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FACULDADE DE FARMÁCIA
UNIVERSIDADE DO PORTO

Ana Cristina Monteiro Pinto

Prediction of the *in vitro* mixture effects of Polycyclic Aromatic Hydrocarbons (PAHs) using the mathematical concepts of concentration addition and independent action

Master's Degree in Clinical, Forensic and Analytical Toxicology

Supervisor: Doutora Diana Dias da Silva

Co-supervisors: Doutora Joana Pinto & Prof Doutora Maria de Lourdes P.A.S Bastos

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Abstract

Exposure to chemicals is one of the main causes of cancer, Polycyclic Aromatic Hydrocarbons (PAHs) being among the main groups of potent carcinogens that are ubiquitous in the environment. The carcinogenicity of PAHs correlates with the type of metabolites produced during the biotransformation depending on the cytochrome P450 (CYP450) isoenzymes involved and the parent compound to be considered. Another factor that greatly contributes to the carcinogenic potential of PAHs is the toxicodynamic and toxicokinetic interactions with other substances. Although these toxics are present in the environment as complex mixtures, little emphasis has been placed on the effects of mixtures of PAHs, but the scarce investigation developed in this area revealed complex and poorly understood interactions, compromising the risk assessment of these substances.

This project aimed at examining the mechanisms and effects of PAH mixtures. In particular, it was intended to elucidate the type and extent of toxicological effects induced by five PAHs and their combinations, and eventually to reveal significant effects (additive, synergistic or antagonistic) arising from the co-occurrence of these compounds.

For this purpose, hepatocytes from Wistar rats (rich in CYP450) were isolated by liver perfusion with collagenase, and cultured in plates overnight. After exposure to phenanthrene (Phe), fluoranthene (F), chrysene (Chry), benzo[b]fluoranthene (B[b]F) and benzo[a]pyrene (B[a]P), at concentrations between 5 nM and 10 mM, individually or in mixture, for 24 or 48 hours, the cytotoxicity was evaluated through the MTT reduction viability test. In an attempt to gain a deep understanding of the potential interactions between PAHs, a binary mixture of B[a]P and B[b]F; a ternary mixture of B[a]P, Phe and Chry; and a quintenary mixture of Phe, F, Chry, B[b]F and B[a]P were tested combining PAHs in proportions similar to those found in sediments of the Tejo River. The results obtained for each PAH were used to calculate the effects of the mixtures, using the additive models of concentration addition and independent action.

The cytotoxic potencies of PAHs found in primary hepatocytes were as follows: Phe>B[a]P>B[b]F>Chry>F, according to the EC_{50} of 2.28 mM, 2.32 mM, 3.19 mM, 5.21 mM, and 6.17 mM, respectively, for 24 hours; and B[a]P>Chry>B[b]F>Phe>F, according to the EC_{50} of 0.52 mM, 1.47 mM, 1.68 mM, 1.84 mM and 4.67 mM, respectively, for 48 hours. Comparisons of predicted responses with the effects observed experimentally for the mixtures, indicate that PAHs interact in an additive, synergistic or antagonistic way, depending on the type of PAHs combination under study, the exposure time (24 or 48 hours) and the complexity of the mixture (binary, ternary or quintenary). The

performance of both prediction models was similar. Notably, substantial mixture effects were observed for the most complex combinations (ternary and quintenary mixtures), even when each PAH was present at levels that individually produced no statistical effect.

Overall, this work provided important knowledge about the magnitude of interactions between PAHs, alerting to possible consequences for human health, in the context of environmental exposure. The present investigation offered, in a relevant way, evidence that the risk assessment strategies for environmental exposure of PAHs may be inefficient if these substances are considered in isolation.

Keywords: Environmental toxicology; Polycyclic Aromatic Hydrocarbons (PAHs); Mixture effects; Risk assessment.

Resumo

A exposição a químicos é uma das principais causas de cancro, estando os hidrocarbonetos aromáticos policíclicos (HAPs) entre os principais grupos de potentes carcinogénicos ubiquitários no meio ambiente. A carcinogenicidade dos HAPs correlaciona-se com o tipo de metabolitos produzidos durante os processos de biotransformação, dependendo das isoenzimas do citocromo P450 (CYP450) envolvidas e do composto-pai em questão. Outro fator que contribui sobejamente para o potencial carcinogénico dos HAPs são as interações toxicodinâmicas e toxicocinéticas com outras substâncias. Embora os HAPs estejam presentes no ambiente como misturas complexas, pouca ênfase tem sido dada aos efeitos de mistura destes tóxicos, mas a escassa investigação desenvolvida nesta área revelou interações complexas e pouco compreendidas, comprometendo a avaliação de risco dessas substâncias.

Este projeto teve como objectivo investigar os efeitos de misturas dos PAHs. Em particular, pretendeu-se elucidar o tipo e a extensão dos efeitos toxicológicos induzidos por cinco PAHs e suas combinações e, eventualmente, revelar efeitos significativos (aditivos, sinérgicos ou antagónicos) decorrentes da coocorrência desses compostos.

Com este intuito, foram isolados hepatócitos de ratazanas Wistar (ricos em CYP450) por perfusão hepática com colagenase, e cultivados em placas durante a noite. Após exposição aos HAPs fenantreno (Phe), fluoranteno (F), criseno (Chry), benzo[b]fluoranteno (B[b]F) e benzo[a]pireno (B[a]P), a concentrações entre 5 nM e 10 mM, individualmente ou em mistura, durante 24 ou 48 horas, foi avaliada a citotoxicidade produzida, através do ensaio de viabilidade de redução do MTT. Numa tentativa de obter uma compreensão profunda das potenciais interações entre os HAPs, foram testadas uma mistura binária de B[a]P e B[b]F; ternária de B[a]P, Phe e Chry; e quintenária de Phe, F, Chry, B[b]F e B[a]P, combinando os HAPs em proporções semelhantes àquelas que foram encontradas em sedimentos do Rio Tejo. Os resultados obtidos para cada HAP foram utilizados para calcular os efeitos das referidas misturas, usando os modelos aditivos de adição de concentração e ação independente.

A potência citotóxica dos HAPs em hepatócitos primários foi a seguinte: Phe>B[a]P>B[b]F>Chry>F, de acordo com os EC_{50} de 2,28 mM, 2,32 mM, 3,19 mM, 5,21 mM, 6,17 mM, respetivamente, às 24 horas; e B[a]P>Chry>B[b]F>Phe>F, de acordo com os EC_{50} de 0,52 mM, 1,47 mM, 1,68 mM, 1,84 mM e 4,67 mM, respetivamente, às 48 horas. A comparação dos efeitos de mistura previstos com os observados experimentalmente indicam que os HAPs interagem de forma aditiva, sinérgica ou antagónica, dependendo do tipo de HAP em consideração, do tempo de atuação (24 ou 48 horas) e da complexidade da mistura (binária, ternária ou quintenária). O desempenho dos modelos de previsão foi semelhante, sem que nenhum se tenha

evidenciado. Notavelmente, foram observados efeitos tóxicos substanciais de mistura para as combinações mais complexas (misturas ternária e quintenária), mesmo quando cada HAP estava presente em quantidade que, individualmente, produzia efeitos negligenciáveis.

Deste modo, este trabalho forneceu conhecimento toxicológico importante sobre a magnitude das interações entre HAPs, alertando para as possíveis consequências para a saúde humana, no contexto da exposição ambiental. A presente investigação ofereceu, de forma relevante, evidência que as estratégias de avaliação de risco para a exposição ambiental aos HAPs podem ser pouco eficientes se estas substâncias forem consideradas isoladamente.

Palavras-chave: Toxicologia ambiental; Hidrocarbonetos Aromáticos Policíclicos (HAPs); Efeitos de mistura; Avaliação de risco.

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Abbreviations

1-MP - 1-Methylphenanthrene

1-OHP - 1-Hydroxy-pyrene

1-OHP - 1-Hydroxy-pyrene

2-OH-FLUO - 2-Hydroxyfluorene

2-OH-NAP - 2-Hydroxynaphthalene

3-OH-Phe - 3-Hydroxyphenanthrene

5-MC - 5-Methylchrysene

9-OH-FLUO - 9-Hydroxyfluorene

AC - Acenaphthene

ACY - Acenaphthylene

AhR - Aryl hydrocarbon receptor

AhRNT - Aryl hydrocarbon receptor nuclear translocator

AKR - Aldo-keto reductase

ANT- Anthracene

B[a]A - Benzo(a)anthracene

B[a]P - Benzo(a)pyrene

B[b]F - Benzo(b)fluoranthene

B[c]Phe - Benzo(*c)*phenanthrene

B[ghi]P - Benzo(g,h,i)perylene

B[j]A - Benz(*j)*aceanthrylene

B[j]F - Benzo(*j)*fluoranthene

B[k]F - Benzo(k)fluoranthene

BB[ah]P - Bibenzo(*a,h)*pyrene

BB[ai]P - Bibenzo(*a,i)*pyrene

BF - Benzofluoranthene

Chry - Chrysene

CP[cd]P - Cyclopenta(*cd)*pyrene

CYP - Cytochrome P450

DB[ah]A - Dibenzo(a,h)anthracene

DB[al]PYR - Dibenzo(a,l)pyrene

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

EH - Epoxide hydrolase

EPA - Environmental Protection Agency

EROD - Ethoxyresorufin O-deethylase

F - Fluoranthene

FLUO - Fluorene

HMW - High-molecular-weight

Hsp90 - Heat shock protein 90

IARC - International Agency for Research on Cancer

IP - Indeno-(1,2,3-c,d)-pyrene

LMW - Low-molecular-weight

MoA - Mode of action

MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAP - Naphthalene

NEC - No effect concentration

PAHs - Polycyclic aromatic hydrocarbons

PEL - Probable effect level

Phe - Phenanthrene

PYR - Pyrene

ROS - Reactive oxigen species

TEL - Threshold effect level

UV - Ultra-violet

WHO - World Health Organization

Chapter I- Introduction

1.1 Introdution

Humans and environment are frequently exposed to a myriad of potentially toxic compounds in a variety of ways, via different routes, both simultaneously and in sequence. The combined action of these compounds with similar or different modes of action (MoA) can affect individual toxicities and lead to potencial synergistic, additive or antagonistic effects on the different biologicals systems (Altenburger et al., 2012; Beyer et al., 2013; Panizzi et al., 2017). In this context, Polycyclic Aromatic Hydrocarbons (PAHs) represent a classe of persistent organic pollutants, which occur ubiquitously in the environment as complex mixtures. They are distributed in the atmosphere, soil and aquatic systems, bioaccumulating in the organisms (Guo et al., 2013; Kim et al., 2013).

