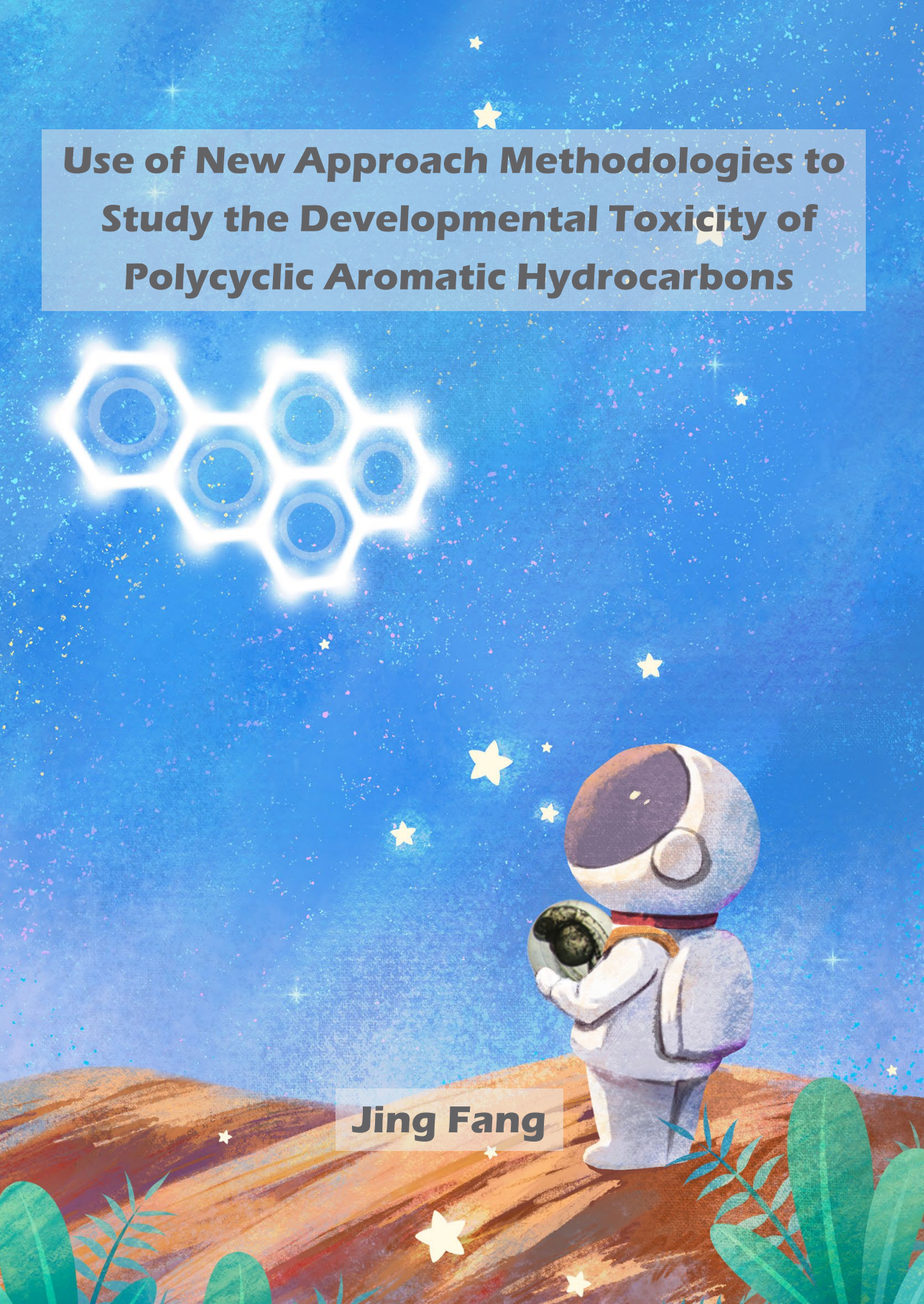
Use of New Approach Methodologies to Study the Developmental Toxicity of Polycyclic Aromatic Hydrocarbons



Jing Fang

Propositions

1. The effect of a methyl substituent on the developmental toxicity of polycyclic aromatic hydrocarbons depends on the site of substitution.
   (this thesis)

2. In vitro and in silico new approach methodologies (NAMs) adequately predict the in vivo developmental toxicity of alkylated polycyclic aromatic hydrocarbons.
   (this thesis)

3. Artificial intelligence makes images and videos accessible to individuals with visual impairments.

4. Organoid technology provides physiologically more relevant models for human disease and drug testing than traditional cell cultures.

5. Action is the remedy for curing fear.

6. Achieving sustainability requires integrating different cultures.

Propositions belonging to the thesis, entitled

Use of new approach methodologies to study the developmental toxicity of polycyclic aromatic hydrocarbons

Jing Fang
Wageningen, 20 June 2023

Use of new approach methodologies to study the developmental toxicity of polycyclic aromatic hydrocarbons

Jing Fang

Thesis committee

Promotor

Prof. Dr I.M.C.M. Rietjens

Professor of Toxicology

Wageningen University & Research

Co-promotors

Prof. Dr P.J. Boogaard

Special Professor, Environmental Health & Human Biomonitoring of Contaminants

Wageningen University & Research

Dr L. Kamelia

Toxicologist

Shell Global Solutions International B.V., The Hague

Other members

Dr M. Rooseboom, Shell Global Solutions International B.V., The Hague

Dr M. Beekhuijzen, Charles River Laboratories, 's-Hertogenbosch

Prof. Dr T.A.J. Murk, Wageningen University & Research

Prof. Dr R. Pieters, HU University of Applied Sciences Utrecht

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Use of new approach methodologies to study the developmental toxicity of polycyclic aromatic hydrocarbons

Jing Fang

Thesis

submitted in fulfilment of the requirements for the degree of doctor
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in the presence of the
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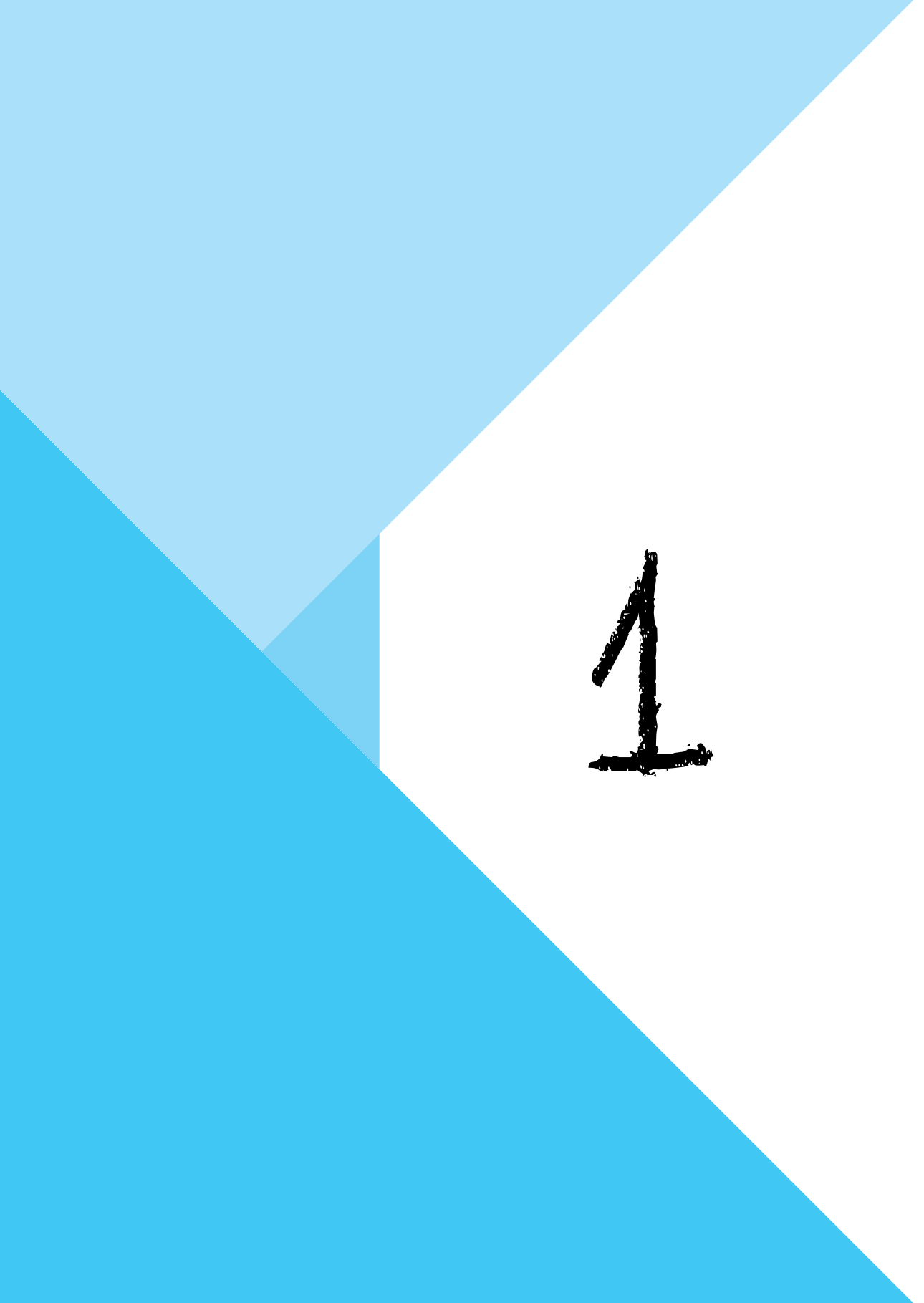


CHAPTER 1.

General Introduction

1. Background and aim of the thesis

Polycyclic aromatic hydrocarbons (PAHs) make up a large group of organic compounds with thousands of individual chemicals (EFSA, 2008). Their molecular structure contains at least three benzene rings (with only carbon and hydrogen atoms) in linear, angular or clustered arrangements (Blumer, 1976). PAHs are derived from natural-biological or anthropogenic sources (Yang et al., 2014). The natural-biological PAHs are generated by plants, algae and microorganism, and are also derived from oil seeps, early diagenesis, forest fires, and volcanoes (Mojiri et al., 2019; Saha et al., 2012, 2009). The anthropogenic sources of PAHs are either petrogenic or pyrogenic. Most pyrogenic PAHs are generated by pyrolysis or incomplete combustion of organic matters such as coal, petroleum and wood (Stout et al., 2015). Petrogenic PAHs derive mainly from crude oil and petroleum products such as gasoline, petroleum gas, kerosene, diesel, heavy fuel oil, gas oil, distillate aromatic extract, lubricating base oil, asphalt and paraffin wax (Saha et al., 2009; Tsitou et al., 2015). Both pyrogenic and petrogenic PAHs are highly complex materials and their chemical composition can vary substantially. Unsubstituted PAHs are abundant in pyrogenic substances while alkylated PAHs (e.g., monomethylated, dimethylated and ethylated PAHs) account for a relatively large proportion of the PAHs present in the petrogenic substances (Hodson, 2017; Sun et al., 2014). This implies that the PAHs present in crude oil and its refined products i.e., petroleum substances, are dominated by alkylated PAHs instead of their unsubstituted/parent PAHs. It is of interest to note that while numerous studies focus on the toxicity of unsubstituted PAHs, limited is known about the toxicity of the alkylated PAHs. However, it was reported that the methylated congeners of some PAHs could induce either higher or lower toxicity compared to their corresponding unsubstituted/parent PAHs (Lin et al., 2015; Turcotte et al., 2011). Thus, it can be hypothesized that the position of the methyl substituent on the aromatic ring of PAHs could either increase or decrease their toxicity such as for example their developmental toxicity.

Regarding petroleum substances, several in vivo studies have reported that the observed developmental toxicity (e.g., increased resorptions and reduced fetal body weights in rats) of petroleum substances might be related to certain polycyclic aromatic hydrocarbons (PACs) known to be present in some petroleum substances i.e., distillate aromatic extract and clarified slurry oil (Feuston et al., 1997, 1989). PACs present in petroleum substances consists of naked/unsubstituted or alkylated PAHs and heterocyclic aromatics (Carrillo et al., 2019; Feuston et al., 1994; Tsitou et al., 2015). Whereas for petroleum substances that are devoid of

or contain very low concentration of PACs, such as low-boiling petroleum substances, bitumen and oxidized asphalt and also all gas-to-liquid products (synthetic analogues of petroleum substances without aromatic compounds), no developmental effects were observed in rats (Boogaard et al., 2021; Kamelia et al., 2021; Whale et al., 2018). Feuston et al. (1994) successfully correlated the developmental toxicity and the 3- to 7-ring PAC content of the petroleum substances. Moreover, a series of studies using a battery of in vitro assays successfully evaluated the in vitro developmental toxicity of some petroleum substances confirming the hypothesis that the observed developmental toxicity is proportional to the amount of 3- to 7-ring PACs that are present in the respective petroleum substances (Kamelia et al., 2019a, 2018, 2017). Thus, it is hypothesized that highly refined petroleum substances, which contain no PACs or extremely low levels of PACs, do not induce developmental toxicity.

The aim of the present thesis was to characterize the in vitro developmental toxicity of methyl PAHs compared to their non substituted analogues and to obtain more insight in the underlying mode(s) of action. Moreover, the hypothesis that the 3- to 7-ring PAC content of petroleum substances is responsible for the observed developmental toxicity upon exposure to petroleum substances was tested in the present thesis by testing the in vitro developmental toxicity of both poorly and highly refined petroleum substances. Finally, a physiologically-based kinetic (PBK) modelling-based reverse dosimetry facilitated read-across from the well-studied unsubstituted 5-ring PAH benzo[a]pyrene (BaP) was performed to predict the in vivo developmental toxicity of a model methylated PAH, namely 8-methyl-BaP (8-MeBaP).

2. Developmental toxicity of PAHs

Pregnant women and their developing fetus may be susceptible to the toxic effects of some PAHs (ATSDR, 1995). The fetus is expected to be more vulnerable to PAHs during the period of organogenesis (Drwal et al., 2019). Most PAHs are lipophilic and hydrophobic, therefore they can easily accumulate in living organisms and also cross the placental barrier, and may affect normal fetal development (Drwal et al., 2019; Perera et al., 2003). Existing epidemiological studies have demonstrated that prenatal exposure to some PAHs is highly associated with developmental effects in humans including fetal growth impairment (Choi et al., 2006), decreased intrauterine growth (Choi et al., 2012), and decreased birth length and weight (Delhommelle, 2010; Perera et al., 2005, 2003). It should be noted that most of the current epidemiological data on PAH-associated developmental toxicity lack information on exposure time and length during pregnancy, exposure concentrations, and the potential

confounding effects of other hazardous chemicals (IARC, 2010; Kennish, 2016). Thus, the definite developmental effects of PAHs, especially individual PAHs including substituted PAHs, on fetuses/embryos upon prenatal exposure remain to be fully elucidated.

To date, developmental toxicity studies using experimental animals are mostly limited to studies on the unsubstituted PAHs and do often not relate to alkylated PAHs. BaP, as the most well-studied PAH, has been reported to induce developmental toxicity in several animal species. For example, prenatal exposure to BaP via inhalation induced decreased fetal survival rate in rats (from the dose of 25 to 100 $\mu g/m^3$) (Archibong et al., 2002), and prenatal exposure to BaP via subcutaneous administration (at a dose of 50 mg/kg) caused significantly increased incidence of resorption and decreased fetal weight in the offspring of pregnant rats (Bui et al., 1986). Beside rats, fish embryos also have been widely used for the developmental toxicity testing of PAHs (see for an overview of developmental toxicity induced by PAHs in fish embryo model systems Table 1). For example, it was reported that BaP induced skeletal malformations to rockfish (*Sebastiscus marmoratus*) embryos (He et al., 2011) and cardiac effects to zebrafish (*Danio rerio*) embryos (Huang et al., 2012). The 4-ring PAH benz[a]anthracene (BaA) induced several malformations in zebrafish embryos such as pericardial edema, yolk sac edema and malformed jaw (Goodale et al., 2013; Incardona et al., 2006; Kühnert et al., 2017; Lin et al., 2015).

Though limited, there are some studies that investigated the developmental toxicity of alkylated PAHs and these studies mainly focused on the alkylated forms of the 3-ring PAH phenanthrene and the 4-ring PAHs BaA and chrysene (Lin et al., 2015; Mu et al., 2014; Turcotte et al., 2011). The alkylated congeners of phenanthrene are generally more potent than the parent/unsubstituted phenanthrene in inducing developmental toxicity in early developmental stages of Japanese medaka and marine medaka, and the observed developmental effects included edema, hemorrhaging and deformations (Turcotte et al., 2011). However, the presence of an alkyl substituent on the aromatic ring of the 4-ring PAHs BaA and chrysene either increased or decreased the potency compared to their unsubstituted/parent compounds to induce developmental toxicity in Japanese medaka embryos, depending on the position of the alkyl substituent (Lin et al., 2015). Overall, given that the limited number of alkylated congeners of PAHs tested for developmental toxicity showed substantial differences in their potencies to induce toxicity, it is of interest to better characterize the influence of alkyl substitution and its regioselectivity on the resulting developmental toxicity.

Table 1. Overview of developmental toxicity induced by some PAHs and alkylated PAHs in fish embryo model systems.

References	Models	Substances	Exposure condition	Effects
Geier et al. (2018a)	Dechorionated zebrafish embryos (Tropical 5D wild type)	123 PAHs including 33 parent PAHs, 22 nitrated PAHs, 17 oxygenated PAHs, 19 hydroxylated PAHs, 14 methylated PAHs, 16 heterocyclic PAHs, and 2 aminated PAHs.	Starting exposure time: 4 hpf Ending exposure time: 120 hpf Solvent: 1% DMSO Concentration: 0.1-50 μM Temperature: 28°C	High-molecular weight PAHs were significantly more developmentally toxic than the low-molecular weight PAHs. Substituted PAHs are often much more developmentally toxic than their parent compounds.
Lin et al. (2015)	Japanese medaka embryos (*Oryzias latipes*)	Chrysene 1-Methyl-chrysene 6-Methyl-chrysene BaA 2-Methyl-BaA 7-Methyl-BaA 2,3-Dimethyl-BaA	Ending exposure time: 17 dpf Concentration: 0-841 μM (chrysene), 0-412.69 μM (1-methyl-chrysene), 0-412.69 μM (6-methyl-chrysene), 0-74.28 μM (2-methyl- BaA), 0-21.85 μM (2,3-dimethyl-BaA), 0-8.76 μM (BaA). 0-41.27 μM (7-methyl-BaA). Temperature: 27°C	Exposure to all PAHs tested caused yolk sac edema, pericardial edema, absence of swim bladder, and craniofacial deformity to Japanese medaka embryos. 2-Methyl-chrysene and 6-methyl-chrysene showed higher embryo toxicity to Japanese medaka embryos than chrysene. 2-Methyl-BaA and 2,3-methyl-BaA showed higher developmental toxicity to Japanese medaka embryos than BaA. 7-Methyl-BaA showed similar embryo toxicity as BaA.
Mu et al. (2014)	Marine medaka (*Oryzias melastigma*)	Phenanthrene	Exposure period: until 25 dpf Solvent: 0.01% DMSO Temperature: 28±2°C Concentration: phenanthrene: nominal concentrations of 100, 200, 400, and 800 μg/L	Phenanthrene induced up to 100% mortality at its highest concentration tested. The major malformation effects of phenanthrene exposure in developing marine medaka embryos included circulation reduction, yolk sac edema, pericardial edema and stretched heart. The EC50 of phenanthrene to induce deformities was 184 μg/L.
Rhodes et al. (2005)	Japanese medaka embryos (*Oryzias latipes*)	BaA 7,12-Dimethyl-BaA Dibenzothiophene Phenanthrene 3,6-Dimethyl-phenanthrene 4,6-Dimethyl-dibenzothiophene	Starting exposure time: 7-8.15 hpf Ending exposure time: 18 dpf Concentration: 0-200 μg/L Temperature: 24±1°C	Dibenzothiophene and 3,6-dimethyl-phenanthrene reduced the hatching-success rate of embryo larval medaka. The tested dimethylated PAHs tended to decrease hatching success. Unsubstituted PAHs (BaA, dibenzothiophene and phenanthrene) induced more signs of blue sac disease (i.e., cranial and skeletal malformation, yolk sac malformation, heart malformation) compared to

				the dimethylated PAHs tested (3,6-dimethylphenanthrene, 4,6-dimethyl-dibenzothiophene and 7,12-dimethyl-BaA).
Turcotte et al. (2011)	Japanese medaka embryos (*Oryzias latipes*)	Phenanthrene 1-Methyl-phenanthrene 7-Ethyl-1-methyl-phenanthrene 1,7-Dimethyl-phenanthrene 2,7-Dimethyl-phenanthrene 2-Ethyl-phenanthrene	Starting exposure time: after fertilization Ending exposure time: 17 dpf Concentration: 0-950 µg/L (phenanthrene), 0-135 µg/L (1-methyl-phenanthrene), 0-175 µg/L (2-ethyl-phenanthrene), 0-145 µg/L (2,7-dimethyl-phenanthrene), 0-102 µg/L (1,7-dimethyl-phenanthrene), Temperature: 26±1°C	Alkylated phenanthrene was more potent in causing edema, hemorrhaging and deformities to Japanese medaka embryos than phenanthrene, except for 7-ethyl-1-methyl-phenanthrene, which was not toxic within its tested concentration range. The developmental toxicity potency to Japanese medaka embryos increased with the number of alkyl substituents.
Weigt et al. (2011)	Zebrafish embryos (wild type AB line)	BaP	Starting exposure time: 2-2.5 hpf Ending exposure time: 3 dpf Solvent: 0.5% DMSO Concentration: 0.25-10 µM Temperature: 26°C	BaP started producing significant developmental toxicity effects from the concentration of 0.5 µM onwards. The developmental toxicity effects induced by BaP in zebrafish embryos at 72 hpf include malformation of the head in 54.4% of total exposed embryos, malformation of tail in 84.1% of total exposed embryos, malformation of tail tip at up to 100% of total exposed embryos, and growth retardation in 75.0% of total exposed embryos.

2.1 Activation of the aryl hydrocarbon receptor (AhR) by PAHs

Until now, the mode of action underlying the developmental toxicity of unsubstituted and especially alkylated PAHs remains to be elucidated. One of the most widely accepted mechanisms proposed for the PAH-induced developmental toxicity proceeds via activation of the aryl hydrocarbon receptor (AhR) (Billiard et al., 2002; Denison and Nagy, 2003; Goodale et al., 2013; Pieterse et al., 2015). The AhR is a ligand dependent transcriptional factor that belongs to the basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) superfamily of proteins (Hahn, 1998). The AhR has a fundamental role in gene regulation, development and cellular homeostasis, but the AhR is also known for its role in the mode of action underlying various toxicities (e.g., carcinogenicity and developmental toxicity) (Hahn, 1998; Stevens et al., 2009). The strong ligand of the AhR, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), is a potent developmental toxicant for many animal species e.g., humans, mice, rats, hamsters, rabbits, monkeys and chickens (Couture and It, 1990). Many PAHs have been reported to be AhR agonists (Machala et al., 2008; Marvanová et al., 2008; Sun et al., 2014). Table 2 summarizes the existing studies that reported AhR-mediated activities of PAHs.

Activation of the AhR upon binding of exogenous chemicals like PAHs regulates amongst others the expression of the cytochrome P450 monooxygenases (CYP450), which could subsequently lead to the biotransformation of PAHs into their reactive metabolites (Hawliczek et al., 2012; Puga et al., 2005). A simplified diagram of the AhR activation by some PAH, and the subsequently induced expression of the relevant CYP genes is presented in Figure 1. Activation of the AhR and the metabolism of PAHs by the CYP enzymes might increase the reactivity of PAHs and could play a role in their developmental toxicity (Goodale et al., 2012).

The physicochemical properties of unsubstituted and methylated PAHs vary with the molecular weight, spatial arrangement of the fused benzene rings, and the position of the methyl substituent. The varied physicochemical properties of unsubstituted and methylated PAHs could result in different potency in AhR activation (Fallahtafti et al., 2012; Lin et al., 2015; Scott et al., 2011; Sun et al., 2014). Table 2 presents an overview of the published AhR-mediated activities of unsubstituted and alkylated PAHs. The presence and position of an alkyl substituent, especially a methyl substituent, on the aromatic ring of PAHs in most cases appeared to increase the potency of the PAHs to activate the AhR (Marvanová et al., 2008; Trilecová et al., 2011).

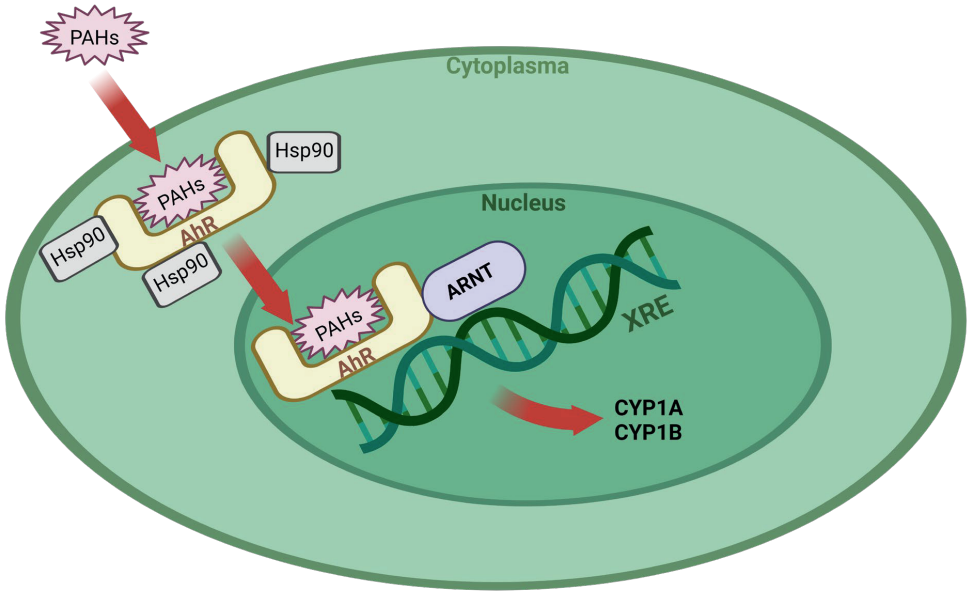


Figure 1. Diagram of the AhR pathway activated by some PAHs and the subsequently induced expression of cytochrome P450 monooxygenases (CYP). *Abbreviations. AhR: aryl hydrocarbon receptor. CYP1A: cytochrome P450 monooxygenases 1A. CYP1B: cytochrome P450 monooxygenases 1B. Hsp90: heat shock protein 90. ARNT: aryl hydrocarbon receptor nuclear translocator. XER: xenobiotic response element.*

Table 2. Overview of the AhR-mediated activities of unsubstituted and methylated PAHs

References	Cell lines, receptors and assays	Compounds	Exposure conditions	Effects
Machala et al. (2001)	Rat hepatoma (H4IIE.luc) cell line, AhR reporter gene assay	30 PAHs including: BaP BaA Dibenz[a,h]anthracene	0-10 μM 6 h, 24 h exposure	BaP, BaA, dibenz[a,h]anthracene induced AhR mediated activities following 6 h and 24 h exposure. 6 h exposure resulted in significant higher AhR-mediated activities than 24 h exposure for all tested PAHs.
Marvanová et al. (2008)	Rat hepatoma (H4IIE.luc) cell line, DR CALUX assay	BaA 1-Methyl-BaA 2-Methyl-BaA 3-Methyl-BaA 4-Methyl-BaA 5-Methyl-BaA 6-Methyl-BaA 7-Methyl-BaA 8-Methyl-BaA 9-Methyl-BaA 10-Methyl-BaA 11-Methyl-BaA 12-Methyl-BaA	0-50 μM 24 h exposure	All monomethylated BaAs were AhR agonists and were more potent than BaA itself, indicating that a methyl-substituent enhanced AhR mediated activity of BaA.
Trilecová et al. (2011)	Rat hepatoma (H4IIE.luc) cell line, DR CALUX assay	BaP 1-Methyl-BaP 3-Methyl-BaP 6-Methyl-BaP 10-Methyl-BaP 11-Methyl-BaP	0-50 μM 24 h exposure	All tested PAHs were AhR agonists. 6-, 10- & 11-Methyl-BaP elicited less or equal AhR-mediated effects compared to BaP. 1- and 3-methyl-BaP elicited higher AhR-mediated effects compared to BaP.

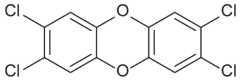
Sun et al. (2014)	Engineered Saccharomyces cerevisiae yeast strain YCM3, AhR reporter gene assay	Phenanthrene 1-Methyl-phenanthrene 2-Methyl-phenanthrene 3-Methyl-phenanthrene 9-Methyl-phenanthrene 3,6-Methyl-phenanthrene	0-100 µM 18 h exposure	All tested monomethylated phenanthrenes were 2 to 5 times more potent for activation of the AhR than phenanthrene.
Vondráček et al. (2017)	Human hepatocellular carcinoma cells, AhR reporter gene assay	BaA Benzo[a]pyrene Dibenz[a,h]anthracene 6-Methyl-BaA 9-Methyl-BaA	0-40 µM 6 h, 24 h exposure	9-Methyl-BaA and 6-methyl-BaA were more potent than their unsubstituted/parent BaA in activating the AhR. For the unsubstituted PAHs, dibenz[a,h]anthracene was more potent than BaP and BaA in activating the AhR.
Lam et al. (2018a)	Rat hepatoma (H4IIE-luc) cell line, AhR reporter gene assay	1-Methyl-chrysene 2- Methyl-chrysene 3- Methyl-chrysene 7-Methyl-BaA 7-Methyl-BaP	0-40 µM 24, 48, 72 h exposure	All tested methylated PAHs were AhR agonists following 24, 48 or 72 h exposure.
Boonen et al. (2020)	Recombinant mouse hepatoma cell line (Hepa1c1c7), AhR CALUX assay	BaP	Concentration range not reported. BaP EC50 in AhR: 1.05×10^{-3} µM 24 h exposure	BaP showed an AhR-mediated agonistic effect.

2.2 Interaction of PAHs with the ER-α and RAR

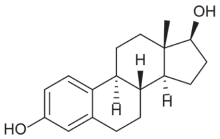
Natural (endogenous) hormones play essential roles in every aspect of the developmental processes, since they are responsible for the maintenance of homeostasis and regulation of the developmental processes of the body (Kummer et al., 2008; Thomas Zoeller et al., 2012). Prenatal exposure to substances that interfere with the production, release, transport, or metabolism of endogenous hormones could result in loss of homeostasis of endogenous hormones and may consequently adversely affect embryonic development. It is known that some PAHs or their metabolites, and especially the ones that closely resemble the natural (endogenous) hormones, could interact with nuclear-hormone-receptors (NHRs) such as for example the estrogen receptor (ER). The ER is a ligand-activated nuclear transcription factor that is involved in the regulation of developmental growth and regulation of sexual and reproductive organs (Bondesson et al., 2015; Jia et al., 2015; Paterni, Iliaria. Granchi et al., 2011). 17β-Estradiol (E2) is the natural ligand of the ER (see the chemical structure of E2 in Figure 2) (Bondesson et al., 2015; Van Lipzig et al., 2005). Some studies reported that PAHs could interfere with ER signaling by exerting both estrogenic and anti-estrogenic effects (see an overview of the ER-mediated activities of PAHs in Table 3). There are two major subtypes of the ER: ER-α and ER-β (Pike et al., 1999). ER-α and ER-β have differential tissue distribution: ER-α is mainly expressed in ovary, uterus, breast and kidney, while ER-β is mainly expressed in ovary, central nervous system, cardiovascular system, lung, reproductive organs of males, and the immune system (Jia et al., 2015). Previous studies using ER knockout mice demonstrated that ER-α and ER-β also have differential biological roles and that the developmental and reproductive effects of ER active chemicals are most often related to the activation of ER-α rather than ER-β (Cooke et al., 1998; Emmen and Korach, 2003; Heine et al., 2000; Krege et al., 1998).

Other NHRs, such as the retinoic acid receptor (RAR), also play important roles in developmental processes of vertebrates (Lammer et al., 1985; Morriss-Kay, 1993; Piersma et al., 2017). Excessive exposure to retinoic acid, the natural ligand of the RAR, induced cardiotoxicity, bent tail, duplicated otoliths and brain deformity in zebrafish embryos (Chen et al., 2008; Herrmann, 1995). However, unlike for the ER-α, information on the interaction between unsubstituted/alkylated PAHs and the RAR is limited. One study using a RAR transactivation assay reported that BaP and BaA could up-regulate the RA-mediated activity (Benišek et al., 2008). This result indicates that the PAHs might also disturb the normal

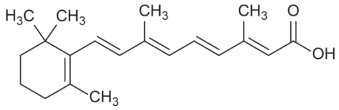
signaling of the RAR which could subsequently result in developmental toxicity. Thus, the role of the RAR in mediating PAH-induced developmental toxicity should be further investigated.



2,3,7,8-Tetrachlorodibenzo (TCDD)



17-β estradiol (E2)



Retinoic acid (RA)

Figure 2. Chemical structure of 2,3,7,8-tetrachlorodibenzo (TCDD), a strong ligand of the AhR;17-β estradiol (E2), the natural ligand of the ER-α; and retinoic acid (RA), the natural ligand of the RAR.

Table 3. Overview of the ER-mediated activities of unsubstituted and methylated PAHs

References	Cell lines, receptors and assays	Compounds	Exposure conditions	Effects
Boonen et al. (2020)	Recombinant human breast cancer cell line (VM7Luc4E2), ER-α CALUX agonist/antagonist assay	BaP	Concentration range not reported. BaP EC50 in AhR: 1.05×10^{-3} µM 24 h exposure	BaP showed both ER-α agonist and antagonist effects.
Charles et al. (2000)	MCF-7 cell line, ER-α CALUX assay	BaP	0-10 µM 24 h exposure	BaP showed strong ER-α agonist activity.
Fertuck et al. (2001)	MCF-7 cell line, ER-α reporter gene expression assay	BaA	0.06-20 µM 24 h exposure	BaA showed weak ER-α agonist activity.
Lam et al. (2018b)	Human breast carcinoma (VM7lucE42) cell line, ER-α reporter gene expression assay (agonist/antagonist)	BaP 7-MeBaP Chrysene 1-Methyl-chrysene 2-Methyl-chrysene 3-Methyl-chrysene	Concentration range not reported. 24 h exposure	BaP showed estrogenic activity (56% of the maximum E2 induction), whereas 7-MeBaP induced no estrogenic effect. The three methylated congeners of chrysene were more potent in inducing estrogenic activity than chrysene itself.

3. Developmental toxicity of petroleum substances

Petroleum substances are produced by the primary atmospheric and vacuum distillation of crude oils and the subsequent refinement processes (Mackerer et al., 2003). Petroleum substances are known as UVCBs (**U**nknown or **V**ariable composition, **C**omplex reaction products and **B**iological materials) due to their highly complex and variable composition. Many petroleum substances are produced/imported at a volume of ≥ 1000 tonnes per year in the European Union (EU), which requires them to be tested for their prenatal developmental toxicity according to the REACH (Registration, Evaluation, Authorization, and Restriction of Chemicals) regulation (ECHA, 2009). The REACH regulation aims at protecting public health and the environment from exposure to hazardous substances. When testing the developmental toxicity of all the petroleum substances that meet the REACH requirements, numerous experimental animals, approximately 560 rodents per test and 784 non-rodent per test (Rovida and Hartung, 2009), will be required for testing according to OECD 414 guideline (OECD, 2018).

As also indicated above, existing evidence suggests that the developmental toxicity of petroleum substances observed in rodents is proportional to their 3- to 7-ring PAC content (Feuston et al., 1997, 1994; Feuston and Mackerer, 1996a, 1996b), and petroleum substances without PAHs do not induce developmental toxicity (Boogaard et al., 2017; Whale et al., 2018). Recent studies using a series of in vitro assays i.e., the mouse embryonic stem cell test (mEST), the zebrafish embryotoxicity test (ZET) and a battery of CALUX reporter gene assays, evaluated the in vitro developmental toxicity of petroleum substances with varying concentrations of PAHs (Kamelia et al., 2019a, 2018, 2017). By comparison to the existing in vivo developmental toxicity data of these petroleum substances, it was concluded that using such a battery of in vitro assays could successfully predict the developmental toxicity of heavier petroleum substances (Kamelia et al., 2019a, 2018, 2017). Moreover, these studies also showed that the observed in vitro developmental toxicity could be partially ascribed to activation of the AhR by 3- to 7-ring PACs present in the petroleum substances (Kamelia et al., 2019b).

Carrillo et al. (2022) and Mackerer et al. (2003) described how several refinement processes applied during the manufacture process of petroleum substances aim at removing the hazardous fractions, such as the 3- to 7-ring PACs. Many of these highly refined petroleum substances (e.g., highly refined base oil, also termed white oils) are widely applied in food packaging and cosmetic production (Pirow et al., 2019). Since all petroleum substances are registered under REACH and most of them are imported at ≥ 1000 tonnes per year in the EU developmental

toxicity testing for these substances is required. However, very limited in vivo data/studies exist for these highly complex substances. Therefore, it is relevant to test the developmental toxicity of not only alkylated PAHs but also of petroleum substances and highly refined petroleum substances with a suitable new approach methodology (NAM).

4. Model compounds used in the present thesis

4.1 PAHs

Existing knowledges on the unsubstituted and methylated PAHs suggested that the position of the methyl substituent on the aromatic ring of PAHs may influence their ability to interfere with nuclear receptors such as the AhR, ER-α or RAR, and their subsequent developmental toxicity (Lin et al., 2015; Marvanová et al., 2008; Sun et al., 2014; Trilecová et al., 2011; Turcotte et al., 2011). In the present thesis we aimed to evaluate the developmental toxicity of a series of selected methylated PAHs relative to that of their corresponding unsubstituted PAHs and the potential underlying mode(s) of action. To this end, both unsubstituted and methylated PAHs were included in the studies, and for methylated PAHs, PAHs with different positions of the methyl substituent on the aromatic rings were selected. However, many of the methylated PAHs are not commercially available mainly due to the difficulties in synthesizing the analytically pure form of most alkylated PAH (Cai et al., 2004). Thus, in the present thesis the model PAHs to be included in the studies were selected based on the following criteria: i) their availability either commercially or through chemical synthesis, ii) the availability of in vitro developmental toxicity data in the mouse embryonic stem cell test (mEST), and iii) structural similarities to unsubstituted PAHs that tested positive for in vitro developmental toxicity in the mEST previously (Kamelia et al., 2020).

Three unsubstituted PAHs, the 4-ring PAH BaA, the 5-ring PAH BaP and the 5-ring PAH dibenz[a,h]anthracene (DB[a,h]A (see for their chemical structures Figure 3) were selected as the model compounds. Among these three unsubstituted PAHs, the in vitro developmental toxicity of two of them, BaP and DB[a,h]A, has been investigated previously in the mEST (Kamelia et al., 2020; Wang et al., 2021). BaP induced in vitro developmental toxicity only upon bioactivation in the mEST, and its major metabolites 3-hydroxy-BaP (3-OHBaP) was shown to contribute to the observed in vitro developmental toxicity; whereas DB[a,h]A could induce in vitro developmental toxicity in the absence of bioactivation (Kamelia et al., 2020).

For the methylated PAHs, 4-methyl-BaA and 3-MeBaP were selected because the presence of a methyl substituent on the position of C4 or C3 on BaA and BaP, respectively, making the molecule of these two methylated PAHs mimic the structural configuration of the developmental toxic metabolites 3-OHBaP. In addition, 8-MeBaA, 9-MeBaA, 7-MeBaP and 8-MeBaP were selected because the presence of a methyl substituent makes them to some extent mimic the molecular dimensions of the unsubstituted DB[a,h]A.

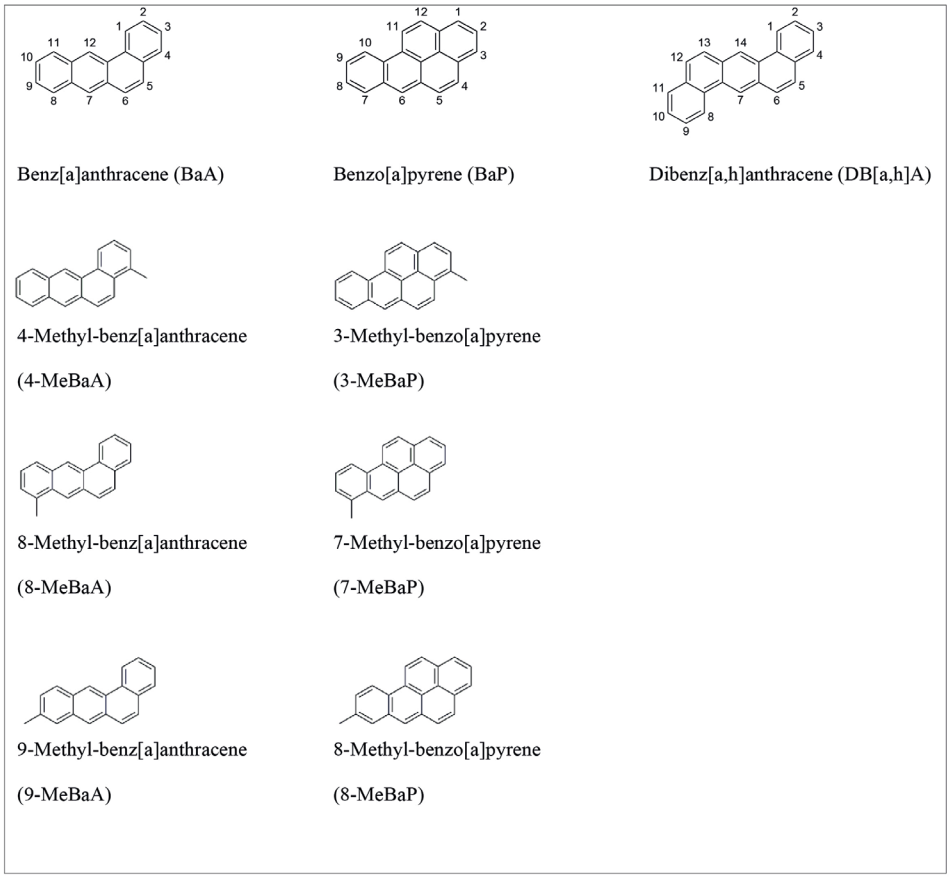


Figure 3. Structure and carbon atom numbering of the selected unsubstituted and methylated PAH model compounds used in the present thesis

4.2 Petroleum Substances

Several recent studies successfully evaluated the in vitro developmental toxicity of some PAC-containing petroleum substances using a series of in vitro assays, including the mEST, ZET and

AhR reporter gene CALUX assay (Kamelia et al., 2021, 2019a, 2018, 2017). In order to test the in vitro developmental toxicity of highly refined petroleum substances and petroleum substances-derived waxes, and also to expand the applicability domain of the battery of the above mentioned in vitro developmental toxicity assays, a series of petroleum substances resulting from different steps in the refinement processes during the manufacture of petroleum substances from crude oil were selected (see for an overview of the manufacture of these petroleum substances Figure 4).

Crude oils primarily undergo atmospheric distillation, yielding atmospheric distillates which are typically used as fuels, and atmospheric residues which contain most PACs. The atmospheric residues may be further distilled under vacuum (Mackerer et al., 2003; HPV, 2012). yielding vacuum distillates (sample SN100-Distillate [SN-100DIS]) and residual oils, which contain 3- to 7-ring PACs (HPV, 2011; Mackerer et al., 2003). To reduce the undesired PAH content in the vacuum distillates, solvent extraction is applied which generates two streams: distillate aromatic extracts (sample SN100-Aromatic extract [SN100-AE]) and waxy raffinate (sample SN100-WR and SN400-WR) (Carrillo et al., 2019), and a residue named waxy brightstock (sample WBS). Distillate aromatic extracts have a relatively high concentration of extracted PAHs and are not subjected to further refinement processes. The waxy raffinate is used as the primary stock for lubricating base oil and waxes. Since waxy raffinate contains paraffins which affect the technical performance at low temperature, these preparations must undergo dewaxing to remove the undesired fractions. Dewaxing of waxy raffinate yields two refinery streams: base oil (sample SN100-base oil [SN100-BO], SN100-lubricating base oil [SN100-LBO], and SN500-LBO) and slack wax (sample SN400-SW) (Carrillo et al., 2022, 2019, 2021; Mackerer et al., 2003).

The lubricating base oil (LBO) can be further refined through single or double hydro-treatment to remove (poly)aromatics, the resulting product is referred to as highly refined base oil (sample SN100-technical white oil (SN100-TWO) and SN100-medicinal white oil (SN100-MWO), also known as white mineral oil, in which the concentration of the undesired PAHs is negligible (Carrillo et al., 2021). The slack wax is solid at room temperature. De-oiling slack wax can result in hard paraffin wax (sample SN400-Hard paraffin wax [SN400-HPW]), which can be further refined to highly refined hard paraffin waxes (sample SN400-HPHPW) (Carrillo et al., 2022, 2021).

Waxy brightstock can undergo a dewaxing process and thus generating brightstock slackwax (sample BSSW). De-oiling of the brightstock slackwax results in microcrystalline wax (MCW), which can be further refined into hydrogenated microcrystalline (HGMCW) by hydrotreatment (Carrillo et al., 2021).

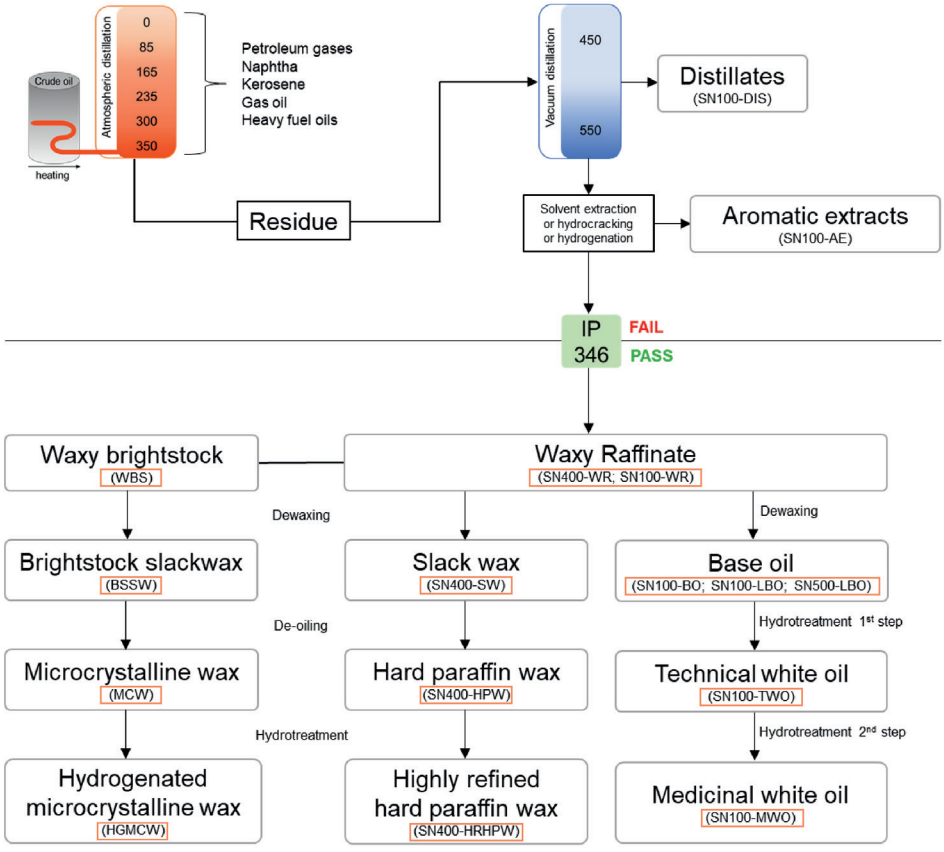


Figure 4. Overview of the manufacturing process of petroleum substances generating the samples included in the present thesis (adopted from Carrillo et al. (2022)). IP346 is a gravimetric method based on a DMSO extraction to determine the total PAC in mineral oils. The % DMSO extract by IP346 method can be used for the prediction of the carcinogenic potential for mineral oils. The cut-off of IP346 is 3% w/w thus samples are considered non-dermally carcinogenic if the % DMSO extract is less than 3% w/w (Carrillo et al., 2022).

5. In vitro assays for testing developmental toxicity

Testing developmental toxicity of chemicals and substances is one of the endpoints that require most experimental animals (Jong et al., 2011). This leads to a high need for alternatives to animal tests for developmental toxicity testing. A large number of assays has been developed

for in vitro developmental toxicity testing (Festag et al., 2007; Kroese et al., 2015; Piersma et al., 2013). A battery of in vitro assays including the mEST, ZET and AhR CALUX reporter gene assay was successfully applied to investigate the developmental toxicity of a series of petroleum substances with systematic changes in the amount and type of PAHs, showing that the results obtained in the in vitro assays matched existing data on their in vivo developmental toxicity (Kamelia et al., 2021, 2019a, 2018, 2017). It was concluded that the battery of in vitro assays was adequate to predict the in vivo developmental toxicity of the tested petroleum substances. Thus, in the present thesis, also in vitro assays were applied to test the developmental toxicity of the newly selected petroleum substances as well as the individual unsubstituted and methylated PAHs. In addition, it was taken into account that some PAHs may require bioactivation to induce developmental toxicity (Fallahtafti et al., 2012; Kamelia et al., 2020). Especially the zebrafish embryos used in the ZET may include bioactivation while the ES-D3 cell line used for the mEST, lacks a metabolism system.

5.1 Zebrafish embryotoxicity test (ZET)

Nowadays, zebrafish embryos are extensively used for evaluating the in vitro developmental toxicity for chemicals and substances (Jarque et al., 2020; Kamelia et al., 2019a; Kroese et al., 2015; Piersma et al., 2013). The ZET is not considered an animal test as long as the zebrafish embryos in the test are not free-feeding, which is typically up to 120 h post fertilization (hpf) (EU, 2010; Sellick, 2011). Comparing to the other in vitro systems for developmental toxicity testing, such as the mEST which is based on a cell line, the ZET utilizes intact organisms in a simple and fast culture system and covers the whole vulnerable developmental stages of the zebrafish embryos (Raldúa et al., 2012; Rothenbücher et al., 2019). This makes the ZET a promising model for evaluating the adverse effects of chemicals and substances. Moreover, the main features of zebrafish embryos during embryogenesis have been extensively studied (Kimmel et al., 1995; Mccollum et al., 2011; Panzica-Kelly et al., 2010; Selderslaghs et al., 2009), and it is found that the embryogenesis in vertebrates is highly conserved making the developmental processes of zebrafish embryos comparable to that of humans (Fukunaga et al., 1995; Sipes et al., 2011). Another advantage of the use of the ZET is that the transparency of the chorion of zebrafish embryos enables a non-invasive observation of the morphological changes. Last but not the least, other advantages of the ZET are that the culturing of zebrafish embryos is very cheap, the ZET experiment is easy to perform, and the duration of the ZET is short (Le Bihanic et al., 2014; Rothenbücher et al., 2019). Up to now, the ZET has been

successfully applied to assess in vitro developmental toxicity of several individual substituted and unsubstituted PAHs.

