AhR to induce CYP1A1 and related enzymes, generating reactive metabolites that form DNA adducts, a key carcinogenic mechanism[420]. The observed dose-dependent variability further underscores the intricacies of its regulatory pathways and its pivotal role in PAH metabolism.

B[b]F demonstrated a strong induction of the AhR-CYP1A1 pathway, with both CYP1A1 mRNA and protein levels increasing in a dose-dependent manner at 5 μM and 50 μM (Figure 6.2., Figure 6.4). Notably, B[b]F elicited the highest AhR activation (Figure 6.5) observed in the luciferase assay, with a 69-fold increase, highlighting its higher potency as an AhR agonist comparing with his congener B[a]P. Unlike B[a]P, the consistent protein expression at lower concentrations suggests a more efficient coupling of transcription and translation for B[b]F, which may reflect differences in their regulatory mechanisms. Colvin et al. (2024)[421] also found B[b]F as a great inducer for CYP1A1 expression in Primary Human Bronchial Epithelial Cell. As a high-molecular-weight PAH with five fused aromatic rings, B[b]F shares structural and functional similarities with B[a]P but has been comparatively less studied. Despite this, several regulatory agencies classify it as a probable human carcinogen[156], underscoring its toxicological relevance.

In contrast, Phe exhibited minimal induction of CYP1A1 mRNA (Figure 6.2 **a**.) and protein levels (Figure 6.4), or AhR luciferase activity (Figure 6.5), underscoring its weaker interaction with the AhR pathway compared to the potent response elicited by B[a]P and B[b]F. This reflects Phe's lower affinity for AhR and its distinct mode of action, already reported [192]. Despite not being classified as carcinogenic by IARC[156], Phe is toxicologically relevant due to its environmental prevalence and ability to modulate the effects of more potent PAHs[422]. Mechanisms such as competitive AhR binding or interference with metabolic pathways likely underlie these modulatory effects. This divergence between Phe and B[a]P highlights the importance of individual PAH properties in mixture toxicity.

When considering the mixtures tested, containing Phe and B[a]P, they consistently activated the AhR-CYP1A1 pathway, with CYP1A1 mRNA levels exceeding 20-fold (Figure 6.2**a**.). However, these inductions were significantly lower than those observed with B[a]P exposure. On the other hand, AhR activation, as measured by the luciferase assay, was similar between the mixtures and B[a]P (Figure 6.5.) indicating comparable receptor activation and nuclear translocation across conditions. As observed by Colvin et al. (2024)[421], the direction and magnitude of some gene expression can vary depending on the specific PAHs present in a mixture. While B[a]P is a potent inducer of CYP1A1 expression, other PAHs, such as Phe, may inhibit or attenuate this induction. This could explain the reduced CYP1A1 mRNA levels observed in mixtures.

Taking now the analyses performed using MEI method, we observed antagonistic interactions at the lowest concentration tested for all mixtures. The reduced CYP1A1 induction in mixtures (Figure 6.2 **a.**), despite strong AhR activity (Figure 6.5.), suggests that antagonistic interactions may stem from competitive effects occurring downstream of AhR activation. Phe, for instance, may compete with B[a]P for binding to shared metabolic pathways or transcriptional machinery, thereby attenuating the full transcriptional or translational activation of CYP1A1. This interference inhibited the metabolic activation of B[a]P without significantly affecting the receptor-level dynamics observed in the luciferase assay. Smith et al. (2022)[423] has shown that, in human hepatic microsomes, multiple PAHs in a mixture can compete as substrates for shared enzymes like CYP1A1, leading to reduced metabolic efficiency. This competitive inhibition likely explains the reduced CYP1A1 induction in mixtures compared to B[a]P , even with strong AhR activity.

AhR mRNA levels showed minimal changes, with a B[a]P fold changes of 0.8 at 50 μM and 1.8 at 5 μM (Figure 6.2 **b**.), indicating limited transcriptional modulation of AhR. Most mixtures did not alter AhR mRNA levels, further emphasizing the stable or even slightly suppressed transcriptional profile of AhR under mixed exposures. This stability contrasts with the strong activation observed in the luciferase assay, suggesting that AhR regulation predominantly occurs at the protein level, with its transcriptional activation playing a minimal role in the observed responses. Some studies have suggested that AhR transcriptional activity is modulated by ligand-specific interactions with co-regulators and transcription factors, fine-tuning receptor functions without necessarily leading to significant changes in mRNA levels. For example, Gargaro et al. (2021)[424] highlight how AhR's interaction with various cofactors can influence its functionality even in the absence of dramatic transcriptional changes.