The health effects of PAHs exposure have been widely studied, mainly because they are potentially carcinogenic, teratogenic and mutagenic agents and, therefore, they are also targeted for risk evaluation (IARC, 1983; 1985; 2010; 2012; Miller and Ramos, 2001; U.S. Environmental Protection Agency, 1993; Yang et al., 2010). In this line, the Environmental Protection Agency (EPA) has promulgated 16 PAHs as high priority pollutants to be analyzed in different environmental matrices, because of their potential to induce toxicity in humans and other organisms, and their prevalence and persistence in the environment (U.S. Environmental Protection Agency, 1993). These PAHs are divided into carcinogenic and non-carcinogenic groups. Accordingly, benzo(a)anthracene (B[a]A), benzo(a)pyrene (B[a]P), benzo(b)fluoranthene (B[b]F), benzo(k)fluoranthene (B[k]F), chrysene (Chry), dibenzo(a,h)anthracene (DB[ah]A), and indeno-(1,2,3-c,d)-pyrene (IP) are considered as probable or possible human carcinogens, i.e., they are classified into groups 2A or 2B, respectively, according to the International Agency for Research on Cancer (IARC). The remaining nine compounds, acenaphthene (AC), acenaphthylene (ACY), anthracene (ANT), benzo(g,h,i)perylene (B[ghi]P), fluoranthene (F), fluorene (FLUO), naphthalene (NAP), phenanthrene (Phe), and pyrene (PYR) are considered non-carcinogens (they fall into group 3 of IARC) (ATSDR, 2005; Banger et al., 2010; IARC, 1983; 1985; 2010; Rousseau et al., 2005; Samet et al., 2019; U.S. Environmental Protection Agency, 2002b).

The toxicity and carcinogenicity of PAHs is closely related to their metabolic activation by xenobiotic metabolizing enzymes of phase I (functionalization) and phase II (conjugation), which transform the PAHs into reactive metabolites that form protein aducts and reactive oxigen species (ROS). These metabolites, such as diol epoxide, if not inactivated during phase II, can also form stable adducts with deoxyribonucleic acid (DNA), leading to fixed mutations in proto-oncogenes or tumor suppressor genes, widely found in chemically induced cancers (Xue and Warshawsky, 2005). In fact, the parent PAHs are not the main responsibles for carcinogenicity, but their metabolites, whose

production is commonly referred to as bioactivation, and is mainly mediated by cytochrome P450 (CYP) during phase I of metabolization (Gao et al., 2018; Moorthy et al., 2015).

From a toxicokinetic/toxicodynamic point of view, PAHs share many of their mechanisms, toxic effects and also the same bioactivation/metabolic pathways. Therefore, it is reasobable that the co-occurrence of these substances may result in toxicities different from those observed when PAHs are present individually. Although the risk of interaction between different PAHs has been clearly pointed out, the lack of systematic information on the effects of the simultaneous occurrence of these substances in the environment, hinders their risk assessment. Knowledge on the dynamics of PAHs individual metabolism might provide important information about the magnitude of their interactions, assisting the anticipation of combined toxic effects and of the health consequences in the context of environmental and human exposures. Recently, metabolomic strategies have been applied to achieve a comprehensive understanding of the toxicological interactions between mixed chemicals on metabolism, revealing biological responses to chemical mixtures and identifying mechanistically relevant biomarkers (Athersuch, 2016; Beyer et al., 2013; Wang et al., 2018).

Chemical mixtures of PAHs might be assessed either by testing the whole mixture (e.g., in effect-based monitoring of surface water) or by predicting the combined risk based on concentration and effect information of the individual components in the mixture (Bopp et al., 2018). In what concerns these approaches, it is nearly impossible to test all putative environmental PAHs combinations and, too often, the data for single chemical assessment (both exposure and toxicity) are unavailable. Additionally, in the majority of risk assessment strategies that take combined effects in consideration, only a limited number of specific toxic endpoints or biomarkers for individual contaminants or simple mixtures are considered (Monosson, 2005). To overcome of such difficulties and predict the potential health effects resulting from human exposure to PAH complexe mixtures, is urgent to combine different approaches, including epidemiology, mixture toxicology and analytical chemistry. In this dissertation these aspects will be considered with emphasis on the patterns of exposure to PAHs and the main approaches available to predict joint effects of PAHs with the same and different MoA.

1.2 Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are ubiquitous environmental polutants, resulting from natural as well as antropogenic sources. Based on their formation processes, PAHs can be classified into pyrogenic, i.e., derived from incomplete combustion of organic matter; petrogenic, i.e., derived from slow maturation of organic matter under geothermal gradient conditions;

or biogenic, i.e., derived from biogenic precursors (Buczyńska et al., 2013). Natural sources of PAHs consist on diagenesis of organic matter, as products of humus conversion by microorganisms, in coal, crude oil, in emissions from forest fires, volcanoes, and hydrotermal processes (Baxter et al., 2014; Choi, 2014; Wang et al., 2016). Among the anthropogenic sources, the petrogenic PAHs include those present in unburned petroleum and its products (gasoline, kerosene, diesel, and lubricating oil), whereas the pyrogenic PAHs are present in high-temperature combustion products produced by pyrolysis or combustion of organic materials (incomplete combustion of fossil fuels during heating processes, in vehicle trafic emissions, in cigarette smoke, and incomplete combustion of organic materials, in particular during waste incinerators) (Bayat et al., 2015; Bojakowska and SokoŁowska, 2001; Slezakova et al., 2013).

PAHs are released into the environment or dispersed in water (Menezes et al., 2015; Nielsen et al., 2015), air (Galarneau, 2008; Ma and Harrad, 2015), soils (Beriro et al., 2016) and sediments (Kim et al., 2008; Xu et al., 2016), and, as a consequence, they are deposited into vegetation, contaminating also the food (Bansal and Kim, 2015; Plaza-Bolaños et al., 2010).

1.2.1 Chemical properties

PAHs are a large group of organic compounds with two or more fused aromatic rings in various structural configurations (linear, angular or cluster arrangements). Their structure rely on angular condensed aromatic rings, possibly as a result of distortions in a region with maximal impact, termed as “fjord” or “bay” regions (Figure 1). The PAHs that display “Fjord” regions are mostly non-planar and reactive. In contrast, PAHs with a “Bay” region are planar and less reactive (Figure 1) (Jiang et al., 2016; Kim et al., 2013; Velasco et al., 2004).

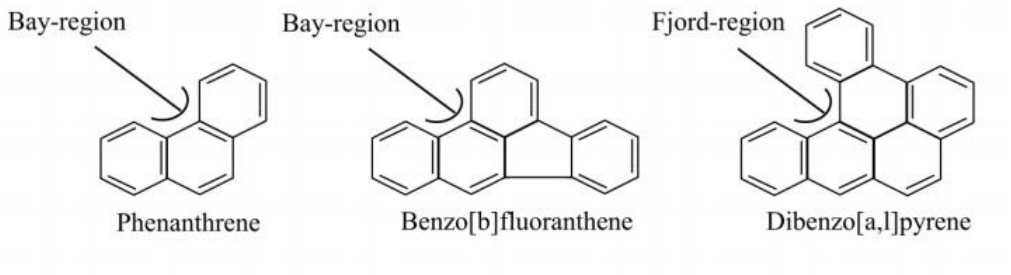


Figure 1. Bay- and Fjord-regions of polycyclic aromatic hydrocarbons (PAHs)

Pure PAHs are generally crystalline solids, ranging from colorless to pale yellow or golden yellow, at ambient temperature (Masih et al., 2012). The physicochemical

properties of PAHs are critical to their biological activity, varying widely with their molecular weight and structure. Many PAHs contain the same number of rings, but their differences in configuration lead to differences in the characteristics (Skupińska et al., 2004). PAHs are hydrophobic organic compounds. The hydrophobicity, bioaccumulation tendency, resistance to biodegradation, and overall environmental persistence, generally increase with increasing molecular weight (Brazkova and Krastanov, 2013; Kim et al., 2013; Srogi, 2007). Also, the volatility of these compounds decreases with increasing molecular weight (Akyüz and Çabuk, 2010; Kim et al., 2013). Based on the molecular structure, PAHs are commonly classified into low-molecular-weight (LMW) PAHs, containing up to four benzene rings (e.g., NAP, AC, ACY, FLUO, ANT, Phe); and high-molecular-weight (HMW) PAHs, with more than four benzene rings (e.g., F, PYR, B[a]P, and benzofluoranthene (BF)). The LMW PAHs are generated at low to moderate temperatures, such those achieved during wood and coal combustion, while the HMW PAHs, which are more stable and toxic, are generated at high-temperature combustion, such as those that originate vehicle emissions (Kim et al., 2013; Masih et al., 2012; Mastral and Callén, 2000).

High melting and boiling points and low vapour pressure are also typical features of PAHs (Table 1). Vapour pressure tend to decrease with increasing molecular weight. On the contrary, PAHs are resistant to oxidation and their reduction increases with increasing molecular weight (Akyüz and Çabuk, 2010; Masih et al., 2012; U.S. Environmental Protection Agency, 2002a).

Solubility varies among PAHs, but in general, PAHs' solubility in water decreases as the molecular weight increases. HMW PAHs are less water-soluble, less volatile and more lipophilic than LMW PAHs. Also, PAHs with a linear arrangement are more likely to be less watter soluble than the angular or *peri*-fused molecules (e.g., ANT is less soluble compared to Phe; Table 1). Alkyl substitution of the aromatic ring also results in an overall decrease of the water solubility of PAHs, although there are some exceptions (e.g., B[a]A is less soluble than either methyl- or ethylbenz[a]anthracene; Table 1). The solubility of PAHs in water is enhanced three to four-fold by a rise in temperature from 5° to 30°C (Giridhar Prabhukumar, 2010).

PAHs also have other properties such as light sensitivity, heat resistance, conductivity, emittability and resistance to corrosion; and possess characteristic ultra-violet (UV) absorbance spectra due to their ring structure, which is especially useful for identification purposes. Most PAHs are also fluorescent, emitting characteristic wavelengths of light when excited (Masih et al., 2010).

