The role of polycyclic aromatic hydrocarbons in developmental toxicity of petroleum substances

Lenny Kamelia

Thesis committee

Promotors

Prof. Dr I.M.C.M. Rietjens
Professor of Toxicology
Wageningen University & Research

Prof. Dr P.J. Boogaard
Special Professor Environmental Health & Human Biomonitoring of Contaminants
Wageningen University & Research
Global Discipline Lead & Manager, Toxicology, Shell Health - Global Risk Sciences Team, SI-SHG/PT, Shell International B.V., The Hague

Other members

Prof. Dr H.F.J. Savelkoul, Wageningen University & Research
Prof. Dr Juliette Legler, Institute for Risk Assessment Sciences (IRAS), Utrecht
Prof. Dr Ivan Rusyn, M.D., Texas A&M University, College Station, USA
Dr Juan-Carlos Carrillo, Shell International B.V., The Hague

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Thesis

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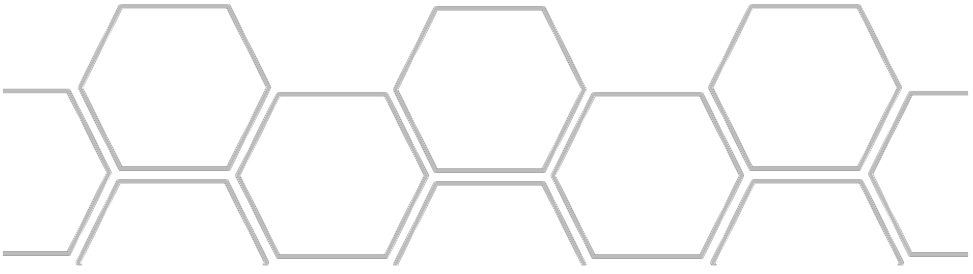
Chapter 1

General introduction



1.1 Background information on REACH legislation and prenatal developmental toxicity testing of petroleum substances

From June 2007 onwards, the European Union (EU) has put into force its new chemical legislation called REACH (Registration, Evaluation, Authorization and Restriction of Chemicals), aiming at greater protection of human health and the environment from the risks of chemical exposure while enhancing the competitiveness of the EU chemical industry (ECHA, 2011). REACH is the first chemical legislation in the world that requires testing for effects on prenatal development on all chemical substances registered or produced in the EU at a volume of ≥100 tonnes/year. Given the large number of experimental animals potentially needed for safety testing of all chemicals falling under this legislation, REACH also promotes and supports the use of alternative testing strategies for the hazard assessment of chemical substances in order to reduce the number of tests on animals (ECHA, 2014).

Petroleum substances (PS) are UVCBs (substances of Unknown or Variable composition, Complex reaction products and Biological materials) and regulated as such under REACH. Since most PS are produced at a volume of far greater than 100 tonnes annually (± 186 currently active registered EINECS numbers), almost all of them will need to be tested for their potential adverse effects on prenatal development. Consequently, this will require a large number of experimental animals and a considerable amount of resources, if the data requirements are fulfilled following the current OECD 414 testing guidelines (OECD, 2001; van der Jagt et al., 2004). Hence, it is highly desirable from both an animal welfare and economic standpoint, and in line with the REACH recommendations, to apply non-animal data (i.e. in vitro alternative assays), and a read-across approach to study prenatal developmental toxicity (PDT) potency of PS.

In vivo studies showed that some PS, particularly the heavy ones that contain relatively high concentrations of polycyclic aromatic hydrocarbons (PAHs), induce PDT in the offspring of pregnant rats (ARCO, 1993; Feuston et al., 1989; Feuston & Mackerer, 1996; Mobil, 1989a). However, when similar PDT studies were conducted with gas-to-liquid (GTL) products, the modern synthetic analogues of PS that are devoid of aromatic compounds, no developmental-related effects were observed (Boogaard et al., 2017). This led to the hypothesis that PDT as observed with some PS is mainly caused by the specific group of PAHs present in these products (Murray et al., 2013; Tsitou et al., 2015).

The present thesis aims to test the hypothesis that PAHs, which may be present in PS, are responsible for the observed in vivo PDT in animal experiments, using a battery of in vitro alternative assays. The resulting in vitro data and findings may be of use to facilitate read-across from PS for which these in vivo PDT data are already available or will be generated under REACH circumstances, to other PS for which in vivo data are lacking. In the long run, this will reduce the animal experimentation and resources needed to study the PDT potency of PS UVCBs, and support the application of the 3Rs (Reduction, Replacement, and Refinement) principle of animal use in toxicological research.

1.2 Petroleum substances and polycyclic aromatic hydrocarbons that may be present in these substances

PS are highly complex hydrocarbon substances, comprising at least hundreds to millions of different hydrocarbon constituents. PS are generated from crude oils by a distillation process, including either atmospheric or vacuum distillation (Figure 1). This process yields several distillates and residues with increasing molecular complexity and composition of hydrocarbons, and they are further categorized into 4 main groups: light distillates (i.e. liquefied petroleum gas/LPG, gasoline, and naphtha), middle distillates (i.e. kerosene, diesel), heavy distillates (i.e. heavy fuel oil/HFO, lubricating oils, and paraffin wax), and residue (i.e. asphalt) (Mackerer et al., 2003). Moreover, the number of carbon atoms in the hydrocarbons present in the petroleum stream increases along with the increase of their boiling points and viscosity (Figure 1). The principle hydrocarbon classes present in PS are categorized into 5 different groups (Figure 2), namely paraffinics (alkanes), isoparaffinics (isoalkanes), olefinics (alkenes), naphthenics (cycloalkanes), and aromatics (monoaromatics, diaromatics, and polycyclic aromatic hydrocarbons/PAHs) (Feder et al., 2013). Paraffinics, isoparaffinics, and naphthenics are saturated hydrocarbons, where olefinics and aromatics are unsaturated hydrocarbons (Speight, 2006).

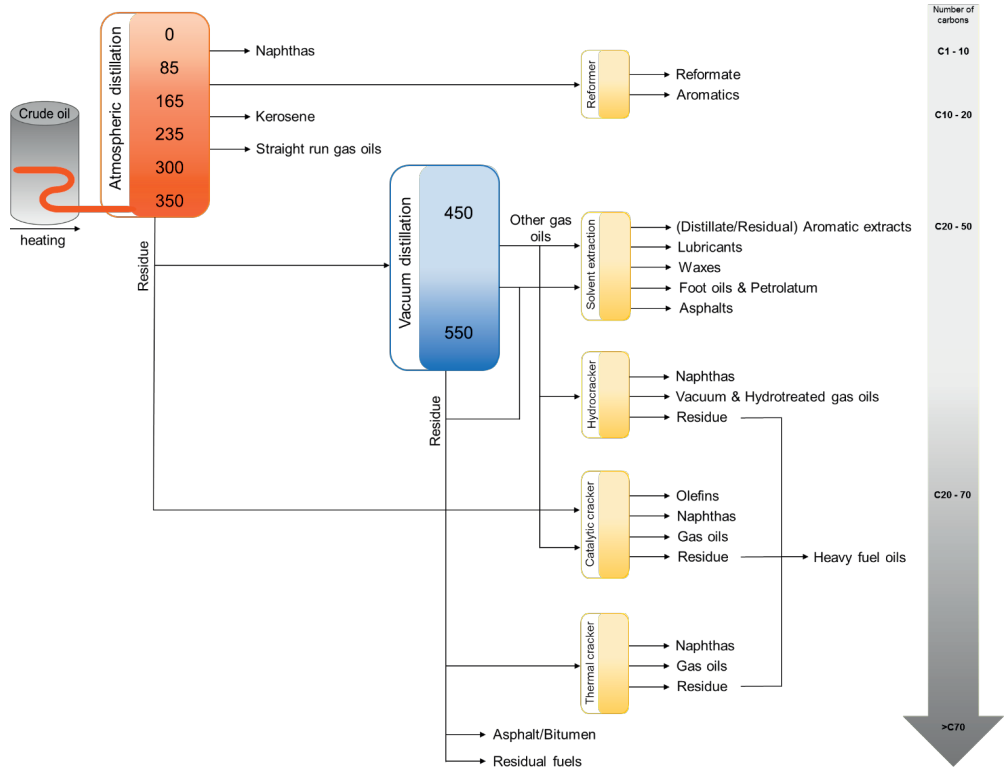


Figure 1 A schematic diagram of petroleum refining processes that transform crude oils into various useful petroleum products, either by atmospheric or vacuum distillation process.

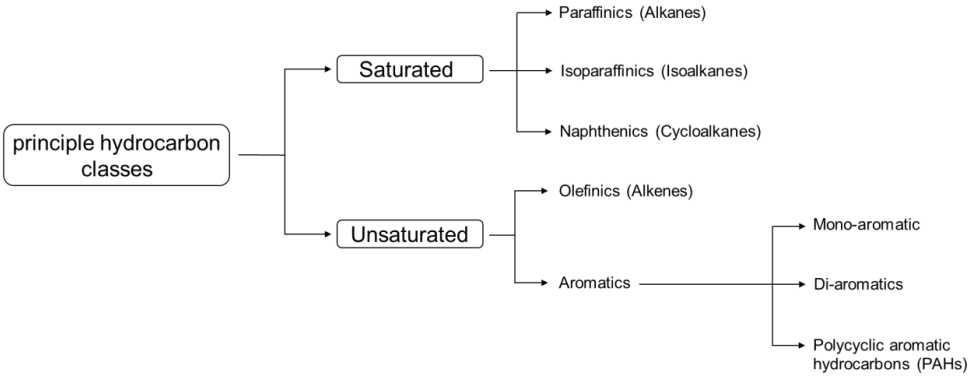
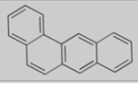
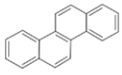
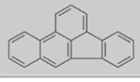
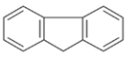
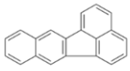
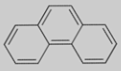
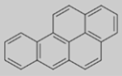
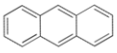
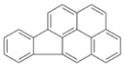
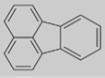
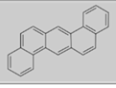


Figure 2 The principle hydrocarbon classes present in PS.

PAHs are composed of only carbon and hydrogen atoms and at least two or more aromatic carbon rings fused in linear, cluster, or angular arrangements (Abdel-Shafy & Mansour, 2016). PAHs can be divided into two categories: low molecular weight (LMW) PAHs that contain up to three fused aromatic rings (e.g. naphthalene, phenanthrene), and high molecular weight (HMW) PAHs that contain at least four or more rings (e.g. pyrene, benzo[a]pyrene, benzo[ghi]perylene). Although PAHs can exist in over 100 different forms (including both unsubstituted and substituted PAHs), most regulations, data analysis/reporting, and analytical measurement of PAHs present in PS focus on only a limited number of PAHs, often referred to as the 16 EPA/EU PAHs-priority lists (Table 1).

Table 1 The 16 EPA/EU PAHs-priority list, including their CAS number, molecular weight, and chemical structure.

Compound	CAS no.	Molecular weight	Structure	Compound	CAS no.	Molecular weight	Structure
Naphthalene	91-20-3	128.17		Benzo[a] anthracene	56-55-3	228.29	
Acenaphthene	83-32-9	154.21		Chrysene	218-01-9	228.29	
Acenaphthylene	208-96-8	152.20		Benzo[b] fluoranthene	205-99-2	252.32	
Fluorene	86-73-7	166.22		Benzo[k] fluoranthene	207-08-9	252.32	
Phenanthrene	85-01-8	178.23		Benzo[a]pyrene	50-32-8	252.32	
Anthracene	120-12-7	178.23		Indeno[1,2,3-cd] pyrene	193-39-5	276.34	
Fluoranthene	206-44-0	202.26		Dibenzo[a,h] anthracene	53-70-3	278.35	
Pyrene	129-00-0	202.26		Benzo[g,h,i] perylene	191-24-2	276.34	

PAHs that may be present in PS, are generally known as PAH of petrogenic origin or petroleum-derived aromatic hydrocarbons and differ substantially from PAHs of pyrogenic origin, which are derived from incomplete combustion of organic material and coal (Pampanin and Sydnes, 2013). PS are manufactured according to physicochemical specifications and their PAH content typically varies, even for samples possessing the

same CAS number, as the chemical composition of each PS is mainly dependent on the source of the crude oil and the processing conditions used to create the stream (Speight, 2006). Heavy distillates, such as HFO and untreated distillates aromatic extracts (DAE), may contain a relatively high concentration of 3- to 7- ring PAHs, when compared to light distillates, such as gas oils that mainly contain 2- to 3- ring PAHs. Different from PS that generally contain PAHs, gas-to-liquid (GTL) products are modern synthetic analogues of PS that typically consist of only saturated hydrocarbons and are virtually devoid of unsaturated and aromatic compounds (Boogaard et al., 2017).

1.3 Prenatal developmental toxicity potency of some PAHs and petroleum substances

Most PAHs and their metabolites can readily cross the placental barrier because of their hydrophobicity and prenatal exposure of pregnant experimental animals to these compounds have resulted in embryo lethality, stillbirths, resorptions, and malformations (Legaverend et al., 1984; Shum et al., 1979; US ATSDR, 1995). Benzo[a]pyrene, a 5-ring PAHs, is the most studied PAHs for the effect on prenatal development in experimental animals. In one study, Bui et al. (1986) have reported that dermal exposure to benzo[a]pyrene (at a dose of 50 mg/kg) of pregnant rats at different stages of gestation increased the number of resorptions and fetal wastage, and decreased the fetal weight. Furthermore, there are publications documenting that some PS, in particular the heavy ones like HFO (Feuston et al., 1989; Hoberman et al., 1995) and distillate aromatic extract (DAE) (Feuston et al., 1996), are also able to induce PDT in pregnant rats exposed dermally to these substances. The main manifestations of this adverse effect include increased incidence of resorptions, reduced number of live fetuses per litter, and decreased fetal body weight. On the contrary, GTL products, the modern synthetic analogues of PS that contain no PAHs but otherwise have similar properties as PS, do not induce any effect in PDT studies or two-generation reproductive toxicity studies (Boogaard et al., 2017). From this evidence, it can be postulated that the ability of some PAH-containing PS to induce PDT in vivo is associated with the presence of PAHs in these products and this association is hypothesized to be causal. In other words, heavier PS containing higher concentrations of PAHs may induce PDT where products with no or low concentration of PAHs will not cause PDT (Tsitou et al., 2015). This postulation is further

supported by a publication by Murray et al. (2013), who showed a strong relationship between PDT induced by some PS and their aromatics content, in particular PAHs. Based on the 21 rat PDT studies on a number of high-boiling PS, including 10 studies on HFOs and 7 studies on gas oils, the authors concluded that PDT as observed with these high-boiling PS is correlated with the amount of 3- to 7- ring PAHs they contain (Murray et al., 2013).

PS are often categorized based on their physicochemical properties such as similar boiling ranges, carbon number ranges, process history (last refining step), and end-uses. Categorization of PS may sometimes be fit-for-purpose depending on the regulatory or industry requirements. For example, the PS categories used for REACH registration consist of 22 product categories, including distillate aromatic extracts (DAE), residual aromatic extracts (RAE), bitumen, oxidised asphalt, gasolines, straight run gas oil (SRGO), vacuum gas oils/hydrocracked gas oil/distillate fuels (VHGO), cracked gas oils (CGO), other gas oils (OGO), MK-1 diesel fuel, HFO, kerosenes, highly refined base oils (HRBO), unrefined/acid treated oils (UATO), lubricant base oil (LBO), foot oils (FO), petroleum gases, other petroleum gases, paraffinic and hydrocarbon waxes (P&H waxes), slack waxes, petrolatums, and sulfur. Within the context of PDT testing of PS under REACH compliance, not all of them have historical data or have been tested for effects on prenatal development in experimental animals. Nonetheless, an overview of available in vivo PDT studies of some PS (within and across product categories), some of which are investigated to a further extent in the present thesis, is provided in Table 2.

Table 2 An overview of in vivo developmental toxicity studies of various PS, within and across product categories, in pregnant rats via dermal route of exposure.

Substances	CAS no.	Rat strain	Exposure day(s)	Dose (mg/kg/day)	Developmental LOAEL and/or NOAEL (mg/kg/day)	Main developmental effects/manifestations	References
Light paraffinic distillate aromatic extract (DAE)	64742-05-8	Crl:CD (SD)	GD 0-19	0, 5, 25, 150, 450	NOAEL: 5	Early resorptions and lower fetal weights	HPV, 2012a
Undiluted heavy paraffinic DAE	64742-04-7	Sprague-Dawley rats	GD 0-19	0, 8, 30, 125	NOAEL: 8 LOAEL: 30	Reduced number of dams with viable fetuses, increased number of dams with resorptions, reduced litter size of viable fetuses, increased percent resorptions, and decreased fetal body weight	HPV, 2012a
F-193 (gas oils intermediate)	64741-43-1	Sprague-Dawley rats	GD 0-19	0, 50, 250, 500	NOAEL: 50 LOAEL: 250	Decreased fetal body weight and embryo/fetal viability, also soft tissue and skeletal alterations	HPV, 2012b
Vacuum tower overheads (vacuum tower condensates)	64741-49-7	Sprague-Dawley rats	GD 0-19	0, 30, 125, 500	NOAEL: 30 LOAEL: 125	Increased number of dams with resorptions, decreased fetal body weight and length, and increased soft tissue anomalies	HPV, 2012b
F-213 (light cycle oil)	64741-59-9	Sprague-Dawley rats	GD 0-20	0, 50, 333, 1000	NOAEL: 50 LOAEL: 333	Decreased number of live fetuses/litter, decreased fetal body weight, and decreased fetal survival	HPV, 2012b
F-228 (atmospheric tower residuals)	64741-45-3	Sprague-Dawley rats	GD 0-20	0, 50, 333, 1000	NOAEL: 333	Decreased fetal body weight	HPV, 2012c
Heavy vacuum gas oil	64741-57-7	Sprague-Dawley rats	GD 0-19	0, 30, 125, 500, 1000	NOAEL: 125	Increased resorptions, decreased fetal body weight, and skeletal malformations at higher doses	HPV, 2012c
F-197 (heavy vacuum gas oil)	64741-57-7	Sprague-Dawley rats	GD 0-19	0, 50, 100, 250	NOAEL: 100	Reduced fetal body weight at the highest dose tested	HPV, 2012c

F-225 (heavy vacuum gas oil)	64741-57-7	Sprague-Dawley rats	GD 0-20	0, 50, 150, 500	NOAEL: 50	Decreased fetal body weight and reduced number of total/live fetuses	HPV, 2012c
F-179 (catalytic cracked clarified oil)	64741-62-4	Crl:CD (SD)	GD 0-19	0, 0.05, 1, 10, 50, 250	NOAEL: 0.05	Increased resorptions, decreased number of live fetuses/litter, and decreased fetal body weight	Hoberman et al., 1995; HPV, 2012c
Clarified slurry oil (catalytic cracked clarified oil)	64741-62-4	Sprague-Dawley rats	GD 0-19	0, 4, 8, 30, 125, 250	NOAEL: 4	Increased resorptions and decreased number of live fetuses/litter	HPV, 2012c
Carbon black oil F-229 (catalytic cracked clarified oil)	64741-62-4	Sprague-Dawley rats	GD 0-19	0, 5, 10, 50	NOAEL: 10	Decreased number of live fetuses/litter and decreased fetal body weight	HPV, 2012c
Heavy coker gas oil (heavy thermal cracked distillates)	64741-81-7	Sprague-Dawley rats	GD 0-19	0, 8, 30, 125, 250	NOAEL: 8	Increased resorptions and decreased fetal body weight	HPV, 2012c
F-274 (heavy thermal cracked distillates)	64741-81-7	Sprague-Dawley rats	GD 0-20	0, 1, 50, 250	NOAEL: 1	Decreased number of live fetuses/litter	HPV, 2012c
F-227 (hydrodesulfurized heavy vacuum gas oil)	64742-86-5	Sprague-Dawley rats	GD 0-20	0, 50, 333, 1000	NOAEL: 50	Decreased number of live fetuses/litter and decreased fetal body weight	HPV, 2012c
F-215 (gas oil intermediate)	68410-00-4	Sprague-Dawley rats	GD 0-20	0, 50, 150, 500	NOAEL: 50	Decreased fetal body weight and decreased fetal survival	HPV, 2012c
F-275 (full range gas oil)	68783-08-4	Sprague-Dawley rats	GD 0-20	0, 50, 250, 500	NOAEL: 50	Decreased number of live fetuses/litter	HPV, 2012c
Heavy atmospheric gas oil (heavy gas oil)	68915-97-9	Sprague-Dawley rats	GD 0-19	0, 8, 30, 125, 500	NOAEL: 30 LOAEL: 125	Decreased fetal body weight and incomplete ossification	HPV, 2012b

1.4 Selection of the model substances and their in vivo developmental toxicity potency

Based on the available knowledge on the PDT potency of some PS and the association of PAHs present in these substances for the observed PDT, the selection of the model substances to be included in the present PhD thesis was based on the following selection criteria:

The selection should include PS and GTL products with and without known developmental toxicity and positive control substances to identify the specific perturbations of relevant biological pathways related to the PDT;
The selected PS should contain a systematic change in the amount and type of PAHs they contain, if possible from the same and different categories of PS, to investigate the role of specific PAHs present in these substances for the observed PDT;
The selected model substances should also include substances that are known not to contain PAHs as their constituents, e.g. GTL products, hence, will serve as negative control substances in the selected in vitro assays for PDT testing of PS UVCBs;
A sufficient number of the PS and GTL products tested should have adequate in vivo developmental toxicity data to allow validation of the predictions for PDT in the selected in vitro assays.

Based on the above criteria, 19 model substances were selected and tested in the present PhD thesis (Figure 3 and Table 3). Of these 19 raw materials of model substances, 2 were gas oils (GOs), 1 was a vacuum tower overhead oil (VTO), 2 were residual aromatic extracts (RAEs), 3 were distillate aromatic extracts (DAEs), 8 were HFOs, 1 was a highly refined base oil (HRBO), 1 was a GTL base oil (GTLb), and 1 was a GTL gas oil (GTLg). The schematic diagram showing where the raw materials of the corresponding PS were generated from the distillation process of crude oils is presented in Figure 1. Moreover, an overview of these model substances, including their CAS number, aromatic ring content, sample code, and in which thesis Chapters they are tested, is provided in Table 3. The selected model substances included extremes regarding their PAH content: HFO (sample #034-HFO; 48 wt.% PAHs), HRBO (sample #145-HRBO; 0 wt.% PAHs) and GTL products (sample #091-GTLb and #092-GTLg; both contain 0 wt.% PAHs), while the PAH content of the other selected-model substances is in-between these extremes (Table 3). Sample #034-HFO represents a heavy distillate from a crude oil refinery process that

contains the highest amount of 3- to 7- ring PAHs among the selected-model substances of the present thesis, where HRBO and GTL products are virtually devoid of aromatics, including PAHs. As mentioned previously, these model substances were also selected based on the availability of their in vivo PDT data in order to enable assessment of the correlation between the in vitro and the in vivo potencies.

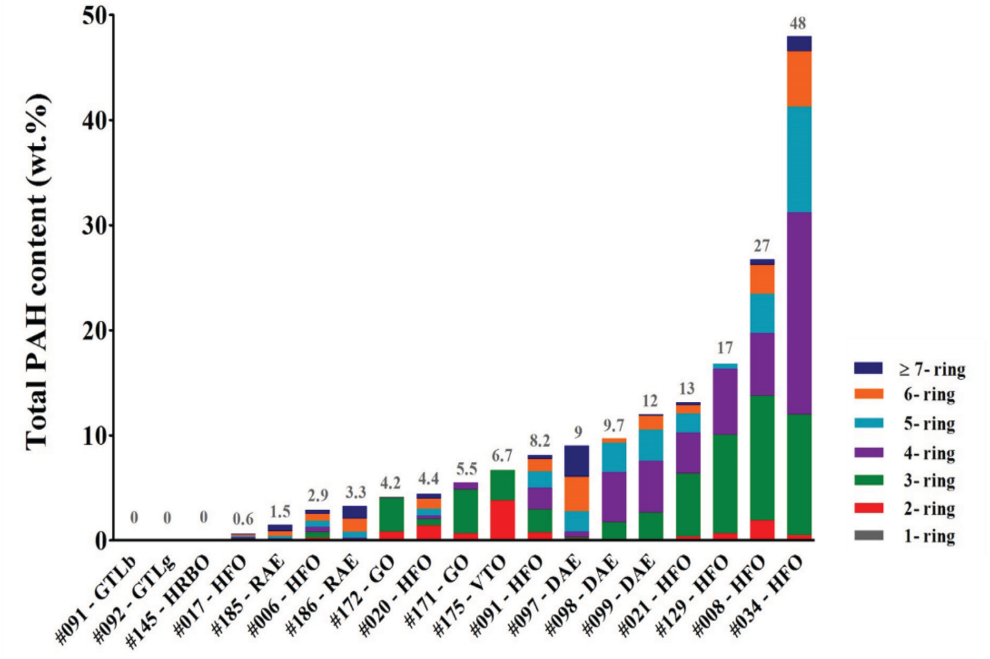


Figure 3 Aromatic Ring Class (ARC) profiles* of PS and GTL products used in the present thesis.

*The weight percent of the DMSO-soluble 1- to ≥7 aromatic-ring compounds present in each sample, from the starting material of 4.0 g sample, as determined by Method II chemical characterization procedure.
Abbreviations. HFO: heavy fuel oil; DAE: distillate aromatic extract; GO: gas oil; VTO: vacuum tower overhead; RAE: residual aromatic extract; GTLb: gas-to-liquid base oil; GTLg: gas-to-liquid gas oil.

Table 3 An overview of model substances tested in the present PhD thesis, including their sample code, product category, CAS number, ARC profile and total weight percent (wt.%) of PAH content.

No.	Sample code	Substance/Category	CAS no.	ARC profile* (wt.%)							Total weight percent (wt.%)	in which thesis Chapter are they tested?
				i	ii	iii	iv	v	vi	≥ vii		
1	#091 - GTLb	Gas-to-liquid base oil	848301-69-9	0	0	0	0	0	0	0	0	2, 3, 4, 5, 6
2	#092 - GTLg	Gas-to-liquid gas oil	848301-67-7	0	0	0	0	0	0	0	0	2, 3, 5
3	#145 - HRBO	Highly refined base oil	8042-47-5	0	0	0	0	0	0	0	0	4
4	#017 - HFO	Heavy fuel oil	92061-97-7	0	8	19	17	21	22	13	0.62	4
5	#185 - RAE	Residual aromatic extract	64742-10-5	0	4	4	4	16	30	42	1.5	2, 3, 5
6	#006 - HFO	Heavy fuel oil	93821-66-0	0	1	22	22	21	21	13	2.9	4
7	#186 - RAE	Residual aromatic extract	91995-70-9	0	0	1	6	18	38	36	3.3	2, 3, 4, 5, 6
8	#172 - GO	Gas oil	64741-43-1	0	20	77	2	0	0	0	4.2	2, 3, 4, 5
9	#020 - HFO	Heavy fuel oil	64741-81-7	3	29	14	7	15	22	10	4.4	4
10	#171 - GO	Gas oil	68915-96-8	0	12	76	12	0	0	0	5.5	2, 3, 5, 6
11	#175 - VTO	Vacuum tower overhead oil	64741-49-7	1	56	43	0	0	0	0	6.7	2, 3, 4, 5, 6
12	#091 - HFO	Heavy fuel oil	68553-00-4	0	9	27	25	19	14	5	8.2	4
13	#097 - DAE	Distillate aromatic extract	64742-04-7	0	2	1	6	22	36	33	9	2, 3, 4, 5, 6
14	#098 - DAE	Distillate aromatic extract	64742-04-7	0	0	18	49	29	4	0	9.7	2, 3, 5
15	#099 - DAE	Distillate aromatic extract	64742-04-7	0	0	22	41	25	11	1	12	2, 3, 5
16	#021 - HFO	Heavy fuel oil	64741-80-6	0	3	46	30	14	6	2	13	4
17	#129 - HFO	Heavy fuel oil	64741-45-3	0	4	55	37	3	0	0	17	4
18	#008 - HFO	Heavy fuel oil	64742-78-5	0	7	44	22	14	10	2	27	4
19	#034 - HFO	Heavy fuel oil	64741-62-4	0	1	24	40	21	11	3	48	2, 3, 4, 5, 6

**The weight percent of the DMSO-soluble 1- to ≥7 aromatic-ring compounds present in each sample, from the starting material of 4.0 g sample, as determined by Method II chemical characterization procedure.*

In the following sections the different PS categories and selected model substances are introduced in some more detail.

1.4.1 Gas oil category: straight run GO and VTO

GO streams are produced either by atmospheric distillation (straight run GO), or by secondary processing of the materials derived from the vacuum distillation of the residuum from the atmospheric distillation of crude oil (other GOs, including VTO) with boiling temperature ranges from 150°C to 450°C (HPV, 2012b). The hydrocarbon constituents present in GO streams include parrafinics (alkanes), naphthenics (cycloalkanes), olefinics (alkenes), and aromatics (e.g. PAHs). The products derived from the secondary processing (e.g. VTO) may have a slightly higher olefin and aromatic content than the straight run GOs (HPV, 2012b). Within the context of PAHs present in GO, the boiling point specifications for these streams limit their aromatics constituents to 1- to 3-ring PAHs and in some cases a small amount of 4-ring PAHs.

In the present thesis, 2 straight run GOs (CAS no. 68915-96-8 and 64741-43-1) derived from the atmospheric distillation and 1 VTO (CAS no. 64741-49-7) from the vacuum distillation of the residuum from the atmospheric distillation of crude oil, were selected and these substances mainly contained 2- to 3-ring PAHs (Figure 3 and Table 3). Available in vivo PDT data showed that a GO (CAS no. 64741-43-1) and VTO (CAS no. 64741-49-7) are able to induce developmental toxicity when applied dermally to pregnant rats during a certain gestation period (ARCO 1993; Mobil 1989a). From those PDT studies, the developmental NOAEL and LOAEL of the GO (CAS no. 64741-43-1) were determined to be 50 and 250 mg/kg bw/day, respectively, and those of the VTO (CAS no. 64741-49-7) were 30 and 125 mg/kg bw/day, respectively (Table 2).

1.4.2 Aromatic extracts category: RAE and DAE

Aromatic extracts are produced as by-products from the extraction of condensed polycyclic aromatic constituents during the production of lubricants and waxes. They are often referred to as either distillate aromatic extracts (DAE) or residual aromatic extracts (RAE), depending on whether they were produced from the extraction of distillate or

residual lubricating oil base stocks (HPV, 2012a). Of the selected aromatic extracts in the present thesis, 3 were DAEs (possessing the same CAS no. of 64742-04-7), and 2 were RAEs (CAS no. 64742-10-5 and 91995-70-9). DAEs or RAEs contain predominantly aromatic hydrocarbons ranging from C15 to C50, and are more complex than the GO streams in terms of carbon number range and PAH content. The boiling temperature of DAEs range from 288 to 584°C and that for the RAEs from 344 to >734°C. In general, DAEs contain higher concentrations of PAHs compared to RAEs. The available data show that concentrations of DMSO-extractable PAH of DAEs ranged from 6.3 - 20.3 wt.% compared to 1.8 - 4.5 wt.% in the RAEs, although RAEs are more viscous than DAEs physically (HPV, 2012a).

With respect to PDT potency of these aromatic extracts, it was observed that dermal exposure of DAE (CAS no. 64742-04-7) to pregnant experimental animals caused various developmental effects, including reduced number of live fetuses, increased number of dams with resorptions, and decreased fetal body weight, resulting in developmental NOAEL and LOAEL values of 8 mg/kg bw/day and 30 mg/kg bw/day, respectively. In the case of RAE (CAS no. 64742-10-5), a PDT study reported by Mobil (1989b) using 3 different experimental doses of 40, 500, 2000 mg/kg bw/day, showed that prenatal dermal exposure of pregnant rats to these substances did not cause substantial developmental effects, resulting in a developmental NOAEL of at least 2000 mg/kg bw/day, with the understanding that PDT potency of individual RAE may vary depending on the amount of PAHs they contain (HPV, 2012b). From this finding, it can be concluded that RAEs are substantially less (or not) developmentally toxic than DAEs, probably due to the relatively lower amount of PAHs present in the RAEs compared to the DAEs, as well as the high viscosity of RAEs reducing absorption of these substances via the exposed-skin. This is in accordance with results of previous studies indicating mammalian toxicity of DAEs to be directly related to their PAH profile where for RAEs, it is related to both their PAH profile and physicochemical properties (HPV, 2012b).

1.4.3 HFO category

HFOs are highly viscous residual streams that consist primarily of the residuum of the refining process (i.e. thermal, catalytic, and hydro cracking processes) after removing

virtually all the higher quality hydrocarbons from the crude oil feedstock (Concawe, 1998). The typical boiling temperature range of HFOs is generally between 350 to 650°C, while they contain constituents with a carbon number range from C20 to ≥ C50. HFOs may contain up to 58% aromatic hydrocarbon content, depending on the type of processing conditions and the nature of the blending stocks used (HPV, 2012c). HFOs represent the most complex PS UVCBs among all PS tested in the present thesis as they are composed of relatively HMW compounds (e.g. HMW PAHs), which makes them more difficult to characterize in detail than the other PS categories.

In general, PS derived from thermal or catalytic cracking processes have a higher olefin and aromatic content (e.g. PAHs) than the straight run streams generated from non-cracking processes (i.e. hydro cracking). For example, catalytically cracked HFO contains more 3- to 5-ring PAHs than HFOs derived from a non-cracked process, such as heavy vacuum gas oil (HPV, 2012c). Of many types of HFOs, the present thesis selected and tested 8 different HFOs having a PAH content range from 0.62 to 48 total wt.% (Figure 3). Of these 8 HFOs, 1 was from atmospheric tower residuals (CAS no. 64741-45-3), 1 was from catalytic cracked clarified oils (CAS no. 64741-62-4), and 1 was from heavy thermal cracked distillates (CAS no. 64741-81-7). For these 3 HFOs of the present study in vivo PDT data were available (Table 2). As shown in Table 2, catalytic cracked clarified oils (CAS no. 64741-62-4) are the most potent substances within the HFO category with respect to PDT in pregnant rats, with NOAEL values ranging from 0.05 to 10 mg/kg bw/day.

1.4.5 HRBO category

HRBO, or white mineral oils, are highly refined mineral oils that consist of saturated hydrocarbons, including alkanes (paraffinics), isoalkanes (isoparaffinics), and cycloalkanes (naphthenics), with carbon numbers ranging from 15 to 25 for light and from 25 to 50 for heavy white mineral oils (Nash et al., 1996). Mineral oils are produced through vacuum distillation at temperatures between 300-600°C. They may be carcinogenic if not sufficiently refined because of the presence of residual HMW PAHs when insufficiently refined (Carrillo et al., 2019). The refining processes used to produce white mineral oils include treatment with acid or hydrogenation which processes both

aim to remove PAHs from the finished products (McKee et al., 1989). In other words, the white mineral oils that are used and available in the market, especially for pharmaceutical, food, and cosmetic applications, should be PAHs-free. Regarding PDT potency of white mineral oils, a rat teratology study conducted by Mobil in 1987 demonstrated that oral, dermal, and pulmonary administration of white mineral oils (CAS no. 8042-47-5) on day 6-19 of gestation did not induce any developmental effects (Mobil, 1987). Since we hypothesize that PAHs that may be present in PS are the main inducers for the observed PDT by some of these substances, the HRBO selected for the present thesis (CAS no. 8042-47-5) represents one of the PAH-free model substances, expected to show no or low effects in any of the in vitro assays of the battery for PDT testing of PS UVCBs.

1.4.6 GTL products

GTL products, the modern synthetic analogues of PS, are synthetic hydrocarbons produced from natural gas as a feedstock using a Fischer-Tropsch process. GTL products primarily contain saturated hydrocarbons (alkanes, isoalkanes, and cycloalkanes) and are virtually devoid of unsaturated and aromatic constituents, and also no sulfur-, oxygen-, or nitrogen-containing constituents are present in these products (Boogaard et al., 2017). Of the main GTL products, the studies of the present thesis included 2 type of these products, namely GTL base oil (C18-C50 branched, cyclic, and linear; CAS no. 848301-69-9) and GTL gas oil (C8-C26 branched and linear; CAS no. 848301-67-7). When both the aforementioned GTL products were evaluated for PDT potency according to the OECD 414 test guidelines, no effects on prenatal development were observed (Boogaard et al., 2017). In other words, both GTL products included in the present thesis tested negative for PDT in in vivo studies.

1.5 Selection of in vitro alternative assays as a battery for PDT testing of PS UVCBs

As previously mentioned, a huge number of experimental animals and a considerable amount of resources would be needed if the data requirement for PDT testing of PS would be fulfilled following the current OECD 414 testing guidelines. Also, PS are highly complex substances, and consequently, the PDT as observed with some of these substances may

be caused by a wide range of modes-of-action. Thus, it is impossible to assess their PDT and the underlying mechanism of the observed PDT using a stand-alone assay. Therefore, the application of robust alternative assays to assess the PDT potency of PS UVCBs is highly relevant. To address this challenge, the present PhD thesis applied a battery of in vitro alternative assays, including the embryonic stem cell test (EST), a panel of CALUX (Chemical Activated LUciferase gene eXpression) reporter gene assays, and the zebrafish embryotoxicity test (ZET), to evaluate the PDT potency (and modes-of-action) of model substances under study: DMSO-extracts of PS and GTL products. The thesis also focussed on a possibility to include biotransformation in the in vitro assays.

1.5.1 Mouse embryonic stem cell test (EST)

The sequel of the validated EST consists of three different assays: the cytotoxicity assay on differentiated mouse 3T3 fibroblasts, the cytotoxicity assay on undifferentiated mouse ES-D3 cells, and the differentiation assay of ES-D3 cells into beating cardiomyocytes (Genschow et al., 2002). The differentiation assay of the EST can be used to evaluate and rank the potency of chemical substances for effects on cell differentiation and development (Seiler and Spielmann, 2011). The differentiation of ES-D3 cells into beating cardiomyocytes proceeds via the formation of so-called embryoid bodies (EBs) in the absence of leukaemia inhibitory factor (LIF) in the EST culture medium. These EBs represent the early embryonic development phase and are capable to spontaneously differentiate into any cell types of endodermal, ectodermal, and mesodermal lineages, including cardiomyocytes (Wobus et al., 1991; Maltsev et al., 1993). The ability of test substances to inhibit the differentiation of ES-D3 cells into beating cardiomyocytes, after 10 days of incubation, is used as the endpoint of the differentiation assay of the EST, reflecting the embryotoxic potential of the respective chemicals (Genschow et al., 2002). A schematic overview of the ES-D3 cell differentiation assay of the EST, as performed in the present thesis, is provided in Figure 4.

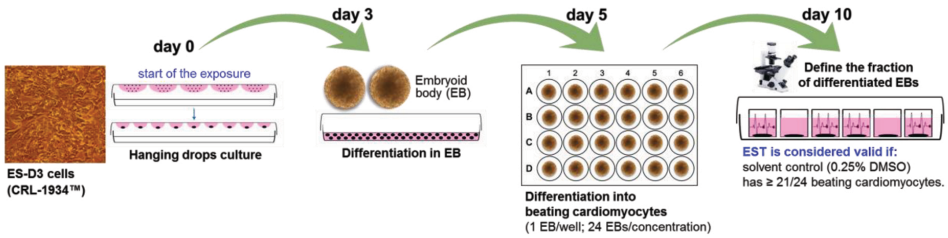


Figure 4 A schematic drawing of ES-D3 cell differentiation assay of the EST performed in Chapter 2, 4, and 6 of the present thesis.

The EST is considered to be one of the most robust and reliable in vitro alternative assays for PDT testing of chemical substances. In the first validation study by the European Centre for Validation of Alternative Methods (ECVAM) (Genschow et al., 2002, 2004), the EST provided 78% accuracy when predicting the embryotoxic potency of 20 reference chemicals (categorized as non, weak, or strong embryotoxic chemicals), as compared to their in vivo PDT potency in rodents. Remarkably, the EST predictivity was increased to 100% when taking only the strong embryotoxic chemicals into account (Genschow et al., 2004). In addition to its high accuracy and sensitivity, the EST offers several other advantages, i.e. it is an animal-free test system since a permanent (stem) cell line is used for the test. In addition, it is less time consuming and cheaper in comparison to other validated assays for PDT testing like the whole embryo culture (WEC) and rat limb bud micromass test (MM). However, the microscopical evaluation of beating cardiomyocytes (at day 10 of the differentiation assay of the EST) is rather subjective since it requires experience and a well-trained person to score the specific beating areas of cardiomyocytes. Consequently, a few attempts have been made to improve the performance and endpoint evaluation of the classical EST, including the addition of quantitative flow cytometry (Seiler et al., 2006) or of transcriptomics approaches (van Dartel and Piersma, 2011). This molecular-based addition is expected to enhance the predictability and applicability domain of the classical EST for assessing developmental toxicity potency of chemical substances (van Dartel and Piersma, 2011).

The differentiation assay of the EST has been widely used over the past decade to evaluate the in vitro PDT potency of different substances, i.e. phthalates (van Dartel et al., 2009a,b), antifungal compounds (Li et al., 2015; Dimopoulou et al., 2018), glycol ethers (de Jong et al., 2011), retinoids (Louisse et al., 2011), phenols (Strikwold et al., 2012), indigenous

plant extracts (Hiben et al., 2019), and several other known developmental toxicants (Piersma et al., 2013). However, the usefulness of the EST to assess PDT potency of highly complex substances, such as PS UVCBs, has not been evaluated yet. Therefore, the present PhD thesis investigates the applicability of the ES-D3 cell viability and differentiation assays of the EST to assess the PDT potency of the DMSO-extracts of PS and GTL products. The obtained in vitro PDT potencies will be compared to available in vivo PDT data in rodents to validate the prediction of PDT for these substances using the EST.

1.5.2 Zebrafish embryotoxicity test (ZET)

The ZET is another emerging alternative method, besides the EST, that is extensively used nowadays to investigate developmental toxicity potency of chemical substances (Brannen et al., 2010; Selderslaghs et al., 2009; Scholz et al., 2008). In the ZET, newly fertilized zebrafish embryos (4-5 hours post fertilization (hpf)) are exposed to different concentrations of a test substance and the development of zebrafish embryos over time is observed and scored according to the general morphology scoring (GMS) system (Beekhuijzen et al., 2015). Zebrafish embryos have a transparent body and chorion, which enables detection of developmental effects on multiple organs during testing (Glaberman et al., 2017). The GMS is scored daily (every 24 hours) to up to 96-144 hpf and the scoring includes the development of eyes, tail, somite, yolk extension, pectoral fin, and mouth; the presence of heartbeat and circulation; the pigmentation of head, tail, and body; and the hatching event. In addition to the assessment using the GMS, some teratogenicity endpoints, including pericardial and yolk sac edemas, malformation of the head, tail, and sacculi/otoliths, and deformation of body shape (i.e. scoliosis) can also be scored after 96 hours exposure to a test substance (Beekhuijzen et al., 2015). By combining the results from both the GMS and teratogenicity endpoints, a so-called Teratogenicity Index (TI) can be determined, a value that is often used to classify teratogenic substances using the ZET (Hersmen et al., 2011; Selderslaghs et al., 2012). The TI is defined as a ratio between the 50% lethal concentration (LC50; based on the GMS) and the 50% effect concentration (EC50; based on the teratogenic endpoints) (Selderslaghs et al., 2012; Beekhuijzen et al., 2015). A schematic representation of the ZET, as performed in the present thesis, is provided in Figure 5.

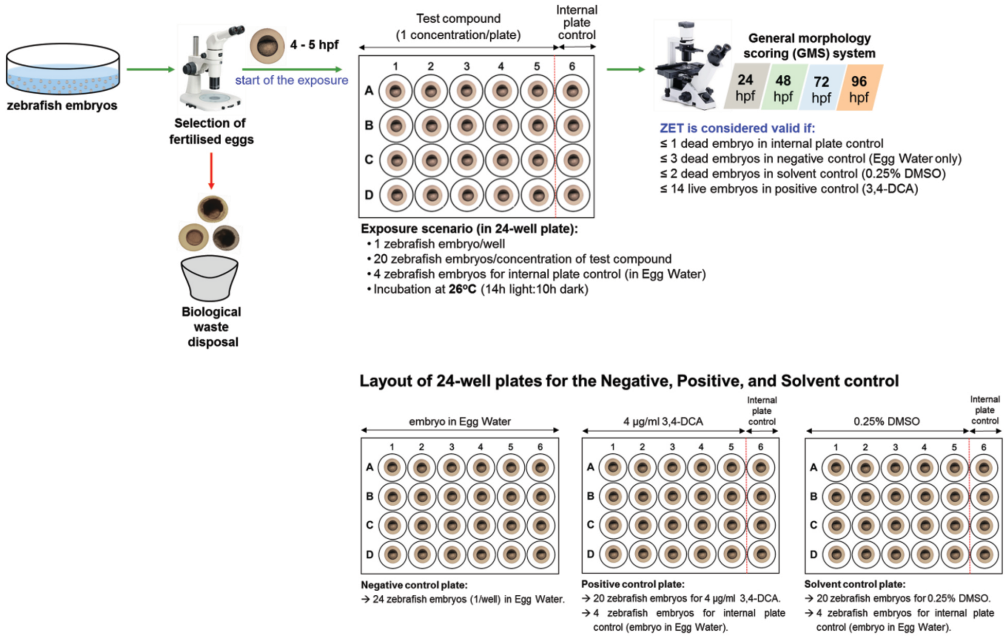


Figure 5 A schematic drawing of the ZET performed in Chapter 5 of the present thesis.