To date, the in vitro developmental toxicity of many PAHs (Dach et al., 2019; Geier et al., 2018a, 2018b; Lin et al., 2015), PAH-containing substances (Kamelia et al., 2021, 2019a), and other chemicals (Adam et al., 2021; Behl et al., 2015; Jomaa et al., 2014; Strähle et al., 2012) have been successfully evaluated in the ZET. Among these studies, scoring of the zebrafish embryo morphology was widely applied. However, in early applications of the ZET there was no standardized protocol for this scoring regarding the endpoint of the developmental toxicity. In order to standardize the morphology scoring, Hermsen et al. (2011) developed an evaluation method named the general morphology scoring (GMS) system for screening embryotoxicity potentials of chemicals in the ZET. This GMS system was further modified and extended by Beekhuijzen et al. (2015), to cover the endpoints of the normal development of zebrafish embryos and also several dysmorphogenic endpoints. In the extended-GMS, the endpoints of the normal development of zebrafish embryos include detachment of tail, somite formation, eye development and pigmentation, movement, circulation, presence of heartbeat, pectoral fins, pigmentation of head and body, pigmentation of tail, hatching, presence of protruding mouth, and yolk extension. The dysmorphogenic endpoints include yolk sac edema, pericardial sac edema, malformation of the tail, deformed body shape, malformation of the head and jaw, and malformation of the sacculi/otoliths. A detailed description of the scoring of the extended-GMS is listed in Table 4. The Table also outlines that the maximum score that can be obtained in the extended-GMS at 96 hpf is 23 with a score of 17 coming from the GMS and an additional score of 5 from the endpoints added in the extended-GMS. This extended-GMS has been successfully applied to predict the in vitro developmental toxicity of some PAH-containing petroleum substances (Kamelia et al., 2021, 2019a). Thus, in the present thesis, the ZET assay with the extended-GMS system was used to evaluate the in vitro developmental toxicity of both individual PAHs (**Chapter 2** and **3**) and petroleum substances samples (**Chapter 4**).

Briefly, the ZET is initiated from 4-5 hours post fertilization (hpf) and terminated at 96 hpf. Zebrafish embryos are exposed (static exposure) to different concentrations of test compounds throughout the whole experiment. The morphology of zebrafish embryos is daily scored based the extended-GMS scoring system.

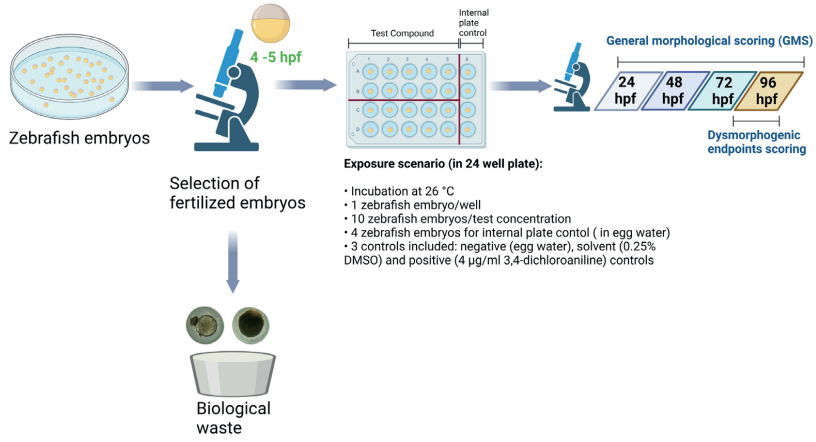


Figure 5. The scheme of the zebrafish embryotoxicity test (ZET).

Table 4. The extended-GMS system as described by Beekhuijzen et al. (2015). This scoring system contains two parts: 1) general development and 2) dysmorphogenic endpoints. In the general development part, endpoints 1-5 are evaluated at 24 hpf, endpoints 1-8 are evaluated at 48 hpf, endpoints 1-11 are evaluated at 72 hpf, and endpoints 1-12 are evaluated at 96 hpf. The 6 dysmorphogenic endpoints are only evaluated at 96 hpf.

No.	**1). General Development Endpoints**	**Scores**			
		24 hpf	**48 hpf**	**72 hpf**	**96 hpf**
1	Detachment of the tail	2	3	3	3
2	Somite formation	1	1	1	1
3	Eye development & pigment	2	3	3	3
4	Movement	1	1	1	1
5	Circulation	1	1	1	1
6	Heartbeat		1	1	1
7	Pigmentation of the head and the body		1	1	1
8	Pigmentation of the tail		1	1	1
9	Hatching			1	1
10	Pectoral fin			1	1
11	Protruding mouth			2	2
12	Yolk extension nearly empty				1
	Total GMS score	**7**	**12**	**16**	**17**
No	**2). Dysmorphogenic endpoints**	**Scores**			
		24 hpf	**48 hpf**	**72 hpf**	**96 hpf**
1	No yolk sac edema				1
2	No pericardial edema				1
3	No malformed tail				1
4	No deformed body shape				1
5	No malformed head and jaw				1
6	No malformed sacculi/otoliths				1
	Total extended-GMS score				**23**

5.2 Chemical Activated LUciferase gene eXpression (CALUX) assays

The underlying mode(s) of action of PAH- and petroleum substance-induced developmental toxicity is complex and poorly understood. However, it is suggested that the activation of the AhR might be involved (Billiard et al., 2006; Feuston et al., 1994; Goodale et al., 2013, 2012; Kamelia et al., 2021, 2018; Tsitou et al., 2015). A previous study which evaluated receptor-mediated activities of PAH-containing petroleum substances in relation to their developmental toxicity showed that the AhR agonist activity of petroleum substances correlated best among the receptors tested with their in vitro developmental toxicity obtained in the mEST ($R^2 = 0.80$; Kamelia et al., 2018). This paper also concluded that compared to the other nuclear receptors tested, e.g., the androgen receptor (AR), progesterone receptor (PR), estrogen- receptor alpha (ER-α) and thyroid receptor beta (TR-β), activation of the AhR plays a more important role in mediating the developmental toxicity of PAH-containing petroleum substances. Thus, testing the AhR-mediated activity of PAH-containing petroleum substances is of importance and informative. Moreover, it was also shown that the AhR agonist activity of the petroleum substances correlated well with their content of 4- and 5-ring PAHs in petroleum substances (Kamelia et al., 2018). Thus, in the present thesis the AhR-mediated activity was investigated for the selected individual 4- and 5-ring PAHs.

Many individual PAHs are also known to interact with the ER-α (see an overview of the ER-α mediated activities of unsubstituted and alkylated PAHs in Table 3). Considering the important role of the ER-α in the developmental processes of many vertebrates (Ahi et al., 2016; Bondesson et al., 2015; Kishida et al., 2001), the role of ER-α in the mode(s) of action underlying the developmental toxicity of individual PAHs (both unsubstituted and methylated) was also investigated.

Beside the abovementioned receptors, the RAR is also known for its important role in mediating developmental toxicity (Piersma et al., 2017). However, very limited studies investigated the role of RAR in PAH-induced developmental toxicity. Thus, the present thesis also assessed the potential of individual 4- and 5-ring unsubstituted and methylated PAHs to act as RAR (ant)agonists.

The Chemical Activated LUciferase gene eXpression (CALUX) assays were employed to investigate the receptor-mediated activities of individual PAHs and petroleum substances. The stably transfected CALUX reporter gene assays have become widely applied assays for

screening the in vitro receptor-mediated activities of chemicals and substances (Kroese et al., 2015; Piersma et al., 2013; Sotoca et al., 2010). Mammalian cells, i.e., the rat H4IIE.luc cell line for the AhR CALUX and the human osteosarcoma cell line (U2OS) for ER-α and RAR, are stably transfected with a firefly luciferase reporter gene that is under control of receptor specific responsive elements (Aarts et al., 1995; van der Burg et al., 2013; Van der Linden et al., 2014). The principle of the CALUX assays is based on the ability of a test compound to bind and consequently activate or inhibit the receptor resulting in activation or inhibition of transcription of the receptor target genes, such as the luciferase reporter gene, which eventually results in modified luciferase activities (Van der Linden et al., 2014; Windal et al., 2018).

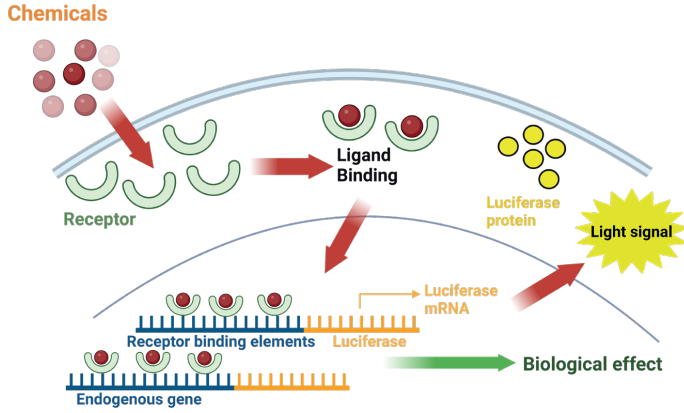


Figure 6. Principle of the CALUX reporter gene assay.

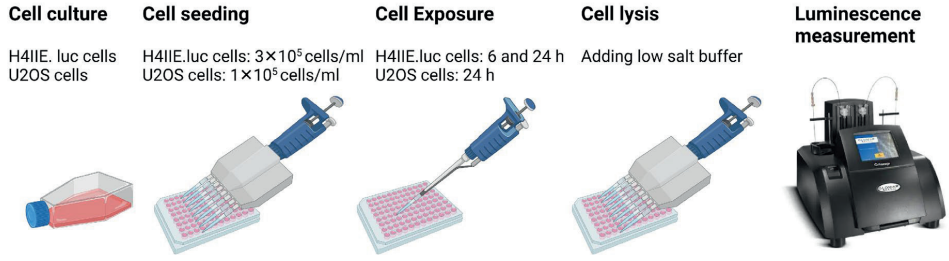


Figure 7. The scheme of the Chemical Activated LUciferase gene eXpression (CALUX) assay.

5.3 Mouse Embryonic Stem Cell Test (mEST)

The mEST is one of the three in vitro assays for developmental toxicity testing validated by the European Center for the Validation of Alternative Methods (ECVAM). The advantage of the

mEST over the other two validated in vitro methods, i.e., the limb bud micromass and the rat whole embryo culture (Genschow et al., 2002; Spielmann et al., 2004) is that only the mEST is an animal-free system. The blastocyst-derived mouse pluripotent embryonic stem cell line (ES-D3) is used for the mEST (Festag et al., 2007; Genschow et al., 2004, 2002). The ES-D3 cells can spontaneously differentiate into beating cardiomyocytes, and the inhibition of this differentiation is considered as the in vitro endpoint for developmental toxicity (Genschow et al., 2004, 2002). In the present thesis, the mEST was performed according to the ten days mEST protocol described by Seiler and Spielmann (2011) with minor modifications (Figure 8). Briefly, the so-called embryoid bodies (EBs) were formed by hanging droplets of an ES-D3 cell solution on the lid of 96-well plates (Day 0). At Day 3 the EBs are collected and cultured in petri-dish plates for another two days. After the culturing, each EB is transferred to a well of the 24-well plates where the EBs are allowed to attach and differentiate into cardiomyocytes for another five days. At Day 10, the number of wells with beating cardiomyocytes is visually counted under the microscope. The in vitro developmental toxicity of test compounds is reflected by the inhibition of differentiation of ES-D3 cells into beating cardiomyocytes in the mEST.

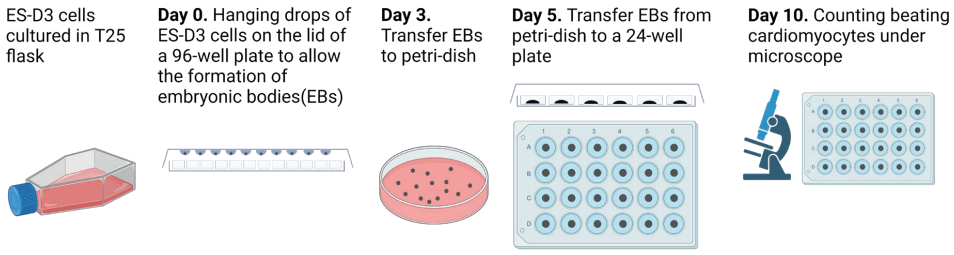


Figure 8. The scheme of the ten days mouse embryonic stem cell test (mEST)

5.4 Physiologically Based Kinetic (PBK) modelling facilitated reverse dosimetry and read-across

Developmental toxicity is one of the most animal-intensive endpoints for toxicity testing. Thus, alternatives to animal tests for developmental toxicity are highly demanded. The aforementioned in vitro assays (i.e., ZET and mEST) could provide in vitro concentration-response curves. However, the in vitro concentration-response curves are inadequate for setting points of departure in risk assessment because this requires in vivo dose-response curves to define a safe exposure level. In vitro concentration-response curves can be converted to the required in vivo dose-response curves using so-called physiologically based kinetic (PBK)

modelling facilitated reverse dosimetry. PBK modelling is an in silico technique and was employed in the thesis to bridge the gap between the in vitro and in vivo situation, and perform quantitative in vitro to in vivo extrapolation (QIVIVE) illustrating how in vitro data can help to reduce the use of experimental animals for the risk assessment of developmental toxicity.

PBK models consist of a set of mathematical equations for quantitatively describing the absorbance, distribution, metabolism and excretion (ADME) of chemicals and their metabolites within the intact organism (Louisse et al., 2011; Rietjens et al., 2011). PBK modelling can be used to predict the internal concentration of a compound and its metabolites in any tissue. To develop a PBK model for a compound of interest, three types of parameters are required as input for the PBK model including 1) physiological parameters (e.g., cardiac output, tissue weight/volumes and tissue blood flows), 2) physico-chemical parameters (e.g., blood-tissue partition coefficient), and 3) kinetic parameters (e.g., kinetic constants for ADME).

Reverse dosimetry approaches based on PBK modelling were previously already shown successful for translation of in vitro concentration-response curves into in vivo dose-response curves (Louisse et al., 2011). The PBK modelling facilitated reverse dosimetry has shown for example adequately predictions for in vivo developmental toxicity based on PBK model based translation of the in vitro concentration-response data of the mEST for compounds like ketoconazole, tebuconazole and all-trans-retinoic acid. (Li et al., 2017, 2015; Louisse et al., 2015), and recently also for the most-well studied PAH, BaP (Wang et al., 2021). It was previously reported that BaP induced in vitro developmental toxicity in the mEST only upon bioactivation, and that its major metabolite 3-OHBaP is responsible for the observed in vitro developmental toxicity (Kamelia et al., 2020). Thus, the in vivo dose response curve for BaP induced developmental toxicity was predicted based on PBK modelling facilitated reverse dosimetry of the in vitro concentration-response curve of 3-OHBaP in the mEST (Wang et al., 2021). This approach was shown to adequately predict the in vivo developmental toxicity of BaP based on in vitro data from the mEST for 3-OHBaP.

Given that in vivo developmental toxicity data for alkylated PAHs are limited or even absent, in the present thesis the same in vitro-in silico approach was applied to characterize the in vivo developmental toxicity of the methylated BaP, 8-MeBaP. To this end, based on read across from BaP, a PBK model was described for 8-MeBaP. It was assumed that, similar to BaP (Kamelia et al., 2020; Wang et al., 2021), for 8-MeBaP developmental toxicity may depend on its 3-hydroxy metabolite 3-OH-8-MeBaP, which was previously already shown to be the major

ring oxidation metabolite of 8-MeBaP (Wang et al., 2022). To verify this hypothesis, in the present thesis the in vitro developmental toxicity of 8-MeBaP and 3-OH-8-MeBaP was evaluated in the mEST. The obtained in vitro concentration-response curve of 3-OH-8-MeBaP was subsequently translated into a predicted in vivo dose-repose curve for the developmental toxicity of 8-MeBaP using PBK modelling based reverse dosimetry.

6. Thesis outline

Chapter 1 of the present thesis presents a general introduction and the aims of the thesis. In this chapter, background information on unsubstituted/alkylated PAHs and petroleum substances is introduced. The developmental toxicity of unsubstituted/alkylated PAHs and petroleum substances, and the role of the AhR and some NHRs in the possible mode(s) of action underlying the developmental toxicity of PAHs and petroleum substances is also introduced. In addition, the test compounds selected for the studies are described, and the alternatives to animal tests to define in vitro developmental toxicity are provided. Finally, it is explained how PBK modeling can help to translate the in vitro developmental toxicity data to in vivo dose response curves.

Chapter 2 evaluates the in vitro developmental toxicity potency of nine unsubstituted and methylated 4- and 5-ring PAHs using the ZET. The influence of the presence and position of a methyl substituent on the aromatic ring of PAHs is discussed. Moreover, the possible mechanisms underlying the different developmental toxicity potencies of unsubstituted and methylated PAHs is discussed in this chapter.

Chapter 3 aims to evaluate the possible mode(s) of action underlying the developmental toxicity of unsubstituted and methylated PAHs. To achieve the aim, the AhR-, ER-α- and RAR-mediated activities of the nine unsubstituted and methylated 4- and 5-ring PAHs already tested in **Chapter 2** are characterized using different CALUX assays. To further investigate the potential role of these receptors in mediating the observed developmental toxicity of PAHs, the tested PAHs that interacted with any of the aforementioned receptors in the CALUX assay are further tested by co-exposing zebrafish embryos to the selected PAHs and the antagonist of the respective receptor.

A series of previous studies has proven the applicability of using a battery of in vitro assays (including the mEST, ZET and AhR CALUX) to evaluate the developmental toxicity of a series of DMSO-extracts of PAH-containing petroleum substances, and this potency was proportional

to the amount of 3- to 7-ring PACs present in these substances (Kamelia et al., 2021, 2019b, 2018, 2017). However, for some highly refined PAHs, which may contain relatively low or negligible concentration of 3- to 7-ring PACs (such as petroleum derived waxes), their developmental toxicity was less evaluated. Thus, in **Chapter 4**, sixteen petroleum substances that underwent different refining processes were tested for in vitro developmental toxicity using the mEST, ZET and AhR CALUX assay.

In **Chapter 5**, to show the potential of the in vitro approach to provide data suitable for risk assessment, the in vitro developmental toxicity of 8-MeBaP and 3-OH-8-MeBaP is characterized in the mEST. The obtained in vitro concentration-response data are subsequently translated into a predicted in vivo dose-repose curve for the developmental toxicity of 8-MeBaP using a developed PBK model (based on read across from the unsubstituted BaP) and PBK modelling based reverse dosimetry.

Chapter 6 presents the general conclusions and an overview of the results of the present thesis. A further discussion including future perspectives following from the results obtained in the present thesis is provided.

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CHAPTER 2.

Developmental toxicity testing of unsubstituted and methylated 4- and 5-ring polycyclic aromatic hydrocarbons using the zebrafish embryotoxicity test

Jing Fang, Shutong Dong, Peter J. Boogaard, Ivonne M.C.M. Rietjens, Lenny Kamelia

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Abstract

The present study evaluates the in vitro developmental toxicity of 4- and 5-ring polycyclic aromatic hydrocarbons (PAHs) including benz[a]anthracene (BaA) and benzo[a]pyrene (BaP) and six of their monomethylated congeners, and dibenz[a,h]anthracene (DB[a,h]A) using the zebrafish embryotoxicity test (ZET). In general, the tested PAHs induced various developmental effects in the zebrafish embryos including unhatched embryos, no movement and circulation, yolk sac and pericardial edemas, deformed body shape, and cumulative mortality at 96 hours post fertilization (hpf). The methyl substituent on different positions of the aromatic ring of the PAHs appeared to change their in vitro developmental toxicity. Comparison to a previously reported molecular docking study showed that the methyl substituents may affect the interaction of the PAHs with the aryl hydrocarbon receptor (AhR) which is known to play a role in the developmental toxicity of some PAHs. Taken together, our results show that methylation can either increase or decrease the developmental toxicity of PAHs, and suggest this may in part relate to effects on the molecular dimensions and resulting consequences for interactions with the AhR.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic compounds comprised of three or more fused benzene rings, containing only carbon and hydrogen atoms (Blumer, 1976; Hodson, 2017; Masood et al., 2017). Most PAHs are formed by incomplete combustion of organic materials through natural-biological or anthropogenic processes (EFSA, 2008). It is known that anthropogenic PAHs have two major sources: petrogenic and pyrogenic (Saha et al., 2012, 2009). Pyrogenic PAHs are formed by incomplete combustion of organic matters and fossil fuels (e.g., wood and coal) (Balmer et al., 2019; Saha et al., 2009). Petrogenic sources of PAHs include crude oil and partially refined crude oil products such as heavy fuel oil, distillate aromatic extract and gas oil (Tsitou et al., 2015). Unsubstituted PAHs are abundant in pyrogenic substances while alkylated PAHs (e.g., monomethylated, dimethylated and ethylated PAHs) account for a relatively large proportion of the PAHs present in the petrogenic substances (Hodson, 2017; Rhodes et al., 2005; Yang et al., 2014). It has been suggested that methylated PAHs may possess different toxicity than their unsubstituted parent compounds (Marvanová et al., 2008; Sun et al., 2014; Turcotte et al., 2011).

While the mutagenic and carcinogenic properties of many unsubstituted/naked and substituted PAHs have been intensively investigated (Fahmy and Fahmy, 1973; Flesher and Lehner, 2016; Hecht et al., 1987; Hoffmann et al., 1974; Santella et al., 1982; Trilecová et al., 2011; Tuyen et al., 2014), there is limited knowledge on the developmental toxicity of these substances, especially for the alkylated PAHs. In epidemiological studies, prenatal exposure to individual PAHs has been linked to increased incidence of miscarriage during early pregnancy (Wu et al., 2010), intrauterine growth retardation (Choi et al., 2008), and decreased birth weight and length (Choi et al., 2006; Delhommelle, 2010; Perera et al., 2005, 2003; Tang et al., 2006). To date, the majority of the in vivo developmental toxicity data of PAHs are from the best studied 5-ring PAH, benzo[a]pyrene (BaP). Prenatal exposure to BaP caused decreased fetal body weight and increased incidences of resorption to the offspring of pregnant rats (Archibong et al., 2002; Bui et al., 1986). Moreover, maternal exposure to BaP and 7,12-dimethyl-benz[a]anthracene (DMBA) before conception was associated with interference with normal fetal growth in mice (Detmar et al., 2008).

To date, the existing knowledge on the developmental toxicity of substituted PAHs mainly relates to some 3- and 4-ring alkylated PAHs. Comparative studies between phenanthrene (PHE) and its alkylated congeners showed that alkylated PHEs exerted higher embryotoxic potency

than PHE itself in the early developmental stage of Japanese medaka and marine medaka (Mu et al., 2014; Turcotte et al., 2011). For chrysene (CHR) and benz[a]anthracene (BaA), the presence of an alkyl substituent either increased or decreased the embryotoxicity relative to the unsubstituted parent PAHs in Japanese medaka embryos (Lin et al., 2015). From these findings, it can be hypothesized that the position of the alkyl substituent on the aromatic ring of PAHs influences the developmental toxicity of these substances.

Further testing of this hypothesis on the developmental toxicity of methylated PAHs, using experimental animals in vivo would be considered unethical given the large number of compounds to be tested. Hence, new approach methodologies (NAMs) would be preferred. The zebrafish embryotoxicity test (referred to as ZET hereafter) is one of the alternative assays widely used to evaluate the in vitro developmental toxicity of chemical substances (Beker van Woudenberg et al., 2013; Dach et al., 2019; Goodale et al., 2013; Hawliczek et al., 2012; Incardona et al., 2006; Jong et al., 2011; Kamelia et al., 2019; Selderslaghs et al., 2009). The ZET is not considered as an animal test if the zebrafish embryos in the test do not exceed 120 hours post fertilization (hpf) (EU, 2010; Sellick, 2011). The ZET makes use of an intact, transparent, and fast-developing vertebrate organism to study the potential effects of chemicals on embryonic development, with the development of zebrafish embryos being highly similar to the embryogenesis of human (Beekhuijzen et al., 2015; Rothenbücher et al., 2019; Sipes et al., 2011). The transparent chorion of zebrafish embryos makes it possible to perform non-invasive observations of the morphological changes in the zebrafish embryos. There are several advantages of the ZET over the other alternative assays for developmental toxicity testing (e.g., the embryonic stem cell test (EST), the whole embryo culture (WEC) and the limb bud micro-mass assay). First of all, in the ZET, zebrafish embryos are exposed to chemicals/substances during the whole vulnerable stage of embryogenesis which provides a more complete evaluation of the chemical's effect on the whole organogenesis period than obtained in other in vitro tests (Rothenbücher et al., 2019). Moreover, the ZET is cheap, fast, easy to conduct and thus enabling a medium-throughput screening of hazards (Le Bihanic et al., 2014a; Rothenbücher et al., 2019). Up to now, the ZET has been successfully applied to assess in vitro developmental toxicity of several individual substituted and unsubstituted PAHs (Dach et al., 2019; Geier et al., 2018; Incardona et al., 2006; Knecht et al., 2013; Kühnert et al., 2017; Turcotte et al., 2011), and many PAH-containing substances (Hedgpeth et al., 2019; Kamelia et al., 2019; Kim et al., 2019; Matson et al., 2008). To assess the effect of the chemical substances on the development of zebrafish embryos, the ZET utilizes a semi-quantitative

method known as the extended general morphology scoring (GMS) system (Beekhuijzen et al., 2015). Exposed zebrafish embryos are scored at specific time-points following exposure to test compounds for embryo lethality, growth retardation and morphological abnormalities based on the endpoints included in the extended-GMS (Beekhuijzen et al., 2015).

The present study aims to test the hypothesis that the developmental toxicity of monomethylated PAHs as compared to the unsubstituted parent PAH is influenced by the position of the alkyl substituent. To this purpose, nine 4- and 5-ring PAHs were evaluated for their potential developmental toxicity in the ZET including: BaA and its three methylated congeners: 4-methyl-benz[a]anthracene (4-MeBaA), 8-methyl-benz[a]anthracene (8-MeBaA), 9-methyl-benz[a]anthracene (9-MeBaA); BaP and its three methylated congeners: 3-methyl-benzo[a]pyrene (3-MeBaP), 7-methyl-benzo[a]pyrene (7-MeBaP), 8-methyl-benzo[a]pyrene (8-MeBaP); and dibenz[a,h]anthracene (DB[a,h]A) (see chemical structures in Supplementary material 1).

2. Materials and Methods

2.1 Test compounds

Benzo[a]pyrene (BaP, CAS No. 50-32-8), benz[a]anthracene (BaA, CAS No. 56-55-3), dibenz[a,h]anthracene (DB[a,h]A, CAS No. 53-70-3), 7-methyl-benzo[a]pyrene (7-MeBaP, CAS No. 63041-77-0), 8-methyl-benzo[a]pyrene (8-MeBaP, CAS No. 63041-76-9), 4-methyl-benz[a]anthracene (4-MeBaA, CAS No. 316-49-4), 8-methyl-benz[a]anthracene (8-MeBaA, CAS No. 2381-31-9), 9-methyl-benz[a]anthracene (9-MeBaA, CAS No. 2381-16-0), and 3,4-dichloroaniline (CAS No. 95-76-1) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). 3-Methyl-benzo[a]pyrene (3-MeBaP, CAS No. 16757-81-6) was synthesized by the Biochemical Institute for Environmental Carcinogens (Grosshansdorf, Germany). All test compounds were with purity of ≥98%. All stocks and dilutions of test compounds were prepared in dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany).

2.2 Zebrafish embryotoxicity test (ZET)

Eggs of wild-type zebrafish (*Danio rerio*) AB line were purchased from the research facility Carus, Wageningen University and Research, The Netherlands. The adult zebrafish were maintained in a flow-through aquarium system at 28°C with 14 h light/10 h dark cycle. Zebrafish eggs were produced via spawning groups by placing adult male and female zebrafish at a ratio of 15:20 in an individual tank several hours before the beginning of the dark cycle on

the day before the test. Collected eggs were rinsed a few times with egg water (prepared as described below), examined under a microscope, and then sorted using a disposable plastic pipette under inverted microscope. Only fertilized embryos that followed normal developmental stages were selected for subsequent experiments while embryos that showed abnormalities, including unfertilized and coagulated embryos, were discarded. The egg water was prepared by adding 10 ml 100-times egg water stock solution (3 g sea salt (Tropic marine, Wartenberg, Germany) in 500 ml demineralized water) to 990 ml demineralized water. The egg water was aerated for around 1 h, adjusted for pH to the range of 7.0–8.0, and stored at 26°C until use.

The ZET was initiated at 4-5 hpf and terminated at 96 hpf. The chorion of zebrafish embryos remains intact when used for exposure at 4-5 hpf. The exposure was performed in 24-well plates (Greiner bio-one, The Netherlands). In each plate, 20 wells of the 24-well plate were used for exposure to test compounds, 10 wells for one concentration of test compound, and the remaining 4 wells were used for the internal plate control (see Supplementary material 2 for the plate layout). Exposure medium was prepared by mixing 400-times concentrated DMSO-dissolved solutions of the test compounds with egg water, and the final concentration of DMSO in egg water was kept at 0.25% v/v. Given that previously the no observed effect concentration (NOEC) for the solvent DMSO in the ZET was determined to be 0.25% v/v, and at concentrations ≥ 0.5% v/v DMSO, there is a possibility that zebrafish embryos would develop tail malformations (i.e. kinked tail) (Selderslaghs et al., 2009), thus the DMSO concentration was kept at a maximum of 0.25 % v/v. The exposure medium was transferred (2 ml/well) into 20 wells of the 24-well plate, and for the internal plate control, 2 ml egg water was added into each of the 4 remaining wells. One zebrafish embryo was then transferred to each well of the 24-well plate (1 embryo/well). The plates were sealed with self-adhesive film covers (Greiner bio-one) to prevent evaporation throughout the exposure period. All test compounds were tested at a range of concentrations up to 50 µM, except for DB[a,h]A which could be dosed up to only 25 µM due to solubility limitation. Three types of controls: negative control (egg water), solvent control (0.25% v/v DMSO in egg water) and positive control (4 µg/ml 3,4-dichloroaniline) were included in each independent experiment. Plates were incubated at 26°C with a photo period of 14 h light/10 h dark. The exposure was static, and the exposure medium was not renewed during the whole experiment up to 96 hpf. Embryos were scored daily (every 24 hours) until 96 hpf for developmental abnormalities and embryo lethality using an inverted microscope, based on the extended general morphological scoring (extended-GMS) system

described by Beekhuijzen et al. (2015). This extended-GMS system includes two parts: 1) general development of zebrafish embryos (GMS), and 2) dysmorphogenic (or malformation) endpoints. The general development scoring in the ZET consists of 12 endpoints with a maximum total score of 17 points, i.e., detachment of tail, somite formation, eye development and pigmentation, movement, circulation, presence of heartbeat, pectoral fins, pigmentation of head and body, pigmentation of tail, hatching, presence of protruding mouth, and yolk extension (see Supplementary material 3). The dysmorphogenic endpoint scoring was done at 96 hpf only and consisted of 6 endpoints (maximum total score of 6 points), i.e., yolk sac edema, pericardial sac edema, malformation of the tail, deformed body shape, malformation of the head and jaw, and malformation of the sacculi/otoliths. Any deviation from normal developmental stages of zebrafish embryos results in a lower extended-GMS score, which corresponds to a certain extent of developmental retardation. It is worth mentioning that the exposure time windows of 0-24, 0-48 and 0-72 hpf did not show substantial differences (i.e. developmental effects on zebrafish embryos) between test compounds and controls (see Supplementary material 4), thus only data from the exposure time window of 0-96 hpf were used for data presentation and analysis. The ZET was considered valid if the following criteria were observed (at 96 hpf): (1) ≤1 dead embryos (out of 4) in the internal plate control of the exposed-plate, (2) ≤1 dead embryos (out of 10) in the negative control plate, (3) ≤1 dead embryos (out of 10) in the solvent control plate, (4) ≤7 alive embryos (out of 10) in the positive control plate (≥30% embryo lethality). Three independent ZET experiments were performed for each test compound.

2.3 Data analysis

The ZET results were expressed as percentage of response of the total GMS score (12 endpoints, including only the general morphology development of the zebrafish embryo, maximum score of 17 points), total extended-GMS score (18 endpoints, including the 12 endpoints for the general morphology development plus 6 dysmorphogenic/malformation endpoints, maximum score of 23 points), and embryo survival at 96 hpf. Results from both GMS and extended-GMS scoring were presented with the extended-GMS covering 6 dysmorphogenic endpoints known to be relevant for assessing the developmental toxicity potency of PAH-containing substances in the ZET (Kamelia et al., 2019, 2021), and the GMS scoring is a standard scoring system for evaluating the general morphology development of zebrafish embryos following exposure to chemical substances.

Figures of concentration-response curves upon exposure to test PAHs in the ZET were made using GraphPad Prism 5.0 (California, USA). Data were fitted to sigmoid concentration-response curves (non-linear regression analysis) with three parameters. Results were presented as mean percentage of response ± standard error of the mean (SEM) from three independent experiments. An unpaired Student's t-test (two tailed) was performed in GraphPad Prism 5.0 to determine the statistical significance of the differences between the solvent control and exposed samples in the GMS, and between the solvent control and exposed samples in the extended-GMS at every tested concentration of each test compound.

To determine the benchmark concentrations (BMC), concentration-response curves obtained in the ZET were fitted to all quantal concentration-response models from the European Food Safety Authority (EFSA) benchmark dose (BMD) modeling web-tool, based on the R-Package PROAST version 66.40 developed by the Dutch National Institute for Public Health and the Environment (RIVM). The quantal models of the ESFA BMD modeling web-tool include two-stage, log logistic, weibull, log probit, gamma, logistic, probit, exponential, and hill models. The benchmark response (BMR) was set to 20% (i.e., BMC20), representing the concentration that induced 20% reduction of either the GMS score or the extended-GMS score in the ZET. A BMR20 was selected as a threshold because 20% was the highest response in the GMS or extended GMS score induced by the test compounds without concomitant embryo lethality. All analyses were performed according to the manual provided on the EFSA BMD modeling web-tool site (https://shiny-efsa.openanalytics.eu/). The performance of each fitted model was evaluated based on the goodness-of-fit, the scaled residuals, and the visual inspection of model fitting. The final BMC20 values were selected from the accepted model with the lowest Akaike's Information Criterion (AIC) (Haber et al., 2018) (see Supplementary material 5).

In addition to concentration-response curves, a heatmap was developed to visualize the effects of each test compound on the endpoints scored in the ZET. To this purpose, the average percentage of incidence (from three independent ZET experiments), obtained by dividing the total number of viable zebrafish embryos displaying effects on a specific endpoint included in the ZET scoring (at 96 hpf) by the total number of viable embryos, was used as data input for the heatmap. An exposure was considered as a 'hit' for a specific endpoint on the heatmap if the average percentage of embryos showing the corresponding effect was equal to or greater than 50% (i.e., ≥50%). The lowest concentration of test compound that caused a "hit" on the heatmap was indicated using a gradient red color code with an increasing shade of red for the decreasing concentrations tested. An unpaired Student's t-test was performed in GraphPad

Prism 5.0 to determine the statistical significance between the percentage of affected embryos at a specific test concentration and that of the solvent control (0.25% v/v DMSO). Results presented in the heatmap represent data from 3 independent ZET experiments.

3. Results

Figure 1 shows the concentration-dependent effects of all tested PAHs in the ZET based on GMS, extended-GMS, and embryo lethality (decreased survival) at 96 hpf. All tested PAHs, except 7-MeBaP, induced concentration-dependent decreases in the GMS and extended-GMS at 96 hpf, where for some test compounds, including BaP and 4-MeBaA, such effects coincided with a decrease in embryo survival at 96 hpf in the ZET. Using the obtained concentration-response curves in the ZET, the BMC20 value for both GMS and extended-GMS were determined and the resulting BMC20s are presented in Table 1. Furthermore, a heatmap (Figure 2) was developed to illustrate the affected endpoints included in the ZET scoring at 96 hpf. Overall, 5 out of 12 general morphology development endpoints were affected following exposure to the test compounds when scored at 96 hpf, which include absence of movement, absence of circulation, unhatched embryos, undeveloped mouth, and yolk extension not nearly empty. Moreover, except for malformed sacculi/otoliths, all dysmorphogenic endpoints, i.e., pericardial sac edema, yolk sac edema, malformation of the tail (i.e., kinked tail), deformed body shape, and deformed head and jaw, were induced by all tested PAHs except 7-MeBaP, at 96 hpf in the ZET (Figure 2). Figure 3 presents representative images of normally developed and malformed zebrafish embryos at 96 hpf (Figure 3A), as well as images of normally developed or malformed embryos observed upon exposure to each PAH at the respective three test concentrations in the range from 5 to 50 μM at 96 hpf (Figure 3B).

Exposure to the unsubstituted parent 4-ring PAH BaA (Figure 1A) induced a concentration-dependent developmental retardation in zebrafish embryos (scored at 96 hpf) with a $BMC20_{extended\text{-}GMS}$ value of 5.29 μM (Table 1). The affected general morphology development endpoints following exposure to BaA included absence of movement, which was observed only at 50 μM, absence of circulation, absence of protruding mouth and yolk extension not empty, which were observed starting at 5 μM (Figures 2 and 3B). Regarding the dysmorphogenic endpoints, exposure to BaA caused pericardial sac edema, yolk sac edema, retarded head and jaw, deformed body shape, and malformed tail (i.e. kinked tail), the starting concentration where these effects occurred at levels exceeding 50% of the embryos can be seen in the heatmap

of Figure 2. Monomethylated congeners of BaA, namely 4-MeBaA, 8-MeBaA and 9-MeBaA, also induced developmental retardations in zebrafish embryos at 96 hpf and their calculated BMC20s for both GMS and extended-GMS are presented in Table 1. The affected endpoints following exposure to monomethylated BaAs are similar to those affected by their parent BaA (Figure 2), with the exception that 9-MeBaA did not induce embryo lethality (Figures 1D and 2) and no malformed tail up to 50 μM (Figures 2 and 3B). In other words, the reduction in the extended-GMS score upon exposure to 9-MeBaA ($BMC20_{extended\text{-}GMS}$: 15.7 μM) occurred at non-lethal concentrations. Exposure to 4-MeBaA and 8-MeBaA caused similar dysmorphogenic effects at the same concentration, i.e., pericardial sac edema, yolk sac edema, deformed body shape retarded head and jaw starting at 5 μM, and kinked tail starting at 15 μM (Figures 2 and 3B). These effects were also comparable to those induced by unsubstituted BaA, although some effects were observed at slightly higher concentrations for these two methylated analogues.

Exposure to BaP and its monomethylated congeners, except 7-MeBaP, induced various developmental effects in zebrafish embryos (scored at 96 hpf; Figures 1, 2, and 3B). As shown in Figure 1E, the parent 5-ring BaP induced a concentration-dependent developmental retardation in zebrafish embryos at 96 hpf with a $BMC20_{extended\text{-}GMS}$ value of 4.31 μM (Table 1). This included the absence of movement and circulation starting at 15 μM, and undeveloped mouth and yolk extension not empty starting at 5 μM (Figure 2). The dysmorphogenic endpoints induced by BaP starting from 5 μM were pericardial sac and yolk sac edema, while effects on the kinked tail, deformed body shape, and retarded head and jaw were observed starting from the concentration of 15 μM (Figure 2). 3-MeBaP disturbed the general development of zebrafish embryos without inducing embryo lethality up to the highest concentration tested ($BMC20_{extended\text{-}GMS}$: 15.1 μM). The absence of movement, unhatched embryos, absence of circulation, undeveloped mouth and yolk extension not empty were the effects observed following exposure to 3-MeBaP in the ZET. In addition, 3-MeBaP caused pericardial sac and yolk sac edema, deformed body shape, and retarded head and jaw starting at the concentration of 15 μM (Figure 2). Exposure to 7-MeBaP did not cause any developmental retardations in zebrafish embryos (Figure 1G), except for the effect on hatching, which occurred mainly at the highest concentration tested (50 μM) (Figure 3B), thus no BMC values could be derived for 7-MeBaP. Of all PAHs tested, 8-MeBaP was the most potent test compound to induce developmental retardation in zebrafish embryos as it has the lowest $BMC20_{extended\text{-}GMS}$ value of 0.38 μM (Table 1). The $BMC20_{extended\text{-}GMS}$ of 8-MeBaP is ~10 times

lower than for its parent BaP (Table 1). The affected general development endpoints of the zebrafish embryos exposed to 8-MeBaP include effects on pectoral fins, absence of movement and circulation, undeveloped mouth and yolk extension not empty, and failure to hatch (Figure 2), which were observed from 0.5 μM onwards. In addition, exposure to 8-MeBaP resulted in pericardial sac and yolk sac edema starting from 0.5 μM, and kinked tail, deformed body shape and retarded head and jaw, starting from 1.5 μM (Figures 2 and 3B).

Of the three unsubstituted 4- to 5-ring PAHs tested, DB[a,h]A was the only PAH that exerted no lethality effect in zebrafish embryos (scored at 96 hpf) up to the highest concentration tested of 25 μM, while showing developmental effects with a $BMC20_{extended\text{-}GMS}$ of 6.71 μM. As shown in Figures 2 and 3B, DB[a,h]A caused failure to hatch starting at 5 μM, whereas the absence of movement, absence of circulation, no protruding mouth, yolk extension not empty were observed at a higher tested concentration starting at 15 μM. In addition, DB[a,h]A also caused several dysmorphogenic effects such as pericardial sac and yolk sac edema starting at 5 μM, and deformed body shape, and retarded head and jaw starting at 15 μM (Figure 2 and 3B).

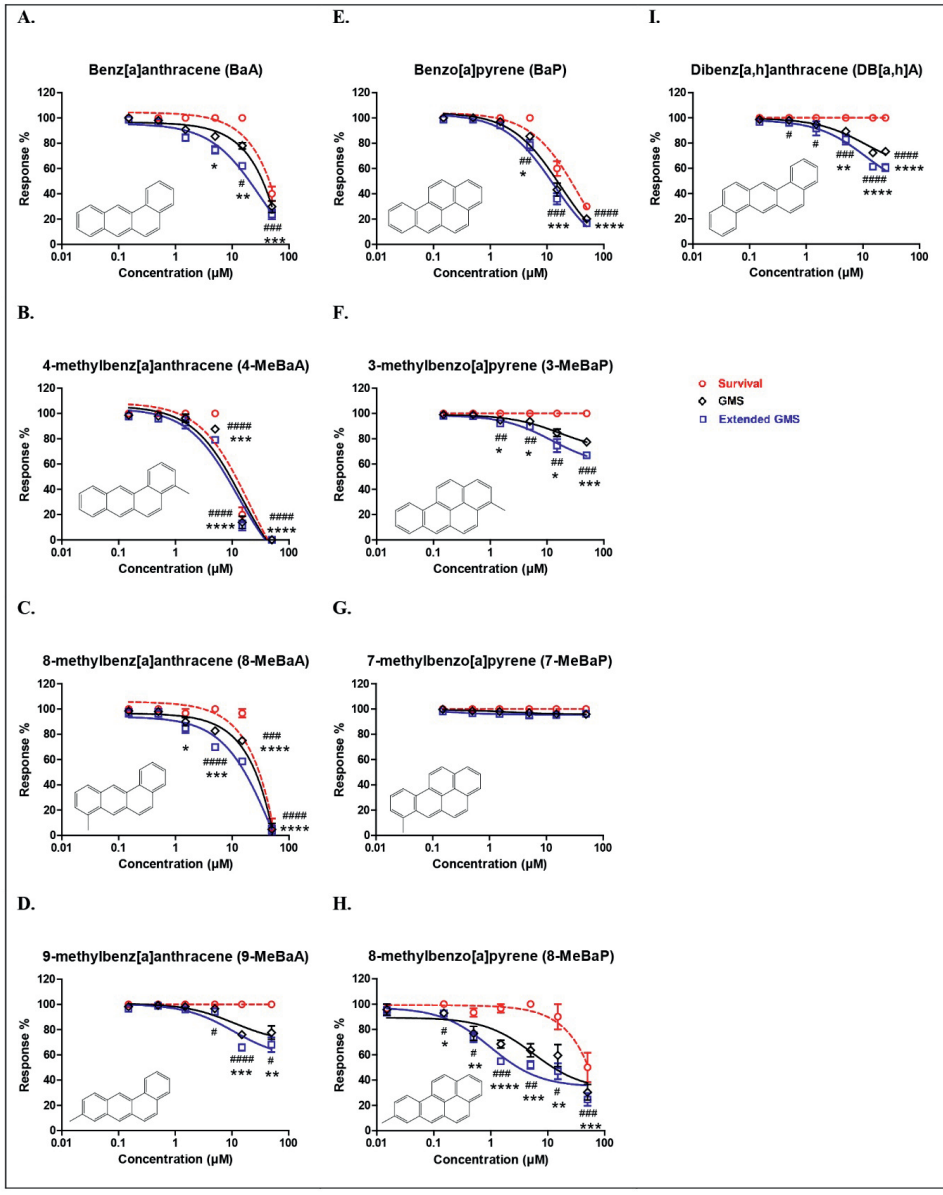


Figure 1. Concentration-dependent effects of the nine PAHs on embryo survival (red dotted line with unfilled circle symbol), GMS (black line with unfilled diamond symbols) and extended-GMS (blue line with unfilled square symbols) at 96 hpf in the ZET. Results represent data from three independent ZET experiments and are presented as mean percentage ± standard error of mean (SEM). The significant differences in GMS between solvent control and exposed samples, and in extended-GMS between solvent control and exposed samples at each tested

concentration are represented by hashtag and asterisk symbols, respectively, in the figure: #/* p<0.05, ##/** p<0.01, ###/*** p<0.001 , ####/**** p<0.0001. *Abbreviations: BaA: benz[a]anthracene; 4-MeBaA: 4-methyl-benz[a]anthracene; 8-MeBaA: 8-methyl-benz[a]anthracene, 9-MeBaA: 9-methyl-benz[a]anthracene; BaP: benzo[a]pyrene; 3-MeBaP: 3-methyl-benzo[a]pyrene; 7-MeBaP: 7-methyl-benzo[a]pyrene; 8-MeBaP: 8-methyl-benzo[a]pyrene; DB[a,h]A: dibenz[a,h]anthracene; GMS: general morphology scoring; 96 hpf: hours post fertilization.*

Table 1. Results from BMD analysis of the ZET data on the GMS and extended-GMS at 96 hpf for the 9 PAHs tested. The percentage of embryo survival at the BMC20s of the concentration response curves for the GMS and extended-GMS score are also listed in this table.

Test compounds	$BMC20_{GMS}$ (µM)	$BMC20_{extended\text{-}GMS}$ (µM)	% of embryo survival at $BMC20_{extended\text{-}GMS}$ values
BaA	20.60	5.29	100
4-MeBaA	6.37	5.34	100
8-MeBaA	14.10	4.81	100
9-MeBaA	28.70	15.70	100
BaP	5.70	4.31	100
3-MeBaP	46.00	15.10	100
7-MeBaP	-	-	100
8-MeBaP	0.71	0.38	93
DB[a,h]A	15.10	6.71	100

Note. The $BMC20_{GMS}$, $BMC20_{extended\text{-}GMS}$ are defined as the benchmark concentration, representing the concentration that induced a 20% reduction of the GMS or extended-GMS score at 96 hpf in the ZET; a dash (-) means there was no effect observed in the ZET up to the highest concentration tested thus BMC20 values could not be defined. *Abbreviations. BaA: benz[a]anthracene; 4-MeBaA: 4-methyl-benz[a]anthracene; 8-MeBaA: 8-methyl-benz[a]anthracene, 9-MeBaA: 9-methyl-benz[a]anthracene; BaP: benzo[a]pyrene; 3-MeBaP: 3-methyl-benzo[a]pyrene; 7-MeBaP: 7-methyl-benzo[a]pyrene; 8-MeBaP: 8-methyl-benzo[a]pyrene; DB[a,h]A: dibenz[a,h]anthracene; $BMC20_{GMS}$: concentration that induces a 20% reduction of GMS score; $BMC20_{extended\text{-}GMS}$: concentration that induce a 20% reduction of extended-GMS score.*

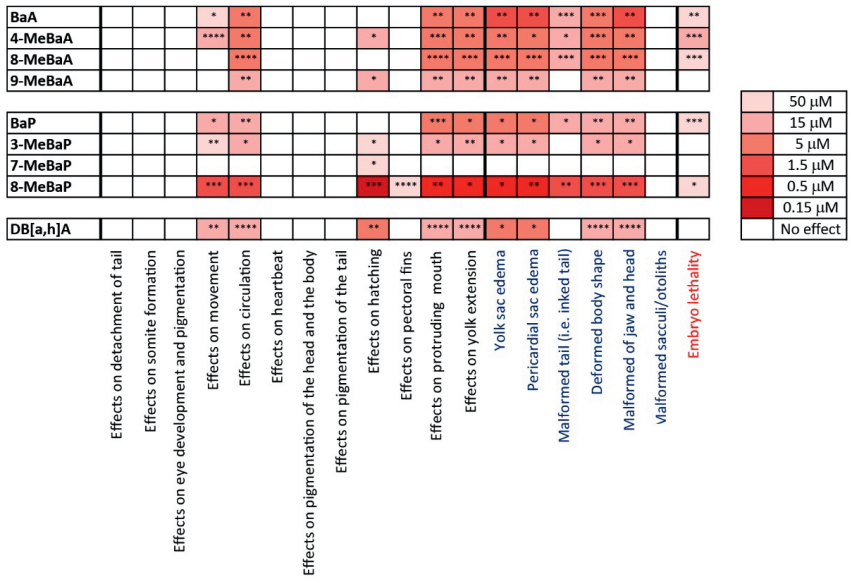
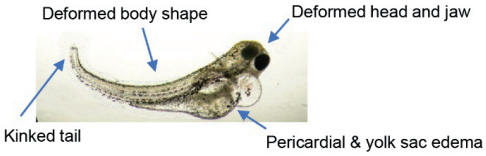


Figure 2. Heatmap summarizing the results in the ZET for the 18 endpoints scored based on the GMS system (black font) and the extended-GMS system (blue font) and for embryo lethality (red font) upon exposure to the 9 PAHs at 96 hpf. An exposure was considered as a 'hit' for a specific endpoint on the heatmap when ≥50% of the viable embryos showed effects on this endpoint scored at 96 hpf. The lowest concentration of the respective test compound that corresponds to a 'hit' is indicated by the color code shown on the right part of the figure. Results presented in the heatmap represent data from three independent experiments. The statistical significance of the difference between the percentage of affected embryos at the concentration as indicated in the heatmap and that of the solvent control is represented by asterisk symbols in the figure: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. *Abbreviations. BaA: benz[a]anthracene; 4-MeBaA: 4-methyl-benz[a]anthracene; 8-MeBaA: 8-methyl-benz[a]anthracene, 9-MeBaA: 9-methyl-benz[a]anthracene; BaP: benzo[a]pyrene; 3-MeBaP: 3-methyl-benzo[a]pyrene; 7-MeBaP: 7-methyl-benzo[a]pyrene; 8-MeBaP: 8-methyl-benzo[a]pyrene; DB[a,h]A: dibenz[a,h]anthracene; GMS: general morphology scoring; hpf: hours post fertilization.*

(A)

Morphology of normally developed zebrafish embryos at 96 hpf (solvent control 0.25% v/v DMSO)



Morphology of malformed zebrafish embryos at 96 hpf

(B)

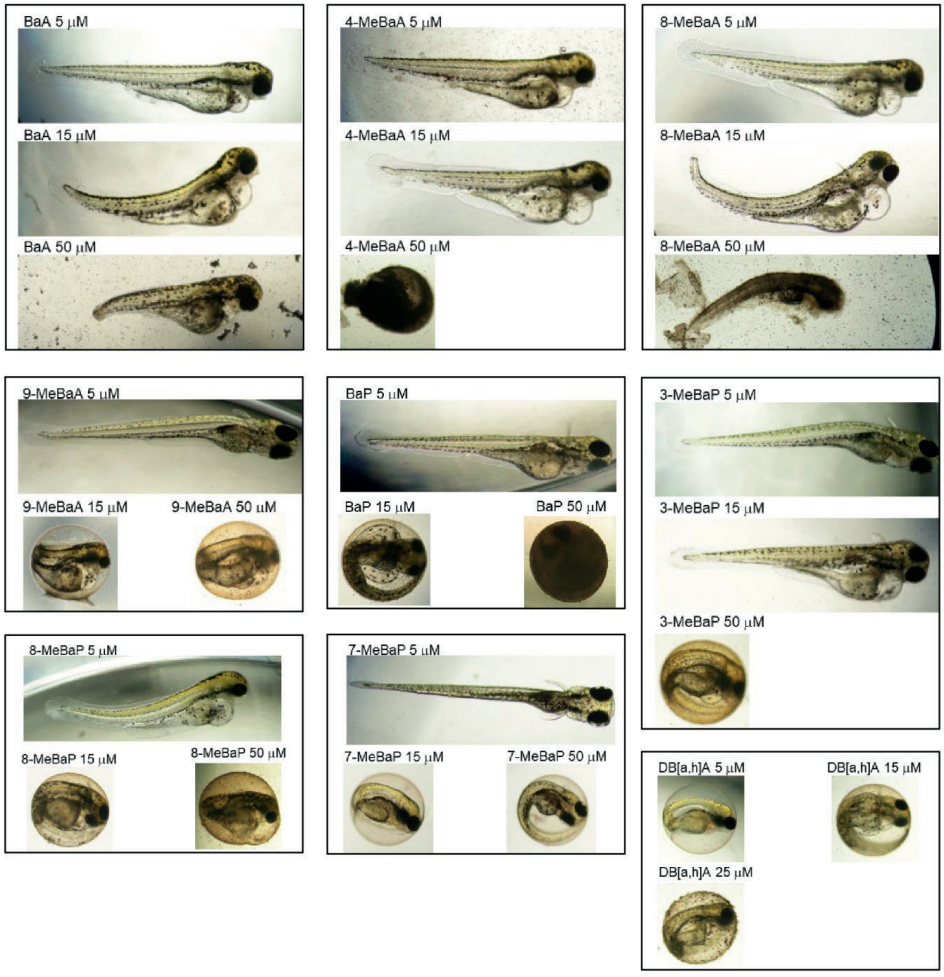


Figure 3. (A) Representative images of normally developed and malformed zebrafish embryos at 96 hpf. (B) Zebrafish morphology at 96 hpf upon exposure to 9 PAHs under study.