Table 1. Physical and chemical properties and carcinogenic group of Polycyclic Aromatic Hydrocarbons (PAHs)

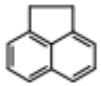
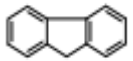
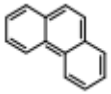
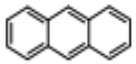
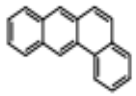
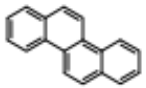
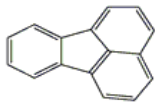
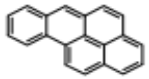
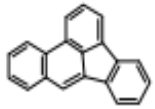
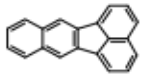
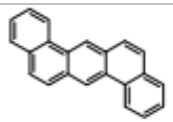
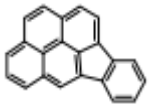
	PAH	Molecular formula	Structure	Molecular weight (g/mol)	Melting point (ºC)	Boiling point (ºC)	Solubility in water (mg/L) 25ºC	Log K_{ow}	Log K_{oc}	Number of rings	Carcinogenic Group (1)
Low Molecular Weight PAHs	Naphthalene (NAP)	$C_{10}H_8$		128.2	80	218	31.8	3.4	3.0	2	3
	Acenaphthylene (ACY)	$C_{12}H_8$		152.2	93	265	16.1	4.1	3.4	3	3
	Acenaphthene (AC)	$C_{12}H_{10}$		154.2	96	279	3.7	3.9	3.7	3	3
	Fluorene (FLUO)	$C_{13}H_{10}$		166.2	117	293	1.98	4.2	3.9	3	3
	Phenanthrene (Phe)	$C_{14}H_{10}$		178.2	100	340	1.2	4.6	4.2	3	3
	Anthracene (ANT)	$C_{14}H_{10}$		148.2	218	340	1.29	4.57	4.4	3	3
	Benzo(a)anthracene (B[a]A)	$C_{18}H_{12}$		228.3	159	435	1.1×10^{-2}	5.84	6.14	4	2B
	Chrysene (Chry)	$C_{18}H_{12}$		228.3	255	448	3×10^{-3}	5.84	5.3	4	2B
	Fluoranthene (F)	$C_{16}H_{10}$		202.3	111	375	0.265	5.22	4.58	4	3

Table 1. Physical and chemical properties and carcinogenic group of Polycyclic Aromatic Hydrocarbons (PAHs) (cont.)

	PAH	Molecular formula	Structure	Molecular weight (g/mol)	Melting point (°C)	Boiling point (°C)	Solubility in water (mg/L) 25°C	Log K_{ow}	Log K_{oc}	Number of rings	Carcinogenic Group (1)
Hight Molecular Weight PAHs	Benzo(a)pyrene (B[a]P)	$C_{20}H_{12}$		252.31	179	496	3.8×10^{-3}	6.4	6.0	5	1
	Benzo(b)fluoranthene (B[b]F)	$C_{20}H_{12}$		252.31	167	393	1.5×10^{-3}	6.6	5.2	5	2B
	Benzo(k)fluoranthene (B[k]F)	$C_{20}H_{12}$		252.31	217	480	7.6×10^{-4}	6.8	5.9	5	2B
	Dibenz(a,h)anthracene (DB[ah]A)	$C_{22}H_{14}$		278.4	262	535	5.1×10^{-4}	6.7	6.1	5	2A
	Indeno(1,2,3-c,d)pyrene (IP)	$C_{22}H_{12}$		276.33	163	534	1.9×10^{-4}	6.6	6.8	6	2B

Log K_{ow}, Octanol-water partition coefficient; Log K_{oc}, Organic carbon partitioning coefficient; (1) carcinogenic groups by International Agency for Research on Cancer (IARC)

1.2.2 Human exposure to PAHs

The main sources of human exposure to PAHs are the air, soil, water and sediment. Due to their physico-chemical properties and lipophilic character, PAHs can be absorbed by the skin, ingested in food, or inhalated, being quickly distributed to the tissues (Zhang et al., 2015). Some exposures may simultaneously involve multiple routes, such as dermal and inhalation exposures from contaminated air, affecting the total dose absorbed (Ravindra et al., 2008). Futhermore, the exposure may not only be acute, but chronic or sequencial. Depending on the volatility and molecular weight of the PAH, and on the atmospheric conditions (ambient temperature, relative humidity, etc.), the substance can adsorb into airborne particulate matter, which increases their persistence in the environment. Aerosols can easily penetrate the human respiratory tract and enter in the blood stream, leading to PAH-initiated carcinogenesis via the formation of PAH-DNA adducts (Delgado-Saborit et al., 2010; Liu et al., 2015; Sánchez et al., 2013; Zhong and Zhu, 2013; Zhu et al., 2009).

Biomarkers of exposure to PAHs

The National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (NIH, 2001). Biomarkers can be classified in several sub-types, mostly classified as biomarkers of exposure, biomarkers of effect, and biomarkers of susceptibility. A biomarker of exposure can be the chemical itself, its metabolites, or products of molecular interaction. Recently, De Craemer *et al.* monitored in adolescents' urine unmetabolized PAHs as biomarkers of environmental exposure (De Craemer et al., 2016). Also, NAP, retene, and Phe have been used as biomarkers to estimate PAHs exposure in wildland firefighters (Navarro et al., 2017). Nevertheless, the most commonly used biomarkers of PAHs exposure are their metabolites and the PAH adducts with DNA or proteins (Castaño-Vinyals et al., 2004).

There is an increasing interest in using biomarker measurements to help correlate the PAHs to the toxic effects on human health. Several biomonitoring studies have been focused in the detection of different biomarkers of PAHs exposure in animal models and in human (De Craemer et al., 2016; Elie et al., 2015; Ferguson et al., 2017; Hu et al., 2012). Urinary PAHs metabolites, predominantly hydroxy PAHs, have been vastly used as biomarkers of PAHs exposure (Ferguson et al., 2017; Hu et al., 2012; Kim et al., 2013). Since pyrene is usually present in many mixtures of PAHs, its metabolite 1-hydroxypyrene (1-OHP) is widely recognized as the biological marker to assess exposure to PAHs (Jakubowski and Trzcinka-Ochocka, 2005; Jongeneelen et al., 1986; Sexton et al., 2011). Nevertheless, since the sources (air, water, soil and food) and compositions of

these mixtures can vary , the biomarker 1-OHP may not reflect the extent of exposure to others PAHs, and as such, the overall exposure to PAHs (Jacob and Seidel, 2002). Thus, the analysis of multiple PAH metabolites may be required. Besides 1-OHP, various hydroxylated PAHs including mono-, poly-, and multihydroxy PAHs have been used as biomarkers in studies exposure to PAHs, such as 2-hydroxynaphthalene (2-OH-NAP), 2-hydroxyfluorene (2-OH-FLUO), 3-hydroxyphenanthrene (3-OH-Phe) and 9-hydroxyfluorene (9-OH-FLUO) (Fernando et al., 2016; Kang and Jeong, 2011; Motorykin et al., 2015; Navarro et al., 2017; Wang et al., 2015; Zhang et al., 2017). Of note, the concentrations of parent PAHs or their metabolites/biotransformation products in an organism depend not only on the external exposure, but also on their absorption, metabolism, bioconversion, detoxification and excretion by the organism (Abdel-Shafy and Mansour, 2016; Kim et al., 2013).

1.2.3 Metabolic activation of PAHs

In general, the main enzyme system involved in the metabolic activation and detoxifation of PAHs is the cytochrome P450 (CYP), primarily by oxidation through CYP1A isoforms (Santana et al., 2018). The CYP family is widely distributed in animal and human cells and tissues. The highest metabolising capacity is present in the liver, followed by the lung, intestinal mucosa, skin and kidneys.

As previously referred, PAHs acquire carcinogenicity after being bioactivated; this occurs by the combined by the conbined actions of CYPs, epoxide hydrolase (EH) and aldo-keto reductase (AKR), forming reactive biotransformation products, which can form DNA adducts, leading to deletions, fusions, translocations, or aneuploidy (Bai et al., 2017; Moorthy et al., 2015; Nebert et al., 2004). The reactive metabolites of PAHs may also induce the formation of protein adducts in cells, which may affect their normal funcitionality.

The metabolism and activation of PAHs is done by phase I and phase II reactions. PAHs are biotransformed by both phase I enzymes which oxidise, reduce or hydrolyse, , and phase II enzymes, which form mainly polar conjugates, more easily excreted (Altenburger et al., 2003; Ewa and Danuta, 2017). Transformation of these compounds involves three major pathways: the CYP1A1/1B1 and EH pathway (CYP/EH pathway), CYP peroxidase pathway, and aldo-keto reductases (AKR) pathway (AKR pathway) (Ewa and Danuta, 2017; Moorthy et al., 2015; Nebert et al., 2004). CYP1A1 and CYP1B1 are highly inducible by PAHs via activation of aryl hydrocarbon receptor (AhR). The AhR is expressed in almost all tissues and highly expressed in liver, adipose tissue (where PAHs accumulate), and bronchial epithelial cells. At the cellular level, the AhR is present in the cytoplasm as a complex with other proteins, such as heat shock protein 90 (Hsp90), p23 and AhR-interacting protein. Having formed a complex with PAHs, Hsp90 is released

and an AhR-PAH complex is translocated into the nucleus. Once in the nucleus, the AhR-PAH complex creates a heterodimer with an AhR nuclear translocator (AhRNT), producing complexe molecules with the hability to interact with major macromolecules (e.g., nucleic acids) (Moorthy et al., 2015). These interactions alter gene expression and lead to the upregulation of CYP enzymes, which target PAHs and iniciate their biotransformation. Therefore, AhR plays an important role in PAHs mediated tumorigenesis (Tarantini et al., 2011; Xue and Warshawsky, 2005).

CYP-mediated epoxidation is often the first step in PAHs biotransformation and begins primarily in endoplasmatic reticulum through CYP. PAHs biotransformation involves the formation of phenols, catechols, quinones, diol-epoxides, o-quinones, and radical cations (Bai et al., 2017; Moorthy et al., 2015; Nebert and Dalton, 2006; Nebert et al., 2004). Further hydroxylation generates active metabolites (PAH-diols) that form DNA adducts by nucleophilic attack (Ewa and Danuta, 2017; Moorthy et al., 2015; Nebert et al., 2004). Peroxidases and some CYP enzymes may also catalyse a one-electron oxidation of PAHs, producing toxic radicals that can be further oxidized to quinone radicals, which are able to induce hight levels of cytotoxicity *via* oxidative stress. The consequent production of ROS can directly affect DNA, lipids and proteins, and initiate carcinogenesis (Käfferlein et al., 2010; Verma et al., 2019). Phase II metabolism of intermediary metabolites is carried out by enzymes such as glutathione-S-transferases, UDP-glucuronyl transferase and sulfotransferases, faciliting metabolic clearance and PAH metabolites excretion (Ewa and Danuta, 2017; Moorthy et al., 2015).