The development of the zebrafish embryo is very similar to embryogenesis in higher vertebrates, including humans, and many molecular pathways are evolutionary conserved between zebrafish and human (Sipes et al., 2011). This makes the ZET a relevant and compelling test system for evaluating the possible effects of chemical exposures on prenatal development with potentially direct relevance to human health, in particular for developmental toxicity endpoints. Recent publications have reported that zebrafish embryos are also capable of bioactivation albeit to a limited extent, due to the expression of cytochrome P450 enzymes during organogenesis (Goldstone et al., 2010; Verbueken et al., 2017), hence, allowing evaluation of the PDT potency of chemical substances in the presence of a biotransformation system. Moreover, the ZET is cost-effective, rapid, and easy to perform. For that reason, the present study includes the ZET as part of the battery of alternative assays designed for PDT testing of PS UVCBs.

The applicability of the ZET to evaluate developmental effects following exposure to chemical substances and complex mixtures has been reported in recent years (Hermsen et al., 2011; Selderslaghs et al., 2012; Teixidó et al., 2019). For example, using a training set of 27 compounds and a standardized protocol, Selderslaghs et al. (2012) showed that

the ZET was able to predict the PDT potency with a sensitivity of 72% and a specificity of 100% when compared to the available human and animal data. The usefulness of the ZET to evaluate PDT potency of individual PAHs (e.g. phenanthrene, pyrene, benzo[a]anthracene, and benzo[a]pyrene) and PAH-containing mixtures has also been reported (Geier et al., 2018; Incardona et al., 2004, 2006, 2011; Wincent et al., 2015). Table 4 shows a summary of the developmental effects induced by PAHs and PAH-containing mixtures using the zebrafish embryo model system.

1.5.3 Chemical Activated Luciferase Gene Expression (CALUX) reporter gene assays

The underlying mechanism by which some PAHs and PS UVCBs induce PDT is poorly understood. Many published studies have suggested the role of the aryl hydrocarbon receptor (AhR) in mediating the toxicity, including developmental toxicity, of PAHs and thus PAH-containing PS (Billiard et al., 2006; Goodale et al., 2013; Puga et al., 2005; Tsitou et al., 2015). The 3- to 7- ring PAHs are able to bind and activate the AhR pathway resulting in upregulation of many downstream genes including the cytochrome P450 enzymes (CYP450s), which further metabolize the PAH parent compounds into their reactive metabolites (Ma, 2001; Mimura and Fujii-Kuriyama 2003; Shimada et al., 2002; Vrabie et al., 2009). In addition to AhR, some PAHs and PAH-containing PS are also able to interact with different nuclear hormone receptors (NHRs), such as the estrogen receptor alpha (ERα) (Kizu et al., 1999; Kummer et al., 2008; Vondráček et al., 2002; Vrabie et al., 2011), due to the structural resemblance of PAHs with the natural ligands of the NHRs. Disturbance of the AhR and/or NHR pathways may eventually affect the embryonic development, particularly during tissue and organ differentiation (Barlow et al., 1999; Colborn et al., 1993; Morreale de Escobar, 2001). In light of this, the present thesis investigated the potential endocrine and dioxin-like activities of model substances under study using a panel of CALUX reporter gene assays. CALUX stands for Chemical Activated Luciferase Gene Expression, and is used since the 90s (Aarts et al., 1995; Bovee et al., 1998; Murk et al., 1996) as a cell-based screening method to study the interaction and effect of certain chemicals of interest, also mixtures, on a specific nuclear receptor. The present study included CALUX assays for activation of the ERα, the progesterone receptor (PR), the androgen receptor (AR), the thyroid receptor beta (TRβ), and the AhR.

Table 4 An overview of developmental toxicity induced by some PAHs and PAH-containing mixtures in the zebrafish embryo model system.

References	Substances	Model	Exposure	Effects
Incardona et al., 2004	naphthalene fluorene dibenzothiophene phenanthrene anthracene pyrene chrysene PAH mixtures: - less weathered oil (LWO: 44.1% naphthalene, 15.7% fluorene, 13.2% dibenzothiophene, 24.2% phenanthrene, and 0.9% chrysene) - more weathered oil (MWO: 13.0% naphthalene, 17.7% fluorene, 12.8% dibenzothiophene, 46.4% phenanthrene, and 4.4% chrysene)	Zebrafish embryos (wild-type AB line)	Start of exposure: 4-8 hpf End of exposure: 96 hpf Concentrations: 0-10 mg/ml; except for Anthracene and Chrysene: 0-5 mg/ml Solvent: 0.2% DMSO Temperature: 28.5°C	naphthalene, anthracene, chrysene: no prominent effect on the development of zebrafish embryos. fluorene, dibenzothiophene, phenanthrene, pyrene: dorsal curvature of the trunk and tail, growth reduction (especially of the head), severe pericardial and yolk-sac edemas. no lethality with any PAH treatment. all PAH-exposed embryos showed delayed or failed inflation of the swim bladder. malformations bradycardia relative embryotoxicity of the PAH mixture is proportional to the amount of phenanthrene or total of phenanthrene-dibenzothiophene in the mixture.
Incardona et al., 2006	pyrene chrysene benz[a]anthracene	Zebrafish embryos (wild-type AB line)	Start of exposure: 4-8 hpf End of exposure: 96 hpf Concentrations: 0-10 mg/ml Solvent: 0.1% DMSO Temperature: 28.5°C	CYP1A induction among tissues and organs. exposure to benz[a]anthracene resulted in cardiovascular defects, intracranial haemorrhage, and dorsal curvature on the body axis. circulation was required to deliver pyrene and chrysene to internal tissues, but Benzo[a]anthracene could diffuse or was transported to internal tissues in a circulation-independent or passive manner.

				pyrene and benz[a]anthracene induced developmental toxicity through the AhR pathway.
Incardona et al., 2011	benzo[a]pyrene benzo[e]pyrene benzo[k]fluoranthene	Zebrafish embryos (wild-type AB line)	Start of exposure: 4-8 hpf End of exposure: 48 hpf Concentrations: 0-10 mg/ml Solvent: 0.1% DMSO Temperature: 28.5°C	tissue-specific patterns of CYP1A induction. exposure to benzo[k]fluoranthene and benzo[a]pyrene resulted in pericardial edema, while benzo[e]pyrene-exposed embryos appeared no different than controls. the primary mode of action for benzo[a]pyrene in zebrafish embryo was the AhR2-dependent pathway, while for benzo[k]fluoranthene it was an AhR2-independent pathway. PAHs were toxic to the embryonic cardiovascular system and the severity varied per individual PAHs.
Gu et al., 2010	phenanthrene fluorene	Zebrafish embryos	Start of exposure: 6 hpf End of exposure: 120 hpf Concentrations: 0-100 µM Solvent: 0.1% DMSO Temperature: 28°C	phenanthrene-exposed embryos: no inflation of swim bladder, axial malformations, pericardial edema, yolk sac edema. fluorene-exposed embryos: axial malformations. reduction of blood stream speed upon exposure to phenanthrene at concentration above 40 µM.
Huang et al., 2012	benzo[a]pyrene	Zebrafish embryos (wild-type TU)	Start of exposure: 0-0.5 hpf End of exposure: 72 hpf Concentrations: 0-2 µM Solvent: 0.1% DMSO	cardiac developmental effects: dose-dependent bradycardia, irregular arrhythmia. induction of CYP1A1, CYP1B1, CYP1C1, and AhR1B. activation of the AhR-mediated pathway
Ren et al., 2012	benzo[a]pyrene benzo[k]fluoranthene	Zebrafish embryos (wild-type AB line)	Start of exposure: 6 hpf End of exposure: 120 hpf Concentrations: 0-10 µM (BaP); 0-50 µM (BkF) Solvent: 0.1% DMSO Temperature: 28°C	pericardial edema, somite deformity, yolk sac edema, weak pigmentation and cumulative mortality. slow heart beat and difficulty in movement. mortality (only benzo[a]pyrene-treated embryos at concentration >1 µm). the toxicity effect of benzo[a]pyrene was in general more pronounced than that of benzo[k]fluoranthene.
Zhang et al., 2012	pyrene	Zebrafish embryos (wild-type TU)	Time of exposure: 0.25 hpf End of exposure: 72 hpf Concentrations: 0-50 nmol/L Solvent: 0.1% DMSO Temperature: 28.5°C	dose-dependent heart abnormalities: pericardial edema and cardiac looping defects. low-level pyrene exposure caused AhR-independent cardiotoxicity in the zebrafish embryos.
Goodale et al., 2013	benz[a]anthracene dibenzothiophene pyrene	Zebrafish embryos (wild-type 5D)	Time of exposure: 6 hpf End of exposure: 48 hpf Concentrations: 0-25 (BaA); 0-50 µM (DBT, Pyrene) Solvent: 1% DMSO	pericardial edema, snout, and jaw malformations. pyrene and benz[a]anthracene-treated embryos: yolk sac edema. dibenzothiophene: axis malformations. benzo[a]pyrene: activation of the AhR-dependent pathway.

			Temperature: 28°C	pyrene and dibenzothiophene: ion transportation & homeostasis pathways.
Wincent et al., 2015	benzo[a]pyrene Soil extracts containing a mix of polycyclic aromatic compounds (PAC), PAHs, and oxy-PAHs	Zebrafish embryos (wild-type AB line)	Time of exposure: 24 hpf End of exposure: 120 hpf Concentrations: 0.004 – 0.5 µg/ml (BaP); Solvent: 0.1% DMSO Temperature: 28°C	soil extracts induced dose-dependent developmental toxicity in the zebrafish embryos: pericardial and yolk-sac edema, no inflation of swimming bladder, body shortening, small eyes, cranio-facial malformations. cumulative mortality. soil extracts were more potent developmental toxicants than benzo[a]pyrene. developmental toxicity is partly mediated through the AhR2. Oxidative stress induction could be another mode of action underlying developmental toxicity of PAHs. developmental effect was proportional to the level of polycyclic aromatic compounds (i.e. PAHs) present in the soil extracts. oxy-PAH containing mixtures were as potent AhR activators and developmental toxicants as PAHs.
Geier et al., 2018	123 PAHs: 33 parent, 22 nitrated, 17 oxygenated, 19 hydroxylated, 14 methylated, 16 heterocyclic, and 2 aminated PAHs.	Dechorionated zebrafish embryo (tropical 5D zebrafish)	Time of exposure: 4 hpf End of exposure: 120 hpf Concentrations: 0.1 - 50 µM Solvent: 1% DMSO Temperature: 28°C	responses to PAHs varied in a structure dependent manner. high-molecular weight PAHs were significantly more developmentally toxic than the low-molecular weight PAHs.

The CALUX reporter gene assays consist of recombinant mammalian cell lines that have been stably transfected with highly specific reporter constructs containing only defined responsive elements and a minimal promotor linked to luciferase (Aarts et al., 1995; Sonneveld et al., 2004). The response elements can be varied depending on the receptors and chemicals of interest that are aimed to be detected using this bioassay. The principle of the CALUX bioassays is based on the ability of the test substances to bind and subsequently activate the transcription of the receptor target genes, which will in turn be proportional to the amount of formed luciferase. In other words, the higher the relative light units (RLUs) of luciferase produced, the higher the activation of the corresponding receptor target genes. A schematic representation of the CALUX bioassay, as performed in the present thesis, is provided in Figure 6. Together, the selected panel of CALUX reporter gene assays may provide novel insights in the possible modes-of-action underlying the PDT effects of the PAHs and PAH-containing PS under study.

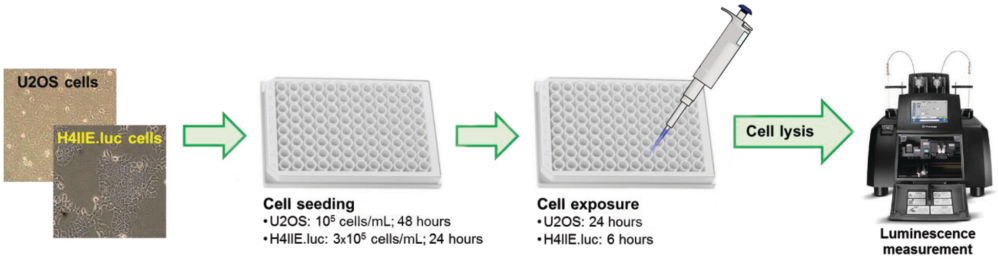


Figure 6 A schematic drawing of the CALUX reporter gene assays performed in Chapter 3 and 4 of the present thesis.

1.5.4 EST coupled with an exogenous biotransformation system

One of the main drawbacks from the current alternative assays, used to evaluate the PDT potency of chemical substances in vitro, including the EST, is the lack of an efficient metabolic activation system and of placental transport. Some chemical substances, such as 2-aminofluorene (AF), 2-acetylaminofluorene (AAF), and valpromide (VPD), require metabolic activation to exert their embryotoxic effects suggesting that the metabolites, and not the parent substances, contribute primarily to the induced-developmental toxicity (Faustman-Watts et al., 1984a,b, 1985, 1986; Juchau et al., 1985; Langsch and Nau

2005). For the model substances under study in the present thesis, it has been reported that some PAHs and PAH-containing PS need to be bioactivated to exert their carcinogenicity and mutagenicity-related effects (Blackburn et al., 1986; Mackerer et al., 2003). Yet, it is still unknown whether this is also the case for these substances to show their potential PDT effects. Hence, to better mimic the in vivo situation and to see if the toxicity potency changes upon biotransformation, the inclusion of a metabolic system in the in vitro assays, such as in the EST, is highly relevant. By this, PDT potency of the DMSO-extracts of PS and GTL products can be evaluated with and without the presence of a (exogenous) biotransformation system.

Over the past decades, various metabolic activation systems that appear suitable to transform parent substances into their reactive metabolites have been proposed, including primary hepatocytes and liver slices, liver S9-fractions, and (induced) liver microsomes (Gomez-Lechon et al., 2008; Hengstler et al., 2001; Jia and Liu, 2007; Richardson et al., 2016; Soldatow et al., 2013). Setting up a metabolic activation system using subcellular liver fractions is easier, less expensive, and less time consuming as compared to the use of fresh/cryopreserved liver preparations. Subcellular liver fractions contain most of the phase I and II metabolizing enzymes although their cofactors are absence (or diluted) (Langsch and Nau, 2005). Hence, relevant cofactors at optimized-concentrations need to be added to establish an optimal biotransformation system in vitro.

Liver S9-mix has been used in combination with the modified Ames test to evaluate the genotoxicity of chemical substances, including some PAHs and PS (Blackburn et al., 1984, 1986; Concawe 2012; Pelroy and Petersen, 1979). The authors have successfully assessed the potential genotoxicity of these substances, upon the combination of a biotransformation system with the Ames test, since the parent substances tested negative for genotoxicity without the presence of a metabolic activation system. However, it is still not clear whether bioactivation is essential for these substances to induce PDT, and if the PDT observed in vivo is caused by the parent substances or their active metabolites or both. In light of this, the present thesis makes an effort to couple an exogenous biotransformation system, using hamster liver microsomes, to the EST, to study the role of metabolism in the observed PDT by PS extracts under study in the EST. Hamster liver microsomes are preferred over rat and mouse liver microsomes, since hamster liver

microsomes were shown to express more cytochrome P450 enzymes relevant for the biotransformation of PAHs and PAH-containing substances (Blackburn et al., 1986; Phillipson and Ioannides, 1989). A schematic drawing describing how the EST and an exogenous biotransformation system using hamster liver microsomes were combined in the present thesis is presented in Figure 7.

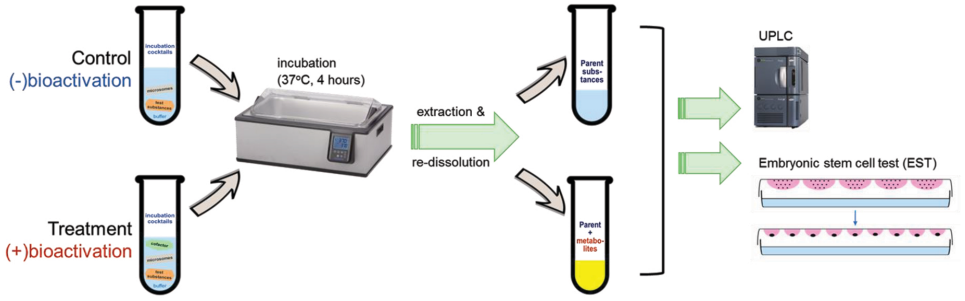


Figure 7 A schematic drawing of coupling of an exogenous biotransformation system with the EST as performed in Chapter 6 of the present thesis.

Outline of thesis

In **Chapter 1** of the thesis, background information on the topic is given and the aim of the thesis is presented, being to test the hypothesis that PAHs that may be present in some PS are responsible for the observed PDT by these substances, using a battery of in vitro alternative assays that include the EST, ZET, and a panel of CALUX reporter gene assays. This objective was defined taking into account the facts that i) within the circumstance of REACH legislation and PDT testing of PS UVCBs, it is highly desirable from both an animal welfare and an economical point of view to apply non-animal data and a read-across approach to study the PDT potency of these substances; ii) the ability of some PS UVCBs to induce PDT is associated with the presence of PAHs in these substances; and iii) PS UVCBs are complex substances, hence, the underlying mechanism for the observed PDT may be caused by a wide range of modes-of-action.

Chapter 2 evaluates the applicability of the EST to evaluate the PDT potency of the DMSO-extracts of 9 PS from different categories of PS varying in their PAH content, and 2 GTL products containing no PAHs but having otherwise similar properties as the PS. Some of these PS are known to induce PDT in the offspring of dermally exposed pregnant experimental animals and this in vivo potency was reported to be correlated with the amount of PAHs in the respective PS. On the other hand, GTL products that are devoid of PAHs did not induce any developmental effects in vivo. In **Chapter 2**, the PDT potency of the aforementioned PS and GTL products is evaluated in the EST. The results obtained in the EST are compared to literature data on the in vivo PDT potency of the respective test substances. Lastly, the in vitro PDT potency is also compared to the PAH content (2- to 7-ring PAHs) present in each PS sample, to see if there is any association between the observed PDT effects in vitro and the amount of specific PAHs present in these substances.

To date, the underlying mechanism of PDT induced by some PS (or PAH-containing PS) is poorly understood. Recent evidence suggests that the interaction of PAHs, present in some PS, with particular NHRs and/or the dioxin (AhR) receptor, may play a role in the PDT induced by these substances. To this end, in **Chapter 3** the possible interference of the same set of test substances as used in **Chapter 2** with different NHRs (ERα, PR, AR, and TRβ) and the AhR were investigated using a panel of CALUX reporter gene assays. The CALUX reporter gene assays used are based on the human osteosarcoma U2OS cell line for ERα, PR, AR, and TRβ CALUX assays and the rat hepatoma H4IIE.luc cell line for the

AhR CALUX assay, in combination with highly specific reporter constructs containing only defined responsive elements and a minimal promotor linked to luciferase. A comparison between the possible endocrine- and/or dioxin-like activities of the test substances and their PAH content is also made to see whether the observed effects correlate with the levels of specific types of PAHs present in these substances.

Chapter 4 challenges the applicability of both the EST and AhR CALUX assay to evaluate the PDT potency of PS to a further extent by testing an additional series of DMSO-extracts of HFOs, heavy PS containing mainly HMW PAHs (3- to 7-ring PAHs), and one HRBO, a highly refined mineral oil containing no aromatics and no PAHs. The correlation between in vitro PDT potency in the EST and 3- to 7-ring PAH content of these PS is characterised to further investigate the possible role of this specific group of PAHs in causing the PDT of the samples studied. Moreover, it is investigated whether the AhR plays a role in the PS-induced inhibition of ES-D3 cell differentiation by testing some PS in the presence or absence of the AhR antagonist, 6,2′,4′-trimethoxyflavone (TMF).

In **Chapter 5**, the PDT potency of the same set of test substances as used in **Chapter 2** is evaluated in the ZET. To this purpose, newly fertilised zebrafish eggs (4-5 hpf) are exposed to different concentrations of the DMSO-extracts of the 9 PS and 2 GTL products and the effects on zebrafish embryo development following exposure are scored over time from 24 to 96 hpf. The outcomes of this study are compared with data on concentration-response effects in the EST and AhR CALUX assays performed in **Chapters 2 and 3**. Furthermore, the outcomes obtained in the ZET are also compared with available in vivo data on PDT effects of these PS UVCBs in rats. Moreover, the PDT potency obtained in the ZET is compared to the specific PAH content (2- to 7-ring PAHs) present in each PS UVCBs under study, to investigate whether any correlation exists between the observed developmental effects in the ZET and the amount of specific PAHs present in these substances.

It is known that some PAHs and thus PAH-containing PS require metabolic activation to exert their genotoxicity and carcinogenicity-related effects. However, it is still unclear whether bioactivation is essential for these substances to induce PDT, and if the observed PDT is caused by the parent substances or their active metabolites or both. In light of this, in **Chapter 6**, we combined an exogenous metabolism system with the EST to compare the in vitro PDT potencies of the DMSO-extracts of PS and GTL products under study, with

and without the presence of biotransformation enzymes. Samples tested in the EST, both with and without metabolic activation included two 5-ring PAHs, namely benzo[a]pyrene and dibenz[a,h]anthracene, the DMSO-extracts of five PS from different product categories: GO, VTO, DAE, RAE, and HFO, and one GTL. By this, the possible contribution of both parent substances and their active metabolites to the observed PDT by these substances in the EST might be elucidated.

An overview of the experimental approaches as described in each Chapter of the present thesis, including the selected in vitro alternative assays and test substances are presented in Figure 8.

Finally, **Chapter 7** summarizes and discusses the findings of the present thesis. In addition, it also provides future perspectives regarding the implications and recommendations for future studies to strengthen and broaden the applicability domain of the battery of in vitro alternative assays that has been evaluated for PDT testing of PS UVCBs, and to fully unravel the role of PAHs in the developmental toxicity of some PS.

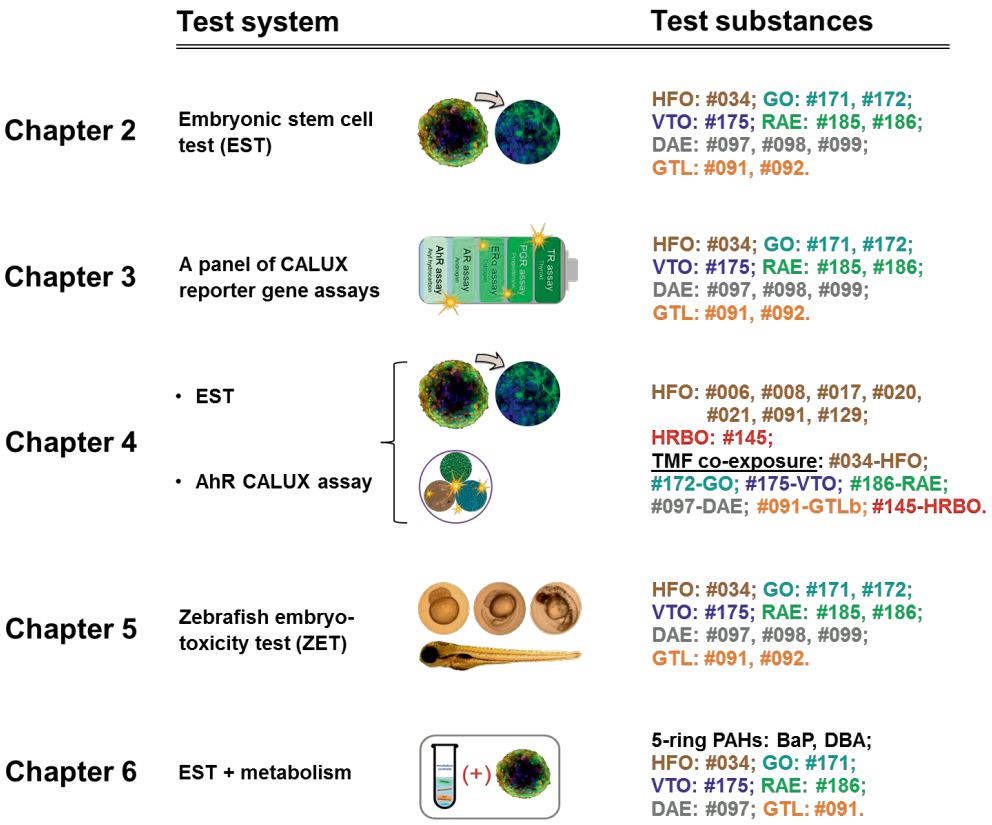


Figure 8 Overview of the experimental approaches as described in each Chapter of the present thesis, including the selected in vitro alternative assays and test substances. Ultimately, the obtained in vitro potencies will be compared to available in vivo PDT data, to assess the applicability of the current battery of in vitro alternative assays to evaluate the PDT potency of PS UVCBs.

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Chapter 2

Prenatal developmental toxicity testing of petroleum substances: application of the mouse embryonic stem cell test (EST) to compare in vitro potencies with potencies observed in vivo

Lenny Kamelia, Jochem Louisse, Laura de Haan, Ivonne M.C.M. Rietjens, Peter J. Boogaard

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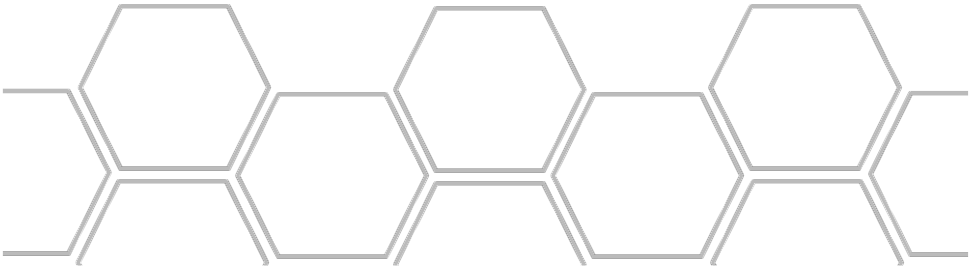


Abstract

Prenatal developmental toxicity (PDT) as observed with some petroleum substances has been associated with the presence of 3–7 ring polycyclic aromatic hydrocarbons (PAHs). In the present study, the applicability of ES-D3 cell differentiation assay of the EST to evaluate in vitro embryotoxicity potencies of PS and gas-to-liquid (GTL) products as compared to their in vivo potencies was investigated. DMSO-extracts of a range of PS, containing different amounts of PAHs, and GTL-products, which are devoid of PAHs, were tested in the ES-D3 cell proliferation and differentiation assays of the EST. The results show that PS inhibited the differentiation of ES-D3 cells into cardiomyocytes in a concentration-dependent manner at non-cytotoxic concentrations, and that their potency was proportional to their PAH content. In contrast, as expected, GTL-products did not inhibit ES-D3 cell vSuppliability or differentiation at all. The in vitro PDT potencies were compared to published in vivo PDT studies, and a good correlation was found between in vitro and in vivo results (R^2: 0.97). To conclude, our results support the hypothesis that PAHs are the primary inducers of the PDT in PS.

1. Introduction

Petroleum streams are regulated as substances under most chemical regulations, including REACH (Registration, Evaluation, Authorisation, and Restriction of Chemicals). Most petroleum substances (PS) are produced at a volume of ≥100 tonnes/year in the European Union (EU), and need to be assessed for prenatal developmental toxicity (PDT) (ECHA, 2009). If the data requirements will be fulfilled following the current Organisation for Economic Co-operation and Development (OECD) testing guidelines, this will involve a large number of experimental animals (van der Jagt et al., 2004). The application of a battery of in vitro alternative assays, including the embryonic stem cell test (EST), could reduce animal experimentation and resources needed for PDT studies of PS (Spielmann, 2009).

Three in vitro alternative methods have been scientifically validated for embryotoxicity testing: the whole embryo culture (WEC), the limb bud micromass (MM), and the embryonic stem cell test (EST) (Genschow et al., 2002, 2004; Piersma et al., 2004; Spielmann et al., 2004). Of these three testing methods, only the EST is an animal-free test system. The EST utilizes the mouse embryonic stem cell line D3, derived from the inner cell mass of the blastocyst (Buesen et al., 2009). The hanging drop culture of ES-D3 cells initiates the formation of so-called embryoid bodies (EBs) that are able to spontaneously differentiate into any cell type of the three germ layers (endoderm, mesoderm, and ectoderm), including cardiomyocytes (Seiler et al., 2006). The ability of chemicals to inhibit the differentiation of ES-D3 cells into contracting cardiomyocytes is exploited as the endpoint of this assay, to evaluate possible developmental toxicity in vitro (Seiler and Spielmann, 2011).

The validated EST consists of the differentiation assay as described above, as well as cytotoxicity assays with ES-D3 cells and Balb/c-3T3 cells, and includes a prediction model for the embryotoxic potency of the test chemical based on the EC50 values obtained in the differentiation assay and the two cytotoxicity assays (Genschow et al., 2004). In the first validation study by the European Centre for the Validation Alternative Methods (ECVAM), the EST provided 78% accuracy when predicting embryotoxicity classes of 20 reference chemicals (classified as non, weak, and strong embryotoxic chemicals), as compared to their in vivo developmental toxicity classification (non, weak, or strong). Remarkably, the predictive ability of the EST increased to 100% when only strongly embryotoxic

chemicals were taken into account (Genschow et al., 2004). Over the past decades, the differentiation assay of the EST has been widely used for in vitro embryotoxicity studies of several compounds, such as glycol ethers (de Jong et al., 2009), phenols (Strikwold et al., 2012), retinoids (Louisse et al., 2011), azoles (fungicides) (Li et al., 2015), and the results have shown a good correlation between in vitro and in vivo potencies. Yet, the applicability of EST to evaluate the in vitro developmental toxicity of complex substances, such as PS, has not been evaluated so far.

PS are UVCBs (substances of Unknown or Variable composition, Complex reaction products, and Biological materials), as they are substances with a variable composition, comprising few to millions of different hydrocarbon compounds. Hydrocarbons present in PS can be divided into five classes: paraffinics (alkanes), isoparafinics (isoalkanes), olefinics (alkenes), naphthenics (cycloalkanes) and aromatics (monoaromatics, diaromatics, and polycyclic aromatic hydrocarbons (PAHs)) (Feder, 2013). PS are produced by the primary distillation process of crude oils with subsequent treatment to alter composition and remove undesired compounds. The refining process generates a wide variety of products, varying from light to heavy distillates, and residues, with increasing molecular complexity and composition of hydrocarbons (Mackerer et al., 2003). For instance, heavy distillates like heavy fuel oil (HFO) and untreated lubricating oils may contain relatively high concentrations of 3–7 ring PAHs when compared to light distillates, such as gasoline or kerosene.

It is known that exposure to individual PAHs, for example, the 5-ring PAH benzo(a)pyrene, decreases fetal body weight and increases the incidence of resorptions in the offspring of pregnant rats (Bui et al., 1986). Dermal exposure to PS, including substances such as gas oils (ARCO, 1993; Feuston et al., 1994; HPV Chemical Challenge Program, 2012a; Mobil, 1989), aromatic extracts (Feuston et al., 1994, 1996; HPV Chemical Challenge Program, 2012b), or clarified slurry oil (Feuston et al., 1989, 1994; Feuston and Mackerer, 1996a; Hoberman et al., 1995; HPV Chemical Challenge Program, 2012c), may also induce PDT. The observed embryotoxicity includes increased incidence of resorptions, reduced number of live fetuses per litter, decreased fetal body weight, and increased incidence of skeletal variations of the fetuses. On the other hand, gas-to-liquid (GTL) products, the modern synthetic analogues of PS, which are devoid of aromatic compounds, do not

induce any effect in PDT studies (Dunster, 2014; Senn, 2014) or two-generation reproductive toxicity studies (Boogaard et al., 2017).

Feuston et al. (1994) reported a correlation between the observed PDT (increased resorptions and decreased fetal body weight endpoints) and the concentrations of 3–7 ring aromatic compounds for a range of PS. Furthermore, the investigators found no significant correlation between developmental toxicity endpoints and the concentrations of non-aromatics, mono-, and diaromatics. In addition, Feuston and Mackerer (1996b) reported that nonaromatic components of PS, such as paraffinics, olefinics, isoparafinics, and naphthenics have low toxicity and are not related to maternal and PDT. Therefore, the ability of some PS to induce PDT in vivo is likely associated with their aromatic composition, mainly their 3–7 ring PAHs (Feuston et al., 1997; Gray et al., 2013; Dalbey et al., 2014). These findings have led to the hypothesis that the observed PDT in heavier PS is due to the presence of 3- to 7-ring PAHs in these products. Consequently, it is also expected that light PS with no PAH or low concentrations of PAHs will not induce PDT (Tsitou et al., 2015).

The present study aims to test the hypothesis that PAHs, which may be present in heavier PS, are responsible for the observed PDT using the differentiation assay of the EST. To this purpose, 9 PS samples: gas oil (GO), vacuum tower overhead (VTO), residual aromatic extract (RAE), distillate aromatic extract (DAE), and heavy fuel oil (HFO), as well as 2 GTL products: GTL base oil and GTL gas oil, were tested and their in vitro potencies obtained were compared to their in vivo potencies, to evaluate the applicability of the differentiation assay of the EST to predict the relative in vivo PDT of PS.

2. Materials and methods

2.1 Samples

The DMSO-extract of 9 PS, with varying concentrations of PAHs, and 2 GTL products were tested in the present study. Of the 9 PS, 2 were gas oils (GO); CAS no. 68915-96-8 and 64741-43-1, 1 was a vacuum tower overhead (VTO); CAS no. 64741-49-7, 2 were residual aromatic extract (RAE); CAS no. 64742-10-5 and 91995-70-9, 3 were distillate aromatic extracts (DAE); possessing the same CAS no. 64742-04-7, and 1 was a heavy fuel oil (HFO);

CAS no. 64741-62-4. Of the 2 GTL products, 1 was a GTL base oil; CAS no. 848301-69-9, and 1 was a GTL gas oil; CAS no. 848301-67-7. The selected samples included extremes regarding their PAH content: HFO and GTL products, and the PAH content of the other 8 samples is in-between these extremes. HFO represents a heavy distillate from the oil refinery process that contains a higher amount of 3–7 ring PAHs, where GTL products are totally devoid of aromatics, including polyaromatics. These samples were also selected based on the availability of their in vivo PDT data in order to enable assessment of the correlation between the in vitro and the in vivo potencies. All petroleum substance samples were kindly provided by Concawe (Brussels, Belgium) and GTL products by Shell International B.V. (The Hague, the Netherlands). An overview of the PAH presents in the test samples, grouped by the number of aromatic rings, and the sample code used throughout the article is provided in Figure 1.

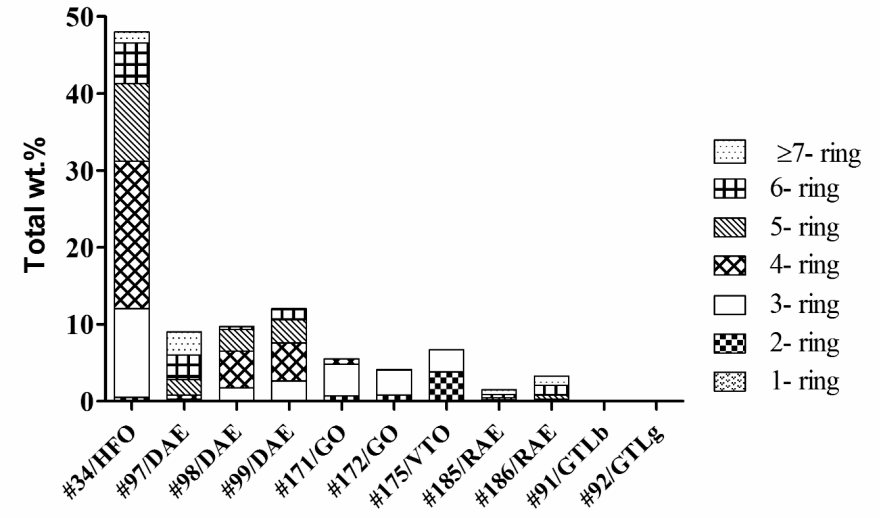


Figure 1 Aromatic Ring Class (ARC) profiles[a] of PS and GTL products used in the present study.

[a]The weight percent of the DMSO-soluble 1- to ≥7 aromatic-ring compounds present in each sample, from the starting material of 4.0 g sample, as determined by Method II chemical characterization procedure (see Section 2.2).

Note. HFO: heavy fuel oil; DAE: distillate aromatic extract; GO: gas oil; VTO: vacuum tower overhead; RAE: residual aromatic extract; GTLb: gas-to-liquid base oil; GTLg: gas-to-liquid gas oil.

2.2 PAH extraction and analysis

The procedures described below, PAH extraction and analysis, were performed at Port Royal Research laboratory. For the extraction, 4.0 g of oil samples were dissolved in 10 ml cyclohexane and extracted twice with 10 ml pre-equilibrated DMSO (mixture of 10:1 DMSO/cyclohexane). This mixture was shaken for 1 min and the PAH-enriched extracts were collected and stored in a 20 ml capped liquid scintillation vial and stored at 4°C.

The aromatic content analysis of each sample was performed according to the method described by Roy et al. (1988) and McKee et al. (2013). This analysis method is also known as the Method II chemical characterization procedure. Briefly, 4.0 g of oil sample was dissolved in 10 ml of cyclohexane, and extracted twice with 10 ml DMSO. The DMSO extracts were combined and diluted with twice the volume of 4% NaCl solution. The diluted DMSO fraction was back extracted with 20 and 10 ml of cyclohexane. The cyclohexane fractions were combined, washed two times with 5 ml of distilled water, and filtered through anhydrous sodium sulphate. The cyclohexane was then evaporated to dryness at 40°C followed by further evaporation at 80°C for 30 min. This residue was then re-dissolved in cyclohexane (~50 mg/ml). The extracts were analysed by gas chromatography with mass spectrometry (GC/MS). Naphthalene, phenanthrene, pyrene, benzo[a]pyrene, benzo[ghi-]perylene, and coronene were used as standards to define the boundaries of retention times for 2-7 ring PAHs. The results are presented as the ARC profile, the weight percent of the DMSO-soluble 1- to ≥7-ring aromatic compounds present in each sample, from the starting material of 4-gram sample.

2.3 Cell line and culture conditions

The pluripotent mouse ES-D3 cell line (ATCC, Wesel, Germany) was maintained in 25 cm^2 polystyrene cell culture flasks (Corning, the Netherlands), pre-coated with 0.1% gelatine, in HyClone AdvanceSTEM™ Low Osmo Dulbecco's Modified Eagle Medium (DMEM) (Fischer Scientific, Landsmeer, the Netherlands). Medium was supplemented with 15% Fetal Bovine Serum (FBS) (ATCC, USA), 2 mM L-glutamine (Invitrogen, The Netherlands), 50 U/ml penicillin (Invitrogen) and 50 µg/ml streptomycin (Invitrogen). Cells were grown at 37°C with 5% CO_2 in a humidified atmosphere and routinely subcultured every

2-3 days using non-enzymatic cell dissociation solution (Sigma-Aldrich, Schnelldorf, Germany) to detach the cells. Cells were kept undifferentiated by the addition of 1000 U/ml murine Leukemia Inhibiting Factor (mLIF) (Sigma-Aldrich).

2.4 ES-D3 cell viability assay (cytotoxicity assay)

The WST-1 assay was performed to determine the cytotoxicity of the test compounds. This assay measures the capability of mitochondrial dehydrogenase enzymes to convert tetrazolium salts into a formazan dye, which directly correlates to the number of metabolically active cells. In short, ES-D3 cells were seeded in 96-well plates (Greiner Bio-One, Alphen a/d Rijn, the Netherlands) at concentrations of 20×10^4 cells/ml (one day exposure) or 10^4 cells/ml (five days exposure) in 100 µl medium (without mLIF) and incubated for one day to facilitate cell adherence. Then, cells were exposed to 100 µl of medium with or without test compounds (3 replicates/concentration) and incubated for one day or five days at 37°C and 5% CO_2 in a humidified atmosphere. All samples were tested at a range of concentrations up to 500 µg raw material/ml, except for sample #034-HFO that could be dosed up to only 50 µg raw material/ml due to solubility issues. The final concentration of solvent, DMSO, was kept at 0.25% (v/v). After the incubation period, 20 µL of WST-1 reagent (Roche Diagnostics, Mannheim, Germany) was added to each well and cells were incubated for 3 h at 37°C and 5% CO_2. Subsequently, the absorbance of the formed formazan was measured at 440 nm using a SpectraMax M2 (Molecular Devices, Sunnyvale, USA). The cell viability of each condition was expressed as the percentage cell viability compared to the solvent control (which was set at 100%). The wells containing culture medium plus WST-1 reagent in the absence of cells were used as a blank (background control). The percentage viability of the blank was set at 0% since no cells were present. At least three independent experiments were conducted for each test compound.

2.5 ES-D3 cell differentiation assay

This assay aims to evaluate the inhibitory effect of test compounds on the differentiation of ES-D3 cells into contracting cardiomyocytes. The ES-D3 cell differentiation assay was

performed according to de Smedt et al. (2008), with minor modifications. The initial step of the differentiation assay is the formation of embryoid bodies (EBs) via hanging drop culture in medium without mLIF. On day 0, droplets of 20 µl of a cell suspension (3.75×10^4 cells/ml), with or without test compound, were placed between the well borders on the inner side of the lid of a 96-well plate. The wells of the 96-well plate were filled with 250 µl of phosphate buffer saline (PBS) (Invitrogen) to create an optimal humidity and to prevent evaporation of the hanging drops. Sterile caps of Eppendorf tubes were placed in the corner of the plates in order to prevent direct contact of the drops with the plate and the plate was subsequently sealed with Micropore tape (3M, Neuss, Germany) to prevent evaporation of the hanging drops. The hanging drop cultures were incubated for three days at 37°C and 5% CO_2. On day 3, the resulting EBs were transferred to 60×15 mm bacteriological petri dishes (Greiner Bio-One) containing 5 ml medium, with or without test compounds. Petri dishes were incubated for another 2 days at 37°C and 5% CO_2 in the presence or absence of the test compounds. On day 5, the EBs were transferred to a 24-well plate (Corning) (1 EB/well), containing 1 ml medium with or without test compound. The EBs in 24-well plates were then incubated for 5 days at 37°C and 5% CO_2. On day 10, the number of wells containing contracting cardiomyocytes was determined by visual inspection using a light microscope. The concentration of solvent in the medium was 0.25% DMSO (v/v) and a solvent control was included in each experiment. The differentiation assay was considered valid if the solvent control had at least 21 out of 24 wells that contained contracting cardiomyocytes. The inhibition of differentiation by the test compound was presented as the fraction of total EBs plated in the 24-well plate and at least three independent experiments were done for each test compound. 5-fluorouracil (Sigma- Aldrich), final concentration 0.065 µg/ml (0.5 µM), was included as a positive control for this assay.

2.6 Benchmark concentration (BMC) and benchmark dose (BMD) derivation

2.6.1 In vitro data

To calculate BMCs, concentration-response curves from the ES-D3 cell differentiation assay were fitted to all dichotomous concentrationresponse models available in Environmental Protection Agency's Benchmark Dose (EPA-BMD) software version 2.6.1

(gamma, logistic, log-logistic, probit, log-probit, weibull, multistage-cancer, multistage, and the quantal-linear models). The benchmark response (BMR) was set at 50%, representing a 50% inhibition of EBs differentiated into contracting cardiomyocytes. 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 benchmark concentration for 50% inhibition on cell differentiation or BMCd50 values used for the invitro-in vivo comparison were selected from the accepted model with the lowest Akaike's Information Criterion (AIC).

2.6.2 In vivo data

The in vivo PDT data of GO (ARCO, 1993; HPV Chemical Challenge Program, 2012a), VTO (HPV Chemical Challenge Program, 2012a; Mobil, 1989), and HFO (Feuston et al., 1989; HPV Chemical Challenge Program, 2012c), were kindly provided by Concawe, and the in vivo data of the DAE were obtained from Feuston et al. (1996) (Table 1). However, no in vivo study of RAE was available. Different developmental toxicity endpoints were reported from these studies, such as increased resorptions, decreased fetal body weight, and skeletal variations. Increased resorptions and decreased fetal body weight, and not skeletal variations, were selected for the BMD analysis, because all studies contained data on incidence of resorptions and fetal body weight, whereas the studies of HFO and DAE did not provide data on skeletal variations. To calculate BMD values, in vivo data from fetal body weight were fitted to the continuous models available in the EPA BMD software version 2.6.1 (continuous data, model fitted included hill, exponential, linear, polynomial, and power models) and the data from the incidence of resorptions were fitted to the dichotomous models of the EPA BMD software (gamma, logistic, log-logistic, probit, log-probit, weibull, multistage-cancer, multistage, and the quantal linear models). The BMR was defined as a 10% decrease in fetal body weight (BMD10-fetal body weight) and a 10% additional incidence of resorptions (BMD10-increased resorptions). The BMD10 values derived from the models with the best fit and lowest AIC were selected and used for the in vitro-in vivo comparison.

2.7 Linear regression analysis

The correlation of in vitro BMCd50 values and in vivo BMD10 values were obtained by performing a linear regression analysis using GraphPad Prism software version 5.0. Using the same approach, the correlation of in vitro BMCd50 values and total or specific PAH content in each sample was also determined. The given R-squared (R^2) value reflects the goodness-of-fit of data to the fitted regression line and was considered statistically significant if the p-value was lower than 0.05.

3. Results

3.1 DMSO extraction and PAH analysis

DMSO-extracts of PS and GTL products that were tested in the cytotoxicity and differentiation assays were analysed for PAH content to determine 1- to ≥7-ring PAH distributions, known as the ARC profile. Based on this analysis, sample #034 (HFO) was shown to contain the highest amount of PAHs (total weight percent: 48%), mainly 3-7 ring PAHs, and the GTL products (#091 and #092) showed to be totally devoid of PAHs (Figure 1). This PAH analysis also revealed that samples #171 (GO), #172 (GO), and #175 (VTO) primarily contain 2-3 ring PAHs, while the major constituents of samples #097 (DAE), #185 (RAE), and #186 (RAE) are 5-7 ring PAHs. Lastly, the other two DAE samples (#098 and #099) contain primarily 3-6 ring PAHs.