Furthermore, ligands like B[a]P may activate post-transcriptional mechanisms that stabilize AhR protein expression or enhance receptor recycling within the cell. These processes maintain robust receptor functionality while keeping mRNA levels stable, a phenomenon also noted in research by Li et al. (2014)[417], which explored how post-transcriptional modifications contribute to AhR regulation, in A549 and HepG2 cells.

These data align with the MEI analysis indicating antagonistic interactions. While receptor activation and nuclear translocation (reflected in the luciferase assay) were comparable between B[a]P Individual and mixtures, the reduced AhR mRNA expression and lower CYP1A1 induction in mixtures suggest competitive or inhibitory effects downstream of AhR activation. This interference could stem from Phe's competition with B[a]P for shared cofactors, transcriptional machinery, or metabolic pathways, contributing to the antagonistic nature of the interactions observed in MEI results.

The absence of antagonistic or synergistic interactions in UGT1A expression (Figure 6.3**c.**) suggests that B[a]P and Phe contribute individually to UGT1A upregulation, potentially through complementary or parallel regulatory pathways, such as AhR and CAR signalling[425,426]. This additive effect may also be influenced by the involvement of UGT2A, another glucuronosyltransferase isoform known to metabolize PAHs[427,428]. UGT2A shares substrate specificity with UGT1A and could contribute to efficient detoxification through parallel pathways, minimizing competition or enhanced activation. Isoform-specific preferences for PAHs might further compartmentalize metabolism, stabilizing the overall response.

For NQO1, a key enzyme in oxidative stress defence, expression levels showed additive behaviour across most mixtures (Figure 6.3 **d.**), emphasizing its importance in mitigating ROS generation and quinone toxicity during PAH metabolism[429]. However, for the 1:1 mixture at doubled concentrations (10 μM and 100 μM total PAHs), antagonistic behaviour was observed, suggesting that elevated exposure levels may surpass the capacity of regulatory networks to sustain NQO1 induction. This antagonism could result from increased metabolic burden, competitive inhibition, or mutual interference between PAHs in activating pathways such as Nrf2. The Nrf2-Keap1/ARE pathway is central to NQO1 regulation, as Nrf2 translocates to the nucleus in response to oxidative stress, driving the transcription of genes involved in antioxidant defence.[430]. For instance, Branco et al. (2021)[178] demonstrated that Phe and B[b]F activate the Nrf2 pathway differently, with B[b]F inducing significant upregulation of glutathione synthesis via Nrf2 signalling, while Phe failed to activate this pathway. This

differential activation could explain the observed antagonistic effects in mixtures, as Phe may interfere with or reduce B[b]F-induced Nrf2 activation when present together, potentially disrupting the balance of antioxidant responses required to mitigate oxidative stress.

In contrast, PXR, a nuclear receptor responsible for activation of a variety of phase I and II enzymes by binding to specific response elements in their promoters, often in response to environmental xenobiotics[431], displayed predominantly antagonistic behaviour across mixtures. This pattern aligns with observations for AhR and CYP1A1, where mutual inhibition or competition among PAHs likely limits receptor activation. PXR activation requires ligand binding to induce transcription of target detoxification genes such as CYPs and UGTs[432], but in mixtures, competitive dynamics may dilute the effective contribution of each PAH. Additionally, the overlapping regulatory functions of PXR and AhR[433], including shared co-factors and metabolic pathways, could further constrain PXR-mediated responses under complex exposures. This antagonism may reflect a cellular strategy to balance detoxification processes and prevent overactivation of metabolic pathways, which could otherwise exacerbate cellular stress or energy demands.

6.5 Conclusion

This study provides valuable insights into the interactions between PAH mixture components with different ratios and their effects on AhR-CYP1A1 pathway activation. While B[a]P showed the strongest induction of CYP1A1 and AhR activity, mixtures revealed significant antagonistic interactions at lower concentrations, likely driven by competitive or inhibitory effects from Phe. These results highlight the complexity of PAH mixture toxicity, where receptor-level activation does not always correlate with downstream transcriptional or enzymatic responses. The reduced CYP1A1 induction in mixtures, despite stable AhR activation, underscores the importance of post-receptor mechanisms, such as competition for metabolic enzymes or transcriptional interference. Furthermore, the stability of AhR mRNA levels and consistent feedback mechanisms suggest protective cellular strategies to prevent overactivation under sustained PAH exposure.