Diet and chemical co-exposures can impact PAH metabolism, including pharmaceuticals and drugs that are inducers of CYP isoforms, or may function as inhibitors or competitors of CYP isoforms. Thus, lifestyle and dietary patterns play an important role in modulating the biotransformation, bioactivation and detoxification of PAHs in humans (Gao et al., 2018).

In short, metabolism of PAHs occurs in all tissues and involves several possible pathways. The metabolic products include epoxide intermediates, dihydrodiols, phenols, quinones and their combinations. While phenols, quinones and dihydrodiols can all be conjugated to form glucuronides and sulfate esters, quinones also form glutathione conjugates. The pathways for metabolic activation of PAHs can form three carcinogens: i) dihydrodiol epoxides requiring CYP-catalyzed oxidations and epoxide hydrolase (CYP/EH pathway); ii) radical cations by cytochrome P450 peroxidase activity (CYP peroxidase pathway); and iii) ortho-quinones via catechols by involving AKRs (AKR pathway) (Ewa and Danuta, 2017; Moorthy et al., 2015).

1.2.4 Biological effects of PAHs

Various studies focused on the impact of PAHs exposure on the environment and human health, such those that aimed to determine the relationship between PAHs exposure and the prevalence of cancer (Defois et al., 2017; Elie et al., 2015; Gao et al., 2018; Kalkhof et al., 2015; Søfteland et al., 2014). One important outcome of such studies is that the effects of specific PAHs on human health mainly depend on the extent of their exposure (acute, chronic, sequential), the concentration of PAHs during exposure, the individual toxicity of the PAHs, the existence of interactions or additive effects, and the route of exposure, i.e., inalation, dermal contact or ingestion (Ma and Harrad, 2015; Ruby et al., 2016).

Short-term health effects include eye and skin irritation, nausea, vomiting and inflammation, while long-term effects may include DNA and protein damage, gene mutation and various cancers (Abdel-Shafy and Mansour, 2016; García-Suástegui et al., 2010; Kim et al., 2013; Srogi, 2007). People who often get exposed to mixtures of PAHs in occupational contexts (e.g., workers in industries using or producing coal or coal products) are more vulnerable to a series of health problems such as increased risk of skin, lung, bladder and gastrointestinal cancers (Armstrong et al., 2004; Kuo et al., 2003; Navarro et al., 2017). Oxidative stress (Wang et al., 2015), diabetes (Yang et al., 2017), inflammation (Ferguson et al., 2017), infertility (Xia et al., 2009), cardiovascular disease (Jomova et al., 2012) and poor fetal development (Sexton et al., 2011), are also some adverse health consequences of PAHs exposures. PAHs also have potential to interfere with hormonal systems, exercising harmful effects on reproduction and immune function (Kim et al., 2013; Kuo et al., 2003; Sexton et al., 2011; Xia et al., 2009).

ANT, B[a]P and NAP are known direct skin irritants and sensitizers, causing allergic skin response in animals and humans (Lawal, 2017). Frequent dermal exposure to NAP may result in redness and inflammation of the skin, while breathing or ingesting large amounts of the substance may result in break down of red blood cells (Srogi, 2007). Phe is one of the 16 PAHs that are considered relevant for environmental monitoring by EPA (U.S. Environmental Protection Agency, 1993) due to? its potential to induce genotoxicity, mutagenicity and neurotoxicity, although it is regarded as non-carcinogenic to humans by IARC (classified in group 3) (IARC, 2010). PAHs having four or more aromatic rings, such as F, one the most abundant PAH pollutants of pyrogenic origin, are more recalcitrant to microbial enzymes. F is also considered non-carcinogenic to humans by IARC classification. Chry, considered as possible carcinogenic, demonstrated positive result for initiating activity and producing skin papailomas and carcinomas when combined with various promoting agents, such as decahydronaphthalene (Biswas and Ghosh, 2014). B[a]P is among the PAHs that have been more extensively studied due to its carcinogenic proprierties. B[b]F is one of PAHs

that are consider possibly carcinogenic to humans, although there are no data that specifically link B[b]F with human cancers (IARC, 2010).

Carcinogenicity of PAHs

Although unmetabolized PAHs can have toxic effects, PAHs themselves are relatively non-reactive chemicals toward macromolecules under physiological condictions. They require metabolic activation in order to exert genotoxicicity, including mutagenicity and tumorigenicity (Yu, 2002). Reactive metabolites of PAHs, like epoxides and dihydrodiols, have ability to bind covalently to cellular proteins and exocyclic amino groups of purines in DNA, forming DNA stable adducts (Abdel-Shafy and Mansour, 2016; Armstrong et al., 2004; Broyde et al., 2011; Ewa and Danuta, 2017; Kim et al., 2013; Wang et al., 2015). Some carcinogenic PAHs are genotoxic by inducing mutations to initiate cancer, while others are not genotoxic but they enhance cancers (Abdel-Shafy and Mansour, 2016). Based on their carcinogenic tendencies, PAHs are classified by the IARC into five groups (group 1, 2A, 2B, 3, or 4), (**Table *2***).

Table 2. Carcinogenicity of polycyclic aromatic hydrocarbons (PAHs) according to International Agency of Research of Cancer (IARC) classification (IARC, 2010; McQueen, 2010).

Group	Definition	PAHs
1	The agent (or mixture) is carcinogenic to humans. The exposure circumstance entails exposures that are carcinogenic to humans	Benzo(a)pyrene (B[a]P)
2A	The agent (or mixture) is probably carcinogenic to humans. The exposure circumstance entails exposures that are probably carcinogenic to humans	Cyclopenta(*cd)*pyrene (CP[cd]P); Dibenz(*a,h)*anthracene (DB[ah]A); Dibenzo(*a,l)*pyrene (DB[al]PYR)

Table 2. Carcinogenicity of polycyclic aromatic hydrocarbons (PAHs) according to International Agency of Research of Cancer (IARC) classification (IARC, 2010; McQueen, 2010) (cont.)

Group	Definition	PAHs
2B	The agent (or mixture) is possibly carcinogenic to humans. The exposure circumstance entails exposures that are possibly carcinogenic to humans	Benz(*j*)aceanthrylene (B[j]A); Benz(*a*)anthracene (B[a]A); Benzo(*b*)fluoranthene (B[b]F); Benzo(*j*)fluoranthene B[j]F; Benzo(*k*)fluoranthene B[k]F; Benzo(*c*)phenanthrene B[c]Phe; Chrysene (Chry); Bibenzo(*a,h*)pyrene (BB[ah]P); Bibenzo(*a,i*)pyrene (BB[ai]P); Indeno(1,2,3-*cd*)pyrene (IP); 5-methylchrysene (5-MC)
3	The agent (mixture or exposure circumstance) is not classificable as to its carcinogenicity to humans.	Anthracene (ANT); Fluoranthene (F); Fluorene (FLUO); Phenanthrene (Phe)
4	The agent (mixture) is probably not carcinogenic to humans.	

The IARC Monographs Programme has reviewed the experimental data for 60 individual PAHs (IARC, 2010) and, based on results, a recent revision of this table simplified the evaluation into four categories (groups 1, 2A, 2B, and 3) (

Table **3**) *(Samet et al., 2019)*.

Table 3. Cancer hazard identification based on streams of evidence (Samet et al., 2019)

Type of evidence					
			Overall evaluation	**Classification based on evidence**	**PAHs**
Cancer in humans*	**Cancer in experimental animals**	**Mechanistic evidence**			
Sufficient	Not necessary	Not necessary	Cancer in humans	Carcinogenic to humans (**group 1**)	Benzo(a)pyrene (B[a]P)
Limited or inadequate	Sufficient	Strong: key characteristics of carcinogens, from exposed humans	Cancer in experimental animals and mechanistic evidence	Probably carcinogenic to humans (**group 2A**)	Cyclopenta(*cd*)pyrene (CP[cd]P); Dibenz(*a,h*)anthracene DB[ah]A; Dibenzo(*a,l*)pyrene (DB[al]PYR)
Limited	Sufficient	Not necessary	Cancer in humans and experimental animals		
Inadequate	Sufficient	Strong: key characteristics of carcinogens, from human cells or tissues	Cancer in experimental animals and mechanistic evidence		
Limited	Less than sufficient	Strong: key characteristics of carcinogens	Cancer in humans and mechanistic evidence		

*Highest strength of evidence for any cancer site(s); † The strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans must specifically be for the tumor sites supporting the classifications of "sufficient evidences in the experimental animals"

Table 3. Cancer hazard identification based on streams of evidence (Samet et al., 2019) (cont.)

Type of evidence					
			Overall evaluation	**Classification based on evidence**	**PAHs**
Cancer in humans*	**Cancer in experimental animals**	**Mechanistic evidence**			
Limited or inadequate	Not necessary	Strong: the agent belongs to a mechanistic class of agents for which one or more members have been classified in group 2A or 1	Mechanistic evidence	Prossibly carcinogenic to humans (**group 2B**)	Benz(*j*)aceanthrylene (B[j]A); Benz(*a*)anthracene (B[a]A); Benzo(*b*)fluoranthene (B[b]F); Benzo(*j*)fluoranthene B[j]F; Benzo(*k*)fluoranthene B[k]F; Benzo(*c*)phenanthrene B[c]Phe; Chrysene (Chry); Bibenzo(*a,h*)pyrene (BB[ah]P); Bibenzo(*a,i*)pyrene (BB[ai]P); Indeno(1,2,3-*cd*)pyrene (IP); 5-methylchrysene (5-MC)
Limited	Less than sufficient	Limited or inadequate	Cancer in humans		
Inadequate	Sufficient	Not necessary	Cancer in experimental animals		
Inadequate	Less than sufficient	Strong: key characteristics of carcinogens	Mechanistic evidence		
Limited	Sufficient	Strong: the mechanism of carcinogenicity in experimental animals does not operate in humans ŧ	Cancer in humans and mechanistic evidence		

*Highest strength of evidence for any cancer site(s); ŧThe strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans must specifically be for the tumor sites supporting the classifications of "sufficient evidences in the experimental animal

Table 3. Cancer hazard identification based on streams of evidence (Samet et al., 2019) (cont.)