3.2 Cytotoxicity assays

Cytotoxicity assays were performed to evaluate the effects of the compounds on the ES-D3 cell viability. The DMSO-extract of PS and GTL products did not decrease cell viability after one day or five days exposure, up to the highest concentration tested, which was 500 µg raw material/ml except for HFO where it was 50 µg raw material/ml (Figure 2). Hence, the benchmark concentration for cell viability or BMCv50 values, representing a 50% reduction in cell viability, could not be determined.

Table 1 In vivo PDT data of PS and GTL products on different developmental toxicity endpoints in rats.

Compounds	CAS No.	Exposure days (method)	Dose (mg/kg bw/day)	No. of dams	No. of resorptions/ No. of implantations	Mean fetal body weight (g) ± SD	Developmental LOAEL and/or NOAEL (mg/kg bw/day)	Reference(s)
Gas oils intermediate (GO)	64741-43-1	GD 0-19 (D)	0 50 250 500	24 24 25 22	16/377 21/360 43/366 105/319	3.47 ± 0.22 3.48 ± 0.23 3.18 ± 0.29 2.99 ± 0.29	LOAEL: 250[a] NOAEL: 50[a]	(ARCO, 1993; HPV Chemical Challenge Program, 2012a)
Vacuum tower overhead (VTO)	64741-49-7	GD 0-19 (D)	0 30 125 500 1000	10 9 10 10 10	10/168 1/142 17/160 93/153 145/161	3.5 ± 0.2 3.5 ± 0.2 3.5 ± 0.2 3.1 ± 0.3 2.8 ± 0.3	LOAEL: 125[b] NOAEL: 30[b]	(HPV Chemical Challenge Program, 2012a; Mobil, 1989)
Distillates aromatic extract (DAE)	64742-04-7	GD 0-19 (D)	0 8 30 125	13 13 14 13	8/189 19/209 58/220 155/182	3.5 ± 0.2 3.5 ± 0.2 3.3 ± 0.2 3.0 ± 0.4	NOAEL: 30[c]	(Feuston et al., 1996; HPV Chemical Challenge Program, 2012b)
Heavy fuel oil (HFO)	64741-62-4	GD 0-19 (D)	0 4 8 30 125 250	10 10 9 9 8 9	14/157 18/166 18/146 98/141 104/107 135/135	3.5 ± 0.2 3.5 ± 0.3 3.4 ± 0.3 2.7 ± 0.6 2.3 ± 0 -	NOAEL: 4[d]	(Feuston et al., 1989; HPV Chemical Challenge Program, 2012c)
Heavy fuel oil (HFO)	64741-62-4	GD 0-19 (D)	0 0.05 1 10 50 250	23 24 24 24 23 23	26/370 20/382 119/343 225/343 322/342 329/329	3.51 ± 0.41 3.54 ± 0.34 2.94 ± 0.33 3.02 ± 0.27 2.62 ± 0.07 -	NOAEL: 0.05[e]	(Hoberman et al., 1995; HPV Chemical Challenge Program, 2012c)
Gas-to-liquid (GTL) gas oil	848301-67-7	GD 5-19 (G)	0 50 200 750	22 23 22 24	13/284 15/312 8/298 20/322	4.15 ± 0.3 4.1 ± 0.3 4.3 ± 0.2 4.15 ± 0.4	NOAEL: 750[f]	(Dunster, 2014)
Gas-to-liquid (GTL) base oil	848301-69-9	GD 6-20 (G)	0 50 200 1000	21 18 19 21	10/272 7/232 9/234 11/276	4.95 ± 0.2 5.0 ± 0.2 4.9 ± 0.3 5.1 ± 0.3	NOAEL: 1000[g]	(Senn, 2014)

[a]LOAEL and NOAEL values were taken from cited studies, based on the increased number of dams with resorptions, decreased fetal body weight, decreased embryo/fetal viability, increase in skeletal alterations.
[b]LOAEL and NOAEL values were taken from cited studies, based on the increased number of dams with resorptions, decreased litter size, decreased fetal body weight and length, increased soft tissue anomalies.
[c]NOAEL value was taken from cited studies, based on the reduced number of viable fetuses per litter, reduced litter size of viable fetuses, increased percent resorptions, and decreased fetal body weight.
[d]NOAEL was taken from cited studies, based on the increased *in utero* death of the embryo/fetus, decreased fetal body weights, and fetal anomalous development.
[e]NOAEL was taken from cited studies, based on the increased incidence of resorptions, decreased number of viable fetuses per litter, decreased fetal body weight, and increased incidence of fetal variations.
[f]NOAEL were taken from cited studies. No toxicological significant changes were detected in the offspring parameters measured at any dose level.
[g]NOAEL were taken from cited studies. No effects on the development of fetuses were observed at any dose level.

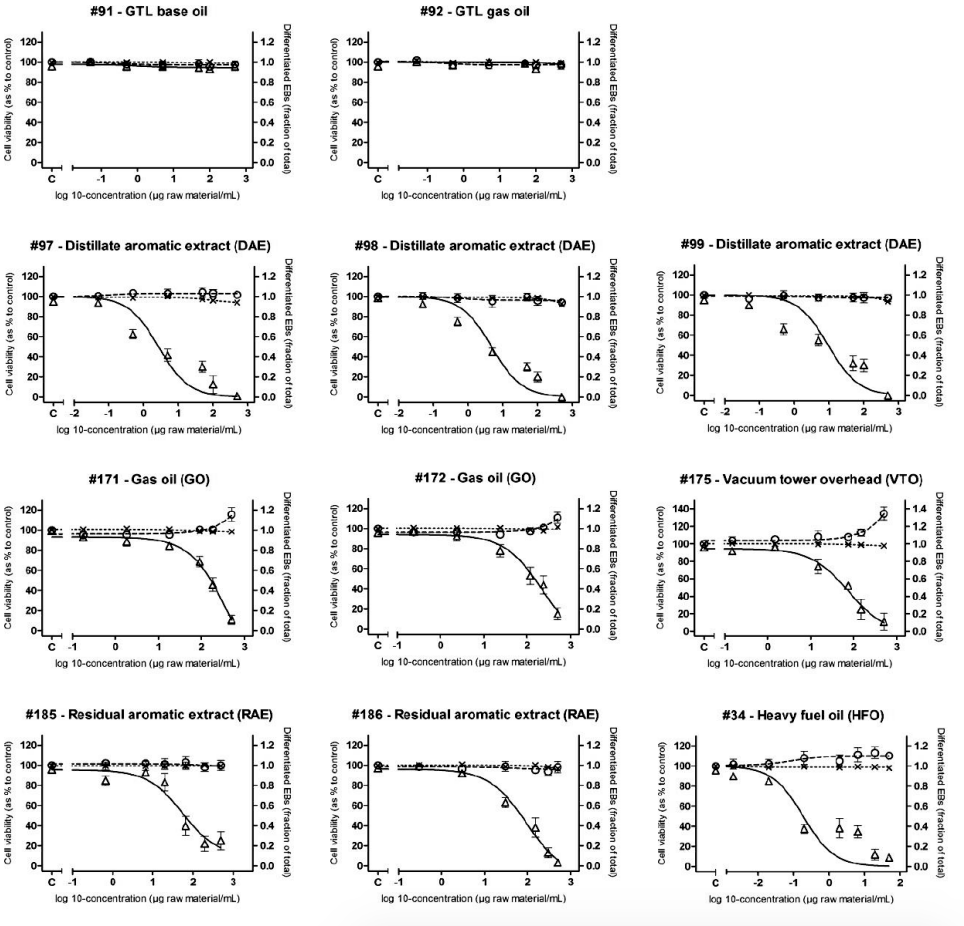


Figure 2 Concentration-dependent effects of DMSO-extracts of PS and GTL products on ES-D3 cell viability upon one-day (x and dotted line) and five-days (○ and dashed line) exposure and on inhibition of ES-D3 cell differentiation into contracting cardiomyocytes (Δ and straight line). Results represent data from at least three independent experiments and are presented as mean ± standard error of the mean (SEM).

3.3 Differentiation assay

To evaluate the in vitro developmental toxicity potency of PS and GTL products, the effects of the compounds on the differentiation of ES-D3 cells into contracting cardiomyocytes in the cell differentiation assay were determined. As depicted in Figure 2, the DMSO extracts of PS inhibited the differentiation of ES-D3 cells in a concentration-dependent manner. On the contrary, the DMSO extracts of both GTL base oil and GTL gas oil did not inhibit the differentiation of ES-D3 cells into contracting cardiomyocytes up to the highest concentration tested (500 µg/ml). The different potencies of the test samples in inhibiting the differentiation of ES-D3 cells, can be clearly seen in Figure 3, in which all concentration-response curves from the ES-D3 cell differentiation assay are plotted together (Figure 3). Among all samples tested, HFO was the most potent in inhibiting the differentiation of ES-D3 cells, followed by DAE, VTO, RAE, and GO. Table 2 presents the BMCd50 values derived from the concentration-response data obtained for the different PS. It is worth mentioning that the BMCd50 values occur at non-cytotoxic concentrations, indicating that the inhibition of ES-D3 cell differentiation is not due to the cytotoxicity.

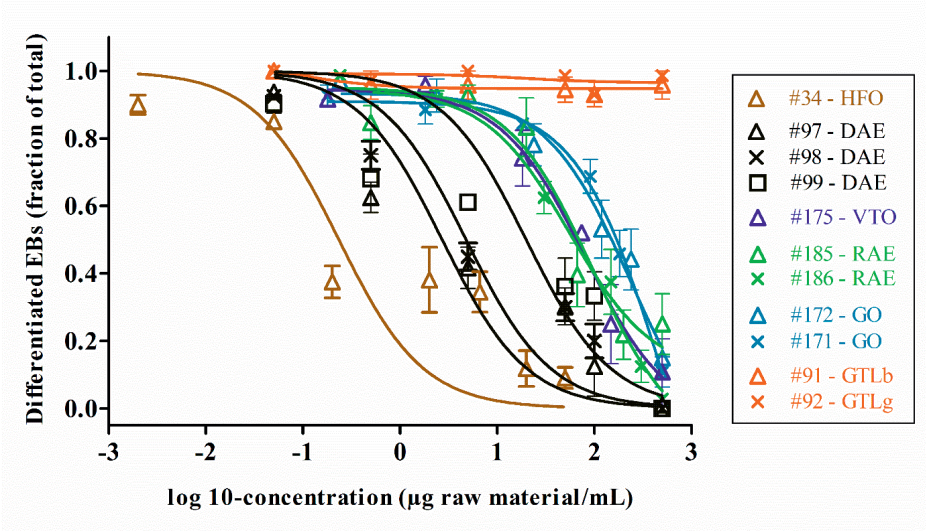


Figure 3 Comparison of the concentration-dependent inhibition of ESD3 cell differentiation upon exposure to DMSO-extracts of PS and GTL products. Results represent data from at least three independent experiments and are presented as mean ± standard error of the mean (SEM).

Table 2 BMCd50 values for inhibition of ES-D3 cell differentiation by the DMSO-extracts of PS samples in the present study.

Sample code	BMCd50 (µg raw material/ml)	% cell viability[a] at BMCd50 values
#34 - HFO	0.47	99.4 (1d) 105 (5d)
#97 - DAE	3.74	98.8 (1d) 102 (5d)
#98 – DAE	5.05	99.2 (1d) 98.5 (5d)
#99 – DAE	7.42	99.1(1d) 98.1(5d)
#171 – GO	171	99.6 (1d) 103 (5d)
#172 – GO	142	101 (1d) 100 (5d)
#175 – VTO	67.2	99.5 (1d) 108 (5d)
#185 – RAE	89.5	99.7 (1d) 101 (5d)
#186 - RAE	95.2	99.6 (1d) 97.5 (5d)

Note. 1d: ES-D3 cell proliferation assay for 1 day exposure; 5d: ES-D3 cell proliferation assay for 5 days exposure; HFO: heavy fuel oil; DAE: distillate aromatic extract; GO: gas oil; VTO: vacuum tower overhead oil; RAE: residual aromatic extract.
[a]The percentage cell viability at BMCd50 values, upon one-day and five-days exposure, were determined by extrapolation to fitted viability curve with linear regression analysis.

3.4 BMD derivation of in vivo data

A literature study on in vivo PDT was performed to obtain data on which the in vivo potency ranking of the test samples could be based. To this purpose, studies conducted in pregnant rats with exposure during gestation days 0-19 were selected. Only in vivo data for GO (#172), VTO (#175), DAE (#097, #098, #099), and HFO (#034) were available. For these substances, data on fetal body weight decrease and increased resorptions were available, which were analysed using the continuous and dichotomous dose-response models, respectively, of the EPA-BMD software. The in vivo data used to calculate the BMD10 values are provided in Table 1. For each compound, the BMD10 with the lowest AIC was selected as the BMD10 value that was used for the in vitro-in vivo comparison. Table 3 provides the BMD10 values of each compound, which corresponds to a 10% decrease in fetal body weight and a 10% additional incidence of resorptions. Considering the BMD10 values for increased resorptions, HFO was the most potent developmental

toxicant in rats, followed by DAE, VTO, and GO. A similar ranking was obtained using the BMD10 values for fetal body weight, except in that case VTO has a lower potency compared to GO.

Table 3 BMD10 values for in vivo PDT of PS in rats (for increased resorptions and decreased fetal body weight).

Substance	Sample code	Route and day of exposure	Dose (mg/kg bw/day)	Reference	BMD10* decreased fetal body weight (mg/kg bw/day)	BMD10* increased resorptions (mg/kg bw/day)
GO	#172	D (GD 0-19)	0, 50, 250, 500	(ARCO, 1993)	245.21[a]	270.05[b]
VTO	#175	D (GD 0-19)	0, 30, 125, 500, 1000	(Mobil, 1989)	332.64[a]	151.16[b]
DAE	#97, #98, #99	D (GD 0-19)	0, 8, 30, 125	(Feuston et al., 1996)	28.53[a]	13.89[b]
HFO	#34	D (GD 0-19)	0, 4, 8, 30, 125, 250	(Feuston et al., 1989)	28.34[a]	9.77[b]
HFO	#34	D (GD 0-19)	0, 0.05, 1, 10, 50, 250	(Hoberman et al., 1995)	20.64[a]	0.32[b]

*BMD10 fetal body weight: dose (mg/kg bw/day) giving 10% decrease in fetal body weight; BMD10 increased resorptions: dose (mg/kg bw/day) giving 10% increase in incidence of resorptions.
[a]BMD10 values were calculated from a dose-response curve using a continuous model (EPA BMD software), and data were taken from cited studies.
[b]BMD10 values were calculated from a dose-response curve using a dichotomous model (EPA BMD software), and data were taken from cited studies.
Note. GD: gestation day; D: dermal exposure; HFO: heavy fuel oil; DAE: distillate aromatic extract; GO: gas oil; VTO: vacuum tower overhead oil; RAE: residual aromatic extract.

3.5 Comparison of in vitro and in vivo developmental toxicity

To compare in vitro and in vivo developmental toxicity potencies, the BMCd50 values from the ES-D3 cell differentiation assay were plotted against the BMD10 values for decrease in fetal body weight and increase in resorptions. The results show that there is a highly significant correlation between the obtained in vitro BMCd50s for inhibition of ES-D3 cell differentiation and the in vivo BMD10s for increase in resorptions, showing an R^2 of 0.97. However, a rather poor correlation was observed when plotting the BMCd50s against the BMD10s for decrease in fetal body weight, showing an R^2 of 0.63 (Figure 4).

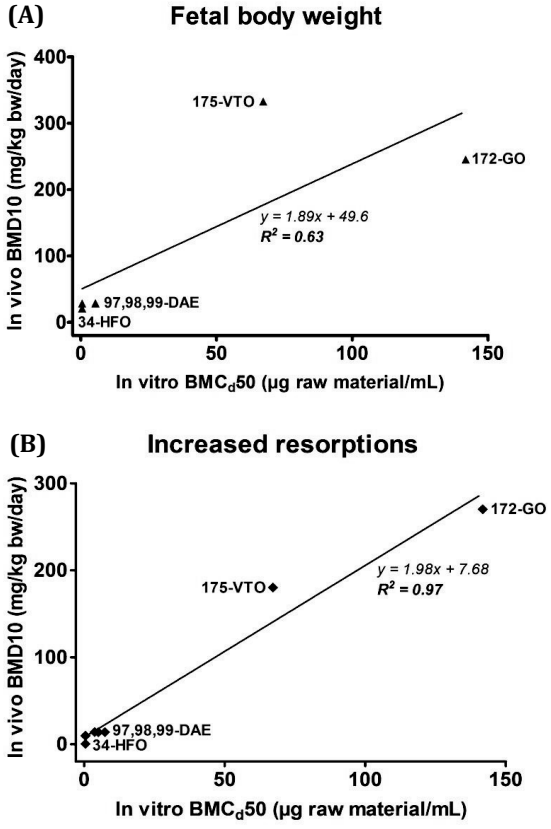
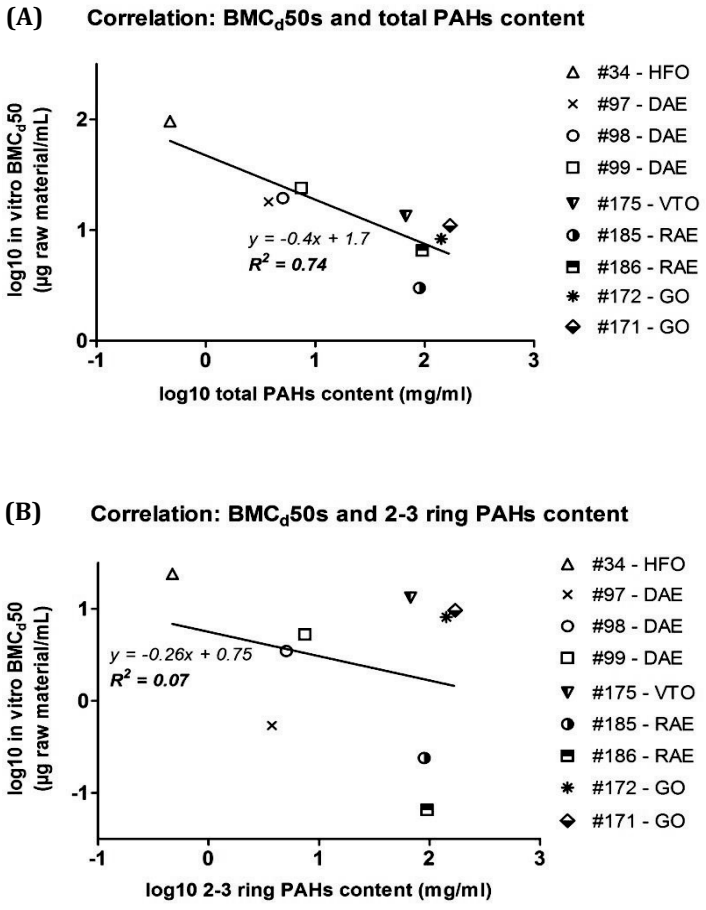


Figure 4 Correlation between in vitro BMCd50 values, obtained from the ES-D3 cell differentiation assay of the EST, and in vivo BMD10 values, based on (A) the decrease in fetal body weight and (B) the increased incidence in resorptions, of four different classes of PS: #034-heavy fuel oil (HFO), #097, #098, #099-distillate aromatic extract (DAE), #175-vacuum tower overhead oil (VTO), and #172-gas oil (GO).

3.6 Relation of in vitro developmental toxicity potencies to PAH contents

The BMCd50 values from the ES-D3 cell differentiation assay were also compared to the PAH content present in each sample, to see if there is any association between the observed effects in vitro and the quantity of either 2-3 ring or 3-7 ring PAHs present. The in vitro BMCd50s did correlate to some extent to the total PAH content, generating an R^2 of 0.74 (Figure 5). Interestingly, a better correlation was obtained when the BMCd50s were compared to only 3-7 ring PAH content, generating an R^2 of 0.81, and no correlation was seen when BMCd50s were compared to 2-3 PAH content (R^2: 0.07).



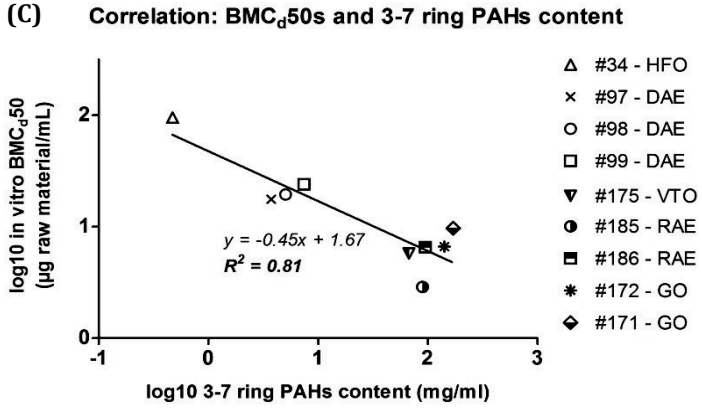


Figure 5 Correlation between in vitro BMCd50 values, obtained from the ES-D3 cell differentiation assay of the EST, with (A) total PAH content, (B) 2-3 ring PAH content, (C) 3-7 ring PAH content present in each sample.

Note. HFO: heavy fuel oil; DAE: distillate aromatic extract; GO: gas oil; VTO: vacuum tower overhead oil; RAE: residual aromatic extract.

4. Discussion and conclusions

Evidence from in vivo studies suggests that PDT as observed with some PS is associated with the presence of PAHs in these products (Feuston et al., 1989, 1994, 1996; Tsitou et al., 2015). Since PDT studies are animal intensive, the application of an in vitro assay, such as the EST, may reduce animal experimentation and resources needed to study the PDT potencies of PS. Hence, the present study aims to evaluate the applicability of the EST to assess in vitro developmental toxicity potencies of PS, by comparing them to their in vivo potencies. Our study shows a good correlation between the in vitro results in the EST and the in vivo PDT data from literature, indicating the potential applicability of the EST to predict in vivo PDT of PS.

The application of the ES-D3 cell differentiation assay of the EST to evaluate the in vitro embryotoxicity potencies of specific chemicals has been reported in several studies (de Jong et al., 2009; Li et al., 2015; Louisse et al., 2011; Strikwold et al., 2012; van Dartel et al., 2009). However, assessment of the usefulness of this assay for determination of PDT potencies of UVCB substances, such as PS has not yet been reported so far. To our knowledge, we are the first to evaluate the effects of PS and GTL products in the ES-D3

cell viability and ES-D3 differentiation assay of the EST (Kamelia et al., 2016). Interestingly, all PS were able to inhibit the differentiation of ES-D3 cells in a concentration-dependent manner at concentrations that did not cause cytotoxicity. In contrast, GTL products did not decreased viability and also did not inhibit the differentiation of the ES-D3 cells.

In order to assess the applicability of the EST to predict in vivo PDT of PS, the BMCd50 values from ES-D3 cell differentiation assay were compared with BMD10 values derived from the in vivo PDT data. The BMCd50s of sample #091 and #092 (GTL products) could not be calculated, and therefore could not be included in this comparison, since no inhibition of ES-D3 cell differentiation was observed upon exposure up to the highest concentration tested (500 µg raw material/ml). This result is in line with the in vivo findings where oral exposure of GTL products to pregnant rats does not induce any PDT effect (Boogaard et al., 2017). Fetal body weight and increased resorptions are the most frequently reported and sensitive endpoints regarding the PDT studies of PS (Murray et al., 2013). Thus, these two endpoints were chosen for the comparison of our results to in vivo studies. The in vitro-in vivo comparison was done with sample #172-GO, #175-VTO, #097, #098, #099-DAE, and #034-HFO, since in vivo data of the other PS are not available. The in vitro potency ranking of PS was found to be the same as the in vivo potency ranking based on the BMD10 for increased resorptions endpoint, with HFO as the most potent one and GO as the least potent. Based on the endpoint fetal body weight, the BMD10 value of VTO was lower than GO, yielding in a different potency ranking as compared to the endpoint increased resorptions. When the developmental NOAEL from the cited studies were used for the comparison, the same potency ranking as observed for the BMD10 for increased resorptions was acquired, with HFO being the most potent and GO the least potent substance (data not shown). Overall, this in vitro in vivo comparison has clearly demonstrated that HFO is the most potent substance with respect to both in vitro and in vivo PDT, and that DAE is more potent than VTO and GO.

A good correlation was observed between BMCd50s and both BMD10s from fetal body weight and increased resorptions endpoints. However, the correlation between BMCd50s and BMD10 values of increased resorptions endpoint was better, generating an R^2 value of 0.97. This suggests that the increased resorptions are a more sensitive endpoint for comparison with the results from the differentiation assay of the EST, than fetal body

weight. This is also corroborated by a study conducted by Murray et al. (2013), where the investigators found a stronger correlation comparing the predicted values of developmental toxicity (PDR10) with increased resorptions than with fetal body weight.

The concentration of PAHs in each petroleum substance may vary, even for samples that have the same CAS number, as the chemical composition is mainly dependent on the source of crude oil and the processing conditions used to create the stream (Speight, 2006). Sample #097, #098, and #099 are distillate aromatic extract (DAE) possessing the same CAS number and approximately the same amount of PAH content. Sample #097 contains mainly 2–7 ring aromatic compounds while sample #098 and #099 contain primarily 3–6 ring PAHs. The results of the ES-D3 cell differentiation assay showed that their relative in vitro potencies, based on the BMCd50s, differed <2 times. Remarkably, for samples that belong to the same class of PS but that possess a different CAS number, such as for gas oil (GO) and residual aromatic extract (RAE), the difference in BMCd50s was even smaller than 1.5-fold. This indicates the suitability of the ES-D3 cell differentiation assay to assess the in vitro embryotoxicity of PS, within and among classes.

The relative differences between in vitro BMCd50s of sample #172-GO, #175-VTO #097, #098, #099-DAE, and #034-HFO, were lower than the relatives differences found in the in vivo situation. For instance, the relative difference between in vitro BMCd50 of sample #172 and #034 is 364-fold, whereas, HFO was >800-times more potent than GO in inducing PDT in vivo (based on the increased resorption endpoint). It should also be noted that the in vivo PDT was observed only at doses that cause maternal toxicity, indicating the developmental effects on fetuses may reflect a secondary outcome to maternal toxicity. Difference in the in vivo and in vitro situation may be caused by differences in kinetics that are present in the in vivo model when compared to the EST model, as main limitations of the EST are the lack of a biotransformation system and of placental transport. Therefore, the coupling of the EST with a metabolic activation system and/or with data from for example an in vitro BeWo placental transport model (Li et al., 2015) could be considered to see whether the developmental toxicity potencies of PS would change after biotransformation, for instance by cytochrome P450 enzymes, or when taking possible differences in placental transport into account.

Sample #185, #097, and #034 contain mainly 3-7 ring aromatic compounds, with a total PAH content of 1.5%, 9%, and 48%, respectively. The in vitro potencies of these 3 samples

increase with increasing concentrations of 3-7 ring PAHs. To further evaluate the association between developmental toxicity and PAH content, a simple linear regression analysis was conducted. A poor correlation was obtained for BMCd50s when plotted against the total 2-3 ring PAH content, with an R^2: 0.07. A higher correlation was obtained when the BMCd50s data were plotted against the total content or 3-7 ring PAHs, with R^2 values of 0.74 and 0.81, respectively. Feuston et al. (1994) demonstrated a relation between certain toxicity endpoints to certain classes of PS composition. The authors stated that developmental toxicity endpoints is more correlated to the concentrations of 3-7 PAHs, where 2-3 ring PAHs was more correlated to skin irritation effects. This supports our hypothesis that PDT, as observed with PS, is associated with the presence of PAHs in these products, particularly 3–7 ring PAHs. Further study is needed to define the contribution and role of individual PAHs or specific types of PAHs in PDT.

The mechanism of action of PAHs and PS in causing developmental toxicity is poorly understood. Some individual PAHs and their metabolites, crude and refined petroleum products are able to bind to the estrogen receptor (ER) and the aryl hydrocarbon receptor (AhR) (Kummer et al., 2008; Machala et al., 2001; van Lipzig et al., 2005; Vrabie et al., 2009), consequently, affecting related biological processes. Previous studies have indicated that only 4-7 ring PAHs may serve as ligand to the AhR but not 3-ring or smaller PAHs (Tsitou et al., 2015). Hence, the interaction of PAHs to AhRs or ERs could be one of the underlying mechanisms with regard to the developmental toxicity.

DMSO extracts polar (or the more polar) constituents from PS, such as cycloalkanes and aromatics, including all PAHs. It is interesting to see that the application of these DMSO-extracts to ES-D3 cells has caused a concentration-dependent inhibition on cell differentiation, even without bio-activation. This suggests that the extraction method used was able to extract most of the active compounds present in each petroleum substance sample. Since the EST has virtually no metabolic capacity, it implies that biotransformation is not necessary to elicit a response of the sample studied in the EST. It may be of interest however to study whether biotransformation would potentiate the effect.

PS with similar physicochemical properties and similar application are usually grouped into categories, such as gasolines, kerosenes, and bitumens. It is impossible to test all PS as they may vary (slightly) in composition. In the present study we showed, for the first

time, that ES-D3 cell viability and differentiation assays of the EST are able to evaluate the embryotoxicity potencies of PS and GTL products, also indicating the applicability for grouping similar substances. Based on the ES-D3 cell differentiation assay results, HFO appears to be the most potent substance followed by DAE, VTO, RAE, and GO. In addition, a good correlation (R^2: 0.97) exists between the BMCd50s, obtained from the ES-D3 cell differentiation assay, and the BMD10s, based on a 10% increase in incidence of resorptions. More samples, especially those that belong to different classes of PS, should be tested to further assess the applicability of the EST in predicting in vitro developmental toxicity of these UVCBs. These findings indicate the potential of the EST for embryotoxicity testing of other UVCBs, which can result in the reduction of animal experimentation regarding the implementation of the REACH legislation.

The present study focuses on hazard not on risk assessment, and, consequently, the relevance of the concentrations we applied in vitro to the in vivo doses related to exposure of pregnant woman were not investigated. Future studies will develop physiologically-based kinetic (PBK) models to assess whether the observed in vitro effects at a certain concentration in the EST are relevant to human exposure. The PBK models also provide a way to investigate to what extent the absence of metabolism and placental transport in the EST contributes to the gap between the two approaches, in vitro and in vivo. In addition, the combination of a biotransformation system with the EST may result in a better predictive ability of the EST. Moreover, more studies are still needed to better understand which type(s) of PAHs that are responsible for the PDT. Recent studies revealed that exposure to PS may induce specific effects on gene expression patterns (Tsitou et al., 2015). Therefore, gene expression studies for mechanistic understanding of developmental toxicity induced by PS will be performed as a next step. In addition to that, a battery of in vitro alternative assays, including the zebrafish embryotoxicity test (ZET) and relevant cell lines, will be integrated in a strategy to fully understand and unravel the association between PAHs in PS and PDT.

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Supplementary data

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Chapter 3

The role of endocrine and dioxin-like activity of extracts of petroleum substances in developmental toxicity as detected in a panel of CALUX reporter gene assays

Lenny Kamelia, Jochem Louisse, Laura de Haan, Anna Maslowska-Gornicz, Hans B. Ketelslegers, Abraham Brouwer, Ivonne M.C.M. Rietjens, Peter J. Boogaard

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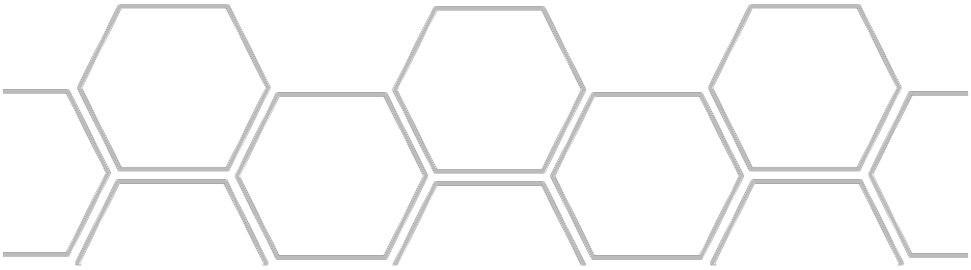

Abstract

Recent evidence suggests that the interaction of polycyclic aromatic hydrocarbons (PAI present in some petroleum substances (PS), with particular nuclear-hormone-receptors and/or the dioxin (aryl hydrocarbon receptor [AhR]) receptor, may play a role in the prenatal developmental toxicity (PDT) induced by these substances. To address this hypothesis, we evaluated the possible endocrine and dioxin-like activity of the dimethylsulfoxide (DMSO)-extracts of 9 PS, varying in PAH content, and 2 gas-to-liquid (GTL) products, containing no PAHs but having similar other properties as PS, using a series of Chemical Activated LUciferase gene eXpression (CALUX) assays. The results show that the extracts of PS tested in this study possess various endocrine and dioxin-like activities and these in vitro potencies are associated with the quantity and type of PAHs they contain. All tested DMSO-extracts of PS show a strong AhR agonist activity and rather weak antiprogesterone, antiandrogen, and estrogenic activities. In the assays that evaluate thyroid-related and antiestrogen activity, only minor effects of specific extracts, particularly those with a substantial amount of 4-5 ring PAHs, i.e. sample #034-HFO, #098-DAE, and #099-DAE, were observed. None of the GTL extracts interacted with the selected receptors. Of all assays, the AhR agonist activity correlates best (R^2: 0.80) with the in vitro PDT of the substances as quantified previously in the embryonic stem cell test, suggesting an important role of the AhR in mediating this effect. Hierarchic clustering of the combined CALUX data clustered the compounds in line with their chemical characteristics, suggesting a PS class-specific effects signature in the various CALUX assays, depending on the PAH profile. To conclude, our findings indicate a high potential for endocrine and dioxin-like activity of some PS extracts which correlates with their in vitro PDT and is driven by the PAHs present in these substances.

1. Introduction

Petroleum substances (PS) are complex materials, defined as UVCBs (substances of Unknown or Variable composition, Complex reaction products, and Biological materials) and comprise hundreds to millions of different hydrocarbon constituents. Several PS may also contain varying amounts of polycyclic aromatic hydrocarbons (PAHs). The type and quantity of PAH constituents in PS vary depending on the source of the crude oil and the processing conditions used to manufacture the material (Speight, 2006). Light PS, such as gasoline, contains very low amounts of PAHs whereas heavier products like heavy fuel oil (HFO) may contain considerable amounts of high-molecular weight PAHs (Mackerer et al., 2003).

Heavy and poorly refined petroleum streams have the potential to show carcinogenicity caused by the level of 3–7 ring PAHs they contain, and some of these substances are also able to induce prenatal developmental toxicity (PDT) in experimental animals (ARCO, 1993; Feuston and Mackerer, 1996; Feuston et al., 1989, 1994, 1996; Hoberman et al., 1995). The main manifestations of this adverse effect include increased incidence of resorptions, reduced number of live fetuses per litter, decreased fetal body weight, and increased incidence of skeletal variations of the fetuses (Feuston and Mackerer, 1996; Feuston et al., 1994; Hoberman et al., 1995). However, reproductive and developmental toxicity studies with gas-to-liquid (GTL) products, modern synthetic analogs of PS, containing only saturated hydrocarbons and devoid of aromatics, showed no PDT or reproductive toxicity (Boogaard et al., 2017; Dunster, 2014; Senn, 2014). Hence, it is hypothesized that heavy and poorly refined PS with relatively high concentrations of certain PAHs may induce PDT while light or highly refined PS with no or very limited amounts of PAHs will not induce PDT (Tsitou et al., 2015).

Recent studies in our lab corroborated this hypothesis since selected PS, varying in PAH constituents and concentrations, were able to induce in vitro PDT as measured in the differentiation assay of the embryonic stem cell test (EST) (Kamelia et al., 2017). The dimethylsulfoxide (DMSO)-extracts of the PS were able to inhibit the differentiation of ES-D3 cells in a concentration-dependent manner and this potency was proportional to their 3- to 7-ring PAH content. This finding strongly suggests that PAHs in PS are primary inducers of the observed PDT induced by these products (Kamelia et al., 2017).

Yet, the underlying mechanism of action of PAHs and some PS in causing developmental toxicity is poorly understood. Several studies have suggested that it may involve activation of the aryl hydrocarbon receptor (AhR) (Billiard et al., 2006; Goodale et al., 2013; Puga et al., 2005), thereby activating the transcription of cytochrome P4501A1 (CYP1A1), P4501A2 (CYP1A2), P4501B1 (CYP1B1), which are key enzymes for the biotransformation of PAHs into their reactive metabolites (Ma, 2001; Mimura and Fujii-Kuriyama 2003; Shimada et al., 2002). It is also known that PAHs present in some petroleum products are able to interact with the nuclear-hormone-receptors (NHRs) (Vrabie et al., 2010, 2011), due to the structural resemblance of PAHs to the natural ligands of the NHRs. For example, (anti)androgenic and (anti)estrogenic activities of PAHs and petroleum products have been reported in the scientific literature over the past decades (Hilscherova et al., 2000; Vrabie et al., 2009, 2010, 2011; Ziccardi et al., 2002). From this increasing evidence, it may be hypothesized that some PS possess endocrine and dioxin-like activity and that this potency is associated with the type and amount of PAHs they contain.

Interference with the function of AhR, steroid and thyroid hormone receptors may result in developmental, reproductive, and endocrine toxicities. Steroid and thyroid hormones play critical roles in all aspects of the fetal developmental process, particularly tissue and organ differentiation (Colborn et al., 1993; Morreale de Escobar, 2001). Hence, prenatal exposure to substances with hormonal or antihormonal activities in this vulnerable time window might affect the homeostasis of endogenous hormones, and as a consequence, exert effects on embryonic development (Barlow et al., 1999).

To test the possible interference with the AhR and different hormone receptors, the DMSO-extracts of 9 PS, with varying types and levels of PAH constituents, and of 2 GTL products, which are devoid of PAHs, were investigated for their potential endocrine and dioxin-like activities using a panel of Chemical Activated LUciferase gene eXpression (CALUX) reporter gene assays. The receptors selected for the present study included the androgen receptor (AR), the estrogen receptor alpha (ERα), the progesterone receptor (PR), the thyroid receptor beta (TRβ), and the AhR. By this, we expect to assess the endocrine and dioxin-like activities and modes of action of PS in relation to their PAH content and PDT potencies.

2. Materials and Methods

2.1 Test compounds

All reference-standard compounds (≥95% purity) for the CALUX agonist and antagonist assays were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands).This includes dihydrotestosterone (DHT; CAS no. 521-18-6), flutamide (CAS no. 13311-84-7), 17b-estradiol (E2; CAS no. 50-28-2), fulvestrant (CAS no. 129453-61-8), progesterone (PGT; CAS no. 57-83-0), mifepristone (RU468; CAS no. 84371-65-3), triiodothyronine (T3; CAS no. 6893-02-3), 1-850 thyroid hormone receptor antagonist (CAS no. 51310-57-3), and benzo[a]pyrene (BaP; CAS no. 50-32-8). All stocks and dilutions of the standards were prepared in DMSO (Merck, Darmstadt, Germany).

DMSO-extracts of 9 PS and 2 GTL products were tested in the present study. The DMSO-extracts were generated according to the extraction procedure described by Kamelia et al. (2017) and Roy et al. (1988), and extraction was performed at Port Royal Research laboratory (Hilton Head, South Carolina). The PAH extraction and analysis procedure that generates DMSO-extracts of PS is generally used to obtain the PAH fraction from the raw material of these substances and this method has been widely used and validated also for mutagenicity and carcinogenicity testing of PS (Blackburn et al., 1986; Clonfero et al., 1996; Concawe, 1994; Mackerer et al., 2003). The raw material of all PS and GTL products, which were used for the DMSO extraction, were kindly provided by Concawe (Brussels, Belgium) and Shell International bv (The Hague, the Netherlands), respectively. These raw materials were: 1 HFO (CAS no. 64741-62-4), 3 distillate aromatic extracts (DAE; all 3 bearing the same CAS no. 64742-04-7), 2 residual aromatic extracts (RAEs; CAS no. 64742-10-5 and 91995-70-9), 2 gas oils (GO; CAS no. 68915-96-8 and 64741-43-1), 1 vacuum tower overhead oil (VTO; CAS no. 64741-49-7), 1 GTL base oil (GTLb; CAS no. 848301-69-9) and 1 GTL gas oil (GTLg; CAS no. 848301-67-7). The PS had varying PAH contents whereas the GTL products were totally devoid of PAHs. An overview of the PAHs present in the DMSO-extracts of PS and GTL, grouped by the number of aromatic rings, is provided in Figure 1.

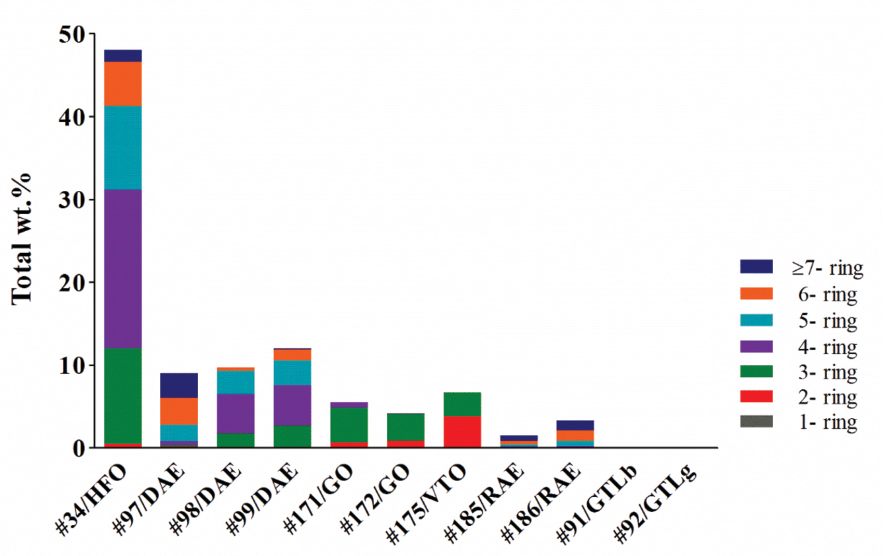

Figure 1 ARC profiles[a] of PS and GTL products tested in this study.

[a]The weight percent of the DMSO-soluble 1- to ≥7 aromatic-ring compounds present in each sample, from the starting material of 4.0 g, as determined by Method II chemical characterization procedure (Kamelia et al., 2017; Roy et al. 1988).
Abbreviations. HFO, heavy fuel oil; DAE, distillate aromatic extract; GO, gas oil; VTO, vacuum tower overhead oil; RAE, residual aromatic extract; GTLb, gas-to-liquid base oil; GTLg, gas-to-liquid gas oil.

2.2 Cell lines and cell culture conditions

Stably transfected human osteosarcoma cell lines (U2OS), expressing AR, ERα, PR, or TRβ, were purchased from BioDetection Systems (BDS, Amsterdam, the Netherlands), and used for the U2OS CALUX assays. U2OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1), a 1:1 mixture of DMEM and F-12 nutrient mixture (Ham) (Gibco, Paisley, UK, No. 31330-038) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, No. F7524), 0.5% minimum essential medium (MEM) nonessential amino acids (MEM NEAA, Gibco, No. 11140-035), and 200 mg/ml geneticin G418 (Gibco, No. 10131-035). Cells were routinely subcultured every 2-3 days, using 0.05% trypsin-EDTA (Gibco) to detach the cells.

The stably transfected rat hepatoma cell line, H4IIE (Aarts et al., 1995), was used for the AhR CALUX assay. H4IIE cells were grown in MEM alpha medium (Gibco, No. 22561-021) supplemented with 10% FBS. All cells were incubated at 37°C with 5% CO_2 in a humidified atmosphere and subcultured every 2-3 days, using 0.05% trypsin-EDTA to detach the cells.

2.3 CALUX reporter gene assays

The CALUX reporter gene assays used in the present study are based on the human osteosarcoma U2OS cell line for U2OS CALUX assays and the rat hepatoma H4IIE cell line for the AhR CALUX assay, in combination with highly specific reporter constructs containing only defined responsive elements and a minimal promotor linked to luciferase (Aarts et al., 1995; Legler et al. 1999; Sonneveld et al., 2004). The principle of these bioassays relies on the ability of the test compound to bind and consequently activate or inhibit the transcription of the receptor target genes. In the U2OS CALUX assays, DMSO-extracts of PS and GTL products were tested at a range of concentrations up to 250 µg raw material/ml or up to the maximum solubility limit of these substances in the assay medium. Subsequently, the cytotoxicity of these concentration ranges was determined in the U2OS Cytotox assay (the method is described in van der Linden et al., 2014) and the results showed that no cytotoxicity was observed upon 24 h exposure to test compounds up to the concentration of 250 µg raw material/ml (data not shown). In the AhR CALUX assay, 5 µg raw material/ml was used as the top tested concentration, since the highest AhR induction was already obtained upon exposure to test compounds at this concentration. For U2OS CALUX assays (AR, ERα, PR, and TRβ), confluent cell cultures were washed with phosphate-buffered saline (PBS, Gibco, No. 10010-023), trypsinised, and then diluted to the proper concentration for cell seeding in DMEM/F-12 medium without phenol red, enriched with 5% dextran-coated charcoal-stripped FBS (DCC FBS, Gibco, No. 12676029) and 0.5% MEM NEAA. This medium is referred to as the assay medium (AM). U2OS cells were seeded in a volume of 100 ml into each of the 60-inner wells of 96-well white plates (Greiner Bio-one, Frickenhausen, Germany) at a density of 10^5 cells/ml. The 36-outer wells of the same 96-well white plate were filled with 200 µl PBS to create an optimal humidity and to limit evaporation from the inner wells. After 24 h of incubation at 37°C/5% CO_2, the old medium was removed using a vacuum-pump and replaced by 100 µl fresh AM/well. Cells were then incubated for another 24 h. Forty-eight hours after cell seeding, cells were exposed to increasing concentrations of test compounds in triplicate by adding 100 µl exposure medium (EM) to each well. The EM for the U2OS agonist assays was prepared by addition of 400 times concentrated stock solutions of the test compounds (dissolved in DMSO) to the AM, and the final concentration of DMSO in the well was kept at 0.25%. The EM for the U2OS antagonist assays was prepared by addition to AM of 800 times concentrated DMSO-stock solutions

of reference-standard agonist compounds to reach a final concentration equal to their EC50 concentration ($2.9x10^{-5}$ µg/ml DHT for the U2OS AR antagonist assay; $3.1x10^{-4}$ µg/ml PGT for U2OS PR antagonist assay; $2.7x10^{-6}$ µg/ml E2 for U2OS ERα antagonist assay; $1.3x10^{-4}$ µg/ml for U2OS TRβ antagonist assay) and adding test compounds also from 800 times concentrated DMSO-stock solutions. The final concentration of solvent, DMSO, was also kept at 0.25%.