Overall, these findings reinforce the need to consider both individual PAH properties and their interactions in mixtures to fully understand their toxicological profiles. The approach used here offers a practical and reliable framework for studying mixture interactions while

adhering to ethical and resource constraints. This methodology provides a foundation for future research into the dynamic and context-dependent effects of PAH mixtures, with implications for risk assessment and environmental health.

7

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

7.1 Concluding Remarks

This work makes a scientific contribution, particularly in the field of environmental ecotoxicology, as it expands the study of complex PAH mixtures, especially their effects on organisms. This work is in line with the goals of the United Nations 2030 Agenda, namely Goal 14, which aims to reduce pollution from various sources, and Goal 3.9, which aims to reduce the number of diseases and deaths related to exposure to hazardous chemicals and pollution.

In this work, several key questions are raised in the previous chapters, based on both mixtures and concentrations of PAHs that are environmentally relevant. The answers to these questions can guide the improvement of risk assessment methods and environmental regulations. Furthermore, the results highlight the significant knowledge gap regarding mixtures of compounds and thus emphasise the need for continued research leading to the creation of new strategies and guidelines based on scientific evidence.

The main objective of this study was, therefore, to understand the mode of action of PAH mixtures and to increase knowledge of the environmental risks associated with them. To this end, biological models, both *in vivo* and *in vitro*, were used to perform more comprehensive mechanistic investigations and to answer the following questions:

- Are PAH mixtures capable of modifying metabolic pathways in a way that has different genotoxic and carcinogenic effects than individual compounds?

- Is it possible to predict the effects/risks of complex PAH mixtures to improve regulations and environmental monitoring in line with the Water Framework and Marine Strategy Framework guidelines?

From a rational point of view, it is reasonable to assume that the combined genotoxic and carcinogenic effects of PAH mixtures are different from those observed for isolated compounds, where synergistic or antagonistic interactions can be observed.

The study begins with the introduction of an innovative method for the extraction of primary fish hepatocytes by pancreatin digestion, which represents a significant advance in the field of ecotoxicology. Unlike the traditional perfusion method, which is complex and expensive, this method has proven to be more cost-effective while maintaining its efficiency and reproducibility, making it accessible for toxicological studies. Primary cell cultures are

widely used for the study of environmental pollutants, including PAHs, due to their sensitivity and ability to maintain key biochemical processes observed *in vivo*.

The extraction results showed that these important properties were retained, demonstrating the success and feasibility of this protocol. The successful application of the method to *Psetta maxima* and *Sparus aurata* expands its potential for application to other marine species and establishes it as a flexible tool in ecotoxicology. This lays the foundation for future research into the toxicity mechanisms of various marine pollutants, which will ultimately improve environmental monitoring and risk assessment. As the high throughput of the method allows the use of a single organism for multiple tests, it is also in line with the 3Rs principle for animal testing.

Two complementary studies were then conducted to investigate the toxicological effects of PAHs: an *in vitro* study with primary hepatocytes of *Sparus aurata* and an *in vitro* study with the same specie. This method allowed a thorough investigation of the mechanisms of toxicity and interactions between Phe, B[a]P, B[b]F and PAHs typically found in the marine environment.

The *in vitro* study with primary hepatocytes showed that B[a]P significantly increased CYP1A1 gene and protein expression, which in turn led to increased DNA damage, ultimately as a result of increased production of reactive metabolites. These results are consistent with research findings on the toxicity of B[a]P. In particular, the 2:1 mixture of Phe:B[a]P caused a significant increase in CYP1A1 mRNA levels and DNA strand breaks with 70% damage. According to these findings, Phe increases the metabolic activation of B[a]P through CYP1A1 induction and thus enhances its genotoxic effect, although it is not considered carcinogenic. Since Phe has no affinity for the AhR, the mechanism causing this induction is still unknown. There may be other pathways, such as CAR or PXR, that need to be further investigated.