Abbreviations. BaA: benz[a]anthracene; 4-MeBaA: 4-methyl-benz[a]anthracene; 8-MeBaA: 8-methyl-benz[a]anthracene, 9-MeBaA: 9-methyl-benz[a]anthracene; BaP: benzo[a]pyrene; 3-MeBaP: 3-methyl-benzo[a]pyrene; 7-MeBaP: 7-methyl-benzo[a]pyrene; 8-MeBaP: 8-methyl-benzo[a]pyrene; DB[a,h]A: dibenz[a,h]anthracene; GMS: general morphology scoring; hpf: hours post-fertilization.

4. Discussion

Alkylated PAHs account for a large proportion of the total PAHs present in crude oil and partially refined petroleum products (Hodson, 2017; Yang et al., 2014). Many studies have correlated the presence of PAHs, including alkylated PAHs, to the developmental toxicity of petroleum substances and PAH-containing mixtures (Barranco et al., 2017; Billiard et al., 2008; Incardona, 2017; Le Bihanic et al., 2014b, 2014a). It was also suggested for some alkylated PAHs that they are more developmentally toxic than their corresponding parent PAHs (Geier et al., 2018; Mu et al., 2014). The present study evaluated the in vitro developmental toxicity of a series of 4- and 5-ring unsubstituted PAHs and some of their monomethylated congeners using the ZET, to investigate the potential consequences of an alkyl substituent at different positions on the aromatic ring of PAHs for the developmental toxicity of these monomethylated PAHs relative to their parent PAHs. Of all PAH tested, 8-MeBaP was the most potent to induce developmental toxicity in zebrafish embryos at non-lethal concentrations, where 7-MeBaP was the only one that tested negative in the ZET. Thus, the position of the alkyl substituent on the aromatic ring of the PAH appeared to influence the in vitro developmental toxicity, either increasing or decreasing it. The PAH model compounds of the present study were selected based on either the availability of in vitro developmental toxicity data in the mouse EST (mEST), or based on structural similarities to PAH model compounds that tested positive for in vitro developmental toxicity in the mEST (Kamelia et al., 2020). In the absence of bioactivation, the 5-ring PAH DB[a,h]A, but not BaP, induced concentration-dependent developmental toxicity in the mEST (Kamelia et al., 2020). BaP was only able to induce in vitro developmental toxicity upon bioactivation, and its major metabolite 3-hydroxybenzo[a]pyrene (3-OHBaP) was shown to be active in the mEST and most likely responsible for the observed developmental toxicity of BaP (Kamelia et al., 2020). Comparison of the chemical structures of the selected model compounds demonstrates that 7-MeBaP, 8-MeBaP, 8-MeBaA and 9-MeBaA mimic the dimensions of DB[a,h]A, and that 4-MeBaA and 3-MeBaP mimic the structural configuration of 3-OHBaP known as the developmental toxic metabolite of BaP

(Kamelia et al., 2020). It has been suggested that the aromatic ring oxidation of PAHs can increase the embryotoxicity towards fish embryos (Fallahtafti et al., 2012; Kamelia et al., 2020), whereas the presence of a methyl substituent on PAHs shifts metabolism from the aromatic ring to methyl chain oxidation (Wang et al., 2020). For BaP it was demonstrated that its developmental toxicity is mediated by its 3-OHBaP metabolite (Kamelia et al., 2020) indicating the need for hydroxylation at C3 of BaP to induce such effects. Thus, the addition of a methyl substituent at the C3 of BaP or C4 of BaA might potentially block the site at which bioactivation to their active metabolite(s) occurs and at the same time provide higher chances for an alternative metabolic pathway at the methyl side chain, thereby reducing the chances on bioactivation and developmental toxicity. BaA and BaP were included as the corresponding unsubstituted PAHs of the selected methylated PAHs under study.

Results obtained in the present study show that except for 7-MeBaP, all the 4- and 5-ring naked and monomethylated PAHs were able to disturb the normal development of zebrafish embryos at 96 hpf, resulting in the occurrence of various developmental retardations and embryo lethality (Figure 2). Several common developmental retardations were observed in zebrafish embryos upon exposure to these naked and methylated PAHs (except for 7-MeBaP), including unhatched embryos, effects on circulation and protruding mouth, yolk sac edema, pericardial edema, malformed head and jaw, and deformed body shape. These findings are consistent with effects previously observed in fish embryos exposed to some other unsubstituted and alkylated PAHs (see an overview of the developmental toxicity induced by some PAHs and alkylated PAHs in fish embryo model systems in Supplementary material 6. For example, exposure of Japanese medaka embryos to the 4-ring PAHs: CHR, BaA and their mono- and di-methylated congeners induced yolk sac and pericardial edemas, absence of swim bladder, and craniofacial deformity (Lin et al., 2015). The authors concluded that the presence of alkyl-substituents on CHR and BaA generally increased their toxicity, except for alkylation at C2 of BaA, and that the observed effects resembled those of dioxin pointing at a specific receptor-based mechanism of toxicity (Lin et al., 2015; Turcotte et al., 2011). Results of the present study corroborate that the monomethyl substituent on the aromatic ring of an unsubstituted PAH can increase but also decrease the potency to induce developmental toxicity, depending on the site of methyl substituent.

In the present study, the extended-GMS scoring system was used as the standard to predict the developmental toxicity of PAHs. The extended-GMS scoring system has been successfully used to evaluate the developmental toxicity of PAH-containing complex materials in the ZET

(Kamelia et al., 2019, 2021). However, the suitability of this approach to assess the potential developmental toxicity of individual PAHs has not been fully evaluated so far. As can be seen from Figure 1, the effects derived from the extended-GMS are notably observed at lower concentrations than the effects quantified by the GMS. This indicates that not only the scoring on general morphology development but also scoring of the dysmorphogenic endpoints contributes to evaluating the developmental toxicity of individual PAHs using the ZET. Given the apparent higher sensitivity, the use of the extended-GMS may prove to be more appropriate to assess the developmental toxicity for the group of PAHs and PAH-containing substances in the ZET.

One of the possible explanations for the different developmental toxicities of the unsubstituted and different monomethylated PAHs, as observed in the present study, is the ability of these substances to interact with different nuclear receptors, including but not limited to the aryl hydrocarbon receptor (AhR), estrogen receptor-α (ER-α) and retinoic acid receptor (RAR). It is known that the AhR, ER-α and RAR play important roles in developmental toxicity in mammals (Barlow et al., 1999; Lammer et al., 1985; Piersma et al., 2017). Exposure to the PAHs under study (except for 7-MeBaP) caused yolk sac edema, pericardial edema and deformed body shape in zebrafish embryos at 96 hpf. Such effects are consistent with the typical observations or manifestations of zebrafish embryos exposed to 3- to 7-ring PAHs and/or PAH-containing substances in the ZET, and are believed to be mediated via the AhR (Billiard et al., 1999; Goodale et al., 2013; Kamelia et al., 2018; Lanham et al., 2014). The AhR is a ligand-activated transcription factor, which belongs to the basic helix-loop-helix Per-Arnt Sim (bHLH-PAS) domain protein family (Hahn, 1998). The AhR has a rather promiscuous ligand-binding site that can be activated by a diverse range of chemical structures (Denison and Nagy, 2003; Mahony et al., 2011; Yaşar et al., 2017). It is known that zebrafish have multiple isoforms of the AhR, namely AhR1a, AhR1b and AhR2 (Aranguren-abadía et al., 2020; Fraccalvieri et al., 2013; Karchner et al., 2005; Scott et al., 2011). Among all subtypes of the AhR in zebrafish, AhR2 plays a dominant role in mediating PAH-induced developmental toxicity, such as in BaP induced AhR2-dependent cardiotoxic effects to zebrafish embryos (Incardona et al., 2011). AhR1b may also be involved since it was suggested to regulate the embryonic development whereas AhR1a was not able to interact with TCDD or TCDD-like chemicals and thus may not have a substantial role in mediating developmental-related effects of PAHs in zebrafish embryos (Fraccalvieri et al., 2013).

The results of the ZET showed that the 5-ring PAHs, BaP and DB[a,h]A, induced different in vitro developmental toxicity potencies (Figure 1 and 2). Given this result, it is of interest to note that while both BaP and DB[a,h]A are known AhR ligands, a recent molecular docking study (Giani Tagliabue et al., 2019) demonstrated that BaP and DB[a,h]A interacted with different parts of the mouse AhR (mAhR) ligand-binding domain (LBD). In that study, BaP was predicted to bind at the bottom of the Bβ, Cα and Dα region of the mAhR, whereas DB[a,h]A was predicted to interact with the Fα/Gβ region and tended to occupy almost the entire volume of the binding cavity due to its bulky and extended molecular structure. Thus, it can be hypothesized that the effect of methylation on the molecular structure of the PAHs leads to different binding to the zebrafish embryo AhR, subsequently resulting in different AhR activation and resulting developmental toxicity. Thus, it can be speculated that 3-MeBaP, 8-MeBaP and 9-MeBaA, in which the methyl substituent extends the diagonal dimension of the molecule making it more comparable to DB[a,h]A, may interact and bind to the Fα/Gβ region of the AhR LBD as does DB[a,h]A (see the possible positioning of the PAHs tested in the Fα/Gβ region of the mAhR LBD in Supplementary material 7), potentially resulting in similar developmental toxicity in the ZET. In contrast to these DB[a,h]A-like PAHs, the addition of a methyl substituent on C7 makes the molecule of 7-MeBaP too bulky at the right-lower end potentially hampering its binding activity to the active site of the AhR (see Supplementary material 7). For the other PAHs tested in the present study, their molecular dimensions might deviate more from that of DB[a,h]A resulting in different binding to the AhR. Altogether, it would be of interest to perform a molecular docking study on the unsubstituted and monomethylated PAHs to investigate their interactions with the zebrafish embryo AhR LBD, and to investigate the ability of unsubstituted and monomethylated PAHs to activate different subtypes of the AhR. Such studies are beyond the objectives of the present study but are considered for future studies.

Published studies have reported AhR-mediated activities of unsubstituted as compared to monomethylated PAHs (Marvanová et al., 2008; Sun et al., 2014; Trilecová et al., 2011; Vondráček et al., 2017). For example, Marvanová et al. (2008) reported that all monomethylated BaAs were more potent than their parent BaA in activating the AhR in rat hepatoma cells, and that of all twelve monomethylated congeners, 9-MeBaA was most potent. In addition, Trilecová et al. (2011) reported that 3-MeBaP was more potent than its parent BaP in activating the AhR in rat hepatoma cells. Results obtained in the present study suggested that 9-MeBaA and 3-MeBaP induced less developmental effects in zebrafish embryos compared to

their corresponding unsubstituted PAHs. This indicates that activation of the AhR may not be the decisive factor in the mode of action underlying the developmental toxicity of the methylated PAHs in the ZET, since results of the present study showed some methylated PAHs to be less and not more potent than the corresponding naked PAHs. Alternative factors may relate to the fact that to induce developmental toxicity, PAHs may need bioactivation as was for example shown for BaP for which the developmental toxicity was ascribed to its 3-OHBaP metabolite (Kamelia et al., 2020). If also for the PAHs of the present study, aromatic ring hydroxylation would be required to generate the toxic metabolite(s), their potency may be dependent on their metabolism and the generation of the respective toxic metabolite(s). So, it should be noted that the ability of unsubstituted and monomethylated PAHs to activate the AhR in vitro may not directly be translated into their ability to induce developmental toxicity, as physiological feedback mechanisms and metabolism are lacking in the AhR reporter gene assays applied. Furthermore, other nuclear hormone receptors such as for example the estrogen receptor-α (ER-α) and the retinoic acid receptor (RAR) may also be involved.

It has been suggested that interference of the monomethyl substituents with binding of the PAHs to the ER-α and RAR may play a role in the modes of action underlying the differences in the developmental toxicity induced by the PAHs (Barlow et al., 1999; Piersma et al., 2017). Some PAHs, including BaP, BaA and DB[a,h]A have been reported to be able to induce estrogenic and/or anti-estrogenic activities (Boonen et al., 2020; Gozgit et al., 2004; Tran et al., 1996; Van Lipzig et al., 2005). In addition, some monohydroxylated BaPs, including 3-OHBaP, 8-OHBaP and 9-OHBaP, were more estrogenic than BaP itself when tested in the T47D.Luc ER-α reporter cell line (Van Lipzig et al., 2005). In zebrafish embryos, disruption of the ER-α signaling pathway via exerting (anti) estrogenic activities may lead to cumulative mortality, unhatched embryos, pericardial edema, kinked tail and craniofacial deformities (Adam et al., 2021; Ahi et al., 2016; Kishida et al., 2001; Malebari et al., 2021; Wester & Vos, 2003). In the present study, similar manifestations were observed in zebrafish embryos following exposure to some PAHs tested. Thus, the role of the ER-α in relation to the developmental toxicity of PAHs and their monohydroxylated metabolites should be investigated to a further extent. Exposure to high concentrations of retinoic acid, the natural ligand of RAR, has been associated with an affected body axis in vertebrates (Maden and Holder, 1992), craniofacial development in rodent embryos (Morriss-Kay, 1993), and failure to hatch (Herrmann, 1995) in zebrafish embryos. Given the fact that some developmental effects such as deformed body shape and

failure to hatch were also observed following exposure to some of the PAHs under study, the role of the RAR in mediating such effects may have to be considered.

Several studies have shown that the expression and activity of several CYP isoforms in zebrafish are low to negligible in embryos up to 96 hpf (Saad et al., 2017). Even so, there are also shreds of evidence showing that the activity of CYP1A appears to have a much earlier onset in the zebrafish than that of the other CYP subfamilies, and that CYP1A in fish embryos could bioactivate some unsubstituted PAHs and alkylated PAHs into intermediate toxic metabolites (Fallahtafti et al., 2012; Hodson, 2017; Timme-Laragy et al., 2008). This could imply that the different developmental toxicities of the PAHs as observed in the ZET of the present study may also be related to their metabolism by the zebrafish embryos. As previously mentioned, DB[a,h]A is able to exert its developmental toxicity without bioactivation as tested in the mEST, whereas BaP was only able to induce developmental toxicity following bioactivation to 3-OHBaP (Kamelia et al., 2020). Given this consideration it is of interest to note that recent studies revealed that in contrast to all other methylated BaPs tested, 7-MeBaP was only converted to a limited extent in incubations with both rat and human liver microsomes indicating that a methyl substituent at C7 of BaP could potentially block its metabolism (Wang et al., 2022). Thus, the incapability of 7-MeBaP to induce developmental toxicity as tested in the ZET might also be attributed to limited metabolism of 7-MeBaP in the zebrafish embryos although this remains open for future research. To add, it is suggested that metabolism may enhance the toxicity of alkylated PAHs in early life stages of fish by the generation of ring-oxidation metabolites (Fallahtafti et al., 2012), and a recent study demonstrated that the alkylation of PAHs shifts metabolism from aromatic ring oxidation to alkyl side chain oxidation (Wang et al., 2020). This could contribute to a potentially lower developmental toxicity upon alkylation of a PAH. Taken together, some PAHs may require metabolic activation to induce their developmental toxicity potency and some do not, and the presence of a methyl substituent on different positions of parent PAHs can significantly influence the biotransformation and thus the resulting toxicity of these substances.

To conclude, the present study evaluated the in vitro developmental toxicity of three unsubstituted and six monomethylated PAHs in the ZET, and the results obtained support the hypothesis that the addition of a methyl group to the unsubstituted parent PAH can either increase or decrease its developmental toxicity depending on the location of the methylation. It is speculated that the effect of the methyl substituent on the molecular dimensions affecting the

interaction with the LBD of the AhR may in part be responsible for the effects of methylation on the in vitro developmental toxicities of the PAHs as observed in the ZET.

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Supplementary material

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CHAPTER 3.

The role of receptor-mediated activities of 4- and 5-ring unsubstituted and methylated polycyclic aromatic hydrocarbons (PAHs) in developmental toxicity

Jing Fang, Danlei Wang, Nynke I. Kramer, Ivonne M.C.M. Rietjens,
Peter J. Boogaard, Lenny Kamelia

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Abstract

The present study evaluated the aryl hydrocarbon receptor (AhR), estrogen receptor-α (ER-α), and retinoic acid receptor (RAR) mediated activities of nine 4- and 5-ring unsubstituted and monomethylated polycyclic aromatic hydrocarbons (PAHs) using a series of Chemical Activated LUciferase gene eXpression (CALUX) assays. The potential role of these aforementioned receptors in relation to the developmental toxicity of these PAHs was further assessed in the zebrafish embryotoxicity test (ZET). The results show that all nine tested PAHs were AhR agonists, benz[a]anthracene (BaA) and 8-methyl-benz[a]anthracene (8-MeBaA) were ER-α agonists, and none of the tested PAHs induced ER-α antagonistic or RAR (ant)agonistic activities. In the AhR CALUX assay, all the methylated PAHs showed higher potency (lower EC50) in activating the AhR than their respective unsubstituted PAHs, implying that the addition of a methyl substituent on the aromatic ring of PAHs could enhance their AhR-mediated activities. Co-exposure of zebrafish embryos with each individual PAH and an AhR antagonist (CH223191) counteracted the observed developmental retardations and embryo lethality to a certain extent, except for 8-methyl-benzo[a]pyrene (8-MeBaP). Co-exposure of zebrafish embryos with either of the two estrogenic PAHs (i.e., BaA and 8-MeBaA) and an ER-α antagonist (fulvestrant) neutralized embryo lethality induced by 50 μM BaA and the developmental retardations induced by 15 μM 8-MeBaA. Altogether, our findings suggest that the observed developmental retardations in zebrafish embryos by the PAH tested may partially be AhR- and/or ER-α-mediated, whereas the RAR seems not to be relevant for the PAH-induced developmental toxicity in the ZET.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) form a group of diverse organic compounds with at least three fused benzene rings, containing only carbon and hydrogen atoms (EFSA, 2008; Masood et al., 2017). PAHs are generated in both natural-biological and anthropogenic processes (i.e., pyrogenic and petrogenic sources) (Balmer et al., 2019; Skoczyńska and de Boer, 2019). Pyrogenic substances comprise mainly unsubstituted parent PAHs, whereas petrogenic substances, such as crude oil and its refined products, contain predominantly alkylated PAHs (Saha et al., 2012, 2009; Skoczynska et al., 2013; Yang et al., 2014).

Some PAHs have been associated with negative health impacts on humans such as prenatal developmental toxicity (Choi et al., 2008, 2006; Perera et al., 2006, 2003; Tang et al., 2006). To date, investigations of the developmental toxicity of PAHs mainly focus on unsubstituted/naked PAHs. For example, prenatal exposure to a 5-ring PAH, benzo[a]pyrene (BaP) resulted in compromised fetal survival, decreased fetal weight and increased incidence of resorptions in the offspring of pregnant rats (Archibong et al., 2002; Bui et al., 1986). However, there is limited knowledge on the developmental toxicity of the substituted (e.g., alkylated) congeners of PAHs. Available studies reported that methylated congeners of the 3-ring phenanthrene (PHE) induced higher embryotoxicity than PHE itself (Mu et al., 2014; Turcotte et al., 2011), and that methylated benz[a]anthracene (BaA) either increased or decreased the embryotoxicity as compared to the unsubstituted parent BaA (Lin et al., 2015).

Our recent study (Fang et al., 2022) evaluated the in vitro developmental toxicity potency of nine unsubstituted and methylated 4- and 5- ring PAHs using the zebrafish embryotoxicity test (ZET). The ZET data showed that addition of a monomethyl substituent could either increase or decrease the in vitro developmental toxicity of PAHs, depending on the location of the methyl-substituent on the aromatic ring of PAH tested. For example, 8-methyl-BaP (8-MeBaP) was more potent than its parent BaP to induce developmental retardation (at non-lethal concentrations) in zebrafish embryos (according to the calculated BMC20s, see Table 1 of Fang et al. 2022), while 7-methyl-BaP (7-MeBaP) tested negative for developmental toxicity in the ZET.

To date, the mode of action underlying the developmental toxicity of PAHs remains unclear, although some studies have suggested the involvement of the aryl hydrocarbon receptor (AhR) in mediating such effects (Goodale et al., 2013; Lammer et al., 2009). The AhR is a cytosolic ligand-activated transcription factor (Denison and Nagy, 2003). AhR activation can further

activate cytochrome P450 monooxygenases (CYP450) which could subsequently lead to the biotransformation of PAHs into their reactive metabolites (Hawliczek et al., 2012; Puga et al., 2005). The PAH-induced AhR activation has been suggested to contribute to the in vitro developmental toxicity of some individual PAHs (Hawliczek et al., 2012; Huang et al., 2012; Incardona et al., 2006) and PAH-containing petroleum substances (Kamelia et al., 2021, 2019a, 2019b, 2018). Besides the AhR, the developmental toxicity observed with some PAHs may also relate to their interaction with other nuclear hormone receptors (NHRs), such as the estrogen receptor-α (ER-α) and the retinoic acid receptor (RAR) (Barlow et al., 1999; Maqbool et al., 2016; Piersma et al., 2017; Van Lipzig et al., 2005). Both ER-α and RAR play an important role in vertebrate embryonic development, and disturbance of these receptors may lead to abnormalities in fetus (Bondesson et al., 2015; Lammer et al., 1985; Mark et al., 2009). Several unsubstituted PAHs, e.g., BaP and BaA, were reported to induce estrogenic and/or anti-estrogenic activities (Boonen et al., 2020; Kirsten C Fertuck et al., 2001; Gozgit et al., 2004; Tran et al., 1996; Van Lipzig et al., 2005). However, data on ER-α- and RAR-mediated activity of especially monomethylated PAHs are limited at present.

Thus, the present study aims to evaluate the AhR-, ER-α- and RAR-mediated activities of a series of unsubstituted and monomethylated PAHs, using reporter gene assays, all in relation to the developmental toxicity potency of these PAHs in the ZET. To that purpose, nine 4- and 5-ring PAHs (including: BaA and its methylated congeners: 4-methyl-BaA (4-MeBaA), 8-methyl-BaA (8-MeBaA), 9-methyl-BaA (9-MeBaA); BaP and its methylated congeners: 3-methyl-BaP (3-MeBaP), 7-MeBaP, 8-MeBaP; and dibenz[a,h]anthracene (DB[a,h]A)) were tested in the ER-α, RAR and AhR Chemical Activated LUciferase gene eXpression (CALUX) reporter gene assays. Moreover, some PAHs were selected and tested in the ZET in the absence or presence of specific receptor antagonists, to further investigate the role of these receptors in mediating PAH-induced developmental toxicity.

2. Materials and Methods

2.1 Test compound

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD, CAS No. 1746-01-6, analytical standard grade), 17β-estradiol (E2, CAS No. 50-28-2, purity ≥ 98%), fulvestrant (CAS No. 129453-61-8, purity ≥ 98%), retinoic acid (RA, CAS No. 302-79-4, purity ≥ 98%), AGN 193109 (CAS No. 171746-21-7, purity ≥ 98%), CH223191 (CAS No. 301326-22-7, purity ≥ 98%), 3,4-dichloroaniline (CAS No. 95–76-1, purity 98%), benzo[a]pyrene (BaP, CAS No. 50-32-8, purity ≥ 96%),

benz[a]anthracene (BaA, CAS No. 56-55-3, purity 99%), dibenz[a,h]anthracene (DB[a,h]A, CAS No. 53-70-3, analytical standard grade), 7-methyl-benzo[a]pyrene (7-MeBaP, CAS No. 63041-77-0, purity 98%), 8-methyl-benzo[a]pyrene (8-MeBaP, CAS No. 63041-76-9, purity ≥ 98%), 4-methyl-benz[a]anthracene (4-MeBaA, CAS No. 316-49-4, purity ≥ 98%), 8-methyl-benz[a]anthracene (8-MeBaA, CAS No. 2381-31-9, purity ≥ 98%), and 9-methyl-benz[a]anthracene (9-MeBaA, CAS No. 2381-16-0, purity ≥ 98%) were purchased from (Merck, The Netherlands). 3-Methyl-benzo[a]pyrene (CAS No. 16757-81-6, purity > 99%) was synthesized by the Biochemical Institute for Environmental Carcinogens (Grosshansdorf, Germany). All stocks and dilutions of test compounds were prepared in dimethyl sulfoxide (DMSO) (Merck, The Netherlands).

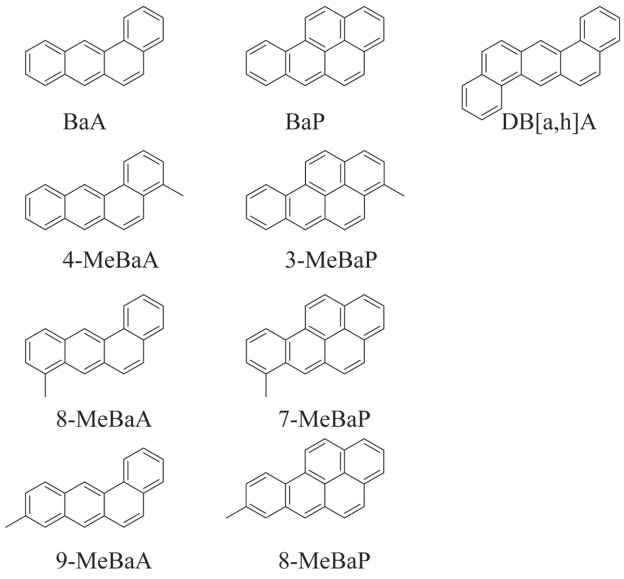

Figure 1. Structure of the nine unsubstituted and methylated PAHs tested in the study.

2.2 CALUX reporter gene assays

2.2.1 Cell line and cell culture conditions

Stably transfected human osteosarcoma cell lines (U2OS), expressing either ER-α (U2OS ER-α CALUX) or RAR (U2OS RAR CALUX) and a firefly luciferase reporter gene under the control of receptor specific responsive elements were kindly provided by BioDetection Systems (Amsterdam, The Netherlands). U2OS cytotox cell line (BioDetection Systems), stably expressing only a firefly luciferase reporter gene without any receptor specific responsive

element was used as a control for luciferase expression in the U2OS-based CALUX reporter gene assay. All the U2OS cell lines were maintained in T75 polystyrene cell culture flasks (Corning, The Netherlands) in Dulbecco's Modified Eagle Medium F-12 nutrient mixture (DMEM/F12; Gibco, Paisley, United Kingdom; catalog no. 31330-038). The DMEM/F12 medium was supplemented with 10% (v/v) fetal bovine serum (FBS; Capricorn, Germany; catalog no. FBS 12A), 0.5% non-essential amino acids (NEEA; Gibco, catalog no. 11140-035) and 200 μg/ml of geneticin G418 (Invitrogen, Breda).

The stably transfected rat hepatoma cell line (H4IIE.luc) was used for the AhR CALUX assay (Aarts et al., 1995). H4IIE.luc cells were cultured in T75 polystyrene cell culture flasks in Minimum Essential Medium (MEM) alpha medium (Gibco; catalog no. 2256-021), supplemented with 10% (v/v) FBS.

All cells were incubated at 37°C with 5% CO_2 in a humidified atmosphere and were routinely sub-cultured two to three times per week using 0.05% trypsin-EDTA (Gibco; catalog no. 15400-054) to detach the cells.

2.2.2 CALUX reporter gene assays

The principle of the CALUX assay relies on the ability of a test compound to bind and consequently activate or inhibit the transcription of the receptor target genes, which eventually results in different luciferase activities (Van der Linden et al., 2014; Windal et al., 2018).

The U2OS CALUX assays (ER-α, RAR, and cytotox) were performed essentially as described before by Kamelia et al. (2018). In brief, the U2OS cells were seeded in the 60-inner wells of 96-well white plates (Greiner Bio-one, Frickenhausen, Germany) at a density of 1×10^4 cells per well in 100 μl assay medium (DMEM/F-12 without phenol red (Gibco; catalog no. 21041-025), supplemented with 5% fetal bovine serum treated with dextran coated charcoal (DCC-FBS, Gibco; catalog no. 12676029) and 0.5% NEAA). The 36-outer wells of the same white 96-well plate were filled with 200 μl PBS to create an optimal humidity and to limit evaporation from the inner 60-wells. After 24 h incubation in a humidified atmosphere at 37°C with 5% CO_2, assay medium was removed using a vacuum pump and replaced with 100 μl assay medium per well. Then cells were incubated at the same condition for another 24 h. Afterwards , cells were exposed to exposure medium containing different concentrations of test compounds in triplicate for 24 h by adding 100 μl of exposure medium to each well. The exposure medium for the U2OS CALUX agonist assay was prepared by addition of 400 times concentrated stock

solutions of the test compounds (dissolved in DMSO) to the assay medium. The exposure medium for the U2OS CALUX antagonist assays was prepared by addition to assay medium of 800 times concentrated DMSO-stock solutions of reference-standard agonist compounds to reach a final concentration equal to their EC50 concentration (1×10^{-5} µM E2 for the ER-α antagonist assay, 2×10^{-1} µM RA for the RAR antagonist assay) and adding test compounds from 800 times concentrated DMSO-stock solutions.

For the AhR CALUX agonist assays, 100 µl of H4IIE.luc cells at a density of 3×10^{4} cells per well were seeded into the 60-inner wells of the 96-wells white plates, and the 36-outer wells of the same white 96-well plate were filled with 200 µl PBS. After 24 h incubation, cells were exposed to exposure medium containing different concentrations of test compounds in triplicate for either 6 h or 24 h. The exposure medium for the AhR CALUX assay was prepared by diluting 400 times concentrated DMSO-stock solutions of the test compounds with pre-conditioned medium, which is the growth medium in which cells were previously grown for 16 to 24 h. The use of the pre-conditioned medium for cell exposure is to avoid a high background luciferase signal induced by tryptophan products present in the fresh medium, which can induce AhR activity and thereby cause false-positive results (Hamers et al., 2000; Vrabie et al., 2009).

After 6 h (AhR CALUX assay) or 24 h (AhR CALUX and U2OS CALUX assays) exposure, cells were washed with 100 µl ½ PBS (PBS : nano-pure water = 1 : 1) and lysed with 30 µl low salt buffer (10 mM Tris (Merck), 2 mM dithiothreitol (DTT, Merck), and 2 mM 1, 2-diaminocyclohexanete triacetic acid monohydrate (Merck); pH 7.8). Afterwards, plates were placed on ice for at least 15 minutes and frozen at -80°C for at least 2 h before measurement.

For the luminescence measurement, plates were thawed at room temperature for 1 h and then shaken for 5 min on a plate shaker. Luciferase activity was measured using a luminometer (Glomax-Multi Detection System, Promega, California) after the addition to each well of 100 µl flash-mix solution. The flash-mix solution consisted of an aqueous solution of (final concentrations) 20 mM tricine (Merck), 1.07 mM $(MgCO_3)_4Mg(OH)_2\cdot5H_2O$ (Merck), 2.67 mM magnesium sulfate ($MgSO_4$, Merck), 0.1 mM ethylenedinitrilotetraacetic acid disodium salt dihydrate (Titriplex III; Merck), 2 mM DTT, 0.47 mM D-luciferin (Synchem UG & Co. KG, Felsberg, Germany), and 5 mM adenosine-5-triphosphate (Duchefa Biochemie bv, Haarlem, the Netherlands).

Three independent experiments were conducted for each test compound in each CALUX assay. Full concentration-response curves of the reference compounds for both agonist and antagonist assays were included in each independent experiment. The final concentration of DMSO was kept at 0.25% (v/v). All test compounds were tested at concentrations up to 50 μM in the U2OS CALUX agonist assays and up to 25 μM in the U2OS CALUX antagonist assay, except for DB[a,h]A. DB[a,h]A was tested at concentrations up to 25 μM in the agonist assay and 15 μM in the antagonist assay due to its solubility limitation. In the AhR CALUX assay, the highest final concentration of all test compounds was 5 μM because the highest AhR induction was already obtained upon exposure to test compounds at this concentration. The following final concentration ranges were used for the reference compounds of the various CALUX assays, TCDD (2.5×10^{-7} - 2.5×10^{-4} μM) for the AhR agonist assay, E2 (1×10^{-7} - 5×10^{-4} μM) for the ER-α agonist assay, fulvestrant (5×10^{-9} - 5×10^{-4} μM) for the ER-α antagonist assay, retinoic acid (5×10^{-4} - 1.5×10^{2} μM) for the RAR agonist assay, and AGN 193109 (1×10^{-4} - 3×10^{1} μM) for the RAR antagonist assay.

2.2.3 Cell viability test

In addition to use of the U2OS cytotox CALUX assay, the cell viability following exposure to test compounds in all CALUX assays was also determined using the WST-1 assay. Briefly, after the same cell seeding and exposure steps outlined in section 2.2.2, 5% (v/v) WST-1 reagent (Roche, Mannheim, Germany) was added in each well of the inner 60 wells of the plates. The plates were further incubated for 3 h at 37°C under a 5% CO_2 atmosphere. After this incubation, the absorbances were measured at 440 and 620 nm using a SpectraMax iD3 (Molecular Devices, San Jose, USA). Three independent experiments were performed for each test compound.

2.3 Co-exposure of zebrafish embryos in the ZET to the selected PAHs and an AhR antagonist or an ER-α antagonist

To further investigate the role of the AhR or ER-α in mediating the PAH-induced developmental toxicity, zebrafish embryos were exposed to the selected PAHs in the presence or absence of an ER-α antagonist (fulvestrant) or an AhR antagonist (CH223191), respectively. The role of RAR in PAH-induced developmental toxicity was not evaluated as none of the PAHs tested induced any agonist or antagonist activity in the RAR CALUX assay (see Result section).

The concentration of the AhR antagonist CH223191 used in the ZET was 1 μM, which was the highest concentration of CH223191 that did not affect the normal development of zebrafish embryos until 96 hpf (see Supporting Information 1). CH223191 was reported to antagonize the AhR without detectable AhR agonist-like activity or estrogenic activity (Kim et al., 2006). Other studies also report that 0.5 μM CH223191 could sufficiently antagonize the AhR-mediated developmental toxicity in 5D zebrafish embryos (Gerlach et al., 2014; McGee et al., 2013).

The concentration of the ER-α antagonist fulvestrant used in the present study was 3 μM, which is considerably higher than the IC50 (0.8 nM) of fulvestrant to antagonize the in vitro ER-α-mediated activity reported previously (Weir et al., 2016). Moreover, a recent study (Adam et al., 2021) showed that 3 μM fulvestrant could sufficiently antagonize the ER-α-mediated developmental toxicity of the estrogenic compound diethylstilboestrol in the ZET.

The ZET was performed as described previously (Kamelia et al., 2019). In short, fertilized eggs of the wild-type zebrafish (*Danio rerio*) AB line were purchased from the research facility Carus, Wageningen University and Research, The Netherlands. The ZET was initiated at 4-5 hours post fertilization (hpf) and terminated at 96 hpf. The exposure was performed in 24-well plates (Greiner bio-one). Twenty wells of the 24-well plates were used for exposure to two concentrations of the test compounds (ten wells/concentration) and the other four wells were used for the internal plate control. The egg water was prepared by adding 10 ml 100-times egg water stock solution (3 g sea salt (Tropic marine, Wartenberg, Germany) in 500 ml demineralized water) to 990 ml demineralized water. The ZET was performed in the absence or presence of the AhR antagonist CH223191 (1 μM) or the ER-α antagonist fulvestrant (3 μM). Exposure medium in the absence of CH223191 or fulvestrant was prepared by adding to egg water the 400-times concentrated DMSO-stock solution of the selected PAHs. Exposure medium for the presence of fulvestrant or CH223191 was prepared by adding to egg water an 800-times concentrated DMSO-stock solution of the selected PAHs and an 800-times concentrated DMSO-stock solution of CH223191 or fulvestrant. The final solvent (DMSO) concentration was kept at 0.25% (v/v). The exposure medium was transferred (2 ml/well) into ten wells of the 24-well plate, and for the internal plate control, 2 ml egg water was added into each of the four remaining wells. Then, one zebrafish embryo was transferred to each well of the 24-well plate. The plates were sealed with self-adhesive film covers (Greiner bio-one) to prevent evaporation and were incubated at 26 °C with a photo period of 14 h light:10 h dark.

For the co-exposure studies in the ZET, the selected PAHs were tested at a concentration of 15 μM and 50 μM, except for 3-MeBaP and DB[a,h]A which were tested at only 15 μM due to solubility limitations. Three types of controls were included in each independent experiment: negative control (egg water), solvent control (0.25% (v/v) DMSO in egg water) and positive control (4 μg/ml 3,4-dichloroaniline). Embryos were scored daily until 96 hpf for developmental abnormalities and embryo lethality using an inverted microscope, based on the extended general morphological scoring (extended-GMS) system described by Beekhuijzen et al., (2015) (See Supporting Information 2). Any deviation from normal development of zebrafish embryos until 96 hpf results in a decreased extended-GMS score, which corresponds to a certain extent of developmental retardation. Each independent ZET was considered valid only if the following criteria were met (at 96 hpf): (1) ≤1 dead embryos (out of 4) in the internal plate control of the exposed-plate, (2) ≤1 dead embryos (out of 10) in the negative control plate, (3) ≤1 dead embryos (out of 10) in the solvent control plate, (4) ≤7 alive embryos (out of 10) in the positive control plate. Three independent experiments were performed for each test compound.

2.4 Co-exposure of H4IIE.luc cells to the selected PAHs and an AhR antagonist in the AhR CALUX assay

The effect of the AhR antagonist CH223191 to antagonize the PAH-induced AhR activation in the AhR CALUX assay was also evaluated. To that purpose, in the AhR CALUX assay, AhR H4IIE.luc cells were exposed to each test compound at its EC50s for either 6 h or 24 h in the absence or presence of 100 μM CH223191. The luciferase induction was subsequently quantified. The EC50 reflects the concentration of test compounds that induce 50% AhR-mediated activity in the AhR CALUX assay and was derived from the obtained concentration-response curve of each PAH tested in the AhR CALUX assay (see Table 1). In addition, cell viability after 24 h exposure to each test compound in the co-exposure experiments (with or without CH223191) was evaluated using the WST-1 assay. Three types of controls were included in each independent experiment: solvent control (0.25 % v/v DMSO), CH223191 only (100 μM), and positive control TCDD (0.25 nM) Three independent experiments were conducted for each test compound in this co-exposure experiment.

2.5 Estimating free concentrations of the nine PAHs tested in the CALUX assays and ZET

Free (unbound) exposure medium concentrations of PAHs in the CALUX assays (AhR, ER-α and RAR) and the ZET were estimated using an in vitro chemical distribution model (Comenges

et al., 2017; Groothuis et al., 2015; Kramer, 2010; Kramer et al., 2022 (manuscript in preparation), 2012). This model was previously used to estimate the free in vitro concentration of the 3-ring PAH PHE (Kramer et al. 2012). In this model, physiochemical properties of the chemical substances, i.e., the octanol-water partition coefficient (K_{ow}) and Henry's law constant (K_H), in combination with test system-specific information (e.g., well-plates dimensions, exposure medium volume, cell density, cell lipid content, serum concentration, exposure temperature) were used as the input parameters to predict the in vitro free concentrations (Supporting Information 8). The output of this in vitro chemical distribution model is: 1) a modeled free fraction of the compound in the assay medium, 2) a modeled fraction bound to serum constituents in the assay medium, 3) a modeled fraction of the compound in the cellular compartment (CALUX cells or zebrafish embryos in our case), 4) a modeled fraction of the compound bound to plastic, and 5) a modelled fraction in the headspace (due to evaporation). The modelled results are presented in Supporting Information 9-12. Moreover, concentration-response curves based on the predicted free fraction in medium were made for the AhR, ER-α and RAR CALUX assays (Supporting Information 13-15).

2.6 Data analysis

The results of luminescence measurements were expressed as relative light units (RLUs). First of all, the fold induction was calculated by dividing the mean value of RLUs from the exposed wells by the mean value of RLUs from the solvent control (0.25% v/v DMSO). For the agonist CALUX assays (AhR agonist, ER-α agonist and RAR agonist CALUX assays), the percentage of luciferase induction was further calculated by setting the maximum fold induction of the corresponding positive controls (TCDD, E2 and RA, respectively) at 100%. For the antagonist CALUX assay (ER-α antagonist and RAR antagonist CALUX assays), the percentage of luciferase induction was calculated by setting the fold induction at the EC50 concentration of the corresponding positive controls (E2 and RA, respectively) at 50%. Results are expressed as mean ± standard error of mean (SEM), obtained from three independent experiments. Figures of concentration-response curves upon exposure to the test compounds were made using GraphPad Prism 5.0 (California, USA). Data were analyzed using non-linear regression and fitted to a sigmoid concentration-response curve with three parameters. EC50 values (effective concentration inducing 50% response) with 95% confidence intervals calculated using Graphpad.

The ZET results were expressed as % of the total extended-GMS score and embryo survival at 96 hpf. Results are presented as mean ± SEM, obtained from three independent experiments. Data were fitted to bar graphs using GraphPad Prism 5.0.

An unpaired Student's *t*-test was performed using GraphPad Prism 5.0 to evaluate the significance ($p < 0.05$) between the % of response in the absence or presence of CH223191 (AhR antagonist) or fulvestrant (ER-α antagonist) in the ZET, and between the response of AhR-mediated activity in the absence or presence of CH223191 in the AhR CALUX assay.

Data from the WST-1 assay were processed by firstly subtracting the absorbance values measured at 620 nm (background) from the values measured at 440 nm. Subtracted data were then expressed as the percentage of cell viability relative to the solvent control (0.25% v/v DMSO, cell viability set as 100%). Results are presented as mean ± SEM, obtained from three independent experiments.

3. Results

It should be noted that the concentrations of test compounds indicated in the present manuscript, e.g., in graphs and tables, are the nominal concentrations. Results of the modelled free concentration in medium for the AhR, ER-α, and RAR CALUX assays, and the ZET are presented in Supporting Information 9-12. Concentration-response curves based on the predicted free fraction in medium for the abovementioned CALUX assays can be found in the Supporting Information 13-15.

3.1 Effects of test compounds on the AhR

In the AhR CALUX agonist assay, exposure time windows of 6 h and 24 h were applied to assess the transient or persistent nature of the AhR induction by the test compounds, respectively. The full concentration-response curves of the AhR-mediated activity for all compounds are shown in Figure 2. Table 1 presents the EC50 values derived from the obtained concentration-response curves, and a qualitive overview of the AhR CALUX results (presented by the "+/-" symbols). As shown in Figure 2, all PAHs tested were able to induce concentration-dependent agonist activities in the AhR CALUX assay following both 6 h and 24 h exposure. For all PAHs, the EC50 values tested at 6 h exposure were lower than those at 24 h exposure which indicates a transient nature of the AhR activation. Based on the EC50 values, upon both 6 and 24 h exposure, 9-MeBaA and 8-MeBaP were the most potent 4-ring and 5-ring PAH tested, respectively. Moreover, all the methylated congeners appear to be more potent than their

respective parent compounds BaA and BaP following both 6 h and 24 h exposure. The cytotoxicity results (Supporting Information 3) showed that no cytotoxicity was observed in the H4IIE.luc cells when exposed to the same concentrations of test compounds as tested in the AhR CALUX.

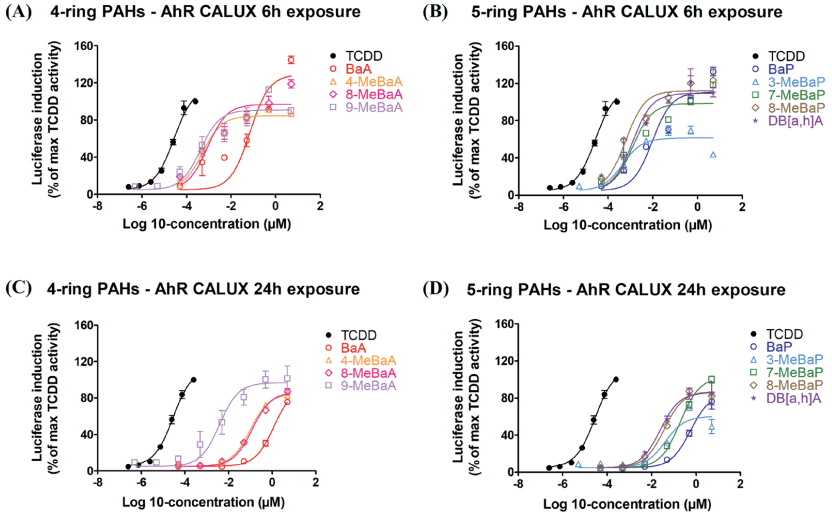

Figure 2. Concentration-dependent effects of TCDD and the nine unsubstituted and methylated PAHs tested in the AhR CALUX agonist assay following 6 h (A, B) or 24 h (C, D) exposure. The highest luciferase induction of the positive control TCDD was set at 100%. Results represent data from three independent experiments and are presented as mean ± SEM.

3.2 Effects of test compounds on the ER-α

In the U2OS CALUX assay, the direct interaction between test compounds and the firefly luciferase enzyme might result in stabilization of the luciferase enzyme which lead to increased luciferase activity without an underlying increased gene expression (Sotoca et al., 2010). Test compounds might also un-specifically inhibit the luciferase induction in the U2OS cells without exerting cytotoxicity. Thus, the U2OS-cytotox CALUX assay (see results in Supporting Information 4) was performed to check and correct for the non-specific induction or inhibition of luciferase activity by each test compound. Results obtained in the ER-α/RAR CALUX assay were then corrected for this non-specific activity by dividing the luciferase induction activity obtained in the U2OS ER-α/RAR assay by the corresponding results obtained in the U2OS-cytotox CALUX assay, for each test compound after which the corrected luciferase induction was calculated. The corrected data are presented in Figure 3 and the uncorrected data are

presented in Supporting Information 5. In addition, the cell viability results obtained in the WST-1 assay (Supporting Information 3) showed that no cytotoxicity was observed in the U2OS cells when exposing to the same concentrations of test compounds as the U2OS CALUX assays. As shown in Figures 3A and 3B, six PAHs (i.e., BaA, 4-MeBaA, 8-MeBaA, 9-MeBaA, BaP and 3-MeBaP) were able to induce estrogenic activity in the ER-α CALUX agonist assay, ranging from 10% (induced by BaP) to 75% (induced by 8-MeBaA) of the maximum E2-induced response. Among these six PAHs, only BaA and 8-MeBaA induced more than 50% luciferase induction, while the other four PAHs induced less than 50% luciferase induction which indicates a rather weak ER-α agonist activity at the concentrations tested. The EC50 values of BaA and 8-MeBaA in the ER-α CALUX agonist assay were 27.00 µM and 11.99 µM, respectively (Table 1). Three 5-ring PAHs (i.e., 7-MeBaP, 8-MeBaP and DB[a,h]A) tested negative in the ER-α CALUX agonist assay. In the ER-α CALUX antagonist assay, none of the PAHs was able to induce substantial anti-estrogenic activity (Figures 3C and 3D).

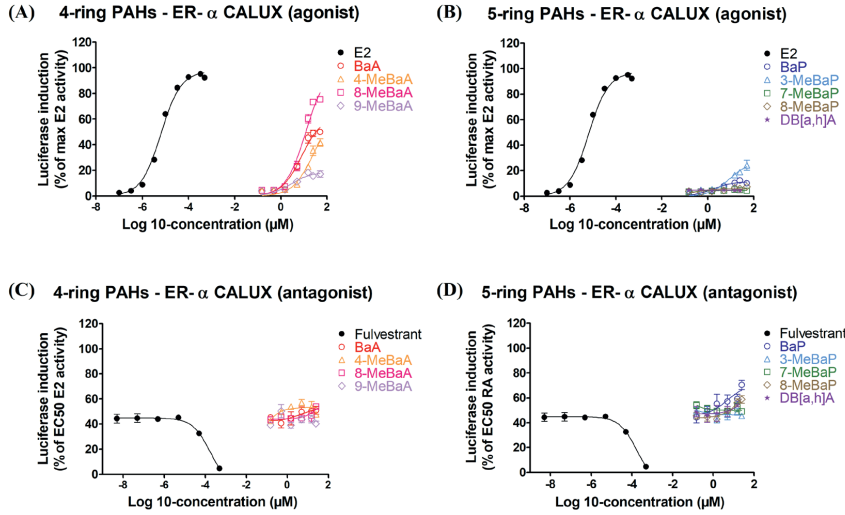

Figure 3. (A-D) Concentration-dependent effects of E2, fulvestrant and the nine unsubstituted and methylated PAHs tested in the ER-α CALUX (agonist/antagonist) assay. The highest luciferase induction of the positive control E2 was set at 100% for the agonist assay, and the luciferase induction at the EC50 of E2 was set at 50% for the antagonist assay. Results represent data from three independent experiments and are presented as mean ± SEM.

3.3 Effects of test compounds on the RAR

The luciferase induction obtained in the RAR CALUX assay (for both agonist and antagonist activity) corrected using the respective results from the U2OS-cytotox CALUX assay is presented in Figure 4 and the uncorrected data are presented in the Supporting Information 5. It can be seen from Figure 4 that none of the PAHs tested was able to induce RAR agonistic or antagonistic activity.

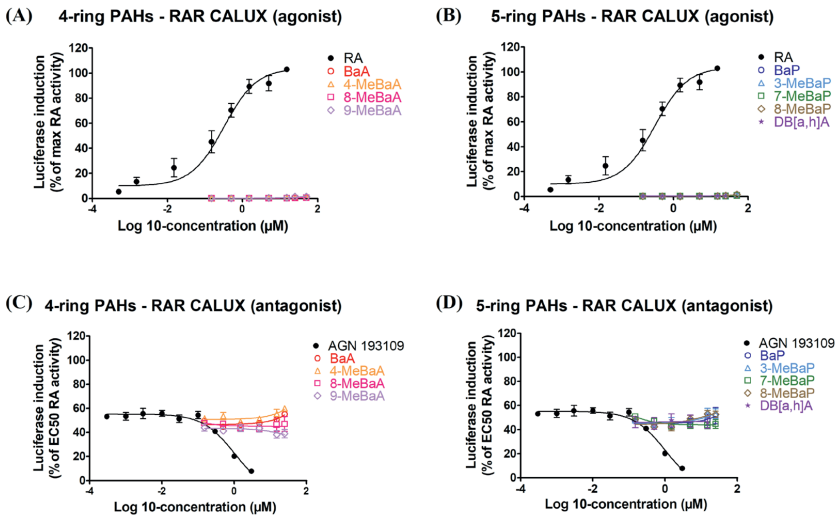

Figure 4. (A-D) Concentration-dependent effects of RA, AGN 193109 and the nine PAHs tested in the RAR CALUX (agonist/antagonist) assay. The highest luciferase induction of the agonist positive control RA was set at 100% for the agonist assay, and the luciferase induction at the EC50 of RA was set as 50% for the antagonist assay. Results represent data from three independent experiments and are presented as mean ± SEM.