For the AhR CALUX assay, 100 µl aliquots of H4IIE cells at a density of $3x10^5$ cells/ml were seeded into the 60-inner wells of 96-well white plates. The cells were incubated overnight (24 h) at 37°C/5% CO_2 and then exposed for 6 h to the test compounds in triplicate. Six hours was chosen, over 24 h, for the exposure time for the AhR CALUX assay because the highest AhR induction by BaP, the positive control of the assay, and DMSO extracts of PS (at the highest tested concentration of 5 µg raw material/ml), was obtained after 6 h of exposure (data not shown). In addition, prior studies by Vrabie et al. (2009) indicated that 6 h is the standard exposure time for the detection of BaP like compounds in the AhR CALUX assay, whereas 24 h could be applied for the detection of more persistent compounds, like PCBs. The EM for the AhR CALUX assay was prepared by diluting the test compounds (from 400 times concentrated DMSO-stock solutions) with preconditioned medium. Pre-conditioned medium is defined as the growth medium in which cells were previously grown for 16-24 h. The use of the preconditioned medium for cell exposure is to avoid high background luciferase signal caused by tryptophan products present in the fresh growth medium, which can induce the AhR activity, and thereby cause false-positive results (Vrabie et al., 2009).

After 6 (AhR CALUX) or 24 h (U2OS CALUX) of exposure, the medium was removed, and cells were washed with 100 µl ½ PBS (1:1 PBS: nanopure water) and then lysed with 30 µl hypotonic low salt buffer: 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. Plates were kept on ice for at least 15 min and subsequently frozen at -80°C for at least 2 h, until use for the luminescence measurement. For the luminescence measurement, plates were thawed at room temperature for about 1 h and then shaken for 3-5 min on a plate shaker. Luciferase activity was determined using a luminometer (Glomax-Multi Detection System, Promega, California) after the addition to each well of 100 µl flashmix solution consisting of an aqueous solution of 20 mM tricine (Sigma-

Aldrich), 1.07 mM $(MgCO_3)_4$ Mg(OH)2.5H_2O (Sigma-Aldrich), 2.67 mM magnesium sulfate (MgSO4, Merck), 0.1 mM ethylenedinitrilotetraacetic acid disodium salt dihydrate (Titriplex III; 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), pH 7.8.

The final concentration of DMSO was kept at 0.25% (v/v) in all CALUX assays of the present study. A full concentration response curve of the reference-standard compound for both agonist and antagonist assay was included in each independent experiment and using the following final concentrations of the respective reference compounds: DHT (2.9 $x10^{-7}$ - 2.2$x10^{-3}$ µg/ml) for the AR agonist assay; flutamide (1.4$x10^{-4}$ - 1.4 µg/ml) for the AR antagonist assay; E2 (2.7 $x10^{-8}$ - 1.4$x10^{-4}$ µg/ml) for the ERα agonist assay; fulvestrant (3.0$x10^{-10}$ - 3.0$x10^{-4}$ µg/ml) for the ERα antagonist assay; PGT (3.1$x10^{-7}$ - 3.1$x10^{-3}$ µg/ml) for the PR agonist assay; RU468 (2.1$x10^{-8}$ - 2.1$x10^{-3}$ µg/ml) for the PR antagonist assay; T3 (6.5 $x10^{-7}$ - 6.5$x10^{-3}$ µg/ml) for the TRβ agonist assay; 1-850 (2.3$x10^{-3}$ - 5.8 µg/ml) for the TRβ antagonist assay; and BaP (1.3$x10^{-5}$ - 1.3$x10^{-1}$ µg/ml) for the AhR agonist assay.

2.4 Data analysis

Luciferase activity per well from CALUX reporter gene assays was expressed in relative light units (RLUs). All data are presented as a percentage luciferase activity relative to maximum response (fold-induction) of the reference-standard agonist compound: DHT/E2/PGT/T3/BaP for the AR/ERα/PR/TRβ/AhR CALUX assay. Data are expressed as mean ± SEM and obtained from at least 4 independent experiments (n ≥ 4). Student's t-test between solvent control (0.25% DMSO) and test compounds was performed using GraphPad Prism 5.0 (California), to determine significant luciferase activity above background.

For the agonistic assays, fold-induction of luciferase activity was calculated by dividing the mean value of RLUs from exposed wells by the mean RLU of the corresponding solvent controls (0.25% DMSO). Luciferase induction as a percentage of maximal DHT/E2/PGT/T3/BaP activity (AR/ERα/PR/TRβ/AhR CALUX assay) was calculated by setting the maximum fold induction of the reference-standard agonist compound (DHT/E2/PGT/T3/BaP) at 100%. For the antagonistic assay, fold induction of luciferase

activity at the EC50 concentration of the reference-standard agonist compound was set at 50%.

Data were analyzed using nonlinear regression in GraphPad Prism 5.0 and fitted to a sigmoid dose-response curve with 3 parameters. From this, EC25/50 or IC25/50 values were obtained, which represent the concentrations for 25%/50% induction or inhibition of luciferase activity upon exposure to the test compounds. The IC25/50 values were calculated from inhibition by reference-standard agonist compounds (DHT/E2/PGT/T3 in the U2OS AR, ERα/PR/TR antagonist assay, respectively), present at their EC50 concentration, and not from their 100% maximal agonist activity. For example, in the U2OS ERα antagonist assay, the IC25 values were determined from the inhibition of $2.7x10^{-6}$ μg/ml (10 pM) E2-induced estrogenic response, not from the 100% of maximal estrogenic activity of E2.

2.5 Linear regression analysis

Correlation of in vitro EC50s/IC50s to specific PAH constituents present in PS and to in vitro PDT potencies of the same substances in the EST (Kamelia et al., 2017), expressed as BMCd50 values were obtained by performing a linear regression analysis (GraphPad Prism 5.0). The given R^2 value reflects the goodness-of-fit of data to the fitted regression line and was considered statistically significant if the p-value was < 0.05.

2.6 Class signatures and luciferase expression profiles analysis

This study applied principal component analysis (PCA), using XLSTAT (Addinsoft, Paris, France), to study the differences and relatedness between experimental groups, based on their responses in the battery of CALUX assays. PCA was used to summarize and simplify the data derived from the luciferase expression profiles of each test compound and transform them into a smaller dataset called principal components (PCs). In the current case, a PC was defined as a mathematically derived combination of test compounds and their luciferase expression profiles in different CALUX assays to describe part of the observed effects. PCA also aims to identify patterns of effects in a multivariate dataset. In this way, the effects signature, which may have been induced by test compounds that

belong to the same class of substances, is visualized in a more simplified and informative way. In addition, a heatmap was developed using Phyton, to evaluate the hierarchic clustering of test compounds based on their activities in the CALUX assays. Euclidean distance and average linkage were selected as metric and clustering method, respectively, for the development of the heatmap. To these purposes (for both PCA and heatmap development), the average luciferase induction factor at the highest tested concentration of each test compound was selected, normalized, log-transformed, and subsequently used as data input for XLSTAT and Phyton. It is worth mentioning that only data from particular CALUX assays: AhR, ERα agonist assays and AR, ERα, PR, TRβ antagonist assays, were included for the analysis. Data from the AR, PR, TRβ agonist assays were considered irrelevant since no substantial agonist or antagonist activity was observed; hence, they were excluded from the analysis.

3. Results

3.1 Effects of PS and GTL products on the AR

The U2OS AR CALUX assay was performed to determine androgenic and antiandrogenic effects of the DMSO-extracts of PS and GTL products after 24 h of exposure. Increasing concentrations of DHT and flutamide were used as reference-standard compounds for the U2OS AR agonist and antagonist assay, respectively. In the U2OS AR agonist assay, no androgenic responses were observed from any of the test compounds at any concentration tested (Figure 2A). The antagonism effects were assessed by incubating the cells with test compounds plus a fixed concentration (EC50) of DHT. The EC50 concentration of DHT, obtained from the AR agonist assay, was $2.9x10^{-5}$ μg/ml (0.1 nM). In the U2OS AR antagonist assay, all DMSO-extracts of PS, except for sample #185-RAE, showed a concentration-dependent antiandrogenic effect (Figure 2B). Sample #186-RAE showed its antagonist effect only at the highest tested concentration, 250 μg/ml. Consequently, the IC50 value, or the concentration for 50% inhibition of luciferase activity, of sample #186-RAE could not be determined. As previously mentioned in section 2.4, the IC50 values in the U2OS AR antagonist assay were calculated from the inhibition of the 0.1 nM DHT-induced androgenic response set at 100%, and not from the maximal androgenic activity of DHT. The same applies for the other antagonist assays conducted

in the present study. Based on their IC50s, listed in Table 1, sample #034-HFO was the most potent antagonist of the AR, in comparison to the other samples. The IC50s of samples that belong to the same class of PS are quite comparable, except for the DAEs. The IC50s of sample #097, #098, and #099 were 70.6, 5.7, and 4.8 μg/ml, respectively. This means that sample #098 and #099 are more potent antagonists of the AR (>14-fold) than sample #097, even though these samples are members of the same class of PS. The discrepancy could partly be explained by the difference in aromatic ring class (ARC) profile and PAH content of these samples, but not by their total PAH content (Figure 1). Also, it is worth mentioning that only non-cytotoxic concentrations of the test compounds were used (data not shown), hence, the observed antagonism effects were not due to cytotoxicity. Furthermore, both GTL extracts, #091-GTLb and #092-GTLg, showed neither agonist, nor antagonist activity in the U2OS AR CALUX assay (up to 250 μg/ml).

3.2 Effects of PS and GTL products on the ERα

To assess (anti) estrogenicity, human U2OS ERα cells were exposed to increasing concentrations of the DMSO-extracts of the PS and the GTL products, in the absence (for agonist assay) or presence (for antagonist assay) of estradiol (E2). As depicted in Figure 2C, the extracts of some PS, including GOs, VTO, and 2 of the DAEs (#098 and #099), were able to activate the ERα. The highest percentage of luciferase induction by those samples is ranging from 15% to 48% of the maximum E2-induced response (Table 1), suggesting weak (partial) agonist ERα activities. A plateau response was not achieved following exposure to the highest possible concentration of test compounds, and testing higher concentration was not feasible due to the solubility limitation of the extracts of these substances in the assay medium. Since the agonist/antagonist responses did not reach 50%, the EC50/IC50 values could not be determined. Instead, EC25/IC25 values were calculated. Although sample #098-DAE and #099-DAE showed some limited agonist activity in the ERα, their luminescence induction did not reach 25% of the maximum E2-induced response, hence, their EC25s could not be calculated. The EC25 values were calculated using GraphPad Prism 5.0 software, assuming a theoretical maximum response of 100%. Altogether, among all tested DMSO-extracts, only sample #171-GO, #172-GO, and #175-VTO showed significant agonist activities in the U2OS ERα agonist assay, giving EC25 values of 118.6, 107.9, and 43.5 μg/ml, respectively.

The antagonistic effects were evaluated by exposing U2OS ERα cells to increasing concentrations of test compounds in combination with the EC50 concentration of E2, $2.7x10^{-6}$ µg/ml (10 pM). Fulvestrant, a known antagonist of the ERα, was used as the reference-standard compound for the antagonist assay. As shown in Figure 2D, weak antagonist effects were seen with 3 out of 9 DMSO-extract of PS: #034-HFO (IC25: 9.7 µg/ml), #098-DAE (IC25: 40.7 µg/ml), and #099-DAE (IC25: 54.1 µg/ml). The antagonistic effects were considered weak because the luminescence activity was reduced just to ≤ ±30% (ranging between 24% and 29%, out of 50% luminescence activity of the EC50 concentration of E2), which occurred only at the highest tested concentration (50 µg/ml for sample #034-HFO and 250 µg/ml for sample #098-DAE and #099-DAE). Again, no agonist or antagonist responses were observed for the DMSO-extract of GTL products.

3.3 Effects of PS and GTL products on the PR

Progestogen activity of the test compounds was determined after exposing the U2OS PR cells to increasing concentrations of the DMSO-extract of PS and of GTL products. PGT, an endogenous ligand of the PR, and mifepristone (RU468) were used as reference-standard compounds for the U2OS PR agonist and antagonist assay, respectively. The results showed that none of the test compounds was able to activate the PR (Figure 2E). On the other hand, as depicted in Figure 2F, all extracts of the PS were able to bind and block the activation of the PR by PGT, resulting in a concentration-dependent reduction of the luciferase activity upon 24 h of exposure in the test for antagonist activity. The IC50s derived from these data reveal that among all samples tested, the DMSO-extract of HFO was the most potent antagonist of the PR, followed by 2 of the DAEs (sample #098 and #099), GOs and VTO, sample #097-DAE, and RAEs. As expected, no PR activity was detected for both GTL extracts (#091-GTLb and #092-GTLg) at any of the concentrations tested (Figure 2E and 2F).

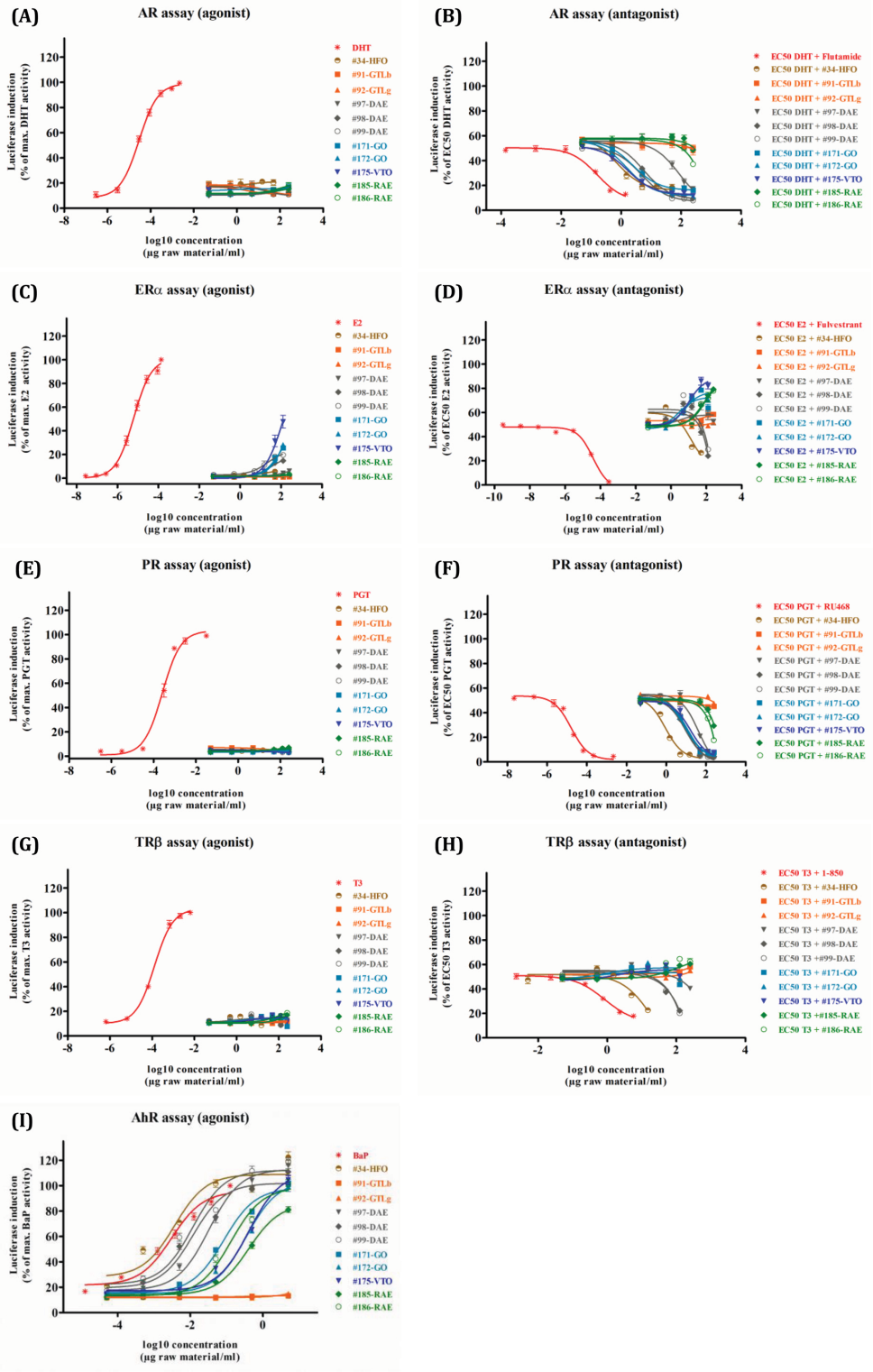
(A)
AR assay (agonist)
Luciferase induction (% of max. DHT activity)
log10 concentration (µg raw material/ml)
DHT
#34-HFO
#91-GTLb
#92-GTLg
#97-DAE
#98-DAE
#99-DAE
#171-GO
#172-GO
#175-VTO
#185-RAE
#186-RAE
(B)
AR assay (antagonist)
Luciferase induction (% of EC50 DHT activity)
log10 concentration (µg raw material/ml)
EC50 DHT + Flutamide
EC50 DHT + #34-HFO
EC50 DHT + #91-GTLb
EC50 DHT + #92-GTLg
EC50 DHT + #97-DAE
EC50 DHT + #98-DAE
EC50 DHT + #99-DAE
EC50 DHT + #171-GO
EC50 DHT + #172-GO
EC50 DHT + #175-VTO
EC50 DHT + #185-RAE
EC50 DHT + #186-RAE
(C)
ERα assay (agonist)
Luciferase induction (% of max. E2 activity)
log10 concentration (µg raw material/ml)
E2
#34-HFO
#91-GTLb
#92-GTLg
#97-DAE
#98-DAE
#99-DAE
#171-GO
#172-GO
#175-VTO
#185-RAE
#186-RAE
(D)
ERα assay (antagonist)
Luciferase induction (% of EC50 E2 activity)
log10 concentration (µg raw material/ml)
EC50 E2 + Fulvestrant
EC50 E2 + #34-HFO
EC50 E2 + #91-GTLb
EC50 E2 + #92-GTLg
EC50 E2 + #97-DAE
EC50 E2 + #98-DAE
EC50 E2 + #99-DAE
EC50 E2 + #171-GO
EC50 E2 + #172-GO
EC50 E2 + #175-VTO
EC50 E2 + #185-RAE
EC50 E2 + #186-RAE
(E)
PR assay (agonist)
Luciferase induction (% of max. PGT activity)
log10 concentration (µg raw material/ml)
PGT
#34-HFO
#91-GTLb
#92-GTLg
#97-DAE
#98-DAE
#99-DAE
#171-GO
#172-GO
#175-VTO
#185-RAE
#186-RAE
(F)
PR assay (antagonist)
Luciferase induction (% of EC50 PGT activity)
log10 concentration (µg raw material/ml)
EC50 PGT + RU468
EC50 PGT + #34-HFO
EC50 PGT + #91-GTLb
EC50 PGT + #92-GTLg
EC50 PGT + #97-DAE
EC50 PGT + #98-DAE
EC50 PGT + #99-DAE
EC50 PGT + #171-GO
EC50 PGT + #172-GO
EC50 PGT + #175-VTO
EC50 PGT + #185-RAE
EC50 PGT + #186-RAE
(G)
TRβ assay (agonist)
Luciferase induction (% of max. T3 activity)
log10 concentration (µg raw material/ml)
T3
#34-HFO
#91-GTLb
#92-GTLg
#97-DAE
#98-DAE
#99-DAE
#171-GO
#172-GO
#175-VTO
#185-RAE
#186-RAE
(H)
TRβ assay (antagonist)
Luciferase induction (% of EC50 T3 activity)
log10 concentration (µg raw material/ml)
EC50 T3 + 1-850
EC50 T3 + #34-HFO
EC50 T3 + #91-GTLb
EC50 T3 + #92-GTLg
EC50 T3 + #97-DAE
EC50 T3 + #98-DAE
EC50 T3 +#99-DAE
EC50 T3 + #171-GO
EC50 T3 + #172-GO
EC50 T3 + #175-VTO
EC50 T3 +#185-RAE
EC50 T3 + #186-RAE
(I)
AhR assay (agonist)
Luciferase induction (% of max. BaP activity)
log10 concentration (µg raw material/ml)
BaP
#34-HFO
#91-GTLb
#92-GTLg
#97-DAE
#98-DAE
#99-DAE
#171-GO
#172-GO
#175-VTO
#185-RAE
#186-RAE

Figure 2 Effects of the DMSO-extracts of PS and GTL products in the selected CALUX reporter gene assays: (A) U2OS AR agonist assay; (B) U2OS AR antagonist assay; (C) U2OS ERα agonist assay; (D) U2OS ERα antagonist assay; (E) U2OS PR agonist assay; (F) U2OS PR antagonist assay; (G) U2OS TRβ agonist assay; (H) U2OS TRβ antagonist assay; (I) H4IIE AhR agonist assay. Data are presented as a percentage of luciferase induction, relative to the maximum-fold luciferase induction by the reference standard agonist/antagonist compound of the corresponding receptor: DHT (AR agonist assay); Flutamide (AR antagonist assay); E2 (ERα agonist assay); Fulvestrant (ERα antagonist assay); PGT (PR agonist assay); RU468 (PR antagonist assay); T3 (TRβ agonist assay); 1-850 (TRβ antagonist assay); BaP (AhR agonist assay). For agonist assays, the maximum-fold luciferase induction of DHT/E2/PGT/T3/BaP was set at 100%, where for the antagonist studies, the fold induction of luciferase at the EC50 concentration of DHT/E2/PGT/T3 was set at 50%. Results represent data from at least 4 independent experiments performed in triplicate and are presented as mean ± SEM.

Abbreviations. AR, androgen receptor; ERα, estrogen receptor alpha; PR, progesterone receptor; TRβ, thyroid receptor beta; AhR, aryl hydrocarbon receptor; DHT, dihydrotestosterone; E2, estradiol; PGT, progesterone; RU468, mifepristone; T3, triiodothyronine; 1-850, thyroid hormone receptor antagonist; BaP, benzo[a]pyrene; HFO, heavy fuel oil; GTLb, gas-to-liquid base oil; GTLg, gas-to-liquid gas oil; DAE, distillate aromatic extract; GO, gas oil; VTO, vacuum tower overhead oil; RAE, residual aromatic extract.

3.4 Effects of PS and GTL products on the TRβ

The ability of the extracts of the test compounds to induce or block the activation of the TRβ was investigated using the U2OS TRβ assay. The results (Figure 2H) indicated that sample #034-HFO and 2 of the DAE samples (#098 and #099) acted as (partial) antagonists of the TRβ, with a reduction of the percentage luminescence activity to 23%, 22%, and 20%, respectively, out of 50% luminescence activity of the EC50 concentration of T3. None of the other extracts, including those of the GTLs, showed either agonist or antagonist effects in the U2OS TRβ assay, indicating their inability to interact with this receptor in vitro (Figure 2G and 2H).

Table 1 Summary of endocrine and dioxin-like activities of the DMSO-extracts of 9 PS and 2 GTL products, derived from their concentration-response curves in different CALUX reporter gene assays in this study.

Substance/compound	AR antagonist assay		ERα antagonist assay		PR antagonist assay		TRβ antagonist assay		ERα agonist assay		AhR agonist assay	
	IC_{50} (µg/ml)	Min. - Max. response (% mean±SEM)	IC_{25} (µg/ml)	Min. - Max. response (% mean±SEM)	IC_{50} (µg/ml)	Min. - Max. response (% mean±SEM)	IC_{50} (µg/ml)	Min. - Max. response (% mean±SEM)	EC_{25} (µg/ml)	Min. - Max. response (% mean±SEM)	EC_{50} (µg/ml)	Min. - Max. response (% mean±SEM)
Reference-standard compound of the respective receptor												
Flutamide	0.32	13 ± 0.9 – 50 ± 2.2	-	-	-	-	-	-	-	-	-	-
Fulvestrant	-	-	1.1×10^{-5}	3 ± 0.2 – 50 ± 0.8	-	-	-	-	-	-	-	-
Mifepristone (RU468)	-	-	-	-	1.8×10^{-5}	5 ± 0.9 – 53 ± 0.6	-	-	-	-	-	-
Thyroid receptor antagonist (1-850)	-	-	-	-	-	-	1.9	18 ± 0.5 – 50 ± 2.2	-	-	-	-
Estradiol (E2)	-	-	-	-	-	-	-	-	5.9×10^{-6}	2 ± 0.5 – 100 ± 0.0	-	-
Benzo[a]pyrene (BaP)	-	-	-	-	-	-	-	-	-	-	3.1×10^{-3}	17 ± 0.8 – 100.0 ± 0.0
Petroleum substances samples												
#34-HFO	0.6	19 ± 2.4 – 56 ± 2.5	9.7	27 ± 0 .3 – 65 ± 1.7	1.1	6 ± 1.3 – 49 ± 0.9	10.6	23 ± 1.1 – 56 ± 2.4	n.a	2 ± 0.2 – 6 ± 0.5	3.7×10^{-3}	21 ± 0.6 – 122 ± 4.5
#97-DAE	70.6	17 ± 1.5 – 57 ± 3.9	n.a	49 ± 1.3 – 65 ± 3.3	32.0	6 ± 0.8 – 54 ± 3.6	n.a	40 ± 1.1 – 60 ± 0.7	n.a	1 ± 0.2 – 6 ± 0.8	3.3×10^{-2}	14 ± 1.1 – 116 ± 4.4
#98-DAE	5.7	10 ± 0.8 – 54 ± 2.0	40.7	24 ± 1.9 – 67 ± 2.1	9.9	4 ± 0.7 – 50 ± 1.0	112.2	22 ± 1.7 – 56 ± 1.7	n.a	2 ± 0.2 – 15 ± 2.0	1.2×10^{-2}	15 ± 1.6 – 111 ± 2.4
#99-DAE	4.8	8 ± 1.0 – 50 ± 1.3	54.1	29 ± 0.9 – 74 ± 1.9	9.8	4 ± 0.9 – 54 ± 1.1	99.4	20 ± 0.8 – 57 ± 1.9	n.a	3 ± 0.6 – 20 ± 1.8	1.2×10^{-2}	17 ± 1.7 – 119 ± 2.9
#171-GO	2.3	16 ± 1.8 – 56 ± 1.5	n.a	50 ± 0.2 – 78 ± 1.2	12.5	8 ± 1.1 – 52 ± 1.1	n.a	44 ± 1.3 – 58 ± 0.9	118.6	2 ± 0.3 – 26 ± 1.9	8.4×10^{-2}	13 ± 0.6 – 102 ± 2.3
#172-GO	1.6	11 ± 1.5 – 55 ± 1.4	n.a	47 ± 2.0 – 80 ± 2.8	13.4	6 ± 0.9 – 51 ± 0.8	n.a	49 ± 1.6 – 62 ± 0.8	107.9	2 ± 0.2 – 28 ± 1.4	3.5×10^{-1}	13 ± 0.6 – 99 ± 4.0
#175-VTO	1.7	12 ± 1.9 – 51 ± 0.4	n.a	50 ± 1.2 – 86 ± 3.3	19.7	8 ± 1.0 – 49 ± 2.0	n.a	47 ± 0.5 – 60 ± 1.2	43.5	2 ± 0.2 – 48 ± 5.4	4.0×10^{-1}	15 ± 1.0 – 105 ± 3.4
#185-RAE	n.a	49 ± 1.8 – 59 ± 2.5	n.a	50 ± 0.2 – 79 ± 1.8	>250	29 ± 1.8 – 51 ± 0.4	n.a	48 ± 1.0 – 61 ± 1.1	n.a	1 ± 0.2 – 3 ± 0.6	4.0×10^{-1}	12 ± 1.3 – 81 ± 2.2
#186-RAE	n.a	37 ± 1.5 – 59 ± 2.2	n.a	47 ± 1.8 – 78 ± 1.5	231.8	18 ± 1.0 – 49 ± 1.0	n.a	50 ± 1.3 – 65 ± 1.6	n.a	1 ± 0.3 – 3 ± 0.8	1.3×10^{-1}	12 ± 0.3 – 101 ± 4.0
Gas-to-liquid products												
#91-GTLb	n.a	50 ± 2.6 – 57 ± 2.1	n.a	52 ± 1.8 – 59 ± 0.6	n.a	45 ± 1.2 – 50 ± 0.8	n.a	50 ± 0.8 – 58 ± 2.3	n.a	1 ± 0.1 – 1 ± 0.2	n.a	12 ± 1.1 – 13 ± 1.3
#92-GTLg	n.a	48 ± 3.5 – 56 ± 3.0	n.a	49 ± 2.3 – 52 ± 2.3	n.a	48 ± 1.8 – 55 ± 1.0	n.a	49 ± 1.2 – 55 ± 2.1	n.a	1 ± 0.1 – 1 ± 0.1	n.a	12 ± 1.0 – 15 ± 1.2

Note. n.a., not available/calculable, as their percentage of luciferase activity was either less than 25% (in the agonist assay) compared with the reference-standard agonist compound: E2 (for U2OS ERα agonist assay) or not lower than 50% (in the antagonist assay) compared with the effect induced by reference-standard antagonist compounds: Flutamide/Fulvestrant/1-850 (for U2OS AR; ERα; and TRβ antagonist assays); (-), not tested. ***Abbreviations.*** HFO, heavy fuel oil; DAE, distillate aromatic extract; GO, gas oil; VTO, vacuum tower overhead oil; RAE, residual aromatic extract; GTLb, gas-to-liquid base oil; GTLg, gas-to-liquid gas oil.

3.5 Effects of PS and GTL products on the AhR

The AhR CALUX assay was conducted to assess the AhR-mediated activity of the DMSO-extracts of PS and GTL products. This in vitro method is commonly used to evaluate the AhR activity of chemicals, including PAHs, as well as crude and refined petroleum products (Aarts et al., 1995; Pieterse et al., 2013; Vrabie et al. 2009). Both BaP and TCDD are widely used as reference standard compound for the AhR CALUX assay, and in this case BaP was chosen over TCDD because BaP is one of the PAH constituents present in the DMSO-extracts of PS samples under study.

Six hours of cell exposure to the DMSO-extracts of these substances resulted in agonist responses by all test compounds, except for the GTL extracts that did not show any activity in the AhR CALUX assay (Figure 2I). The highest luminescence responses, relative to the maximum BaP induction, were ranging from approximately 81% (sample #185-RAE) to 122% (sample #034-HFO). Based on the EC50s (Table 1), the DMSO extract of HFO was the strongest activator/ligand of the AhR, followed by those of the DAEs, GOs, and VTO, and the RAEs. Given that the DMSO-extract of all PS showed clear AhR agonist activity, they were not tested for AhR antagonism.

3.6 Relation of in vitro agonist/antagonist potencies to specific PAH content

To assess whether there is any association between the agonist/antagonist effects of the substances and their PAH content, IC50/EC50 values from the CALUX assays were compared with the amount of PAHs present in the extract of each PS. Test compounds that showed no substantial activity, for which no IC50/EC50 values could be defined, such as the GTL extracts and sample #185-RAE in the U2OS AR antagonist assay, were not included in this comparison. Likewise, no correlation analysis was applied to the results from the U2OS ERα and U2OS TRβ assays, because too few test compounds showed either agonist or antagonist effects to perform a reliable analysis. For three CALUX assays where substantial agonist or antagonist effects were observed (i.e. the U2OS AR antagonist assay (Figure 2B), the U2OS PR antagonist assay (Figure 2F) and the AhR agonist assay (Figure 2I), data correlations were made with the amount of PAHs (ranging from 2- to 7-ring) present in PS, in total giving 15 combinations/assay for which the correlation between PAH content and IC50/EC50 values were determined. The results for all these

comparisons are provided in the Supplementary material. The goodness-of-fit of regression analysis is expressed in the R^2 value and the most relevant correlations with the highest R^2 are presented in Figure 3. Figure 3 reveals that good correlations were obtained, especially between the IC50s of the U2OS AR antagonist assay and the 2- to 3-ring PAH content present in DMSO-extracts of the PS (R^2: 0.98; Figure 3A). Further, a good correlation was seen between the IC50s of the U2OS PR antagonist assay and the 2- to 5-ring PAH content, generating an R^2 of 0.97 (Figure 3B). Finally, the observed EC50s for agonist effects in the AhR CALUX assay were best correlated to the 3- to 7-ring PAH content in the corresponding samples (R^2: 0.90; Figure 3C).

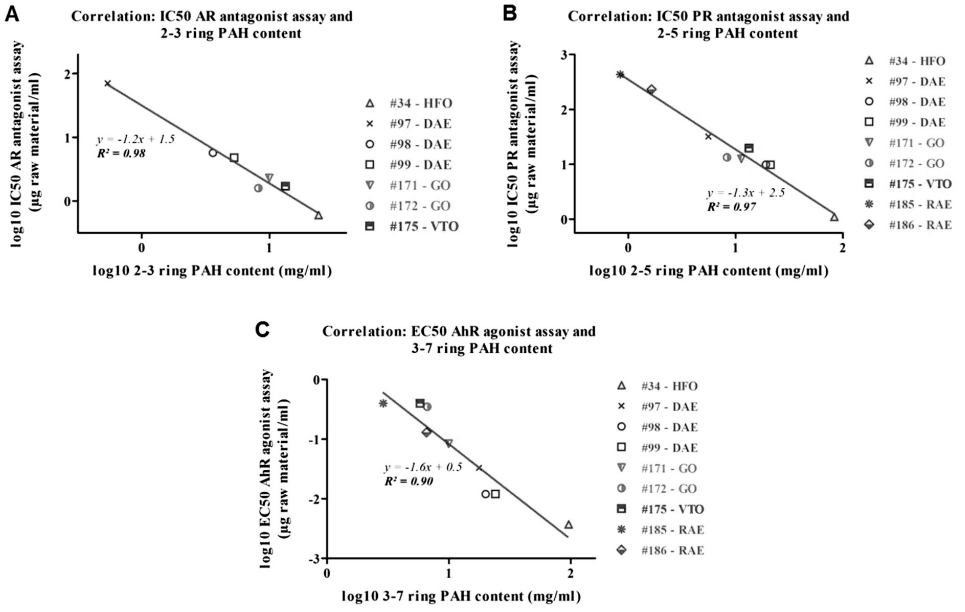

Figure 3 Correlation between in vitro agonist/antagonist potencies (EC50s/IC50s), obtained from (A) U2OS AR antagonist assay; (B) U2OS PR antagonist assay; (C) H4IIE AhR agonist assay; and specific PAH content in PS samples.

Abbreviations. HFO, heavy fuel oil; DAE, distillate aromatic extract; GO, gas oil; VTO, vacuum tower overhead oil; RAE, residual aromatic extract.

3.7 Relation of in vitro developmental toxicity of PS extracts to their steroid and dioxin-like activity

The results of the CALUX assays were also compared with previous results obtained in the EST, (Kamelia et al., 2017), an in vitro assay for developmental toxicity. Also for this comparison, only results from the U2OS AR antagonist, U2OS PR antagonist, and AhR agonist CALUX assays were included since most of the extracts were only active in these assays and showed no or marginal activity in the other CALUX assays. The best correlation, among the 3 comparisons (Figure 4), was obtained between the EC50s from the AhR agonist activity and the BMCd50s EST, with an R^2 of 0.80 (Figure 4C). In contrast, there were no meaningful correlations found when plotting the BMCd50s from the EST against the IC50s from either the AR (R^2: 0.02; Figure 4A) or the PR (R^2: 0.38; Figure 4B) antagonist assay.

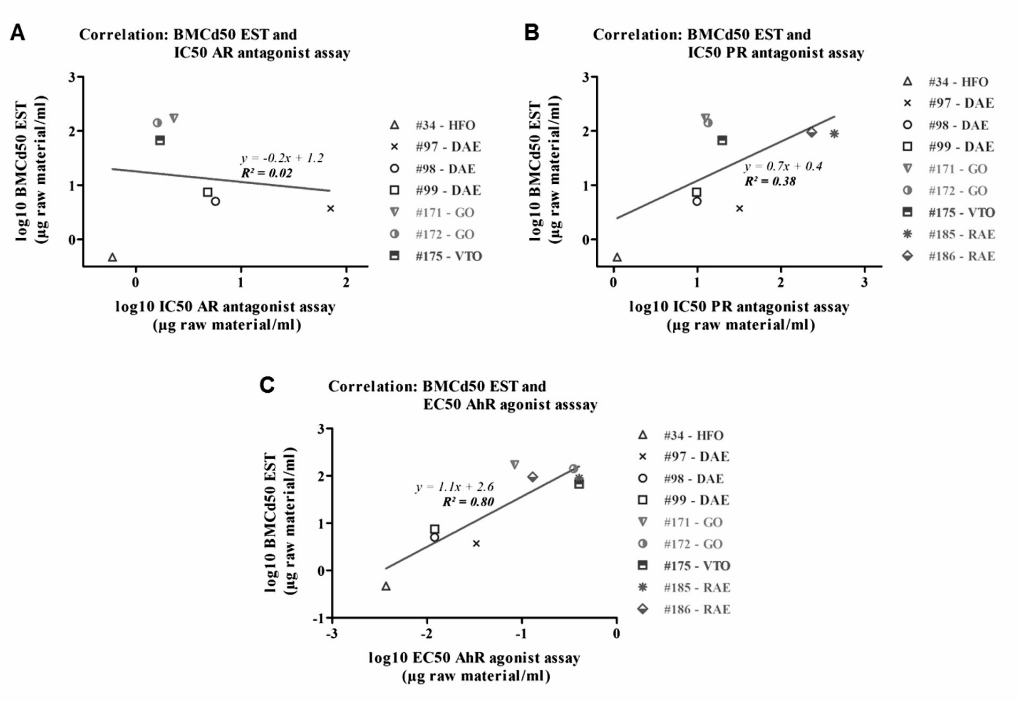

Figure 4 Correlation between in vitro developmental toxicity of PS in the EST (expressed as BMCd50s), obtained from our previous study (Kamelia et al., 2017), and in vitro agonist/antagonist potencies (expressed as EC50s/IC50s), obtained from (A) U2OS AR antagonist assay; (B) U2OS PR antagonist assay; (C) H4IIE AhR agonist assay of this study.

3.8 Class signatures and luciferase expression profiles in CALUX assays

To integrate the results obtained in the various assays, a PCA analysis was performed based on the average luciferase induction factor at the highest tested concentration of the extracts of the PS and the GTL products. This PCA analysis revealed that 91.44% of all variance of responses in the 6 CALUX assays could be explained using only 2 PCs (PC1 and PC2) (Figure 5). PC1 accounts for the largest possible variance in the dataset (66.08%), where PC2 captures as much as the remaining variation as possible (25.36%). PC1 and PC2 were selected, over other PCs, as together they captured most variation of effects (91.44%), among the data. The combination of other PCs, e.g. PC1 and PC3 (73.24%) or PC1 and PC4 (67.27%), projected less variation and had a minor contribution to the total variances of luciferase activity induced by the test compounds.

As shown in the biplot matrix of the PCA (Figure 5), sample #034-HFO that contains the highest amount of PAHs (48% wt.) was fully separated from all other test compounds. The DAE samples with similar PAH content (#098 and #099) were clustered together while the other DAE, sample #097, with distinct PAH constituents (relatively higher level of high-molecular weight PAHs than the other DAE) was located in the middle of 3 different PS classes: GOs, RAEs, and DAEs. Moreover, the GO and RAE extracts were also grouped together, and so did the GTLs. The PCA clearly discriminates the PS in line with the differences in their PAH composition. Thus, the PCA indicates that the battery of CALUX assays is able to differentiate between the PS in a way that is in line with the differences in their PAH composition.

PC1 of the PCA biplot matrix contains a positive contribution of the results from several antagonist assays (U2OS AR, ERα, PR, and TRβ antagonist assays), while results from agonist assays (U2OS ERα and AhR agonist assays) give a negative contribution to this PC. In the PC2, positive contributions mostly come from the results of the U2OS ERα antagonist, U2OS TRβ antagonist, U2OS ERα agonist, and H4IIE AhR agonist assays. Correlation between active variables in the PCA can be interpreted in terms of the vector angle; a narrow angle reflects positively linked variables while wide angles depict variables that are unrelated to each other. For example, antagonist activities in the AR assay are positively correlated with the activities in the PR antagonist assay, and so are the antagonist effects in the ERα and TRβ and the agonist activities in the AhR and ERα. A

more detailed description regarding correlations between variables and factors in this PCA is provided in the Supplementary material.

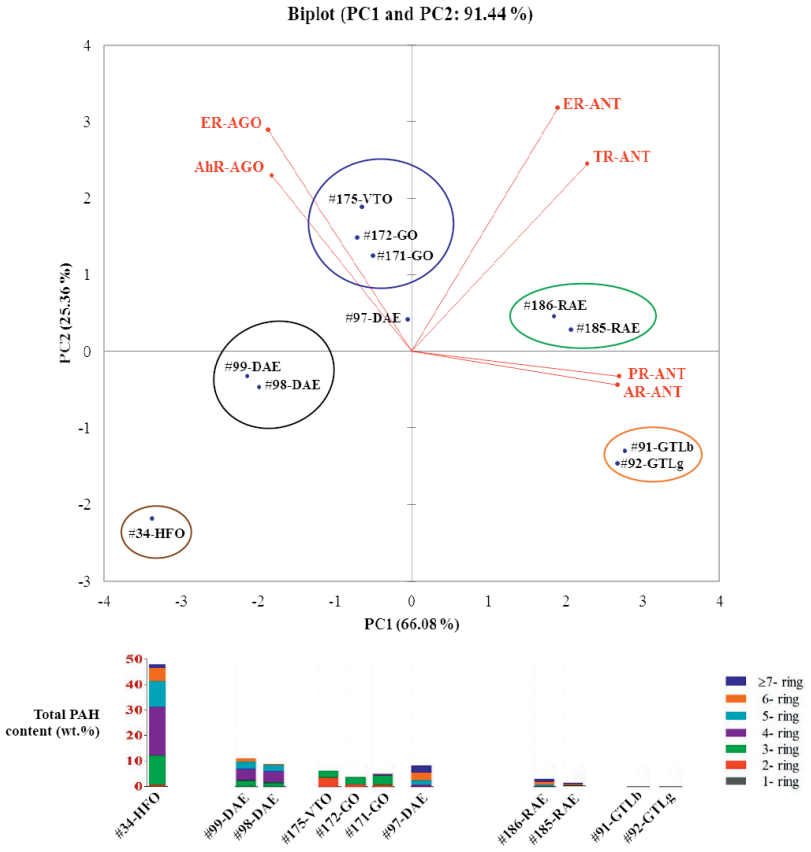

Figure 5 PCA based on luciferase expression profiles induced by the DMSO-extracts of PS and GTL products in 6 different CALUX assays: AhR; ERα agonist assays and ERα; TRβ; PR; AR antagonist assays. The average luciferase induction factor at the highest tested concentration of each test compound, from at least 4 independent experiments, is used as data input for the PCA. An overview of total PAH content (wt.%) and ARC profiles of every test compound are also presented below the PCA biplot.

Abbreviations. AhR-AGO, AhR agonist assay; ERα-AGO, ERα agonist assay; AR-ANT, AR antagonist assay; PR-ANT, PR antagonist assay; ERα-ANT, ERα antagonist assay; TRβ-ANT, TRβ antagonist assay; PC: principle component; HFO, heavy fuel oil; GTLb, gas-to-liquid base oil; GTLg, gas-to-liquid gas oil; DAE, distillate aromatic extract; GO, gas oil; VTO, vacuum tower overhead oil; RAE, residual aromatic extract.

To further evaluate the class signatures of the different samples in the battery of CALUX assays, hierarchic clustering was performed. This resulted in a heatmap that visualizes the luciferase expression profile of each test compound's extract in the aforementioned 6 CALUX assays (Figure 6A). A qualitative overview of the results from our previous study (Kamelia et al., 2017) in which the same test samples were investigated in the EST was included in the figure to show whether these substances tested positive or negative for in vitro developmental toxicity. Information on the ARC profiles and PAH content of each sample was also included in Figure 6A. The heatmap reveals that 2 main clusters of test compounds can be distinguished on the basis of their luciferase expression pattern in the battery of CALUX assays. The GTL extracts are grouped in cluster 1 (green cluster) and all the extracts of the PS are clustered in cluster 2 (red cluster), as displayed in Figure 6A. Within cluster 2, test samples that belong to the same class of substances are clustered together: GOs, the 2 most similar PAH profiles of the 3 DAEs (#098 and #099), and the RAEs.

Further, VTO appears to be in the same cluster with the GOs, as a result of having similar PAH profiles and of inducing similar effects in the CALUX assays. Thus, also the hierarchic clustering indicates that the battery of CALUX assays is able to differentiate between the PS in a way that is in line with the differences in their PAH composition. Finally, Figure 6B also presents the hierarchic clustering based on the results of only the AhR agonist, ERα agonist, PR antagonist, and ERα antagonist CALUX assays resulting in the same pattern as observed when using data from the whole battery, indicating that the AhR, ERα, and PR drive the clustering.