The *in vivo* study with juvenile *S. aurata* showed complex interactions between Phe and B[a]P, both individually and in mixtures. However, the results confirmed the *in vitro* results and showed that the 2:1 mixture of Phe:B[a]P significantly increased GST3 mRNA expression (up to 7-fold). However, this increase was only observed at the mRNA level without a corresponding increase in enzymatic activity. In addition, a multidimensional scaling analysis (MDS) showed that B[a]P and the mixture with a higher proportion of B[a]P were strongly associated with detoxification mechanisms, while Phe was more closely associated with oxidative stress responses.

AhR is primarily activated by PAHs to produce their toxic effects. Then, when the AhR binds to PAHs, it changes its shape and translocates to the nucleus where it dimerises with the aryl hydrocarbon receptor nuclear translocator (ARNT). After binding to xenobiotic-responsive elements (XREs) in promoter regions of target genes, this AhR-ARNT complex triggers the transcription of phase I and phase II detoxification enzymes, including glutathione S-transferase (GST) and cytochrome P450 1A1 (CYP1A1). In the metabolic processing of PAHs, this regulatory cascade is crucial as it influences both the detoxification of the compounds and the production of potentially reactive metabolites that can increase toxicity.

However, although the 42-day *in vivo* study revealed activation at the genetic level, this did not necessarily correspond to an observed increase in enzyme activity. This result emphasises the role of post-transcriptional regulation, which may include translational regulation, control of mRNA stability, alternative splicing and RNA processing. Furthermore, it is possible that prolonged exposure has led to a new equilibrium state that reflects a complex adaptive response over time, thus highlighting the observed differences in short- and long-term effects.

This discrepancy between enzyme activity and gene expression illustrates the complexity of biological systems and the need for a comprehensive approach to toxicological assessments. On the one hand, mRNA analysis provides valuable information on cellular responses; on the other hand, enzyme activity is influenced by post-transcriptional and post-translational regulatory mechanisms and often shows up as the end result. To gain a deeper understanding of the mechanisms of toxicity and interactions of PAHs in complex mixtures, these results emphasise the importance of integrating multiple levels of biological regulation.

The concentrations used in the sub chronic *in vivo* study were lower than those used *in vitro,* as the aim of the *in vivo* study was to reflect environmentally relevant exposure levels and thus to assess the effects of PAH mixtures after long-term exposure to low concentrations. The results demonstrate the risks of long-term exposure to pollutants as reflected in the erythrocyte nuclear abnormalities (ENAs), which reflect persistent chromosomal damage that has increased despite the activation of cellular defence mechanisms.

These results challenge traditional toxicological paradigms, which often assess PAHs separately and may underestimate the hazards associated with complex environmental mixtures. The results show that non-carcinogenic PAHs can actively modify the toxicity of carcinogenic PAHs through additive or synergistic interactions, rather than being merely

passive components. This calls for a paradigm shift in environmental risk assessment (ERA) that emphasises the importance of the composition of mixtures.

Subsequently, the mechanistic results obtained in the models using *S. aurata* as a biological model were analysed and validated using the HepG2 cell line. The aim was to clarify the role of AhR pathway in mediating the toxicological effects of PAH mixtures. Therefore, recognised methods such as RT-qPCR, Western blot and luciferase reporter gene assays were used to assess gene expression, protein levels, and receptor activation, respectively, to extend the current understanding of the molecular processes regulated by AhR.

Interestingly, the results in HepG2 cells differed from those of previous studies. Most of the interactions were purely additive or antagonistic, with Phe attenuating the effects of B[a]P. The Phe:B[a]P 2:1 mixture, for example, which had previously shown synergistic effects, behaved antagonistically or additively in this model. Since the compounds belong to the same chemical class, the theoretical interactions were calculated using the CA model. However, the final calculation of the Mixture Interaction Index (MEI) may contain biases due to the limitations of the CA model, namely the assumption of linearity and the non-assumption that different mechanisms of action can co-exist.

In addition, the immortalized HepG2 cell line is only able to reproduce the biochemical pathways *in vivo* to a limited extent, for example due to the loss of functional specialisation or the fact that they are programmed for continuous growth. This characteristic may have contributed to the observed differences. In addition, the same fish species were used in previous *in vitro* and *in vivo* studies. However, when trying to validate the results in HepG2, a difference was observed at the cellular level (from primary cells to immortalized cells) as well as interspecific variation that could show differences, for example, at the level of CYP1A or AhR isoforms as well as in other signalling pathways.