Type of evidence					
			Overall evaluation	**Classification based on evidence**	**PAHs**
Cancer in humans*	**Cancer in experimental animals**	**Mechanistic evidence**			
Inadequate	Sufficient	Strong: the mechanism of carcinogenicity in experimental animals does not operate in humans †	Mechanistic evidence	Not classifiable as to its carcinogenicity to humans (**group 3**)	Anthracene (ANT); Fluoranthene (F); Fluorene (FLUO); Phenanthrene (Phe)
All other situations not listed above					

*Highest strength of evidence for any cancer site(s); † The strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans must specifically be for the tumor sites supporting the classifications of "sufficient evidences in the experimental animals"

Group 1 consists of substances with carcinogenic potential for humans and B[a]P, supported by sufficient toxicological data both in animls and humans, is the only member. This category is used whenever there is sufficient evidence of carcinogenicity in humans. In addition, this category may apply when there is both strong evidence in exposed humans that the agent exhibits key characteristics of carcinogens and sufficient evidence of carcinogenicity in experimental animals. In group 2A, the agent is probably carcinogenic to humans and a major example in this group is DB[ah]A. Although, the list contains more PAHs in this category, they are not among the EPA priority contaminants. In group 2B, the agent is possibly carcinogenic to humans and this category generally applies when exist a limited evidence of carcinogenicity in humans, sufficient evidence of carcinogenicity in experimental animals or strong evidence that the agent exhibits key characteristics of carcinogens. B[a]A and B[b]F are some examples of PAHs in this group. Finally, from the previous classification, group 3 (not classifiable) and group 4 (probably not carcinogenic to humans) have been combined. When epidemiological studies do not find a positive association between the compound and cancer in humans, the agent should be classified in group 3. An evaluation as group 3 is not an assumption of non carcinogenicity, but it might mean that the agent has unknown carcinogenic potential and cannot be considered definitely safe. AC, FLUO, Phe, ANT, F, PYR, and B[ghi]P are some of the 45 PAHs included in this group (IARC, 2010; Samet et al., 2019).

PAHs require enzymatic metabolic activation to exert their carcinogenic effects, and one important pathway proceeds through a three-step sequence resulting in the formation of diol epoxides, which react with DNA to produce adducts that can cause mutations and initiate the carcinogenic process. Adduct formation is the result of a covalent binding between reactive electrophilic substances and the nucleophilic sites in DNA and proteins. The biological activity of these compounds is connected with their structural features, in particular with "fjord" and "bay" regions. Molecules with "fjord" regions (e.g., dibenzo[a,l]pyrene (DB[al]PYR)) are generally non-planar and bind preferentially to adenine nucleotides, while PAHs with a "bay" region (e.g., B[a]P and B[b]F) are planar and bind to guanine nucleotides. Though their reactivity depends on the density of electron charges, geometric distortions in molecules also influence charge distribution and indirectly its reactivity. Furthermore, increasing the non-planarity of PAHs lowers their capability of being metabolized to reactive forms and, therefore, to produce DNA-damaging adducts (Ewa and Danuta, 2017; Gao et al., 2018; Xue and Warshawsky, 2005).

1.3 Combination effects of PAHs

Human populations are exposed to a variety of mixtures of potentially toxic PAHs, all too often at very low concentrations. Due to the ubiquity of PAHs, it is rare to find these compounds isolated in the environment, and is very common to also find them in combination with other toxic compounds. PAHs mixtures in complexe environmental matrices are very common and the behaviour of such mixtures may lead to antagonistic, additive or synergistic interactions between the compounds. In this regard, Martins et al, conduced an experimental study to deepen the understanding on the interaction effects between carcinogenic and non-carcinogenic PAHs in a model of marine fish (*Dicentrarchus labrax*) (Martins et al., 2015). The laboratory assays were carried out for 28 days, under ecologically relevant parameters, i.e., fish were exposed to realistic concentrations (taking into account their Threshold Effects Level (TEL) and Probable Effects Level (PEL)) of carcinogenic/non-carcinogenic PAHs. The sediments were spiked with low-moderate concentrations (250-800 ng/g) of Phe (non-carcinogenic) and B[b]F (carcinogenic to experimental animals). Both PAHs induced hepatic histopathological changes that indicate metabolic failure and inflammation, especially in animals exposed to the mixtures of both drugs. Phe elicited biochemical changes better related to oxidative stress (lipid peroxidation, glutathione decline, and increased glutathione S-transferase activity) and CYP induction, whereas B[b]F disrupted metabolic responses and defences. Mixed PAHs yielded lesions and responses that, altogether, are compatible with the AhR-dependent pathway (the basis of PAH mutagenicity), potentially generating supra-additive effects. Their results demonstrate that environmental guidelines may not apply

to mixtures by underestimating adverse effects and that the true risk of PAHs toxicity in realistic circunstances may be overlooked (Martins et al., 2015).

Pushparajah et al. (2017) investigated synergistic or antagonistic interactions of binary mixtures of B[a]P and five others PAHs, i.e., DB[ah]A; F; B[b]F; dibenzo[a,l]pyrene (D[al]P); and 1-methylphenanthrene (1-MP), in the upregulation of CYP1 activity and mRNA levels, using precision-cut rat liver slices. Precision-cut rat liver slices were incubated with benzo[a]pyrene (0.5 or 1.0 μM) alone or in combination with a range of concentrations of a second PAH, and ethoxyresorufin O-deethylase (EROD), CYP1A1 and CYP1B1 mRNA levels determined. They found that the concurrent incubation of B[a]P with either DB[ah]A (0-0,25 μM) or F (0-100 μM) for 24 hours led to a synergistic interaction at least at low concentrations, and that EROD activity was statistically higher than the added effects of the individual compounds. B[b]F (0-1 μM) and D[al]P (0-100 μM) gave rise to antagonism at high concentrations only, whereas 1-MP (0-100 μM) had no effect at all concentrations studied. When CYP1A1 mRNA levels were monitored, B[b]F gave rise to an antagonistic response when incubated with B[a]P, whereas all other compounds displayed synergism, with 1-MP being the least effective.

In fact, mixtures of PAHs may exhibit significantly different toxicities when compared with their individual components; this happens for a variety of reasons, including competition for receptors, metabolic modulation, and altered bioavailability (Altenburger et al., 2003; Cedergreen, 2014). Since the toxicity of the PAHs may depend on their biotransformation into toxic metabolites, the interactions at the level of the metabolic enzymes that occur in the mixture settings, might origin that one PAH enhance or decrease the toxicity of another PAH. Unfortunatly, the study of mixtures effects of PAHs is often difficult, since most toxicological data are related to the individual compounds and oftentimes is very difficult to know all constituents of the mixtures at which humans and organisms are exposed to. In addition, PAHs have long-term effects and they can act sequentially or simultaneously as a mixture.

Of concern, some studies have shown toxic effects in mixtures of various PAHs present at regulatory admissible levels (Ba et al., 2015; Martins et al., 2015; Pushparajah et al., 2017). Since it is impossible to test all the conceivable mixtures, it is, at least, important to be able to predict the type of interaction between PAHs, to more reliably define the admissible levels for single/combined exposure to PAHs.

Methodologies to assess mixture effects

In general, due to the spacial and temporal variability of the composition of mixtures present in the environment or in a given organism, it is unrealistic to directly assess the toxicity of all possible combinations of substances (Qin et al., 2011). It is commonly accepted by the scientific community that, if all the components of a given mixture are

known, their toxicity can be predicted based on the individual toxicities of the components, provided the ratio between them is known (Qin et al., 2011). Yet, predicting the effects of these mixtures from the effects of single compounds poses a major challenge (Olmstead and LeBlanc, 2005).

There are two main additive approaches to predict the combined effect of substances, namely concentration addition (CA), also called dose addition or Loewe additivity; and independent action (IA), also called response additivity or Bliss independence (Altenburger et al., 1996; Bliss, 1939; Goldoni and Johansson, 2007; Loewe and Muischnek, 1926). These two reference models assume no interaction between compounds and are used to describe the joint toxicity based on the MoA of single compounds. The CA model is applied when two or more compounds with similar MoA affect the same target/endpoint of toxic action, being the sum of toxicity of similarly acting chemicals, scaled to reflect their relative toxicities. The IA model assumes that two or more chemicals affect the same endpoint but through dissimilar MoA, and their effects are statistically independent of each other. However, the MoA of the compounds in the chemical mixtures might be unknown and, in these cases, both the CA and IA models are applied to predict the mixture effect.

The concept of CA was originally introduced by Loewe and Muischnek (1926) and can be mathematically explained by the equation 1 (Loewe, 1927; Loewe and Muischnek, 1926), where $ECx_{(mix)}$ is the predicted concentration of the mixture that induces x% effect; p_i is the relative fraction of component i in the mixture; and ECx_i is the concentration of the substance i provoking a certain effect x when applied alone:

$$ECx_{(mix)} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1} \qquad (equation\ 1)$$

The concept of IA was first applied to biological data by Bliss (1939), and can be mathematically explained by the equation 2, where E_{mix} is the effect of the mixture of n compounds; and E_i is the effect of the substance i when applied singly:

$$E_{mix} = 1 - \prod_{i=1}^{n} [1 - E_i] \qquad (equation\ 2)$$

In experimental data where the mixture effect deviates from the additive models estimates, synergisms (i.e., when the effect caused by exposure to two or more chemicals at one time results in the effect greater than the sum of the effects of individual

chemicals) or antagonisms (i.e., when the combined effect of two or more compounds is less than the individual effects) are at play (Altenburger et al., 2003; Jonker et al., 2005).