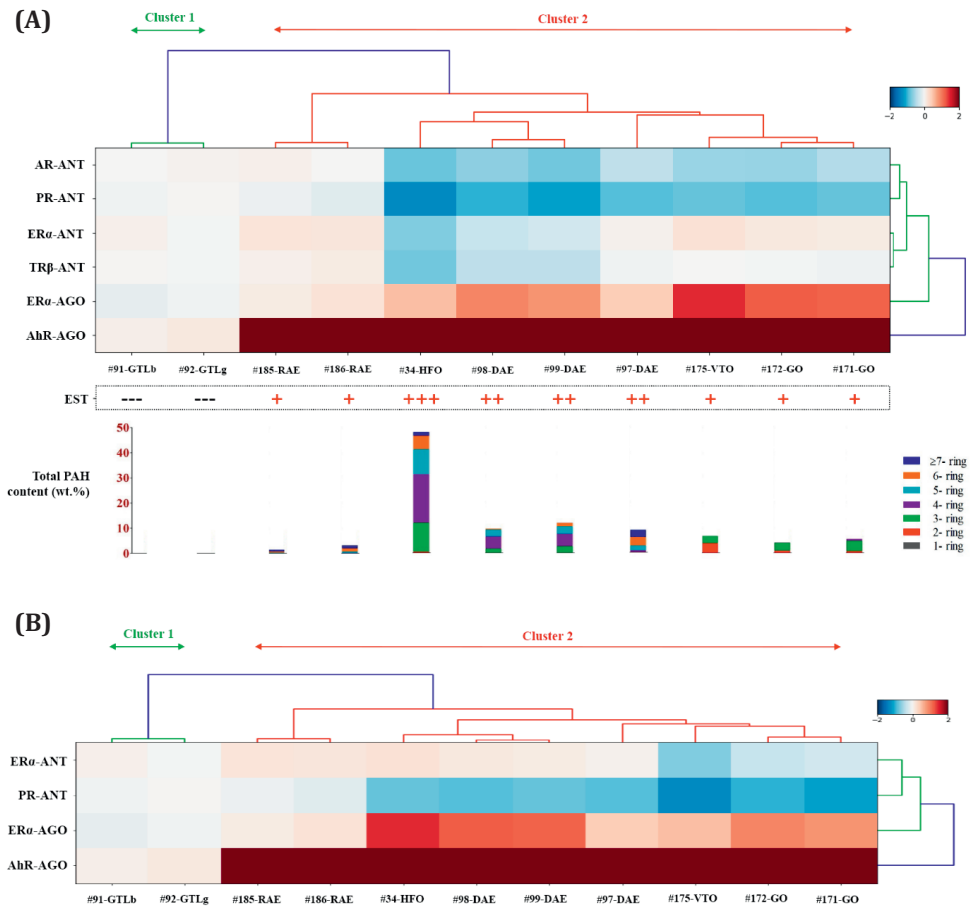

Figure 6 Hierarchic clustering, illustrated by heatmap summarizing luciferase expression profiles of the DMSO-extract of PS and GTL products in (A) 6 different CALUX assays: AhR; ERα agonist assays and ERα; TRβ; PR; AR antagonist assays; (B) 4 different CALUX assays: AhR; ERα agonist assays and ERα; PR antagonist assays. The average luciferase induction factor at the highest tested concentration of each test compound, from at least 4 independent experiments, is used as data input for the development of the heatmap. An overview of results in the EST from our previous study (Kamelia et al., 2017), total PAH content (wt.%), and ARC profiles of every test compound are presented in addition to the data from CALUX assays. Note. Based on the calculated in vitro developmental toxicity potency, expressed as BMCd50s, the EST positive results differ 3 orders of magnitude: +++ (HFO), ++ (DAEs), and + (GOs, VTO, and RAEs), where EST negative result is reflected by the --- symbol (GTL).

4. Discussion and conclusions

To obtain insight in possible modes of action underlying the PDT of PS, the endocrine and dioxin-like activity of DMSO extracts of PS were evaluated using the AR, ERα, PR, TRβ, and AhR CALUX assays. It was evident that the DMSO-extract of each PS shows different responses in the various CALUX assays, suggesting a specific profile of effects exhibited by each of them. This was confirmed by PCA and hierarchic clustering of the combined data, which clustered the samples in line with their chemical characteristics. This reflects that extracts from the same class of PS induced a similar pattern of endocrine and dioxin-like activities. Another prominent finding is that sample #034-HFO, containing the highest amount of PAHs (48% wt.) was the most potent (ant)agonist in all assays where activity was observed. In contrast, extracts of the GTL samples, containing no aromatics, show no activity at all in any of the CALUX assays. The fact that the PCA and hierarchic clustering grouped the samples with comparable PAHs composition closer to one another suggests that the observed effects are related to the types and levels of PAHs present in these substances.

The DMSO-extracts of heavy PS, such as HFO and DAEs, containing a relatively high concentration of high-molecular weight 4- to 7-ring PAHs, acted as antiandrogens, antiprogestogens, antiestrogens, (weak) antithyroids, and strong agonists of the AhR in the investigated assays. DMSO-extracts of light PS, like GOs and VTO, mainly comprising low-molecular weight 2- to 3-ring PAHs, showed a rather weak antiandrogenicity, antiprogesteronicity, estrogenicity, and AhR-mediated activity. In contrast to the other PS samples, the DMSO-extracts of the RAEs showed almost no activity in the CALUX assays apart from a notable activity in the AhR agonist assay. This may partly be explained by the fact that the RAEs investigated in the present study are low in total PAH content (1.5%–3.3% of 5- to 7-ring PAHs), thus, were not able to induce a remarkable activity. Further, the current findings are in line, to some extent, with those of previous studies reported in the literature where antiandrogenicity, (anti)estrogenicity, and dioxin-like activity of some individual PAHs and various petroleum products were reported. To facilitate comparison of our data to those in the literature, Table 2 presents a summary of endocrine and dioxin-like activity of PAHs and PS, as reported in the literature, also indicating where results were in line with the data from the present study (marked with [+] in the last column). In addition to what has been reported before, and because the thyroid hormone

system is strongly involved in developmental processes (Bernasconi et al., 2015), the present study also included the TRβ CALUX assay. However, although disturbance of the thyroid system can be an important mode of action in developmental toxicity (Haddow et al., 1999; Jomaa et al., 2014), none of the products showed a meaningful interaction with the respective receptor, which is in concordance with results in vivo as reported by Fowles et al. (2016). This may be due to the fact that disturbance of thyroid hormone signaling may occur at levels different from receptor binding and also because the TRs are known to be less promiscuous than other nuclear receptors (Williams and Franklyn, 1994).

One of the interesting findings is that the extracts of 2 of the DAE samples #098 and #099, showed both estrogenicity and antiestrogenicity. This contradictory effect has several possible explanations. First, these samples might belong to the group of so-called mixed-agonists/antagonists and may behave differently depending on the provided conditions, such as the concentration used for cell exposure. It seems that they are able to activate the ERα at low concentrations, and antagonize the corresponding receptor activity at a very high concentration. Second, the observed agonist/antagonist effect may be caused by different PAH constituents present in these 2 substances. For instance, the agonist effects could be promoted by low-molecular weight PAHs whilst the antagonist activity might be induced by high-molecular weight PAHs.

Table 2 Summary of endocrine and dioxin-like activity of PAHs and petroleum products, as reported in the literature.

References	Substances	Receptor and Model/Method	Exposure	Effects	Remarks	Results of the present study are in line with the following published literature?
Vinggaard et al. (2000)	Phenanthrene Anthracene Fluoranthene Chrysene Pyrene Benz[a]anthracene Benzo[a]pyrene 7,12-dimethylbenz[a]anthracene	AR – CHO cells	0 – 10 µM	AR antagonist	Phenanthrene, Anthracene, Pyrene: antagonize the AR activity just at the highest tested concentration.	[+] **Note:** Some individual 3- to 5- ring PAHs induce AR antagonist activity. DMSO-extracts of PS that contain mainly 3-5 rings PAHs: sample #98 and #99, also show AR antagonist effect.
Kizu et al. (2003)	Anthracene Pyrene Chrysene Benzo[k]fluoranthene Benzo[a]pyrene	AR – LNCaP cells	0 - 100 µM	AR antagonist (except for anthracene and pyrene)	Anthracene and Pyrene show no AR-mediated activity.	
Kizu et al. (2000)	C-heavy oil crude extracts	AR - LNCaP cells	0 – 10 µg/ml	AR antagonist	The anti-androgen effect is associated with particular petrogenic PAHs, such as Benzo[a]anthracene, Benzo[k]fluoranthene, and Benzo[a]pyrene, present in this product.	[+] **Note:** All DMSO-extracts of PS of the present study antagonize the AR activity, as shown in the U2OS AR antagonist assay (Figure 2B).
Vrabie et al. (2010)	7 refined petroleum products: gasoline, kerosene, distillate marine grade A, engine oil, bilge oil, bunker oils. 4 crude oils: Arabian, Romanian, Oseberg, Hollmix crude oil.	AR – YAS (yeast androgen screen) assay	0 – 200 mg/L	AR antagonist	Heavy petroleum products: bilge oil, distillate marine grade A oil, and bunker oils; also Romanian crude oil show anti-androgenic effects while light petroleum products: kerosene and gasoline, do not.	
Charles et al. (2000)	Benzo[a]pyrene (and its hydroxy metabolites)	ERα – MCF7 cells	0 – 10 µM	ERα agonist	Hydroxy metabolites of BaP, including 3OH-BaP, 9OH-BaP, and 9,10OH-BaP, act as ERα agonist.	[+] **Note:** Some of the 3-, 4-, and 5-ring PAHs are estrogenic. DMSO-extracts of PS, which majorly comprise of 3-ring PAHs: GOs and VTO; or a mix of 3-5 ring PAHs: DAEs, demonstrate (weak) estrogen activity in the U2OS ERα agonist assay (Figure 2C).
Kummer et al. (2008)	Fluoranthene Benz[a]anthracene Benzo[a]pyrene Benzo[k]fluoranthene	ERα – rat uterotrophic assay	10 mg/kg bw/day	ERα agonist (except for Benzo[k]fluoranthene)	Benzo[k]fluoranthene shows no ERα-mediated activity.	
van Lipzig et al. (2005)	Chrysene (and its hydroxy metabolites) Benzo[a]pyrene (and its hydroxy metabolites)	ERα – T47D cells	0 – 250 µM	ERα agonist	The estrogenic effects are ER-mediated and mainly induced by their hydroxy metabolites.	

Vrabie et al. (2011)	7 refined petroleum products: gasoline, kerosene, distillate marine grade A, engine oil, bilge oil, bunker oils. 4 crude oils: Arabian, Romanian, Oseberg, Hollmix crude oil.	ERα and ERβ – U2OS cells	0 – 100 mg/L	ERα and ERβ agonist	All oils, except two refined (gasoline and kerosene) and one crude oil (Hollmix), induce estrogenic responses.	[+] **Note:** In line with our findings, DMSO-extracts of light petroleum streams, such as GOs and VTO, show weak estrogenicity, where DMSO-extracts of heavy petroleum products show anti-estrogen activity.
Kizu et al. (1999)	Nakhodka heavy oil C-heavy oil	ER – MCF7 cells	0 – 10 µg/ml	ER antagonist	ER-mediated antagonist activity.	[+] **Note:** DMSO-extracts of heavy PS, including sample #34-HFO, #98-DAE, #99-DAE, induce anti-estrogenic effects in the U2OS ERα antagonist assay (Figure 2D).
Arcaro et al. (2001)	Clarified slurry oil Belridge heavy crude oil Lost Hills light crude oil	ER – MCF7 cells	0 – 10 mg/L	ER antagonist	The anti-estrogenic potency increases with increasing concentration of polycyclic aromatic compounds (PACs).	
Ssempebwa et al. (2004)	Waste crankcase oil	ER – MCF7 cells	0 – 25 mg/L	ER antagonist	The ER antagonist effect is mainly driven by PAHs constituent present in this product.	
Machala et al. (2001)	30 individual PAHs (3-6 ring PAHs)	AhR – H4IIE cells	0 – 10 µM	AhR agonist	4-7 ring PAHs are strong activators of the AhR.	[+] **Note:** 3- to 7- ring PAHs are able to activate the AhR. All DMSO-extracts of PS of the present study, which contain a blend of 3- to 7-ring PAHs show AhR-mediated activity.
Pieterse et al. (2013)	25 individual PAHs (2-5 ring PAHs)	AhR – H4IIE cells	0 – 100 µM	AhR agonist	3-5 ring PAHs efficiently bind and activate the AhR but not 2-ring PAHs, such as naphthalene.	
Vondracek et al. (2017)	19 individual PAHs (3-6 ring PAHs)	AhR – AZ-AhR cells[a]	0 – 10 µM	AhR agonist	High potency AhR ligands by 4-5 ring PAHs; low potency AhR ligands by 3- and 6- ring PAHs.	
Ziccardi et al. (2002)	32 refined petroleum products: gasolines, diesels, jet fuels, lubricating oils, fuel oils, weathered-oil products, commercial oil products	AhR – H1L1.1c2 cells	0 - up to 10^7 pg ligand concentration/ well	AhR agonist	Most petroleum products induce AhR activity, except jet fuels and some fuel oils.	[+] **Note:** In concordance with our results, DMSO-extracts of heavy PS (HFO and DAE) act as a stronger ligand of the AhR than the extracts of light petroleum products (GOs and VTO).
Vrabie et al. (2009)	7 refined petroleum products: gasoline, kerosene, distillate marine grade A, engine oil, bilge oil, bunker oils. 4 crude oils: Arabian, Romanian, Oseberg, Hollmix crude oil.	AhR – H4IIE cells	0 – 100 mg/L	AhR agonist	All oils induced AhR-mediated activity. Crude oils is a stronger AhR activator than the refined petroleum products.	

[a]*The AZ-AhR cells used in this study are based on human hepatocellular carcinoma (HepG2) cells. The AhR-inducing potencies differ from all other data based on the rat H4IIE cellular model.* ***Note.*** AR: androgen receptor; ERα: estrogen receptor alpha; AhR: aryl hydrocarbon receptor; CHO: chinese hamster ovary cells; LNCaP: human prostate carcinoma cells; MCF-7: human breast cancer cells; T47D: human mammary gland cells; U2OS: human osteosarcoma cells; H4IIE: rat hepatoma cells; AZ-AhR: human hepatocellular carcinoma cells HepG2; H1L1.1c2: mouse hepatoma cells.

This study evaluates the possible estrogenic activity of DMSO-extracts of PS and GTL in the U2OS ERα assay, and not in the U2OS ERβ assay. Vrabie et al. (2011) reported a greater effect on ERβ than on ERα-mediated gene expression in the U2OS ERβ and ERα assays, respectively, upon exposure to increasing concentrations of some crude and refined oil products. However, a plot of the maximum response in the U2OS ERα assay versus that in the ERβ assay as reported by Vrabie et al. (2011) reveals that the in vitro ERα and in vitro ERβ-mediated level of gene expression correlate quite well (R^2: 0.82, data not shown). Nonetheless, given the relative tissue distribution of ERα and ERβ, with ERα being more predominant in tissues relevant in development (Brandenberger et al., 1997; Lemmen et al., 1999), as well as the fact that ERα was reported to play a dominant role in mediating reproductive or developmental-related effects (Bondensson et al., 2015; Couse and Korach, 2004), it was concluded that testing the DMSO-extracts of PS and GTL in the U2OS ERα would be most relevant for the battery of tests for an alternative testing strategy for developmental toxicity. Furthermore, the correlation between the ERα and ERβ response indicates that the use of both assays in such a battery may not be needed.

From PCA and hierarchic clustering, it appears that sample #097-DAE induces a different pattern of in vitro potencies compared with other samples that belong to the same class of PS. The most likely explanation for this observation is the large difference in PAH content of sample #097 in comparison to the other 2 PS in this class, #098-DAE and #099-DAE, which is reflected by the observed differences in steroid and dioxin-like activity. The content and concentration of PAHs in each PS may vary, even for samples possessing the same CAS number, as the chemical composition is mainly dependent on the source of crude oil and the processing condition to create the stream (Speight, 2006). Hence, the type, structure, and concentration of particular PAH constituents in these PS extracts play an essential role in determining their steroid and dioxin-like activity.

Of all assays, especially the agonist activity in the AhR CALUX assay correlated well with the in vitro PDT of the same substances as quantified previously in the EST (Kamelia et al., 2017). The potency from both in vitro assays is also proportional to their 3- to 7-ring PAH content (EST; R^2: 0.81 and AhR CALUX assay; R^2: 0.91). This means that the relative PDT and dioxin-like activity of the extracts of the present PS may be induced by the same PAH constituents belonging to the class of 3- to 7-ring PAHs. The AhR CALUX assay was also one of the most important parameters driving the hierarchic clustering and PCA. These

findings may imply that knowing the dioxin-like activity of PS may help to predict their relative PDT potencies. Beyond that, the AhR activation could be one of the possible modes of action underlying PDT of some PS. AhR-mediated developmental toxicity has also been reported in several studies where developmental effects were witnessed upon exposure to some individual PAHs and a mixture of PAHs in the zebrafish model (Billiard et al., 2006; Goodale et al., 2013; Wincent et al., 2015). Besides the selected transcription factors, namely AR, ERα, PR, TRβ, and AhR, it has been suggested that PAHs can also interact with other nuclear receptors, such as the retinoic acid receptor (RAR; Benisek et al., 2011) and peroxisome proliferator-activated receptors (PPAR; Kim et al., 2005). The developmental effects induced by some PAH-containing PS are mainly associated with increased incidence of resorptions (prenatal loss) and decreased fetal body weight, and not so much with malformations of the fetuses (ARCO, 1993; Feuston et al., 1989, 1994, 1996; Feuston and Mackerer, 1996; Hoberman et al., 1995; Mackerer et al., 2003). It is known that in developmental toxicity, retinoic acid pathways play a major role in causing malformations (Lammer et al., 1985). Hence, it would be interesting to assess the role of the other receptors, such as RAR and PPAR, in developmental toxicity induced by some PS although these cannot be presumed upfront to be involved.

In recent years, the use of a battery of in vitro assays to study the mechanism of toxicity (Piersma et al., 2013), in our case PDT, is emerging. To our knowledge, we are the first who combined the aforementioned CALUX assays, in a panel, to study the possible underlying mechanisms of PDT by PS. The reason for choosing only the AR, ERα, PR, TRβ, and AhR, is that these receptors are pivotal in the most frequently affected biological pathways in relation with the developmental toxicity induced by this group of substances. The results of the present study indicate that for the PS, of all CALUX assays, the AhR CALUX assay would be the most suitable assay to be included in an in vitro test battery since it gives the best correlation with the EST and dominates the grouping of the different PS in line with their chemical class. This AhR CALUX assay should not be used as a stand-alone, but rather be part of an alternative in vitro test battery that we are developing to study the PDT potency (and modes of action) of PS.

Altogether, DMSO-extracts of 9 PS evaluated in this study show diverse in vitro endocrine and dioxin-like activities in the tested assays and their in vitro potencies could be associated with the quantity and type of their PAH content. The DMSO extracts of the GTL

samples, which contain no aromatics, are unable to activate any of the selected receptors, which strengthen the hypothesis that PAHs are causing the observed effects. It would be of interest for future work to evaluate the activity of individual PAHs present as major constituents in the DMSO-extracts of PS samples, in both the reporter gene assays of this study as well as in in vitro developmental toxicity assays such as the ES-D3 differentiation assay of the EST, as shown before to detect the developmental toxicity of the DMSO-extracts of PS samples (Kamelia et al., 2017). Obviously this has to await until further chemical characterization of the complex PAH mixtures in these substances to identify their major PAH constituents. Of all CALUX assays applied, the AhR assay appears to be the most useful for determining receptor-mediated activities of PS (UVCBs) that are relevant for developmental toxicity. The question may arise on the relevance of the current results on the in vitro endocrine and dioxin-like activity of PS for the in vivo situation. The CALUX assays are merely in vitro screening assays that provide information on receptor (in)activation but do not necessarily reflect what happens in vivo since physiological feedback mechanisms and metabolism are lacking in these in vitro systems. However, the results of especially the AhR CALUX assay correlated with the results from the in vitro EST for developmental toxicity, and the data in the EST were previously shown to correlate with available in vivo data on PDT of the selected petroleum samples (R^2: 0.97; Kamelia et al., 2017). This supports the hypothesis that especially the dioxin-like activity observed with the DMSO-extracts of some PS may play a role in the actual in vivo outcome with regard to the PDT potency. In future research, models including the zebrafish embryo test, *Caenorhabditis elegans*, and toxicogenomics approaches will be investigated for their potential integration in a strategy to further unravel the association between PAHs in PS and their PDT.

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Supplementary data

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Chapter 4

In vitro prenatal developmental toxicity induced by some petroleum substances is mediated by their 3- to 7-ring PAH constituent with a potential role for the aryl hydrocarbon receptor (AhR)

Lenny Kamelia, Laura de Haan, Hans B. Ketelslegers, Ivonne. M.C.M. Rietjens, Peter J. Boogaard

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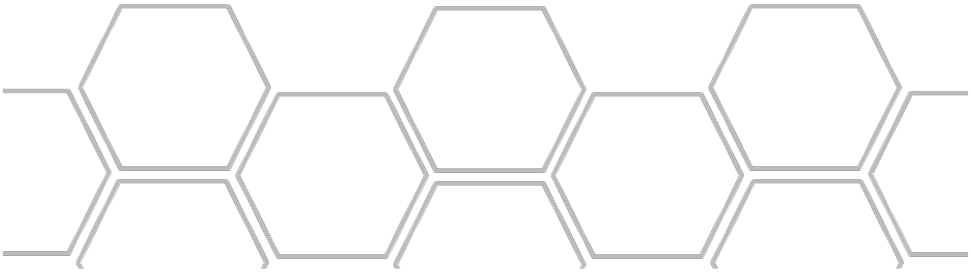


Abstract

To test the hypothesis that 3-7 ring polycyclic aromatic hydrocarbons (PAHs) are responsible for the prenatal developmental toxicity (PDT) as observed with some petroleum substances (PS), the present study evaluates the PDT potency of DMSO-extracts of 7 heavy fuel oils (HFO), varying in their PAHs content, and 1 highly refined base oil (HRBO), containing no aromatics, in the embryonic stem cell test (EST). All DMSO-extracts of HFO inhibit ES-D3 cell differentiation in a concentration-dependent manner and their potency is proportional to the amount of 3-7 ring PAHs they contain. The DMSO-extracts of HFOs also show aryl hydrocarbon (AhR)-mediated activities, as tested in the AhR-CALUX assay. Contrarily, the HRBO-extract tested negative in both assays. Co-exposure of ES-D3 cells with selected DMSO-extracts of PS and the AhR-antagonist trimethoxyflavone, successfully counteracted the PS-induced inhibition of ES-D3 cell differentiation, confirming the role of the AhR in mediating the observed PDT of PS extracts in the EST. A good correlation exists when comparing the in-vitro with the in-vivo PDT potencies of the PS under study. Altogether, our findings corroborate the hypothesis that PS-induced PDT is caused by 3-7 ring PAHs present in these substances and that the observed PDT is partially AhR-mediated.

1. Introduction

Since 2007, the REACH (Registration, Evaluation, Authorisation, and Restriction of Chemicals) legislation requires substances that are produced in the European Union (EU) at a volume of ≥100 tonnes/year to be tested for prenatal developmental toxicity (PDT) (ECHA, 2009). This implies that a large number of chemical substances, including complex substances such as all petroleum products, will require PDT testing. Petroleum substances (PS) are complex hydrocarbon substances with unknown or variable composition (UVCBs), comprising at least hundreds to millions of different hydrocarbons per substance, including polycyclic aromatic hydrocarbons (PAHs). Testing for PDT is one of the most animal- and resource-intensive testing endpoints in the field of toxicology (van der Jagt et al., 2004), since ~2500-3000 experimental animals are needed to test just one substance following the current OECD 414 testing guideline (OECD, 2001). Within the circumstance of PS and REACH legislation, PDT testing for these substances (186 currently active registered EINECS numbers, all with a volume >1000 tonnes/year) would require an exceptionally large number of experimental animals and a considerable amount of resources; for the PDT endpoint alone, this means testing in two species per substance, which would lead to the use of thousands of test animals (both rats and rabbits) and between >1M€ testing cost per PS. Such a situation is not desirable for both industry and regulators, as the REACH goals are to ensure a high level of protection for human health and the environment while enhancing competitiveness of the industry as well as enhancing innovation, including the promotion of alternative test methods to reduce animal testing (www.echa.europa.eu). Therefore, both regulators and industry have a vested interest both from animal welfare and financial standpoints (in view of industry competitiveness and innovation) to develop and apply an alternative approach for this endpoint, based on a battery of non-animal based test methods. Such alternative testing strategies may include the embryonic stem cell test (EST), which was recently shown able to detect the PDT potencies of PS (Kamelia et al., 2017). This may assist in the grouping of PS into a limited number of categories and/or in facilitating the selection of worst-case representatives per PS category for in vivo testing to fill remaining data gaps (Kamelia et al., 2017). Such alternative testing strategies will significantly minimize the required animal tests by facilitating read-across from PS for which in vivo PDT data are already available or are being generated under REACH.

To date, there are four in vitro alternative assays that have been scientifically validated for PDT testing, namely the EST (Genschow et al., 2004), the whole embryo culture (WEC; Piersma et al., 2004), the limb bud micromass (MM; Spielmann et al., 2004), and the zebrafish embryotoxicity test (OECD 2011a,b; Busquet et al., 2014). The EST is favoured over the other assays since no animal is involve in the test (animal-free test system). Moreover, a considerable amount of literature has been published in the last decade on the applicability of the EST to predict relative PDT potency of different groups of chemicals, including phenols (Strikwold et al., 2012), glycol ethers (de Jong et al., 2011), azoles (Li et al., 2015; Dimopoulou et al., 2018), retinoids (Louisse et al., 2011), and PS UVCBs (substances of Unknown or Variable composition, Complex reaction products and Biological materials) (Kamelia et al., 2017). This made the EST become one of the most used in vitro alternative assays for investigating the potential adverse effect of chemicals and complex substances on prenatal development.

Available in vivo data on the PDT potency of PS show that some PS, especially those comprising a high concentration of PAHs, induce PDT in experimental animals (Feuston et al., 1989; 1996; 1997; Hoberman et al., 1995) whilst their gas-to-liquid (GTL) analogues, which are synthetic products that are completely devoid of aromatics, do not induce PDT (Boogaard and Roberts, 2015; Boogaard et al., 2017). This evidence suggests that the observed PDT for some PS may be caused by mainly certain types of PAHs present in these products (Murray et al., 2013). Further studies suggested that this PDT may be especially due to the 3- to 7-ring PAHs (Tsitou et al., 2015).

These experimental in vivo data are consistent with our recent in vitro findings (Kamelia et al., 2017), where a series of PS extracts, varying in PAH content, induced in vitro PDT, as quantified in the EST, with this potency being proportional to the amount of 3- to 7-ring PAHs present in these substances. Moreover, activation of the aryl hydrocarbon receptor (AhR), quantified in the so-called AhR CALUX assay, was identified as one of the possible mode-of-actions underlying the PDT of the PS (Kamelia et al., 2018). On the contrary, DMSO-extracts of GTL products (GTL base oil and GTL gas oil) that contain no PAHs were negative in both the EST (Kamelia et al., 2017) and the AhR CALUX assay (Kamelia et al., 2018). Altogether, EST as a stand-alone assay was proven to be able to predict the in vitro PDT potency of the PS extracts, within and across categories, in agreement with their in vivo potency (Kamelia et al., 2017). Yet, it is of importance to strengthen the usefulness of

the EST and also the AhR CALUX assay, as part of the battery of in vitro alternative assays that we are currently developing, for PDT testing of PS. This can be done by testing more PS with a systematic variation in the amounts of PAHs in both the EST and the AhR CALUX assay.

Therefore, the present study aims to further test the hypothesis that PDT by some PS is induced by the group of 3- to 7-ring PAHs present in these substances and that the observed PDT is AhR-mediated. To this purpose, a series of heavy fuel oil (HFO; with systematic variation in 3-7 ring PAH content, from 0.62 to 27 total wt.%; Figure 1), and one highly refined base oil (HRBO; contains no PAHs) were tested in the EST and the AhR CALUX assay. This series of substances was selected because it includes a series with a systematic variation in PAH content, being substances containing a range of 3-7 ring PAHs and extremes regarding their PAH content (with and without PAHs). HFO is one of the heavy petroleum products that may contain high amounts of 3-7 ring PAHs and is known to be able to induce PDT in vivo (Feuston et al., 1989; Hoberman et al., 1995). Whereas, HRBO consists primarily of saturated hydrocarbons, mainly paraffinic or naphthenic (Nash et al., 1996), and was found to be negative in both reproductive and PDT studies (Mobil 1987a,b). The relative in vitro PDT potencies as obtained from the EST were then compared with the AhR-mediated activities as measured in the AhR CALUX assay. Furthermore, the correlation between in vitro PDT potencies in the EST and 3- to 7-ring PAHs was investigated to see the role of this specific group of PAHs in causing the PDT of the samples studied. Finally, some PS and one GTL product were selected and tested in the ES-D3 cell differentiation assay in the absence or presence of the AhR antagonist 6,2′,4′-trimethoxyflavone (TMF), to investigate whether the PS-induced inhibition of ES-D3 cell differentiation was indeed AhR-mediated.

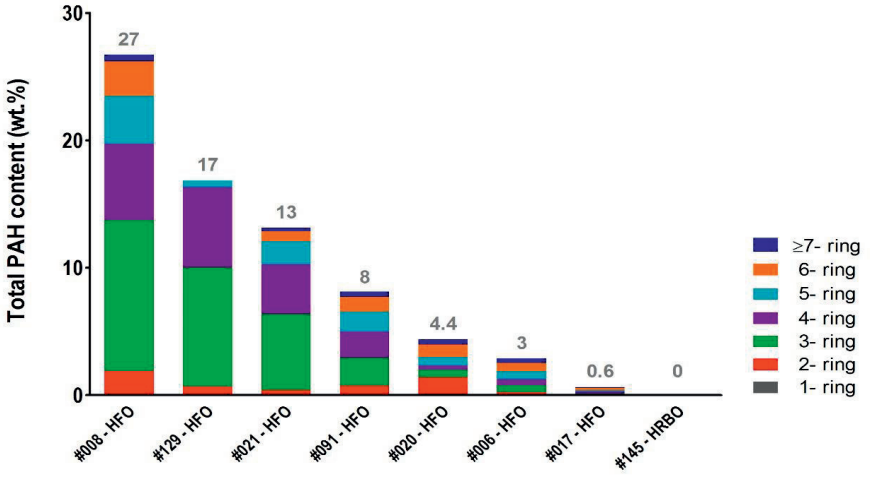


Figure 1 An overview of Aromatic Ring Class (ARC) profiles of the various PS and GTLb tested in the present study. The ARC profile represents the weight percent of the DMSO-soluble 1- to ≥7 aromatic-ring compounds present in each test compound, from the starting raw material of 4.0 gram sample, determined by the Method II chemical characterization procedure described previously by Roy et al., 1988.

Abbreviations. HRBO: highly refined base oil; HFO: heavy fuel oil; DAE: distillate aromatic extract; VTO: vacuum tower overhead oil; GTLb: gas-to-liquid base oil; AhR: aryl hydrocarbon; EST: embryonic stem cell test.

2. Materials and Methods

2.1 Test compounds

5-Fluorouracil (5-FU; CAS no. 51-21-8), 6,2′,4′-trimethoxyflavone (TMF; CAS no. 720675-90-1), benzo[a]pyrene (BaP; CAS no. 50-32-8), and methoxyacetic acid (MAA; CAS no. 625-45-6) were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). All stocks and dilutions were prepared in dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany).

DMSO-extracts of 11 PS (with systematic variation in their PAH content; Figure 1) and 1 GTL base oil (GTLb; containing no PAHs) were tested in the present study. The DMSO-extracts were generated according to the extraction procedure described by Roy et al. (1988). The extraction procedure was done at Port Royal Research laboratory (Hilton Head, South Caroline, USA). The raw material of all PS and GTL products, which were used for the DMSO extraction, were kindly provided by Concawe (Brussels, Belgium) and Shell

International bv (The Hague, the Netherlands), respectively. Of these raw materials, 8 were heavy fuel oils (HFO; CAS no. 92061-97-7, 93821-66-0, 64741-81-7, 68553-00-4, 64741-80-6, 64741-45-3, 64742-78-5, 64741-62-4), 1 was highly refined base oil (HRBO; CAS no. 8042-47-5), 1 was a distillate aromatic extract (DAE; CAS no. 64742-04-7), 1 was a vacuum tower overhead oil (VTO; CAS no. 64741-49-7), and 1 was a GTLb (CAS no. 848301-69-9). An overview of the type and level of PAHs present in the DMSO-extracts of the aforementioned PS and GTL product, grouped by the number of the aromatic rings, is presented in Figure 1.

2.2 Embryonic stem cell test (EST)

DMSO-extracts of the above-mentioned test compounds (7 HFOs and 1 HRBO) were tested in both the ES-D3 cell viability and differentiation assay of the EST. In addition to that, some DMSO-extracts of PS and GTL product were selected and tested in the ES-D3 cell differentiation assay with TMF co-exposure (see section 2.4 for the details), to see whether the observed PDT activity in the EST are indeed AhR-mediated. These selected samples included sample #034-HFO, #097-DAE, #175-VTO, #172-GO, #186-RAE, #145-HRBO, and #091-GTLb; and were selected because together they represent the different categories of PS, namely the HFOs and HRBO of the present study, but also DAE, GO, VTO, and RAE; and the GTL product that we have tested so far in the EST (Kamelia et al., 2017).

2.2.1 ES-D3 cell culture

The pluripotent mouse ES cells line D3 (ES-D3; ATCC® CRL-1934™, Wesel, Germany) was cultured in 25 cm^2 polystyrene cell culture flasks (Greiner Bio-One, Alphen a/d Rijn, the Netherlands), pre-coated with 0.1% gelatine (for at least 1 hour prior to use). ES-D3 cells were grown in HyClone AdvanceSTEMTM Low Osmo Dulbecco's Modified Eagle Medium (DMEM) (GE Healthcare, Eindhoven, the Netherlands), supplemented with 15% fetal bovine serum (FBS) ES cell qualified (ATCC, USA), 2 mM L-glutamine (Invitrogen, Thermo Fisher Scientific, Breda, The Netherlands), 50 U/ml penicillin (Invitrogen), and 50 µg/ml streptomycin (Invitrogen). Cells were incubated at 37°C with 5% CO_2 in a humidified atmosphere and subcultured every 2-3 days using non-enzymatic cell dissociation solution

(Sigma-Aldrich) to detach the cells. Murine Leukemia Inhibiting Factor (mlIF; Sigma-Aldrich) at a concentration of 1000 U/ml was added into the growth medium to maintain the pluripotency of ES-D3 cells.

2.2.2 ES-D3 cell viability assay of the EST

For the ES-D3 cell viability assay, cells were seeded in a volume of 100 µl into each of the 60-inner wells of 96-well plates (Greiner Bio-One) at concentrations of $20x10^4$ cells/ml (1-day exposure) or 10^4 cells/ml (5-days exposure) and incubated for one day to facilitate cell adherence. The 36-outer wells of the same 96-well plate were filled with phosphate buffer saline (PBS; Invitrogen) to limit evaporation from the inner wells. After 24 hours of cell seeding, cells were exposed to increasing concentrations of test compounds in triplicate by adding 100 µl exposure medium to each well. The exposure medium was prepared by mixing the 400 times concentrated stock solutions of the test compounds (dissolved in DMSO) to the growth medium (without mLIF). The final concentration of solvent, DMSO, was kept at 0.25% (v/v). Cells were then incubated for one day or five days at 37°C and 5% CO_2 in a humidified atmosphere. All samples were tested at a range of concentrations up to 500 µg raw material/ml, except for sample #021-HFO and #129-HFO that were tested at concentrations up to 125 µg raw material/ml, and sample #008-HFO that was tested at concentrations up to 50 µg raw material/ml, due to their solubility limitation in the aqueous medium. After the incubation of one or five days, 20 µl of WST-1 reagent (Roche Diagnostics, Mannheim, Germany) was added to each well and cells were incubated for 3 hours at 37°C and 5% CO_2. WST-1, a stable tetrazolium salts, will be cleaved into a soluble formazan by the succinate-tetrazolium reductase enzyme that is only active in viable cells. Hence, the amount of formed formazan directly correlates to the number of metabolically active cells in the culture. The absorbance of the formed formazan was measured at 440 nm (background at 620 nm) using a SpectraMax M2 (Molecular Devices, Sunnyvale, USA). The cell viability of each well was expressed as the percentage cell viability compared to that of the solvent control 0.25% DMSO (which was set at 100%). The wells containing culture medium plus WST-1 reagent in the absence of cells were used as a blank (background control). The percentage viability of the blank was set at 0% since no cells were present. At least three independent experiments were done for ES-D3 cell viability assay of each test compound.

2.2.3 ES-D3 cell differentiation assay of the EST

The ES-D3 cell differentiation assay of the EST was performed as previously described by Kamelia et al. (2017). In brief, the initial step of the differentiation assay is the formation of embryoid bodies (EBs) via hanging drop culture in growth medium without mLIF. At the start of the assay (day 0), droplets of 20 µl of a cell suspension ($3.75x10^4$ cells/ml), with or without test compound, were placed between the well borders on the inner side of the lid of a 96-well plate. All wells of the 96-well plate were filled with 250 µl of PBS to create an optimal humidity and to prevent evaporation of the hanging drops. Sterile caps of Eppendorf tubes were placed in the corner of the plates in order to prevent direct contact of the drops with the plate and the plate was then sealed with Micropore tape (3M, Neuss, Germany). The hanging drop cultures were incubated for three days at 37°C and 5% CO_2. On day 3, the resulting EBs were collected and transferred to 60x15 mm bacteriological petri dishes (Greiner Bio-One) containing 5 ml medium, with or without test compound. Petri dishes were incubated for another two days at 37°C and 5% CO_2 in the presence or absence of the test compounds. On day 5, the EBs were transferred to a 24-well plate (Greiner Bio-One) (1 EB/well), containing 1 ml medium with or without test compound. The EBs in 24-well plates were then incubated for 5 days at 37°C and 5% CO_2. On day 10, the number of wells containing beating cardiomyocytes was determined by visual inspection using a light microscope. The ES-D3 cell differentiation assay was considered valid if the solvent control in each experiment (0.25% DMSO) had at least 21 out of 24 wells that contained beating cardiomyocytes. The inhibition of differentiation by the test compound was presented as the fraction of total EBs plated in the 24-well plate. The final concentration of solvent in the medium was kept at 0.25% (v/v) throughout the 10 days experiment. In addition to solvent control, 0.065 µg/ml 5-fluorouracil (5-FU,Sigma-Aldrich) was included in each experiment as a positive control of the assay. At least three independent experiments were conducted for each of the test compound.

2.3 Aryl hydrocarbon (AhR) CALUX assay

AhR-mediated activity of the DMSO-extracts of 7 HFOs and 1 HRBO was evaluated in the AhR CALUX assay, which was performed essentially as described before by Kamelia et al. (2018). In short, H4IIE.luc cells at a density of $3x10^5$ cells/ml were seeded into the 60-inner

wells of 96-well white plate 24 hours prior to exposure. Afterwards, cells were exposed to increasing concentrations (0.05 – 5 µg raw material/ml) of the HFO and HRBO extracts for 6 hours. Cells were lysed upon exposure and the luciferase induction activity was measured using a luminometer (Glomax-Multi Detection System, Promega, California, USA) after the addition to each well of 100 µl flash mix solution (20 mM tricine, 1.07 mM (MgCO3)4 Mg(OH)2.5H2O, 2.67 mM magnesium sulfate (MgSO4), 0.1 mM ethylenedinitrilotetraacetic acid disodium salt dihydrate (Titriplex III), 2 mM dithiothreitol (DTT), 0.47 mM D-luciferin, and 5 mM adenosine-5-triphosphate). The amount of the produced-luminescence quantifies the AhR-mediated activity induced by the respective test substance. BaP, a strong AhR agonist, was used as the positive control in this assay (range of used concentrations: $1.3x10^{-5}$ - $1.3x10^{-1}$ µg/ml). BaP was chosen over TCDD as the reference-compound of the AhR assay of the present study because BaP is more representative for the PAH present in the PS extracts under study. DMSO was used as solvent control and the final concentration of the DMSO was kept at 0.25% (v/v). At least 3 independent experiments were performed for every test compound in the AhR CALUX assay.

2.4 Co-exposure of cells in the ES-D3 cell differentiation assay of the EST and the AhR CALUX assay to the selected PS extracts and an AhR antagonist

In an attempt to clarify the role of AhR in mediating the PDT of PS in the EST, ES-D3 cells were co-treated with the mixture of the selected PS extract and a potent antagonist of the AhR, 6,2′,4′-trimethoxyflavone (TMF; final concentration 3 µg/ml or 10 µM). The concentration of TMF used in the present study was 10 µM, a concentration shown to display a potent AhR antagonist activity in vitro, as previously reported by Murray et al. (2010). To this purpose, the DMSO-extracts of 6 PS with variable PAH content and belonging to different PS categories: #034-HFO, #097-DAE, #175-VTO, #172-GO, #186-RAE, #145-HRBO (the latter containing no PAHs); and 1 GTL product (containing no PAHs): #091-GTLb, were selected and tested in the ES-D3 cell differentiation assay of the EST with and without TMF co-exposure. These samples were selected as they represent the different categories of PS, namely HFO, DAE, GO, VTO, RAE, and HRBO; and a GTL product that we have tested so far in the EST (Kamelia et al., 2017). The exposure medium for TMF co-exposure was prepared by mixing the PS DMSO-extracts (800 times concentrated stock solution) and TMF (also from 800 times concentrated DMSO-stock solution) in the EST

medium without mLIF. All samples, except for #091-GTLb, were tested at their BMCd80-90 concentrations, obtained from the ES-D3 cell differentiation assay, (#034-HFO: 20; #097-DAE: 100; #175-VTO: 150; #172: 250; #186: 150 µg raw material/ml) because it is known from our previous study (Kamelia et al., 2017) that this concentration is not cytotoxic for the ES-D3 cells but able to inhibit the differentiation of ES-D3 cells into beating cardiomyocytes. BMCdx is defined as the benchmark concentration that corresponds to x% inhibition of ES-D3 cell differentiation into beating cardiomyocytes in the EST, as determined using the BMD software US-EPA version 2.6.1 (see the section 2.4 for the details). The tested concentrations for sample #145-HRBO and #091-GTLb were 250 µg raw material/ml, the highest possible tested concentration based on its solubility. When AhR activation would play a major role in the in vitro PDT, TMF co-exposure is expected to counteract the inhibition on ES-D3 cell differentiation induced by the aforementioned PS extracts. After the preparation of the exposure medium (with and without TMF), the ES-D3 cell differentiation assay of the EST was performed according to the protocol described in section 2.2.3 of the current manuscript. DMSO alone (0.25%), TMF alone (3 µg/ml), and DMSO with TMF co-exposure were used as controls of this experiment and the final concentration of DMSO was kept at 0.25% (v/v). In addition, as an extra control, methoxyacetic acid (MAA; with and without TMF co-exposure) was also included in the experiment. MAA is one of the chemicals that is known to inhibit the ES-D3 cell differentiation via a mode of action that is potentiated via the Na^+/H^+ antiporter blocker amiloride (Louisse et al., 2011), and not proceeding via the AhR. In other words, MAA induces developmental toxicity but not mediated via the AhR, hence, the co-exposure of MAA with TMF should not counteract the in vitro PDT potency of MAA in the EST. The final concentration of TMF and MAA was 3 µg/ml (10 µM) and 0.18 mg/ml (2 mM), respectively. At least 3 independent experiments were done for each test compound in the ES-D3 cell differentiation assay with TMF co-exposure.

In addition, the effect of the AhR antagonist TMF was studied in the AhR CALUX assay to see whether the presence of TMF is also able to neutralize the AhR-mediated activity induced by some of the DMSO-extracts of PS under study. To this end, H4IIE.luc cells were exposed to the EC50-80 concentration of the PS extracts (Appendix C), as determined from the AhR CALUX assay of the present and our previous study (Kamelia et al., 2018), in both the absence and presence of TMF (final concentration 3 µg/ml or 10 µM). The EC50 is defined as the concentration that corresponds to 50% AhR-mediated activity induced by

the test compound in the AhR CALUX assay, as determined using the GraphPad Prism software version 5.0 (California, USA). Sample #145-HRBO and #091-GTLb, for which an EC50 value could not be established since they showed no AhR-mediated activity in the AhR CALUX assay, were tested at the concentration of 5 µg raw material/ml, the highest tested concentration used in the AhR CALUX assay (present study and Kamelia et al., 2018). Moreover, MMA, at the same concentration (2 mM) as tested above in the ES-D3 cell differentiation assay, was also tested in the AhR CALUX assay with TMF co-exposure. At least 3 independent experiments were done for each test compound in the AhR CALUX assay with TMF co-exposure

2.5 Data analysis (BMC and EC value derivation)

Figures of concentration-response curves upon exposure to the DMSO-extracts of 7 HFOs and 1 HRBO in the EST were made using GraphPad Prism 5.0 (California, USA). For this purpose, data from ES-D3 cell viability (1-day and 5-days exposure) and differentiation assays of the EST were analysed using non-linear regression analysis of GraphPad and fitted to a sigmoid dose-response curve with 3 parameters. In parallel, Benchmark Dose (BMD) software version 2.6.1 from US-EPA was used to calculate the benchmark concentration (BMC) that corresponds to 50% decrease of ES-D3 cell viability (BMCv50) or 50% inhibition of ES-D3 cell differentiation into beating cardiomyocytes (BMCd50). For the BMCd50 determination, concentration-response curves from ES-D3 cell differentiation assay were fitted to all dichotomous concentration-response models (gamma, logistic, log-logistic, probit, log-probit, weibull, multistage-cancer, multistage, and the quantal-linear models) available in the BMD software (Appendix A). The benchmark response (BMR) was set at 50%, representing a 50% inhibition of cardiomyocytes differentiation. 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 BMCd50 values were selected from the accepted model with the lowest Akaike's Information Criterion (AIC) (Kamelia et al., 2017).