3.4 Counteracting effects of PAH-induced developmental toxicity in the ZET by the AhR antagonist (CH223191)

As depicted in Figures 5 and 6, all PAHs tested, except 7-Me-BaP, induced developmental retardations in zebrafish embryos, shown by reduction of extended-GMS score and embryo survival in the ZET at 96 hpf. The current results are consistent with the ZET results we reported previously (Fang et al., 2022). As shown in Figure 5A, the extended-GMS score upon exposure to 15 µM BaA was not significantly affected with by the addition of CH223191, however, the severity of several developmental retardations, e.g., pericardial edema, yolk sac edema, and deformed body shape, induced by 15 µM BaA was slightly reduced in the presence of

CH223191 (Figure 6A). Furthermore, CH223191 fully counteracted the embryo lethality induced by 50 μM BaA (Figure 5A) but had no effect on the observed developmental retardations (Figure 6A). For 4-MeBaA (Figure 5B), the addition of CH223191 significantly counteracted only the embryo lethality induced at the concentration of 15 μM but not of 50 μM 4-MeBaA. Similar to BaA, even though the addition of CH223191 did not result in a higher extended-GMS score following exposure to 15 μM 8-MeBaA in the ZET (Figure 5C), the severity of pericardial edema, yolk sac edema, deformed body shape, and deformed head and jaw were reduced (Figure 6C). The addition of CH223191 successfully counteracted the effect of unhatched embryos and reduced the severity of yolk sac edema induced by 15 μM and 50 μM 9-MeBaA (Figured 5D, 6D).

Both embryo lethality and developmental retardations of the parent compound BaP were significantly counteracted by CH223191 as shown by an increase in the extended-GMS score and the survival rates, however, a slightly enlarged yolk sac could still be observed in the zebrafish embryos when co-exposed with 50 μM BaP and CH223191 (Figures 5E, 6E). For 3-MeBaP, the addition of CH223191 counteracted all developmental retardations induced by this test compound. 7-MeBaP tested negative in the ZET, with or without the presence of an AhR antagonist. Of the nine tested PAHs, 8-MeBaP was the most potent one to induce developmental retardations in zebrafish embryos (Fang et al., 2022), and as shown in Figures 5H & 6H, its developmental toxicity in the ZET could not be counteracted by the addition of 1 μM CH223191. Finally, the observed developmental toxicity induced by 15 μM DB[a,h]A in the ZET was successfully counteracted by the addition of CH223191 (Figures 5I, 6I).

The AhR antagonist CH223191 used in the present study was previously reported to be a selective AhR antagonist ligand for TCDD (Zhao et al., 2010). To ensure that the antagonist can be used to counteract PAH mediated effects on the AhR, the antagonizing effect of CH223191 on the observed PAH-induced AhR activity in the AhR CALUX assay was investigated by co-exposing the H4IIE.luc cells with each PAH at their EC50s, in the absence or presence of 100 μM CH223191. Results obtained (Figure 7) show that CH223191 successfully counteracted the AhR-mediated gene expression induced by each PAH tested in the AhR CALUX assay after both 6 h and 24 h exposure, without inducing cytotoxicity (Supporting Information 3). This indicates that CH223191 is not only able to inhibit TCDD-induced AhR-mediated activities, but also PAH-induced AhR-mediated gene expression.

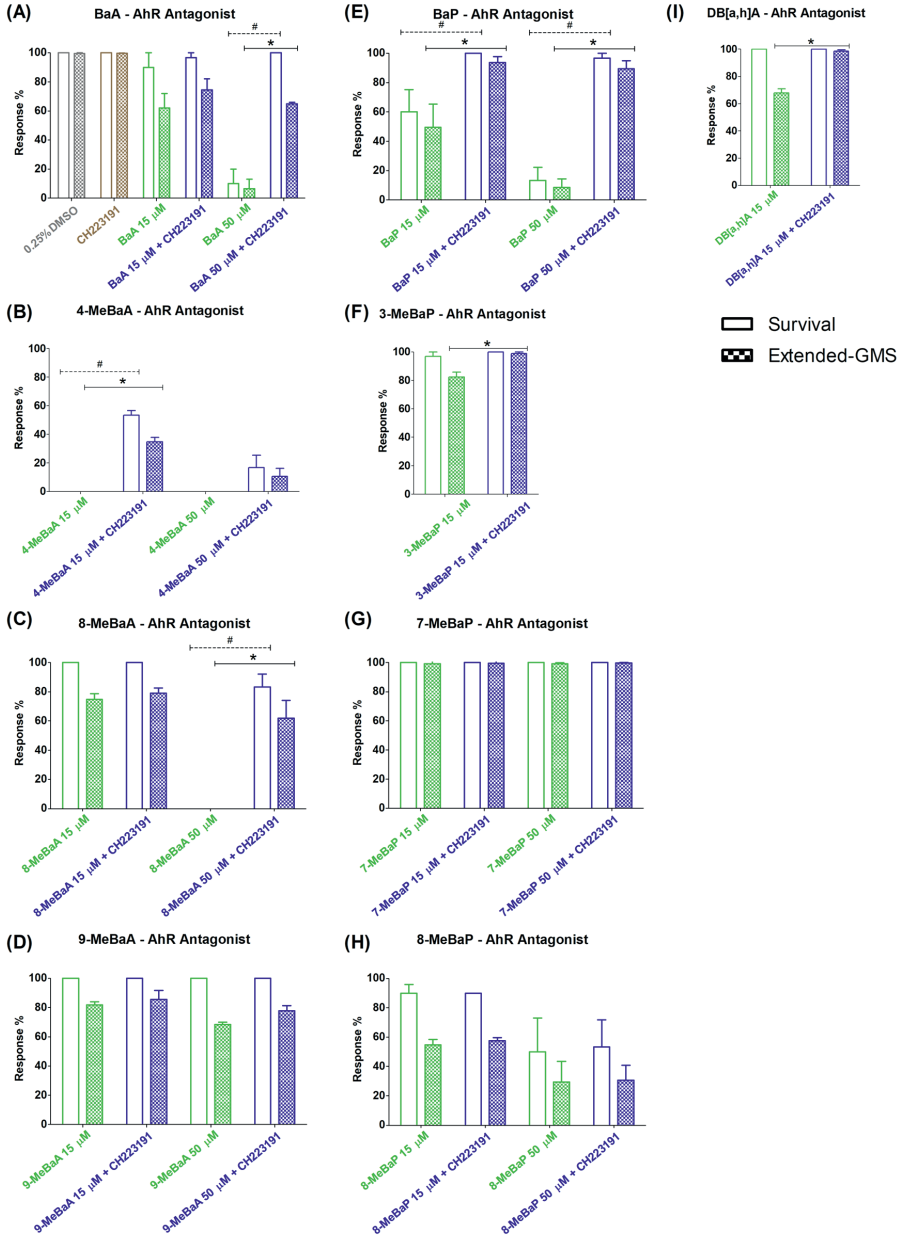

Figure 5. (A-I) Effects of the nine PAHs on embryo survival (open bars) and extended-GMS (shaded bars) with the absence (green bars) or presence (blue bars) of CH223191 (an AhR antagonist). 0.25% DMSO (grey bars) and only CH223191 (brown bars) were used as controls for the experiment. Results represent data from three independent experiments and are

presented as mean ± SEM. The significant differences ($p < 0.05$) in embryo survival and extended-GMS in the absence or presence of CH223191 are represented by hashtag (#) and asterisk (*) symbols, respectively.

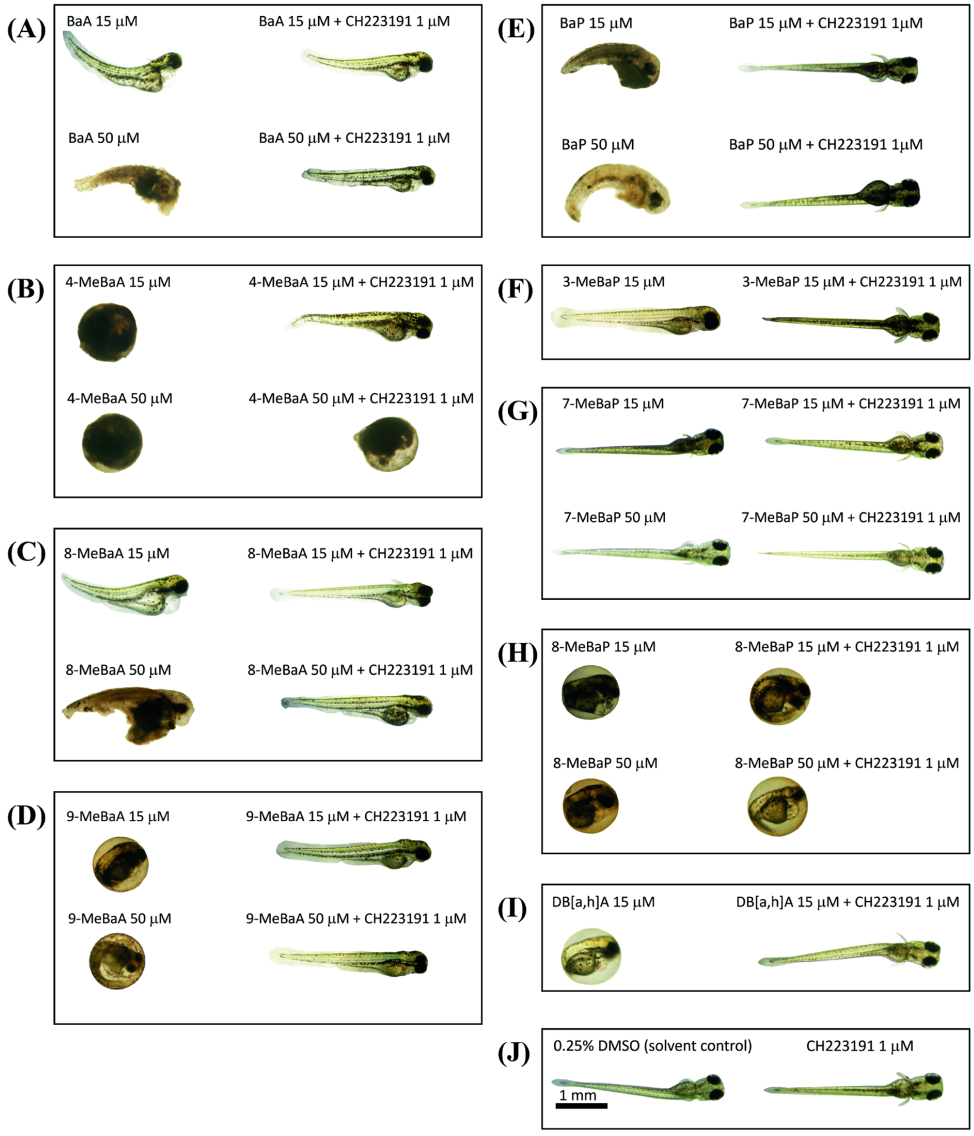

Figure 6. **(A-I)** Zebrafish embryos morphology at 96 hpf upon exposure to nine PAHs under study with and without CH223191 at 96 hpf. **(J)** Representative images of zebrafish embryos exposed to 0.25% DMSO (solvent control) and 1 µM CH223191 at 96 hpf. Scale bar = 1 mm.

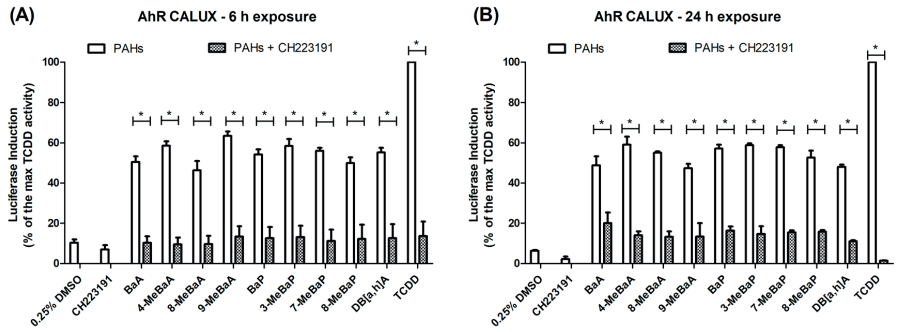

Figure 7. Counteracting effect of CH223191 on the AhR-mediated gene expression by the nine PAHs following **(A)** 6 h or **(B)** 24 h exposure in the AhR CALUX assay. 0.25% v/v DMSO, CH223191 only (100 μM) and TCDD (0.25 nM) were used as controls for the experiment. Results represent data from three independent experiments and are presented as mean ± SEM. The significant difference ($p < 0.05$) in response (i.e., luciferase induction), in the absence or presence of CH223191, are represented by asterisk (*) symbols.

3.5 Counteracting effects of PAH-induced developmental toxicity in the ZET by the ER-α antagonist (fulvestrant)

PAHs under study that showed substantial estrogenic activities (≥50% response of max E2 activity; Figure 3A) in the ERα CALUX assay, i.e., BaA and 8-MeBaA, were tested in the ZET, in the absence or presence of 3 μM of the ER-α antagonist fulvestrant. As shown in Figure 8, co-exposure of zebrafish embryos with fulvestrant did not counteract the developmental retardations induced by 15 μM of BaA, but fully counteracted the embryo lethality induced by 50 μM BaA at 96 hpf although most developmental retardations observed e.g., pericardial and yolk sac edemas were still present or not affected by the addition of fulvestrant (Figure 9A). For 8-MeBaA, at 96 hpf fulvestrant significantly counteracted the developmental retardations induced by 15 μM 8-MeBaA (e.g., reduced severity of pericardial and yolk sac edemas), but did not counteract the embryo lethality induced by 50 μM BaA (Figures 8 and 9B).

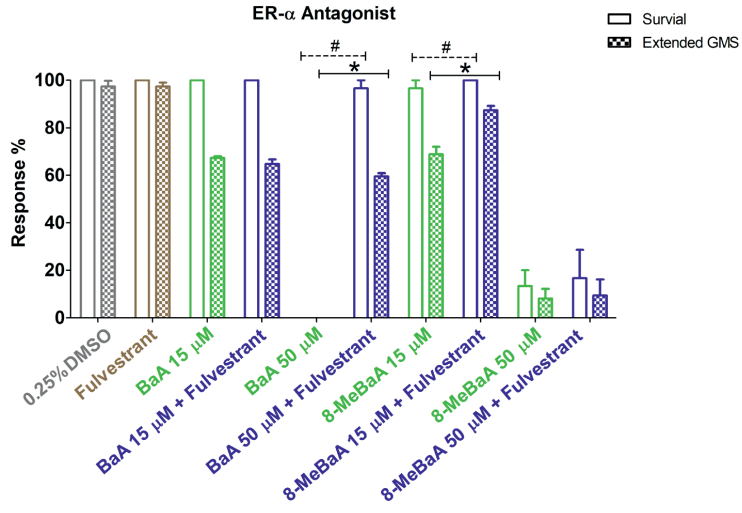

Figure 8. Effects of BaA and 8-MeBaA on embryo survival (open bars) and extended-GMS (shaded bars) with the absence (green bars) or presence (blue bars) of fulvestrant (a potent ER-α antagonist). 0.25% DMSO (grey bars) and fulvestrant (brown bars) were used as controls for the experiment. Results represent data from three independent experiments and are presented as mean ± SEM. The significant differences ($p < 0.05$) in embryo survival and extended-GMS in the absence or presence of fulvestrant are represented by hashtag (#) and asterisk (*) symbols, respectively.

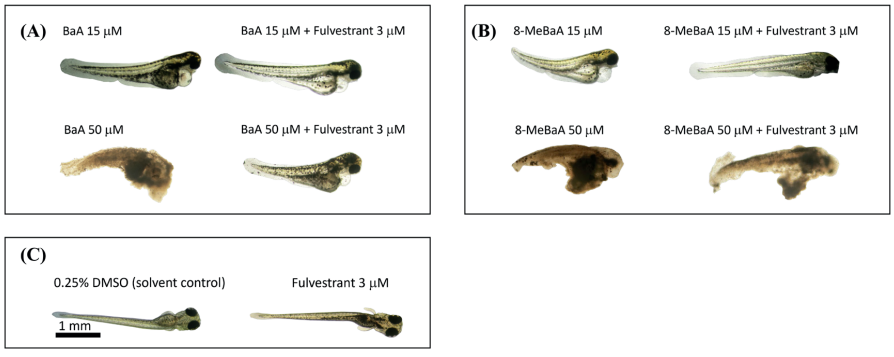

Figure 9. (A-B) Zebrafish embryo morphology at 96 hpf upon exposure to BaA and 8-MeBaA with the absence or presence of fulvestrant. **(C)** Representative images of zebrafish embryos exposed to 0.25% (solvent control) and 3 μM fulvestrant at 96 hpf. Scale bar = 1 mm.

Table 1. Overview of the AhR and ER-α mediated activities of the nine unsubstituted and methylated PAHs in the CALUX reporter gene assays under study, and their in vitro developmental toxicity in the ZET (Fang et al., 2022)

Test compound	AhR CALUX agonist assay (6h exposure)		AhR CALUX agonist assay (24h exposure)		ER -α agonist CALUX assay		ZET [a]
	EC50 (μM)	95% confidence intervals	EC50 (μM)	95% confidence intervals	EC50 (μM)	95% confidence intervals	BMC20 (μM)
BaA	0.013 **[+++]**	0.0037 - 0.047	1.27 **[++]**	0.98 - 1.63	27.00 **[+]**	21.52 - 33.88	5.29 **[++]**
4-MeBaA	0.00081 **[+++]**	0.00041 - 0.0016	0.14 **[+++]**	0.096 - 0.21	NA **[-]**		5.34 **[++]**
8-MeBaA	0.00080 **[+++]**	0.00038 - 0.0017	0.17 **[+++]**	0.12 - 0.26	11.99 **[+]**	8.080 - 17.85	4.81 **[++]**
9-MeBaA	0.00051 **[+++]**	0.00025 - 0.0010	0.0036 **[+++]**	0.0017 - 0.0076	NA **[-]**		15.7 **[+]**
BaP	0.0046 **[+++]**	0.0020 - 0.010	0.78 **[+++]**	0.53 - 1.14	NA **[-]**		4.31 **[++]**
3-MeBaP	0.0030 **[+++]**	0.00085 - 0.011	0.20 **[+++]**	0.076 - 0.50	NA **[-]**		15.1 **[+]**
7-MeBaP	0.0010 **[+++]**	0.000523 - 0.0020	0.15 **[+++]**	0.11 - 0.19	NA **[-]**		NA **[-]**
8-MeBaP	0.00035 **[+++]**	0.00016 - 0.00075	0.048 **[+++]**	0.032 - 0.071	NA **[-]**		0.38 **[+++]**
DB[a,h]A	0.00070 **[+++]**	0.00030 - 0.0016	0.035 **[+++]**	0.020 - 0.060	NA **[-]**		6.71 **[++]**

Note: NA (not applicable) means the compound induced less than 50% luciferase induction in the AhR or ER-α agonist CALUX assays, or less than 20% reduction of the extended-GMS in the ZET. The EC50 of the AhR and ER-α agonist CALUX assays, and the in vitro developmental toxicity in the ZET (expressed as BMC20) were grouped as follows: + (10 μM

< EC50/BMC20 < 50 μM), ++ (1 μM < EC50/BMC20 < 10 μM), +++ (EC50/BMC20 < 1 μM); the negative results were presented by the '-' symbol. [a] The BMC20 value of each test compound in the ZET was taken from Fang et al. (2022).

4. Discussion

The present study evaluated the AhR, ER-α and RAR-mediated activities of a series of 4- and 5-ring unsubstituted and monomethylated PAHs using CALUX reporter gene assays, and the potential role of these receptors in mediating the observed PAH-induced developmental toxicity in the ZET. The results demonstrate that all tested PAHs induced a strong AhR agonist activity in the AhR CALUX assay; no (ant)agonist activity in the RAR CALUX assay; no anti-estrogenic activity in the ER-α antagonist CALUX assays; and only two out of nine PAHs tested, i.e., BaA and 8-MeBaA, were able to induce estrogenic activity (≥50% of E2 max activity) in the ER-α agonist CALUX assay. It is worth mentioning that in the AhR CALUX assay, all monomethylated 4- and 5-ring PAHs tested induced stronger AhR-mediated activities compared to their respective unsubstituted parent PAHs. The role of AhR and ER-α in PAH-induced developmental toxicity was further investigated by co-exposing zebrafish embryos to the selected PAHs and an AhR antagonist (CH223191) or an ER-α antagonist (fulvestrant). The co-exposure to PAHs with CH223191 reduced the PAH-induced developmental toxicity in the ZET to various degrees, except for 8-MeBaP for which the developmental toxicity was not significantly counteracted by CH223191. Moreover, the observed embryo lethality and developmental retardations induced by the estrogenic PAHs i.e., BaA and 8-MeBaA, could be counteracted to a certain extent by the addition of fulvestrant in the ZET. These findings suggested that both ER-α and/or AhR may partially be involved in mediating the PAH-induced developmental toxicity observed in the ZET.

All tested PAHs induced AhR-mediated activities after both 6 h and 24 h exposure with EC50 values being substantially lower after 6 h of exposure than after 24 h of exposure, indicating a transient AhR activation. A similar transient AhR activation was observed previously for individual PAHs (Machala et al., 2001) and PAH-containing substances (Kamelia et al., 2021). The transient AhR activation is considered to be a protective adaptive response to many ligands, including PAHs, whereas persistent AhR induction may affect normal biological processes including embryonic development in vertebrates (Hankinson, 1995; Larigot et al., 2018; Puga et al., 2002). Additionally, our data is in line with the data reported in published studies using yeast, rat, mice or human cell lines (Boonen et al., 2020; Lam et al., 2018; Machala et al., 2001;

Marvanová et al., 2008; Sun et al., 2014; Trilecová et al., 2011; Vondráček et al., 2017), where monomethylated PAHs were shown to be more potent AhR inducers than their parent PAHs (see an overview of the reported receptor-mediated activities induced by unsubstituted and alkylated PAHs in Supporting Information 6). This indicates that the addition of a methyl substituent on the aromatic ring of PAHs could increase the AhR-mediated activity of these substances compared to their parent PAHs. However, such an observation does not always result in an increased in vitro developmental toxicity potency of these substances. Our previous study (Fang et al., 2022) showed that the addition of a methyl substituent on the aromatic ring of a PAH, depending on its position, could either increase or decrease the developmental toxicity of the corresponding PAHs. Taken together, the AhR activation by PAHs, as quantified in the AhR CALUX assay, should not directly be translated into their developmental toxicity potency because the AhR reporter gene assay is merely a cell-based screening assay that lacks the physiological feedback mechanisms and metabolism.

Developmental retardations, including yolk sac edema, pericardial edema and delayed development such as unhatched embryos, were noted following exposure to the (un)substituted PAHs under study. These manifestations are consistent with the typical AhR mediated developmental effects observed in zebrafish embryos upon exposure to some PAHs and/or PAH-containing substances (Billiard et al., 1999; Fang et al., 2022; Goodale et al., 2013; Kamelia et al., 2019a; Knecht et al., 2017; Puga et al., 2005; Wincent et al., 2015). To further evaluate the role of the AhR in mediating the PAH-induced developmental toxicity in the ZET, co-exposure experiments were conducted where zebrafish embryos were exposed to each individual PAH with or without the AhR antagonist, CH223191. CH223191 has been reported to antagonize the TCDD-induced AhR-mediated activity without AhR agonist activity or estrogenic activity in HepG2 human hepatoma cells and MCF-7 human breast cancer cells (Kim et al., 2006). However, another study reported that CH223191 had high selectivity to the TCDD related AhR ligands and might not inhibit the AhR-mediated activity induced by PAHs (Zhao et al., 2010). In this previous study the AhR-dependent reporter gene expression by TCDD at 1 nM was almost fully inhibited by CH223191 at 10 mM, while the activity of PAHs at equally effective concentrations of 0.1 to 1 mM was not inhibited. This apparent selectivity for TCDD may be due to the experimental conditions chosen with an 10,000 fold excess of CH223191 to TCDD and an only 10 to 100 fold excess of CH223191 to the PAHs. Thus, this apparent selectivity for TCDD may have been due to the higher ratio between inhibitor and agonist, while the lower ratio between inhibitor and agonist in the case of PAHs hampers effective inhibition.

Thus, in the present study CH223191 was tested at 100 mM. The co-exposure experiment in the AhR CALUX assay (Figure 7) shows that CH223191 could not only inhibit TCDD-induced AhR-mediated gene expression, but also PAH-induced AhR-mediated gene expression. This indicates that CH223191 is a suitable AhR antagonist to evaluate the potential role of the AhR in PAH-induced developmental toxicity in both the AhR CALUX assay and the ZET. Results obtained in the ZET show that the presence of CH223191 substantially reduced the severity or even fully counteracted the developmental retardations i.e., yolk sac edema, pericardial edema and deformed body shape, induced by seven out of nine PAHs tested i.e., BaA, 4-MeBaA, 8-MeBaA, 9-MeBaA, BaP, 3-MeBaP and DB[a,h]A. This observation is in line with the results of several studies which reported that the knockout of AhR2, the primary isoform of AhR responsible for PAH-induced developmental toxicity in zebrafish, abated TCDD- and PAH-induced pericardial and yolk sac edemas in zebrafish embryos (Billiard et al., 2006; Goodale et al., 2012; Incardona et al., 2006; Van Tiem and Di Giulio, 2011). 8-MeBaP was the only PAH for which the observed developmental retardations in zebrafish embryos were not substantially neutralized by the addition of CH223191. 8-MeBaP is the most potent BaP methylated congener to induce AhR-mediated activity as tested in the AhR CALUX, which suggests that a higher concentration of CH223191 or a more potent AhR antagonist may be needed to counteract the observed retardations induced by this substance in the ZET. However, changes to the experimental design appeared not practically possible because 1 μM was already the highest concentration of CH223191 that did not affect the normal development of zebrafish embryos until 96 hpf (see Supporting Information 1). Overall, our results support the notion that the developmental toxicity as observed with some PAHs is partially AhR-mediated.

Of the nine PAHs tested, only BaA and 8-MeBaA showed substantial estrogenic activity in the ER-α CALUX assay. Some discrepancy of (anti)estrogenic activities of PAHs tested between results obtained in the present study and available literature data was noted (see an overview of the ER-α-mediated activities induced by unsubstituted and alkylated PAHs in Supporting Information 6. For example, BaP was reported to be a strong estrogenic compound (Boonen et al., 2020; Charles et al., 2000; K. C. Fertuck et al., 2001), while in the present study BaP showed only a weak estrogenic activity. A possible explanation for this discrepancy might be related to the different metabolic capabilities present in the different used cell lines. The human breast carcinoma (VM7lucE42) cell line used by Lam et al. 2018a contains CYP450, which might result in the bioactivation of BaP into its major hydroxylated metabolite 3-hydroxy-BaP (3-OHBaP), which was shown to induce more estrogenicity than its parent BaP (Charles et al.,

2000; Kamelia et al., 2020). Thus, a higher estrogenic activity might be observed for BaP when using a test system that is capable of metabolic activation, which does not hold for the U2OS cell line used in the present study.

The ER-α antagonist fulvestrant only counteracted the embryo lethality induced by 50 μM BaA and the developmental retardations induced by 15 μM 8-MeBaA such as craniofacial deformities and kinked tail which are the typical ER-α mediated developmental effects in zebrafish (Adam et al., 2021; Ahi et al., 2016; Kishida et al., 2001; Wester and Vos, 2003). One of the possible explanations for this observation could be that 8-MeBaA has a lower EC50 than BaA in activating the ER-α in the ER-α CALUX assay (Table 1), pointing at a potential higher binding affinity of 8-MeBaA for ER-α than of BaA, hence, the ER-α fulvestrant counteracted the developmental retardations induced by 8-MeBaA only at a lower concentration (i.e., 15 μM) while being potent enough to counteract the BaA induced effects at 50 μM.

Published studies reported that the well-characterized RAR endogenous ligand, retinoic acid, can induce developmental toxicity e.g., causing craniofacial malformation in rodent embryos (Morriss-Kay, 1993) and failure to hatch in zebrafish embryos (Herrmann, 1995). None of the nine PAHs under study showed RAR agonist or antagonist activity, indicating that the PAH-induced developmental toxicity might be RAR-independent. Besides AhR, ER-α and RAR, other receptors such as the androgen receptor (AR) also play a role in the developmental processes (Kelce and Wilson, 1997). Previous studies showed that the DMSO-extracts of some PAH-containing substances induced AR antagonist effect in the U2OS AR antagonist CALUX assay (Kamelia et al., 2018). However, the IC50s for this AR antagonist activity showed no meaningful correlation with the 4-5 ring PAHs content ($R^2 = 0$) present in those substances. Moreover, a poor correlation ($R^2 = 0.02$) was found between the IC50s obtained in the AR antagonist CALUX assay of the DMSO-extracts of PAH-containing substances and their corresponding in vitro developmental toxicity potency, as evaluated in the mEST (Kamelia et al., 2018, 2017). Based on the above findings, it can be assumed that the AR does not play an important role in mediating the developmental toxicity induced by the 4- and 5- ring PAHs. Therefore, the AR-CALUX assay was not included in the present study.

Nominal concentrations of chemicals with hydrophobic property such as PAHs were reported to be significantly higher than their free concentrations in in vitro test systems, including the ZET (Groothuis et al., 2015; Knöbel et al., 2012). Lipophilic compounds may partition to the

cell culture medium constituents, e.g., serum protein and lipid, and to the plastic, which consequently affect their biologically effective concentration at the molecular target in vitro. In light of this, an in vitro chemical distribution model developed by Kramer et al (Kramer et al., 2012, 2022 (manuscript in preparation); Groothuis et al., 2015) was used to estimate actual concentrations of the nine PAHs in the CALUX assays and ZET applied (Supporting Information 9-12). The outcome of these model predictions shows that in the CALUX assays, free concentrations of the tested PAHs are substantially lower than their nominal concentrations, and that a large portion of the PAHs is predicted to bind to the cells and the plastic well-plates. For the ZET which contains no serum in the exposure medium (i.e., egg water), PAHs were predicted to bind to the zebrafish embryos and the plastic well-plates. However, it should be noted that the relative potencies of the nine PAHs to induce AhR-mediated or ER-α mediated activities remain the same when using the modeled free fraction of PAHs in the CALUX assays instead of the nominal concentrations (Supporting Information 13-15). The considerable differences between the free available concentration and the nominal concentration should be taken into consideration when using in vitro assay results for example for reverse dosimetry to predict in vivo dose response curves.

To conclude, the present study evaluated the in vitro receptor-mediated activities of nine (un)substituted PAHs, and the role of these receptors in mediating the observed developmental retardations by these substances in the ZET. Obtained results show that all nine tested PAHs were strong AhR ligands and only two out of nine PAHs induce substantial estrogenic activity, whereas none of the tested PAHs showed anti-estrogenic activity or interacted with the RAR or showed anti-estrogenic activity. Co-exposure experiments reveal that developmental retardations observed in zebrafish embryos following exposure to the selected PAHs were counteracted to a certain extent by the addition of an AhR or ER-α antagonist. This observation supports the hypothesis that the AhR and/or ER-α may play a role in mediating the developmental toxicity of PAHs. Moreover, the addition of a methyl substituent on the aromatic ring of PAHs appeared to result in increased in vitro AhR-mediated activity, which was not necessarily accompanied by increased but in some cases rather by decreased in vitro developmental toxicity, indicating that AhR activation does not present the only mode of action underlying in vitro developmental toxicity in the ZET.

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Supplementary materials

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CHAPTER 4.

Evaluating the developmental toxicity potency of a series of petroleum substances extracts using new approach methodologies (NAMs)

Jing Fang, Ivonne M.C.M. Rietjens, Juan-Carlos Carrillo, Peter J. Boogaard, Lenny Kamelia

Manuscript in preparation

Abstract

The present study evaluates the in vitro developmental toxicity and the possible underlying mode of action of DMSO extracts of a series of highly complex petroleum substances in the mouse embryonic stem cell test (mEST), the zebrafish embryotoxicity test (ZET) and the aryl hydrocarbon receptor reporter gene assay (AhR CALUX assay). Results show that two out of sixteen samples tested, both being poorly refined products which may contain a substantial amount of 3- to 7-ring polycyclic aromatic compounds (PACs), induced persistent AhR activation in the AhR CALUX assay, and concentration-dependent developmental toxicity in both mEST and ZET. The other samples tested representing highly refined petroleum substances and petroleum-derived waxes (containing typically a very low amount or no PACs at all) were negative for these three endpoints, pointing at their inability to induce developmental toxicity in vitro. It is concluded that the refining processes applied during the production of highly refined petroleum products, such as solvent extraction and hydrotreatment which focus on the removal of undesired constituents, including 3- to 7-ring PACs, abolish the in vitro developmental toxicity. In conclusion, the results support the hypothesis that 3- to 7-ring PACs are the primary inducers of the developmental toxicity induced by some (i.e., poorly refined) petroleum substances, and that the observed effect is partially AhR-mediated.

1. Introduction

Petroleum substances are primarily produced by either atmospheric or vacuum distillation of crude oil, generating a range of products with various specifications and usage purposes (Mackerer et al., 2003; Concawe, 2017). Petroleum substances are highly complex hydrocarbon materials, also known as UVCB substances (**U**nknown or **V**ariable composition, **C**omplex reaction products and **B**iological materials). The principle hydrocarbon classes present in petroleum substances are categorized into five groups, namely paraffinics (alkanes), isoparaffinics (isoalkanes), olefinics (alkenes), naphthenics (cycloalkanes), and aromatics (Feuston et al., 1994; Mackerer et al., 2003). Aromatics present in petroleum substances include monoaromatics, diaromatics and polyaromatics such as polycyclic aromatic compounds (PACs) (Feuston et al., 1994; Mackerer et al., 2003; Tsitou et al., 2015). When referring to ≥3 ring polyaromatics found in petroleum substances the term PACs is used to encompass three types of structures: naked/unsubstituted PAHs, alkylated PAHs and heterocyclic aromatics (Feuston et al., 1994; Tsitou et al., 2015; Carrillo et al., 2019). The term PAH is often used as surrogate for the entire PAC fraction (Andersson, 2009; Kamelia et al., 2017; Wang et al., 2020, 2022)

The type and quantity of aromatic constituents in petroleum substances vary depending on the source of the crude oil feedstock and processing conditions applied during their manufacturing process (Mackerer et al., 2003; Tsitou et al., 2015; Carrillo et al., 2022). For example, aromatic extracts, obtained upon solvent extraction of distillates or the residue from a vacuum tower, contain a relatively high concentration of dimethyl sulfoxide (DMSO)-extractable aromatics (6.6% - 20.3%, mostly 3- to 7-ring PACs) (HPV, 2012; Carrillo et al., 2021). Whereas, highly refined petroleum products, such as highly refined base oil (HRBO) and petroleum derived-waxes, obtained upon dewaxing, deoiling and/or hydrotreatment of base oil process, contain mainly highly alkylated 1- to 2-ring aromatics because the hazardous 3- to 7-ring aromatic compounds in these products are removed during the refinement (Figure 1) (Mackerer et al., 2003; Carrillo et al., 2019, 2021, 2022; Pirow et al., 2019). At comparable refining conditions, the level of DMSO extractable-aromatics may be used as surrogate of the amount of PAC content of a substance and highly refined substances yield less DMSO extractables. Such highly refined petroleum substances and petroleum derived-waxes are generally considered as inert and of high purity, thus are used for various applications including cosmetic production, pharmaceutical production, and food packaging materials (Pirow et al., 2019).

The developmental toxicity which is observed in animal studies with heavier petroleum substances, such as heavy fuel oil and aromatic extracts, is associated with the presence of polyaromatics (mainly 3- to 7-ring compounds) in these substances (Feuston et al., 1989, 1994; Murray et al., 2013; Tsitou et al., 2015). Recent studies (Kamelia et al., 2017, 2018, 2019a, 2019b, 2021) showed that the developmental toxicity of PAC-containing petroleum substances was successfully evaluated using a battery of in vitro alternative assays, comprising the mouse embryonic stem cell test (mEST), the zebrafish embryotoxicity test (ZET), and the aryl hydrocarbon receptor CALUX reporter gene assay (AhR CALUX assay). It was demonstrated that the developmental toxicity induced by DMSO extracts of some petroleum substances was proportional to the level of 3- to 7-ring PACs present in these extracts, in agreement with the aforementioned in vivo observations. Also, it was shown that the observed in vitro developmental toxicity in both mEST and ZET was partially AhR-mediated (Kamelia et al., 2017, 2018, 2019a, 2019b, 2021). Since the highly refined petroleum products mainly contain 1- to 2-ring aromatics, and contain low or no levels of 3-7 ring PACs (Carrillo et al., 2022) thus they are not expected to induce developmental toxicity.

The present study aims to extend the applicability domain of the battery of tests beyond the previously tested petroleum samples by evaluating the in vitro developmental toxicity and the underlying mode of action to induce developmental toxicity of DMSO extracts of an additional series of petroleum substances using the AhR reporter gene assay, mEST and ZET. In total, sixteen samples with varying degrees of refinement were tested in the present study (Figure 1), which include distillates, aromatic extracts, highly refined petroleum substances, and petroleum substances-derived waxes. Two out of sixteen selected samples were a distillate and an aromatic extract, which are not extensively refined, thus containing relatively high amounts of 3- to 7-ring PACs. The other fourteen samples were further treated by various refinement processes to remove the undesired 3- to 7-ring PACs, thus they typically contain either low amounts or no biologically active aromatics. The results are expected to broaden the applicability domain of the new approach methodologies (NAMs) previously applied (Kamelia et al., 2017, 2018, 2019a, 2019b, 2021) and allow the assessment of the developmental toxicity of not only petroleum substances with relatively high PAC levels but also of highly refined petroleum substances and petroleum-derived waxes with low level or devoid of PACs.

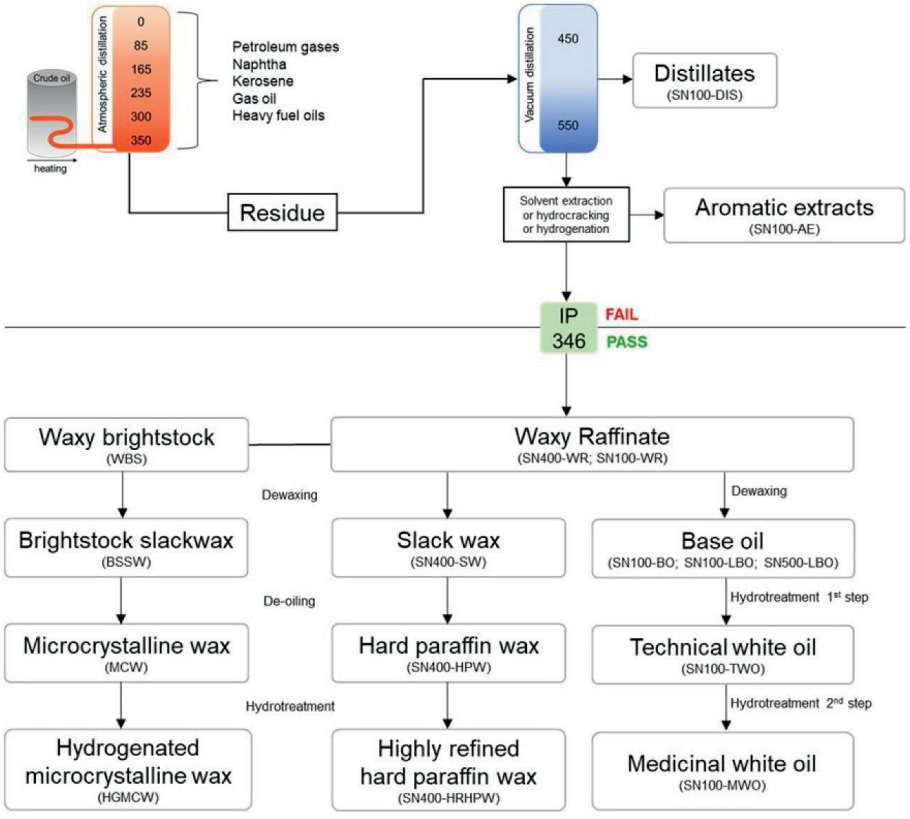

Figure 1. Flowchart of manufacturing process of paraffinic petroleum products. In brackets are the grades (viscosities related to the produced base oil) that were tested. *Abbreviations. SN100-DIS: SN100-Distillate, SN100-AE: SN100-Aromatic extract, SN100-WR: SN100-Waxy raffinate, SN400-WR: SN400-Waxy raffinate, SN100-BO: SN100-base oil, SN100-LBO: SN100-lubricating base oil, SN500-LBO: SN500-lubricating base oil, SN100-TWO: SN100-Techinical white oil, SN100-MWO: SN100-Medicinal white oil, SN400-SW: SN400-Slackwax, SN400-HPW: SN400-Hard paraffin wax, SN400-HRHPW: SN400-Highly refined hard paraffin wax, WBS: Waxy brightstock, BSSW: Brightstock slackwax, MCW: Microcrystalline wax, HGMCW: Hydrogenated microcrystalline wax.*

2. Materials and Methods

2.1 Test compound

Benzo[a]pyrene (BaP; CAS no. CAS No. 50–32-8), 5-fluorouracil (CAS no. 51-21-8) and 3,4-dichloroaniline (CAS no. 95-76-1) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). All stocks and dilutions of test compounds were prepared in dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany).

DMSO extracts of the sixteen complex petroleum substances (Figure 1) were tested, including a distillate (sample SN100-DIS, CAS No. 64741-50-0), an aromatic extract (sample SN100-AE, CAS No. 64742-05-8), waxy raffinates (samples SN100-WR and SN400-WR; CAS No. 64741-89-5), base oils (sample SN100-LBO, CAS. No 64742-65-0; sample SN500-LBO, CAS. No 64742-65-0; sample SN100-BO, CAS No. 64742-56-9), a technical white oil (sample SN100-TWO, CAS No. 8042-47-5), a medicinal white oil (sample SN100-MWO, CAS No. 8042-47-5), a slackwax (sample SN400-SW, CAS No.64742-61-6), a hard paraffin wax (sample SN400-HPW, CAS No. 8002-74-2), a highly refined hard paraffin wax (sample SN400-HPHPW, CAS No.8002-74-2), a waxy brightstock (sample WBS, CAS No. not available), a brightstock slackwax (sample BSSW, CAS No. not available), a microcrystalline wax (sample MCW, CAS No. 63231-60-7), and a hydrogenated microcrystalline wax (sample HGMCW, CAS No. 64742-60-5).

2.2 DMSO extraction procedure

To generate DMSO extracts of the substances tested, the method previously described by Roy et al. 1988, McKee et al. 2014 and Kamelia et al. 2017 was applied. Briefly, 4.0 g of each test material was dissolved in 10 ml cyclohexane (CAS No. 110-82-7, Sigma-Aldrich) and then extracted twice with 10 ml DMSO. The extract was collected in capped brown glass vial and stored at 4^{o}C until use.

2.3 Aryl hydrocarbon (AhR) CALUX reporter gene assay

2.3.1 Cell line and cell culture conditions

The stably transfected rat hepatoma cell line (H4IIE.luc) was used for the AhR CALUX assay (Aarts et al., 1995). H4IIE.luc cells were cultured in 75 cm^2 polystyrene cell culture flasks (Corning, The Netherlands) with Minimum Essential Medium (MEM) alpha medium (Gibco, Paisley, UK, catalog no. 2256-021) which was supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich, The Netherlands) at 37°C with 5% CO_2 in a humidified atmosphere. All cells were routinely sub-cultured using 0.05% trypsin-EDTA (Gibco) for every 2 to 3 days.

2.3.2 AhR CALUX reporter gene assay

In the present study, the H4IIE.luc AhR CALUX reporter gene assay was performed to evaluate the AhR-mediated activity of the test compounds. First, H4IIE.luc cells were washed with phosphate-buffered saline (PBS; Gibco) and trypsinized with 0.05% trypsin-EDTA when reaching 80-90% confluency. Then, 100 µl of cell suspension at a density of 3×10^5 cells/ml was seeded into each of the 60-inner wells of white 96-well plates (Greiner Bio-one, Frickenhausen, Germany); the 36-outer wells of the same white 96-well plate were filled with 200 µl PBS to limit evaporation from the inner wells. Cells were incubated for 24 h at 37°C with 5% CO_2. After the incubation, cells were exposed to exposure medium for either 6 h or 24 h, in triplicate. The exposure medium was prepared by adding 400 times concentrated test compounds dissolved in DMSO into pre-conditioned medium. Pre-conditioned medium was the growth medium harvested from cells which have been already cultured for 16 – 24h. The use of pre-conditioned medium helps to reduce the high background luciferase signal induced by tryptophan products present in fresh medium (Hamers et al., 2000). After 6 h or 24 h exposure, cells were washed with 100 µl ½ PBS (PBS: nano-pure water = 1:1) and lysed with 30 µl low salt buffer (LSB; 10 mM Tris (Sigma-Aldrich), 2 mM dithiothreitol (DTT, Sigma-Aldrich), and 2 mM 1, 2-diaminocyclohexanete triacetic acid monohydrate (Sigma-Aldrich); pH 7.8). Afterwards, plates were placed on ice for at least 15 minutes and frozen at -80°C for at least 120 minutes before luminescence measurement.

For the luminescence measurement, a luminometer (Glomax-Multi Detection System, Promega, California) was used. Before measurement, plates were thawed at room temperature for 1 h and placed on a plate shaker for around 5 min. Then, 100 µl flash-mix solution was added to each well. The flash-mix solution contained 20 mM tricine (Sigma-Aldrich), 1.07 mM $(MgCO_3)_4Mg(OH)_2\cdot5H_2O$ (Sigma-Aldrich), 2.67 mM magnesium sulfate ($MgSO_4$, Merck), 0.1 mM ethylenedinitrilotetraacetic acid disodium salt dihydrate (Merck), 2 mM DTT (Sigma-Aldrich), 0.47 mM D-luciferin (Synchem UG & Co. KG, Felsberg, Germany), and 5 mM adenosine-5-triphosphate (Duchefa Biochemie bv, Haarlem, The Netherlands).

All test compounds were tested in the AhR CALUX assay at concentrations up to 5 µg/ml, and the final concentration of the solvent DMSO was kept at 0.25% (v/v). A full concentration-response curve of the positive control BaP up to the concentration of 1.26×10^{-1} µg/ml was included in each independent experiment. Three independent experiments were conducted for each test compound.

2.4 Mouse embryonic stem cell test (mEST)

2.4.1 Cell line and cell culture conditions

The pluripotent mouse ES-D3 cell line (ATCC, Wesel, Germany) was used for the mEST. ES-D3 cells were cultured in 25 cm^2 polystyrene cell culture flasks (Corning, The Netherlands). The flasks were pre-coated with 0.1% (w/v) gelatine for at least 1 h before use. ES-D3 cells were cultured in mouse ES cell basal medium (ATCC, USA) supplemented with 15% FBS, ES cell qualified (ATCC, USA), 50 U/ml penicillin with 50 µg/ml streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.5 % non-essential amino acids (NEEA; Invitrogen), and 0.1 mM 2-mercaptoethanol (Gibco) at 37°C with 5% CO_2 in a humidified atmosphere. Cells were kept undifferentiated by the addition of 1000 U/mL murine Leukemia Inhibiting Factor (mLIF; Sigma-Aldrich). The ES-D3 cells were routinely sub-cultured every 2-3 days by using non-enzymatic cell dissociation solution (Sigma-Aldrich).

2.4.2 ES-D3 cell viability assay of the mEST

The water-soluble tetrazolium (WST-1) assay was performed to determine the cell viability following exposure to test compounds. The principle of the assay is based on the ability of metabolically active cells to convert the tetrazolium salt WST-1 into formazan dye by mitochondrial dehydrogenase (Ngamwongsatit et al., 2008). First, 100 µl ES-D3 cell solution at a density of 2×10^5 cells/ml (1-day exposure) or 1×10^4 cells/ml (5 days exposure) was seeded into the 60-inner wells of 96-well plates (Greiner Bio-One) in the absence of mLIF. Then, cells were incubated for 24 h at 37°C with 5% CO_2 in a humidified atmosphere to allow cell adherence. After the incubation, ES-D3 cells were exposed to increasing concentrations of test compounds by adding 100 µl exposure medium to each well. The exposure medium was prepared by mixing the 400 times concentrated test compounds in DMSO into ES-D3 growth medium (without mLIF). All test compounds were tested in the ES-D3 cell viability assay at concentrations up to 250 µg/ml, and the final concentration of the solvent DMSO was kept at 0.25% (v/v). The cells were exposed to each concentration of the test compounds in triplicate and incubated for 1 or 5 days at 37°C with 5% CO_2. After the incubation for 1 or 5 days, 10 µl of WST-1 reagent (Roche, Woerden, The Netherlands) was added into each well, then cells were further incubated for 3 h at 37°C with 5% CO_2. Finally, the absorbance of the formed formazan was measured at 440 nm (background at 620 nm) using a SpectraMax iD3 (Molecular

Devices, San Jose, USA). Three independent experiments were conducted for each test compound.

2.4.3 ES-D3 cell differentiation assay of the mEST

The ES-D3 cell differentiation assay of the mEST was performed as previously described by Kamelia et al. 2017. On day 0, droplets of 20 µl ES-D3 cell solution (3.75×10^4 cells/mL) with or without test compounds were placed between the well borders on the inner side of the lid of a 96-well plate. To create an optimal humidity and prevent evaporation of the hanging drops, each well of the 96-well plates was filled with 250 µl PBS. Sterile caps of Eppendorf tubes were placed in the corner of the plates to prevent direct contact of the hanging drops with the plate, and the plate was then sealed with Micropore Tape (3M, Neuss, Germany). After 72 h incubation at 37°C with 5% CO_2 in a humidified atmosphere, or on day 3, the formed embryoid bodies (EBs) were collected and transferred to non-tissue culture treated petri dishes (diameter 6 cm, Greiner), containing 5 ml of medium with or without test compounds. The petri dishes were then incubated for another 48 h (2 days) at 37°C with 5% CO_2. On day 5, the EBs were transferred to a 24-well plate (Corning), 1 EB per well, containing 1 ml of medium with test compound. The 24-well plates were incubated for 5 days at 37°C with 5% CO_2. On day 10, the number of wells containing contracting cardiomyocytes was determined by visual inspection using a light microscope. 0.25% v/v DMSO (solvent control) and 0.3 µM 5-fluorouracil (positive control) were included in each independent experiment. The ES-D3 cell differentiation assay was considered valid if the solvent control in each experiment had at least 21 out of 24 wells that contained contracting cardiomyocytes. Three independent experiments were conducted for each test compound.