Chapter II - Objectives

From a toxicokinetic/toxicodynamic point of view, PAHs share many of their mechanisms, toxic effects and also the same metabolic pathways for bioactivation. Therefore, it is reasonable that the cooccurrence of these substances may result in toxicities different from those observed when PAHs are present individually. Althought the risk of interaction between different PAHs has been clearly pointed out, the lack of systematic information on the effects of the simultaneous occurrence of these substances in the environment, hinders their risk assessment. The present project aimed to scrutinize the effects of PAHs mixtures. The motto of this investigation was to modulate the proportions and the number of different PAHs, in order to elucidate the type and extent of toxicological effects induced by different PAHs and their mixtures, eventually revealing significant effects from the cooccurrence of these compounds. To increase the understanding of toxicological interations between these PAHs, the following specific objectives were outlined:

- Assess whether the hepatotoxic effects of three carefully selected PAHs mixtures could be accurately predicted, based on the toxicological information of the individual PAHs, using the CA and IA models;
- Determine if there are significant mixing effects when the individual components are present in the mixtures in low, "realistic" concentrations, even if in isolation they do not produce measurable effects;
- Establish whether the mixtures are additive, synergistic or antagonistic, comparing the experimental observations with the expectations obtained according to the two additivity models, CA and IA.

Chapter III- Material and methods

3.1 Chemicals

All chemicals used in this study were of analytical grade. B[a]P (≥96%), B[b]F (98%), Phe (98%), F (98%), and Chry (98%) were acquired from Sigma-Aldrich (Lisbon, Portugal). Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich (Lisbon, Portugal) and all the cell culture reagents from Gibco® (Alfagene, Lisbon, Portugal).

3.2 Animals

This study was performed at the highest standards of ethics after approval by the local Ethical Committee for the Welfare of Experimental Animals (University of Porto-ORBEA) and by the national authority *Direção-Geral de Alimentação e Veterinária* (DGAV). Housing and all experimental procedures were performed (or supervised) by investigators accredited for laboratory animal use in accordance with the Portuguese and European legislation (law DL 113/2013, Guide for Animal Care; Directives 86/609/EEC and 2010/63/UE) under strict supervision of veterinary physicians. Female Wistar Han rats with a body weight of 150-250 g were kept in sterile facilities under controlled temperature (20±2 °C), humidity (40–60%), and light (12 h-light/dark cycle) conditions, and were fed with sterile standard rat chow and tap water *ad libitum*. Isolation of hepatocytes was always conducted between 8:00 and 10:00 a.m., with the surgical procedures being performed after rat anaesthesia and analgesia induced by an i.p. injection of a combination of 100 mg/kg ketamine (Clorketam® 1000, Vétoquinol, France) and 20 mg/kg xylazine (Rompun® 2%, Bayer HealthCare, Germany), and maintained through inhalation of isoflurane vapour (IsoVet® 1000 mg/g, B. Braun VetCare, Germany).

3.3 Isolation of primary rat hepatocytes

Isolation of hepatocytes was performed using a modified two-step perfusion of the liver, as previously described by Dias da Silva (2017) with some modifications. The liver was perfused *in situ* via the portal vein, with a sterile EGTA-buffer at 37 °C, for approximately 8–10 min. The chelator promoted the irreversible cleavage of the hepatic desmosomes (junctional complexes) through calcium sequestration. The EGTA-buffer consisted of 155 mL glucose solution (9 g/L *d*-glucose), 25 mL Krebs-Henseleit-buffer (60 g/L NaCl, 1.75 g/L KCl, and 1.6 g/L KH_2PO_4; adjusted to pH 7.4), 25 mL HEPES-buffer I (60 g/L HEPES; adjusted to pH 8.5), 18.5 mL MEM non-essential amino acid solution (100×), 18.5 mL MEM Amino Acids (5×) solution, 2.5 mL glutamine solution (7 g/L *l*-glutamine), and 1 mL EGTA-solution (47.5 g/L EGTA; dissolved by addition of NaOH, adjusted to pH 7.6). Subsequently, hepatic collagen was hydrolysed by liver perfusion for 10–15 min with a sterile collagenase buffer supplemented with calcium (collagenase cofactor), at 37

°C. The collagenase buffer consisted of 77.5 mL glucose solution, 12.5 mL Krebs-Henseleit-buffer, 12.5 mL HEPES-buffer I, 7.5 mL MEM non-essential amino acid solution (100×), 7.5 mL MEM amino acids (5×) solution, 2.5 mL $CaCl_2$ solution (19 g/L $CaCl_2.2H_2O$), 1.25 mL glutamine solution, and ~300 U/mL collagenase type IA from *Clostridium histolyticum* (dissolved immediately before use). After perfusion, the liver was dissected, removed from the animal, and the hepatic capsule gently disrupted in a sterile suspension buffer [124 mL glucose solution, 20 mL KH-buffer, 20 mL HEPES-buffer II (60 g/L HEPES; adjusted to pH 7.6), 15 mL MEM non-essential amino acid solution (100×), 15 mL MEM amino acids (5×) solution, 2 mL glutamine solution, 1.6 mL $CaCl_2$ solution, 0.8 mL $MgSO_4$ solution (24.6 g/L $MgSO_4.7H_2O$), and 400 mg bovine serum albumin (BSA)]. The obtained suspension was purified by three low-speed centrifugations at 50 *g*, for 2 min, at 4 °C. The viability of isolated hepatocytes was always above 85%, as assessed by the trypan blue exclusion method.

3.4 Culture of primary rat hepatocytes

A suspension of 5×10^5 viable cells/mL in culture medium was seeded onto the central 60 wells of 96-well plates (5×10^4 cells/well; BD Falcon, Enzifarma, Lisbon, Portugal) pre-coated with collagen G (Biochrom Ltd.). Cell culture medium consisted of William's E medium (Sigma-Aldrich, Lisbon, Portugal) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 5 μg/mL insulin solution from bovine pancreas (Sigma-Aldrich, Lisbon, Portugal), 50 nM dexamethasone (Sigma-Aldrich, Lisbon, Portugal), 1% antibiotic solution (10,000 U/mL penicillin; 10,000 μg/mL streptomycin), 100 μg/mL gentamicin, and 250 ng/mL amphotericin B. After seeding, primary rat hepatocytes were left to adhere overnight at 37 °C, in an atmosphere of 5% CO_2. On the next day, cells were exposed to PAHs.

3.5 Exposure of cells to PAHs

Stock solutions of PAHs and their mixtures were prepared in dimethyl sulfoxide (DMSO), stored protected from light at -20 °C in glass vials, and freshly diluted in cell culture medium on the day of the experiment. Primary rat hepatocyte cultures were exposed to sixty-four concentrations of each PAH and their mixtures (from 5 nM to 10 mM), previous to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay. PAHs exposures were performed for 24h or 48h, at 37 °C, in a humidified, 5% CO_2 atmosphere. Solvent and negative controls were always assessed parallel to PAHs incubations. In any circumstance the percentage of solvent in the medium was higher than 0.05%. Comparison between solvent (DMSO at the maximum

concentration tested) and negative controls (cells incubated in cell culture medium only) showed no statistically significant differences in viability(p>0.05).

3.6 Mixture testing

In this work, a binary mixture of B[a]P and B[b]F was selected due to the similarity of these PAHs (both have 5 rings and they are carcinogens or potencial carcinogens, respectively); a ternary mixture of B[a]P, Phe and Chry was selected due to their dissimilarity (these PAHs display a structure with 5, 3 and 4 rings, respectively); and a quintenary mixture of B[a]P, Phe, Chry, B[b]F and F was selected due to its complexity; in all cases, PAHs were combined in proportions similar to those that were found in sediments of the Tejo River (Martins et al., 2012). Accordingly, mixture stock solutions were prepared combining the individual components in the proportions shown in Table ***4 .***. A wide range of working concentrations was then extemporaneously produced in cell culture medium, using the *fixed mixture ratio design*, as described by Altenburger et al. (2000) and Backhaus et al. (2000). Briefly, the stock solution of each mixture was diluted in series, keeping the proportion between each constituent unchanged. Serial dilutions covered a wide range of concentrations, so that a complete concentration-response relationship could be obtained.

Table 4 . Concentrations of Polyclyclic Aromatic Hydrocarbons (PAHs) found in the Tejo river sediment (Martins et al., 2012), on which were based the design of a binary mixture of benzo(a)pyrene (B[a]P) and benzo(b)fluoranthene (B[b]F); a ternary mixture of B[a]P, phenanthrene (Phe) and chrysene (Chry); and a quintenary mixture of benzo(a)pyrene (B[a]P), phenanthrene (Phe), chrysene (Chry), benzo(b)fluoranthene (B[b]F) and Fluoranthene (F).

		Fraction in the mixture		
PAHs	**ng PAH/g sediment**	**Binary**	**Ternary**	**Quintenary**
Phe	72.40	-	0.40	0.11
B[a]P	70.20	0.47	0.39	0.11
F	402.10	-	-	0.61
Chry	37.60	-	0.21	0.06
B[b]F	79.60	0.53	-	0.12

3.7 Cell viability evaluation by the MTT reduction assay

The cytotoxicity of the PAHs, individually or in mixture, was evaluated in primary rat hepatocytes seeded onto 96-well plates using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, as described before (Dias-da-Silva et al., 2015). The assay assesses cell viability indirectly, by measuring the activity of redutases that convert soluble yellow MTT into purple insoluble formazan salts. Since MTT can only be reduced when these enzymes are active, the reaction is used as an indicator of cellular metabolic competence and, therefore, viability. As previously described (Dias da Silva et al., 2017), after incubation with PAHs, the cell culture medium was aspirated from the plate, and 100 μL of 1 mg/mL MTT solution was added to each well. The cells were subsequently incubated at 37 ° C, for 1 hour. Then, the MTT solution was aspirated and the intracellular purple formazan crystals dissolved with 100 μL of DMSO. The plate was shaken for 15 minutes, protected from light. The absorbance of the coloured solution was measured at 550 nm, directly on the plate, using a BioTek Synergy™ HT (BioTek Instruments, Inc.). The results were normalized with positive (1% Triton X-100) and negative controls, and plotted as the percentage of cell death *versus* concentration (mM).

3.8 Prediction of mixture effects

The mixture effects were calculated using two widely used additivity models, the CA model and the IA model (da Silva et al., 2014; Dias-da-Silva et al., 2015; Dias da Silva et al., 2013a; Dias da Silva et al., 2013b). Assuming that the effect of a mixture with $\boldsymbol{n}$ components is additive by CA, the effect of the mixture is calculated by $\boldsymbol{ECmix} = [\sum \boldsymbol{pi}/\boldsymbol{ECi}]^{-1}$, where $\boldsymbol{ECmix}$ is the concentration of the mixture that causes an effect of defined magnitude, $\boldsymbol{pi}$ is the fraction of each individual compound in relation to the total concentration of the mixture needed to produce the determined effect, and $\boldsymbol{ECi}$ is the concentration of each individual compound that can produce that same effect. The mixture effect is estimate by IA according to $Emix = Emax[1 - \prod[1 - Fi(ci)/Emax]]$, where $\boldsymbol{Emax}$ is the maximum measured effect and $\boldsymbol{Fi\ (ci)}$ the average effect predicted by the regression model, for each PAH.