For the AhR CALUX assay, the AhR-mediated activity of DMSO-extracts of 7 HFOs and 1 HRBO was presented as a percentage luciferase activity relative to the maximum response (fold-induction) induced by the reference-compound of the assay, BaP. Data from the AhR

CALUX assay were then analysed using non-linear regression in GraphPad Prism 5.0 and fitted to a sigmoid dose-response curve with 3 parameters to determine of what-so-called EC50 value. The EC50 value (50% effective concentration) represents the concentration for 50% induction of luciferase activity upon exposure to the respective test compound.

2.6 Linear regression analysis

Correlation between in vitro PDT potency of DMSO-extracts of PS (expressed as BMCd50s; obtained from the ES-D3 cell differentiation assay of the EST) and AhR-mediated activity (expressed as EC50s; derived from the AhR CALUX assay) were made using linear regression analysis in GraphPad Prism 5.0. To see whether the observed PDT potency in the EST or AhR-mediated activity of PS extracts is induced by particular group of PAH constituent, correlation between BMCd50s-EST or EC50s-AhR and specific PAH content (ranging from 2- to 7- ring PAH level) present in each PS extract was also conducted using GraphPad Prism 5.0. The obtained R^2 value reflects the goodness-of-fit of data to the fitted regression line and was considered significant if the p-value was ≤ 0.05.

3. Results

3.1 In vitro developmental toxicity of the DMSO-extracts of 7 HFOs and 1 HRBO in the EST

DMSO-extracts of 7 HFOs and 1 HRBO were evaluated for their in vitro PDT potency in the EST. As shown in Figure 2, all DMSO-extracts of HFOs induced concentration-dependent inhibition of the differentiation of the ES-D3 cells into cardiomyocytes in the EST at non-cytotoxic concentrations. The calculated BMCd50s, benchmark concentration for 50% inhibition of ES-D3 cell differentiation, derived from these data are presented in Table 1. The HFO-extract that was most potent with respect to inhibition of the ES-D3 cell differentiation was sample #008 with a BMCd50 value of 1.04 µg raw material/ml (Figure 2; Table 1). Sample #129, #021, and #091 were the other three potent HFO-extracts after sample #008, and their BMCd50 values ranged from 3.47 - 14.7 µg raw material/ml (Table 1). These in vitro PDT potencies are followed by that of sample #020-HFO (BMCd50: 69.8 µg raw material/ml), sample #006-HFO (BMCd50: 177.6 µg raw material/ml), and lastly

sample #017-HFO (BMCd50: 273.6 µg raw material/ml). In contrast, the DMSO-extract of HRBO did neither cause cytotoxicity nor inhibition of differentiation of ES-D3 cells into cardiomyocytes up to the highest tested concentration of 500 µg raw material/ml (Figure 2). Since no cytotoxicity was observed upon 1-day or 5-days exposure to the DMSO-extracts of the aforementioned test compounds, the benchmark concentration for 50% reduction on ES-D3 cell viability (BMCv50 values), could not be determined. The BMCd50 value for sample #145-HRBO could not be determined either as no inhibition of ES-D3 cell differentiation was observed.

3.2 AhR-mediated activity of the DMSO-extracts of 7 HFOs and 1 HRBO in the AhR CALUX assay

The AhR-mediated activity of the above-mentioned DMSO-extracts of 7 HFOs and 1 HRBO was investigated in the AhR CALUX agonist assay. All DMSO-extracts of the 7 HFOs were able to bind and activate the AhR, showing concentration-dependent agonist activities in the AhR CALUX assay (Figure 3). These concentration-response curves were then used to derive the EC50 values, which are listed in Table 1. The EC50 value is defined as the concentration that induces 50% of the AhR-mediated activity, relative to the reference-standard agonist compound of the AhR, BaP, in the AhR CALUX agonist assay. Based on their EC50s (Table 1), the DMSO-extract of sample #008-HFO was the most potent AhR agonist, where the group of samples #020, #006, #017-HFO were the weakest AhR agonists among all HFO-extracts under study. In contrast to the agonist activities of the HFO-extracts, sample #145-HRBO did not show any notable effect in the AhR CALUX assay, indicating its inability to interact with this receptor in vitro (Figure 3), and as a consequence, no EC50 value could be derived for this sample (Table 1).

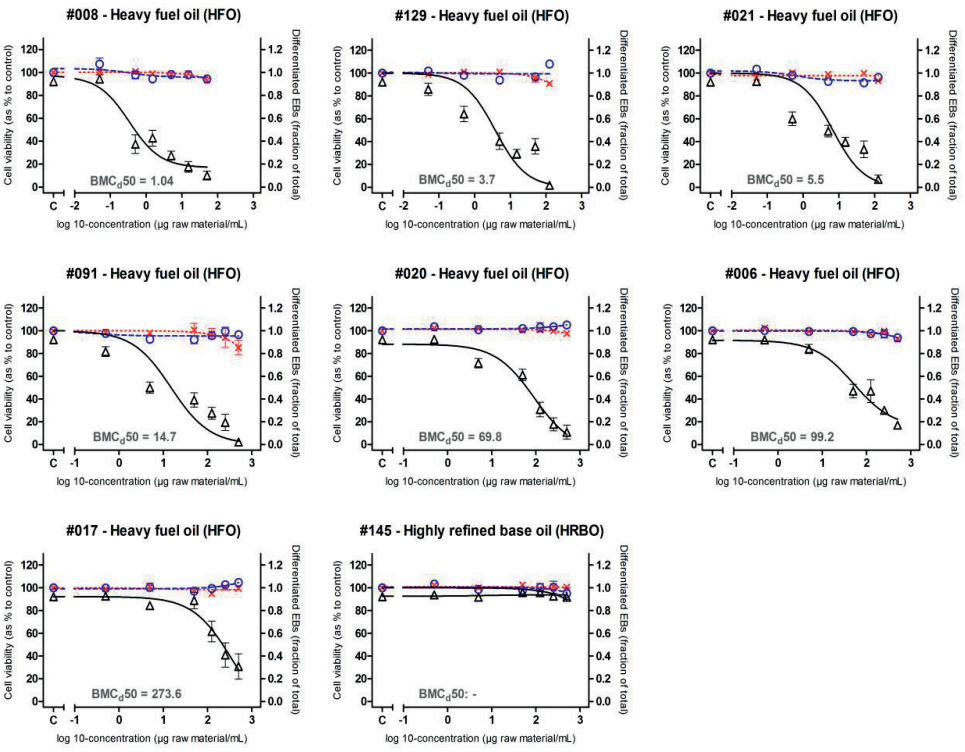


Figure 2 Concentration-dependent effects of the DMSO-extracts of 7 HFOs and 1 HRBO in the ES-D3 cell viability assay upon 1-day (x and red dotted-line) or 5-days (o and blue dashed-line) exposure and in the ES-D3 cell differentiation assay (Δ and black continuous-line) of the EST. The benchmark concentration for 50% inhibition on cell differentiation in the EST, BMCd50 value, is also presented. Results represent data from at least three independent experiments and are presented as Mean ± Standard Error of the Mean (SEM).

Abbreviations. EBs: embryoid bodies; HFO: heavy fuel oil; HRBO: highly refined base oil; BMCd50: the benchmark concentration for 50% inhibition on cell differentiation into beating cardiomyocytes in the ES-D3 cell differentiation assay of the EST.

Table 1 An overview of BMCd50 and EC50 values of the DMSO-extracts of PS (listed from the most to the least potent substances) tested in the present ES-D3 cell differentiation assay of the EST and AhR CALUX assay, respectively (Appendix D and E).

Sample code	Substance/ Compound	Total PAH content (wt. %)	Major group of PAH constituent	EST-BMCd50 (µg raw material/ml)	AhR CALUX-EC50 (µg raw material/ml)
#008-HFO	heavy fuel oil	27	3-6 ring PAH	1.0	8.0×10^{-3}
#129-HFO	heavy fuel oil	17	3-5 ring PAH	3.7	5.8×10^{-2}
#021-HFO	heavy fuel oil	13	3-6 ring PAH	5.5	3.3×10^{-2}
#091-HFO	heavy fuel oil	8	3-6 ring PAH	14.7	2.6×10^{-2}
#020-HFO	heavy fuel oil	4.4	2-7 ring PAH	69.8	3.3×10^{-1}
#006-HFO	heavy fuel oil	3	3-7 ring PAH	99.2	4.6×10^{-1}
#017-HFO	heavy fuel oil	0.62	3-7 ring PAH	273.6	2.1×10^{-1}
#145-HRBO	highly refined base oil	0	no PAH	(-)[a]	(-)[b]

[a]The calculated BMCd50 value is above the highest tested concentration of 500 µg raw material/ml.
[b]The calculated EC50 value is above the highest tested concentration of 5 µg raw material/ml.

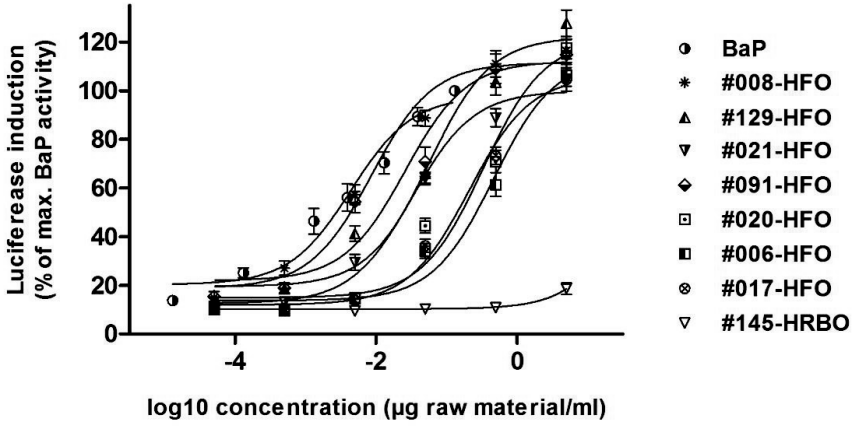


Figure 3 Effects of the DMSO-extracts of 7 HFOs (varying in PAH content) and 1 HRBO (no PAH) in the AhR CALUX agonist assay. Data are presented as a percentage of luciferase induction, relative to maximum-fold luciferase induction by BaP, the reference-standard agonist compound of the AhR in the present study. Each test compound was tested in triplicate in four independent experiments and results are presented as Mean ± SEM.

Abbreviations. AhR: aryl hydrocarbon receptor; CALUX: chemical activated luciferase gene expression; BaP: benzo[a]pyrene; HFO: heavy fuel oil; HRBO: highly refined base oil.

3.3 Relation of in vitro developmental toxicity potencies of PS extracts to their AhR-mediated activities

The BMCd50 values, obtained from the ES-D3 cell differentiation assay of the EST, were plotted against the EC50 values, derived from the AhR CALUX assay, to see if there is any association between the observed PDT effects and AhR-mediated activities of the DMSO-extracts of the PS under study. As shown in Figure 4A, a good correlation exists between in vitro PDT potencies and AhR-mediated activities of the HFO PS extracts (R^2: 0.85; Figure 4A). In a subsequent analysis, the data obtained for the HFO PS extracts of the present study were combined with the data previously obtained when testing other categories of PS in the EST and AhR CALUX assay. To this end the BMCd50s and EC50s of the following PS extracts: #034-HFO, #097-DAE, #098-DAE, #099-DAE, #171-GO, #172-GO, #175-VTO, #185-RAE, and #186-RAE, were taken from our previous published studies Kamelia et al., 2017 and Kamelia et al., 2018, respectively. Figure 4B shows the results obtained and reveals that a good correlation also exists across PS categories (R^2: 0.77; Figure 4B), although the correlation obtained when comparing the BMCd50s and EC50s among PS extracts that belong to the same HFO PS category (Figure 4A) appears slightly better. Test compounds that showed no substantial activity in either the EST and AhR CALUX assay, for which no BMCd50 or EC50 value could be derived, were not included in any of the correlation analysis presented, including sample #145-HRBO, #091-GTLb, and #092-GTLg.

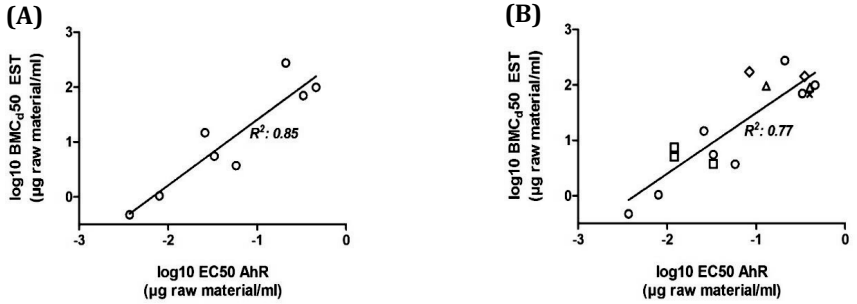


Figure 4 Correlation between in vitro PDT potency in the ES-D3 cell differentiation assay of the EST (expressed as BMCd50s) and AhR-mediated activity (expressed as EC50s) as measured in the AhR CALUX agonist assay, of the DMSO-extracts of (A) only HFO samples, n:8, 1 PS category; or (B) all PS samples: 8 HFOs (○), 3 DAEs (□), 2 GOs (◊), 1 VTO (x), 2 RAEs (Δ), n:16, 5 PS categories.

Note. The EST and AhR-mediated activity data of sample #034-HFO, #097-DAE, #098-DAE, #099-DAE, #171-GO, #172-GO, #175-VTO, #185-RAE, #186-RAE, were taken from our previous published study Kamelia et al., 2017; 2018.
BMCd50: the benchmark concentration for 50% inhibition on cell differentiation into beating cardiomyocytes in the ES-D3 cell differentiation assay of the EST.
EC50: concentration that induce 50% of the AhR-mediated activity, relative to the reference-standard agonist compound of the AhR, BaP, in the AhR CALUX agonist assay.
Abbreviations. HFO: heavy fuel oil; DAE: distillate aromatic extract; GO: gas oil; VTO: vacuum tower overhead oil; RAE: residual aromatic extract.

3.4 Relation of in vitro developmental toxicity potencies of PS extracts in the EST to their specific PAH content

In vitro PDT potencies of PS extracts, both from the present and our previous study (Kamelia et al., 2017), were compared with their PAH content to see whether the observed effect in the EST is induced by a particular group of PAHs present in these substances. For this purpose, BMCd50 values were plotted against the amount of PAHs (ranging from 2- to 7-ring PAHs) present in the respective PS extract, in total giving 15 combinations of comparisons for which the correlation between BMCd50s and PAH content could be determined. The details for all these comparisons are provided in the Supplementary Materials (Appendix F), and only the results with the most relevant correlation and highest R^2, which reflect the goodness-of-fit of data in this regression analysis, are presented in Figure 5A and 5B. As shown in Figure 5A, the first comparison was made between the BMCd50s of the HFO PS samples (1 PS category, n: 8) and their PAH content. From this, the observed in vitro PDT potencies of HFO-extracts in the EST were best correlated with the 3- to 7-ring PAHs they contain (R^2: 0.95). When this comparison was taken a step further, including all 6 PS categories tested so far: HFO, DAE, RAE, GO, VTO, HRBO; and GTL products (in total n: 16; Figure 5B), the best correlation, among the 15 comparisons (Supplementary Material; Appendix F), was again obtained between the BMCd50s and the total 3- to 7-ring PAHs present in each of the PS extract, with an R^2 of 0.85 (Figure 5B).

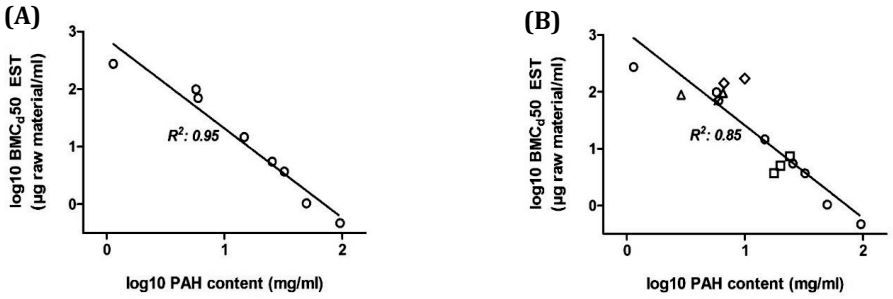


Figure 5 Correlation between in vitro PDT potency in the ES-D3 cell differentiation assay of the EST (expressed as BMCd50s) and 3- to 7-ring PAH content present in (A) each of the HFO samples, n: 8, 1 PS category; or (B) each of all PS samples: 8 HFOs (○), 3 DAEs (□), 2 GOs (◊), 1 VTO (x), 2 RAEs (Δ), n:16, 5 PS categories.

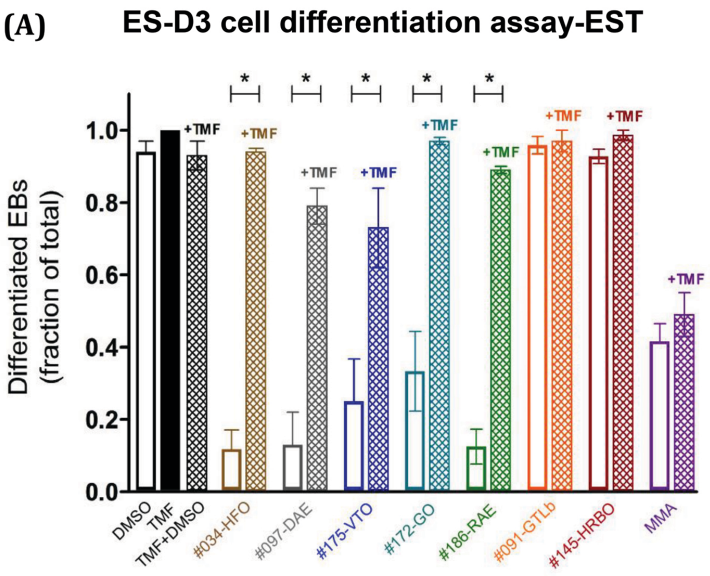
Abbreviations. HFO: heavy fuel oil; DAE: distillate aromatic extract; GO: gas oil; VTO: vacuum tower overhead oil; RAE: residual aromatic extract.

3.5 Neutralization of PS-induced inhibition of ES-D3 cell differentiation in the EST and AhR-mediated activity in the AhR CALUX assay by the AhR-antagonist 6,2′,4′-trimethoxyflavone (TMF)

To evaluate whether the PS-induced inhibition of ES-D3 cell differentiation into cardiomyocytes in the EST was AhR-mediated, we selected DMSO-extracts of different PS categories and one GTL product, and tested them in the ES-D3 cell differentiation assay in the presence and absence of the AhR antagonist 6,2′,4′-trimethoxyflavone (TMF). The selected PS (varying in PAH content and originating from different PS categories) and GTL (no PAHs) extracts included #034-HFO, #097-DAE, #175-VTO, #172-GO, #186-RAE, #145-HRBO, and #091-GTLb, which were respectively tested at the concentration of 20, 100, 150, 250, 150, 250, and 250 µg raw material/ml, known from previous studies to represent the concentrations resulting in 80-90% inhibition of ES-D3 cell differentiation by the respective samples. The results show that for all selected PS the induced inhibition of ES-D3 cell differentiation in the EST is counteracted by the AhR antagonist TMF (Figure 6A). The DMSO-extract of sample #091-GTLb, was not able to inhibit the ES-D3 cell differentiation into cardiomyocytes in either the absence or presence of TMF. In addition to the PS and GTL extracts, a known developmental toxicant MAA (Welsch et al., 1987; Clarke et al., 1992; Louisse et al., 2011) was also tested in the ES-D3 cell differentiation

assay with and without TMF co-exposure. MAA was chosen since it is known not to act via the AhR pathway when inducing developmental toxicity (Louisse et al., 2011), hence, the addition of TMF would be expected to not change the in vitro PDT potency of MAA in the EST. The concentration of MAA tested amounted to 0.18 mg/ml, a concentration shown previously to inhibit the differentiation of ES-D3 cell differentiation while not being cytotoxic (Louisse et al., 2011). As illustrated by the last two bars in Figure 6, MAA shows an inhibition of ES-D3 cell differentiation into cardiomyocytes in the EST regardless of the presence or absence of TMF.

The effect of the AhR antagonist TMF was also studied in the AhR CALUX assay to see whether the presence of TMF is also able to neutralize the AhR-mediated activity induced by some of the DMSO-extracts of PS under study. As shown in Figure 6B, TMF successfully counteracted the AhR-mediated induction of luciferase activity by the DMSO-extract of sample #034-HFO, #097-DAE, #175-VTO, #172-GO, and #186-RAE. In contrast and as expected, the DMSO-extract of sample #091-GTLb, #145-HRBO, as well as MAA were not able to induce AhR-mediated activity in either the absence or presence of TMF, since these compounds are known not to have AhR agonist activity.



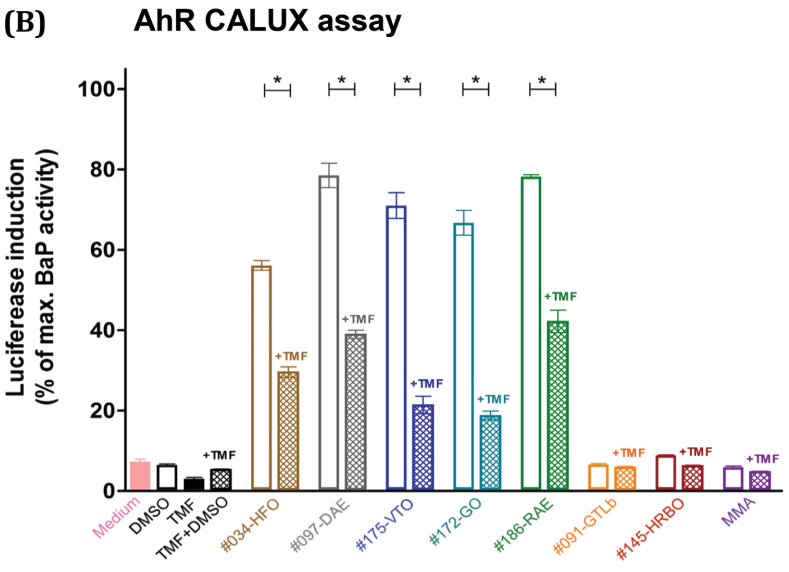


Figure 6 Counteracting effect of 6, 2, 4-trimethoxyflavone (TMF) on (A) the inhibition of ES-D3 cell differentiation and (B) AhR-mediated induction of gene expression by DMSO-extracts of some selected PS (varying in PAH content): #034-HFO, #097-DAE, #175-VTO, #172-GO, and #186-RAE. The DMSO-extracts of sample #145-HRBO and #91-GTLb, which contain no PAH, were also tested with TMF co-exposure. All test compounds were tested in the absence or presence of TMF, a potent AhR inhibitor (Murray et al., 2010). DMSO (0.25%), TMF only, and DMSO with TMF co-exposure were used as controls for this experiment. MAA was tested as an extra control, because MAA is one of the chemicals that is known to inhibit the ES-D3 cell differentiation via a mode of action that is potentiated via the Na^+/H^+ antiporter blocker amiloride (Louisse et al., 2011), and does not proceed via the AhR. Hence, TMF should not counteract the effects of MAA in the EST. Results represent data from at least three independent experiment and are presented as Mean ± SEM. Student's t-test was performed to evaluate the significance ($p < 0.05$) of the difference in response in the absence or presence of TMF.

4. Discussion and conclusions

Prior studies, both in vivo (Feuston et al., 1989; Hoberman et al., 1995) and in vitro (Kamelia et al., 2017), have shown a strong relationship between developmental toxicity induced by some (unrefined) high-boiling petroleum substances (HBPS), such as HFO, and the level of high-molecular-weight (HMW) PAHs they contain. This role of HMW PAHs in the developmental toxicity of PS is corroborated by data from PDT studies conducted with HRBO (Mobil, 1987b) and GTL products (Boogaard and Roberts, 2015; Boogaard et al., 2017), which are a similar HBPS and an analogue non-petroleum based products, respectively, that do not contain PAHs and for which no developmental toxicity effects were observed. In other words, the observed PDT for some PS seems to be caused by HMW PAHs present in these products (Murray et al., 2013: Kamelia et al., 2017). To further test this hypothesis, the present study evaluates the applicability of the EST to assess PDT potency of the DMSO-extracts of 7 HFOs, varying in a systematic way in their PAH content and all originating from one single PS category, and 1 HRBO (containing no PAHs), relative to the amount of 3- to 7-ring PAHs they contain. All DMSO-extracts of the selected series of HFO induced concentration-dependent inhibition of ES-D3-cell differentiation into cardiomyocytes in the EST at non-cytotoxic concentrations and this potency appeared to be proportional with the amount of 3- to 7-ring PAHs they contain (R^2: 0.95). Moreover, the DMSO-extracts of the HFOs also showed AhR-mediated activities, as tested in the AhR CALUX assay. Co-exposure of ES-D3 cells with the selected DMSO-extracts of HFO and the AhR antagonist trimethoxyflavone (TMF), successfully counteracted the HFO-induced inhibition of ES-D3 cell differentiation into cardiomyocytes, confirming the role of the AhR in mediating the observed PDT of these substances in the EST. In contrast, the DMSO-extract of HRBO, which is virtually devoid of PAHs, showed no effect at all when tested in the EST (with and without TMF co-exposure) or in the AhR CALUX assay. These results confirm that 3- to 7-ring PAHs, present in the PS extracts of the present study, are plausibly responsible for the observed PDT in the EST, and that this adverse effect is mediated by AhR activation.

In vitro PDT potency of the DMSO-extracts of HFOs, as obtained in the EST, is in line with in vivo findings where dermal exposure of HFO to pregnant rats caused various developmental effects, including decreased fetal body weight, reduced number of live fetuses per litter, and increased incidence of resorptions (Feuston et al., 1989; Hoberman

et al., 1995; Feuston & Mackerer 1996). In contrast to HFO, exposure of HRBO (contain no PAHs) to animal experiments via three different route of administrations: oral, pulmonary, and dermal; did not induce any PDT effects (Mobil, 1987b). This is also in concordance with results of the present study, where exposure to the DMSO-extract of HRBO did not induce any effects in the EST, thus testing negative for in vitro PDT Despite this excellent concordance between the obtained in vitro and in vivo findings, one should also remember to take into account the kinetic differences that exist between in vitro and in vivo approaches when comparing the obtained potencies. The EST model lacks an optimal biotransformation system and placental transport, as compared to the in vivo situation, which consequently affects the uptake and bioavailability of test substances. Hence, it is of interest for future research to evaluate the role of both metabolism and placental transport to the PDT potency of these substances, to further narrow the gap between in vivo and in vitro studies. Furthermore, it is worth mentioning that the present study is the second attempt we made to evaluate the applicability of the EST to predict the PDT potency of PS UVCBs. In the first study (Kamelia et al., 2017), the DMSO-extracts of 9 PS and 2 GTL products were evaluated for their in vitro PDT potency in the EST, and a very good correlation between in vitro and in vivo findings was obtained (R^2: 0.97; Kamelia et al., 2017). Having said that, based on the results from the in vitro-in vivo comparisons, described in both the present and our previous study (Kamelia et al., 2017), it can be concluded that the EST successfully predicts the relative in vivo PDT potency of PS and GTL products. In view of this, the EST shows a great promise as one of the in vitro alternative assays for PDT testing of PS extracts, as it proves to adequately predict their PDT potency, within and across categories.

Several in vivo studies have reported the association between developmental toxicity induced by some HBPS, such as HFO, and specific types of PAHs they contain (Feuston et al., 1994; Murray et al., 2013). One example, based on the 21 rat developmental toxicity studies on a number of high-HBPS; including 10 studies of HFOs and 7 studies of GOs), Murray and his co-workers (2013) concluded that developmental toxicity of HBPS is associated with the presence and amount of 3- to 7-ring PAHs present in these products. This is in agreement with our current results where a very good correlation exists between the observed PDT potency (expressed as BMCd50s) of PS extracts under study, within (HFO only, R^2: 0.95) and across (among 6 PS categories, R^2: 0.85) categories, and the amount of 3- to 7-ring PAHs they contain. For instance, comparing the BMCd50s of sample within 1

PS category: #017-HFO (total wt.% 3-7 ring PAHs = 0.62%) and #006-HFO (total wt.% 3-7 ring PAHs = 3%), it is evident that sample #006 is a more potent substance for inhibition of ES-D3 cell differentiation into cardiomyocytes than sample #017, as the amount of 3-7 ring PAHs in sample #006 is tripled compared to that in sample #017. To conclude, there is a good correlation between in vitro PDT potencies of HFO in the EST and their 3-7 ring PAH content (Figure 5A). Even when including other PS categories to this comparison, a good correlation still holds (Figure 5B). Thus, it can be said that the higher the 3- to 7-ring PAH content of a PS, the more potent it is to induce in vitro PDT.

In contrast to 3- to 7-ring PAH, it is suspected that 2-ring PAHs are unlikely to induce developmental toxicity-related effects (Feuston et al., 1994; Murray et al., 2013). Corroborating this notion, PS extracts of the present study that predominantly contained 2-ring PAHs as a part of their PAH constituents (sample #171, #172, #175, #020; the ARC profiles of these samples is provided in Appendix B) indeed showed a lower potency in the EST in comparison to the other PS samples without 2-ring PAH constituents. This is illustrated by comparison of the BMCd50 values of samples #175-VTO and #091-HFO, which both contain a comparable total PAH content with sample #175-VTO containing more 2-ring PAHs than sample #091-HFO. As a consequence, sample #175-VTO induced in vitro PDT in the EST to a lesser extent compared to sample #091-HFO, which comprises mainly 3-7 ring PAHs. This finding obviously points out the contribution of 3-7 ring PAH constituents present in the PS extracts under study for their observed PDT in the EST. Altogether, it can be concluded that the specific types and amount of PAHs present in each PS extract, play a pivotal role in determining their PDT potency. In light of this, subsequent research of the present study will focus on the role and contribution of each PAH ring-group, i.e. 3-, 4-, 5-, 6-, 7-ring PAHs, in PDT.

To date, the modes-of-action underlying PDT of PAHs and PS is not completely understood. Prior studies have reported a role of AhR in mediating developmental toxicity of some individual and complex mixtures of PAHs (Incardona et al, 2004; Huang et al., 2012; Zhang et al., 2012; Goodale et al., 2013; Geier et al., 2018a,b). Disruption of the AhR-signalling pathway may result in the disturbance of the embryonic development, resulting in altered cardiac development that leads to an increased risk of early life stage mortality in the developing embryo (Denison et al., 2011; Careira et al., 2015; Doering et al., 2018). Supporting this notion, the present study shows a good correlation when comparing in

vitro PDT potency (expressed as BMCd50s) of PS extracts to AhR mediated CALUX activity (expressed as EC50s), both within (R^2: 0.85) and across categories (R^2: 0.77). This suggests an important role of the AhR for the observed PDT of these substances. Based on these observations, some PS extracts were selected and tested in the ES-D3 cell differentiation assay in the presence of the AhR antagonist TMF, to see whether the inhibition of ES-D3 cell differentiation into cardiomyocytes in the EST is indeed AhR-mediated. Our results show that TMF counteracts the PS-induced inhibition of ES-D3 cell differentiation, confirming that the AhR is involved in the observed PDT by the selected PS extracts (from different PS categories) in the EST. This also means that the ES-D3 cell differentiation assay of the EST contains a relevant pathway for the PS-induced embryotoxicity, thus, supporting the intention of using the EST as one of the in vitro alternative assays for PDT testing of PS UVCBs. However, one should remember that some individual PAHs may also induce embryotoxicity independent of the AhR. For example, pyrene and dibenzothiophene are known to cause developmental toxicity in the zebrafish embryo and this occurs independently of the AhR (Zhang et al., 2012; Goodale et al., 2013), while on the other hand, the AhR is crucial for the embryotoxicity of BaP (Huang et al., 2012; Goodale et al., 2013). This may also reflect a difference in the underlying mechanism of fish and mammalian PDT. Hence, the contribution and role of individual PAHs or specific groups of PAHs present in the PS extracts under study for their observed PDT in the mammalian test system should be further investigated.

A study by de Jong et al. (2014) showed that one of the AhR-activators, TCDD, was not able to inhibit cardiomyocyte differentiation (ESTc) but osteoblast differentiation (ESTo) of ES-D3 cells in the EST. The authors also explained and discussed that the embryotoxic effect of TCDD could only be detected using the ESTo, but not the ESTc, due to the specific mechanism of action of TCDD to induce developmental toxicity, which is via the inhibition of osteogenesis. Hence the embryotoxicity potency of TCDD cannot be captured by the cardiac differentiation assay of the EST. In the case of the present study, it is concluded that the PDT induced by some PAHs-containing PS, is partly mediated via the AhR (Billiard et al, 2006; Incardona et al., 2005; Kamelia et al., 2018; Vrabie et al., 2009). Surely, the interference of the AhR-related pathway may not be the only mode-of-action underlying the developmental toxicity potency of PS, although results of the present study indicate that the AhR plays a role in mediating this adverse effect, in particular for this group of complex substances. Nonetheless, other cellular receptors may play a role as well. It has been

reported that the retinoic acid receptor (RAR) (Louisse et al., 2011) and the estrogen receptor alpha (ERα) (Adam et al., 2019), known to be present also in the ES-D3 cells of the EST, may also be involved in modes-of-action underlying developmental toxicity. Thus, their role in PS-mediated developmental toxicity remains to be investigated.

Despite the great promise of the EST (Rohdewel et al., 2001; Seiler and Spielmann, 2011) to assess the embryotoxicity potency of different chemical substances including PS UVCBs, this assay also has some limitations, such as the lack of prominent metabolic activity and of placental transport. Hence, the usefulness of another in vitro PDT assay that is capable of metabolic activation, such as the ZET (Selderslaghs et al., 2009; Geier et al., 2018a,b) or *C. elegans* models (Boyd et al., 2012; Hunt, 2017), should be considered to explore testing the same PS extracts under study. Previous studies (Kühnert et al., 2017; Le Fol et al., 2017; Verbueken et al., 2017; Geier et al, 2018) showed that zebrafish embryos, to some extent, progressively express metabolizing enzymes, thereby, making them an in vitro model that may be more metabolically competent for evaluating developmental toxicity-related effects. This makes the ZET a suitable in vitro model to be added to the testing battery that we are currently developing for PDT testing of PS UVCBs.

α-naphtoflavone (ANF) is a well-known and commonly used AhR inhibitor in both in vivo and in vitro studies (Gaziewicz & Rucci, 1991; Merchant et al., 1993; Timme-Laragy et al., 2007). However, the present study selected 6,2',4'-trimethoxyflavone (TMF), and not ANF, to study the role of AhR in mediating the observed effects in both the EST and AhR CALUX assay because of the relatively higher selectivity of TMF as an AhR inhibitor. This was shown by Murray and his co-workers reporting that TMF displays a potent AhR antagonist activity while having no partial AhR-agonist activity, in contrast to other documented antagonists of AhR, including ANF, that possess a partial weak agonist activity besides their AhR-antagonism capability (Murray et al., 2010). Because of this higher selectivity, we selected TMF to characterize the effect of an AhR antagonist on the PDT effects induced by some DMSO-extracts of PS and GTL products. So we tested whether the PS-induced PDT, as observed in the EST, and the AhR-mediated gene expression induced by these substances, as quantified in the AhR CALUX assay, would be counteracted by the addition of TMF as an AhR antagonist. As expected, co-exposure of ES-D3 cells (EST) or H4IIE.luc cells (AhR CALUX assay) with the selected DMSO-extracts of PS or GTL product and TMF, successfully

counteracted the PS-induced inhibition of ES-D3 cell differentiation into cardiomyocytes and AhR-mediated induction of gene expression by these substances.

MAA-induced inhibition of ES-D3 cells differentiation in the EST is caused by acid overload following the decreased of the intracellular pH (pHi) of embryonic cells upon exposure to MAA, affecting embryoid bodies (EBs) formation and ES-D3 cell differentiation processes (Louisse et al., 2010). Hence, in line with the results of the present study, the ability of MAA to inhibit the differentiation of ES-D3 cells would not be resolved by the addition of the AhR antagonist TMF.

The presence and expression of protein receptors in the ES-D3 cell line used for the EST of the present study are not fully known. Using the same cell line and assay, a study by Louisse et al. (2011) showed that the ES-D3 cell differentiation assay contains RAR-related pathways since it successfully predicted the embryotoxicity of retinoids and other RAR-agonists. Likewise, Adam et al. (2019) demonstrated that the ES-D3 cell line also contains ER-mediated pathways. However, it should be remembered that AhR-mediated, as shown by the results of the present study, RAR-mediated, and ER-mediated are not the only pathways involved in mediating developmental toxicity. Hence, the characterization of receptor proteins expressed in the ES-D3 cell line, in particular for embryotoxicity-related pathways, may also be one of the interests for future research to fully explore the applicability of the EST for in vitro PDT testing. Furthermore, it is known that some PAHs and PS require metabolic activation to exert their genotoxicity and carcinogenicity (Blackburn et al., 1986; Mackerer et al., 2003). However, it is still unknown whether bioactivation is essential for these substances to induce PDT, and also whether the cytochrome P450 enzymes CYP1A and CYP1B, which are the responsible enzymes for metabolizing PAHs into their reactive genotoxic metabolites, are present and active in the ES-D3 cells. Whether the PDT induced by the PS under study, as observed in the EST, is induced by the parent or PAH-metabolites or both, remains a topic of interest for future studies.

One may argue on the relevance of using the DMSO-extracts instead of the neat materials of PS for in vitro testing of these substances. However, the neat material of PS, in particular the heavy ones like HFO, are extremely poorly soluble in aqueous media as they are highly lipophilic. As a consequence, a special approach was taken to ensure that these test materials are introduced in the in vitro test system in such a way that they are bioavailable.

In the present study, the PAH extraction method (based on the Method II chemical characterization procedure) as described by Roy et al., 1988 was used to generate so-called DMSO-extracts of PS. The application of DMSO extracts, which contain the polar fraction of the PS including 3-to 7-ring PAH, to test PS in in vitro systems is a standard approach in the industry, and has been widely applied and validated for in vitro carcinogenicity and mutagenicity testing of PS (Concawe, 1994; Blackburn et al., 1986). Data on aliphatic HRBO and GTL products which do not show PDT effects in in vivo studies support the fact that the DMSO-extract indeed contains the biologically active fraction of the PS and no potential developmental effects are missed as the non-extracted molecules are proven to be non-developmental toxic. A recent published data show that the non-extracted fractions from the DMSO-extraction procedure of PS contain mainly saturated hydrocarbons (e.g., aliphatics/alkanes) and highly alkylated PAHs (e.g., >6 carbon atoms of the alkyl chain length) (Carrillo et al., 2019). It is known that the aforementioned group of saturated hydrocarbons does not cause developmental toxicity in experimental animals (Boogaard et al., 2017; Feuston and Mackerer, 1996; Murray et al., 2013; Tsitou et al., 2015). In addition, the HRBO and GTL products, which do not contain PAHs, do not show PDT effects in in vivo studies. Furthermore, for the highly alkylated PAHs, their bioavailability is limited since they are not soluble in aqueous medium (Wang et al., 2019). Altogether, based on the present and previous EST results (Kamelia et al., 2017), the use of the DMSO-extracts of PS in the EST proved to adequately capture the desirable and expected PDT effects of these substances, as compared to their in vivo potencies. Thus, the application of the DMSO-extracts of these PS for cell exposure, is relevant and appropriate for in vitro PDT testing of these substances and unlikely to miss potential developmental effects.

Recent studies by Geier et al. (2018a) and Wincent et al. (2015) using the ZET showed that some substituted PAHs, including nitrated, oxygenated, and alkylated PAHs are often more developmental toxic than their parent naked PAHs. Indeed, little is known on the developmental toxicity potency of the substituted PAHs, where for carcinogenicity and mutagenicity endpoints, it is known that some methylated PAHs are more genotoxic than their unsubstituted analogues (Santella et al. 1982). Taking this into account, the role of substituted PAHs that may be present in PS extracts under study for the PS-induced PDT is not negligible, and hence, should be considered for future research.

In summary, these new findings support earlier studies to identify the applicability of the EST to assess in vitro PDT of PS, within and across categories. DMSO-extracts of 7 HFOs, varying in 3- to 7-ring PAH content, inhibited ES-D3 cell differentiation in a concentration- and 3- to 7-ring content-dependent manner where the HRBO extract, containing no polycyclic aromatic compounds, showed no effect at all. This corroborates the hypothesis that 3- to 7-ring PAHs are the main inducers of PDT by some PS. In addition, the PS-induced inhibition of ES-D3 cell differentiation into cardiomyocytes in the EST was neutralized following the co-exposure with the AhR antagonist TMF, confirming that the observed PDT by the tested PS in the ES-D3 cell differentiation assay of the EST is partially mediated via the AhR. PS are complex substances (UVCBs), hence, it should be well-noted that their underlying mechanism to induce embryotoxicity may not be due to a single mode-of-action. Still, it can be hypothesized that PS that belong to the same PS category (regardless of their PAH content and profile) may act via similar modes-of-action in mediating their PDT. Therefore, the next step of our research will focus on the use of a targeted transcriptomic approach that may eventually provide mechanistic insight for the underlying PDT caused by different PS. Moreover, PDT testing for substances like PS is particularly difficult and poses a huge challenge due to their complex compositional-origin, which is highly variable depending on the source of crude oil and processing conditions used to create the steam (Speight, 1996). Having said that, the use of a battery instead of a stand-alone assay, which can capture the relevant modes-of-action of PS UVCBs in inducing the PDT, and for now includes the EST and the AhR CALUX, should be used and can be applied for batch screening of PS, and for prioritization purposes in an intelligent testing strategy to minimize and eventually replace animal testing for the evaluation of the PDT potency of these substances, within and across product categories. In practice, this will be achieved by applying a battery of assays to identify the worst-case representatives for each PS category for further in-vivo testing where needed as a last resort. The resulting data and findings will also facilitate read-across from PS for which these in vivo data will be generated (the worst case approach), or historical data are already available, to other PS for which in vivo data are lacking. Read-across and grouping are the commonly used alternative approaches for data gap filling in registrations of chemical substances under the REACH legislation. Based on the read-across assessment framework (RAAF) proposed by ECHA in 2017 (ECHA, 2017), the read-across approach for multi-constituent substances, e.g. UVCBs, can possibly be done in two ways, namely a constituent-based approach and/or a category-based

approach, and the latter is a more suitable approach for read-across purpose of PS UVCBs. Such an approach has been used in recent years for grouping and prioritization purposes, also to evaluate the risk of PS exposure to human and environment (Grimm et al., 2017; Redman et al., 2012, 2014). For example, in 2014, Redman et al. has introduced a modeling framework called PETRORISK, which utilizes representative hydrocarbon constituents (or hydrocarbon block method/HBM) (King et al., 1996; Redman et al., 2012) as data-input to evaluate environmental risk and human exposure to complex PS. In light of this, the evaluation on how our approach by combining the bioactivity-profiling data, obtained from the current battery of in vitro alternative assays for PDT testing of PS, with analytical characterization data of the corresponding PS, might be relevant and can be used as a basis for future risk assessment of these highly complex substances (UVCBs), replacing their in vivo PDT testing, remains a task that we are going to perform in the near future.

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Supplementary data

Appendix A

Results from concentration-response modeling of the data on ES-D3 cell differentiation assay of EST for DMSO-extracts of PS.

Table A1 Results from a BMD analysis of the data on ES-D3 cell differentiation assay of **sample #008 – heavy fuel oil (HFO)**. The table presents the benchmark concentration for 50% effect on differentiation (BMCd50) and the 95% benchmark dose lower confidence limit (BMCdL50) values for a BMR of 10% extra risk with characteristics of the model fit. The selected model (BMD/BMDL < 3, lowest AIC) is given in bold.

Model type	Risk Type	BMFR	Restricted model	No of para-meters	p-value (goodness of fit)	AIC	BMCd50 (µg raw material/ml)	BMCdL50 (µg raw material/ml)	BMD/ BMDL
Gamma	Extra	0.1	yes	2	0	196.49	9.90	6.62	1.50
Logistic	Extra	0.1	no	2	0	203.17	15.91	11.00	1.45
LogLogistic	Extra	0.1	yes	2	0.0012	174.33	1.44	0.87	1.66
LogProbit	**Extra**	**0.1**	**no**	**3**	**0.1406**	**167.52**	**1.04**	**0.51**	**2.02**
Multistage	Extra	0.1	yes	2	0	196.49	9.90	6.62	1.50
Multistage Cancer	Extra	0.1	yes	2	0	196.49	9.90	6.62	1.50
Probit	Extra	0.1	no	2	0	204.77	19.30	14.44	1.34
Weibull	Extra	0.1	yes	2	0	196.49	9.90	6.62	1.50
Quantal Linear	Extra	0.1	na	2	0	196.49	9.90	6.62	1.50

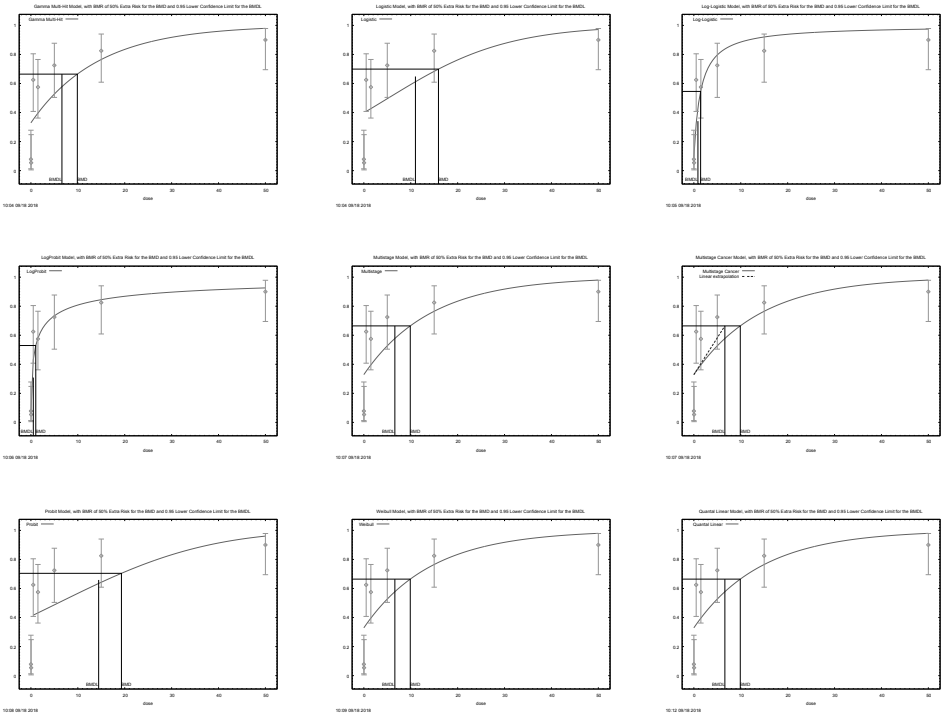


Table A2 Results from a BMD analysis of the data on ES-D3 cell differentiation assay of **sample #129 – heavy fuel oil (HFO)**. The table presents the benchmark concentration for 50% effect on differentiation (BMC_d50) and the 95% benchmark dose lower confidence limit (BMC_dL50) values for a BMR of 10% extra risk with characteristics of the model fit. The selected model (BMD/BMDL < 3, lowest AIC) is given in bold.