2.5 Zebrafish Embryotoxicity Test (ZET)

Fertilized eggs of wild-type zebrafish (*Danio rerio*) AB line were purchased from the research facility Carus, at Wageningen University and Research, The Netherlands. The ZET was performed as previously described (Kamelia et al., 2019a). Briefly, the zebrafish embryos were exposed to the test compounds at 4-5 hours post fertilization (hpf). 24-well plates (Greiner Bio-One) were used for the exposure: twenty wells were used for exposure to two concentrations of test compounds (ten wells for each concentration) and the other four wells were used for the internal plate control. The exposure medium was prepared by mixing 400 times concentrated DMSO stock solutions of test compounds with egg water. The egg water was prepared by

adding 10 ml 100-times egg water stock solution (3 g sea salt (Tropic marine, Wartenberg, Germany) in 500 ml demineralized water) to 990 ml demineralized water. All test compounds were tested in the ZET at concentrations up to 250 μg/ml, except for SN100-AE, which was tested up to 25 μg/ml because at such concentration this test substance already induced 100% cumulative mortality at 96 hpf. The exposure medium was transferred (2 ml/well) into twenty wells of the 24-well plate, and 2 ml egg water was added into each of the 4 remaining wells for the internal plate control. One zebrafish embryo was transferred to each well of the 24-well plate (1 embryo/well). The plates were sealed with self-adhesive film covers (Greiner) to prevent evaporation and were incubated at 26 °C with a photo period of 14 h light:10 h dark. Three controls were included in each independent experiment: solvent control (0.25% v/v DMSO), positive control (4 μg/ml 3,4-dichloroaniline) (Sigma-Aldrich), and negative control (egg water only). Embryos were scored daily using an inverted microscope until 96 hpf for embryo lethality and developmental abnormalities, based on the extended general morphological scoring (extended-GMS) system described by Beekhuijzen et al. 2015. This extended-GMS system includes two parts: 1) general development of zebrafish embryos (GMS), and 2) dysmorphogenic endpoints. The GMS consists of twelve endpoints i.e., detachment of tail, somite formation, eye development and pigmentation, movement, circulation, presence of heartbeat, pectoral fins, pigmentation of head and body, pigmentation of tail, hatching, presence of protruding mouth, and yolk extension. The dysmorphogenic endpoint scoring consisted of six endpoints i.e., yolk sac edema, pericardial sac edema, malformation of the tail, deformed body shape, malformation of the head and jaw, and malformation of the sacculi/otoliths (Supplementary material 1). Any deviation from normal development of zebrafish embryos results in a lower extended-GMS score, corresponding to a certain extent of developmental retardation. The ZET was considered valid if the following was observed (at 96 hpf): (1) ≤ 1 dead embryo (out of 4) in the internal plate control of the exposed plate; (2) ≤ 1 dead embryos (out of 10) in the negative control plate; (3) ≤ 1 dead embryos (out of 10) in the solvent control plate; (4) ≤ 7 live embryos (out of 10) in the positive control plate.

2.6 Data Analysis

Figures of concentration-response curves in the AhR CALUX assay, ZET and mEST were generated using GraphPad Prism 5.0 (California, USA). Data were fitted into sigmoid concentration-response curves using non-linear regression analysis with three parameters. Results were presented as mean percentage of response ± standard error of the mean (SEM)

from at least three independent experiments. It should be noted that except for SN100-DIS, SN100-AE, SN100-LBO and SN500-LBO, other petroleum samples applied induced no effect at the highest concentration i.e., 250 μg/ml, in both mEST and ZET, thus they were only tested at three concentrations: 0.5 (low), 125 (middle) and 250 (high) μg/ml in the mEST and ZET.

For the AhR CALUX assay, the results of luminescence measurement were expressed as relative light units (RLUs). All data are expressed as induction factor by dividing the mean RLUs of exposed wells to the mean RLUs of the corresponding solvent control (0.25% v/v DMSO). EC50 (effective concentration inducing 50% response) values were calculated using Graphpad from the concentration-response curves of the AhR CALUX assay. For the mEST, the results of cell viability were expressed as the percentage of cell viability as compared to the solvent control which was set as 100%. Data from the ES-D3 cell differentiation assay were expressed as the percentage of fraction of total differentiated EBs into contracting cardiomyocytes, out of the total EBs plated in the 24-well plate. For the ZET, results were expressed as percentage response of the total extended-GMS score (maximum 23) and as embryo survival at 96 hpf.

To determine the benchmark concentrations (BMC), concentration-response curves obtained in the mEST and ZET were fitted to all quantal concentration-response models from the European Food Safety Authority (EFSA) benchmark dose (BMD) modelling web-tool (https://shiny-efsa.openanalytics.eu/), based on the R-Package PROAST version 66.40 developed by the Dutch National Institute for Public Health and the Environment (RIVM). The quantal models of the ESFA BMD modelling web-tool include two-stage, log logistic, weibull, log probit, gamma, logistic, probit, exponential, and hill models. The benchmark response (BMR) was set to 50% (i.e., BMC50), representing the concentration that induced 50% inhibition of EBs differentiated into contracting cardiomyocytes in the mEST, 50% reduction of the embryo survival in the ZET, or 50% reduction in the extended-GMS score in the ZET. The performance of each fitted model was evaluated based on the goodness-of-fit, the scaled residuals, and the visual inspection of model fitting. The final BMC50 values were selected from the accepted model with the lowest Akaike's Information Criterion (AIC) (Haber et al., 2018) (Supplementary material 3).

2.7 Correlation analysis

Correlation between the AhR mediated activities (EC50) and in vitro developmental toxicity potencies in mEST or ZET (BMC50) of the DMSO extracts of the petroleum substances was tested using the linear regression approach in GraphPad Prism 5.0. Data obtained in the present study were combined with data of previous studies (Kamelia et al., 2017, 2018, 2019a), which tested the in vitro developmental toxicity of samples of other categories of petroleum substances using the AhR CALUX assay, mEST and ZET. No EC50 or BMC50 could be derived for petroleum substances that induced no substantial effects in the AhR CALUX assay, mEST and mEST, and therefore these samples were not included in the correlation test. The obtained R^2 reflects the goodness-of-fit of data to the fitted regression, and the compared data were considered as significantly different if the p value was lower than 0.05.

3. Results

3.1 Effects of the DMSO extracts of sixteen petroleum substances in the AhR CALUX assay

The AhR-mediated activities of the DMSO extracts of sixteen petroleum substances were evaluated in the AhR CALUX assay with two exposure time windows, 6 h and 24 h. The concentration-response curves of the AhR-mediated activities of all test samples are shown in **Figure 2**. Results show that samples SN100-DIS and SN100-AE (with EC50-6 h of 0.0065 μg/mL and 0.0052 μg/mL, respectively; with EC50-24 h of 0.76 μg/mL and 0.47 μg/mL, respectively) were the most potent test substances under study to induce AhR-mediated activity in the AhR CALUX assay following 6 h and 24 h exposure. Two samples, namely SN100-LBO and SN500-LBO, induced AhR-mediated activities following 6 h exposure (see their EC50s in Table 1), but such activities were not observed after 24 h exposure, indicating a transient AhR activation in the AhR CALUX assay. The other twelve samples tested negative in the AhR CALUX assay after 6 h and 24 h exposure.

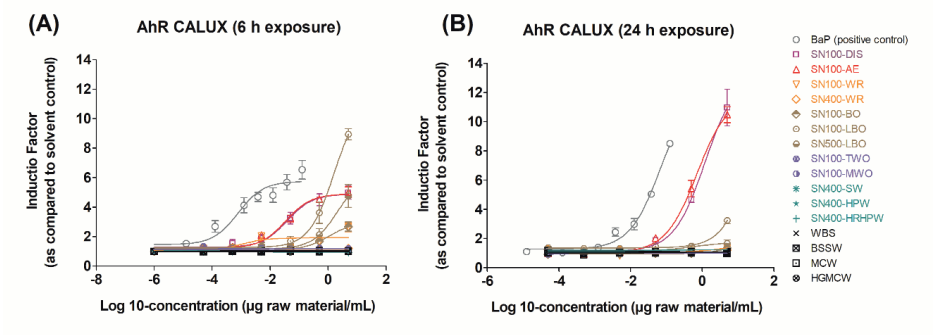

Figure 2. Concentration-dependent effects of BaP (positive control) and the DMSO extracts of sixteen petroleum substances in the AhR CALUX assay following **(A)** 6 h and **(B)** 24 h exposure. Results represent data from three independent experiments and are presented as mean ± SEM.

3.2 Effects of the DMSO extracts of sixteen petroleum substances in the mEST

The in vitro developmental toxicity of the test samples was first evaluated in the mEST. As illustrated in Figure 3, none of the test samples induced cytotoxicity to the ES-D3 cells following 1- or 5-days exposure, up to the highest concentration tested (250 µg/ml). Only two test samples: SN100-DIS (Figure 3A) and SN100-AE (Figure 3B) inhibited the differentiation of the ES-D3 cells into contracting cardiomyocytes. The BMC50 values derived from the concentration-response curves of SN100-DIS and SN100-AE in the ES-D3 cell differentiation assay were 15.3 µg/ml and 3.21 µg/ml, respectively (**Table 1**). Samples SN100-LBO and SN500-LBO inhibited the ES-D3 cell differentiation with ~20% reduction at the highest tested concentration of 250 µg/ml, thus their BMC50s were above 250 µg/ml. The other samples tested negative in the ES-D3 cell differentiation assay.

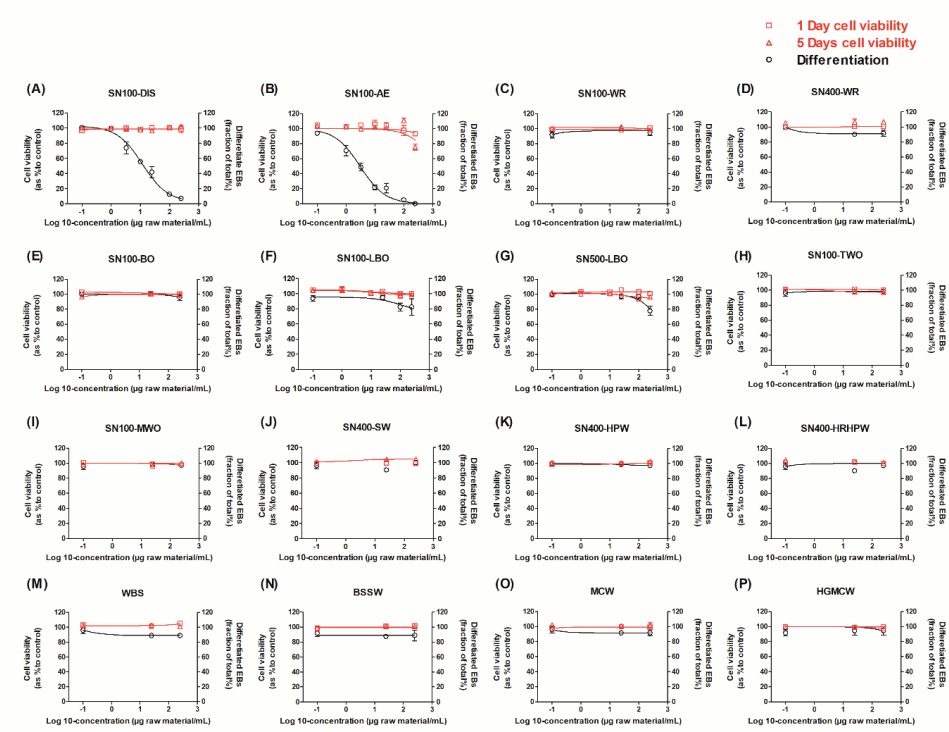

Figure 3. Concentration-dependent effects of the DMSO extracts of sixteen petroleum substances on ES-D3 cell viability upon 1 day (red line with unfilled square symbols) and 5 days (red line with unfilled triangle symbols) exposure, and on inhibition of ES-D3 cell differentiation into contracting cardiomyocytes (black line with round symbols). Results represent data from at least three independent experiments and are presented as mean ± SEM.

3.3 Effects of DMSO extracts of the sixteen petroleum substances in the ZET

The ZET was the second assay applied to investigate the in vitro developmental toxicity of the DMSO extracts of the sixteen petroleum substances under study. **Figure 4** shows the concentration-dependent effects of the DMSO extracts of sixteen petroleum substances in the ZET based on embryo survival and the extended-GMS at 96 hpf. The results show that only two test compounds: SN100-DIS and SN100-AE induced substantial reduction in both the embryo survival and the extended-GMS responses at 96 hpf. The BMC50 values for embryo survival and extended-GMS, derived from the concentration-response curves of SN100-DIS and SN100-AE in the ZET, are listed in **Table 2**. The calculated BMC50 value of SN100-DIS for embryo survival was 18.1 µg/ml and that for the extended-GMS was 9.72 µg/ml. The BMC50 value of SN100-AE for embryo survival was 7.41 µg/ml and that for the extended-

GMS was 3.91 µg/ml. Sample SN100-LBO induced ~20% reduction in the extended-GMS response just at the highest tested concentration of 250 µg/ml, thus its BMC50 was above 250 µg/ml. The other thirteen samples tested negative in the ZET. Furthermore, a heatmap (**Figure 5**) was generated to better visualize the affected endpoints induced by samples SN100-DIS and SN100-AE in the ZET. As depicted in **Figure 5**, the most affected endpoints upon exposure to SN100-DIS and SN100-AE include the absence of movement and circulation, unhatched embryos, yolk extension was not fully emptied, pericardial sac and yolk sac edema, and cumulative mortality.

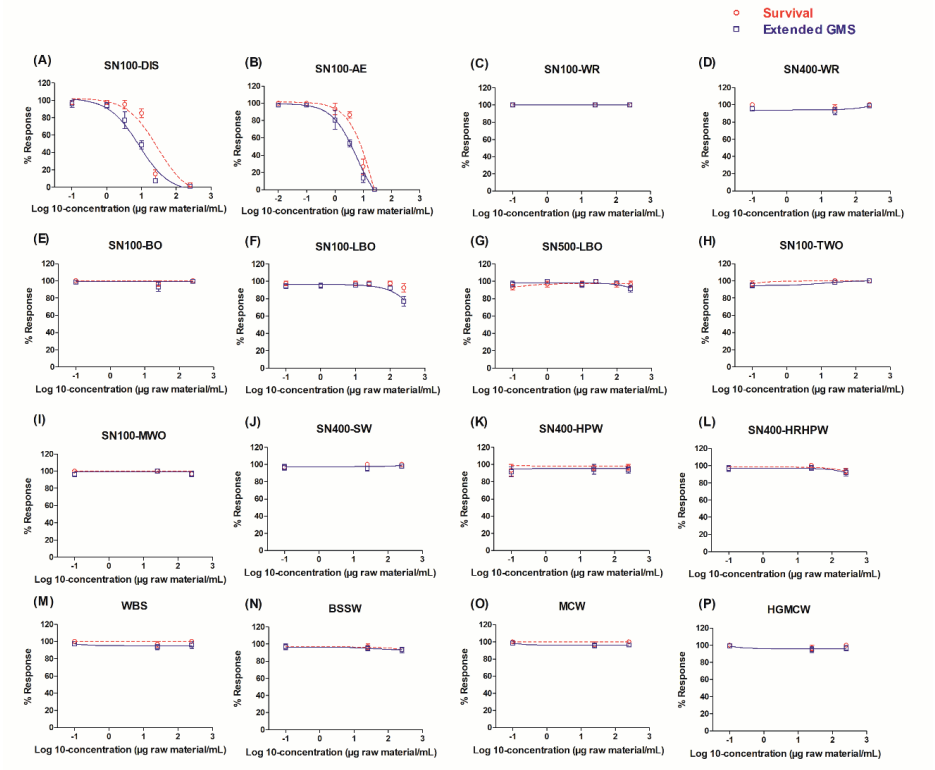

Figure 4. Concentration-dependent effects of the DMSO extracts of the sixteen petroleum substances on embryo survival (red line with unfilled round symbols) and extended-GMS (blue line with unfilled square symbols). Results represent data from at least three independent experiments and are presented as mean ± SEM.

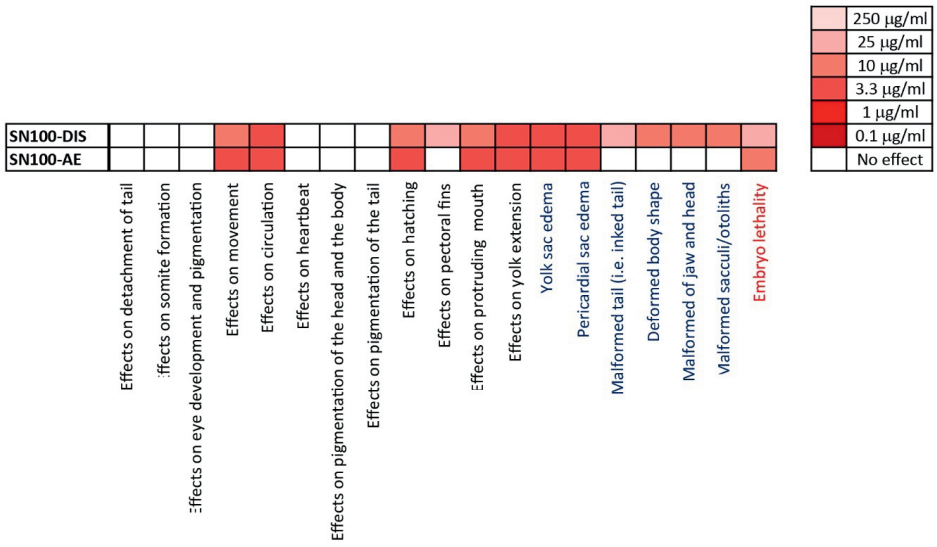

Figure 5. Heatmap summarizing the results in the ZET for the 18 endpoints scored based on the general morphology development (black font), dysmorphogenic (blue font) and for embryo lethality (red font) endpoints, upon exposure to the DMSO extracts of sample SN100-DIS and SN100-AE at 96 hpf. An exposure was considered as a 'hit' for a specific endpoint on the heatmap when ≥50% of the viable embryos showed effects on this endpoint scored at 96 hpf. The lowest concentration of the respective test compound that corresponds to a 'hit' is indicated by the color code shown on the right part of the figure. Results presented in the heatmap represent data from at least three independent experiments.

3.4 Correlation between in vitro potencies obtained in the AhR CALUX assay and/or mEST and ZET

To investigate the potential relationship between the AhR-mediated activities and the in vitro developmental toxicity of the DMSO extracts of the petroleum substances in the mEST/ZET, a correlation analysis was performed by combining the AhR CALUX assay, mEST and ZET results obtained in the present study with results obtained in a series of previous studies (Kamelia et al., 2017, 2018, 2019a) which tested different categories of petroleum substances samples including gas oil (GO), vacuum tower overhead (VTO), residual aromatic extracts (DAE), and heavy fuel oil (HFO) in the same in vitro assays. It should be noted that since the previous studies only tested the AhR mediated activities of petroleum substances following 6 h exposure, the correlation test was only performed between the in vitro potencies in mEST or

ZET and AhR mediated activities following 6 h exposure. Moreover, in the previous studies when testing other categories of petroleum substances in the ZET (Kamelia et al., 2019a), the BMC50 was determined based on GMS instead of extended-GMS. Therefore, to be consistent with this previous study, BMC50s based on GMS were also calculated for the samples that tested positive in the ZET in the present study (i.e., poorly refined SN100-DIS and SN100-AE). Their BMC50s based on the GMS are listed in Table 1, and the concentration-response curves on embryo survival, GMS and extended-GMS of these two samples are presented in Supplementary material 2.

The results (Figure 6) show that when the data of the present study are combined with those from previous studies by Kamelia et al. (2017, 2018, 2019a), the current correlation between the AhR-mediated activities (following 6 h exposure) and the in vitro developmental toxicity potencies in the ZET (R^2 = 0.73) or mEST (R^2 = 0.73) matches the correlation defined previously (mEST and AhR CALUX assay: R^2 = 0.80; ZET and AhR CALUX assay: R^2 = 0.65) (Kamelia et al., 2017, 2018, 2019a).

Table 1. Overview of the EC50 and BMC50 values of the DMSO extracts of sixteen petroleum substances under study in the AhR CALUX assay, mEST and ZET.

Test compound	EC50 of AhR CALUX agonist assay (6h exposure, µg/ml)	EC50 of AhR CALUX agonist assay (24h exposure, µg/ml)	BMC50 of the mEST (µg/ml)	BMC50 of the ZET (µg/ml)		
				Embryo lethality	GMS	Extended-GMS
SN100-DIS	0.0065	0.35	15.3	18.1	11.63	9.72
SN100-AE	0.0052	0.21	3.21	7.41	4.63	3.91
SN100-WR	-	-	-	-	-	
SN400-WR	-	-	-	-	-	
SN100-BO	-	-	-	-	-	
SN100-LBO	0.80	-	>250 µg/ml [a]	>250 µg/ml [a]	-	
SN500-LBO	0.54	-	>250 µg/ml [a]	-	-	
SN100-TWO	-	-	-	-	-	
SN100-MWO	-	-	-	-	-	

SN400-SW	-	-	-	-	-	
SN400-HPW	-	-	-	-	-	
SN400-HPHPW	-	-	-	-	-	
WBS	-	-	-	-	-	
BSSW	-	-	-	-	-	
MCW	-	-	-	-	-	
HGMCW	-	-	-	-	-	

Note: "-" means the sample was tested negative in the AhR CALUX assay, mEST and ZET.

[a] The calculated BMC50s were higher than the highest tested concentration of 250 µg/ml.

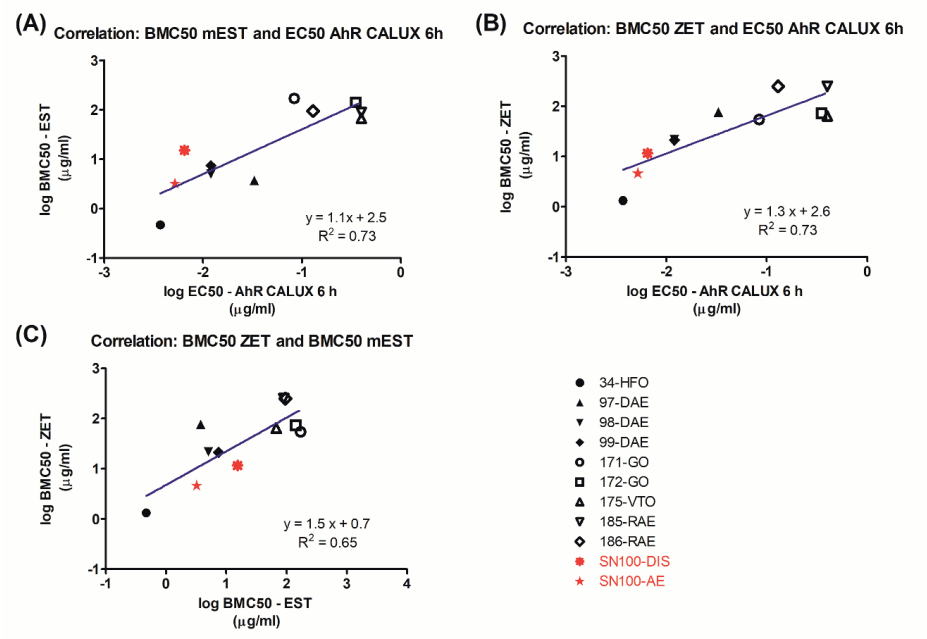

Figure 6. Correlation between the AhR mediated activities following 6 h exposure (expressed as EC50s) of the DMSO extracts of petroleum substances as tested in the AhR CALUX assay and their in vitro developmental toxicity potency as tested in the (**A**) mEST (expressed as BMC50s) and **(B)** ZET (expressed as BMC50s); and (**C**) correlation between the in vitro developmental toxicity potency in the mEST and ZET. *Note: the in vitro data of samples 34-HFO, 97-DAE, 98-DAE, 99-DAE, 171-GO, 172-GO, 175-VTO, 185-RAE and 186-RAE were taken from Kamelia et al., 2017, 2018, 2019a.*

4. Discussion

Previous studies have shown the usefulness of a battery of in vitro assays to evaluate the developmental toxicity of PAC-containing substances including highly complex petroleum substances extracts (Kamelia et al., 2017, 2018, 2019a, 2019b, 2021). The present study aimed to extend the usefulness of such a battery of in vitro assays including the AhR CALUX assay, mEST and ZET for developmental toxicity testing of a series of petroleum substance extracts from different product categories and varying in their PAC content. To this end, DMSO extracts of sixteen test compounds, including two poorly refined petroleum substances, seven highly refined petroleum substances and seven petroleum-derived waxes were tested. Results obtained show that the two poorly refined petroleum substances i.e., SN100-DIS and SN100-AE, induced persistent AhR activation in the AhR CALUX assay and concentration-dependent in vitro developmental toxicity in both mEST and ZET. Moreover, two base oil extracts (i.e., SN100-LBO and SN500-LBO) induced only transient AhR activation following 6 h exposure since such activity was not seen upon 24 h exposure in the AhR CALUX assay. The remaining petroleum substances and petroleum-derived waxes tested, containing either very low levels or no PACs at all, tested negative in all assays applied (AhR CALUX assay, mEST and ZET). Due to the absence of in vivo developmental toxicity data on the actual samples, it is not possible to directly assess the correlation between in vitro developmental toxicity potency, obtained in mEST and ZET, and potencies observed in vivo. Nonetheless, our results demonstrate that the applied test battery does not only capture the in vitro developmental toxicity induced by DMSO extracts of PAC-containing petroleum substances, but also the absence of developmental toxicity-related effects for substances containing virtually no aromatics and no PACs, such as highly refined oils and petroleum-derived waxes.

Sample SN100-DIS is a vacuum distillate, produced by the vacuum distillation of the residuum from atmospheric distillation of crude oil, and belongs to the petroleum substance category of untreated/acid-treated oils. Vacuum distillates that have not been subject to further refining processes may thus contain hazardous 3- to 7-ring PACs (HPV, 2012). Subsequent refining processes of the vacuum distillate, such as solvent extraction, results in two streams: aromatic extract (sample SN100-AE) containing typically high levels of 3- to 7-ring PACs, and waxy raffinate (samples SN100/400-WR) which serves as the primary feedstock for producing base oils and wax substances (HPV, 2012; Carrillo et al., 2021, 2022). In line with the fact that the samples SN100-DIS and SN100-AE were not subject to further refining and are expected to contain substantial amounts of 3- to 7 ring PACs, both samples were able to induce in vitro

developmental toxicity in the ZET and mEST. The waxy raffinate samples tested (samples SN100-WR and SN400-WR), which contain less than 3% wt.% total PACs after the refining processes since they passed the IP346 test (Figure 1) were negative in all assays applied in the present study. The positive outcomes for SN100-DIS and SN100-AE are in line with in vivo studies which reported that the developmental toxicity induced by some petroleum substances such as heavy fuel oil is highly associated with their 3- to 7-ring PAC content (Feuston et al., 1994; Murray et al., 2013), and also with results from a series of in vitro studies which confirm the hypothesis that the 3- to 7-ring PAC content of petroleum substances plays an important role in mediating the developmental toxicity of petroleum substances substances (Kamelia et al., 2017, 2018, 2019a, 2019b, 2021). In addition, the correlation observed between the AhR mediated activity (following 6 h exposure) and the in vitro developmental toxicity in the ZET or mEST when combining results of SN100-DIS and SN100-AE with petroleum substances from other categories matches with the correlation defined previously by Kamelia et al., 2017, 2018, 2019a. Altogether, the results support the hypothesis that the AhR plays important role in mediating developmental toxicity of petroleum substances relative high levels of 3-7 PAC (Kamelia et al., 2018, 2019b). The EC50 values of SN100-DIS and SN100-AE obtained in the AhR CALUX assay following 24 h exposure were higher than the EC50 values following 6 h exposure, this indicates that persistent AhR activation by these two substances since such effects remain even after 24h exposure. It is known that persistent AhR activation such as the activity induced by strong AhR ligand TCDD could lead to various adverse effects including developmental toxicity, while transient AhR induction is required for maintaining normal cell homeostasis (Hankinson, 1995; Puga et al., 2002; Mitchell and Elferink, 2009; Larigot et al., 2018). However, a previous study by Kamelia et al. 2018 only measured the AhR-mediated activity after 6 h but not 24 h exposure, hence comparison and correlation between the in vitro developmental toxicity of the petroleum substances and their sustained AhR activation thus cannot be further evaluated. Therefore, it seems of interests to include the AhR CALUX assay with two exposure time windows (i.e., 6 h and 24 h) in the proposed in vitro testing strategy for developmental toxicity testing of highly complex petroleum substances.

Dewaxing of waxy raffinates separates the waxes from the oils, thus yielding two streams namely slack wax (sample SN400-SW) and base oil (samples SN100-BO, SN100-LBO and SN500-LBO). The major hydrocarbon components of slack wax are paraffins and low levels of highly alkylated 1- to 2-ring aromatics (CONCAWE, 2017; Carrillo et al., 2019). Slack wax could be de-oiled into hard paraffin wax (sample SN400-HPW), and then generate highly

refined hard paraffin wax (sample SN400-HRHPW) by hydrotreatment. All these petroleum substances-derived waxes (i.e., SN400-SW, SN400-HPW, SN400-HRHPW) tested negative in the AhR CALUX assay, mEST and ZET. Furthermore, DMSO extracts of the base oil samples tested (samples SN100-BO, SN100-LBO and SN500-LBO) induced no developmental toxicity in the ZET or mEST. However, it is interesting to observe that two of the three base oil samples, SN100-LBO and SN500-LBO, induced only a transient AhR activation in the AhR CALUX assay following 6 h exposure. The predominant class of aromatics in base oils is formed by non-hazardous highly alkylated 1- or 2-ring aromatics (Carrillo et al., 2022). Thus, the transient AhR activation induced by lubricating base oil could be explained by the presence of a type of highly alkylated 1- or 2-ring aromatics in these substances. HRBO are produced by hydrotreating the base oils, either once (SN100-TWO) or twice (SN100-MWO), respectively. Both SN100-TWO and SN100-MWO tested negative in the AhR CALUX assay, mEST, and ZET in the present study, which is in line with previous studies that reported that HRBO tested negative for developmental toxicity both in vivo and in vitro (Feuston et al., 1994; Kamelia et al., 2020). Waxy brightstock, a heavy lubricating stock, is derived from vacuum residuum (Wauquier, 1995). Waxy brightstock substances could be further processed into brightstock slackwax and microcrystalline wax by dewaxing, deoiling and/or hydrotreatment. All these petroleum substances-derived waxes (samples WBS, BSSW, MCW, HGMCW) tested negative in the AhR CALUX assay, mEST, and ZET. Altogether, our results indicate that refining processes efficiently reduced the amounts of hazardous PACs (mainly 3- to 7-ring PACs) in waxy raffinates and the subsequent products to a level that induced no longer developmental toxicity even if 1- or 2- ring aromatics are present.

It should be mentioned that the refined petroleum substances generated from the same crude oil feedstock could have a different viscosity, for example SN100 products have lower viscosity compared to SN400/500 products. Petroleum substances with higher viscosity may contain predominantly high molecular weight substituted PACs (e.g., with long alkyl side chains) because high molecular weight is related to alkyl chain length (Carrillo et al., 2022). On the other hand, the hazardous aromatics are typically the 3- to 7-ring PACs with no or low degree of alkylation, that can be efficiently and selectively removed from feedstock during the refining processes (e.g., solvent extraction and hydrotreatment). However, the highly alkylated 1- or 2-ring aromatics are not removed completely and may remain in the raffinate. They are intrinsic constituents of some petroleum substances, particularly those with high viscosity but do not pose developmental concerns (Carrillo et al., 2022).

Most petroleum substances are produced or registered at a volume of ≥1000 tonnes/year thus are required to be tested for their effects on prenatal development under the EU REACH (Registration, Evaluation, Authorization, and Restriction of Chemicals) regulation. If this is all done according to the current OECD TG 414 (OECD, 2018), then a huge number of experimental animals (rodent and non-rodent) and resources would be required. Currently, there is no standardized testing method established for assessing the developmental toxicity of highly complex materials like petroleum substances, as it is still unclear what lowest level of total 3- to 7-ring PACs in the petroleum substances would induce in vivo developmental toxicity. It is worth noting that the samples tested negative for in vitro developmental toxicity in this study are all raffinates or derived from raffinates (the primary feedstock for producing base oils and wax substances), and all pass the IP346 test (Figure 1). IP346 is a gravimetric method based on a DMSO extraction to determine the total PAC in base oils (also known as mineral oils). The percentage of DMSO extracts by IP346 method can be used for the prediction of the carcinogenic potential for mineral oils. The cut-off of IP346 is 3% w/w thus samples are considered non-dermally carcinogenic if the % DMSO extract is less than 3% w/w (Carrillo et al., 2022). The group of 3- to 7-ring PACs (naked and/or lowly alkylated) as shown here, is not only associated with mutagenicity/carcinogenicity of heavy petroleum substances, but also developmental toxicity. Therefore, it can be assumed that the usefulness of the IP346 test could be extended beyond the carcinogenicity assessment of mineral oils (and other relevant streams) since it may also capture the developmental toxicity of these substances as well. Altogether, the proposed in vitro testing battery (i.e., AhR CALUX assay, mEST and ZET) in combination with the simple yet robust analytical method IP346 test may be applied to evaluate the developmental toxicity potency of particularly petroleum substances derived from raffinates (i.e., the refined petroleum substances and waxes) and raffinates themselves. Such a joint approach (i.e., combination of the IP346 test with the proposed in vitro testing strategy) might provide a more reliable prediction than using only the battery of in vitro assays, and help with screening the in vitro developmental toxicity of petroleum substances from different categories to set priorities for subsequent in vivo testing. The applicability of such a joint approach would need to be further investigated.

To conclude, the present study extends the usefulness of a test battery, including the AhR CALUX assay, mEST and ZET for the developmental toxicity testing (and mode-of-action investigation) of extracts of highly complex petroleum substances, produced by different refining processes. Our results suggest that the highly refined petroleum products with

extremely low level or devoid of PACs do not induce developmental toxicity and confirm that the persistent activation of the AhR is relevant to the in vitro developmental toxicity of the unrefined petroleum substances obtained in the mEST and ZET. Data obtained from the proposed in vitro testing strategy together with the IP346 test could be further used to facilitate read-across from petroleum substances for which in vivo data are already available, to the petroleum substances for which in vivo data are lacking, thereby ultimately contributing to reducing the number of experimental animals required to assess developmental toxicity of petroleum substances regulated under REACH.

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Supplementary material 1

Table S1. The extended-GMS system as described by Beekhuijzen et al. (2015). This scoring system contains two parts: 1) general development and 2) dysmorphogenic endpoints. In the general development part, endpoints no. 1-5 are evaluated at 24 hpf, endpoints 1-8 are evaluated at 48 hpf, endpoints 1-11 are evaluated at 72 hpf, and endpoints 1-12 are evaluated at 96 hpf. The 6 dysmorphogenic endpoints are only evaluated at 96 hpf.

No.	**1). General Development Endpoints**	**Scores**			
		24 hpf	**48 hpf**	**72 hpf**	**96 hpf**
1	Detachment of the tail	2	3	3	3
2	Somite formation	1	1	1	1
3	Eye development & pigment	2	3	3	3
4	Movement	1	1	1	1
5	Circulation	1	1	1	1
6	Heartbeat		1	1	1
7	Pigmentation of the head and the body		1	1	1
8	Pigmentation of the tail		1	1	1
9	Hatching			1	1
10	Pectoral fin			1	1
11	Protruding mouth			2	2
12	Yolk extension nearly empty				1
	Total GMS score	7	12	16	17
No	**2). Dysmorphogenic endpoints**	**Scores**			
		24 hpf	**48 hpf**	**72 hpf**	**96 hpf**
1	No yolk sac edema				1
2	No pericardial edema				1
3	No malformed tail				1
4	No deformed body shape				1
5	No malformed head and jaw				1
6	No malformed sacculi/otoliths				1

Supplementary material 2

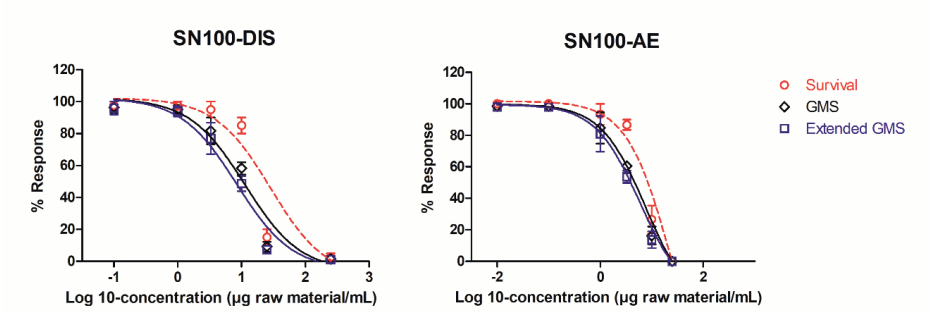


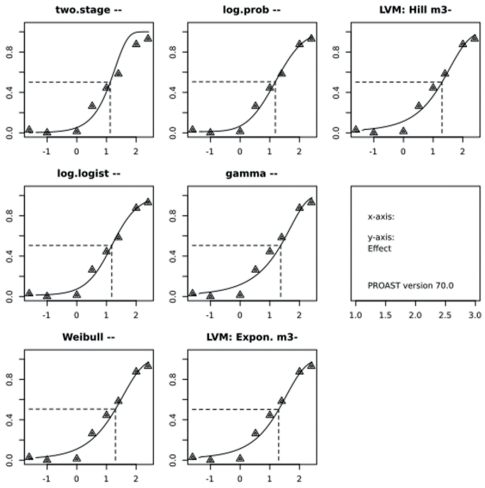
Figure S2. Concentration-dependent effects of sample SN100-DIS and SN100-AE on embryo survival (red dotted line with unfilled circle symbol), GMS (black line with unfilled diamond symbols) and extended-GMS (blue line with unfilled square symbols) at 96 hpf in the ZET. Results represent data from 3 independent ZET experiments and are presented as mean percentage ± standard error of mean (SEM).

Supplementary material 3

Results from BMD analysis of the EST and ZET data for petroleum substances samples SN1—DIS and SN100-AE tested in the present study, based on the obtained EST (cell differentiation) and ZET (embryo lethality, GMS and extended-GMS at 96 hpf) data. Concentration-response curves obtained in the EST or ZET were fitted to all quantal concentration-response models (i.e., two-stage, log logistic, weibull, log probit, gamma, logistic, probit, exponential, and hill models) available in the EFSA BMD modelling web-tool (https://shiny-efsa.openanalytics.eu/). For BMC50 determination, the benchmark response (BMR) was set at 50%, representing the concentration that induces a 50% reduction of cell differentiation in the EST, and embryo lethality, GMS or extended GMS at 96 hpf in the ZET.

Table. S2 (A) Results from BMD analysis of the data on EST of SN100-DIS based on the EST cell differentiation data. The table presents characteristics of fitted models, the AIC values, and the benchmark concentration for 50% effect (BMC50). The selected model (with lowest AIC) is heighted in yellow.

model	No.par	loglik	AIC	accepted	BMDL	BMDU	BMD	conv
null	2	-128.68	261.36		NA	NA	NA	NA
full	9	-66.56	151.12		NA	NA	NA	NA
two.stage	4	-82.73	173.46	no	NA	NA	13.3	yes
log.logist	4	-68.71	145.42	yes	9.64	24.0	15.1	yes
Weibull	4	-70.33	148.66	no	NA	NA	20.1	yes
log.prob	4	-68.39	144.78	yes	9.79	24.1	15.3	yes
gamma	4	-71.45	150.90	no	NA	NA	23.3	yes
LVM: Expon. m3-	4	-70.97	149.94	no	NA	NA	20.0	yes
LVM: Hill m3-	4	-70.95	149.90	no	NA	NA	20.0	yes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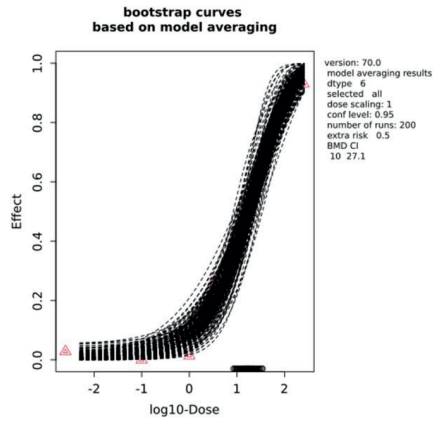
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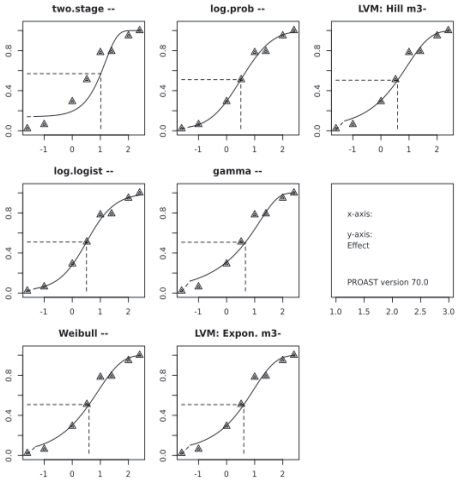
Table. S2 (B) Results from BMD analysis of the data on EST of SN100-AE based on the EST cell differentiation data. The table presents characteristics of fitted models, the AIC values, and the benchmark concentration for 50% effect (BMC50). The selected model (with lowest AIC) is heighted in yellow.

model	No.par	loglik	AIC	accepted	BMDL	BMDU	BMD	conv
null	2	-132.20	268.40		NA	NA	NA	NA
full	9	-68.98	155.96		NA	NA	NA	NA
two.stage	4	-81.04	170.08	no	NA	NA	10.30	yes
log.logist	4	-70.05	148.10	yes	1.77	5.71	3.21	yes
Weibull	4	-70.51	149.02	yes	2.03	7.07	3.93	yes
log.prob	4	-69.87	147.74	yes	1.76	5.82	3.21	yes
gamma	4	-71.66	151.32	no	NA	NA	4.68	yes
LVM: Expon. m3-	4	-70.83	149.66	yes	2.43	7.28	4.09	yes
LVM: Hill m3-	4	-70.77	149.54	yes	2.00	7.22	3.92	yes



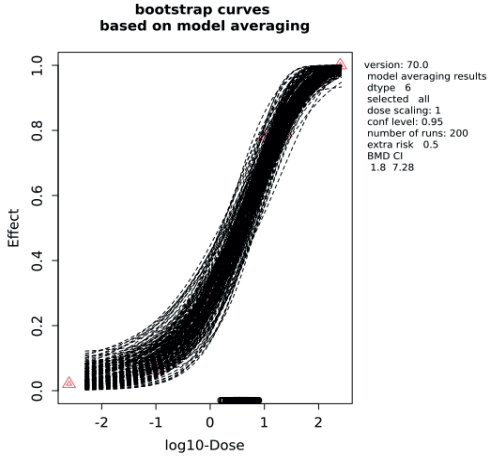
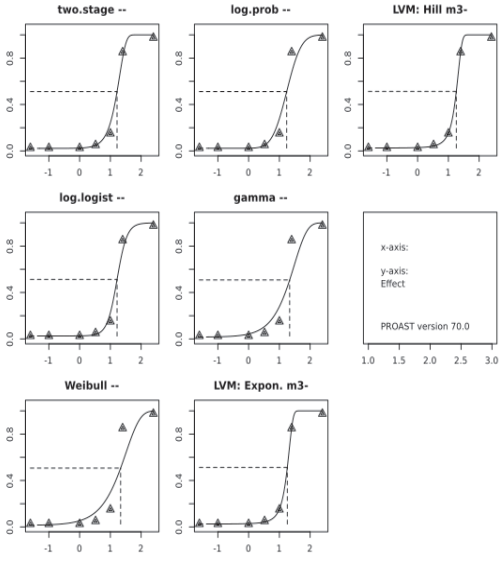


Table. S2 (C) Results from BMD analysis of the data on EST of SN100-DIS based on the embryo lethality data in the ZET. The table presents characteristics of fitted models, the AIC values, and the benchmark concentration for 50% effect (BMC50). The selected model (with lowest AIC) is heighted in yellow.

model	No.par	loglik	AIC	accepted	BMDL	BMDU	BMD	conv
null	2	-42.77	89.54		NA	NA	NA	NA
full	8	-15.12	46.24		NA	NA	NA	NA
two.stage	4	-16.03	40.06	yes	11.9	24.3	16.5	no
log.logist	4	-16.24	40.48	yes	10.8	27.2	16.5	yes
Weibull	4	-18.71	45.42	no	NA	NA	21.5	yes
log.prob	4	-16.98	41.96	no	NA	NA	17.3	yes
gamma	4	-18.76	45.52	no	NA	NA	21.6	yes
LVM: Expon. m3-	4	-15.69	39.38	yes	12.2	24.2	18.1	yes
LVM: Hill m3-	4	-15.69	39.38	yes	12.2	23.9	18.1	yes

BMC50 = (18.1+18.1)/2=18.1



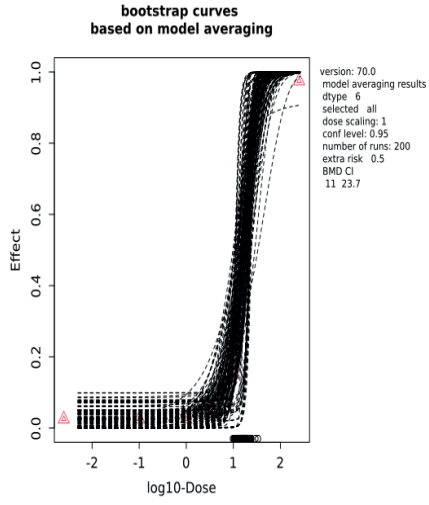
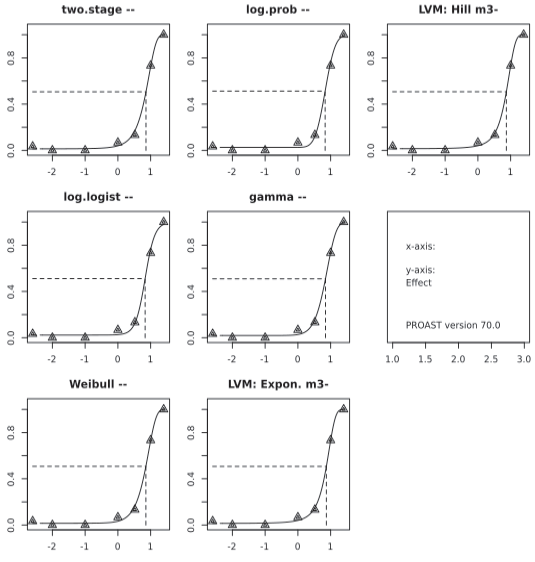


Table. S2 (D) Results from BMD analysis of the data on EST of SN100-AE based on the embryo lethality data in the ZET. The table presents characteristics of fitted models, the AIC values, and the benchmark concentration for 50% effect (BMC50). The selected model (with lowest AIC) is heighted in yellow.

model	No.par	loglik	AIC	accepted	BMDL	BMDU	BMD	conv
null	2	-41.56	87.12		NA	NA	NA	NA
full	8	-13.66	43.32		NA	NA	NA	NA
two.stage	4	-14.17	36.34	yes	4.71	10.8	7.21	yes
log.logist	4	-14.58	37.16	yes	4.11	10.4	6.79	yes
Weibull	4	-14.21	36.42	yes	4.51	10.7	7.15	yes
log.prob	4	-14.52	37.04	yes	4.10	10.3	6.80	yes
gamma	4	-14.34	36.68	yes	4.36	10.5	6.96	yes
LVM: Expon. m3-	4	-14.13	36.26	yes	4.69	10.9	7.41	yes
LVM: Hill m3-	4	-14.13	36.26	yes	4.69	10.9	7.40	yes

BMC50 = (7.41 +7.40)/2 = 7.41



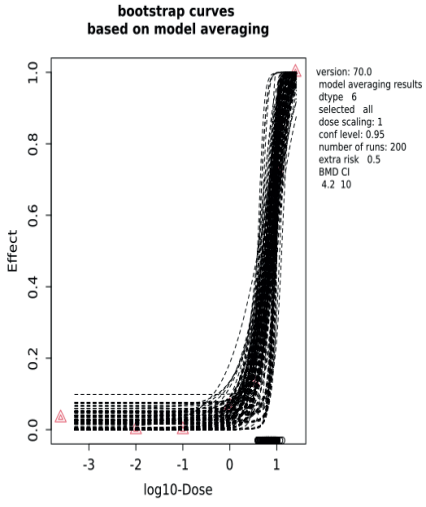
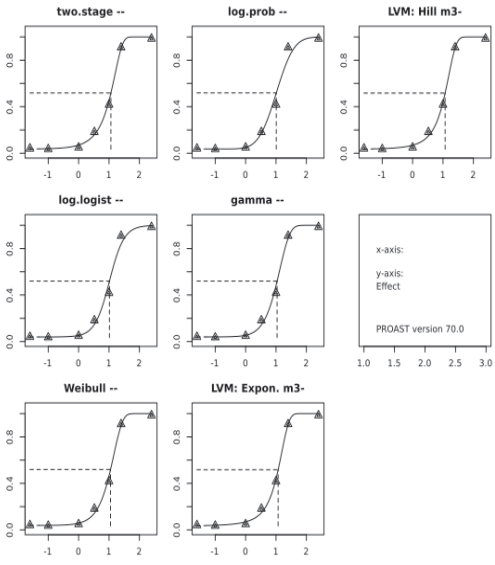


Table. S2 (E) Results from BMD analysis of the data on EST of SN100-DIS based on the GMS data in the ZET. The table presents characteristics of fitted models, the AIC values, and the benchmark concentration for 50% effect (BMC50). The selected model (with lowest AIC) is heighted in yellow.

model	No.par	loglik	AIC	accepted	BMDL	BMDU	BMD	conv
null	2	-46.31	96.62		NA	NA	NA	NA
full	8	-20.52	57.04		NA	NA	NA	NA
two.stage	4	-20.88	49.76	yes	6.56	18.4	11.5	yes
log.logist	4	-21.06	50.12	yes	5.76	18.5	10.3	yes
Weibull	4	-20.90	49.80	yes	6.55	18.1	11.2	yes
log.prob	4	-21.33	50.66	yes	5.63	19.4	10.2	yes
gamma	4	-20.96	49.92	yes	6.49	18.0	10.9	yes
LVM: Expon. m3-	4	-20.88	49.76	yes	6.75	18.6	11.7	yes
LVM: Hill m3-	4	-20.88	49.76	yes	6.74	18.6	11.7	yes

BMC50 = (11.5 + 11.7 + 11.7)/3 = 11.63



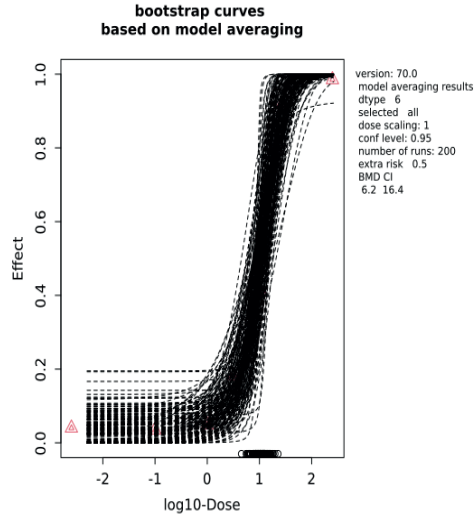
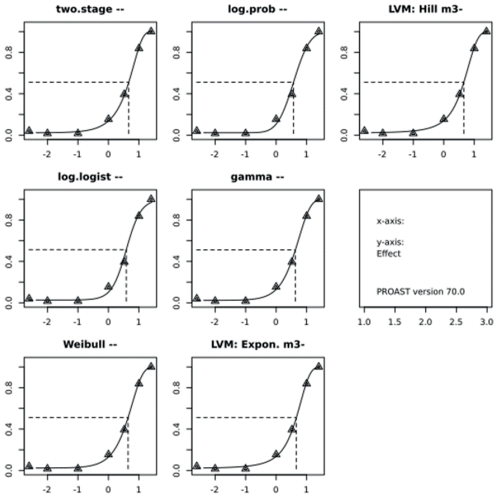


Table. S2 (F) Results from BMD analysis of the data on EST of SN100-AE based on the GMS data in the ZET. The table presents characteristics of fitted models, the AIC values, and the benchmark concentration for 50% effect (BMC50). The selected model (with lowest AIC) is heighted in yellow.

model	No.par	loglik	AIC	accepted	BMDL	BMDU	BMD	conv
null	2	-45.39	94.78		NA	NA	NA	NA
full	8	-18.73	53.46		NA	NA	NA	NA
two.stage	4	-18.87	45.74	yes	2.53	7.68	4.63	yes
log.logist	4	-19.30	46.60	yes	2.10	6.75	3.85	yes
Weibull	4	-18.88	45.76	yes	2.40	7.36	4.40	yes
log.prob	4	-19.23	46.46	yes	2.07	6.67	3.73	yes
gamma	4	-18.90	45.80	yes	2.43	7.20	4.29	yes
LVM: Expon. m3-	4	-18.94	45.88	yes	2.48	7.85	4.60	yes
LVM: Hill m3-	4	-18.94	45.88	yes	2.47	7.84	4.53	yes



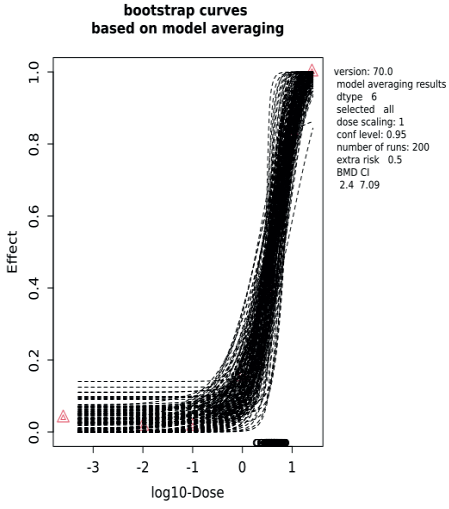
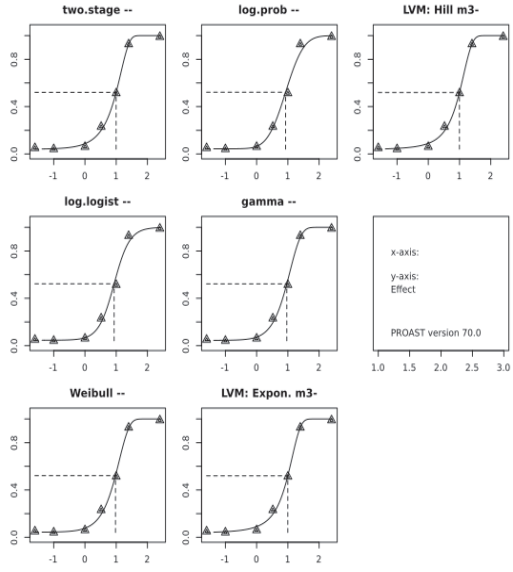


Table. S2 (G) Results from BMD analysis of the data on EST of SN100-DIS based on the extended-GMS data in the ZET. The table presents characteristics of fitted models, the AIC values, and the benchmark concentration for 50% effect (BMC50). The selected model (with lowest AIC) is heighted in yellow.

model	No.par	loglik	AIC	accepted	BMDL	BMDU	BMD	conv
null	2	-47.18	98.36		NA	NA	NA	NA
full	8	-21.51	59.02		NA	NA	NA	NA
two.stage	4	-21.76	51.52	yes	5.41	16.2	9.93	yes
log.logist	4	-21.89	51.78	yes	4.68	15.5	8.58	yes
Weibull	4	-21.76	51.52	yes	5.31	15.8	9.50	yes
log.prob	4	-22.07	52.14	yes	4.60	15.9	8.48	yes
gamma	4	-21.78	51.56	yes	5.34	15.6	9.25	yes
LVM: Expon. m3-	4	-21.79	51.58	yes	5.47	16.5	9.95	yes
LVM: Hill m3-	4	-21.79	51.58	yes	5.46	16.5	9.93	yes

BMC50 = (9.93+9.50)/2=9.72



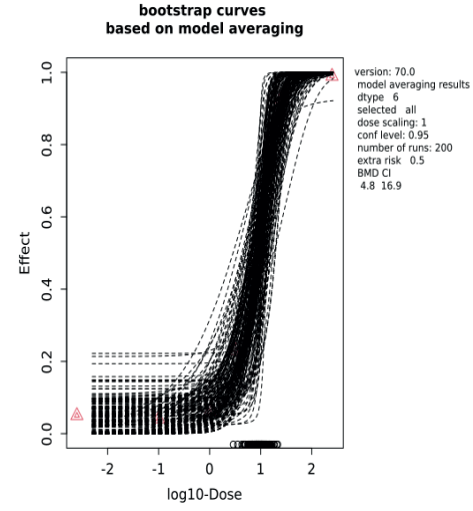
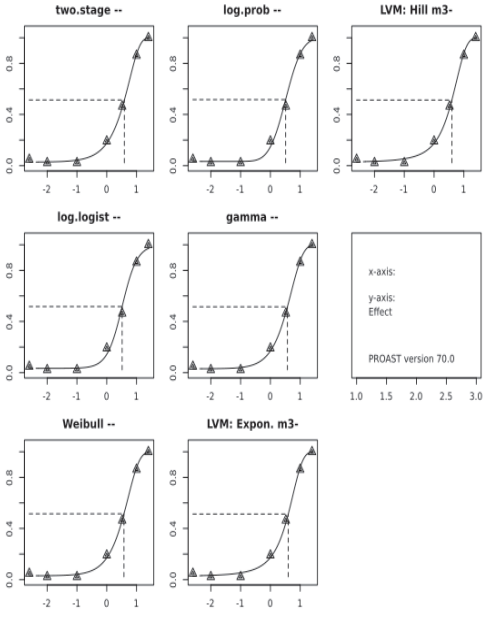
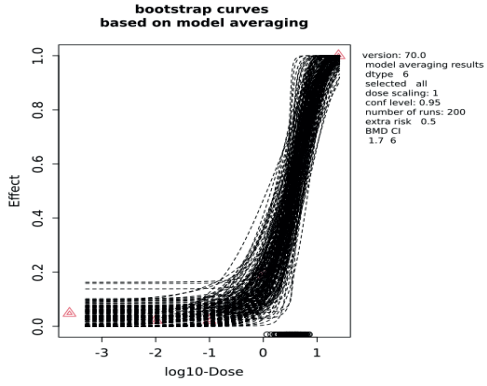


Table. S2 (H) Results from BMD analysis of the data on EST of SN100-AE based on the extended-GMS data in the ZET. The table presents characteristics of fitted models, the AIC values, and the benchmark concentration for 50% effect (BMC50). The selected model (with lowest AIC) is heighted in yellow.

model	No.par	loglik	AIC	accepted	BMDL	BMDU	BMD	conv
null	2	-46.26	96.52		NA	NA	NA	NA
full	8	-19.85	55.70		NA	NA	NA	NA
two.stage	4	-20.00	48.00	yes	2.17	6.91	3.91	yes
log.logist	4	-20.36	48.72	yes	1.72	5.92	3.29	yes
Weibull	4	-20.01	48.02	yes	1.96	6.61	3.79	yes
log.prob	4	-20.25	48.50	yes	1.70	5.82	3.25	yes
gamma	4	-20.01	48.02	yes	2.03	6.43	3.73	yes
LVM: Expon. m3-	4	-20.10	48.20	yes	2.03	7.03	3.98	yes
LVM: Hill m3-	4	-20.10	48.20	yes	2.03	7.03	3.97	yes





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CHAPTER 5.