3.9 Statistical Analysis

In the MTT redution assay, each concentration was tested in triplicate, in four independent experiments (unless otherwise indicated). The results were normalized by the negative and positive controls, and adjusted to the dosimetric Logit model which was chosen based on a statistical goodness-of-fit principle: y = θmax/(1 + exp[−θ1 − θ2 *

log(x)]), where $\boldsymbol{\theta max}$ is the maximal observed effects, $\boldsymbol{\theta 1}$ is the parameter for location, $\boldsymbol{\theta 2}$ is the slope parameter and $\boldsymbol{x}$ is the concentration of the PAH. To compare concentration-response curves, the overlapping of the 95 % confidence intervals was used in addition to the extra sum-of-squares *F* test. Solvent and negative control values were compared by the Student's *t* test. In all cases, significance was accepted at *p* values <0.05. All statistical calculations were performed using GraphPad Prism software, version 7.0 (GraphPad Software, San Diego, CA, USA).

Chapter IV - Results and Discussion

4.1 The cytotoxic potency of PAHs changes with the time of exposure

Studies that have examined the cytotoxicity of Phe, F, Chry, B[b]F and B[a]P are mostly focused on cancer end points (Jarvis et al., 2014; Nwagbara et al., 2007; Smith et al., 2019).

Considering that the liver is, par excellence, the metabolizing organ of xenobiotics, and given the importance of the bioactivation of PAHs for the expression of their toxicity, the experimental model of primary rat hepatocytes, which are rich in CYP450, was selected to evaluate the profile of cytotoxicity of three mixtures, from simple to complex, of five PAHs, namely, Phe, F, Chry, B[b]F and B[a]P; and although most investigations concerning PAHs are carried out on fish (Silva et al., 2002), the use of other species, such as the rat, can provide an additional source of diversity to the current studies and a more robust model for translability to human. This experimental model proved to be very robust for this type of experiments, allowing a large number of replicates, which are necessary for the mixture studies. In addition, since these are cells directly extracted from a living organism, they provide a more reliable representation of the liver *in vivo* than the traditional cell lines that accumulate mutations along the passages.

To accurately estimate the effects of a well-defined composition mixture, detailed and reliable information on the individual effects of each component is necessary (Martins et al., 2012; Martins et al., 2015). Therefore, a comprehensive range of concentrations (64 concentrations; from 5 nM to 10 mM) was tested for all PAHs individually, through the MTT reduction test, allowing to obtain the most possible complete curves (ideally, from 0% to 100% effect). Figure 2 and Figure 3 show the cytotoxicity curves obtained for the individual compounds after incubation at 24 and 48 hours, respectively, and 5 shows the EC50 of the PAHs at these selected time-points.

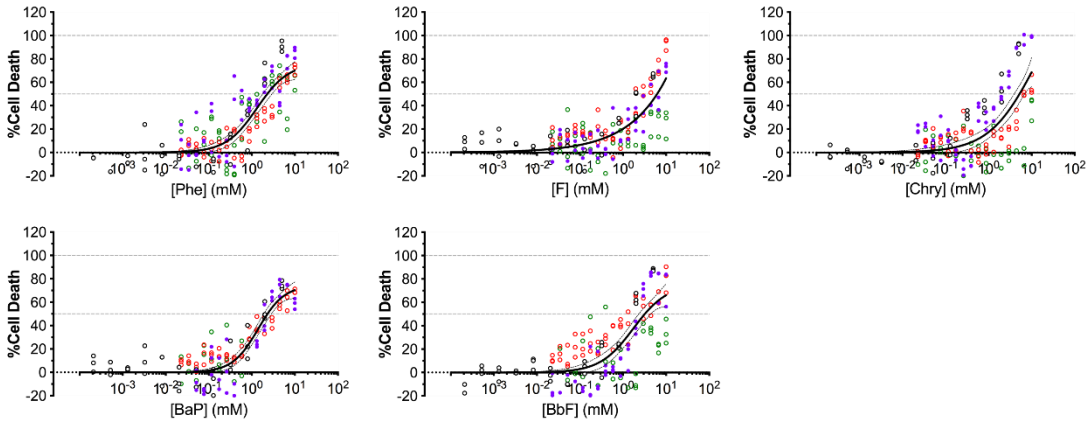


Figure 2- Cytotoxicity caused by phenanthrene (Phe), fluoranthene (F), chrysene (Chry), benzo(b)fluoranthene (B[b]F) and benzo(a)pyrene (B[a]P) in primary rat hepatocytes, as evaluated by the MTT reduction assay, after incubation for 24 hours, at 37 ° C. The results are

presented as percentage of cell death in relation to the negative control and are from a minimum of four independent experiments (each experiment represented by dots of different colours), performed in triplicate. The curves were fitted to the dosimetric Logit model. The dashed lines represent the upper and lower limits of the 95% confidence interval of the best estimate of the mean responses. The dotted lines represent 50 and 100% of the effect.

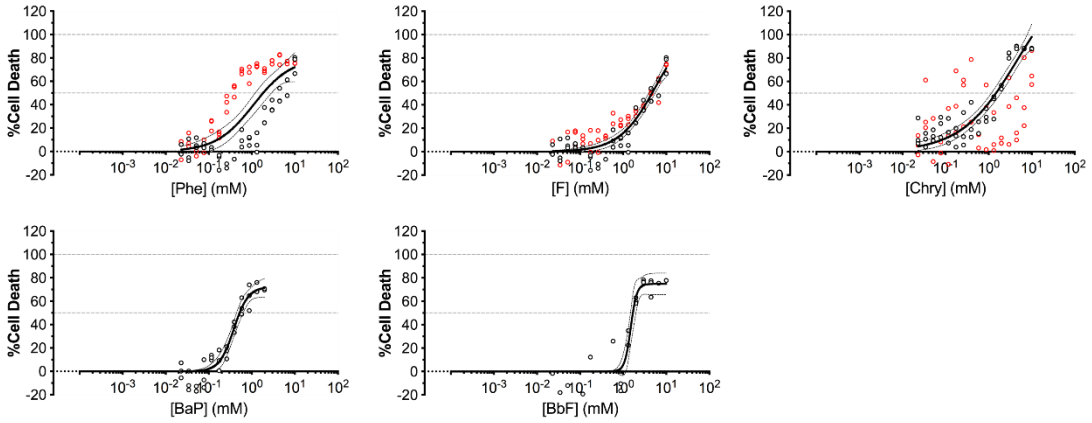


Figure 3- Cytotoxicity caused by phenanthrene (Phe), fluoranthene (F), chrysene (Chry), benzo(b)fluoranthene (B[b]F) and benzo(a)pyrene (B[a]P) in primary rat hepatocytes, as evaluated by the MTT reduction assay, after incubation for 48 hours, at 37 ° C. The results are presented as percentage of cell death in relation to the negative control and are from one or two independent experiments (each experiment represented by dots of different colours), performed in triplicate. The curves were fitted to the dosimetric Logit model. The dashed lines represent the upper and lower limits of the 95% confidence interval of the best estimate of the mean responses. The dotted lines represent 50 and 100% of the effect.

Table 5. Cytotoxicity caused by phenanthrene (Phe), fluoranthene (F), chrysene (Chry), benzo(b)fluoranthene (B[b]F) and benzo(a)pyrene (B[a]P) in primary rat hepatocytes, as evaluated by the MTT reduction assay, after incubation for 24 or 48 hours, at 37 ° C.

PAHs	EC_{50} (mM)		Ratio EC_{50} 24/ 48 hours
	24 hours	48 hours	
Phe	2.28	1.84	1.24
F	6.17	4.67	1.32
Chry	5.21	1.47	3.54 (p<0.001)
B[b]F	3.19	1.68	1.90 (p<0.01)
B[a]P	2.32	0.52	4.46 (p<0.001)

EC_{50}, Half maximal effective concentration; Phe, Phenanthrene; F, Fluoranthene; Chry, Chrysene; B[b]F, Benzo(b)Fluoranthene; B[a]P, Benzo(a)Pyrene

According to the results observed at 24 hours, the most potent PAH was Phe (EC50 2.28 mM), with a cytotoxic potency very similar to that of B[a]P (EC50 2.32 mM).

This result is in line with a study of Wolińska et al. (2011) in zebrafish (*Danio rerio*) larvae, where Phe and B(a)P exposure caused a similar toxic responses (the mean of No Effect Concentration (NEC) were 5.16±0.45µmol·l-1 (B[*a*]P) and 4.88±0.13µmol·l-1 (Ph)), in spite of the use of a different model (the animal *in vivo*) and viability test (DEBtox 2.0.1). In our study Phe is approximately three times more potent than F (EC50 6.17 mM), the least toxic PAH. The cytotoxic potency of the compounds was as follows: Phe>B[a]P>B[b]F>Chry>F. These results go against the expected, since generally, PAHs with higher molecular weight and greater number of aromatic rings display higher toxicity (Abdel-Shafy and Mansour, 2016). Herein, similar results were obtained for LMPAHs (e.g., Phe) and HMPAHs (e.g., B(a)P).

Interestingly, when the exposure time was extended to 48 hours, all compounds showed greater toxicity, according to the respective EC50. Previous studies corroborate this observation. For instance, Harris et al. (2013) verified that cytotoxicity observed in HT-29 collon cells exposed to B(a)P and F, increased with exposure time. In our study, the difference observed between the two incubation periods for the various substances, may be due to bioactivation, as in the 48 hour-exposures, a greater amount of metabolites is expected, which are possibly more toxic than the parent compound, having an important impact on the EC50 variation between 24 to 48 hours. In fact, the prolonged exposure may allow more time for extensive metabolism to occur, and probably caused the accumulation of ROS, via CYP-pathway. Elevated ROS contributes to the oxidative stress and cytotoxicity of PAHs (Ranjit et al., 2016). In addition, the relative cytotoxic potency of the PAHs changed according to the following order: B[a]P>Chry> B[b]F>Phe>F. To this, much contributed the increase of the individual cytotoxic potencies of Chry, B[a]P and B[b]F, by about 3.5, 1.9 and 4.5 times, respectively ($p < 0.01$). It is well acknowledged that the increase of toxicity of Chry and B[b]F involves metabolic activation (RAIS, 1994a; b), and recently B[a]P and they metabolite benzo(a)pyrene-7,8-dihydrodiol 9,10-epoxide have been involved in B[a]P-induced hepatocarcinogenesis (Souza et al., 2016) . On the other hand, Phe (EC50 1.84 mM) was approximately four times less toxic than the most potent PAH tested, i.e., B[a]P (EC50 0.52 mM), and twice more toxic than F (EC50 4.67 mM), which again proved to be the least toxic of the five PAHs tested. The absence of a bay region in F can explain its lower toxicity when compared to other PAHs (Ewa and Danuta, 2017).