Model type	Risk Type	BMFR	Restricted model	No of para-meters	p-value (goodness of fit)	AIC	BMCd50 (µg raw material/ml)	BMCdL50 (µg raw material/ml)	BMD/ BMDL
Gamma	Extra	0.1	yes	2	0.0007	186.51	25.60	17.74	1.44
Logistic	Extra	0.1	no	2	0.0002	190.51	38.15	28.23	1.35
LogLogistic	Extra	0.1	yes	2	0.0036	180.25	10.13	5.64	1.80
LogProbit	**Extra**	**0.1**	**no**	**3**	**0.1297**	**174.62**	**3.66**	**1.51**	**2.43**
Multistage	Extra	0.1	yes	2	0.0007	186.51	25.60	17.74	1.44
Multistage Cancer	Extra	0.1	yes	2	0.0007	186.51	25.60	17.74	1.44
Probit	Extra	0.1	no	2	0.0003	190.52	41.05	31.27	1.31
Weibull	Extra	0.1	yes	2	0.0007	186.51	25.60	17.74	1.44
Quantal Linear	Extra	0.1	na	2	0.0007	186.51	25.60	17.74	1.44

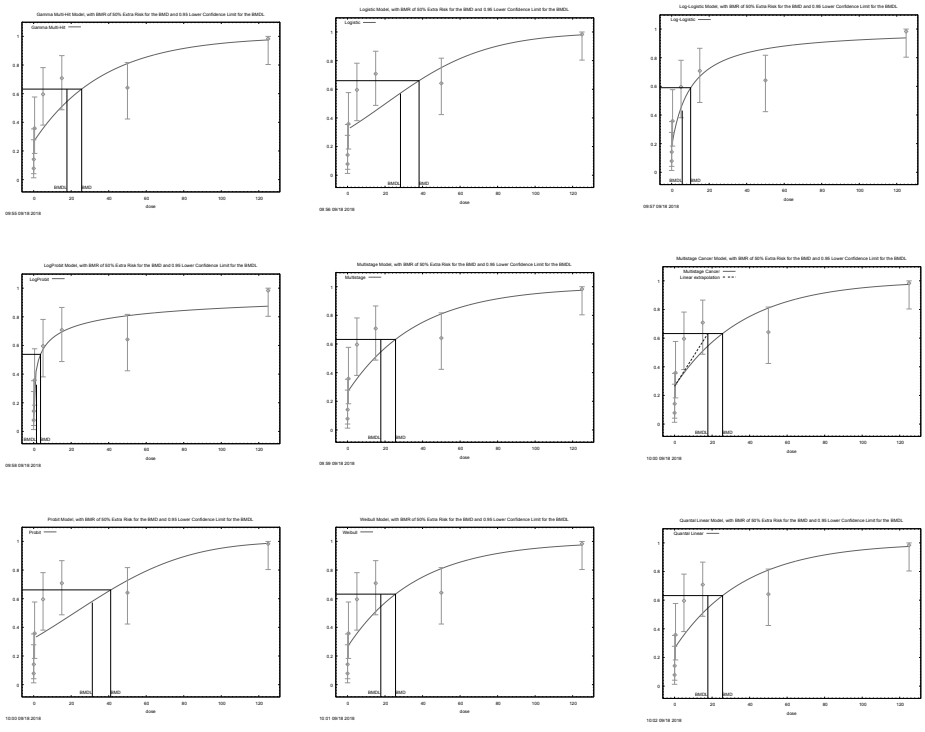


Table A3 Results from a BMD analysis of the data on ES-D3 cell differentiation assay of **sample #021 – heavy fuel oil (HFO)**. The table presents the benchmark concentration for 50% effect on differentiation (BMC_d50) and the 95% benchmark dose lower confidence limit (BMC_dL50) values for a BMR of 10% extra risk with characteristics of the model fit. The selected model (BMD/BMDL < 3, lowest AIC) is given in bold.

Model type	Risk Type	BMFR	Restricted model	No of para-meters	p-value (goodness of fit)	AIC	BMCd50 (µg raw material/ml)	BMCdL50 (µg raw material/ml)	BMD/ BMDL
Gamma	Extra	0.1	yes	2	0.0043	188.99	31.78	22.29	1.43
Logistic	Extra	0.1	no	2	0.0012	193.33	46.36	34.76	1.33
LogLogistic	Extra	0.1	yes	2	0.0154	183.96	14.84	8.33	1.78
LogProbit	**Extra**	**0.1**	**no**	**3**	**0.2518**	**178.16**	**5.53**	**2.43**	**2.27**
Multistage	Extra	0.1	yes	2	0.0043	188.99	31.78	22.29	1.43
Multistage Cancer	Extra	0.1	yes	2	0.0043	188.99	31.78	22.29	1.43
Probit	Extra	0.1	no	2	0.001	193.80	50.49	39.46	1.28
Weibull	Extra	0.1	yes	2	0.0043	188.99	31.78	22.29	1.43
Quantal Linear	Extra	0.1	na	2	0.0043	188.99	31.78	22.29	1.43

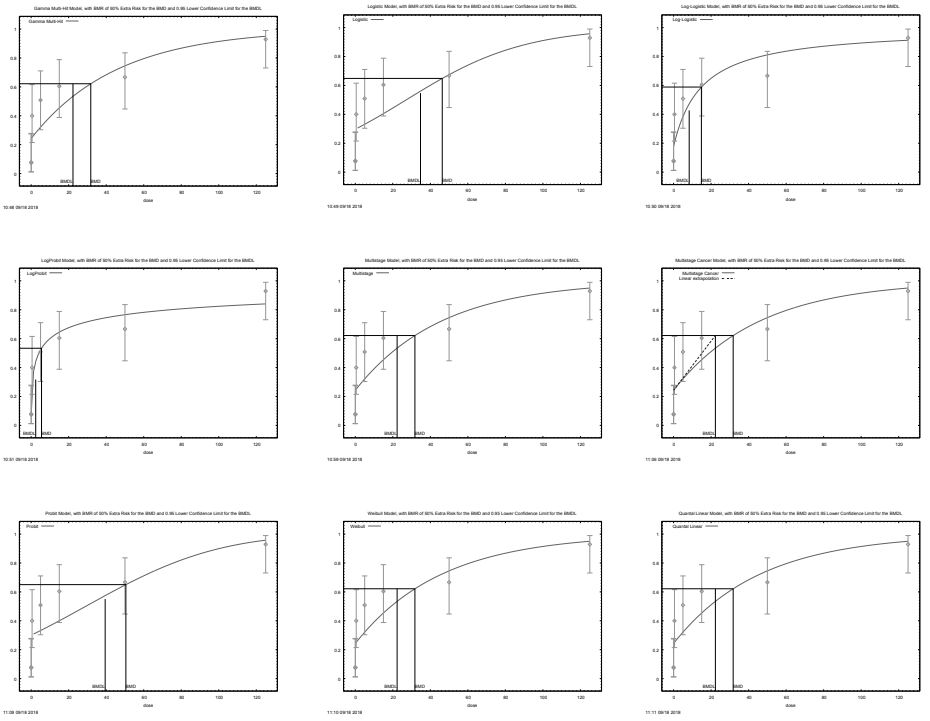


Table A4 Results from a BMD analysis of the data on ES-D3 cell differentiation assay of **sample #091 – heavy fuel oil (HFO)**. The table presents the benchmark concentration for 50% effect on differentiation (BMC_d50) and the 95% benchmark dose lower confidence limit (BMC_dL50) values for a BMR of 10% extra risk with characteristics of the model fit. The selected model (BMD/BMDL < 3, lowest AIC) is given in bold.

Model type	Risk Type	BMFR	Restricted model	No of para-meters	p-value (goodness of fit)	AIC	BMCd50 (µg raw material/ml)	BMCdL50 (µg raw material/ml)	BMD/ BMDL
Gamma	Extra	0.1	yes	2	0.022	175.79	95.77	70.06	1.37
Logistic	Extra	0.1	no	2	0.0062	180.01	134.64	104.14	1.29
LogLogistic	Extra	0.1	yes	2	0.0576	172.94	46.43	25.78	1.80
LogProbit	**Extra**	**0.1**	**no**	**3**	**0.3888**	**169.22**	**14.69**	**5.88**	**2.50**
Multistage	Extra	0.1	yes	2	0.022	175.79	95.77	70.06	1.37
Multistage Cancer	Extra	0.1	yes	2	0.022	175.79	95.77	70.06	1.37
Probit	Extra	0.1	no	2	0.0042	180.93	148.65	118.17	1.26
Weibull	Extra	0.1	yes	2	0.022	175.79	95.77	70.06	1.37
Quantal Linear	Extra	0.1	na	2	0.022	175.79	95.77	70.06	1.37

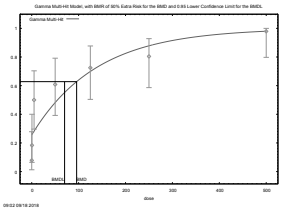
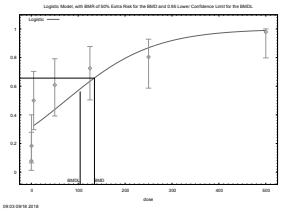
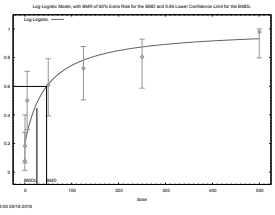
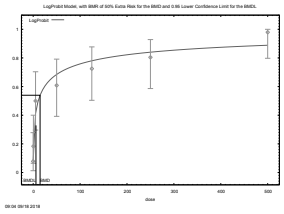
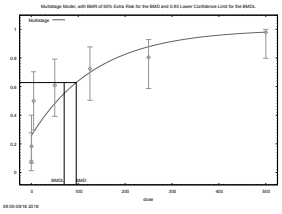
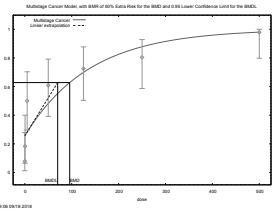
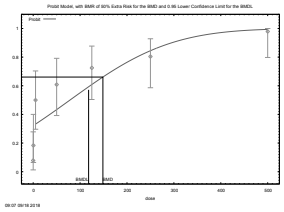
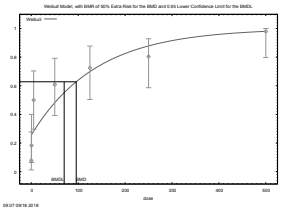
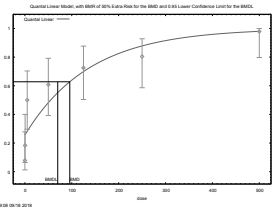
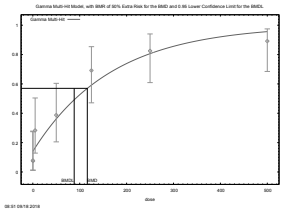
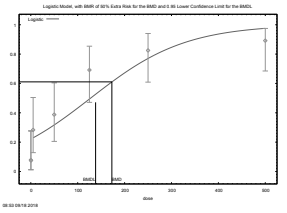
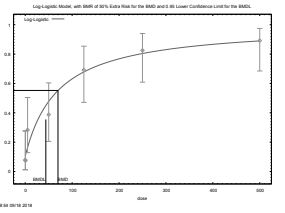










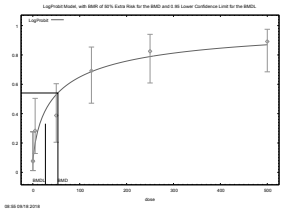
Table A5 Results from a BMD analysis of the data on ES-D3 cell differentiation assay of **sample #020 – heavy fuel oil (HFO)**. The table presents the benchmark concentration for 50% effect on differentiation (BMC_d50) and the 95% benchmark dose lower confidence limit (BMC_dL50) values for a BMR of 10% extra risk with characteristics of the model fit. The selected model (BMD/BMDL < 3, lowest AIC) is given in bold.

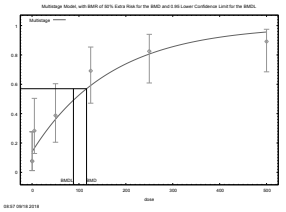
Model type	Risk Type	BMFR	Restricted model	No of para-meters	p-value (goodness of fit)	AIC	BMCd50 (µg raw material/ml)	BMCdL50 (µg raw material/ml)	BMD/ BMDL
Gamma	Extra	0.1	yes	2	0.1789	165.89	116.22	88.24	1.32
Logistic	Extra	0.1	no	2	0.003	175.35	172.85	138.19	1.25
LogLogistic	**Extra**	**0.1**	**yes**	**2**	**0.5732**	**162.53**	**69.84**	**43.90**	**1.59**
LogProbit	Extra	0.1	no	3	0.4956	164.30	53.54	26.87	1.99
Multistage	Extra	0.1	yes	2	0.1789	165.89	116.22	88.24	1.32
Multistage Cancer	Extra	0.1	yes	2	0.1789	165.89	116.22	88.24	1.32
Probit	Extra	0.1	no	2	0.0021	177.41	195.05	160.93	1.21
Weibull	Extra	0.1	yes	2	0.1789	165.89	116.22	88.24	1.32
Quantal Linear	Extra	0.1	na	2	0.1789	165.89	116.22	88.24	1.32

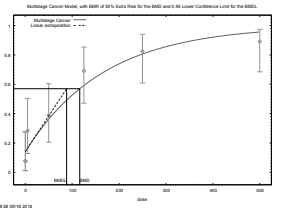


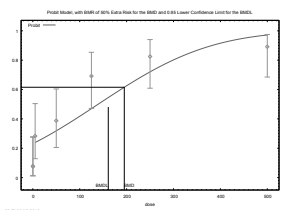


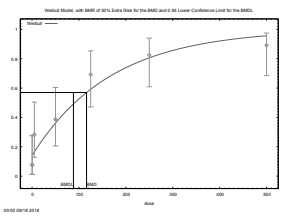












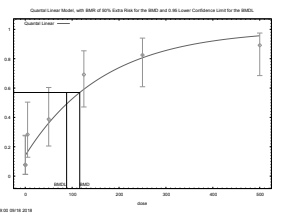


Table A6 Results from a BMD analysis of the data on ES-D3 cell differentiation assay of **sample #006 – heavy fuel oil (HFO)**. The table presents the benchmark concentration for 50% effect on differentiation (BMC_d50) and the 95% benchmark dose lower confidence limit (BMC_dL50) values for a BMR of 10% extra risk with characteristics of the model fit. The selected model (BMD/BMDL < 3, lowest AIC) is given in bold.

Model type	Risk Type	BMFR	Restricted model	No of para-meters	p-value (goodness of fit)	AIC	BMCd50 (µg raw material/ml)	BMCdL50 (µg raw material/ml)	BMD/ BMDL
Gamma	Extra	0.1	yes	2	0.1237	177.22	155.89	117.37	1.33
Logistic	Extra	0.1	no	2	0.0044	186.33	233.35	186.29	1.25
LogLogistic	**Extra**	**0.1**	**yes**	**2**	**0.7249**	**171.98**	**99.21**	**63.88**	**1.55**
LogProbit	Extra	0.1	no	3	0.8529	172.54	81.98	43.14	1.90
Multistage	Extra	0.1	yes	2	0.1237	177.22	155.89	117.37	1.33
Multistage Cancer	Extra	0.1	yes	2	0.1237	177.22	155.89	117.37	1.33
Probit	Extra	0.1	no	2	0.0039	186.74	246.73	202.68	1.22
Weibull	Extra	0.1	yes	2	0.1237	177.22	155.89	117.37	1.33
Quantal Linear	Extra	0.1	na	2	0.1237	177.22	155.89	117.37	1.33

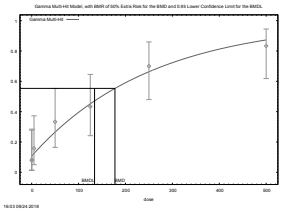
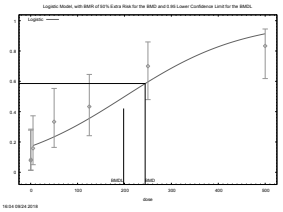
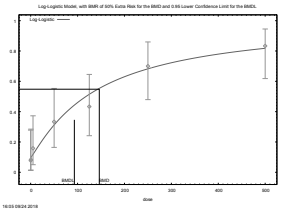
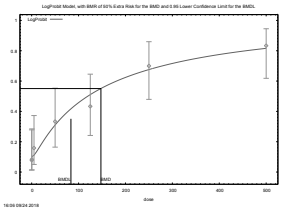
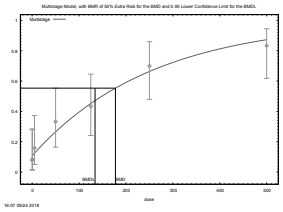
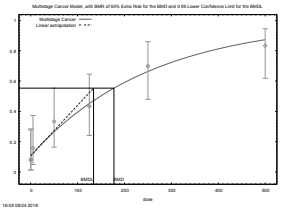
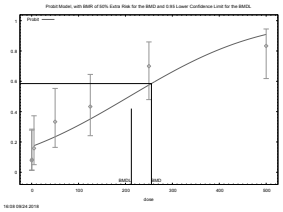
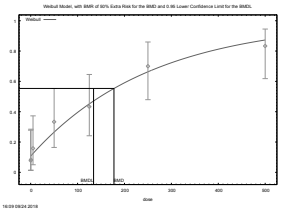
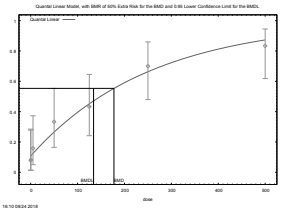
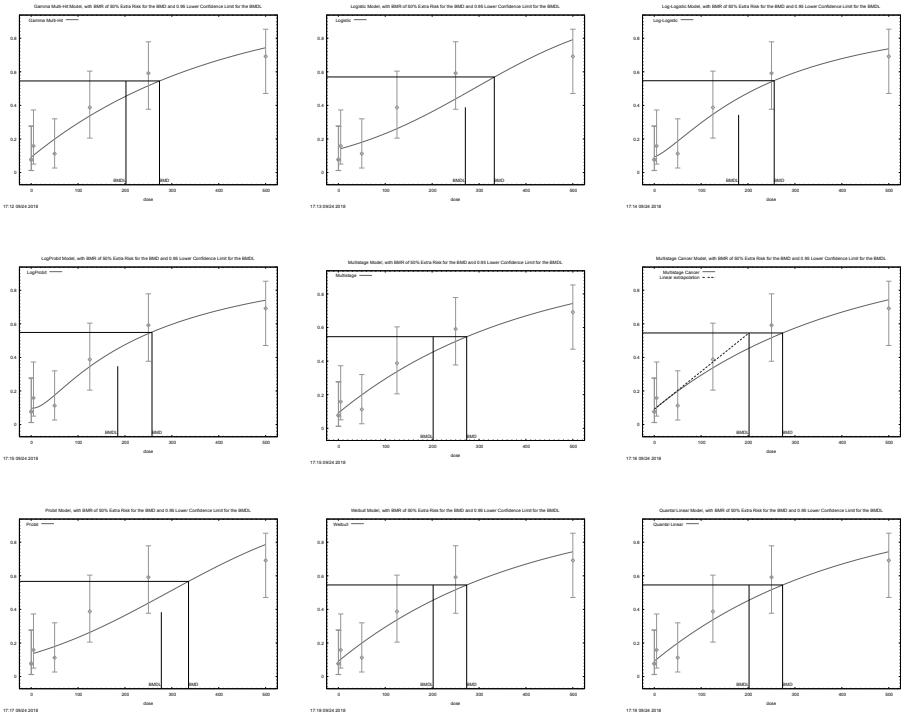










Table A7 Results from a BMD analysis of the data on ES-D3 cell differentiation assay of **sample #017 – heavy fuel oil (HFO)**. The table presents the benchmark concentration for 50% effect on differentiation (BMC_d50) and the 95% benchmark dose lower confidence limit (BMC_dL50) values for a BMR of 10% extra risk with characteristics of the model fit. The selected model (BMD/BMDL < 3, lowest AIC) is given in bold.

.	Risk Type	BMFR	Restricted model	No of para-meters	p-value (goodness of fit)	AIC	BMCd50 (µg raw material/ml)	BMCdL50 (µg raw material/ml)	BMD/ BMDL
Gamma	**Extra**	**0.1**	**yes**	**2**	**0.6669**	**165.22**	**273.63**	**202.16**	**1.35**
Logistic	Extra	0.1	no	2	0.1531	170.05	332.53	270.80	1.23
LogLogistic	Extra	0.1	yes	2	0.6106	166.63	255.76	179.88	1.42
LogProbit	Extra	0.1	no	3	0.6247	166.51	257.67	184.62	1.40
Multistage	**Extra**	**0.1**	**yes**	**2**	**0.6669**	**165.22**	**273.63**	**202.16**	**1.35**
Multistage Cancer	**Extra**	**0.1**	**yes**	**2**	**0.6669**	**165.22**	**273.63**	**202.16**	**1.35**
Probit	Extra	0.1	no	2	0.1742	169.66	335.55	277.55	1.21
Weibull	**Extra**	**0.1**	**yes**	**2**	**0.6669**	**165.22**	**273.63**	**202.16**	**1.35**
Quantal Linear	**Extra**	**0.1**	**na**	**2**	**0.6669**	**165.22**	**273.63**	**202.16**	**1.35**



Appendix B

Aromatic ring class (ARC) profiles of PS and GTL products that have been tested so far in the battery of in vitro alternative assays that we are developing (listed from highest to the lowest PAH content).

Compound	ARC profile[a] (wt.%)							Total (wt.%)
	1-ring	2-ring	3-ring	4-ring	5-ring	6-ring	≥ 7-ring	
#034-HFO	0	1	24	40	21	11	3	48
#008-HFO	0	7	44	22	14	10	2	27
#129-HFO	0	4	55	37	3	0	0	17
#021-HFO	0	3	46	30	14	6	2	13
#099-DAE	0	0	22	41	25	11	1	12
#098-DAE	0	0	18	49	29	4	0	9.7
#097-DAE	0	2	1	6	22	36	33	9
#091-HFO	0	9	27	25	19	14	5	8.2
#175-VTO	1	56	43	0	0	0	0	6.7
#171-GO	0	12	76	12	0	0	0	5.5
#020-HFO	3	29	14	7	15	22	10	4.4
#172-GO	0	20	77	2	0	0	0	4.2
#186-RAE	0	0	1	6	18	38	36	3.3
#006-HFO	0	1	22	22	21	21	13	2.9
#185-RAE	0	4	4	4	16	30	42	1.5
#017-HFO	0	8	19	17	21	22	13	0.62
#091-GTLb	0	0	0	0	0	0	0	0
#092-GTLg	0	0	0	0	0	0	0	0
#145-HRBO	0	0	0	0	0	0	0	0

[a] The weight percent of the DMSO-soluble 1- to ≥7 aromatic-ring compounds present in each substance, from the starting raw material of 4 gram sample, as determined by Method II chemical characterization procedure (Roy et al., 1988; Kamelia et al., 2017).

Abbreviations. GTLb: gas-to-liquid base oil; GTLg: gas-to-liquid gas oil; HRBO: highly refined base oil; HFO: heavy fuel oil; DAE: distillate aromatic extract; GO: gas oil; VTO: vacuum tower overhead oil; RAE: residual aromatic extract.

Appendix C

An overview of BMCd50 and EC50 values of the DMSO-extracts of PS and GTL products that have been tested so far in the ES-D3 cell differentiation assay of the EST and AhR CALUX assay, respectively (listed from highest to the lowest PAH content).

Sample code	Substance/ Compound	Total PAH content (wt. %)	Major group of PAH constituent	EST-BMCd50 (µg raw material/ml)	AhR CALUX-EC50 (µg raw material/ml)
#034-HFO	heavy fuel oil	48	3-7 ring PAH	0.47	3.7×10^{-3}
#008-HFO	heavy fuel oil	27	3-6 ring PAH	1.04	8.0×10^{-3}
#129-HFO	heavy fuel oil	17	3-5 ring PAH	3.7	5.8×10^{-2}
#021-HFO	heavy fuel oil	13	3-6 ring PAH	5.5	3.3×10^{-2}
#099-DAE	distillate aromatic extract	12	3-6 ring PAH	7.40	1.2×10^{-2}
#098-DAE	distillate aromatic extract	9.7	3-5 ring PAH	5.05	1.2×10^{-2}
#097-DAE	distillate aromatic extract	9	5-7 ring PAH	3.74	3.3×10^{-2}
#091-HFO	heavy fuel oil	8	3-6 ring PAH	14.7	2.6×10^{-2}
#175-VTO	vacuum tower overhead oil	6.7	2-3 ring PAH	67.2	4.0×10^{-1}
#171-GO	gas oil	5.5	2-3 ring PAH	171	8.4×10^{-2}
#020-HFO	heavy fuel oil	4.4	2-7 ring PAH	69.8	3.3×10^{-1}
#172-GO	gas oil	4.2	2-3 ring PAH	142	3.5×10^{-1}
#186-RAE	residual aromatic extract	3.3	5-7 ring PAH	95.2	1.3×10^{-1}
#006-HFO	heavy fuel oil	3	3-7 ring PAH	99.2	4.6×10^{-1}
#185-RAE	residual aromatic extract	1.5	5-7 ring PAH	89.5	4.0×10^{-1}
#017-HFO	heavy fuel oil	0.62	3-7 ring PAH	273.6	2.1×10^{-1}
#091-GTLb	gas-to-liquid base oil	0	no PAH	(-)[a]	(-)[b]
#092-GTLg	gas-to-liquid gas oil	0	no PAH	(-)[a]	(-)[b]
#145-HRBO	highly refined base oil	0	no PAH	(-)[a]	(-)[b]

[a]The calculated BMCd50 value is above the highest tested concentration of 500 µg raw material/ml.
[b]The calculated EC50 value is above the highest tested concentration of 5 µg raw material/ml.
Note. BMCd50 (EST) and EC50 (AhR CALUX) values of sample #034-HFO; #097-DAE; #098-DAE; #99-DAE; #171-GO; #172-GO; #175-VTO; #185-RAE; #186-RAE; #091-GTLb; #092-GTLg are taken from our previously published studies Kamelia et al., 2017; 2018.

Appendix D

Results from ES-D3 cell viability and ES-D3 differentiation assays of various DMSO-extracts of PS. 0.25% (v/v) DMSO was used as solvent control for both assays. The results of ES-D3 cell viability are presented as mean % cell viability as compared to solvent control. For ES-D3 cell differentiation assay, data are provided as mean fraction of total differentiated EBs. Results represent data from at least three independent experiment and are presented as mean ± standard error mean (SEM).

Table D1 Results from ES-D3 cell viability and ES-D3 differentiation assays of **sample #145 – highly refined base oil (HRBO).**

Concentration (µg raw material/mL)	1 day – cell viability assay		5 days – cell viability assay		Cell differentiation assay	
	Mean	SEM	Mean	SEM	Mean	SEM
0 (DMSO)	100	0.59	100	0.96	0.92	0.02
0.5	102.35	1	103.24	1.45	0.94	0.03
5	100.04	0.5	98.57	2.91	0.92	0.03
50	102.45	0.92	97.9	3.08	0.96	0.03
125	98.61	0.32	100.38	3.5	0.96	0.03
250	101.31	1.37	100.48	5.26	0.93	0.02
500	100.57	1.16	94.92	5.15	0.92	0.03

Table D2 Results from ES-D3 cell viability and ES-D3 differentiation assays of **sample #017 – heavy fuel oil (HFO).**

Concentration (µg raw material/mL)	1 day – cell viability assay		5 days – cell viability assay		Cell differentiation assay	
	Mean	SEM	Mean	SEM	Mean	SEM
0 (DMSO)	100.00	0.59	100.00	0.96	0.92	0.02
0.5	99.17	0.70	99.81	1.77	0.93	0.01
5	100.66	3.52	100.51	1.30	0.84	0.03
50	97.85	2.32	97.06	1.69	0.89	0.07
125	94.98	2.61	99.44	3.14	0.34	0.05
250	100.48	1.32	102.73	2.73	0.14	0.06
500	99.59	2.40	104.74	2.09	0.08	0.03

Table D3 Results from ES-D3 cell viability and ES-D3 differentiation assays of **sample #006 – heavy fuel oil (HFO).**

Concentration (µg raw material/mL)	1 day – cell viability assay		5 days – cell viability assay		Cell differentiation assay	
	Mean	SEM	Mean	SEM	Mean	SEM
0 (DMSO)	100.00	0.59	100.00	0.96	0.92	0.01
0.5	102.52	1.77	100.38	2.54	0.84	0.04
5	99.59	0.59	99.44	2.61	0.47	0.06
50	99.31	1.33	99.45	0.68	0.47	0.10
125	97.35	1.74	97.61	1.72	0.30	0.02
250	99.80	0.89	97.34	3.58	0.17	0.03
500	92.76	1.96	93.95	3.28	0.92	0.01

Table D4 Results from ES-D3 cell viability and ES-D3 differentiation assays of **sample #020 – heavy fuel oil (HFO).**

Concentration (µg raw material/mL)	1 day – cell viability assay		5 days – cell viability assay		Cell differentiation assay	
	Mean	SEM	Mean	SEM	Mean	SEM
0 (DMSO)	100.00	0.59	100.00	0.96	0.92	0.02
0.5	102.41	1.89	103.64	1.63	0.93	0.02
5	102.06	1.90	101.09	2.25	0.72	0.04
50	100.53	1.99	102.04	2.13	0.61	0.05
125	100.88	3.15	103.09	3.98	0.31	0.06
250	99.98	0.68	103.62	2.73	0.18	0.06
500	97.70	2.26	105.21	1.08	0.11	0.06

Table D5 Results from ES-D3 cell viability and ES-D3 differentiation assays of **sample #091 – heavy fuel oil (HFO).**

Concentration (µg raw material/mL)	1 day – cell viability assay		5 days – cell viability assay		Cell differentiation assay	
	Mean	SEM	Mean	SEM	Mean	SEM
0 (DMSO)	100.00	0.59	100.00	0.96	0.92	0.02
0.5	98.91	1.67	97.75	2.10	0.82	0.04
5	98.14	3.07	92.57	2.74	0.50	0.05
50	100.69	5.75	92.23	3.52	0.39	0.06
125	97.56	4.44	95.83	2.08	0.27	0.05
250	94.25	8.90	99.58	3.62	0.19	0.07
500	85.22	6.39	96.63	2.75	0.02	0.02

Table D6 Results from ES-D3 cell viability and ES-D3 differentiation assays of **sample #021 – heavy fuel oil (HFO).**

Concentration (µg raw material/mL)	1 day – cell viability assay		5 days – cell viability assay		Cell differentiation assay	
	Mean	SEM	Mean	SEM	Mean	SEM
0 (DMSO)	100.00	0.59	100.00	0.96	0.92	0.02
0.05	96.11	0.69	103.48	1.60	0.93	0.02
0.5	100.22	1.39	98.21	1.57	0.60	0.06
5	99.02	1.76	92.80	1.96	0.49	0.05
15	n.a	n.a	n.a	n.a	0.40	0.04
50	99.94	1.38	91.57	1.71	0.33	0.07
125	93.43	2.54	96.27	3.04	0.07	0.04

n.a: data is not available as the specific concentration was not tested in the ES-D3 cell viability assay.

Table D7 Results from ES-D3 cell viability and ES-D3 differentiation assays of **sample #129 – heavy fuel oil (HFO).**

Concentration (µg raw material/mL)	1 day – cell viability assay		5 days – cell viability assay		Cell differentiation assay	
	Mean	SEM	Mean	SEM	Mean	SEM
0 (DMSO)	100.00	0.59	100.00	0.96	0.92	0.02
0.05	98.97	1.57	101.82	2.93	0.86	0.05
0.5	101.03	1.35	98.23	1.16	0.64	0.07
5	101.06	2.62	93.95	0.96	0.40	0.07
15	n.a	n.a	n.a	n.a	0.29	0.04
50	95.90	3.91	96.76	1.74	0.36	0.07
125	90.90	2.94	108.01	3.04	0.02	0.01

n.a: data is not available as the specific concentration was not tested in the ES-D3 cell viability assay.

Table D8 Results from ES-D3 cell viability and ES-D3 differentiation assays of **sample #008 – heavy fuel oil (HFO).**

Concentration (µg raw material/mL)	1 day – cell viability assay		5 days – cell viability assay		Cell differentiation assay	
	Mean	SEM	Mean	SEM	Mean	SEM
0 (DMSO)	100.00	0.59	100.00	0.96	0.92	0.02
0.05	100.49	0.25	107.36	5.25	0.94	0.04
0.5	100.92	2.41	97.92	3.45	0.38	0.08
1.5	98.94	0.84	94.45	1.83	0.43	0.07
5	98.56	2.13	98.25	2.90	0.28	0.04
15	99.14	0.92	98.02	3.00	0.18	0.05
50	93.43	2.36	94.44	2.88	0.10	0.04

Appendix E

Results from AhR CALUX assay of the DMSO-extracts of 7 HFOs and 1 HRBO tested in the present study. Results represent data from at least three independent experiment and are presented as mean ± standard error mean (SEM).

Concentration (µg/ml)	Benzo[a]pyrene		#017-HFO		#006-HFO		#020-HFO		#091-HFO		#021-HFO		#129-HFO		#008-HFO		#145-HRBO	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
1.30E-05	13.73	0.92																
5.00E-05			10.51	0.71	10.06	0.85	11.09	0.99	15.35	1.69	9.96	1.09	10.59	0.49	15.05	2.48	11.33	1.15
1.30E-04	25.18	2.02																
5.00E-04			10.46	0.38	9.66	0.82	10.27	0.59	18.94	2.06	13.07	1.08	18.46	1.89	27.04	2.96	10.13	0.98
1.30E-03	46.38	5.26																
3.90E-03	56.1	5.62																
5.00E-03			13.85	1.15	14.9	1	14.51	0.64	54.27	4.38	29.44	3.27	41.27	3.13	57.24	4.12	9.54	0.84
1.30E-02	70.32	4.54																
3.90E-02	89.35	3.79																
5.00E-02			36.32	2.64	34.14	3.03	44.49	3	70.78	5.99	64.17	2.29	65.97	4.62	88.71	3.28	10.31	1.39
1.30E-01	100	0																
5.00E-01			73.24	3.51	61.39	4.89	70.86	4.55	108.99	6.04	88.86	3.68	103.47	5.32	111.05	5.49	10.83	0.84
5.00E+00			104.61	1.6	106.3	4.49	117.66	4.52	114.69	6.2	104.64	4.82	127.65	5.46	116.4	4.83	18.62	2.35

Appendix F

Table F1 Correlation between in vitro developmental toxicity potency in the EST (BMCd50s) and specific PAH content (2- to 7-ring PAHs) present in each of the HFO sample under study (n:8).

Sample code	ii-iii	ii-iv	ii-v	ii-vi	ii-vii	iii-iv	iii-v	iii-vi	iii-vii	iv-v	iv-vi	iv-vii	v-vi	v-vii	vi-vii
#034-HFO	24.2	62.9	83.2	93.9	96.8	61.9	82.3	92.9	95.8	59.0	69.7	72.6	31.0	33.9	13.6
#017-HFO	0.3	0.5	0.8	1.1	1.2	0.4	0.7	1.0	1.1	0.5	0.7	0.9	0.5	0.7	0.4
#006-HFO	1.3	2.6	3.8	5.0	5.8	2.6	3.8	5.0	5.7	2.5	3.7	4.5	2.4	3.2	2.0
#020-HFO	3.8	4.4	5.7	7.7	8.5	1.8	3.2	5.1	6.0	1.9	3.9	4.8	3.3	4.1	2.8
#091-HFO	5.9	10.0	13.1	15.4	16.2	8.5	11.6	13.9	14.8	7.2	9.5	10.3	5.4	6.2	3.1
#021-HFO	12.7	20.5	24.2	25.7	26.3	19.8	23.4	25.0	25.5	11.4	13.0	13.5	5.2	5.7	2.1
#129-HFO	20.1	32.6	33.7	33.7	33.7	31.3	32.3	32.3	32.3	13.6	13.6	13.6	1.0	1.0	0.0
#008-HFO	27.5	39.4	47.0	52.4	53.5	35.6	43.2	48.6	49.7	19.4	24.8	25.9	13.0	14.0	6.5
R square	0.88	0.94	0.95	0.94	0.94	0.95	0.96	0.95	0.95	0.95	0.93	0.92	0.58	0.51	0.76

Table F2 Correlation between in vitro developmental toxicity potency in the EST (BMCd50s) and specific PAH content (2- to 7-ring PAHs) present in each of the PS sample under study (n:16), including 8 HFOs, 2 RAEs, 3 DAEs, 2 GOs, and 1 VTO.

Sample code	ii-iii	ii-iv	ii-v	ii-vi	ii-vii	iii-iv	iii-v	iii-vi	iii-vii	iv-v	iv-vi	iv-vii	v-vi	v-vii	vi-vii
#034-HFO	24.2	62.9	83.2	93.9	96.8	61.9	82.3	92.9	95.8	59.0	69.7	72.6	31.0	33.9	13.6
#185-RAE	0.2	0.4	0.8	1.7	3.0	0.2	0.7	1.6	2.9	0.6	1.5	2.8	1.4	2.6	2.2
#186-RAE	0.1	0.5	1.7	4.2	6.5	0.5	1.7	4.2	6.5	1.6	4.1	6.5	3.7	6.1	4.9
#171-GO	9.9	11.3	11.3	11.3	11.3	9.9	9.9	9.9	9.9	1.4	1.4	1.4	0.0	0.0	0.0
#172-GO	8.2	8.3	8.3	8.3	8.3	6.6	6.6	6.6	6.6	0.2	0.2	0.2	0.0	0.0	0.0
#175-VTO	13.3	13.3	13.3	13.3	13.3	5.8	5.8	5.8	5.8	0.0	0.0	0.0	0.0	0.0	0.0
#097-DAE	0.5	1.6	5.6	12.1	18.0	1.3	5.2	11.7	17.6	5.0	11.5	17.5	10.4	16.4	12.4
#098-DAE	3.6	13.4	19.2	20.0	20.0	13.4	19.2	20.0	20.0	15.6	16.4	16.4	6.6	6.6	0.8
#099-DAE	5.3	15.1	21.1	23.8	24.0	15.1	21.1	23.8	24.0	15.8	18.5	18.7	8.6	8.9	2.9
#017-HFO	0.3	0.5	0.8	1.1	1.2	0.4	0.7	1.0	1.1	0.5	0.7	0.9	0.5	0.7	0.4
#006-HFO	1.3	2.6	3.8	5.0	5.8	2.6	3.8	5.0	5.7	2.5	3.7	4.5	2.4	3.2	2.0
#020-HFO	3.8	4.4	5.7	7.7	8.5	1.8	3.2	5.1	6.0	1.9	3.9	4.8	3.3	4.1	2.8
#091-HFO	5.9	10.0	13.1	15.4	16.2	8.5	11.6	13.9	14.8	7.2	9.5	10.3	5.4	6.2	3.1
#021-HFO	12.7	20.5	24.2	25.7	26.3	19.8	23.4	25.0	25.5	11.4	13.0	13.5	5.2	5.7	2.1
#129-HFO	20.1	32.6	33.7	33.7	33.7	31.3	32.3	32.3	32.3	13.6	13.6	13.6	1.0	1.0	0.0
#008-HFO	27.5	39.4	47.0	52.4	53.5	35.6	43.2	48.6	49.7	19.4	24.8	25.9	13.0	14.0	6.5
R square	0.27	0.45	0.62	0.73	0.79	0.49	0.68	0.80	0.85	0.83	0.80	0.75	0.58	0.47	0.38

Chapter 5

Prenatal developmental toxicity testing of petroleum substances using the zebrafish embryotoxicity test

Lenny Kamelia, Sylvia Brugman, Laura de Haan, Hans B. Ketelslegers, Ivonne M.C.M. Rietjens, Peter J. Boogaard

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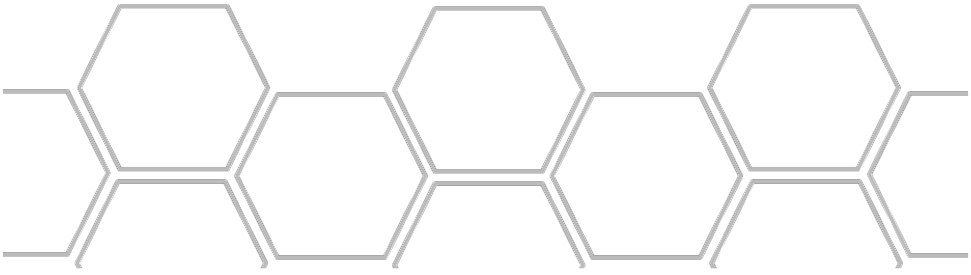


Abstract

The present study evaluates the applicability of the zebrafish embryotoxicity test (ZET) to assess prenatal developmental toxicity (PDT) potency of the DMSO-extracts of 9 petroleum substances (PS), with variable polycyclic aromatic hydrocarbon (PAH) content, and 2 gas-to-liquid (GTL) products, without any PAHs but otherwise similar properties to PS. All PS extracts induced concentration-dependent PDT as quantified in the ZET and this potency is associated with their 3-5 ring PAH content. In contrast and as expected, GTL products did not induce any effect in the ZET. The potencies obtained in the ZET correlated with those previously reported for the embryonic stem cell test (EST) (R^2: 0.61), while the correlation with potencies reported in in vivo studies was higher for the EST (R^2: 0.85) than the ZET (R^2: 0.69). Combining the results of the ZET with those previously reported for the EST (Kamelia et al., 2017), the aryl hydrocarbon (AhR) CALUX assay (Kamelia et al., 2018), and the PAH content, ranked and clustered the test compounds in line with their in vivo potencies and chemical characteristics. Our findings indicate that the ZET does not outperform the EST as a stand-alone assay for testing PDT of PS, but confirm the hypothesis that PAHs are the major inducers of PDT by some PS, while they also indicate that the ZET is a useful addition to a battery of in vitro tests able to predict the in vivo PDT of PS.

1. Introduction

Petroleum substances (PS) are UVCBs (substances of Unknown or Variable composition, Complex reaction products and Biolog-ical materials) and regulated as such under the European Union (EU) REACH legislation (Registration, Evaluation, Authorisation, and Restriction of Chemicals). REACH has been implemented in the EU since 2007 to improve the protection of human health and the environment from the risks of chemical exposure. REACH legislation requires substances, including PS, that are manufactured or marketed in the EU at a volume of ≥100 tons/year to be tested for prenatal developmental toxicity (PDT) (ECHA, 2009). This is a new requirement that was not present in the previous legislation, hence PDT testing would be required for all existing substances produced over a volume of ≥100 tons/year. One of the consequences is that most PS (186 currently active registered EINECS numbers, all with a volume of > 1000 tons/year) will need to be tested for their potential adverse effect on prenatal development according to the Organisation for Economic Co-operation and Development (OECD) 414 testing guideline. Theoretically, this would require substantial numbers of experimental animals (> 2500 animals/test/compound; OECD, 2001) and involve a considerable amount of resources, both monetary and in terms of capacity (van der Jagt et al., 2004; Rovida et al., 2011). Therefore, the use of a battery of in vitro alternative assays, such as the zebrafish embryotoxicity test (ZET; Sipes et al., 2011; Strähle et al., 2012) and/or the embryonic stem cell test (EST; Spielmann, 2009), to group PS into a limited number of categories and/or to facilitate read-across from PS for which in vivo PDT data are already available, may reduce the number of animals and resources needed to study PDT potencies of PS.

The embryonic stem cell test (EST; Genschow et al., 2004), the whole embryo culture (WEC; Piersma et al., 2004), the limb bud micromass (Spielmann et al., 2004), and the zebrafish embryotoxicity test (ZET; OECD 2011a,b; Busquet et al., 2014), are the four in vitro alternative methods that have been validated for PDT testing. In the past decades, the ZET and EST have been the most used in vitro alternative assays to assess PDT of chemicals compared to the other two alternative methods (Incardona et al., 2004; de Jong et al., 2009; Selderslaghs et al., 2009; Louisse et al., 2011; Hawliczek et al., 2012; Kuske et al., 2012; Strikwold et al., 2012; Goodale et al., 2013; Li et al., 2015; Dimopoulou et al., 2018). The EST does not require harvesting of cells from animals since a permanent stem cell line is used. The validated EST consists of three different assays, including a cytotoxicity assay on differentiated T3 fibroblasts, a cytotoxicity assay on undifferentiated

ES-D3 cells, and a differentiation assay of ES-D3 cells into beating cardiomyocytes (Rohwedel et al., 2001; Genschow et al., 2002). The differentiation assay of the EST can be used to rank the potency of chemicals and define concentration-response curves for effects on cell differentiation and development (Seiler and Spielmann, 2011).