In general, the results obtained in this study show that the proportions and presence of certain PAHs can modify the overall effect expected for the mixture. Results thus highlight the gap in the current environmental guidelines, which are based only on the total allowed concentrations of PAHs in water or sediments and do not take into account the composition of the mixtures. Furthermore, the results also highlight the complexity of analysing chemical mixtures, as it is challenging to fully predict the interactions and toxic effects of the various combinations of compounds that may occur in the environment. In addition to this complexity, the results have also shown that there are inter-specific differences, as different

species are differently sensitive and handle exposure to mixtures differently, which introduces an additional layer of complexity that makes it difficult to generalize the results.

This emphasizes the critical need for more complex risk assessment frameworks that take into account biological variability among exposed organisms as well as the particular composition of PAH mixtures and total concentrations. To better reflect real-world situations and guarantee the efficient protection of aquatic ecosystems, future environmental guidelines should incorporate interspecies sensitivity and mixture-specific toxicity data.

7.2 Future Perspectives

The knowledge of the toxicological interactions of PAHs and their mixtures can be expanded by the basis that this study provides for future investigations. Below are some important recommendations for further research:

Chronic exposures and long-term effects: Since organisms in the environment are continuously exposed to pollutants at relatively low doses, in order to mimic these conditions, future studies should focus on the effects of long-term and low-dose exposure to PAHs. Prolonged exposures can cause cumulative and potentially irreversible effects, such as changes in gene expression, DNA damage and an increased risk of cancer. Assessing these long-term effects, particularly through chronic *in vivo* studies, would provide important new information on how toxicity develops beyond the acute assays that have been most commonly used to date.

Species variability: As observed in this study, interspecies variability is a factor that needs to be considered when predicting the effects of PAH mixtures. Future studies should therefore investigate how different species, particularly marine species, respond to PAH mixtures, taking into account differences in metabolic processes, cellular responses and susceptibility between species.

Advanced Metabolite Profiling and Pathway Analysis: The combination of analytical methods such as gas chromatography and mass spectrometry (GC-MS) would allow the characterization of metabolites produced during the biotransformation of PAHs, thus expanding the available data libraries. If they can be accurately identified and quantified, they would provide more information on specific metabolic pathways. The interpretation of reactive metabolite formation could serve as a fingerprint associated with each PAH or mixture, which could ultimately contribute to the discovery of new biomarkers of exposure and effect.

Non-linear interactions: With improved computational modelling and more accurate statistical techniques, such as artificial intelligence models, researchers can attempt to modulate the nature of the interactions and predict the additive, antagonistic or synergistic effects of PAHs and other environmental substances. Given the specific composition of mixtures and the potential impact of non-linear interactions, it is also crucial to investigate how different proportions and combinations of compounds alter the observed toxic effects.

Risk assessment models: The creation of risk models that consider the interactions between PAHs with other pollutants such as plastics, metals, pharmaceuticals, among others, which would make the scenario even more realistic, since there are multivariate interactions between the different pollutants in the environment. The accuracy of risk assessment and monitoring techniques can be improved by using advanced predictive modelling based on high-quality data from *in vitro* and *in vivo* studies to improve the extrapolation of results to broader environmental scenarios.

"**Omics**" **approaches:** The use of "omics" technologies (genomics, proteomics and metabolomics) to find specific biomarkers of exposure and effect will help to better understand the molecular mechanisms behind the interaction of different compounds. These methods can provide comprehensive insights into the metabolic, protein and genetic changes caused by exposure to complex PAH mixtures.

Public health and ecology: By studying the effects on food chains and ecosystems, public health and ecology can integrate considerations of the transmission of PAH effects from marine organisms to humans. This includes examining the toxicological effects at different trophic levels as well as the potential combined effects of PAH bioaccumulation on human health and marine ecosystems. With the help of these guidelines, a more comprehensive understanding of the effects of PAH mixtures and their consequences for ecological and human health can be developed.

These directions can help build a broader approach to understanding the effects of PAH mixtures and their implications for human and ecological health. By combining experimental methods with advanced analytical tools, such as predictive models and "omics" technologies, it is possible to obtain a more accurate and integrated understanding of environmental risks, contributing to the development of more effective public policies grounded in robust scientific evidence.

8 References

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