Predicting the developmental toxicity of 8-methyl-benzo[a] pyrene by physiologically-based kinetic (PBK) modelling-facilitated reverse dosimetry and read across from benzo[a]pyrene

Danlei Wang, Jing Fang, Miaoying Shi, Lenny Kamelia, Ivonne M.C.M Rietjens, Peter J. Boogaard

Manuscript in preparation

Abstract

The present study predicts an in vivo dose response curve for developmental toxicity of 8-methyl-benzo[a]pyrene (8-MeBaP) in rats using PBK model facilitated reverse dosimetry and read across from BaP. Given the lack of in vivo animal data, the present model and predictions for 8-MeBaP were validated using read across from the predicted and reported in vivo data on the kinetics and developmental toxicity of BaP. In line with BaP, in vitro developmental toxicity of 8-MeBaP in the mouse embryonic stem cell test (mEST) was shown to require metabolic activation to its 3-hydroxy metabolite (3-OH-8-MeBaP). To facilitate extrapolation of the in vitro data on the developmental toxicity of 3-OH-8-MeBaP to an in vivo dose response curve a PBK model for 8-MeBaP with a sub-model of 3-OH-8-MeBaP was built based on the previously developed PBK model for BaP that was validated with in vivo toxicokinetic data in rats. The predicted half maximal effect dose (ED_{50})for developmental toxicity of 8-MeBaP that was derived from the predicted in vivo dose response curve was 1.3-fold lower to that of BaP for which the predicted value was in line with the available in vivo data on developmental toxicity. The present study illustrates how new approach methodologies (NAM) can be applied to predict developmental toxicity of a methyl substituted PAH for which in vivo data are not available, by PBK modeling facilitated read across from a non-substituted PAH for which in vivo data have been reported. The method also elucidates how a methyl substituent could affect the kinetics and subsequent developmental toxicity of a non-substituted PAH.

1. Introduction

Petroleum substances on the EU market are required to be evaluated for their effect on prenatal developmental toxicity in accordance with the REACH (Registration, Evaluation, Authorization, and Restriction of Chemical) legislation (EC 2006; ECHA 2009). Given the large number of experimental animals potentially needed for safety testing of chemical substances according to the current OECD 414 guideline (OECD 2018) , the use of alternative testing strategies for the hazard assessment of chemical substances is promoted and supported (ECHA 2014). The development and application of alternative testing strategies for assessing the developmental toxicity of highly complex petroleum substances are highly relevant and important in view of next generation risk assessment (NGRA).

Developmental toxicity as observed with some petroleum substances has been associated with the presence of certain polycyclic aromatic compounds (PACs), including polycyclic aromatic hydrocarbons (PAHs), in these products (Dalbey et al. 2014; Feuston et al. 1996; Feuston et al. 1997; Kamelia et al. 2017; Murray et al. 2013). The hazardous aromatic substances in petroleum-derived products comprise 3- to 7-ring polycyclic aromatic hydrocarbons (PAHs) that may be unsubstituted or lowly alkylated (Carrillo et al. 2022). Limited studies on developmental toxicity of the 3- to 7- ring PAHs have been reported in experimental animals. The offspring of pregnant rats exposed to benzo[a]pyrene (BaP), a 5 ring PAH, was affected with regard to embryo lethality, fetal body weight and incidence of resorptions (Archibong et al. 2002; Bui et al. 1986). Developmental toxicity of BaP was shown in the mouse embryotic stem cell test (mEST) to require metabolic activation to its major metabolite 3-hydroxy-benzo[a]pyrene (3-OHBaP) (Kamelia et al. 2020). Recent findings in the zebrafish embryotoxicity test (ZET) showed that methyl substitution on PAHs causes developmental retardation in zebrafish embryos, compared to effects induced by their unsubstituted parent PAHs. The most potent PAH was 8-methyl-benzo[a]pyrene (8-MeBaP) with a BMC_{20} value that was 10 times lower than that of BaP (Fang et al. 2022). 3-Hydroxy-8-methyl-BaP (3-OH-8-MeBaP) was the major oxidative aromatic ring metabolite of 8-MeBaP in both rat liver microsomal and S9 incubations (Wang et al. 2022) as shown in Figure 1. It is hypothesized that 8-MeBaP follows a similar bioactivation pathway as BaP with regard to the induction of developmental toxicity.

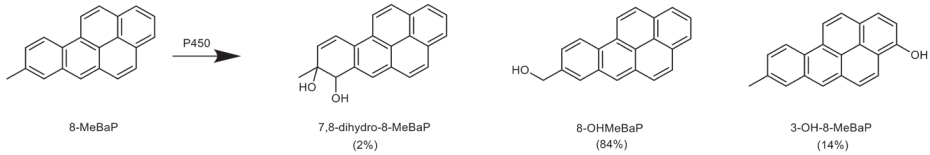

Figure 1. Metabolic profile of 8-MeBaP by liver microsomes in rats (Wang et al. 2022). The percentage values shown in the brackets were calculated from the concentration of the metabolite divided by the total concentration of metabolites formed in microsomal incubations.

Our recent study supported an in vitro-in silico approach to predict the in vivo developmental toxicity of BaP in rats using physiologically-based kinetic (PBK) model-facilitated reverse dosimetry (Wang et al. 2021). The predicted blood concentrations of BaP and 3-OHBaP were sufficiently validated by comparison to results from toxicokinetic studies in rats (Marie et al. 2010; Moreau and Bouchard 2015). The prediction of the developmental toxicity of BaP upon oral exposure was based on the concentration response curves of 3-OHBaP obtained in the mEST, and the predicted in vivo dose response curves for developmental toxicity of BaP matched the literature available dose response curves from in vivo developmental toxicity studies (Archibong et al. 2002; Bui et al. 1986), thereby validating the alternative testing strategy. The aim of the present study was to predict the developmental toxicity of 8-MeBaP using PBK model facilitated reverse dosimetry and read across from BaP based on the same in vitro-in silico approach previously shown successful for BaP (Wang et al. 2021). This will facilitate further read across in the hazard and risk assessment of alkyl substituted PAHs for which developmental toxicity data have not been reported.

2. Material and Method

2.1 Materials

BaP (≥99%) was obtained from Sigma Aldrich (St.Louis, United States). 3-OHBaP was purchased from TRC Canada (Toronto, Canada). 8-MeBaP (≥98%) and 3-OH-8-MeBaP (≥99%) were custom-synthesized at the Biochemical Institute for Environmental Carcinogens ((Groβhansdorf, Germany).

The mouse embryonic multipotent stem cell line ES-D3 (CRT-1934™), cell basal medium (SCRR-2011™) and fetal bovine serum (SCRR-30-2020™) were obtained from ATCC (Wesel, Germany). 2 mM L-glutamine and 50 µg/ml penicillin-streptomycin were supplied by Invitrogen (Carlsbad,

United States). Cell dissociation solution, 5-fluorouracil (5-FU), 2-mercaptoethanol, non-essential amino acids, cell proliferation reagent WST-1 and murine Leukemia Inhibiting Factor (LIF) were bought from Sigma Aldrich (Schnelldorf, Germany).

Pooled liver S9 from male Sprague Dawley rats was purchased from Tebu-Bio (Heerhugowaard, The Netherlands). Uridine 5'diphosphoglucuronic acid (UDPGA) was obtained from Carbosynth (Compton, United Kingdom). Magnesium chloride, dimethyl sulfoxide (DMSO), trifluoroacetic acid (TFA), and acetonitrile were purchased from Merck (Darmstadt, Germany). Alamethicin and 3'-phosphate 5'-phosphosulfate (PAPS) lithium salt were bought from Sigma Aldrich (Zwijndrecht, The Netherlands).

2.2 Methods

2.2.1 In vitro concentration response towards 8-MeBaP and 3-OH-8-MeBaP in the mEST

2.2.1.1 Cell culture

The multipotent mouse ES-D3 cell line was maintained in 0.1% gelatin precoated 25 cm^2 polystyrene cell culture flasks in 5 ml supplemented cell basal medium. The medium was supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 50 µg/ml penicillin-streptomycin, 0.5 mM 2-mercaptoethanol and 1% non-essential amino acids. The ES-D3 cell line was sub-cultured each 2-3 days and maintained at 37 °C with 5% CO_2 in a humidified atmosphere. Cell dissociation solution was used to detach the cells. 1000 U/ml LIF was added to keep the cells undifferentiated.

2.2.1.2 ES-D3 cell differentiation and cytotoxicity assay

The developmental toxicity of the test compounds was assessed by the inhibitory effect on the differentiation of cells of the ES-D3 cell line into contracting cardiomyocytes. The assay was performed essentially as previously described (Kamelia et al. 2020). The cells were cultured on the lid of a 96 well plate in 45 hanging drops (volume 20 µl/drop) at a concentration of 37500 cells/ml. The 96 wells were filled with 250 µl PBS to maintain the humidity. Sterile Eppendorf lids were placed at the corners of the 96 well plate to prevent the hanging drops from contacting the 96 well plate. The plates were sealed with micropore tape and incubated at 37 °C with 5% CO_2 in a

humidified atmosphere for 72 hours. After 72 hours, 5 ml exposure medium was prepared for each concentration of each test compound with 0.25% DMSO in a 60×15mm petri dish. The formed embryonic bodies (EBs) in the hanging drops on the lid of the 96 well plate were washed off with exposure medium and collected in the petri dish. The petri dish was subsequently incubated at 37 °C with 5% CO_2 in a humidified atmosphere for 48 hours. After 48 hours, the EBs were transferred to a 24 well plate filled with 1 ml exposure medium per well. Each well contained 1 EB. The 24 well plates were incubated at 37 °C with 5% CO_2 in a humidified atmosphere for 120 hours. The number of EBs with contracting cardiomyocytes and the undifferentiated EBs were counted under a microscope. In the solvent (DMSO) control, the presence of more than 21 EBs with contracting cardiomyocytes out of 24 EBs indicates a valid assay. In each experiment, 0.5 µM (0.065 µg/ml) 5-FU was used as a positive control. The result of the differentiation assay is expressed as the fraction of differentiated EBs of the total number of EBs.

The viability of the ES-D3 cells following 1-day and 5-day exposure to the test compounds was measured with the WST-1 assay. To this end 100 µl cell suspension were seeded in the inner 60 wells of 96 well plates at a concentration of 200000 cells/ml for 1-day exposure and 10000 cells/ml for 5-day exposure and incubated at 37 °C with 5% CO_2 for 24 hours. After 24 hours, 100 µl exposure medium with 0.25% DMSO and the required concentration of the test compounds, solvent control, and positive control was added to the cells in triplicate. The plates were incubated at 37 °C with 5% CO_2 for 1 day and 5 day until measurement. After the exposure period, 20 µl of the cell proliferation reagent WST-1 were added to each well that contained cells and incubated for 3 hours at 37 °C with 5% CO_2. The absorbance of each plate was measured at 440nm and 620nm with a SpectraMax iD3 (San Jose, USA). The result was expressed as cell viability percentage following incubation with the test compounds compared to the solvent control, calculated from the measured absorbance at 440nm minus the background absorbance at 620nm.

2.2.1.3 Data analysis

The concentration response data for the test compounds in the EST were presented in three curves including curves for 1-day cytotoxicity, 5-day cytotoxicity and differentiation. The obtained curves were fitted using the inhibition equation log (inhibitor) vs response (three parameters) option using GraphPad Prism software 5.0 (California, USA). The concentration of the test compound that

resulted in 50% inhibitory effect on differentiation and cytotoxicity was derived and expressed as IC_{50}.

2.2.2 Obtaining kinetic parameters for metabolism of 8-MeBaP and 3-OH-8-MeBaP

The oxidative metabolism of 8-MeBaP mediated by P450 enzymes in rat liver microsomes was characterized in our previous study (Wang et al. 2022). The kinetic parameters including K_m and V_{max}, obtained by fitting the Michaelis-Menten equation, for formation of 3-OH-8-MeBaP and for formation of the sum of all other metabolites were used in the PBK model developed for 8-MeBaP and are presented in Table 1.

Table 1. Kinetic parameter values for liver metabolism of 8-MeBaP and 3-OH-8-MeBaP in rat

[*] *Data were derived from the study of Wang et al (2022).*

Reaction	Kinetic parameters			
	V_{max}[a]	**K_m[b]**	**Scaled V_{max}[c]**	**Scaled V_{max}[d]**
8-MeBaP to 3-OH-8-MeBaP*	*0.07*	11.55	0.19	2
8-MeBaP to other metabolites*	0.43	8.07	1.18	11
	V_{max}[e]	**K_m[b]**	**Scaled V_{max}[c]**	**Scaled V_{max}[d]**
Glucuronidation of 3-OH-8-MeBaP	6.92	15.58	51.9	470
Sulfation of 3-OH-8-MeBaP	0.15	26.07	1.16	10

[a] *nmol/min/mg microsomal protein*
[b] *μM*
[c] *μmol/h/g liver*
[d] *μmol/h/liver*
[e] *nmol/min/mg S9 protein*

2.2.2.1 Glucuronidation of 3-OH-8-MeBaP

The conjugation of 3-OH-8-MeBaP with glucuronic acid was studied in incubation with rat liver S9 in vitro. The incubation time and S9 protein concentration were optimized to ascertain linearity of glucuronidation with time and the amount of S9 protein. The incubation was performed with a 200 µl mixture in glass vials, and consisted of (final concentrations) 0.1 mM Tris buffer (pH 7.4), 0.1 mg/ml pooled rat liver S9, 3 mM UDPGA, 5 mM $MgCl_2$, 0.025 mg/ml alamethicin, and 1-100 µM 3-OH-8-MeBaP. The final concentration of substrate vehicle (DMSO) in the incubation

mixture was 1% (v/v). The incubation mixtures without substrate were preincubated at 37°C for 1 min. The reaction was initiated by adding substrate to the incubation mixtures that were subsequently incubated at 37°C for 20 min, after which the reaction was terminated by adding 100 µl ice-cold acetonitrile. The incubation mixture was subsequently centrifuged at 3717g at 4°C for 5 minutes. The supernatant was collected for Ultra Performance Liquid Chromatography (UPLC) analysis.

2.2.2.2 Sulfation of 3-OH-8-MeBaP

The conjugation of 3-OH-8-MeBaP by sulfation was studied in incubations with rat liver S9 in vitro. The incubation time and S9 protein concentration were optimized to ascertain linearity of the sulfation reaction with respect to time and amount of protein. The 200 µl incubation mixture consisted of (final concentrations) 0.1 mM Tris buffer (pH 7.4), 0.1 mg/ml rat liver S9, 0.2 mM PAPS and 1-100 µM 3-OH-8-MeBaP. After 1-min preincubation of the incubation mixture without substrate, the reaction was initiated by adding substrate from a 100 times concentrated stock solution in DMSO (final concentration 1% DMSO (v/v)). The incubation mixture was incubated at 37°C for 70 min after which 100 µl ice-cold acetonitrile was added to the incubation mixture to terminate the reaction. The incubation mixture was subsequently centrifuged at 3717g at 4°C for 5 minutes. The supernatant was collected for subsequent UPLC analysis.

2.2.2.3 UPLC Analysis

An Acquity UPLC system with a photodiode array (PDA) detector was used to quantify the metabolites formed in the glucuronidation and sulfation incubations. The supernatants of the incubation samples were injected onto an Acquity UPLC BEH® C18 column (21 × 50mm, 1.7µm, Waters, Milford, MA). The total running time per sample was 22 min with a flow rate of 0.6 ml/min. The temperature of column and auto sampler was 40°C and 10°C respectively. The injection volume was 3.5 µl and the detection wavelength ranged from 190 to 400 nm. Two eluents were used including A (nanopure with 0.1% (v/v) TFA) and B (acetronitrile with 0.1% (v/v) TFA). The gradient elution started from 90% A and 10% B applied from 0.0 min to 0.5 min, which was changed to 10% A and 90% B from 0.5 to 15.5 min and then kept at 10% A and 90% B from 15.5 min to 18.5 min, and changed back to 90% A and 10% B from 18.51 to 22min.

2.2.2.4 Data analysis

The concentration of the glucuronidated and sulfated metabolites formed in incubations of 3-OH-8-MeBaP and pooled rat liver S9 were quantified and further used to calculate the velocity of the enzymatic reaction expressed in pmol/min/mg S9 protein. The curve reflecting the substrate (3-OH-8-MeBaP) concentration dependent velocity of the metabolite formation was fitted by the Michaelis-Menten equation using GraphPad Prism 5 (California, USA). The kinetic constants derived included the K_m (μM) and V_{max} (nmol/min/mg S9 protein).

2.2.3 PBK model for 8-MeBaP in rats

The PBK model for 8-MeBaP was developed based on the model for BaP that was previously validated by comparison of predictions made to available in vivo toxicokinetic data (Wang et al. 2021). Figure 2 presents the conceptual model that simulates absorption, distribution, metabolism and excretion of 8-MeBaP in rats. The model includes several organ and tissue compartments that are connected to the systematic circulation. The model of 8-MeBaP contains a sub-model for 3-OH-8-MeBaP since the developmental toxicity of 8-MeBaP needs bioactivation to 3-OH-8-MeBaP as shown in the present study by results obtained in the mEST.

Exposure to 8-MeBaP was described in the model for the intravenous, intratracheal and oral route. The uptake of 8-MeBaP was assumed to be complete and to occur with (1) a transport rate Kd=1000000h^{-1} from needle to blood, (2) an absorption rate Kt=1h^{-1} from trachea to lung and (3) an absorption rate Ka=1h^{-1} from stomach to gastrointestinal (GI)-tract followed by uptake form the GI tract compartments into the liver with a kabin (Papp*SAin) of 0.003 L/hr (Sun et al. 2002; Zhang et al. 2018). The intestine was divided to 7 sub-compartments (Wang et al. 2021; Zhang et al. 2018). The permeability of coefficient (Papp in vivo) of 8-MeBaP was assumed to be the same as BaP being 0.02 dm/hr when 8-MeBaP is taken up from the intestine to liver (Sun et al. 2002).

In liver, 8-MeBaP was assumed to be metabolized by cytochrome P450 enzymes to 3-OH-8-MeBaP and other metabolites, including the side chain hydroxylated metabolite 8-hydroxymethyl-BaP (8-OHMeBaP) (Figure 1) and dihydrodiols (Wang et al. 2022). 3-OH-8-MeBaP was subsequently modelled to be conjugated by glucuronidation and sulfation in the liver. The kinetic constants for these conjugation reactions were determined in incubation experiments described in

section 2.2.2, the kinetic constants for the microsomal cytochrome P450 catalyzed reactions were taken from literature(Wang et al. 2021). The scaling factors from rat liver microsomes and rat liver S9 to the whole liver were 45 mg microsomal protein/g liver and 125 mg S9 protein/g liver, respectively (Houston and Galetin 2008). The oxidative metabolism of 8-MeBaP and conjugation of 3-OH-8-MeBaP in lung was considered to be negligible, based on what was found for BaP (Wang et al. 2021).

Table 2 summarizes the physiochemical parameters of 8-MeBaP and 3-OH-8-MeBaP and the physiological parameter values including body weight, fractional tissue volumes and fractional tissue blood flow of rats (Brown et al. 1997; Crowell et al. 2011; Marie et al. 2010; Moreau and Bouchard 2015) used in the PBK model. The tissue:blood partition coefficients of 8-MeBaP and 3-OH-8-MeBaP were calculated using the Berezhkovskly method in the quantitative in vitro to in vivo extrapolation (QIVIVE) tool developed by Wageningen Food Safety Research (WFSR) (https://wfsr.shinyapps.io/wfsrqivivetools/)(Berezhkovskiy 2004; Punt et al. 2021), and are also presented in Table 2.

Given the lipophilic properties of 3-OH-8-MeBaP, a blood protein compartment was included to prevent 3-OH-8-MeBaP from partitioning into the fat tissue. The unbound fraction (fub, in vivo) of 3-OH-8-MeBaP in the blood compartment was calculated using the Lobell and Sivarajah method in the previously mentioned QIVIVE tool developed by WFSR (Lobell and Sivarajah 2003). Biliary excretion of 8-MeBaP and 3-OH-8-MeBaP to feces was included with an assumed excretion constant Kb=1 from liver to feces.

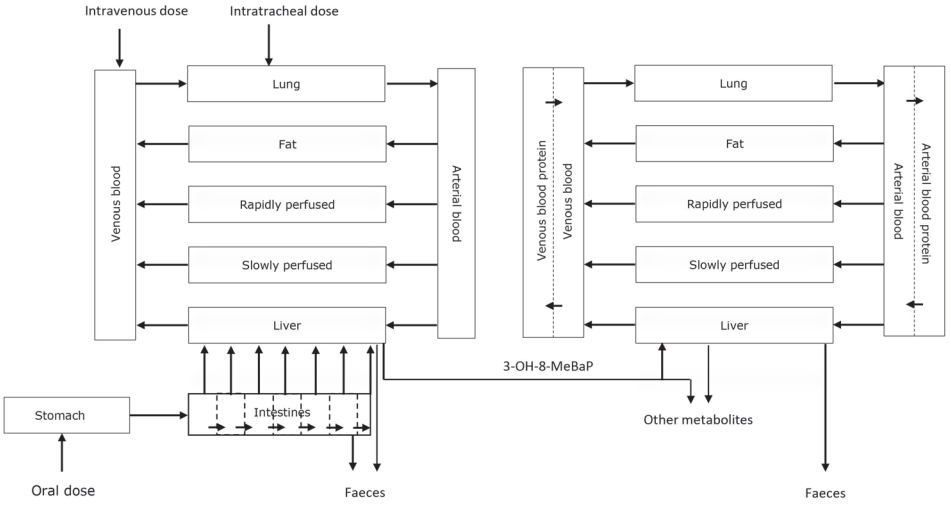

Figure 2. Structure of the PBK model of 8-MeBaP with a sub-model for 3-OH-8-MeBaP in rats, in analogy to the PBK model previously developed and validated for BaP and its active metabolite 3-OHBaP(Wang et al. 2021)

Table 2. Physiological, anatomical and physicochemical parameter values for BaP and 3-OHBaP for the rat PBK model.

Model parameter	Symbol	Value	Reference
Physiological parameters			
Body weight	BW	0.245[a]	Marie et al. (2010)
Fractional tissue volumes			Moreau and Bouchard (2015)
Fat	VFc	0.065	DeJongh
Liver	VLc	0.037	
Lung	VLuc	0.005	
Arterial blood	VABc	0.0257	
Venous blood	VVBc	0.0514	
Rapidly perfused tissue	VRc	0.2159	
Slowly perfused tissue	VSc	0.6	
Cardiac output (mL/s)	QC	$15*BW^{0.74}$	
Fractional tissue blood flows			
Fat	QFc	0.07	
Liver	QLc	0.183	
Lung	QLuc	1	
Rapidly perfused tissue	QRc	0.4	
Slowly perfused tissue	QSc	0.347	
Physicochemical parameters			
8-Methyl-benzo[a]pyrene			
Molecular weight	MW8MBaP	266.34	
LogP		6.7	XLogP3
Fraction unbound	f_{ub}	0.003	Calculated according to Lobell and Sivarajah (2003)
Tissue:blood partition coefficients			
Fat	PF8MBAP	434.55	Berezhkovskiy
Liver	PL8MBAP	12.85	
Lung	PLu8MBAP	14.65	
Rapidly perfused tissue	PR8MBAP	12.85	
Slowly perfused tissue	PS8MBAP	7.13	
3-hydroxy-8-methyl-benzo[a]pyrene			
Molecular weight	MW3-OH8MBaP	282.34	
LogP		6.3	XLogP3
Fraction unbound	f_{ub}	0.004	Calculated according to Lobell and Sivarajah (2003)
Tissue:blood partition coefficients			
Fat	PF3OH8MBAP	392.83	Berezhkovskiy
Liver	PL3OH8MBAP	12.55	
Lung	PLu3OH8MBAP	14.31	
Rapidly perfused tissue	PR3OH8MBAP	12.55	
Slowly perfused tissue	P3OH8MBAP	6.98	

[a] Median of body weights of rats in these two studies

2.2.4 Evaluation and sensitivity analysis of the PBK model for 8-MeBaP

Given the lack of in vivo toxicokinetic data of 8-MeBaP, the predictions from the developed PBK model were evaluated by comparison to the data from model predictions and in vivo studies for BaP which is structurally similar to 8-MeBaP. The predicted blood concentration versus time curves of 8-MeBaP, were, as those for BaP, evaluated by comparison to in vivo toxicokinetic data reported in literature for BaP (Marie et al. 2010; Moreau and Bouchard 2015).

The sensitivity analysis of the PBK model was performed to determine the influential parameters on the predicted maximum blood concentration (C_{max}) of 3-OH-8-MeBaP. Three exposure routes including intravenous, intratracheal and oral uptake of 8-MeBaP were evaluated. The normalized sensitivity coefficients (SC) were calculated using the following equation (1): $SC = \{(C' - C)/(P' - P)\} \cdot (P/C)$. In this formula, P represents the initial value of a parameter and P' the modified value with 10% increase. C represents the initial value of C_{max} of 3-OH-8-MeBaP and C' the modified value caused by the 10% increase of P. Each parameter was modified independently while other parameters remain unchanged. When changing the fractional tissue blood flows, the total blood flow of rat, liver, rapidly perfused tissue and slowly perfused tissue was maintained at 1.

2.2.5 Reverse dosimetry and read across to predict developmental toxicity of 8-MeBaP

The in vivo dose-response curve for the developmental toxicity of 8-MeBaP was assumed to be dependent on the C_{max} of unbound 3-OH-8-MeBaP in the maternal rat blood ($C_{ub,\ in\ vivo}$). It was reported that the blood concentration of BaP in maternal and foetal blood are comparable in pregnant rats (Withey et al. 1993), therefore, the same assumption was applied for 8-MeBaP eliminating the need to include a placental barrier and separate foetal compartment in the model. The relationship between the in vivo 3-OH-8-MeBaP concentration in maternal blood ($C_{in\ vivo}$) and the in vitro 3-OH-8-MeBaP concentration in the mEST ($C_{in\ vitro}$) was described by the following equation (2): $C_{in\ vivo} * f_{ub,\ in\ vivo} = C_{in\ vitro} * f_{ub,\ in\ vitro}$. The fraction unbound in blood ($f_{ub,\ in\ vivo}$) of 3-OH-8-MeBaP was calculated as 0.004 (Table 2) based on the methodology mentioned in section 2.2.3. The fraction unbound in the mEST ($f_{ub,\ in\ vitro}$) was assumed to be half of the $f_{ub,\ in\ vivo}$ based on the ratio between the protein content in rat blood plasma (7.5%) and the mEST assay medium (15%). Upon oral uptake of 8-MeBaP, the C_{max} of 3-OH-8-MeBaP was described in relation to the ODOSE (mg/kg bw) in the PBK model by the following equation (3): C_{max} (3-OH-8-MeBaP) = 0.0839* ODOSE, which was derived from the PBK model predicted linear relationship between the dose and C_{max}. The unbound concentration in

vitro ($C_{ub,\ in\ vitro}$) inducing the developmental toxicity response in mEST was converted using equation (2) to an unbound concentration in blood ($C_{ub,in\ vivo}$) that was further converted by the PBK model to a predicted oral dose.

3. Results

3.1 In vitro concentration response in the mEST

The developmental toxicity of 8-MeBaP and 3-OH-8-MeBaP was assessed in vitro by quantifying the inhibitory effect of these model compounds on the differentiation of ES-D3 cells into beating cardiomyocytes in the mEST. The concentration-response curves for the cytotoxicity and differentiation assays of the mEST for (a) 3-OH-8-MeBaP and (b) 8-MeBaP as well as for (c) 3-OHBaP and (d) BaP, the latter for comparison, are presented in Figure 3. 8-MeBaP appeared to cause a reduction in both differentiation and cell viability on Day 5 with an IC_{50} of 0.83 µM and 1.74 µM, respectively, indicating the effect on differentiation to be mainly due to cytotoxicity instead of reflecting developmental toxicity of 8-MeBaP. For 3-OH-8-MeBaP a concentration dependent inhibition of the number of beating cardiomyocytes was observed with an IC_{50} of 1.40 µM without accompanying cytotoxicity. 3-OHBaP inhibits ES-D3 cell differentiation with an IC_{50} of 1.51 µM in a concentration-dependent manner at non-cytotoxic concentrations , while BaP itself is inactive in this differentiation assay.

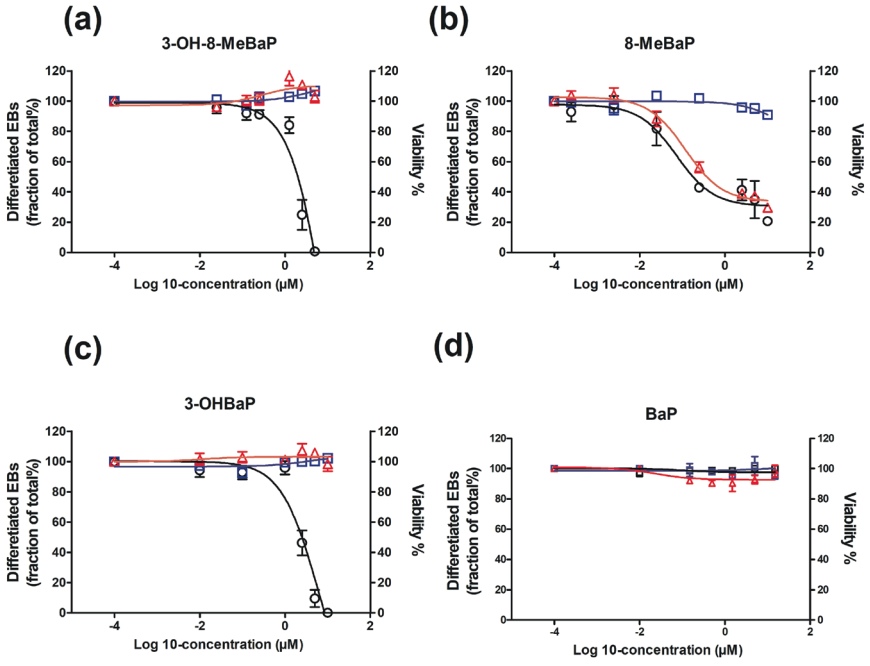

Figure 3. Concentration response curves for the effect of (a) 3-OH-8-MeBaP, (b) 8-MeBaP, (c) 3-OHBaP and (d) BaP on differentiation of ES-D3 cells into beating cardiomyocytes (black open circle and black solid line), and the accompanying 1-day cytotoxicity (blue open square and blue solid line), and 5-day cytotoxicity (red open triangle and red solid line). The presented data are the result from at least three independent experiments, shown as mean ± SEM.

3.2 Kinetics of 8-MeBaP and 3-OH-8-MeBaP in rats

The kinetics for the cytochrome P450 mediated metabolism of 8-MeBaP in rats was described for the formation of 3-OH-8-MeBaP and for the sum of other metabolites. The kinetic parameters for formation of 3-OH-8-MeBaP and for formation of the sum of other metabolites are presented in Table 1, and were obtained from our previous study (Wang et al. 2022). The V_{max} values for 3-OH-8-MeBaP formation and for the sum of the formation of other metabolites were 0.07 and 0.43 nmol/min/mg microsomal protein, respectively, amounting to 2 and 11 μmol/h/liver using the scaling factor described in section 2.2.3 and converting minutes to hours, nmol to μmol and assuming a liver weight of 9.1 g.

The UPLC analysis of the microsomal or S9 incubations showed an elution time for 3-OH-8-MeBaP of 9.32 min with a maximum wavelength of 260.8 nm. In the incubations with 3-OH-8-MeBaP as substrate, pooled rat liver S9 and UDPGA as co-factor, the glucuronide metabolite was detected at a retention time of 6.56 min with a maximum wavelength of 306.2 nm representing a peak that was not present in the control without UDPGA. In the incubations with 3-OH-8-MeBaP as substrate, pooled rat liver S9 and PAPS as co-factor, the sulfated metabolite eluted at 6.60 min with a maximum wavelength at 304.3nm.

The substrate concentration dependent glucuronidation and sulfation of 3-OH-8-MeBaP in incubations with rat liver S9 are shown in Figure 4. The kinetic curves were fitted with the Michaelis Menten equation to define the parameters V_{max} and K_m that are presented in Table 1. The V_{max} of the hepatic glucuronidation and sulfation of 3-OH-8-MeBaP were 6.92 and 0.15 nmol/min/mg S9 protein, respectively, amounting to 470 and 10 µmol/h/liver using the scaling factor described in section 2.2.3 and converting minutes to hours, nmol to µmol and assuming a liver weight of 9.1 g.

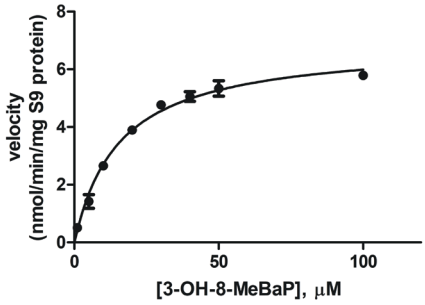

Figure 4. Substrate concentration-dependent kinetic curves of (a) glucuronidation (b) sulfation of 3-OH-8-MeBaP in incubations with rat liver S9 and the respective cofactors. Symbols represent the mean of three independent experiments, and the error bars represent the standard error of the mean (SEM).

3.3 Evaluation of the PBK model of 8-MeBaP and sensitivity analysis

The prediction of the time dependent blood concentration of 8-MeBaP and 3-OH-8-MeBaP in rats was evaluated by the reported in vivo toxicokinetics of BaP and was compared with the predictions for BaP and 3-OHBaP by the previously developed PBK model for BaP. Figure 5 shows the blood concentration of 8-MeBaP and 3-OH-8-MeBaP upon (a) intravenous (b)

intratracheal (c) oral exposure to 10mg 8-MeBaP/kg bw in rats in comparison with the predictions for blood concentration of BaP and 3-OHBaP upon an equimolar dose of BaP. The predicted time-dependent blood concentration curves of 8-MeBaP and 3-OH-8-MeBaP were well in line with the predictions and the data reported in the literature for BaP and 3-OHBaP. Table 3 presents the maximum blood concentration C_{max} of 8-MeBaP and 3-OH-8-MeBaP in comparison to BaP and 3-OHBaP predicted by the PBK models. The C_{max} of BaP was predicted to be 1.2-, 1.3- and 2.1-fold higher than the C_{max} of 8-MeBaP upon intravenous, intratracheal and oral exposure, respectively. The C_{max} predicted for 3-OH-8-MeBaP for each exposure route was identical to that predicted for 3-OHBaP resulting in a ratio of 1. The lower Cmax values for 8-MeBaP than for BaP accompanied by comparable C_{max} values for their 3-OH metabolites can be ascribed to faster overall metabolic clearance of 8-MeBaP due to the contribution of methyl side chain oxidation, while the rate of aromatic hydroxylation at C3 is comparable.

Table 3. Maximum blood concentration (C_{max}) of 3-OH-8-MeBaP in rats predicted by the PBK model for 8-MeBaP in comparison to C_{max} of 3-OHBaP predicted by the model for BaP upon intravenous, intratracheal and oral exposure of rats to 10mg/kg bw.

Route of exposure	C_{max} 3-OH-8-MeBaP, µM	C_{max} 3-OHBaP, µM	C_{max} 3-OH-8-MeBaP / C_{max} 3-OHBaP	C_{max} 8-MeBaP, µM	C_{max} BaP, µM	C_{max} 8-MeBaP / C_{max} BaP
intravenous	0.68	0.68	1.0	7.08	8.23	0.86
intratracheal	0.65	0.65	1.0	3.97	5.14	0.77
oral	0.97	0.96	1.0	1.79	3.75	0.48

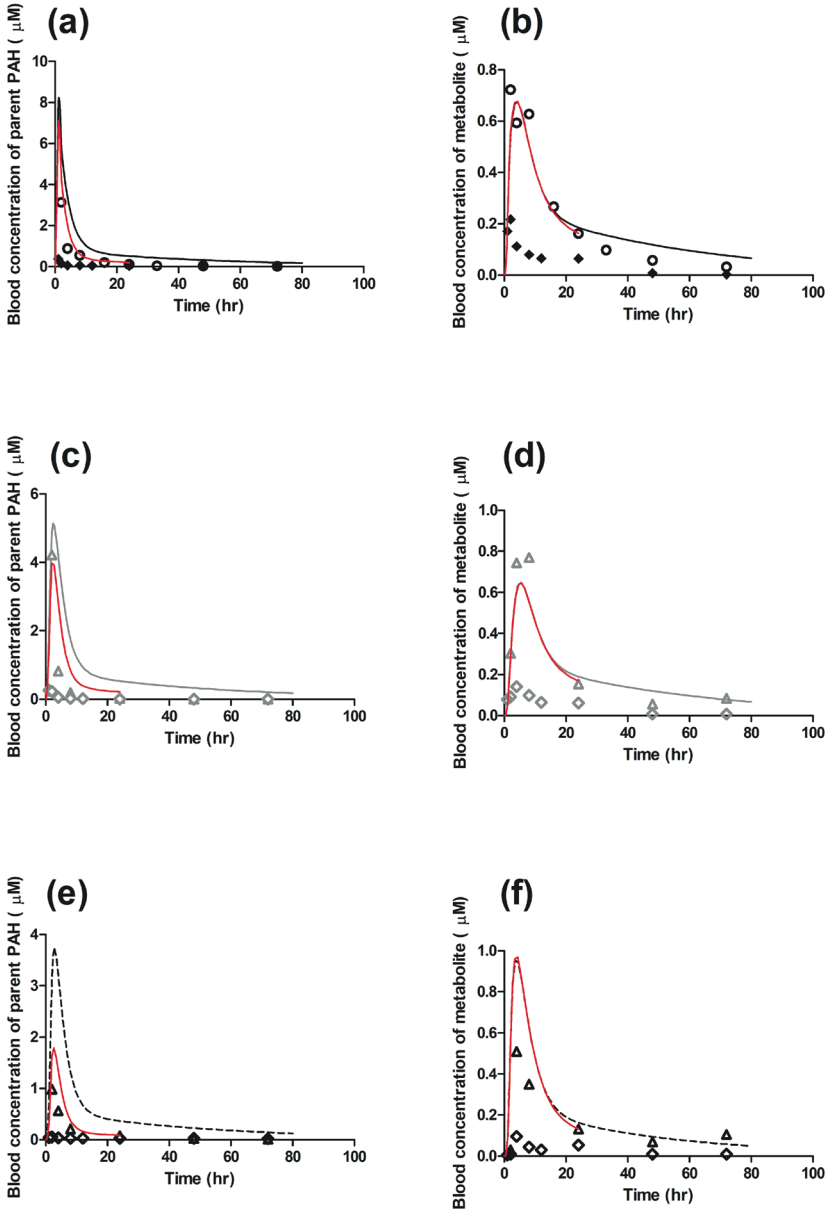

Figure 5. PBK model based predicted time dependent blood concentration of 8-MeBaP and 3-OH-8-MeBaP in rats (red curves) and, for comparison, the previously PBK model predicted blood concentrations for the structurally related substance BaP and its metabolite 3-OHBaP (black dotted curves). The dose level was 10 mg/kg bw. Figure (a) and (b) show the predicted

blood concentrations of 8-MeBaP and 3-OH-8-MeBaP (red solid lines), respectively, and that of BaP and 3-OHBaP (black solid lines), together with the reported in vivo data of BaP and 3-OHBaP from Marie et al. (black open circles) (2010) and Moreau and Bouchard (black filled diamond) (2015) upon intravenous exposure. Figure (c) and (d) present the predicted blood concentrations of 8-MeBaP and 3-OH-8-MeBaP (red solid lines), respectively, and, for comparison, those of BaP and 3-OHBaP (grey solid lines), together with the reported in vivo data of BaP and 3-OHBaP from Marie et al. (grey open triangles) (2010) and Moreau and Bouchard (grey open diamond) (2015) upon intratracheal exposure. Figure (e) and (f) demonstrate the predicted blood concentrations of 8-MeBaP and 3-OH-8-MeBaP (red solid lines), respectively, and that of BaP and 3-OHBaP (black dashed lines), together with the reported in vivo data of BaP and 3-OHBaP from Marie et al. (black open triangles) (2010) and Moreau and Bouchard (black open diamond) (2015) upon oral exposure. The results obtained reveal that the prediction for 8-MeBaP and 3-OH-8-MeBaP, in spite of differences in the kinetics for metabolism and log Kow values that dominate distribution, are close to the predictions for BaP and its metabolite 3-OHBaP and also in line with the in vivo data available for BaP and 3-OHBaP.

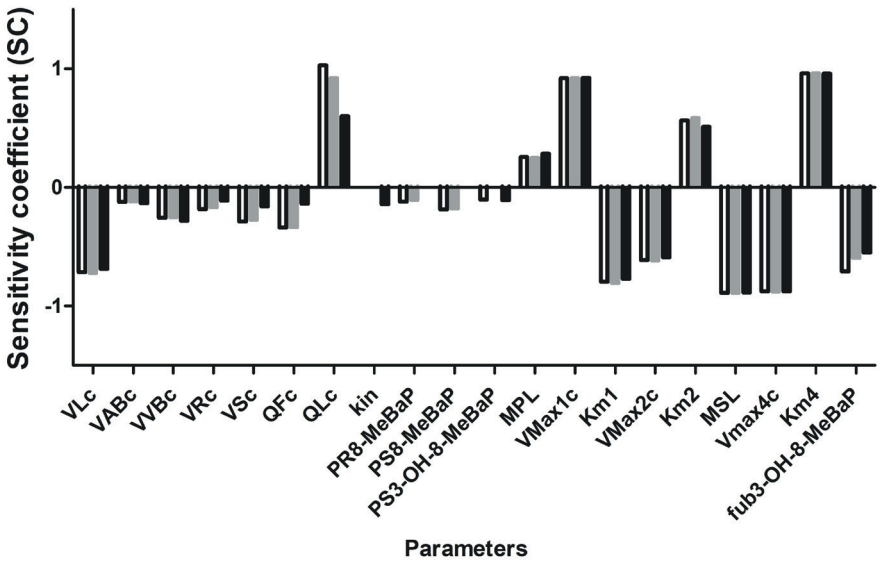

Figure 6. Sensitivity coefficients (SC) of PBK model parameters for the predicted Cmax of 3-OH-8-MeBaP in rat blood after intravenous (white open bars), oral (black filled bars) or

intratracheal (grey filled bars) administration of 10 mg/kg bw 8-MeBaP. Model parameters with an absolute SC of ⩾0.1 are shown. VLc = fraction of liver tissue, VABc = fraction of arterial blood, VVBc = fraction of venous blood, VRc = fraction of rapidly perfused tissue, VSc = fraction of slowly perfused tissue, QFc = fraction of blood flow to fat, QLc = fraction of blood flow to liver, kin = transfer rate to next compartment within the intestines, PR8-MeBaP = rapidly perfused tissue:blood partition coefficient of 8-MeBaP, PS8-MeBaP = slowly perfused tissue: blood partition coefficient of 8-MeBaP, PS3-OH-8-MeBaP = slowly perfused tissue:blood partition coefficient of 3-OH-8-MeBaP, MPL = microsomal protein content in liver, Vmax1c = maximum rate of 3-OH-8-MeBaP formation in liver, Km1= Michaelis-Menten constant for metabolism of 8-MeBaP to 3-OH-8-MeBaP in liver, Vmax2c = maximum rate formation of other metabolites in liver, Km2= Michaelis-Menten constant for metabolism of 8-MeBaP to other metabolites, MSL = S9 protein content in liver, Vmax4c = maximum rate of glucuronidation of 3-OH-8-MeBaP formation in liver, Km4= Michaelis-Menten constant for glucoronidation 3-OH-8-MeBaP in liver, fub3-OH-8-MeBaP = fraction unbound of 3-OH-8-MeBaP

Figure 6 demonstrates the results of a sensitivity analysis to define the parameters that influence the predictions of in vivo blood concentration of 3-OH-8-MeBaP at a dose of 10 mg/kg bw 8-MeBaP to the largest extent. The calculated sensitivity coefficients (SCs) are presented for intravenous (white open bars), intratracheal (grey filled bars) and oral (black filled bars) exposure. Only the parameters with normalized absolute SCs that were greater than 1 are presented. The most influential parameters with an absolute SC value higher than 0.5 include the fractional tissue volume of liver (VLc), the fractional tissue blood flow of liver (QLc), the metabolic parameters for oxidative conversion of 8-MeBaP to 3-OH-8-MeBaP and of 8-MeBaP to other metabolites (Vmax1c, Km1, Vmax2c, Km2), the S9 protein content of the liver (MSL), the metabolic parameters for glucuronidation of 3-OH-8-MeBaP (Vmax4c, Km4) and the value of the fraction unbound of 3-OH-8-MeBaP (fub, in vivo).