Of note, the greater slope of the regression lines of some substances at 48 hours (e.g., B[b]F and B[a]P), compared to those at 24 hours, seems to mean that for smaller increments of concentration, largeer increments in toxicity are observed. It is also to be noted that, not all substances are similar in the maximum effect (i.e., not all PAHs cause 100% mortality). To achieve the maximum effect in the MTT assay, the highest test concentration of Phe, F, B(a)P and B(b)F would have to be increased; unfortunately, this

is not possible without the parallel increase in the concentration of the solvent (DMSO). The alternative would be to increase the concentration of the stock solutions of these drugs to allow the increasing of the solvent diluition in the medium. However, this was also not possible because the PAHs would exceed the solubility threshold and, therefore, precipitate.

4.2 Mixtures of PAHs produce different effects, depending on their composition, complexity and exposure time

In an attempt to gain a deeper understanding on the potential interactions between PAHs, herein was tested i) a binary mixture consisting of two similar PAHs, as B[a]P and B[b]F are compounds with carcinogenic potencial and with 5 rings; ii) a ternary mixture consisting of distinct PAHs in its composition: B[a]P displays five rings, Phe displays three rings, and Chry dispalys four rings in its composition; and iii) a quintenary mixture, therefore of greater complexity, consisting of PAHs of three (Phe), four (Chry and F) and five rings (B[a]P and B[b]F). All mixture designs combined the PAHs in proportions similar to those found in the sediments of the Tejo river (Martins et al., 2012). The results obtained at 24 and 48 hours are presented in the Figure 4.

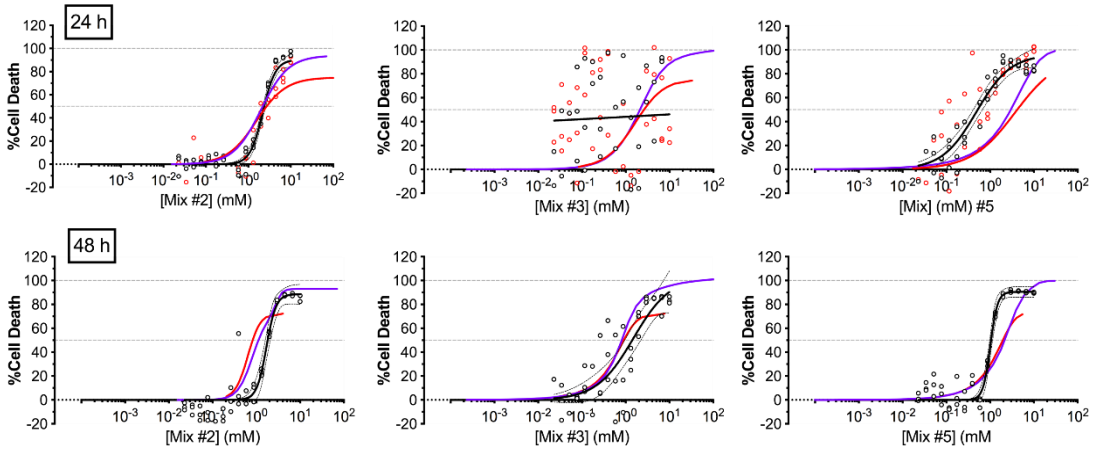


Figure 4 - Predicted and observed cytotoxicity for three different mixtures (binary, ternary and quintenary mixtures, as described in the Material and Methods section; Table 4 **.**) of polycyclic aromatic hydrocarbons, in primary rat hepatocytes, after incubations of 24 or 48 hours, at 37 ° C. The experimental effects were obtained by the MTT redution assay. The effects of the additive combination were predicted using the concentration addition (CA; solid red line) or the independent action (IA; solid purple line) models. The results obtained experimentally (solid black line) are presented as the percentage of cell death in relation to the negative control, and are from a minimum of one independent experiment (each experiment represented by dots of different colours), performed in triplicate. The curves were fitted to the Logit model. The dashed lines represent the upper and lower limits of the 95% confidence interval of the best estimate of the mean responses. The dotted lines represent 50 and 100% effect.

Regarding the experimental effects at 24 hours, in the binary mixture there was an antagonism (effect obtained was smaller than the expected) for concentrations up to EC50, which are the most relevant in terms of environemental exposure. In fact, the concentrations present in the environment do not exceed these values; for example, B[a]P is generally present in the surface water at levels below 0.27 μg/L (WFD, 2000). The antagonism observed in the binary mixture was consistent, as it was maintained at 48 hours (Figure 4). Since these PAHs share toxicocinetic and metabolic pathways (Jarvis et al., 2014), it is expected that the co-occurrence of B[a]P and B[b]F may result in competition for the same bioactivation pathways, reducing the production of toxic metabolites and, consequently, resulting in antagonism (Pushparajah et al., 2017).

In the ternary combination, it was not possible to infer the behaviour of the mixture based on the results obtained at 24 hours, since a satisfactory concentration *versus* effect relationship was not produced. Thus, more experiments (only two independent experiments were carried out) would be needed to confirm the cytotoxicity profile of this mixture at these experimental settings. However, if these results are confirmed, mechanistic studies, such as metabolic and metabolomic studies, would be necessary to understand why it is not possible to obtain a sigmoid concentration *versus* response profile. With the increase of the test time, however, it was possible to observe an additive effect, as the effect predicted by CA is within the entire confidence interval of the results obtained; and the effects predicted by IA fall within this range for the biologically relevant concentrations. According to the assumptions of additivity, this means that the compounds do not interact with each other, i.e., the presence of a component of the mixture does not disturb the toxicokinetic and toxicodynamic pathways of the others. Noteworthy, B[a]P interacts with B[b]F antagonically (see results of binary mixture), but does not appear to interact with Phe or Chry.

Finally, in the quintenary mixture there was a marked synergism (obtained effects greater than the expected; $p<0.001$) for the entire range of concentrations, at 24 hours. This synergistic effect is very relevant because, in fact, complex mixtures are the most prevalent in the environment, as humans and wildlife are co-exposed to a myriad of compounds, and not just to one or two substances. At 48 hours, despite a synergism for the effect concentrations above 20% (approximately) for the highest concentrations, for the lowest concentrations, and therefore the most relevant, there was an antagonism. However, critically looking at the results presented, it is possible to see that, with more experiments, it might be also possible to obtain an additive effect for the lower part of the curve, since the data obtained seem to be well described by the additivity models. In this case, Logit may not be the most suitable model for the lower concentration range of the response curve (although it perfectly describes the upper part of the curve; and

statistically it was not possible to find a model that better described these results). Again, the differences that exist between the mixture at different incubation periods, may be due to the fact that, at 24 hours, we are mainly facing interactions between parent compounds and at 48 hours there is already a greater contribution from interactions between metabolites.

As far as we are aware, no studies have been found to assess the cytotoxicity of PAHs combinations in primary rat hepatocytes, with most of the studies focusing in the mechanisms of carcinogenesis of PAH mixtures in skin (Hughes and Phillips, 1990), liver, lung and kidneys (Jarvis et al., 2014). In this line, Pushparajah et al. (2017) investigated in precision-cut rat liver slices the interactions of binary mixtures of B(a)P with five structurally diverse PAHs [i.e., DB(a,h)A; F; B(b)F; DB(a,l)PYR, and 1-methylphenanthrene (1-MP)] at the CYP1 activity. Concurrent incubation of B(a)P with DB(a,h)A or F led to a synergistic interaction. In contrast, B(b)F and DB(a,l)PYR gave rise to an antagonistic response when incubated with B(a)P, whereas 1-MP had no effect at all concentrations studied.

4.3 Applicability of the additivity models: significant combination effects were observed for ternary and quintenary mixtures, even when each PAH was present at levels exerting no cytotoxicity

The models CA and IA are used to predict the toxicity of mixtures based on the MoA of individual compounds (Dias da Silva et al., 2013b). It is traditionally accepted that CA properly predicts effects of compounds with a similar MoA, while IA provides better results for mixtures of compounds with different MoA. In this study, these models were unable to predict the toxicity of most of the mixtures tested, suggesting the presence of synergistic and/or antagonistic effects, which, in most cases, anticipate toxicokinetic and toxicodynamic interactions (Versieren et al., 2016). In order to elucidate the mechanisms inherent to the toxicity and differences observed, it would be very important to conduct mechanistic studies such as those that were initially planned for this work, but unfortunately, due to the current pandemic circunstances that we face, were not carried out in time to be included in this discussion. Regardless, based on the assumptions of additivity and synergism, it is, however, possible to conclude about the existence of significant effects for the mixtures displaying synergism (quintenary mixture) or additivity (ternary mixture), even when each of their constituents is present at irrelevant levels of toxicity. This phenomenon has been previously described (da Silva et al., 2014; Dias da Silva et al., 2013a; Dias da Silva et al., 2017) and is of paramount importance, since it clearly demonstrates the risk to which humans and environment are exposed to and the need for urgent intervention by regulatory authorities.

Chapter V - Conclusions

This work provided important information about the magnitude of the interactions between PAHs and the possible consequences for human and ecosystem health. Synergisms were observed for the more complex mixture (consisting of B[a]P, Phe, Chry, B[b]F and F) and additive effects arose for the ternary mixture of B[a]P, Phe and Chry. This means that, for these combinations, significant mixture effects are achieved, even when each PAH is present in an amount that individually produces negligible effects. This observation is of serious concern since this type of PAHs combination is the most representative of the current exposure scenarios (humans and animals are concurrently exposed to a plethora of compounds). Thus, the existing guidelines to regulate exposure to PAHs may be unrealistic in the context of environmental risk assessment, as they assume that this risk is substantially underestimated, by considering these substances in isolation. New risk assessment strategies for exposure to PAH mixtures are needed to change this paradigm.

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