3.4 Prediction of in vivo dose response curves from in vitro concentration response curves

Based on the developed PBK model of 8-MeBaP following the oral route, the in vitro concentration response curve for the effect of 3-OH-8-MeBaP on cell differentiation in the mEST (black curve in Figure 3a) was translated to an in vivo dose response prediction for the developmental toxicity of 8-MeBaP (Figure 7). The predicted oral doses of 8-MeBaP were

calculated from the in vitro concentration of 3-OH-8-MeBaP in the mEST using the equations provided in section 2.2.5 with correction for the difference in protein binding in the in vitro and in vivo situation. The ED50 value derived from of the predicted dose response curve for 8-MeBaP is presented in Table 4 together with the previously reported predicted and experimental ED50 values for BaP. The previously predicted ED50 value for BaP (red line in Figure 7) (Wang et al. 2021) was 1.4-fold higher than the ED50 value for 8-MeBaP being 10.5 mg/kg bw that was derived from the predicted dose response curve for single oral exposure to 8-MeBaP (green curve in Figure 7). In addition, the concentration response curve obtained in the present study for 3-OHBaP in the mEST (black curve in Figure 3c) was also translated to a dose response prediction for BaP using the published PBK model of BaP (Wang et al. 2021). The ED50 of BaP thus obtained from the predicted in vivo dose response curve (blue curve in Figure 7) was 14.0 mg/kg bw and only 0.9-fold lower than the ED50 of 15.1 mg/kg bw derived from the previously reported predicted dose response curve for BaP (red curve in Figure 7) (Wang et al. 2021) and 1.3-fold higher than the value derived from the reported in vivo study on developmental toxicity of BaP of 10.8 mg/kg bw (black dotted line in Figure 7) (Archibong et al. 2002).

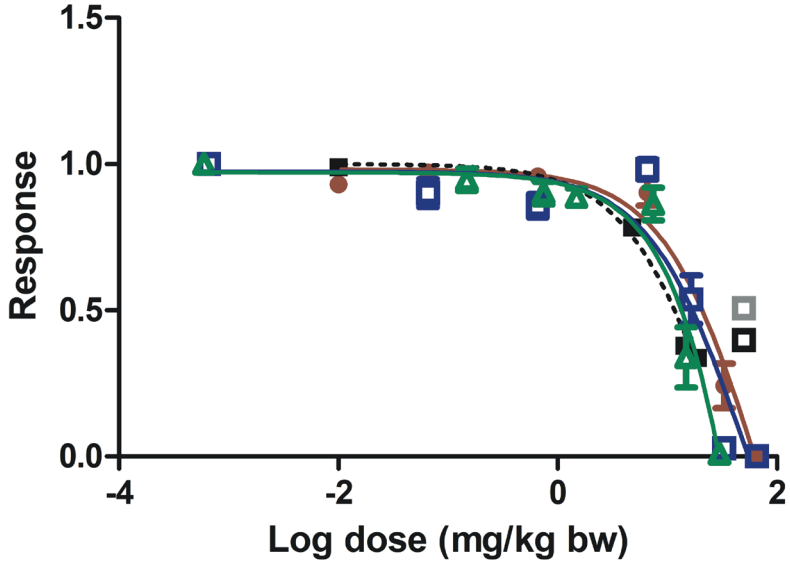

Figure 7. The predicted dose response curve for developmental toxicity of 8-MeBaP (green open triangles and green solid line) and BaP (blue open squares and blue solid line) obtained

by reverse dosimetry of the mEST differentiation data reported in the present study for 3-OH-8-MeBaP (Figure 3a) and 3-OHBaP (Figure 3c), in comparison to the predicted dose response curve previously predicted by the same method for BaP (red filled circle and red solid line) and the dose response data reported by Archibong et al. (2002) for an in vivo developmental toxicity study for BaP (black filled squares and black dashed line). The data from the in vivo study reported by Bui et al. (1986) are shown as grey open square for 6-day exposure and as black open square for 3-day exposure. Each symbol represents the mean of the response and the error bars represent the standard error of the mean (SEM).

Table 4. ED_{50} obtained from the predicted dose response curve of 8-MeBaP in comparison to the ED_{50} from thsse model prediction and in vivo data for BaP upon oral exposure in rats. The dose response curves were constrained with bottom (constant equal to 0) and top (constant equal to 100).

Study	The present study		Wang et al, 2022	Archibong et al 2002
Compound	8-MeBaP	BaP	BaP	BaP
EC_{50} (mg/kg bw)	10.5	14.0	15.1	10.8

4. Discussion

The present study developed a PBK model for 8-MeBaP in rats, a methylated analogue of BaP for which no in vivo animal data on developmental toxicity are available. The model was based on the well-validated model of BaP and includes intravenous, intratracheal and oral exposure routes. Both models of 8-MeBaP and BaP included a sub-model of their major phenolic metabolite, 3-OH-8-MeBaP and 3-OHBaP respectively that was shown able to induce in vitro developmental toxicity in the mEST, in contrast to their parent compounds that tested negative for in vitro developmental toxicity in the mEST. Biotransformation of PAHs plays an important role in their subsequent developmental toxicity (Carrillo et al. 2022; Fang et al. 2022; Incardona et al. 2006; Kamelia et al. 2020). Previously it was already shown that the methyl substitution of BaP shifts oxidative metabolism to the side chain at the cost of the aromatic ring. Especially in the metabolism of 8-MeBaP by rat liver microsomes, the metabolic efficiency in formation of 8-OHMeBaP was 5.3-fold higher than the formation of the aromatic ring oxidation metabolites. In spite of the extensive conversion of 8-MeBaP to 8-OHMeBaP by side chain hydroxylation, the metabolic efficiency (defined at V_{max}/K_m) for formation of 3-OH-8-MeBaP from 8-MeBaP was 5.9 µl/min/mg protein and comparable to that of the conversion of BaP to 3-OHBaP being 7.8 µl/min/mg protein (Wang et al. 2022). This resulted in comparable predicted blood concentration versus time curves for 3-OH-8-MeBaP and 3-OHBaP upon dosing an equimolar amount of the parent PAH, with a similar C_{max}. The relatively overall oxidative metabolism of 8-MeBaP compared to BaP as a result of the extensive side chain hydroxylation did result in faster metabolic clearance resulting in a relatively lower C_{max} of 8-MeBaP than for BaP at an equimolar dose with the ratio C_{max} 8-MeBaP/C_{max} BaP ranging from 0.48 to 0.86. These differences were observed for all three route of exposure.

The developed PBK model for 8-MeBaP was used to translate the in vitro concentration response curve for its 3-OH metabolite in the mEST into in vivo dose response predictions for developmental toxicity upon oral exposure of rats to 8-MeBaP. Developmental toxicity of 8-MeBaP was assumed to be caused by metabolic activation to 3-OH-8-MeBaP by cytochrome P450 enzymes following the same pathway as previously elucidated for BaP (Kamelia et al. 2020; Wang et al. 2021). Results of the present study reveal that both 3-OH metabolites affected the differentiation of the ES-D3 cells in the differentiation assay of the mEST with an IC_{50} for 3-OH-8-MeBaP that was 1.1-fold, lower than that of 3-OHBaP while BaP and 8-MeBaP both tested negative in the mEST.

Translation of the in vitro data to an in vivo dose response curve predicted the in vivo ED_{50} for developmental toxicity to be only 1.3-fold lower than that of BaP. The lower difference in ED_{50} than in the IC_{50} in the mEST is related to differences in the kinetics of the two PAHs with the relative conversion to the toxic 3-OH metabolite being less effective for 8-MeBaP for which the catalytic efficiency for formation of the 3-OH metabolite is 0.8-fold lower than for BaP.

Thus based on the mEST and the predicted in vivo curves for developmental toxicity the potency of 8-MeBaP appears to be respectively 1.1- and 1.3-fold higher than that of BaP. The higher potency of 8-MeBaP as compared to BaP is in line with results of the ZET (Fang et al. 2022) where 8-MeBaP appeared also more potent than BaP albeit by a factor 10. This different relative potency of 8-MeBaP compared to BaP in the mEST and mEST-based in vivo predictions reported in the present study and the ZET may be due to differences in the cytochrome P450 enzymes involved in metabolic activation in the zebrafish embryo's as compared to the rat liver microsomes and/or in the underlying mode of action underlying the developmental toxicity in zebrafish and the mouse ES-D3 cells of the mEST.

Due to the lack of in vivo toxicokinetic and toxicity data, the current model predictions for blood concentration and developmental toxicity of 8-MeBaP could only be evaluated by comparison to data for the related BaP. It seems reasonable to assume that the new approach methodology (NAM) that was proven valid for BaP, and uses PBK model facilitated QIVIVE of data from the mEST to predict in vivo dose response curves for developmental toxicity, will also provide reliable data for the developmental toxicity of the related compound 8-MeBaP. Nevertheless it would be of interest to validate the model and the QIVIVE predictions when in vivo data in rats would become available in the future. Until such data become available, the present study illustrates how NAMs can be applied to predict developmental toxicity of a methyl substituted PAH for which in vivo data are not available, by PBK modeling facilitated read across from a non-substituted structural analogue for which in vivo data have been reported. The method also elucidates how a methyl substituent could affect the kinetics and subsequent developmental toxicity of a non-substituted PAH. The PBK model facilitated read across may be of use for further evaluation of other PAHs that need bioactivation to induce developmental toxicity. The study may contribute to the need of reducing the use of animal testing for hazard assessment of alkyl substituted PAHs.

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Code availability: Model code available in the supplementary material

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Supplementary material

;Date:09-06-2022

;Purpose: PBK model 8-methyl-benzo[a]pyrene and 3-hydroxy-8-methyl-benzo[a]pyrene, built with literature, in vitro and in silico derived parameter values{Marie, 2010 #933}{Marie, 2010 #933}

;Species: Rat (gender mixed)

;Compiled by: Danlei Wang, based on PBK models for BaP that included submodels for 3OHBaP

;===

;Physiological parameters

;===

;tissue volumes (Crowell et al. (2011), based on Brown et al. (1997))

BW = 0.245 {Kg} ; body weight rat (variable, dependent on study)

VFc = 0.065 ; fraction of fat tissue

VLc = 0.037 ; fraction of liver tissue

VLuc= 0.005 ; fraction of lung tissue

VABc =0.0257 ; fraction of arterial blood

VVBc = 0.0514 ; fraction of venous blood

VRc = 0.2159 ; fraction of richly perfused tissue (= 1 - (VFc + VLc + VLuc + VABc + VVBc+ VSc))

VSc = 0.6 ; fraction of slowly perfused tissue

VF = VFc*BW {L or Kg} ; volume of fat tissue (calculated)

VL = VLc*BW {L or Kg} ; volume of liver tissue (calculated)

VLU = VLuc*BW {L or Kg} ; volume of lung tissue (calculated)

VAB = VABc*BW {L or Kg} ; volume of arterial blood (calculated)

VVB = VVBc*BW {L or Kg} ; volume of venous blood (calculated)

VR = VRc*BW {L or Kg} ; volume of richly perfused tissue (calculated)

VS = VSc*BW {L or Kg} ; volume of slowly perfused tissue (calculated)

;---

;blood flow rates (Crowell et al. (2010), based on Brown et al. (1997))

QC = 15*BW^0.74 {L/hr} ; cardiac output

QFc = 0.07 ; fraction of blood flow to fat

QLc = 0.183 ; fraction of blood flow to liver

QLuc = 1 ; fraction of blood flow to lung

QRc = 0.4 ; fraction of blood flow to richly perfused tissue

QSc = 0.347 ; fraction of blood flow to slowly perfused tissue

QF = QFc*QC {L/hr} ; blood flow to fat tissue (calculated)

QL = QLc*QC {L/hr} ; blood flow to liver tissue (calculated)

QLu = QLuc*QC {L/hr} ; blood flow to lung tissue (calculated)

QS = QSc*QC {L/hr} ; blood flow to slowly perfused tissue (calculated)

QR = QRc*QC {L/hr} ; blood flow to richly perfused tissue (calculated)

;---

;Intestinal lumen volumes, surfaces, absorption rates, transfer rates

;Papp, Caco-2 = 3.8 {x1E-6 cm/sec} ;in vitro, from Caco-2 cells (Goth-Goldstein et al. (1999)

PappCaco2=-5.42 ; Log Papp, Caco-2

;Log (Papp,in vivo) = 0.6836*Log(PappCaco-2)-0.5579 (Sun et al. 2002)

Papp=10^(0.6836*PappCaco2-0.5579)*3600/10 ; apparent intestinal permeability coefficient in vivo {dm/hr}

; 7-compartment model for GI-tract based on model bisphenol A and 17β-estradiol by Zhang et al., (2018)

Vin = 0.0012 ; volume for each compartment of intestines {L}

SAin = 0.134 ; surface area {dm2}

kin = 4.17 ; transfer rate to next compartment within the intestines {/hr}

;kabin1 = Papp*SAin ; absorption rate constant {L/hr}

Vin1 = Vin ; volume of intestine compartment 1 {L}

SAin1 = SAin ; surface area of intestine compartment 1 {dm2}

kabin1 = Papp*SAin1 ; absorption rate constant of intestine compartment 1 {L/hr}

kin1 = kin ; transfer rate to intestine compartment 2 {/hr}

Vin2 = Vin ; volume of intestine compartment 2 {L}

SAin2 = SAin ; surface area of intestine compartment 2 {dm2}

```
kabin2  = Papp*SAin2     ; absorption rate constant of intestine compartment 2  {L/hr}
kin2  = kin                      ; transfer rate to intestine compartment 3  {/hr}

Vin3  =  Vin                     ; volume of intestine compartment 3  {L}
SAin3=  SAin                     ; surface area of intestine compartment 3  {dm2}
kabin3  = Papp*SAin3             ; absorption rate constant of intestine compartment 3  {L/hr}
kin3  = kin                      ; transfer rate to intestine compartment 4  {/hr}

Vin4  =  Vin                     ; volume of intestine compartment 4  {L}
SAin4 = SAin                     ; surface area of intestine compartment 4  {dm2}
kabin4  = Papp*SAin4     ; absorption rate constant of intestine compartment 4  {L/hr}
kin4  = kin                      ; transfer rate to intestine compartment 5  {/hr}

Vin5  =  Vin                     ; volume of intestine compartment 5  {L}
SAin5 = SAin                     ; surface area of intestine compartment 5  {dm2}
kabin5  = Papp*SAin5     ; absorption rate constant of intestine compartment 5  {L/hr}
kin5  = kin                      ; transfer rate to intestine compartment 6  {/hr}

Vin6  =  Vin                     ; volume of intestine compartment 6  {L}
SAin6 = SAin             ; surface area of intestine compartment 6  {dm2}
kabin6  = Papp*SAin6     ; absorption rate constant of intestine compartment 6  {L/hr}
kin6  = kin                      ; transfer rate to intestine compartment 7  {hr}

Vin7  =  Vin                     ; volume of intestine compartment 7  {L}
SAin7  = SAin                    ; surface area of intestine compartment 7  {dm2}
kabin7 = Papp*SAin7              ; absorption rate constant of intestine compartment 7  {L/hr}
kin7   = kin                     ; transfer rate to co {/hr}

kfe = 0.27                       ; transfer rate to faeces {/hr} taken from Crowell et al. (2011)
;=====================================================================
;Physicochemical parameters
;=====================================================================
```

;partition coefficients 8MBaP (tissue:blood)

PF8MBaP = 434.55 ; fat/blood partition coefficient

PL8MBaP = 12.85 ; liver/blood partition coefficient

PLu8MBaP = 14.65 ; lung/blood partition coefficient

PR8MBaP= 12.85 ; rapidly perfused tissue/blood partition coefficient

PS8MBaP = 7.13 ; slowly perfused tissue/blood partition coefficient

;---

;partition coefficients 3-OH8MBaP (tissue:blood)

PF3OH8MBaP = 392.83 ; fat/blood partition coefficient of 3-OH8MBaP

PL3OH8MBaP = 12.55 ; liver/blood partition coefficient of 3-OH8MBaP

PLu3OH8MBaP = 14.31 ; lung/blood partition coefficient of 3-OH8MBaP

PR3OH8MBaP = 12.55 ; rapidly perfused tissue/blood partition coefficient of 3-OH8MBaP

PS3OH8MBaP = 6.98 ; slowly perfused tissue/blood partition coefficient of 3-OH8MBaP

;==========

;Kinetic parameters

;==========

;Metabolism liver

;MPL: scaling factor of rat liver microsomes (mg microsomal protein /g liver)

MPL=45 ; mg microsomal protein/g liver Reference: (Houston & Galetin (2008))

;Maximum rate of metabolism of 8MBaP to 3-OH8MBaP, measured in vitro in present study

VMax1c= 0.07 {nmol/min/mg microsomal protein} ; 8MBaP -> 3-OH8MBaP

;Maximum rate of metabolism scaled to liver

VMax1 = VMax1c/1000*60*MPL*VL*1000 {µmol/hr/liver}

;Michaelis-Menten constant for metabolism of 8MBaP to 3-OH8MBaP measured in vitro in present study

Km1 = 12 {uM}

;---

;Maximum rate of metabolism of 8MBaP to remaining metabolites measured in present study

VMax2c = 0.43 {nmol/min/mg microsomal protein} ; (8MBaP-->remaining metabolites)

;Maximum rate of metabolism scaled to liver

VMax2 = VMax2c/1000*60*MPL*VL*1000 {µmol/hr/liver}

;Michaelis-Menten constant for metabolism of 8MBaP to remaining metabolites measured in vitro in present study

Km2 = 8 {uM}

;----------

;Sulfonation of 3-OH8MBaP

;MSL: scaling factor of rat liver S9 (mg S9 protein /g liver)

MSL = 125 ; mg S9 protein/g liver (Houston and Galetin, 2008)

;Maximum rate of metabolism for sulfation of 3-OH8MBaP determined in vitro in present study

VMax3c= 0.15 {nmol/min/mg S9 protein}

VMax3 = VMax3c/1000*60*MSL*VL*1000 {µmol/hr/liver}

;Michaelis-Menten constant for sulfation of 3-OH8MBaP determined in vitro in present study

Km3 = 26 {µM}

;----------

;Glucuronidation of 3-OH8MBaP

;Maximum rate of metabolism glucuronidation of 3-OH8MBaP determined in vitro in present study

Vmax4c= 6.92 {nmol/min/mg S9 protein} ;

Vmax4 = Vmax4c/1000*60*MSL*VL*1000 {µmol/hr/liver}

; Michaelis-Menten constant for glucuronidation of 3-OHBaP determined in vitro

Km4 = 16 {µM}

;==========

;Run settings

;==========

;Exposure parameters

;Molecular weight

MW8MBaP = 266.34 ; Molecular weight 8MBaP

```
MW3OH8MBaP = 282.34                                    ; Molecular weight 3-OH8MBaP

;IV dose = given IV dose in mg/kg bw
IVDOSEmg = 0                                           {mg/kg bw}
IVDOSEumol2 = IVDOSEmg*1E-3/MW8MBaP*1E6                {µmol/ kg bw}
;IVDOSEumol2 = given iv dose recalculated to µmol/kg bw
IVDOSEumol=IVDOSEumol2*BW                              {µmol}  ; intravenous dose

;oral dose = given oral dose in mg/kg
ODOSEmg = 0                                            {mg/kg bw}
ODOSEumol2 = ODOSEmg*1E-3/ MW8MBAP*1E6                 {µmol/kg bw}
ODOSEumol=ODOSEumol2*BW;                       {µmol}  ; oral dose

;intratracheal dose = given intratracheal dose in mg/kg
ITDOSEmg = 0                                           {mg/kg bw}
ITDOSEumol2 = ITDOSEmg*1E-3/MW8MBaP*1E6                {µmol/ kg bw}
;ITDOSEumol = given intratracheal dose recalculated to µmol/kg bw
ITDOSEumol=ITDOSEumol2*BW                      {µmol}  ; intratracheal dose


;Time
Starttime = 0                  ; in hr
Stoptime = 24                  ; in hr

;====================================================================
;Model calculations
;====================================================================
;Exposure routes

Frequency = 24    {h}          ; duration between doses, in hours
Repetitions = 1                ; total number of daily doses

;Intravenous, needle
```

```
;ANe  = amount 8MBaP in needle
      ANe'  = -kd*ANe + iv_input'
      Init ANe = 0

      Iv_input' = IF time <= Repetitions * Frequency THEN pulse(IVDOSEumol, 0, Frequency) ELSE 0
      Init iv_input = 0


kd=1000000        {/h}       ;kd, the transport rate from needle to blood

; intratracheal
;ATr = amount 8MBaP in trachea
     ATr'  = -kt*ATr + it_input'
      Init ATr  = 0

      It_input' = IF time <= Repetitions * Frequency THEN pulse(ITDOSEumol, 0, Frequency) ELSE 0
      Init it_input = 0


kt = 1                {h}       ; absorption from trachea to lung, maximum value assumed

;-------------------------------------------------------------------------------------------------------------
;Stomach compartment
;Ast  = amount of BaP remaining in stomach, µmol
      Ast'  = -ka*Ast + oral_input'
      Init Ast = 0

      oral_input' = IF time <= Repetitions * Frequency THEN pulse(ODOSEumol, 0, Frequency) ELSE 0
      Init oral_input = 0

ka = 1                {h}       ; Absorption constant stomach to GI-tract maximum value assumed

;-------------------------------------------------------------------------------------------------------------
```

```
;intestinal compartment, divided in 7 sub compartments
;Ain1  = Amount 8MBaP in intestine compartment 1 (µmol)
Cin1   = Ain1/Vin1
Ain1'  = ka*Ast - kin1*Ain1 - kabin1*Cin1
Init Ain1  =  0

;Ain2 = Amount 8MBaP in intestine compartment 2 (µmol)
Cin2  = Ain2/Vin2
Ain2' = kin1*Ain1 - kin2*Ain2 - kabin2*Cin2
Init Ain2  = 0

;Ain3 = Amount 8MBaP in intestine compartment 3 (µmol)
Cin3  = Ain3/Vin3
Ain3' = kin2*Ain2 - kin3*Ain3 - kabin3*Cin3
Init Ain3  = 0

;Ain4 = Amount 8MBaP in intestine compartment 4 (µmol)
Cin4  = Ain4/Vin4
Ain4' = kin3*Ain3 - kin4*Ain4 - kabin4*Cin4
Init Ain4  = 0

;Ain5 = Amount 8MBaP in intestine compartment 5 (µmol)
Cin5  = Ain5/Vin5
Ain5' = kin4*Ain4 - kin5*Ain5 - kabin5*Cin5
Init Ain5  = 0

;Ain6= Amount 8MBaP in intestine compartment 6 (µmol)
Cin6  = Ain6/Vin6
Ain6' = kin5*Ain5 - kin6*Ain6 - kabin6*Cin6
Init Ain6  = 0

;Ain7= Amount 8MBaP in intestine compartment 7 (µmol)
Cin7  = Ain7/Vin7
```

```
Ain7' = kin6*Ain6 - kin7*Ain7 - kabin7*Cin7

Init Ain7  = 0


;Aco = Amount 8MBaP in colon (µmol)

Aco' = kin7*Ain7- kfe*Aco

Init Aco  = 0

ACco' = kin7*Ain7

Init ACco  = 0    ; cumulative amount reaching colon

;-------------------------------------------------------------------------------------------------------------

;feces

;AFA = amount 8MBaP in feces (µmol)

AFe' = kfe*Aco + Kb*AL8MBAP

Init AFe  = 0

Kb = 1                    ; excretion constant liver to faeces via bile maximum value assumed

;-------------------------------------------------------------------------------------------------------------

;liver compartment


;AL8MBAP = Amount of 8MBaP in liver tissue (µmol)


AL8MBAP' =   kabin1*Cin1 + kabin2*Cin2 + kabin3*Cin3 + kabin4*Cin4 + kabin5*Cin5 + kabin6*Cin6 +
kabin7*Cin7 + QL*( CAB8MBAP - CVL8MBAP) - AMMO'- AMMT1' - Kb*AL8MBAP

Init AL8MBAP = 0

CL8MBAP = AL8MBAP/VL

CVL8MBAP = CL8MBAP/PL8MBAP


;AMMO = amount of 8-methyl-BaP metabolized to metabolite 3-OH-8-methyl-Benzo[a]pyrene

     AMMO' =VMax1*CVL8MBAP/(Km1 + CVL8MBAP)

      init AMMO = 0


;AMMT1 = amount of 8MBaP metabolized to other metabolites

     AMMT1' = Vmax2*CVL8MBAP/(Km2 + CVL8MBAP)

      init AMMT1 = 0

;-------------------------------------------------------------------------------------------------------------
```

```
;fat compartment

;AF8MBAP = Amount of 8MBaP in fat tissue (µmol)
      AF8MBAP' = QF*( CAB8MBAP -CVF8MBAP)
      Init AF8MBAP = 0
      CF8MBAP = AF8MBAP/VF
      CVF8MBAP = CF8MBAP/PF8MBAP
;-----------------------------------------------------------------------------------------------------------
;tissue compartment richly perfused tissue

;AR8MBAP = Amount of 8MBaP in richly perfused tissue (µmol)
      AR8MBAP' = QR*( CAB8MBAP -CVR8MBAP)
      Init AR8MBAP = 0
      CR8MBAP = AR8MBAP/VR
      CVR8MBAP = CR8MBAP/PR8MBAP
;-----------------------------------------------------------------------------------------------------------
;tissue compartment slowly perfused tissue

;AS8MBAP = Amount of 8MBaP in slowly perfused tissue (µmol)
      AS8MBAP' = QS*( CAB8MBAP -CVS8MBAP)
      Init AS8MBAP = 0
      CS8MBAP = AS8MBAP/VS
      CVS8MBAP = CS8MBAP/PS8MBAP
;-------------------------------------------------------------------
;lung blood compartment

;ALU8MBAP = Amount of 8MBaP in lung blood, µmol
      ALU8MBAP' = kt*ATr+ QLU*(CVB8MBAP - CVLU8MBAP)
      Init ALU8MBAP = 0
      CLU8MBAP = ALU8MBAP/VLU
      CVLU8MBAP = CLU8MBAP/PLU8MBAP
;-------------------------------------------------------------------
; venous blood
```

```
;AVB8MBAP = amount of 8MBaP in venous blood, µmol

    AVB8MBAP' = Ane*kd + (QF*CVF8MBaP + QL*CVL8MBaP  + QS*CVS8MBaP + QR*CVR8MBaP) -
QLU*CVB8MBAP

    Init AVB8MBAP = 0

    CVB8MBAP = AVB8MBAP/VVB

    AUCVB8MBAP' = CVB8MBAP

    init AUCVB8MBAP = 0


;---------------------------------------------------------------------

; arterial blood


;AAB8MBAP = amount of 8MBaP in arterial blood, µmol

    AAB8MBAP' = QLU*(CVLU8MBAP -CAB8MBAP)

    Init AAB8MBAP = 0

    CAB8MBAP = AAB8MBAP/VAB

    AUCAB8MBAP' = CAB8MBAP

    init AUCAB8MBAP = 0


CB8MBaPtot =CVB8MBAP + CAB8MBAP


;=====================================================================

;3-hydroxy-8-methyl-benzo[a]pyrene submodel

;=====================================================================

;feces

;AFA = amount 3OH8MBaP in feces (µmol)

A3OH8MFe' = kfe*Aco + Kc*AL3OH8MBAP

Init A3OH8MFe  = 0

Kc = 1                    ;excretion constant liver to faeces via bile maximum value assumed

;-----------------------------------------------------------------------------------------------------------

;liver compartment


;AL3OH8MBAP = Amount of 3-OH8MBaP in liver tissue (µmol)
```

```
      AL3OH8MBAP' =  AMMO' +  QL*( CAB3OH8MBAP - CVL3OH8MBAP) - AMMT2' - AMMT3' -
Kc*AL3OH8MBAP

      Init AL3OH8MBAP = 0

      CL3OH8MBAP = AL3OH8MBAP/VL

      CVL3OH8MBAP = CL3OH8MBAP/PL3OH8MBAP


;AMMT2 = Amount of 3-OH8MBaP sulfonated

     AMMT2' = Vmax3*CVL3OH8MBAP/(Km3 + CVL3OH8MBAP)

     Init AMMT2  = 0


;AMMT3 = Amount of 3-OH8MBaP glucuronidated

     AMMT3' = Vmax4*CVL3OH8MBAP/(Km4 + CVL3OH8MBAP)

     Init AMMT3  = 0


;----------------------------------------------------------------------------------------------------------

;fat compartment


;AF3OH8MBAP = Amount of 3-OH8MBaP in fat tissue (µmol)

      AF3OH8MBAP' = QF*( CAB3OH8MBAP -CVF3OH8MBAP)

      Init AF3OH8MBAP = 0

      CF3OH8MBAP = AF3OH8MBAP/VF

      CVF3OH8MBAP = CF3OH8MBAP/PF3OH8MBAP

;----------------------------------------------------------------------------------------------------------

;tissue compartment richly perfused tissue


;AR3OH8MBAP = Amount of 3-OH8MBaP in richly perfused tissue (µmol)

      AR3OH8MBAP' = QR*( CAB3OH8MBAP -CVR3OH8MBAP)

      Init AR3OH8MBAP = 0

      CR3OH8MBAP = AR3OH8MBAP/VR

      CVR3OH8MBAP = CR3OH8MBAP/PR3OH8MBAP

;----------------------------------------------------------------------------------------------------------

;tissue compartment slowly perfused tissue
```

```
;AS3OHBAP = Amount of 3-OH8MBaP in slowly perfused tissue (µmol)

      AS3OH8MBAP' = QS*( CAB3OH8MBAP -CVS3OH8MBAP)

      Init AS3OH8MBAP = 0

      CS3OH8MBAP = AS3OH8MBAP/VS

      CVS3OH8MBAP = CS3OH8MBAP/PS3OH8MBAP

;---------------------------------------------------------------------

;lung compartment


;ALU3OH8MBAP = Amount of 3-OH8MBaP in lung tissue, µmol

      ALU3OH8MBAP' = QLU*(CVB3OH8MBAP -CVLU3OH8MBAP)

      Init ALU3OH8MBAP = 0

      CLU3OH8MBAP = ALU3OH8MBAP/VLU

      CVLU3OH8MBAP = CLU3OH8MBAP/PLU3OH8MBAP

;---------------------------------------------------------------------

; venous blood


;AVB3OH8MBAP = amount of 3-OH8MBaP in venous blood, µmol

      AVB3OH8MBAP' = ( (QF*CVF3OH8MBAP + QL*CVL3OH8MBAP  + QS*CVS3OH8MBAP +
QR*CVR3OH8MBAP) - QLU*CVB3OH8MBAP)*fub3OH8MBAP

      Init AVB3OH8MBAP = 0

      CVB3OH8MBAP = AVB3OH8MBAP/VVB

      AUCVB3OH8MBAP' = CVB3OH8MBAP

      init AUCVB3OH8MBAP = 0


fub3OH8MBAP = 0.004                                    ; 3-OH8MBaP fraction unbound in blood

;---------------------------------------------------------------------

; venous blood protein


;AVBP3OH8MBAP = amount of 3-OH8MBaP in bound to venous blood protein, µmol

      AVBP3OH8MBAP' = ( (QF*CVF3OH8MBAP + QL*CVL3OH8MBAP  + QS*CVS3OH8MBAP +
QR*CVR3OH8MBAP) - QLU*CVB3OH8MBAP)*fb3OH8MBAP

      Init AVBP3OH8MBAP = 0

      CVBP3OH8MBAP = AVBP3OH8MBAP/VVB
```

```
    AUCVBP3OH8MBAP' = CVBP3OH8MBAP
    init AUCVBP3OH8MBAP = 0
fb3OH8MBaP = 1-fub3OH8MBAP                    ; 3-OH8MBaP fraction bound to blood protein
;-----------------------------------------------------------------
; arterial blood

;AAB3OH8MBAP = amount of 3-OH8MBaP in arterial blood, µmol
    AAB3OH8MBAP' = (QLU*(CVLU3OH8MBAP -CAB3OH8MBAP)) * fub3OH8MBAP
    Init AAB3OH8MBAP = 0
    CAB3OH8MBAP = AAB3OH8MBAP/VAB
    AUCAB3OH8MBAP' = CAB3OH8MBAP
    init AUCAB3OH8MBAP = 0

;-----------------------------------------------------------------
; arterial blood protein

;AABP3OH8MBAP = amount of 3-OH8MBaP bound to arterial blood protein, µmol
    AABP3OH8MBAP' = (QLU*(CVLU3OH8MBAP - CAB3OH8MBAP))* fb3OH8MBAP
    Init AABP3OH8MBAP = 0
    CABP3OH8MBAP = AABP3OH8MBAP/VAB

CB3OH8MBaPtot = CVB3OH8MBAP + CVBP3OH8MBAP + CAB3OH8MBAP + CABP3OH8MBAP

;=====================================================================
;Mass balance calculations for 8-methyl-Benzo[a]pyrene
Total8MBAP = oral_input + iv_input + it_input
Calculated8MBAP = Ain1 + Ain2 + Ain3 + Ain4 + Ain5 + Ain6 + Ain7 + Aco + AFe + AL8MBAP +
AF8MBAP +AS8MBAP + AR8MBAP + AAB8MBAP + AVB8MBAP +ALU8MBAP + AMMO  + AMMT1 +
ANe  + Ast + ATr

ERROR8MBAP=(Total8MBAP-Calculated8MBAP)/(Total8MBAP+1E-30)*100
MASSBBAL8MBAP=Total8MBAP-Calculated8MBAP + 1
;=====================================================================
```

;Mass balance calculations for 3-hydroxy-8-methyl-benzo[a]pyrene sub-model

Total3OH8MBAP = AMMO

Calculated3OH8MBAP = A3OH8MFe + AL3OH8MBAP + AF3OH8MBAP +AS3OH8MBAP + AR3OH8MBAP + AAB3OH8MBAP + AABP3OH8MBAP+AVB3OH8MBAP + AVBP3OH8MBAP + ALU3OH8MBAP + AMMT2 + AMMT3

ERROR3OH8MBAP=(Total3OH8MBAP-Calculated3OH8MBAP)/(Total3OH8MBAP+1E-30)*100

MASSBBAL3OH8MBAP=Total3OH8MBAP-Calculated3OH8MBAP + 1

;===

CHAPTER 6.

General Discussion and future perspectives

1. Overview of the main finding of the thesis

Many studies have associated exposure to some PAHs with developmental toxicity in animal species and even humans (Archibong et al., 2002; Bui et al., 1986; Choi et al., 2008, 2006; Perera et al., 2012; Tang et al., 2006). Moreover, the presence of aromatics, especially the 3- to 7-ring polycyclic aromatic compounds (PACs), in heavier petroleum substances has been associated with the developmental toxicity induced by these substances (Feuston et al., 1994, 1989; Murray et al., 2013; Tsitou et al., 2015). PACs consist of naked or lowly alkylated polycyclic aromatic hydrocarbons (PAHs) and heterocyclic aromatics. Compared to the unsubstituted/naked PAHs, little is known about the potential developmental toxicity of alkylated PAHs, including methylated PAHs. Existing evidence shows that alkylation of PAHs may have a substantial influence on the developmental toxicity of PAHs (Geier et al., 2018a; Lin et al., 2015; Wagner, 2016). Under REACH, high volume chemical substances (⩾100 tonnes/year) produced in or imported into the EU market require developmental toxicity testing according to the OECD TG 414. Considering that testing for developmental toxicity according to the OECD 414 testing guidelines (OECD, 2018) is animal-intensive, new approach methodologies (NAMs) would be preferred (ECHA, 2009; OECD, 2018). In the present thesis, the in vitro developmental toxicity of a series of methylated PAHs compared to their corresponding unsubstituted parent PAHs, and the possible underlying mode(s) of action were evaluated using NAMs (**Chapter 2 and 3**). Moreover, the hypothesis that the developmental toxicity induced by some petroleum substances is due to their 3- to 7-ring PAC content was tested by using a battery of in vitro assays to test the developmental toxicity of some selected (highly) refined petroleum substances (**Chapter 4**). Finally, physiologically-based kinetic (PBK) modelling-based reverse dosimetry facilitated read-across of the unsubstituted PAH benzo[a]pyrene (BaP) was applied to predict the in vivo developmental toxicity of 8-methyl-BaP by quantitative in vitro to in vivo extrapolation (QIVIVE) (**Chapter 5**).

In **Chapter 2**, to get a better understanding of the influence of methyl substitution on the developmental toxicity of PAHs, the in vitro developmental toxicity of a series of 4- and 5-ring PAHs including benz[a]anthracene (BaA) and its three methylated congeners: 4-methyl-BaA (4-MeBaA), 8-methyl-BaA (8-MeBaA), 9-methyl-BaA (9-MeBaA); benzo[a]pyrene (BaP) and its three methylated congeners: 3-methyl-BaP (3-MeBaP), 7-methyl-BaP (7-MeBaP), 8-methyl-BaP (8-MeBaP); and dibenz[a,h]anthracene (DB[a,h]A), was assessed using the zebrafish embryo toxicity test (ZET). The results show that except for 7-MeBaP, all the tested

PAHs (both methylated and unsubstituted PAHs) induced various developmental effects to zebrafish embryos at 96 hours post fertilization (hpf), including no movement, no circulation, no protruding mouth, not empty yolk extension, pericardial and yolk sac edemas, deformed body shape, deformed head and jaw, and cumulative mortality. Comparison of the in vitro developmental toxicity potencies of the tested PAHs demonstrated that the methylated PAHs induced either increased or decreased developmental toxicity potency relative to their corresponding unsubstituted PAHs. One of the possible explanations for the different developmental toxicities of the tested 4- and 5-ring unsubstituted and methylated PAHs is their ability to interact with the aryl hydrocarbon receptor (AhR), which is known to play an essential role in mediating the developmental toxicity of many PAHs (Hawliczek et al., 2012; Huang et al., 2012; Incardona et al., 2006). It is speculated that the position of a monomethyl substituent on the aromatic ring of PAHs changes their molecular dimension thereby affecting their interactions with the ligand binding domains (LBDs) of the AhR and subsequently results in the different in vitro developmental toxicities. Altogether, the addition of a methyl substituent to the corresponding unsubstituted PAH can either increase or decrease its developmental toxicity depending on the position of the methyl substituent on the aromatic rings of the PAHs, and this may in part relate to effects on the molecular dimensions and resulting consequences for interactions with different nuclear receptors involved in developmental toxicity, including the AhR.

Existing evidence suggests that the AhR plays an important role in mediating the developmental toxicity of some PAHs (Goodale et al., 2013; Incardona et al., 2011). Besides the AhR, some studies also suggested the involvement of some nuclear hormone receptors, such as the estrogen receptor alpha (ER-α) and the retinoic acid receptor (RAR) in mediating PAH-induced developmental toxicity (Barlow et al., 1999; Maqbool et al., 2016; Piersma et al., 2017; Van Lipzig et al., 2005). Therefore, in **Chapter 3** the AhR-, ER-α- and RAR-mediated activities of the same series of unsubstituted and methylated PAHs tested in **Chapter 2** were evaluated using Chemical Activated LUciferase gene eXpression (CALUX) reporter gene assays. The results show that all PAHs tested were AhR agonists and all methylated PAHs were more potent to activate the AhR compared to their corresponding unsubstituted PAHs, suggesting that the presence of a methyl substituent enhanced the AhR mediated activity of their corresponding unsubstituted PAHs. Two of the tested PAHs, BaA and 8-MeBaA, induced estrogenic activity; none of the PAHs induced anti-estrogenic activity; and none of the PAHs interacted with the RAR. To further investigate the role of the AhR and ER-α in PAH-induced developmental

toxicity in the ZET, the PAHs that interacted with the AhR or ER-α were selected and tested in the ZET in the absence or presence of an AhR or ER-α antagonist. Prior to testing the PAHs in the presence of the AhR antagonist CH223191, the AhR antagonist CH223191 was first tested in the AhR CALUX assay in the presence of the selected PAHs to show that it is a suitable antagonist for PAH-induced AhR mediated activities. Co-exposure of H4IIE.luc cells in the AhR CALUX assay to CH223191 and each of the PAHs successfully counteracted the tested PAH-induced AhR mediated activities. This suggested that CH223191 is a suitable antagonist for evaluating PAH-induced AhR activation and the resulting developmental toxicity. Co-exposure of zebrafish embryos to the nine tested PAHs and the AhR antagonist CH223191 showed that except for 8-MeBaP, the embryo lethality and/or developmental retardations (including yolk sac and pericardial edemas, deformed body shape and kinked tails) induced by the other tested PAHs in the ZET were reduced by CH223191. Co-exposure of the two estrogenic PAHs (i.e., BaA and 8-MeBaA) with the ER-α antagonist fulvestrant counteracted either embryo lethality or developmental retardations induced by the two estrogenic PAHs. Altogether, these findings suggested that both the ER-α and AhR are involved in mediating PAH-induced developmental toxicity observed in the ZET, whereas RAR appears to be not relevant. Moreover, comparison of the AhR activating potency of the tested PAHs and their developmental toxicity potency in the ZET (**Chapter 2**) showed that the potency of PAHs to activating the AhR cannot be directly translated into their developmental toxicity most likely because the AhR mediated gene expression is not the only decisive mechanism of PAH-induced developmental toxicity, and because the AhR reporter gene assay is merely a cell-based screening assay that lacks the physiological feedback mechanisms and active metabolism compared to the ZET. Last but not the least, our findings imply that the ZET is able to capture the role of the AhR and ER-α in the mode of action underlying the developmental toxicity of both naked and related methylated PAHs in the ZET bioassay.

A series of studies (Kamelia et al., 2017, 2018, 2019a, 2019b, 2021) has shown the usefulness of a battery of in vitro assays, consisting of the AhR CALUX assay, the ZET and also the mouse embryonic stem cell test (mEST), in detecting the developmental toxicity of some PAC-containing petroleum substances. To broaden the applicability domain of such an in vitro testing strategy in detecting the developmental toxicity of petroleum substances, in **Chapter 4** we evaluated the in vitro developmental toxicity of not only the petroleum substances with relatively high 3- to 7-ring PAC content which are dominated by alkylated PAHs (Tsitou et al., 2015; Yang et al., 2014), but also some highly refined petroleum substances and petroleum-

derived waxes using the above mentioned battery of in vitro assays. To this end, in total sixteen samples of petroleum substances from different product categories and varying in their PAH content were tested, including a poorly refined distillate and aromatic extract, extensively refined petroleum substances and petroleum-derived waxes. Results obtained showed that only the two poorly refined distillate and aromatic extract samples induced concentration-dependent effects in the mEST and ZET, and induced persistent AhR activation in the AhR CALUX assay. Two base oil samples were only able to induce transient AhR activation in the AhR CALUX assay. The other twelve samples, including highly refined petroleum substances and petroleum-derived waxes, tested negative in the AhR CALUX assay, mEST and ZET. Altogether, our results suggest that the refining processes applied during the production of highly refined petroleum substances and petroleum-derived waxes extensively reduces or even fully removes the undesired 3- to 7-ring PACs. As a result, the highly refined petroleum substances and petroleum-derived waxes do not induce in vitro developmental toxicity.

To further enable the use of NAMs in the developmental toxicity testing of methylated PAHs in **Chapter 5**, a rat physiologically-based kinetic (PBK) model was developed for 8-MeBaP to predict the in vivo developmental toxicity based on its in vitro developmental toxicity in the mEST and read-across from its corresponding unsubstituted BaP (Wang et al., 2021). It was hypothesized that, similar to BaP which requires bioactivation into 3-OHBaP to induce developmental toxicity, 8-MeBaP would also induce developmental toxicity via its major ring oxidation metabolite 3-OH-8-OHBaP. To verify this hypothesis, 8-MeBaP and 3-OH-8-MeBaP were tested for their in vitro developmental toxicity using the mEST. The results show that 8-MeBaP tested negative in the mEST whereas its metabolite 3-OH-8-MeBaP induced in vitro developmental toxicity in the mEST with an IC50 of 1.40 μM which is 1.1-fold lower than that of 3-OHBaP (IC50 = 1.51 μM). The PBK model developed for 8-MeBaP was based on BaP and consisted of eight compartments, including venous blood, arterial blood, fat tissue, liver tissue, lung tissue, rapidly perfused tissue, slowly perfused tissue, stomach and intestines. The time-dependent blood concentrations of 8-MeBaP and 3-OH-8-MeBaP upon intravenous, intratracheal and oral exposure were predicted and compared to those of BaP and 3-OHBaP respectively. The comparison indicated that the predicted time-dependent blood concentration of 8-MeBaP and 3-OH-8-MeBaP were in line with the predictions and the data reported in the literature for BaP and 3-OHBaP. The PBK model was subsequently used for reverse dosimetry to translate the in vitro concentration-response curve of 3-OH-8-MeBaP derived from the mEST into a predicted in vivo dose-response curve for developmental toxicity upon oral exposure to

8-MeBaP. The predicted ED50 for 8-MeBaP was 10.5 mg/kg bw, which was 1.3-fold lower than the predicted ED50 for BaP (14 mg/kg bw). Altogether, our results show the possibility of utilizing PBK model based QIVIVE and read-across to convert a PBK model from BaP, a PAH with available in vivo developmental toxicity data, to a PBK model for its methylated congener 8-MeBaP, an alkylated PAH for which such in vivo data are absent, and to use this PBK model to predict the in vivo developmental toxicity by PBK model based reverse dosimetry of data obtained in the mEST, which could further enable risk assessment for this alkylated PAH based on NAMs.

To conclude, this thesis presents the in vitro developmental toxicity of 4- and 5-ring unsubstituted and methylated PAHs and the possible underlying mode of action all characterized using in vitro assays. The developmental toxicity of some highly refined petroleum substances and petroleum-derived waxes was also investigated using a battery of in vitro assays (i.e., AhR CALUX, mEST and ZET) to broaden the applicability domain of this battery of in vitro assays in testing developmental toxicity of petroleum related products. Moreover, an in vitro – in vivo approach based on PBK model facilitated QIVIVE and read-across was developed based on previous results for the well-studied BaP to predict the in vivo developmental toxicity of a selected methylated BaP, 8-MeBaP.

2. General discussion and future perspectives

The present thesis evaluated the developmental toxicity of some 4- and 5-ring unsubstituted and related methylated PAHs, the influence of a methyl substituent on the developmental toxicity of PAHs, and the possible modes of action underlying their developmental toxicity using in vitro assays. Moreover, the usefulness of using an in vitro testing strategy to evaluate the developmental toxicity potency of highly refined petroleum substances with low level or devoid of PACs was also investigated. Last but not the least, PBK modeling facilitated reverse dosimetry based on read-across from the unsubstituted BaP to one of its methylated congeners 8-MeBaP was performed to predict the developmental toxicity of this methylated PAH. In the following sections the obtained results are further discussed with emphasis on the current study limitations and recommendations for future directions.

2.1 Influence of a monomethyl-substituent on developmental toxicity of PAHs and the possible underlying modes of action

In this section, the influence of a monomethyl substituent on the developmental toxicity of PAHs will be discussed. Moreover, the role of the AhR, ER-α and RAR in mediating the developmental toxicity of unsubstituted and methylated PAHs, and other possible modes of action underlying the PAH-induced developmental toxicity are also discussed.

2.1.1 Structure-toxicity relationships of unsubstituted and methylated PAHs

Results of **Chapter 2** suggested that the presence of a methyl substituent on the aromatic ring of BaA and BaP, depending on the position on the aromatic ring, could either increase or decrease the in vitro developmental toxicity in the ZET. The addition of a methyl substituent on the aromatic ring of PAHs influences their physico-chemical properties such as for example their lipophilicity which will be increased. Studies have shown that chemicals with higher lipophilicity can more easily penetrate cell membranes, which might result in more accumulation in the developing fetuses leading to greater developmental toxicity at a given dose level (Bleeker et al., 2003; Incardona et al., 2006; Wassenaar and Verbruggen, 2021). The structure-toxicity relationship of the nine PAHs tested in **Chapter 2** was evaluated by plotting their BMC20 for the extended general morphology scoring ($BMC20_{extended\text{-}GMS}$) in the ZET (**Chapter 2**) versus their octanol–water partition coefficient (log K_{ow}, calculated by using EPI-Suite 4.1). The obtained result (**Figure 1**) demonstrates that there is a poor correlation between the in vitro developmental toxicity potencies of the tested PAHs and their $\log K_{ow}$ ($R^2 = 0.1228$), implying that the increased lipophilicity caused by the presence of a methyl substituent cannot provide a full explanation for the different developmental toxicity potencies of the tested PAHs. It is reported that a methyl substituent might change the mode of action underlying PAH-induced developmental toxicity (Scott et al., 2011), and therefore several possible mechanisms potentially involved in causing the differences in the developmental toxicity of the unsubstituted and methylated PAHs tested are discussed in some more details in the following sections.

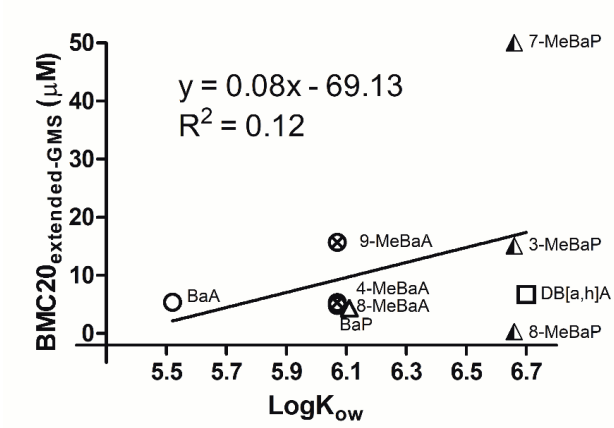

Figure 1. Correlation between the $BMC20_{extended\text{-}GMS}$ for the in vitro developmental toxicity of the tested PAHs in the ZET and their $logK_{ow}$ (calculated by using EPI-Suite 4.1).

2.1.2 The role of the AhR in developmental toxicity of unsubstituted and methylated PAHs

The full mechanism of PAH-induced developmental toxicity remains to be elucidated, although existing evidence suggests that the interaction between PAHs and the AhR and some nuclear hormone receptors (NHRs) including but not limited to the ER-α and RAR may play a role in this developmental toxicity. It is known that the AhR, ER-α and RAR are important in mammalian reproduction and fetal development (Barlow et al., 1999; Göttel et al., 2014; Hahn et al., 2009; Piersma et al., 2017), and thus interference with these receptors might lead to adverse effects on embryonic development.

Many PAHs have been reported to be AhR agonists, and it was also suggested that methylated PAHs are generally more potent than their corresponding unsubstituted PAHs in activating the AhR (Marvanová et al., 2008; Trilecová et al., 2011). Existing studies also demonstrated that the developmental toxicity observed upon exposure to some PAHs is accompanied by activation of the AhR (Goodale et al., 2013; Hawliczek et al., 2012; Matson et al., 2008; Timme-Laragy et al., 2007). However, comparison of the potency of the nine tested PAHs in activating the AhR (EC50 derived from the AhR CALUX assay in **Chapter 3**) and in inducing in vitro developmental toxicity (BMC20 derived from the ZET in **Chapter 2**) demonstrates that the increased AhR-mediated activity due to the addition of a methyl-substituent does not always result in an increased in vitro developmental toxicity of PAHs (**Figure 2**, $R^2 = 0.04$). 7-MeBaP, for example, was a potent AhR agonist in the AhR CALUX assay but it was not able induce developmental toxicity in the ZET. Moreover, results of co-exposure of zebrafish embryos to

the tested PAHs and an AhR antagonist CH223191 demonstrated that the AhR antagonist CH223191 can only partially but not fully inhibit the developmental retardations and embryo lethality induced by these PAHs. Altogether, these results suggested that the ability of PAHs to activating the AhR in cell-based assays cannot be directly translated to their ability to induce in vitro developmental toxicity, and that the PAH-induced developmental toxicity is only partly mediated by the AhR. The discrepancy between the AhR-mediated activities and the developmental toxicity of the unsubstituted and methylated PAHs tested could be due to the involvement of other nuclear receptors and/or can also in part be explained by the fact that the CALUX bioassays lack the physiological feedback mechanisms and metabolism that are present in the zebrafish embryos.

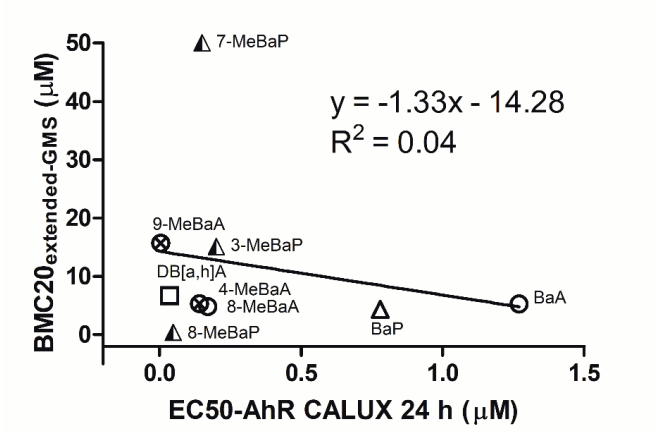

Figure 2. Correlation between the $BMC20_{extended\text{-}GMS}$ for the in vitro developmental toxicity of the tested PAHs in the ZET and their EC50 for AhR-mediated activities following 24 h exposure in the ZET (expressed as EC50-AhR CALUX 24 h).

In addition, it was reported that the differences in AhR-dependent developmental toxicity potencies of unsubstituted and methylated PAHs may be related to their interaction with different isoforms of the AhR (Goodale et al., 2012; Lille-Langøy et al., 2021). Zebrafish (*Danio rerio*) has three different isoforms of the AhR, namely AhR1a, AhR1b and AhR2, and these isoforms acquired different functional roles (Andreasen et al., 2002; Karchner et al., 2005). AhR2 is the predominant isoform present in zebrafish embryos and is required for the TCDD-induced toxicity in zebrafish; moreover, activation of AhR2 could subsequently induce CYP1A activity (Goodale et al., 2012; Souder and Gorelick, 2019). Two of the unsubstituted PAHs tested under study, BaA and BaP, induced AhR2-dependent developmental toxicity, implying

that some PAHs may induced AhR2-dependent developmental toxicity (Garner et al., 2013; Incardona et al., 2011, 2006; Knecht et al., 2017). AhR1b is functional in regulating the embryo development (Billiard et al., 2006; Timme-Laragy et al., 2008). Some studies report AhR1a as the nonfunctional receptor in zebrafish, as TCDD does not bind to it (Billiard et al., 2006; Goodale et al., 2012). However, Garner et al. (2013) reported that AhR1a may provide a protective effect towards PAH-induced toxicity in zebrafish (*Danio rerio*). In this study, knockdown of AhR1a using morpholine rejection exacerbated the embryo toxicity induced by BaP, benzo[k]fluoranthene and fluoranthene; moreover, increased CYP1A activity was observed when AhR1a was inhibited. Therefore, in contrast to AhR2 which mediated the developmental toxicity of TCDD and some PAHs in zebrafish embryos, the AhR1a may have a protective role in PAH-induced but not TCDD-induced toxicity. A recent study (Lille-Langøy et al., 2021) tested the ability of unsubstituted and methylated chrysene to transactivate the Atlantic cod AhR1a and AhR2a in vitro. The results showed that the tested methylated chrysenes were more potent agonists of both AhR1a and AhR2a than chrysene itself, and that the tested methylated chrysenes showed different potencies to activate the AhR1a and AhR2a, which might be related to their different molecular structure caused by the presence of the methyl substituent. Considering that the function of AhR isoforms is species-specific (Lille-Langøy et al., 2021), it would be of interest to investigate the interaction between methylated and unsubstituted PAHs and the AhR isoforms in zebrafish (*Danio rerio*) embryos to better understand the effect of methyl substituent on PAH-induced AhR-dependent developmental toxicity.

Besides interaction with different isoforms of the AhR, the different developmental toxicity of the tested PAHs (**Chapter 2**) might be related to their interaction with different parts of the AhR ligand-binding domain (LBD). In the present study, BaP and DB[a,h]A induced different developmental toxicity in the ZET. BaP disturbed the normal development of zebrafish embryos with concomitant embryo lethality at 96 hpf, whereas DB[a,h]A disturbed the normal development of zebrafish embryos without inducing embryo lethality up to the highest concentration tested. It was reported that BaP and DB[a,h]A bind to different parts of the mouse AhR LBD and this different binding mode within the mouse AhR LBD might be due to their different molecular dimension (Giani Tagliabue et al., 2019). Thus, it can be hypothesized that the effect of methylation on the molecular structure of PAHs leads to different binding modes to the zebrafish embryo AhR LBD, and might subsequently result in different AhR mediated effects and developmental toxicity.

Altogether, results of **Chapter 2** and **3** support the hypothesis that the observed developmental toxicity induced by the nine tested PAHs is partially mediated by the AhR. However, there are still several questions arising from the current findings that need to be addressed in the future. First of all, what are the potencies of the tested PAHs to activate the different AhR isoforms in zebrafish (*Danio rerio*) embryos, and how does the methyl-substituent influence the ability to bind to these isoforms? Secondly, which AhR isoforms of zebrafish (*Danio rerio*) embryos are responsible for the in vitro developmental toxicity of each of the tested PAHs? What is the role of different parts of the AhR LBD in mediating PAH-induced developmental toxicity and how do the PAHs interact with the AhR LBD? And finally, what are the differences in the downstream effects of this potentially differential AhR receptor activation by different (methylated) PAHs, and how does that relate to their developmental toxicity?

2.1.3 The role of ER-α and RAR in developmental toxicity of unsubstituted and methylated PAHs

Results of **Chapter 3** showed that the in vitro developmental toxicity of the two estrogenic PAHs, BaA and 8-MeBaA in the ZET could be partly counteracted by the ER-α antagonist fulvestrant, suggesting that this developmental toxicity of these estrogenic PAHs may be partially ER-α mediated. In **Chapter 3**, only the two PAHs that induced estrogenic activities in the U2OS ER-α CALUX assay were selected and further tested in the ZET in the absence or presence of the ER-α antagonist fulvestrant. It should be noted that the U2OS cell line applied in the ER-α CALUX assay is incapable of metabolic activation. Thus, the U2OS ER-α CALUX only evaluated the estrogenic activity of PAHs themselves but not of their metabolites. It is generally accepted that metabolism plays an important role in the developmental toxicity of some PAHs such as BaP (Kamelia et al., 2020; Wang et al., 2021) with the metabolites produced by ring hydroxylation being responsible for the observed developmental toxicity (Fallahtafti et al., 2012). Moreover, a previous study suggested that some hydroxylated BaPs, including 3-OHBaP, 7-OHBaP, 8-OHBaP and 9-OHBaP, were more estrogenic than BaP itself when tested in the T47D.Luc ER-α reporter cell line (Van Lipzig et al., 2005). This observation may be due to the fact that with an additional hydroxyl moiety the PAHs may better resemble the endogenous ligand for the ER-α, estradiol. Therefore, it is recommended to also test the nine PAHs under study using the T47D.Luc cell line or any other cell lines with metabolic capability to evaluate the estrogenic activity of not only the parent PAHs but also their major metabolites.

This will require custom synthesis of the respective hydroxy PAHs as was done for 3-OH-8-MeBaP in **Chapter 5** of the thesis.

Considering the importance of the RAR in vertebrate embryonic development, the role of the RAR in mediating PAH-induced developmental toxicity was also evaluated in the thesis. Results (**Chapter 3**) suggested that the tested PAHs were not able to interact with RAR, thus RAR appeared to be not relevant in PAH-induced developmental toxicity. Our results are in line with those from a previous study which tested the RAR mediated activity of 26 PAHs including BaP, BaA and DB[a,h]A, showing that none of the tested PAHs could activate the RAR (Beníšek et al., 2011, 2008). However, it was demonstrated that instead of acting directly as RAR ligands, PAHs indirectly affected retinoic acid-mediated signaling (Beníšek et al., 2011, 2008; Berntssen et al., 2015). This in vitro disruption of retinoid signaling caused by PAHs might be related to the PAH metabolites formed by CYP induction (Beníšek et al., 2011). As discussed previously, the U2OS cell line is metabolically inactive, thus similarly to the ER-α, it is recommended to test the nine PAHs using cell lines with a metabolically active system to further understand the influences of not only parent PAHs but also their metabolites on the retinoic acid-mediating signaling.

2.1.4 The role of metabolism in developmental toxicity of PAHs

Existing evidence suggests that metabolism plays an important role in the developmental toxicity of PAHs. PAHs that act as AhR agonists can activate cytochrome P450 monooxygenases (CYP450) which could subsequently lead to bioactivation of PAHs into developmentally toxic metabolites (Fallahtafti et al., 2012; Hodson, 2017; Timme-Laragy et al., 2008). Results of **Chapter 5** together with those from previous studies (Kamelia et al., 2020; Wang et al., 2021) showed that in the mEST in addition to BaP also its methylated analogue 8-MeBaP, can exert developmental toxicity only upon its bioactivation to the corresponding 3-OH metabolite . The ring hydroxylation metabolites 3-OHBaP and 3-OH-8-MeBaP did induce in vitro developmental toxicity in the mEST, while the parent PAHs tested negative. In line with this result, another study suggested that metabolism may enhance the toxicity of alkylated phenanthrenes in early life stages of fish by the generation of ring-oxidation metabolites (Fallahtafti et al., 2012). Given this toxicity of the ring hydroxylated metabolites it is of interest to note that a previous study reported that the presence of a methyl substituent on the aromatic ring of BaP shifted the metabolism from aromatic ring oxidation to aromatic chain oxidation (Wang et al., 2022). However, comparison of the in vitro developmental toxicity between BaP

and its three methylated congeners (i.e., 3-MeBaP, 7-MeBaP and 8-MeBaP) in the ZET implies that despite the metabolic shift from aromatic chain to side chain oxidation, the presence of a methyl substituent on BaP does not always reduce its developmental toxicity. This may be related to the observation also presented by Wang et al. (2022) that the presence of a methyl substituent at C8 could increase the rate of metabolism compared to that of BaP, whereas a methyl substituent at C7 potentially blocked the metabolism. As a result, the rate of formation and the resulting endogenous concentrations of the metabolite from ring oxidation may not always decrease when side chain oxidation increases as a result of a methyl substituent. Altogether, for the PAHs that require metabolism to exert their developmental toxicity, the presence of a methyl substituent on different positions of the unsubstituted PAHs can significantly influence the biotransformation and the resulting developmental toxicity. Besides metabolism-dependent developmental toxicity, some PAHs such as DB[a,h]A could exert developmental toxicity in the mEST without bioactivation implying a metabolism-independent mode of action underlying the developmental toxicity of some PAHs (Kamelia et al., 2020). At the present state-of-the-art it is hard to predict upfront which PAHs require bioactivation and which do not, and this remains a topic of interest for future studies.

Many studies reported that CYP1A induction was observed upon exposure to PAHs like BaP, BaA, pyrene, chrysene, and benz[b]anthracene (Billiard et al., 2006; Incardona et al., 2011, 2006; Timme-Laragy et al., 2008). Comparing to other CYP subfamilies, CYP1A activity appears to have a much earlier onset in the developing zebrafish embryos, while at the same time CYP1A is considered to be responsible for the bioactivation of PAHs into their developmentally toxic metabolites (Fallahtafti et al., 2012; Hodson et al., 2007; Hodson, 2017; Saad et al., 2017, 2016). Based on these observations, it has been commonly accepted that CYP1A activity is highly associated with mediating PAH-induced toxicity. However, several studies reported that inhibition of CYP1A decreased the CYP1A activity but exacerbated the developmental toxicity induced by several PAHs including BaP, β-naphthoflavone and retene in fish embryos (Billiard et al., 2006; Timme-Laragy et al., 2007; Wassenberg and Di Giulio, 2004). These findings imply that at least in fish embryos CYP1A might have protective effects against PAH-induced developmental toxicity, in contrast to the commonly accepted hypothesis for both mammalian and invertebrate systems that CYP1A activity increases the toxicity of PAHs via bioactivation (Fallahtafti et al., 2012; Ma and Lu, 2007; Uno et al., 2001). Meanwhile, inhibition of CYP1A activity might shift the metabolism of PAHs to produce other toxic

metabolites (Hodson et al., 2007) suggesting the involvement of other CYP isoforms such as CYP1B, CYP1C, CYP1D in mediating the developmental toxicity of PAHs.

Altogether, the existing evidence shows that the developmental toxicity induced by PAHs may be either metabolism-dependent or independent. Regarding the metabolism-dependent pathway, the position of the methyl substituent could influence the biotransformation and subsequently result in different developmental toxicities of the substituted as compared to the respective naked PAH. Methylation can either increase or decrease the developmental toxicity of the PAH. This may result from side chain oxidation replacing aromatic ring oxidation, the blocking of the hydroxylation site by the methyl substituent, or effects on the overall rate of metabolism resulting in increased concentrations of the toxic metabolite. Moreover, it is suggested that in mammalian model systems, the higher developmental activity of hydroxylated PAHs as compared to the parent PAHs indicates the importance of CYP1A in PAH-induced developmental toxicity, while in fish CYP1A activity may have protective effects against PAH-induced developmental toxicity rather than mediating the developmental toxicity. It is possible that CYP1A is not the exclusive enzyme involved in the biotransformation of PAHs, and the role of other CYP enzymes that are present in zebrafish should also be taken into consideration. Therefore, it is recommended for future research to also focus on the role of each of the CYP isoforms in individual PAH-induced developmental toxicity.

2.1.5 Other modes of action

Despite the fact that results of the present thesis demonstrate that the developmental toxicity induced by the nine unsubstituted and methylated PAHs is partially mediated by the AhR and/or ER-α, and that metabolism plays an important role for some but not all of the PAHs in exerting developmental toxicity, it should be noted that other modes of action have also been proposed. For example, exposure of zebrafish embryos to phenanthrene caused cardiac defects via perturbing the calcium pathway (Zhang et al., 2013), and exposure to dibenzothiophene and pyrene affected the normal development of zebrafish by disturbing the ion balance (Goodale, 2013). These mechanisms were not evaluated in the present thesis but could be considered when conducting future research.

2.2 Mixture effects of PACs

Chapter 2 and **Chapter 3** of the present thesis evaluated the in vitro developmental toxicity of individual PAHs and the possible underlying modes of action. However, PAHs always occur

as mixtures, and the petroleum substances tested in **Chapter 4** are good examples of highly complex materials with varying concentration of PACs. Lam et al. (2018) reported that a concentration addition model for predicting the potency of PAH mixtures to activate the AhR could not always provide correct predictions of the real AhR-mediated activity of the self-designed mixtures tested in the H4IIE-luc bioassay. This study suggested that PAH mixtures may exert additive and also nonadditive-inhibitory effects. Billiard et al. (2004) suggested that in case of a binary mixture of PAHs, binary mixtures of two PAHs with strong or two PAHs with weak CYP1A induction potency showed additive effects, whereas binary mixtures of a PAH with strong and a PAH with weak CYP1A induction potency showed synergistic effects. However, the PAH-containing materials such as petroleum substances are more complex than those lab-designed PAH mixtures. Petroleum substances contain from few to thousands of hydrocarbons including PACs with varying potency in activating the AhR and CYP enzymes. These features of petroleum substances increase the difficulty to evaluate the interactions between individual PACs in a mixture content. Thus, before defining mixture effects of PACs, it is important to first determine the potency of individual PAHs in mediating the developmental toxicity and the underlying modes of action as well as the nature of the PAHs actually present in a mixture.

2.3 Characterization of PAH content present in poorly and highly refined petroleum substances

REACH legislation requires prenatal developmental toxicity testing for chemicals/substances produced in or imported to the EU at a volume of more than 100 tonnes a year, which include petroleum substances (ECHA, 2009). Developmental toxicity testing is one of the most animal and resource demanding test endpoints. According to OECD TG 414, testing one petroleum substance might require more than 3000 animals (two species, one rodent and one non-rodent; Shell, 2019). This poses demands for alternatives to animal tests to reduce the number of animals used in prenatal developmental toxicity testing. A series of previous studies (Kamelia et al., 2021, 2019b, 2018, 2017) successfully evaluated the in vitro developmental toxicity of some petroleum substances extracts using NAMs i.e., a combination of the AhR CALUX assay, the mEST and the ZET, and concluded that the observed in vitro developmental toxicity of the petroleum substances is proportional to their 3- to 7-ring PAC content. **Chapter 4** of the present thesis aimed to broaden the applicability domain of this testing strategy by testing not only poorly refined petroleum substances with relatively high concentration of 3- to 7-ring PACs,

but also some highly refined petroleum substances and petroleum-derived waxes with either very low levels or devoid of PACs.

To investigate the role of PACs in the developmental toxicity of petroleum substances, the aromatic ring class (ARC) profile, which presents the weight percent of the DMSO-soluble 1 to 7 aromatic ring compounds present in a petroleum substance has generally been quantified (Kamelia et al., 2019a, 2019b, 2018, 2017). A linear correlation between the total or specific PAC content of a petroleum substance provided by ARC profile and the corresponding in vitro developmental toxicity potency has been observed in both in vitro and in vivo studies (Feuston et al., 1994; Kamelia et al., 2019a, 2019b, 2018, 2017; Murray et al., 2013). Our results together with results of previous studies (Kamelia et al., 2019b; Mobil, 1987) indicate that some petroleum substances, such as the highly refined base oil samples which undergo extensively refining processes during production and are devoid of PACs, did not induce in vitro or in vivo developmental toxicity. However, the lack of in vivo developmental toxicity data of the highly refined petroleum samples tested in the present thesis, makes it difficult to evaluate whether the negative results from the in vitro testing adequately predicts the absence of their in vivo developmental toxicity. Nevertheless, results of **Chapter 4** showed that the DMSO extracts of highly refined petroleum substances tested negative for in vitro developmental toxicity and it should be noted that these samples are all derived from raffinates and could also pass the IP346 test. IP346 is a gravimetric method based on a DMSO extraction to determine the total PAC in base oils. The percentage of DMSO extracts by IP346 method can be used for the prediction of the carcinogenic potential for mineral oils. If the DMSO extract % is less than the cut-off of IP346 which is 3% w/w, samples are considered non-dermally carcinogenic (Carrillo et al., 2022). Altogether, it implies the results of the IP346 test can also be used in combination with the proposed in vitro testing strategy (i.e., AhR CALUX assay, mEST and ZET) to evaluate the developmental toxicity of the highly refined petroleum substances derived from raffinates (i.e., the refined petroleum substances and waxes) and raffinates themselves.

2.4 Limitations and future recommendations of evaluating developmental toxicity using in vitro assays

New approach methodologies (NAMs) were applied in the present thesis to evaluate the developmental toxicity of individual PAHs and petroleum substances. In the present section limitations and future recommendations for the NAMs applied in the present thesis either as an individual test or as an integrated testing strategy are discussed.

2.4.1 ZET for individual PAHs

Zebrafish (*Danio rerio*) embryos are a popular vertebrate model for assessing developmental toxicity of many chemicals and substances (Adam et al., 2021; Dach et al., 2019; Hawliczek et al., 2012; Kamelia et al., 2019a; Meyer-Alert et al., 2019; Wolinska et al., 2011). The close similarity between the zebrafish embryo development and human embryogenesis makes this model relevant for developmental toxicity evaluation (Rothenbücher et al., 2019; Sipes et al., 2011). Moreover, like mammals including human, zebrafish embryos respond to xenobiotics with both phase I and phase II metabolism (de Souza Anselmo et al., 2018, 2017). Two of the PAHs tested in this thesis, BaP and 8-MeBaP, required bioactivation to exert in vitro developmental toxicity (**Chapter 5**; Wang et al., 2021). When testing BaP and 8-MeBaP in the mEST which lacks a metabolic system, both PAHs were not able to inhibit the differentiation of ES-D3 cells into beating cardiomyocytes at non cytotoxic concentrations indicating that BaP and 8-MeBaP tested negative in the mEST. Their 3-hydroxy metabolites 3-OHBaP and 3-OH-8-MeBaP however did induce in vitro developmental toxicity in the mEST. However, when testing BaP and 8-MeBaP in the ZET (**Chapter 2**) both of them affected the normal development of zebrafish embryos suggesting that zebrafish embryos are capable of bioactivation of PAHs into their developmentally toxic metabolites. However, it is also reported that zebrafish embryos up to 96 hpf show low biotransformation capacity, substantially lower than that of humans, which might hamper the accuracy of predicting the developmental toxicity of PAHs for humans in the ZET (Giusti et al., 2019; Saad et al., 2017). Giusti et al., (2019) developed a novel methodology combining the ZET with an in vitro pre-metabolic bioactivation system. In this method, chemicals are first incubated with rat liver microsomes at 37 °C for 1 h, then zebrafish embryos at 72 hpf are exposed for 48 h to the metabolic mixture diluted in Danieau's medium (Giusti et al., 2019). This method successfully improved the predictivity of the toxicity of parathion because the incubation of parathion with rat liver microsomes generated paraoxon, the active metabolite causing toxicity. This combination of the ZET with such a mammalian metabolic system could also improve the accuracy of the ZET to predict the developmental toxicity of PAHs, especially for the PAHs that require bioactivation to become developmentally toxic.

In **Chapter 3**, the role of the AhR in the developmental toxicity of the selected PAHs was evaluated by exposing the zebrafish embryos to the selected PAHs in the presence or absence of an AhR antagonist CH223191. Before testing in the ZET, the counteracting effects of CH223191 on PAH-induced AhR mediated activity was evaluated in the AhR CALUX assay.

Results of these preliminary tests (data not shown) demonstrated that 100 μM CH223191 could not inhibit the induction of AhR gene expression by the highest concentration of the PAHs tested in the AhR CALUX assay (i.e., 5 μM), therefore lower concentrations, namely the EC50 values of the tested PAHs in the AhR CALUX assay were selected for further testing to facilitate competition by the antagonist. Results show that 100 μM CH223191 effectively inhibited the AhR gene expression induced by the PAHs at their EC50 values in the AhR CALUX assay, implying that a high ratio between CH223191 and the tested PAHs is required to achieve full inhibition on the AhR-mediated activity. This excess in the concentration of CH223191 over that of the tested PAHs might also be required for obtaining even stronger inhibition of the PAH induced developmental toxicity in the zebrafish embryos than what was detected in Chapter 3. However, increasing the concentration of CH223191 appeared not feasible as 1 μM was the highest concentration of CH223191 not to affect the normal development of the zebrafish embryos until 96 hpf (**Chapter 3**, Supporting Information 1). Therefore, it is recommended for future studies to consider using a morpholino approach to knock out the AhR in the zebrafish embryo to study the role of the AhR, or any other receptor of interest, in the developmental toxicity of PAHs in the ZET.

2.4.2 CALUX reporter gene assays for individual PAHs

For individual PAHs tested in the present thesis, their AhR-, ER-α- and RAR-mediated activities were evaluated using the corresponding CALUX reporter gene assays. The PAHs that interacted with any of the aforementioned receptors in the CALUX assays were further tested in the ZET by exposing the zebrafish embryos to these PAHs in the absence and presence of the antagonist of the respective receptor. Thus, the CALUX assays were used as a screening tool to identify the ability of a compound to interact with any of these receptors but was not used to predict the in vitro or even in vivo developmental toxicity of the test compound. This is because the cell-based CALUX reporter gene assays lack the physiological feedback mechanisms and metabolism system which are present in the in vivo situation. A hormone homeostatic system is maintained by positive and negative feedback loops. The CALUX assays only provide information on whether chemicals can interact with certain receptors but cannot evaluate whether the chemical could interfere with the hormone homeostasis and subsequently result in adverse effects including developmental toxicity in vivo.

2.4.3 A battery of in vitro assays for detecting developmental toxicity of petroleum substances

The battery of vitro assays applied in **Chapter 4** (i.e., AhR CALUX, mEST, ZET) was proposed by Kamelia et al., (2021, 2019a, 2018, 2017) and shown to successfully evaluate the in vitro developmental toxicity of petroleum substances extracts with varying PAC content. Results of **Chapter 4** indicate that such a battery of in vitro assays could not only detect the developmental toxicity of the poorly refined petroleum substances (with a substantial level of 3- to 7-ring PACs), but could also capture the absence of developmental toxicity of the highly refined petroleum substances and petroleum-derived waxes, which contain a low level or are devoid of PACs. Unfortunately, due to the lack of relevant in vivo data, the accuracy of this prediction for the in vivo developmental toxicity of highly refined petroleum substances and petroleum-derived waxes based on the battery of in vitro tests cannot be evaluated. But still other studies reported that no PACs were detected in the highly refined petroleum samples, and that for the samples with available developmental toxicity data, no in vitro and/or in vitro developmental toxicity was detected. For example, one highly refined base oil sample (CAS No. 8042-47-5) tested negative for developmental toxicity both in vivo and in vitro (Kamelia et al., 2019b; Mobil, 1987). In addition, two base oil samples (CAS No. 64742-65-0 and 64742-70-7) with no measurable PACs tested negative for in vivo developmental toxicity in rats via dermal exposure (HPV, 2011a), and 5 petroleum derived wax samples (CAS No. 64742-61-6, 8002-74-2, 64742-51-4, 63231-60-7 and 8009-03-8) contained no PACs (HPV, 2011b). Altogether, these data, together with the results of the present thesis, suggested that the highly refined petroleum substances and petroleum-derived waxes are devoid of or contain only no measurable concentration of PACs, and therefore do not pose a developmental toxicity hazard, in line with what was observed in the battery of in vitro assays.

As the organ that connects the mother and fetus, the placenta is essential in supporting the normal growth of the fetus by transferring nutrients and oxygen from mother to fetus (Gude et al., 2004). Toxicants able to cross the placenta barrier and to damage the fetus are assumed to induce direct developmental toxicity (Dugershaw et al., 2020). Thus the placental transfer rate of potentially toxic compounds is considered as an important kinetic parameter of influence on the developmental toxicity in vivo. However, the in vitro assays for developmental toxicity testing in the present study, i.e., mEST and ZET, lack such a placental transport system which implies that these assays do not consider the placental transport of compounds. To solve this issue, the battery of in vitro tests could be extended to include in vitro bioassays that enable detection of placental transfer and of effects of test chemicals on this transfer. For this one might

consider model systems that represent the placental barrier. For example, evaluating the placental transfer of PAHs or petroleum substances in the in vitro BeWo transport model (Dimopoulou et al., 2018) might help to characterize the role of placental transport in the developmental toxicity PAHs and petroleum substances.

Regarding the modes of action underlying the developmental toxicity induced by petroleum substances, an AhR-mediated effect was previously identified as an important one based on the good correlation (R^2= 0.80) observed between the EC50s in the AhR CALUX assay and the BMC50s in the mEST (Kamelia et al., 2018). However, a good correlation between the AhR mediated activity and the in vitro developmental toxicity of petroleum substances does not necessarily imply that activation of the AhR is the only decisive factor. Results of **Chapter 2** suggested that besides the AhR, also the ER-α can play a role in the developmental toxicity of some PAHs. Given this potential role of the ER-α it is also of interest to note that Kamelia et al. (2018) reported that several petroleum substances were able to interact with the ER-α (both agonistic and antagonistic) even though these interactions seem to not match with their corresponding in vitro developmental toxicity tested in the mEST. Altogether, these findings suggest that the petroleum substances or PAH mediated activities of other receptors which show "lower" correlation with the developmental toxicity should not be neglected.

2.4.4 Relevance of the in vitro assays applied in the present thesis for human risk assessment

One of the ultimate goals of using NAMs is to contribute to the 3Rs (replacement, reduction, refinement) of use of experimental animals in toxicological human risk assessment. Thus, the usefulness of the three in vitro assays (i.e., AhR CALUX assay, EST and ZET) in human risk assessment is discussed in this section.

For traditional risk assessment, points of departure (PODs) are in most cases derived from animal data. Regarding the mEST, several studies have provided the proof-of-principle that in vitro concentration-response curves derived from the mEST can be translated into dose response curves for in vivo developmental toxicity using PBK modelling facilitated reverse dosimetry (Li et al., 2015; Louisse et al., 2017; Wang et al., 2021). With the predicted in vivo developmental toxicity curves, PODs could be obtained for risk assessment to determine acceptable daily intake values (ADI) applying uncertainty factor for interspecies differences, interindividual differences and potentially also an extra uncertainty factor for the fact that the POD was derived via an alternative testing strategy, bringing extra uncertainties. When the

PBK model used for the reverse dosimetry would be based on human kinetics one could consider eliminating the uncertainty factor of 4.0 for the interspecies differences in kinetics, which is part of the overall default uncertainty factor of 10 for interspecies differences. Using the mEST for predicting PODs for human risk assessment should also consider the fact that the mEST does not take metabolism into consideration, so the assay would have to be combined preferably with a metabolic system of human origin such as human liver microsomes. The models referred to above which used rat liver microsomal incubations in combination with the mEST (Kamelia et al., 2020) may provide a suitable model to overcome this limitation.

Considering the relevance of the CALUX reporter gene cell-based assays for human risk assessment it is importance to consider whether the receptors integrated into the assay are of human origin or not. The ER-α and RAR CALUX assays used in the present study are based on the human U2OS cell line stably transfected with human ER-α or RAR (Mertl et al., 2014), while the AhR CALUX assay is based on the recombinant rat H4IIE cell line stably transfected with mouse AhR (Garrison et al., 1996). In cases where the receptor is not of human origin it has to be kept in mind that species differences in the nature of the receptor my exist, as already outlined above for the AhR in mouse, human and zebrafish. It also has to be considered that due to the lack of physiological feedback mechanisms and a metabolism system which are present in the in vivo situation, the interaction between test compounds and a certain receptor cannot be translated directly to an ability to disturb the hormone balance in vivo. Moreover, as discussed previously (**sections 2.1.1, 2.1.2, 2.1.3, 2.1.4**), the developmental toxicity of individual PAHs is complex and not due to the interaction with a single receptor. Therefore, results from the CALUX reporter gene assays could not be used to provide PODs that can be used in human risk assessment on the developmental toxicity of PAHs, not even when the respective CALUX cells are based on human receptors.

The ZET is the only in vitro assay applied in the present thesis that utilizes intact developing organisms to evaluate the in vitro developmental toxicity. The zebrafish genes share orthologs with around 70% of human genes, and many essential biological pathways are conserved across zebrafish and human (Rothenbücher et al., 2019; Sipes et al., 2011). These advantages make zebrafish a good tool for screening the developmental hazard of chemicals (Adam et al., 2021; Dach et al., 2019; Gebrelibanos Hiben et al., 2019; Geier et al., 2018b, 2018a; Incardona et al., 2006). However, for human risk assessment, whether the ZET can be used as a valid alternative remains controversial. First of all, zebrafish embryos (until 96 hpf) have very limited

metabolism compared to humans or mammalian experimental animals such as mice or rats (Rothenbücher et al., 2019; Saad et al., 2017, 2016; Van Steijn et al., 2019). Moreover, the liver, which plays a central role in mediating xenobiotic metabolism, is not fully developed in zebrafish embryos until 120 hpf, while zebrafish embryos are only exposed in the ZET to test compounds from 4 to 96 hpf. This suggests that the toxicokinetics of compounds in zebrafish embryos might be different from that in human or rodents due to the limited metabolism in zebrafish embryos until 96 hpf. However, so far it is still unclear that to what extent the zebrafish embryo metabolism is different from that in human or rodents. In addition to differences in metabolism, different exposure routes between mammals and zebrafish might also influence the kinetics of compounds while it may also hamper QIVIVE. Mammals such as mice and rats are generally exposed to test compounds via diet, injection or inhalation exposure, whereas zebrafish embryos are immersed in egg water with test compounds and absorb test compounds directly from the medium. The different exposure routes between zebrafish embryos and mammals makes it difficult to determine what an internal dose in zebrafish embryos is equivalent to that of mammals. This implies that the PODs for risk assessment could not be derived by QIVIVE when the ZET is used. Considering the differences of metabolism and routes of exposure between zebrafish embryos and human/mammals, it is suggested to test the in vitro developmental toxicity using a battery of tests such as for example the battery consisting of the AhR CALUX assay, mEST and ZET, rather than using the ZET as a stand-alone assay. Altogether, although the ZET has been generally accepted as a good screening tool for identifying compounds with potential developmental hazards, using the ZET as alternative to animal testing in human risk assessment is limited by the knowledge gaps such as information on the different kinetics between zebrafish embryos and mammals.

2.5 PBK modeling facilitated QIVIVE and read-across

Chapter 5 present a good example of how to use PBK modeling facilitated QIVIVE and read-across from BaP, a PAH with available in vivo developmental toxicity data, to predict the developmental toxicity of its methylated congener 8-MeBaP for which in vivo data on developmental toxicity are absent. The PBK model previously developed for BaP was validated based on available in vivo toxicokinetic data of BaP in rats (Wang et al., 2021). Subsequently, PBK modeling facilitated reverse dosimetry adequately translated a concentration–response curve for 3-OHBaP obtained in the mEST into an in vivo dose–response curve for developmental toxicity of BaP in rats upon single or repeated dose exposure (Wang et al., 2021). The read-across from BaP to 8-MeBAP enabled developing a PBK model for 8-MeBaP based

on the hypothesis that BaP and 8-MeBaP have a similar bioactivation pathway and both induce developmental toxicity via their 3-OH metabolite.

Besides alkylated PAHs, the aromatics present in petroleum substances also include heterocyclic aromatic compounds (S-, N- or O- atom containing PACs). Similar to alkylated PAHs, limited toxicological data exits for heterocyclic aromatic compounds. But several studies suggest that some heterocyclic aromatic compounds are more developmental toxic than the naked PAHs as tested in zebrafish embryo models (Chlebowski et al., 2017; Geier et al., 2018a; Wincent et al., 2015). Thus, the PBK model developed for predicting in vivo developmental toxicity of BaP and 8-MeBaP might be also further extended to heterocyclic aromatic compounds.

The predicted in vivo ED_{50} for developmental toxicity of 8-MeBaP was 1.3-fold lower than that of BaP. The in vitro developmental toxicity of their corresponding 3-OH metabolites tested in the mEST, revealed an IC50 for 3-OH-8-MeBaP (IC50 = 1.40 μM) that was 0.9-fold lower than that for 3-OHBaP (IC50 = 1.51 μM). However, neither the in vivo prediction nor the in vitro developmental toxicity tested in the mEST shows the same potency difference as observed for the in vitro developmental toxicity potency of BaP and 8-MeBaP tested in the ZET (**Chapter 2**), with the BMC20 of 8-MeBaP being 10 times lower than that for BaP. This difference could be explained by the differences between the ZET and mEST. For the mEST, a specific endpoint, inhibition of ES-D3 cell differentiation into cardiomyocytes is used as readout for in vitro developmental toxicity. Whereas for the ZET, any morphological changes upon exposure to test compounds or embryo lethality are included in the evaluation of the in vitro developmental toxicity. However, many chemicals including PAHs could also induce non-specific toxicity in the ZET, such as embryo lethality before 24 hpf and malformations induced by hypoxia, which can hamper the accuracy of predicting developmental toxicity using the ZET. Moreover, the differences between the metabolic CYP enzymes in zebrafish embryos and the absence of metabolic activation in the mEST could also lead to different developmental toxicity potencies.

By using PBK facilitated reverse dosimetry, in vivo dose-response curves for developmental toxicity for BaP and 8-MeBaP were predicted from the in vitro concentration response curves for their 3-OH metabolites obtained in the mEST. With the predicted in vivo dose-response curves of BaP and 8-MeBaP, a $BMDL_{10}$ (lower confidence limit of the benchmark dose causing 10% extra effect above background) could be calculated as point of departure (POD) for the risk assessment of these PAHs. The $BMDL_{10}$ of BaP was 1.97 mg/kg bw and that of 8-MeBaP

was 1.64 mg/kg bw (calculated using the EFSA BMD modeling web-tool, with 95 % confidence interval). According to EFSA, (2008), the average dietary exposure to BaP across European countries is 3.9 ng/kg bw per day assuming a body weight of 60 kg. Based on these data a Margin of Safety (MOS) was calculated for BaP by using the equation MOS=BMDL/EDI. The MOS for BaP amounted to ~500,000, which indicates a low priority of risk management for the developmental toxicity of BaP for the general population across European countries. Although there is no reported estimated daily intake of 8-MeBaP upon dietary exposure in any populations available, it is estimated from the MOS of BaP that 8-MeBaP might also have low priority for risk management for the developmental toxicity. Altogether, this result implies that BaP poses little developmental risk for humans exposed to BaP only via dietary exposure. It should be noted that this is only a rough estimation for the developmental toxicity risk of BaP and 8-MeBaP, as humans could also be exposed to PAHs via smoking and occupational exposure which might pose a developmental risk. Moreover, many PAHs including BaP and 8-MeBaP are also genotoxic and carcinogenic, thus the PBK model developed for predicting in vivo developmental toxicity of BaP and 8-MeBaP could be also further extended to evaluate their in vivo genotoxicity, which could further improve the risk assessment of PAHs.

3. Conclusion

To conclude, the present thesis evaluated the in vitro developmental toxicity of nine 4- and 5-ring PAHs and the possible underlying modes of action using the ZET and CALUX reporter gene assays. Obtained results demonstrated that the presence of a monomethyl substituent could either increase or decrease the developmental toxicity of PAHs, and that the PAH-induced developmental toxicity may partially be AhR- and/or ER-α-mediated. The thesis also presented a good example of how to predict developmental toxicity of a methyl substituted PAH for which in vivo data are not available, by PBK modeling facilitated read-across from a non-substituted structural analogue for which in vivo data have been reported. Moreover, the applicability domain of an in vitro testing strategy, consisting of AhR CALUX assay, mEST and ZET, was extended from petroleum substances containing relatively high level of PAHs to highly refined petroleum substances and petroleum-derived waxes with low level or devoid of PAHs. Altogether, the present thesis provides new insights on the developmental toxicity of PAHs and petroleum substances using both in vitro and in silico methods, thereby contributing to the 3Rs of use of experimental animals in toxicological hazard and risk assessment

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CHAPTER 7.

Summary

Summary

The developmental toxicity of petroleum substances has been associated with their content of 3- to 7-ring polycyclic aromatic compounds (PACs) consisting of naked or lowly, short-chain alkylated heterocyclic aromatics and polycyclic aromatic hydrocarbons (PAHs). Petrogenic petroleum substances are dominated by alkylated PAHs instead of by their unsubstituted/parent PAHs, and limited is known about the developmental toxicity of alkylated PAHs. Though limited, shreds of evidence suggests that the presence of a methyl substituent on the aromatic ring could either increase or decrease the developmental toxicity of PAHs. The aim of the present thesis was to investigate the influence of the presence and position of a methyl substituent on the aromatic ring of PAHs on their developmental toxicity, and the possible mode(s) of action underlying this toxicity. This was investigated by testing the developmental toxicity of three unsubstituted 4- and 5-ring PAHs and six of their methylated analogues using the zebrafish embryo toxicity test (ZET). Moreover, the present thesis investigated the usefulness of a battery of in vitro assays including the aryl hydrocarbon receptor Chemical-Activated LUciferase gene eXpression assay (AhR CALUX assay), the mouse embryonic stem cell test (mEST) and the ZET to evaluate the developmental toxicity of not only petroleum substances with relatively high level of PACs, but also of some highly refined petroleum substances and petroleum-derived waxes with low level or devoid of PACs. Finally, a rat physiologically- based kinetic (PBK) model was developed for 8-methyl-benzo[a]pyrene (8-MeBaP) and used for quantitative in vitro to in vivo extrapolation (QIVIVE) of data for its metabolite 3-OH-8-MeBaP in the mEST to predict the in vivo developmental toxicity of 8-MeBaP based on read-across from its corresponding unsubstituted benzo[a]pyrene (BaP).

Chapter 1 provides the basic information on PAHs and petroleum substances and their developmental toxicity. The selected model compounds tested in the thesis, including both individual PAHs and petroleum substances, are described. In addition, the in vitro assays for developmental toxicity testing and the in vitro-in silico approach to predict the developmental toxicity based on read-across, were introduced, together with the aim of the thesis.

Chapter 2 tested the hypothesis that the developmental toxicity of monomethylated PAHs as compared to the unsubstituted parent PAH is influenced by the position of the methyl substituent on the aromatic ring. The results show that except for 7-methyl-benzo[a]pyrene (7-MeBaP), all the eight tested PAHs induced developmental toxicity to zebrafish embryos at 96 hours post fertilization (hpf), and the methyl substituent on different positions of the aromatic

ring of the PAHs could either increase or decrease the in vitro developmental toxicity. It is speculated that the effect of a methyl substituent on the molecular dimension of the PAHs influences the interaction with the different ligand binding domains of the AhR, subsequently resulting in differences in developmental toxicity potency.

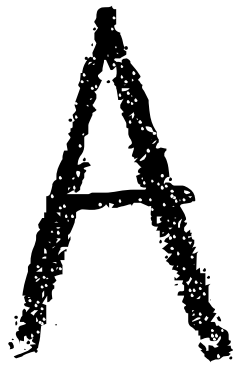
Chapter 3 evaluated the possible role of three receptors: the AhR, the estrogen receptor alpha (ER-α) and the retinoic acid receptor (RAR) in mediating the developmental toxicity of unsubstituted and monomethylated PAHs in the ZET. To achieve this, the AhR-, ER-α- and RAR-mediated activities of the unsubstituted and monomethylated PAHs were evaluated in the different CALUX reporter gene assays. The results show that all PAHs tested were AhR agonists, and all monomethylated PAHs more potently activated the AhR compared to their corresponding unsubstituted PAH, suggesting that the presence of a methyl substituent enhanced the AhR-mediated activity of PAHs. Two of the tested PAHs, benz[a]anthracene (BaA) and 8-methyl-benz[a]anthrancene (8-MeBaA) induced estrogenic activity; none of the PAHs induced anti-estrogenic activity; and none of the PAHs interacted with the RAR. PAHs that interacted with the AhR and ER-α were further tested in the ZET in the absence or presence of an AhR antagonist (CH223191) or an ER-α antagonist (fulvestrant). The results of the co-exposure experiment in the ZET revealed that the developmental toxicity induced by the selected PAHs, except for 8-MeBaP, was partly reduced by the presence of CH223191 or fulvestrant. Altogether these findings support the hypothesis that the AhR and/or ER-α is involved in mediating PAH-induced developmental toxicity as observed in the ZET, whereas the RAR appears to be not relevant.

In **Chapter 4** the developmental toxicity of sixteen petroleum substances with different degree of refinement (from poorly to highly refined) was tested using a battery of in vitro assays, consisting of the AhR CALUX assay, mEST and ZET. The results show that only the two poorly refined substances i.e., one aromatic extract and one distillate, which are not subject to any refinement process to remove their 3- to 7-ring PAC content, induced persistent AhR activation and in vitro developmental toxicity in the mEST and ZET. The other fourteen samples which were all highly refined petroleum substances or petroleum-derived waxes with typically a very low amount or no PACs at all, tested negative for their developmental toxicity in the mEST and ZET. The results confirm the hypothesis that the 3- to 7-ring PACs are the primary inducers of the developmental toxicity induced by some petroleum substances (i.e., poorly refined). The results also showed the usefulness of the in vitro testing strategy to detect

the developmental toxicity of not only poorly refined petroleum substances but also the absence of such an effect for highly refined petroleum substances.

Chapter 5 describes the development of a rat PBK model for 8-MeBaP, for which in vivo developmental toxicity data are lacking, based on read-across from its corresponding unsubstituted BaP for which in vivo data are available. The PBK model predicted the plasma concentration of 8-MeBaP and its developmental toxic metabolite 3-OH-8-MeBaP upon intravenous, intratracheal and oral exposure. Due to the lack of in vivo toxicokinetic data of 8-MeBaP, the predicted plasma concentration of 8-MeBaP and 3-OH-8-MeBaP were evaluated by comparing to the reported in vivo toxicokinetic data of BaP. This comparison revealed that the predicted time-dependent blood concentration of 8-MeBaP and 3-OH-8-MeBaP were in line with the predictions and the data reported in the literature for BaP and 3-OHBaP with somewhat lower maximum blood concentrations for 8-MeBaP than for BaP related to its more rapid clearance due to side-chain oxidation. The PBK model was subsequently used for reverse dosimetry translating the in vitro concentration-response developmental toxicity of 3-OH-8-MeBaP tested in the mEST to in vivo predicted developmental toxicity of 8-MeBaP upon single oral exposure. The predicted ED50 of 8-MeBaP obtained from the predicted in vivo dose response curve was 0.75-fold lower than the predicted ED50 for BaP, which is in line with the results obtained in **Chapter 2** that 8-MeBaP is more potent than BaP in inducing developmental toxicity in the ZET. Although lack of in vivo toxicokinetic and developmental toxicity data of 8-MeBaP hampers further model validation, the study illustrates how new approach methodologies (NAMs) can be applied for methylated PAHs for which no in vivo developmental toxicity data are available, thereby providing a way to reduce the use of experimental animals for the testing for developmental toxicity within the REACH regulation and risk assessment.

Overall, our thesis demonstrates that the use of NAMs provides insight in the developmental toxicity of both individual PAHs and highly complex petroleum substances, thereby ultimately contributing to reduce the number of animals required to assess developmental toxicity.



Appendix

Acknowledgement
Biography
List of Publications
Overview of completed training activities

Acknowledgement

Five years ago, as a master student who had just been awarded a PhD scholarship by the China Scholarship Council, I was full of longing for my future life. Looking back on my PhD life, it was full of ups and downs, with both joys and sorrows. But through it all, my heart is always filled with gratitude.

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For all the co-authors of my experimental chapters of this thesis (Dr Nynke I. Kramer, Dr Juan-Carlos Carrillo and Dr Miaoying Shi), I appreciate your contribution to my project. To Nynke, thank you very much for teaching me the in vitro chemical distribution model you developed, I appreciate for your kindness and patience. To Juan, thank you very much for providing valuable knowledge on the IP346 method and petroleum production, and your kind feedbacks for my thesis. To Miaoying, thank you very much for not only helping with arranging the in vivo studies, but also for being such a great colleague and friend. Moreover, I would like to extend my gratitude to my MSc students Shutong Dong and Cornelia Lang. In particular, I am thankful to Shutong for her contribution to the data presented in Chapter 2 of the present thesis. It was delightful to work with both of you.

I would like to thank Dr Sylvia Brugman (Department of Animal Science, WUR), Tom Spanings and Menno ter Veld (CARUS-ARF, WUR) for providing zebrafish embryos and

expert advice on zebrafish embryo husbandry. It was not easy for me to conduct the zebrafish embryo experiment at TOX especially during the COVID time, thanks to your kind support. The zebrafish embryo toxicity test was a very essential part of my whole PhD thesis, I would not make it without your kind help.

At the same time, I would like to express my gratitude to my thesis assessment committee (Dr Martijn Rooseboom, Dr Manon Beekhuijzen, Prof. Dr Tinka Murk, and Prof. Dr Raymond Pieters) for accepting the invitation of assessing this thesis and spending time evaluating it. Your efforts are highly appreciated.

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In 2019, Lenny initiated the PAClub at TOX with the members of Lenny, Danlei Wang, Maartje Rietdijk and me. PAClub was organized to share knowledge and solving questions of PAC related research. I am very grateful to have such an amazing team, providing support and help during the most challenging phase of my research. Maartje, thank you for being such a kind friend and colleague, I miss the small talks we had both on and off campus. Although it's a bit late, I still want to congratulate you on becoming a mother. I wish you a joyful and blissful life ahead. Danlei, words cannot express my love and gratitude for you. From the very beginning, we were office mates, later we became neighbors at Nobelweg, eventually we became good friends. Thanks for always listening to me and taking care of me by cooking nice foods, bringing me medicine when I was sick, and giving me your self-cultivated potted plant (the good news is that it is still alive). I wish you all the best with your new job and hope you achieve your life goal soon: becoming an independent and wealthy woman.

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I would like to express my appreciation to my paranymphs Qiuhui and Merel. To my dear Qiuhui AKA Kiwi, you hoped (forced) me to write a one-page acknowledgment for you. I am not doing this not because I am lazy, but because our friendship cannot be measured by the length of acknowledgment. We met each other at Wageningen in 2019, but before that, we have

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Most importantly, I am extremely grateful to my family. 感谢所有的家人对我无私的支持，爱与鼓励。尤其是妈妈（史常青女士）,大舅（史涛先生），小姨妈（史常芬女士），小舅小舅妈（史征先生，董建霞女士），以及孙大爷（孙京栋先生），感谢您们对我无微不至的关照，总是能够尊重和倾听我的想法。在我读博期间，即使与家相隔万里，您们的爱与支持也一直常伴在我身旁。感谢我的姥姥姥爷陪伴我长大，希望我可以令您们感到骄傲和自豪。同时我也要感谢我的公公婆婆（李晶先生与翟丽女士），感谢您们对我和绪君的小家庭的支持与关心，让我们能够自由的选择和追逐我们的未来。在此衷心的感谢我的家人们！

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Biography

Jing Fang was born on 8th August 1993, in Tsingtao, Shandong Province, China. Jing obtained her Bachelor of Science degree in Food Quality and Security, from Shandong Agricultural University, China in 2016. In the meantime, she obtained her 2nd Bachelor of Science degree in International Food Business Management from Royal Agricultural University, United Kingdom. In the summer of 2016, Jing came to the Netherlands for the Master of Science study (Applied Food Safety) at Wageningen University and Research (WUR). She did her MSc thesis entitled "Assessment of the effects of polycyclic aromatic hydrocarbons (PAHs) on the estrogen receptor alpha in relation to their developmental toxicity potency" at the Toxicology department of WUR. After the MSc thesis, Jing did an internship in Merieux Nutrisciences. Jing started her PhD at the Toxicology department of WUR under the supervision of Prof. Dr Ivonne M.C.M. Rietjens and Prof. Dr Peter J. Boogaard. Her PhD is sponsored by the China Scholarship Council, and supported by Operationeel Programma Kansen voor West II (EFRO) (KVW-00181). During her PhD, she followed the post-graduate education program in Toxicology which will allow her to be registered as European Registered Toxicologist in the future.

List of Publications

Fang J., Dong S., Boogaard P, Rietjens I., Kamelia L.(2022). Developmental toxicity testing of unsubstituted and methylated 4- and 5-ring polycyclic aromatic hydrocarbons using the zebrafish embryotoxicity test. *Toxicology in Vitro*. 80, 105312.

Fang J., Wang D., Kramer N., Rietjens I., Boogaard P., Kamelia L. (2023). The role of receptor-mediated activities of 4- and 5-ring unsubstituted and methylated polycyclic aromatic hydrocarbons (PAHs) in developmental toxicity. *Journal of Applied Toxicology*.

Fang J., Rietjens I., Carrillo J., Boogaard P., Kamelia L. (2023). Evaluating the developmental toxicity potency of a series of petroleum substances extracts using new approach methodologies (NAMs) (Manuscript in preparation)

Wang D., **Fang J.**, Shi M., Kamelia L., Rietjens I., Boogaard P. (2023). Predicting in vivo developmental toxicity of 8-methyl-benzo[a]pyrene using PBK facilitated read-across (Manuscript in preparation)

Overview of completed training activities

Discipline specific activities

Molecular TOX	Vrije Universiteit Amsterdam	2019
Introduction Laboratory Animal Science	Utrecht University	2019
Pathobiology	Utrecht University	2019
Organ TOX	Radboud UMC	2020
Reproduction Toxicology	Utrecht University	2021
Toxicogenomic	Maastricht University	2021
Epidemiology	Vrije Universiteit Amsterdam	2021

Conferences

61st Annual Meeting Society of Toxicology (SOT) and ToxExpo, poster, virtual, 2022

21st International Congress of the European Society of Toxicology In Vitro (ESTIV), poster, Spain, 2022

General courses

Start to Teach	WGS	2019
Brain Training	WGS	2019
Introduction to R	VLAG	2019
Applied Statistics	VLAG	2019
PhD Week	VLAG	2019
Philosophy and Ethics of Food Science and Technology	VLAG	2022

Other activities

Preparation of research proposal	TOX	2019
Environmental TOX	TOX	2020
General TOX	TOX	2020
Scientific presentation at Division of Toxicology	TOX	2020-2021

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