The ZET is another emerging alternative method for PDT testing. It makes use of a developing vertebrate organism to investigate the embryotoxicity potential of substances based on the notion that the development of the zebrafish embryo is very similar to the embryogenesis in higher vertebrates, including humans (Sipes et al., 2011). According to EU Directive 2010/63/EU, the ZET is considered an alternative method since the test does not exceed 5 days post-fertilization (dpf) and zebrafish embryos are considered free-living larvae during this time period (EU, 2010). The ZET makes use of newly fertilized eggs and a general morphology scoring (GMS) system, which includes the evaluation of mortality, and the development of tail, somite, heartbeat and eyes to assess the effects of chemicals on the development of zebrafish embryos (Hermsen et al., 2011; Jomaa et al., 2014; Beekhuijzen et al., 2015). The ZET offers several advantages over the EST and the other in vitro alternative assays for PDT testing. First, the ZET uses a complete embryo for the test and the chorion of a zebrafish embryo is transparent or is absent from around 2 dpf, facilitating detection of multiple developmental effects on multiple organs during testing (Glaberman et al., 2017). Good predictability, possibility for large-scale high-throughput hazard screening, short duration, ease to perform, and low cost make the ZET one of the most used alternative assays in in vitro PDT testing (Scholz et al., 2008; Kari et al., 2007; Brannen et al., 2010; Hill et al., 2011).

PS are complex materials, comprising hundreds to millions of different hydrocarbon constituents, including polycyclic aromatic hydrocarbons (PAHs). PAHs that are present in PS are generally known as petrogenic-origin or petroleum-associated hydrocarbons and differ substantially from PAHs of pyrogenic origin (Pampanin and Sydnes, 2003). The level and type of PAH constituents may vary depending on the source of the crude oil and the processing conditions used to manufacture the raw material (Speight, 2006). For instance, heavy PS, e.g. heavy fuel oil (HFO), untreated lubricating oils, and distillate aromatic extract (DAE), contain high amounts of high-molecular-weight (HMW) PAHs, i.e. 4- to 7-ring PAHs, where light PS, such as gas oil (GO), mainly contain low molecular weight (LMW) PAHs, i.e. 2-3 ring PAHs. In contrast to PS, which generally contain PAHs as one of their constituents, gas-to-liquid (GTL) products are modern synthetic analogues

of PS that typically consist of only saturated hydrocarbons and are virtually devoid of unsaturated and aromatic compounds (Boogaard et al., 2017).

Some PAH-containing PS have been reported to induce PDT in vivo and show effects in in vitro systems designed to test for PDT. Moreover, their potency has been shown to be associated with the presence of 3- to 7-ring PAHs in these products (Feuston et al., 1997; Mackerer et al., 2003; Gray et al., 2013; Kamelia et al., 2017). Animal studies have provided data on developmental toxicity effects induced by some PS, including increased incidence of resorptions (prenatal loss), decreased number of live fetuses/litter, and decreased fetal body weight (Mobil, 1989; ARCO, 1993; Hoberman et al., 1995; Feuston et al., 1996). On the other hand, GTL products, which contain no PAHs but have similar other properties to PS, were negative when tested in PDT studies as well as in two-generation reproductive toxicity studies (Dunster, 2014; Senn, 2014; Boogaard et al., 2017). Altogether, these data led to the hypothesis that the PDT as observed with certain PS is induced by particular 3-7 ring PAH constituents present in these products. In other words, heavier PS containing high concentrations of HMW PAHs may induce PDT where products with no or low concentrations of PAHs will not induce PDT (Tsitou et al., 2015; Kamelia et al., 2017).

This hypothesis is supported by our recent findings, where the EST and a panel of CALUX reporter gene assays were used to evaluate the PDT potency and possible underlying mechanism of PDT of the DMSO-extracts of PS and GTL products (Kamelia et al., 2017, 2018). In the EST, DMSO-extracts of 9 PS, varying in PAH level and content, were able to inhibit the differentiation of ES-D3 cells into beating cardiomyocytes, where GTL products that contain no PAHs showed no effects (Kamelia et al., 2017). Moreover, a pronounced aryl hydrocarbon (AhR)-mediated activity was found upon exposure to increasing concentrations of DMSO-extracts of the same PS, as tested in the AhR CALUX assay. This AhR-mediated activity correlated well with the in vitro PDT potency in the EST, suggesting an important role of the AhR in mediating this effect (Kamelia et al., 2018). These earlier results corroborated the notion that PAHs are the primary inducers of PDT in some PS and that the AhR may play an important role in the underlying mode of action. The present study aims to evaluate the PDT potency of the same substances, DMSO-extracts of 9 PS and 2 GTL products, in the ZET in order to investigate the suitability of the ZET to predict relative in vivo PDT of these PS extracts.

2. Materials and Methods

2.1 Test compounds

DMSO-extracts of 9 PS, varying in PAH content and concentration, and 2 GTL products, which contain no PAHs but have similar other properties to PS, were the model substances tested. The DMSO-extracts of the PS and GTL products were generated using the extraction procedure described by Roy et al. (1988), explained in some more detail in Section 2.2. The raw material of all PS and GTL products that were used for the DMSO extraction were provided by Concawe (Brussels, Belgium) and Shell International bv (The Hague, The Netherlands), respectively. These raw materials were: 1 heavy fuel oil (HFO; CAS no. 64741-62- 4), 3 distillate aromatic extracts (DAE; all 3 possessing the same CAS no. 64742-04-7), 2 residual aromatic extracts (RAE; CAS no. 64742-10-5 and 91995-70-9), 2 gas oils (GO; CAS no. 68915- 96-8 and 64741-43-1), 1 vacuum tower overhead oil (VTO; CAS no. 64741-49-7), 1 GTL base oil (GTLb; CAS no. 848301-69-9), and 1 GTL gas oil (GTLg; CAS no. 848301-67-7). An overview of the PAHs present in each PS and GTL product of the present study, grouped by the number of aromatic rings, is provided in Figure 1 (see also Appendix D).

2.2 PAH extraction and analysis

Extraction and analysis of the PS and GTL products was performed essentially as described before by Roy et al. (1988), and was carried out at Port Royal Research Laboratory (Hilton Head, South Carolina, USA). This extraction and analysis procedure is commonly used to obtain and quantify the PAH fraction from the raw material of PS (also known as Method II chemical characterization procedure), and has been widely used and validated also for mutagenicity and carcinogenicity testing of PS (Blackburn et al., 1986; Concawe, 1994; Clonfero et al., 1996; Mackerer et al., 2003). In brief, 4.0 g of PS or GTL product sample was dissolved in 10 ml of cyclohexane and extracted twice with 10 ml DMSO. The DMSO extracts were combined and diluted with twice the volume of 4% NaCl solution. The diluted DMSO fraction was back extracted with 20 and 10 ml of cyclohexane. The cyclohexane fractions were combined, washed twice with 5 ml of distilled water, and filtered through anhydrous sodium sulphate. The cyclohexane was then evaporated to dryness at 40°C followed by further evaporation at 80°C for 30 min. This residue was then

re-dissolved in cyclohexane (~50 mg/ml). The extracts were analyzed by gas chromatography with mass spectrometry (GC/MS). Naphthalene, phenanthrene, pyrene, benzo(a)pyrene, benzo[ghi-]perylene, and coronene were used as standards to define the boundaries of retention times for 2-7 ring PAHs. The results are presented as the aromatic ring class (ARC) profile, the weight percent of the DMSO-soluble 1- to ≥ 7-ring aromatic compounds present in each sample, from the starting raw material of 4.0 g (Figure 1).

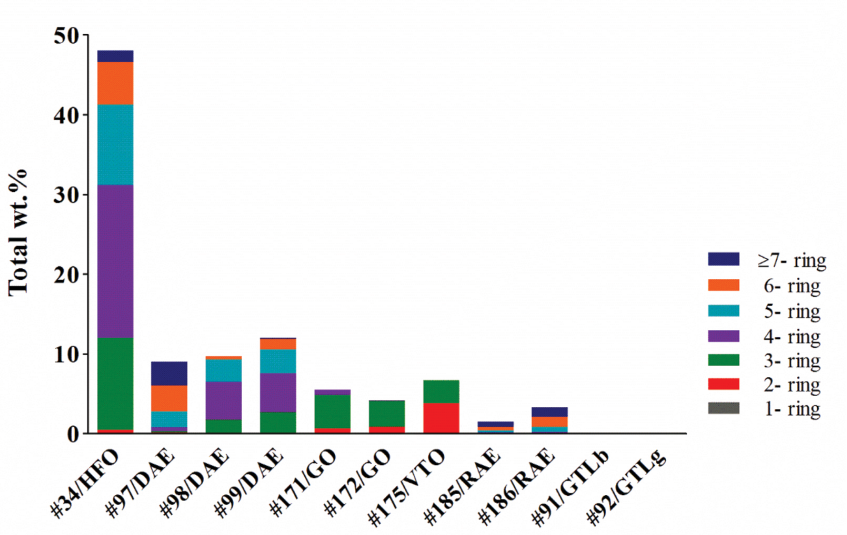


Figure 1 Aromatic ring class (ARC) profiles of 2 GTL products (contain no aromatics) and 9 PS (vary in PAH content, starting from 1.5% to 48% of total weight PAHs) tested in the present study. ARC profiles represent the weight percent of the DMSO-soluble 1- to ≥ 7 aromatic-ring compounds present in each PS and GTL product sample, from the starting raw material of 4.0 g, as determined using the Method II chemical characterization procedure described in detail by Roy et al. (1988).

Abbreviations. HFO, heavy fuel oil; DAE, distillate aromatic extract; GO, gas oil; VTO, vacuum tower overhead oil; RAE, residual aromatic extract; GTLb, gas-to-liquid base oil; GTLg, gas-to-liquid gas oil.

2.3 Zebrafish embryotoxicity test (ZET)

Wild-type adult zebrafish (*Danio rerio*) AB line were obtained from the research facility Carus, Wageningen University and Research, The Netherlands, and maintained in a flow-through aquarium system at 27°C with 14 h light/10 h dark cycle. The maintenance of

adult zebrafish was done in accordance with the protocols of the Zebrafish Handbook. Zebrafish eggs were produced via spawning groups by placing male and female fish (at a ratio of 1:2) in an individual spawning tank equipped with spawn traps several hours prior to the onset of darkness on the day before the test. A minimum of three parallel spawning tanks was set for the egg production for each independent experiment. Mating, spawning, and fertilization take place within 30 min to 1 h after the onset of the light cycle.

Spawned eggs were collected, rinsed a few times with egg water (1.5 ml salt stock solution in 1 L distilled water), and incubated at 26°C until further use for egg selection. The salt stock solution was prepared by dissolving 40 g "Instant Ocean" sea salt (Blacksburg, Virginia, USA) in 1 liter distilled water. The egg water was aerated for several hours, adjusted for pH (range of pH 7-8), and kept at 26-28°C prior to use. Egg water was used throughout the ZET as the assay medium. Egg selection was done by sorting the zebrafish embryo at the 8- to 32-cell stage using a stereomicroscope and disposable pipette, choosing embryos that followed a normal development, which were pooled in Petri dishes for further use. Zebrafish embryos with obvious anomalies, such as coagulated embryos, and unfertilized eggs, were discarded. The ZET was initiated at 4-5 h post fertilization (hpf) and terminated at 96 hpf, as this covers the entire organogenesis in a zebrafish embryo. The exposure was performed in 24-well plates (Greiner Bio-one, Frickenhausen, Germany) in combination with a self-adhesive film cover (Sigma-Aldrich, Zwijndrecht, The Netherlands). 4-5 hpf was chosen as the start time of exposure as this covers the same development stages as in the developmental toxicity studies performed according to the OECD 414 guideline in rat and rabbit (Beekhuijzen et al., 2015). Hence, this allows comparison of results between the ZET and in vivo PDT studies.

Twenty wells of the 24-well plate were used for exposure to one concentration of test compound and the other four wells were used for the internal plate control. Every well of the internal plate control, in the exposed-plate, contained a zebrafish embryo in egg water (negative control), and if more than 1 dead embryo, out of 4 embryos, was observed in these internal plate control wells, the plate was rejected and the results from the respective plate were not used. The exposure medium was prepared by mixing the 400-times concentrated stock solutions of the test compounds (dissolved in DMSO) with egg water. The exposure medium was then transferred into 20 wells of the 24-well plate, at 2 ml exposure medium/well, and for the internal plate control, 2 ml egg water was added

into each of the 4 remaining wells. The plate was sealed with self-adhesive film cover to prevent evaporation of test compound throughout the exposure period (up to 96 hpf). All samples were tested at a range of concentrations up to 250 µg raw material/ml, except for sample #034-HFO, which could be dosed up to only 15 µg raw material/ml due to solubility limitations in egg water. Solvent controls (0.25% v/v DMSO; Merck, Darmstadt, Germany), positive controls (4 µg/ml 3,4-dichloroaniline; Sigma-Aldrich), and negative controls (egg water only) were included in each independent experiment. Plates were incubated at 26°C with a photo period of 14 h light:10 h dark. Embryos were scored daily (every 24 h) for developmental abnormalities and cumulative mortality using the inverted microscope until 96 hpf (the daily assessment time points correspond to 28, 52, 76, and 100 hpf), based on the extended general morphological scoring (GMS) system described by Beekhuijzen et al. (2015). Deviation from normal developmental stages, for example incomplete detachment of tail, incomplete development of eyes, fin, and mouth, unhatched embryos, results in a lower total GMS value corresponding to a certain extent of developmental retardation. A comparison of results from the daily morphological assessment using the GMS reveals that the most significant results upon exposure to test compounds were achieved with an exposure time window of 0-96 hpf (Appendix E1). The GMS used for the exposure time window of 0-96 hpf is based on the 96 hpf endpoints, as listed and described in detail by Beekhuijzen et al. (2015). Exposure time windows of 0-24/0-48/0-72 hpf did not show notable differences between solvent control and test compounds, hence the GMS data of 0-96 hpf were used for further data analysis and comparison, including comparison with published in vivo data or PAH content present in each PS sample (Section 2.7). The ZET results of each test compound were presented as fraction of GMS at 96 hpf compared to that of the solvent control (0.25% DMSO). The ZET was considered valid if the following was observed: ≤ 1 dead embryo (out of 4) in the internal plate control of every exposed-plate; ≤ 3 dead embryos (out of 24) in the negative control plate (at least 87.5% survival rate); ≤ 2 dead embryos (out of 20) in the solvent control plate (0.25% DMSO); ≤ 14 live embryos (out of 20) in the positive control plate (4 µg/ml 3,4-dicholoaniline; exposure to positive control should result in a minimum of 30% mortality by 96 hpf). At least 4 independent ZET experiments were performed for each test compound in which the same stock dilutions were used to prepare the concentration ranges tested. A graphical illustration of the ZET performed in the present study and the

layout of 24-well plates for the negative, positive, and solvent controls are shown in Appendix H.

2.4 Embryonic stem cell test (EST)

The in vitro developmental toxicity data of the DMSO-extracts of 9 PS and 2 GTL products, as tested in the EST, were taken from our previous study (Kamelia et al., 2017). In short, the effects on cell viability and cell differentiation upon exposure to increasing concentrations of test compounds were evaluated in the ES-D3 cell viability and ES-D3 cell differentiation assays of the EST.

2.5 Aryl hydrocarbon (AhR) CALUX reporter gene assay

Data for the AhR-mediated activity of the DMSO-extracts of 9 PS and 2 GTL products, as tested in the AhR CALUX assay, were taken from our recently published study (Kamelia et al., 2018). Briefly, H4IIE.luc cells were exposed to increasing concentrations of the DMSO-extracts of the aforementioned substances for 6 h. The luciferase induction activity upon exposure was then measured, and the amount of produced luminescence was used to quantify the AhR-mediated activity induced by the respective test compound.

2.6 Data analysis

Concentration-response curves upon exposure to the DMSO-extracts of PS and GTL products in the ZET were made using GraphPad Prism 5.0 (California, US). Here, data were fitted to a sigmoid concentration-response curve with three parameters. These curves were used only for graphical illustration of the obtained results, and not for the determination of benchmark concentration (BMC) values. Results obtained from the ZET were expressed as fraction of total GMS score at 96 hpf compared to the solvent control (0.25% DMSO) and are presented as mean ± standard error of the mean (SEM). The DMSO concentration was kept at 0.25% v/v throughout the ZET experiments.

Benchmark concentration (BMC) derivation of in vitro *developmental toxicity data*

Results from the ZET were analyzed using the Benchmark Dose software (BMD software US-EPA version 2.6.1) to obtain the benchmark concentration at a benchmark response (BMR) of 50% effect (BMC50). For this purpose, concentration-response curves from the ZET were fitted to all dichotomous concentration-response models (gamma, logistic, log-logistic, probit, log-probit, weibull, multistage-cancer, multistage, and the quantal-linear models; Appendix A) available in the BMD software US-EPA version 2.6.1. The BMR was set to 50%, representing 50% developmental retardation (BMC50) in the ZET upon exposure to the DMSO-extracts of PS and GTL products. The performance of each fitted model was evaluated based on the goodness-of-fit, the scaled residuals, and the visual inspection of model fitting. To allow comparison to data obtained previously in the EST (Kamelia et al., 2017), the respective concentration-response curves of the EST were analyzed in a similar manner. The BMC50 values thus obtained (Table 1), which account for 50% inhibition of cell differentiation (EST; BMCd50-EST) or 50% developmental retardation (ZET; BMC50-ZET), were selected from all accepted models based on the lowest Akaike's Information Criterion (AIC) (Kamelia et al., 2017; Haber et al., 2018) and were further used for the in vitro-in vitro (ZET to EST; ZET to AhR CALUX assay) and the in vitro-in vivo comparisons.

Benchmark dose (BMD) modeling of in vivo developmental toxicity data

In vivo data, derived from published PDT studies available for some of the PS of the present study, were used to determine the in vivo benchmark dose (BMD) values of the corresponding PS. In vivo PDT data of GO (ARCO, 1993) and VTO (Mobil, 1989) were kindly provided by Concawe, and the in vivo data of the HFO and DAE were obtained from Feuston et al. (1996) and Hoberman et al. (1995), respectively. Different developmental toxicity endpoints were reported in these studies, such as increased resorptions, number of live fetuses/litter, decreased fetal body weight, and skeletal variations. Increased resorptions, number of live fetuses/litter, decreased fetal body weight, and not skeletal variations, were selected for the BMD analysis, because all studies contained data on incidence of resorptions, number of live fetuses/litter, and fetal body weight, whereas the studies of HFO and DAE did not provide data on skeletal variations. To determine BMD

values, in vivo data for fetal body weight and number of live fetuses/litter were fitted to the continuous models (hill, exponential, linear, polynomial, and power models) and the data from the incidence of resorptions were fitted to the dichotomous models (gamma, logistic, log-logistic, probit, log-probit, weibull, multistage-cancer, multistage, and the quantal-linear models), of the BMD software US-EPA version 2.6.1. For these BMD analyses, the BMR was set to 10%, which represents a 10% decrease in fetal body weight (BMD10-fetal body weight), 10% decrease in number of live fetuses/litter (BMD10- live fetuses/litter; Appendix B1), and a 10% additional incidence of resorptions (BMD10-increased resorptions). 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 BMD10 values (Table 1) derived from the models with the best fit and lowest AIC were selected and further used for the in vitro-in vivo comparison.

2.7 Correlation analysis

Correlation of in vitro potencies obtained from three distinct in vitro assays: ZET, EST, and AhR CALUX assay

Using a linear regression approach (in GraphPad Prism 5.0), the correlation between in vitro potencies obtained from three different assays that evaluate the PDT potency and underlying mechanism of PDT of PS and GTL products, i.e. the ZET, EST, and AhR CALUX assay, were determined. Data for the EST and AhR-mediated activity were taken from our previous studies (Kamelia et al., 2017; 2018; Section 2.4 and 2.5, respectively). The correlation analyses were conducted between in vitro potencies (BMC50s/EC50s) of the test compounds between each duo of the three different in vitro assays, including ZET and EST, ZET and AhR CALUX assay, and EST and AhR CALUX assay. The given R-squared (R^2) value reflects the goodness-of-fit of data to the fitted regression line and was considered statistically significant if the p-value was lower than 0.05.

Table 1 Overview of in vivo and in vitro developmental toxicity data upon exposure to PS and GTL products.

Compound	CAS no.	In vivo data[a]			In vitro data		
		BMD10 increased resorptions (mg/kg bw/day)[b]	BMD10 number of live fetuses/litter (mg/kg bw/day)[c]	BMD10 fetal body weight (mg/kg bw/day)[d]	BMC50 ZET (µg/ml)	BMCd50 EST (µg/ml)[g]	EC50 AhR CALUX (µg/ml)[h]
#34 - HFO	64741-62-4	0.32	0.59	20.64	1.32	0.47	0.0037
#97 - DAE	64742-04-7	13.89	44.38	28.53	76.71	3.74	0.033
#98 - DAE	64742-04-7	13.89	44.38	28.53	21.82	5.05	0.012
#99 - DAE	64742-04-7	13.89	44.38	28.53	21.23	7.42	0.012
#171 - GO	68915-96-8	n.a	n.a	n.a	54.53	171	0.084
#172 - GO	64741-43-1	270.05	174.04	245.21	73.13	142	0.35
#175 - VTO	64741-49-7	151.16	144.03	332.64	64.77	67.2	0.40
#185 - RAE	64742-10-5	n.a	n.a	n.a	>250[f]	89.5	0.40
#186 - RAE	91995-70-9	n.a	n.a	n.a	>250[f]	95.2	0.13
#91 - GTLb	848301-69-9	(-)[e]	(-)[e]	(-)[e]	(-)	(-)	(-)
#92 - GTLg	848301-67-7	(-)[e]	(-)[e]	(-)[e]	(-)	(-)	(-)

[a]*In vivo* data for determination of the BMD10 values from different developmental toxicity endpoints were taken from Hoberman et al. (1995) for the HFO; Feuston et al. (1996) for the DAE; ARCO (1993) for the GO; and Mobil (1989) for the VTO.
[b]BMD10 increased resorptions: dose (mg/kg bw/day) giving 10% increase in incidence of resorptions. BMD10 values were calculated from a dose-response curve using a dichotomous model (BMD software US-EPA) and data were taken from cited studies.
[c]BMD10 number of live fetuses/litter: dose (mg/kg bw/day) giving 10% decrease in live fetuses/litter. BMD10 values were calculated from a dose-response curve using a continuous model (BMD software US-EPA) and data were taken from cited studies.
[d]BMD10 fetal body weight: dose (mg/kg bw/day) giving 10% decrease in fetal body weight. BMD10 values were calculated from a dose-response curve using a continuous model (BMD software US-EPA) and data were taken from cited studies.
[e]Negative for prenatal developmental toxicity studies; data were taken from Senn (2014) for the GTLb and Dunster (2014) for the GTLg.
[f]The calculated BMC50 values are above the highest tested concentration of 250 µg/ml.
[g]The BMCd50s-EST is taken from Kamelia et al., 2017.
[h]The EC50s-AhR CALUX is taken from Kamelia et al. (2018).
HFO, heavy fuel oil; DAE, distillate aromatic extract; GO, gas oil; VTO, vacuum tower overhead; RAE, residual aromatic extract; GTLb, gas-to-liquid base oil; GTLg, gas-to-liquid gas oil; n.a, data not available; (–), negative; AhR, aryl hydrocarbon receptor; CALUX, chemical activated luciferase gene expression.

Correlation between in vitro developmental toxicity potency in the ZET and the in vivo developmental toxicity data or the PAH content

In vitro potencies obtained in the ZET were compared to potencies derived from in vivo studies to see whether any correlation exists between in vitro and in vivo PDT potencies. To this end, the BMC50-ZET values were plotted against the BMD10 values, obtained as described in Section 2.6. No relevant report/literature on an in vivo developmental toxicity study was available for the RAE samples, and for that reason they were not included in the in vitro-in vivo comparison. Furthermore, correlations between in vitro potencies in the ZET and specific PAH constituents present in each PS sample were also investigated. All these correlation analyses were done by performing a linear regression analysis in GraphPad Prism 5.0. The given R2 value reflects the goodness-of-fit of data to the fitted regression line and was considered statistically significant if the p-value was lower than 0.05.

2.8 Data integration and visualization in ToxPi GUI 2.0

ToxPi Graphical User Interface 2.0 (ToxPi GUI 2.0; Reif et al., 2010; Marvel et al., 2018) was used for data integration, visualization, and comparison of effect-signatures from different in vitro assays and the chemical analysis of the 9 PS and 2 GTL products. The in vitro potencies (BMC50s/EC50s) in the three different assays (EST, ZET, and AhR CALUX assay), and total PAH content (mg/ml) present in each test compound, were used as point of departure (POD) for ToxPi data input. In cases where no effects were observed, for example the GTL products showed negative results in all of the selected assays, the highest tested concentration was used as the POD value for the respective test compound. Briefly, the POD values (BMC50s/EC50s/total PAH content) of each test compound were listed and inversely normalized on a 0-1 scale. The value of 0 represents the lowest bioactivity in the corresponding in vitro assay (the least potent compound of all in the corresponding in vitro assay). The value of 1 represents the highest bioactivity or the most potent test compound of all in a given data set. For the chemical analysis data (PAH profiles), the value of 0 represents the test compound that contains the lowest PAH content, i.e. GTL products (0% PAHs), and the value of 1 represents the test compound with the highest PAH content, which is sample #034-HFO (48% PAHs). These normalized

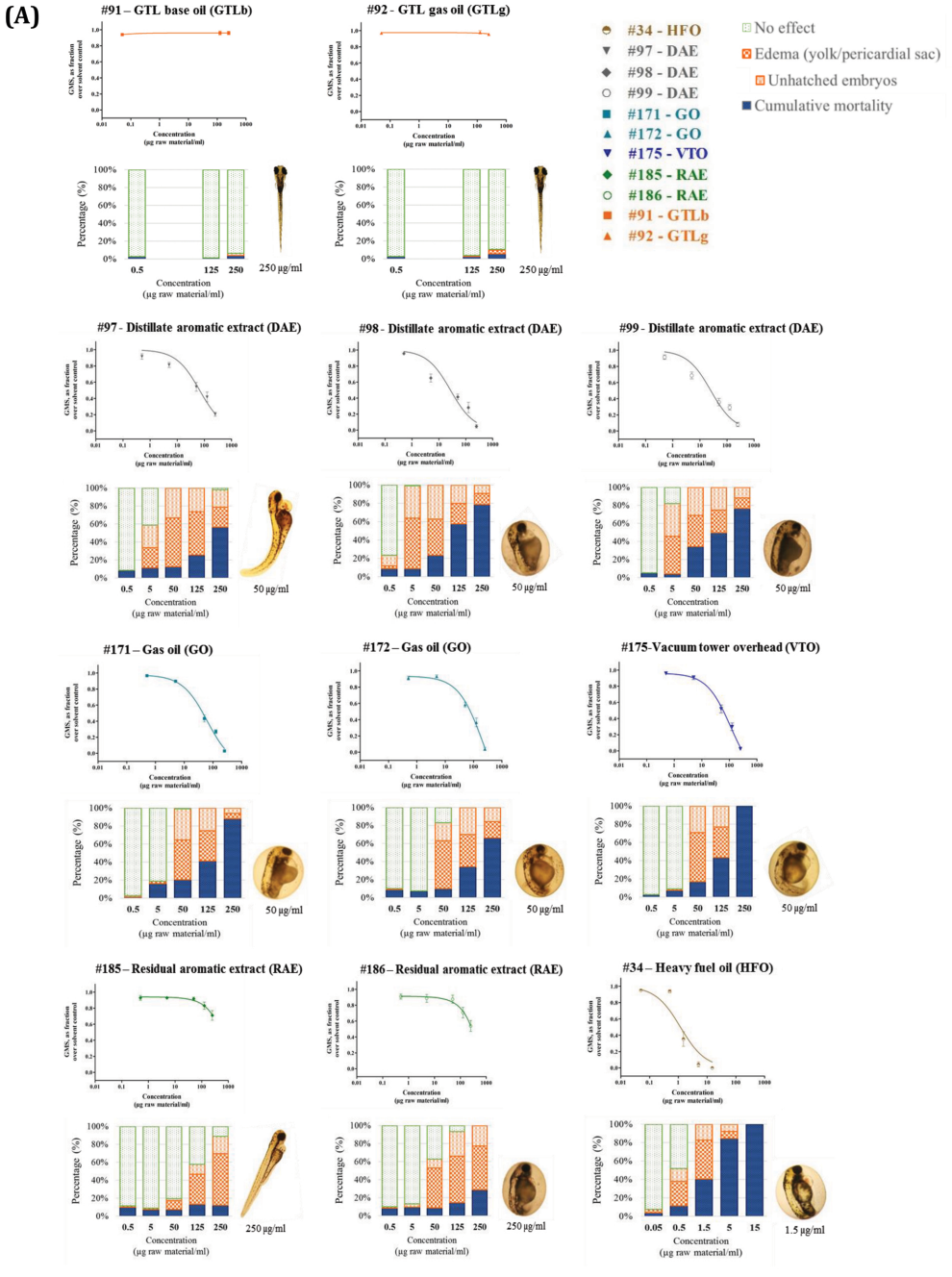
POD values were then used as quantitative inputs for bioactivity profiling in ToxPi (Grimm et al., 2016). Further, the ToxPi score was calculated based on the equation provided below, and from this the bioactivity profiling (ToxPi pie-charts), hierarchical clustering, and chemical rank were generated. The terms POD min and POD max that were used to calculate the ToxPi score represent the lowest and highest POD, respectively, observed within one corresponding assay of the present study, i.e. within the ZET. The average method was used for the development of the hierarchical clustering in ToxPi.

$$ToxPi\ score = 1 - \frac{log10\ POD - log10\ POD\ min.}{log10\ POD\ max. - \log 10\ POD\ min.}$$

3. Results

3.1 Zebrafish embryotoxicity test (ZET)

As shown in Figure 2A, exposure to increasing concentrations of the DMSO-extracts of PS induced concentration-dependent developmental retardation in zebrafish embryos (scored at 96 hpf), while both GTL products showed no effects (data are provided in Appendix C1). The effects induced by PS in the ZET at this time point include the absence of circulation and movement, delayed development (i.e. unhatched embryos), deformed body shape (dorsal curvature), the reduction of body and tail length, and malformations of the heart and yolk sac (Figure 2B). Figure 2A presents the 100% stacked-bar graph illustrating the percentage of particular developmental anomalies at 96 hpf, including pericardial and yolk sac edemas (in both hatched and unhatched embryos) and cumulative mortality, below the concentration-response curves of the GMS. It should be noted that the deformed body shape and the reduction of body and tail length can only be observed when the embryo has hatched at 96 hpf.


(A)
#91 – GTL base oil (GTLb)
#92 - GTL gas oil (GTLg)
#34 - HFO
#97 - DAE
#98 - DAE
#99 - DAE
#171 - GO
#172 - GO
#175 - VTO
#185 - RAE
#186 - RAE
#91 - GTLb
#92 - GTLg
No effect
Edema (yolk/pericardial sac)
Unhatched embryos
Cumulative mortality
#97 - Distillate aromatic extract (DAE)
#98 - Distillate aromatic extract (DAE)
#99 - Distillate aromatic extract (DAE)
#171 – Gas oil (GO)
#172 – Gas oil (GO)
#175-Vacuum tower overhead (VTO)
#185 – Residual aromatic extract (RAE)
#186 – Residual aromatic extract (RAE)
#34 – Heavy fuel oil (HFO)
GMS, as fraction over solvent control
Concentration (µg raw material/ml)
Percentage (%)
50 µg/ml
250 µg/ml
1.5 µg/ml

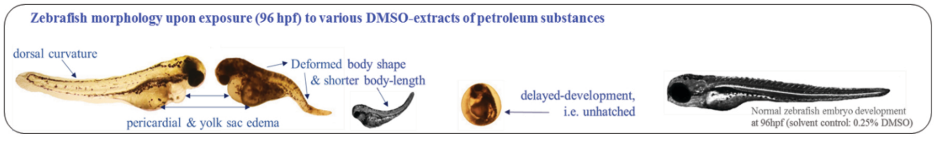

(B)
Zebrafish morphology upon exposure (96 hpf) to various DMSO-extracts of petroleum substances
dorsal curvature
Deformed body shape
& shorter body-length
pericardial & yolk sac edema
delayed-development,
i.e. unhatched
Normal zebrafish embryo development
at 96hpf (solvent control: 0.25% DMSO)

Figure 2 Effects of PS and GTL products on zebrafish embryo development. (A) Concentration-dependent effects of DMSO-extracts of PS and GTL products on zebrafish embryo development at 96 hpf, based on the extended general morphology scoring (GMS) system. Results represent data from at least four independent experiments (n ≥ 4) and are presented as mean ± standard error of the mean (SEM). Bar graph illustrating the typical developmental effects upon exposure to PAH-containing substances, in addition to what was scored using the GMS, including pericardial and yolk sac edemas, delayed development, i.e. unhatched embryos, and cumulative mortality were also scored at 96 hpf and are presented below the concentration-response curve of each test compound. Results, as illustrated in the bar graph, are presented as percentage of embryos with the aforementioned developmental effects based on n ≥ 4 with 20 embryos/concentration/ experiment. (B) Zebrafish morphology at 96 hpf upon exposure to various DMSO-extracts of PS.

Abbreviations. HFO, heavy fuel oil; DAE, distillate aromatic extract; GO, gas oil; VTO, vacuum tower overhead oil; RAE, residual aromatic extract; GTLb, gas-to-liquid base oil; GTLg, gas-to-liquid gas oil; hpf, hours post fertilization.

As a consequence, only the occurrence of pericardial and yolk sac edema, unhatched embryos, and cumulative mortality were noted and presented here in the stack-bar graph of Figure 2A. From the stacked-bar graph, it is clear that the percentage of cumulative mortality increases with the concentration of the test compounds, except for the RAE samples. The main manifestations upon exposure to increasing concentrations of RAE samples were pericardial and yolk-sac edemas. It is worth mentioning that the high incidence of pericardial and yolk sac edemas induced by heavy PS like HFO and DAEs happened at a lower concentration, ranging from 0.5 (HFO) to 5 µg/ml (DAE), than for the light PS, such as GO and VTO, for which the effect occurred only from 50 µg/ml onwards.

Using the concentration-response curves of the GMS, the BMC50-ZET value was determined and the results were listed in Table 1 and presented in Figure 3. Among all samples tested, sample #034-HFO appeared to be the most potent substance in inducing the developmental retardation of zebrafish embryos, followed by two of the DAE samples (#098 and #099), then the GO, VTO, and sample #097-DAE, and lastly the RAE samples (Figure 3). For RAE samples, a decline in the GMS occurred only at the highest tested concentrations (Figure 2A and 3), thus, the obtained BMC50 values of these two samples

were above the highest tested concentration of 250 µg/ml (Table 1). The BMC50 values for both of the GTL products could not be determined as they did not induce any effects in the ZET.

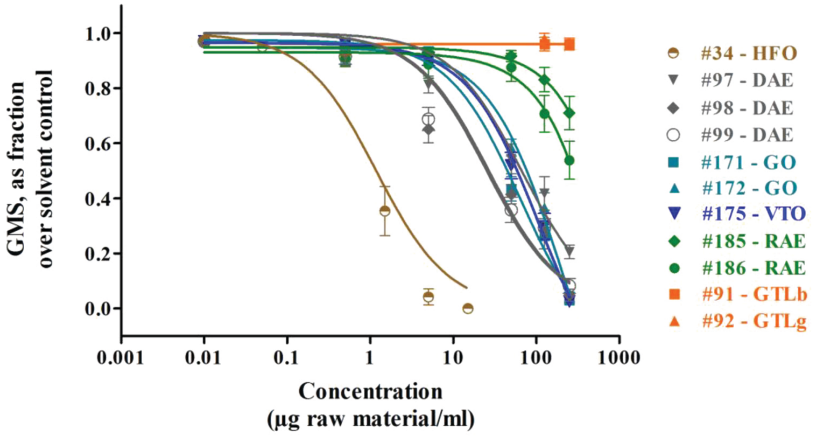


Figure 3 Comparison of the concentration-dependent inhibition of zebrafish embryo development in the ZET (at 96 hpf) upon exposure to DMSO-extracts of PS and GTL products. Results represent data from at least four independent experiments (n ≥ 4) and are presented as mean ± standard error of the mean (SEM).

Abbreviations. HFO, heavy fuel oil; DAE, distillate aromatic extract; GO, gas oil; VTO, vacuum tower overhead oil; RAE, residual aromatic extract; GTLb, gas-to-liquid base oil; GTLg, gas-to-liquid gas oil; hpf, hours post fertilization

3.2 Correlation between in vitro developmental toxicity in the ZET and EST, and between the in vitro PDT in the ZET or EST and the observed effects in the AhR-mediated gene expression assay testing the same substances

The results obtained in the ZET were compared to results obtained in our previous studies (Kamelia et al., 2017, 2018) for the same DMSO-extracts of PS and GTL products tested in the EST and AhR CALUX assay. Figure 4 presents the correlation between the results obtained in these in vitro assays. The in vitro potencies in the ZET had a moderate correlation with either the EST (R^2: 0.61; Figure 4A) or the AhR CALUX assay (R^2: 0.66; Figure 4B). Further, a good correlation also exists between the in vitro potencies in the EST and the AhR CALUX assay, showing an R^2 value of 0.80 (Kamelia et al., 2018; data and graph are provided in Appendix E).

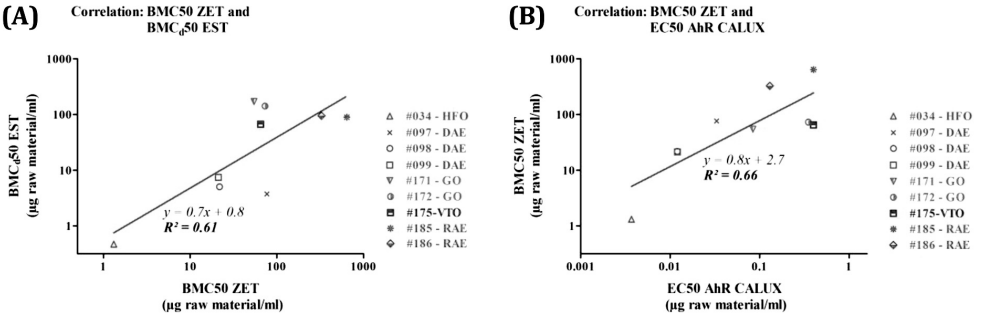


Figure 4 Correlation between (A) developmental toxicity potency of PS in the ZET and the EST; (B) developmental toxicity potency of PS in the ZET, expressed as BMC50s, and agonist activity of the same substances in the AhR CALUX assay, expressed as EC50s. Data of the PS in the EST and AhR CALUX assay were taken from our previous published studies Kamelia et al. 2017 and 2018, respectively.

Abbreviations. HFO, heavy fuel oil; DAE, distillate aromatic extract; GO, gas oil; VTO, vacuum tower overhead oil; RAE, residual aromatic extract; GTLb, gas-to-liquid base oil; GTLg, gas-to-liquid gas oil.

3.3 Correlation between in vitro developmental toxicity potency in the ZET and potencies observed *in vivo*

Linear regression analysis was performed to see whether a correlation exists between the obtained in vitro potencies in the ZET and published in vivo developmental toxicity data of the selected PS samples. For this purpose, the BMC50s-ZET were plotted against the BMD10 values for increased resorptions, number of live fetuses/litter, and fetal body weight endpoints. As depicted in Figure 5A, the in vitro potencies in the ZET correlate best with the developmental effect reflected by the number of live fetuses/litter (R^2: 0.91), and also correlate well with the increased resorptions endpoint (R^2: 0.80). However, a very poor correlation was obtained when plotting the BMC50s-ZET with the BMD10s of the fetal body weight endpoint, resulting in an R^2 of 0.35 (Figure 5A). To sum up, good correlations exist between in vitro potencies in the ZET and potencies observed in vivo, giving an average R^2 for in vitro-in vivo correlation (from three different PDT endpoints) of 0.69.

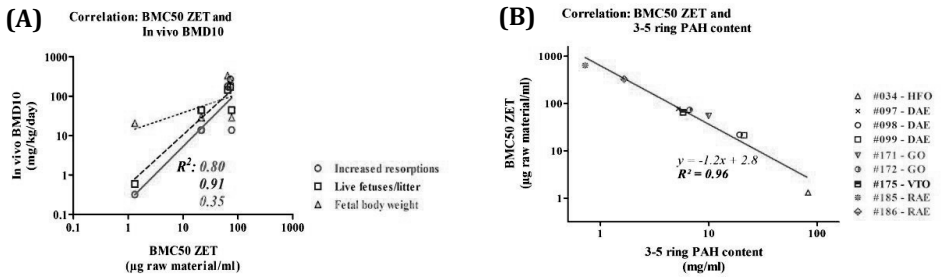


Figure 5 (A) Correlation between BMC50 values, obtained from the ZET, and in vivo BMD10 values based on three different developmental toxicity endpoints: increased incidence of resorptions, number of live fetuses/litter, and fetal body weight; (B) Correlation between PDT potencies in the ZET (BMC50s) and 3-5 ring PAH content present in each PS sample BMD10: the benchmark dose for 10% decrease in fetal body weight (BMD10-fetal body weight), 10% decrease in number of live fetuses/litter (BMD10-live fetuses/litter), and a 10% additional incidence of resorptions (BMD10-increased resorptions), as determined using the BMD software (US-EPA); BMC50: the benchmark concentration at which the fraction of GMS is reduced by 50% in the ZET or EST.

Abbreviations. HFO, heavy fuel oil; DAE, distillate aromatic extract; GO, gas oil; VTO, vacuum tower overhead oil; RAE, residual aromatic extract; GTLb, gas-to-liquid base oil; GTLg, gas-to-liquid gas oil.

3.4 Relation of in vitro developmental toxicity potency in the ZET to specific PAH content present in each PS sample

The in vitro potencies obtained from the ZET, expressed as BMC50s, were also compared to the amount and type of PAHs in each of the PS samples to see if there is any relation between the observed in vitro effects and their PAH content. Data correlations were made with the amount of 2- to 7-ring PAHs present in PS samples, in total giving 15 combinations/assay for which the correlation between PAH content and BMC50s were determined. The results and details for all of these correlation analyses are provided in Appendix F. The goodness-of-fit of correlation analysis is expressed in the R^2 value and the most relevant correlation with the highest R2 is presented in Figure 5B. The in vitro potencies in the ZET were best correlated to the amount of 3- to 5-ring PAHs present in the corresponding PS sample (R^2: 0.96; Figure 5B).

3.5 Bioactivity profiling and grouping of the DMSO-extracts of PS and GTL products

Figure 6 shows the bioactivity profiling (ToxPi pie-charts) and grouping of the DMSO-extracts of 9 PS and 2 GTL products based on the data obtained from the three different in vitro assays: ZET, EST, and AhR CALUX assay, and the chemical analysis (PAH content) of the respective test compound. In the resulting ToxPi pie-charts (Figure 6; top), each slice is associated with a specific variable (bioassay/chemical analysis data), and the area covered by the slice is proportional to the relative activity (defined by ToxPi score equation, see Section 2.8) of the test compound within this data set. The red slice in the pie-chart represents data from the EST (BMC50 EST; Kamelia et al., 2017), the green slice represents data from the AhR CALUX assay (EC50 AhR CALUX assay; Kamelia et al., 2018), the blue slice reflects data from the ZET (BMC50 ZET), and the yellow slice portrays the level of PAHs (mg/ml) present in each test compound. It is worth mentioning that the pie-chart was developed by giving the same weight to each dataset: EST, AhR CALUX assay, ZET, and PAH content, in each slice of the pie-chart. This means, each dataset has 25% weight in the pie-chart to make a total of 100%. Looking closely at the bioactivity profiles illustrated by the ToxPi pie-charts, similarities in bioactivity profiles of test compounds that belong to the same class of substances were also observed (Figure 6; top).

ToxPi also derives a global chemical rank based on the cumulative ToxPi score (Figure 6; bottom). The cumulative ToxPi score was calculated based on the sum and average of all 4 slice areas (see Section 2.8 for the ToxPi score equation and determination), in which each area represents the ToxPi score from one input-variable (EST/AhR CALUX assay/ZET/PAH content). A high ToxPi score reflects a higher activity (a more potent) and a low ToxPi score reflects a lower activity (a less potent) of the corresponding test compound in all of the assays used for the present study. As shown in Figure 6 (bottom), the global chemical rank showed that sample #034-HFO (ToxPi score: 1) was the most active/potent compound, where both of the RAEs (ToxPi score: 0.16-0.20) were the least active test compounds of the present study. Furthermore, the DAEs, GOs, and VTO were ranked in between the HFO and RAE, with DAEs (ToxPi score: 0.45-0.54) being more active/potent compared to the GOs and VTO (ToxPi score: 0.22-0.28). Both of the GTL products showed no effects in any of the assays: EST, AhR CALUX assay, and ZET, while they also contain no PAH (0% PAHs), hence their cumulative ToxPi score was 0. As a consequence, they occupy the two lowest ranks in the global chemical rank. Altogether, in

the ToxPi score, albeit based on four endpoints, test compounds were ranked proportionally according to their PAH level from the highest, HFO, to the lowest PAH content, RAEs. The list of cumulative ToxPi scores presented in Figure 6 is provided in Appendix G.

In addition to the bioactivity profiling (ToxPi pie-charts) and global chemical rank also a hierarchical clustering was obtained from ToxPi GUI 2.0. The dendrogram of the hierarchical clustering (Figure 6; top) reveals that two main clusters of test compounds can be distinguished on the basis of their potencies in the three different in vitro assays and their PAH content. The GTL extracts are grouped in one cluster and all PS extracts are grouped in another cluster (Figure 6; top). Within the cluster of PS extracts, test compounds that belong to the same class of substances are grouped together, for example DAEs and RAEs. VTO appears to be in the same cluster with the GOs, as a result of having similar PAH profiles (Figure 1) and of inducing similar effects in the ZET, EST, and AhR CALUX assay.

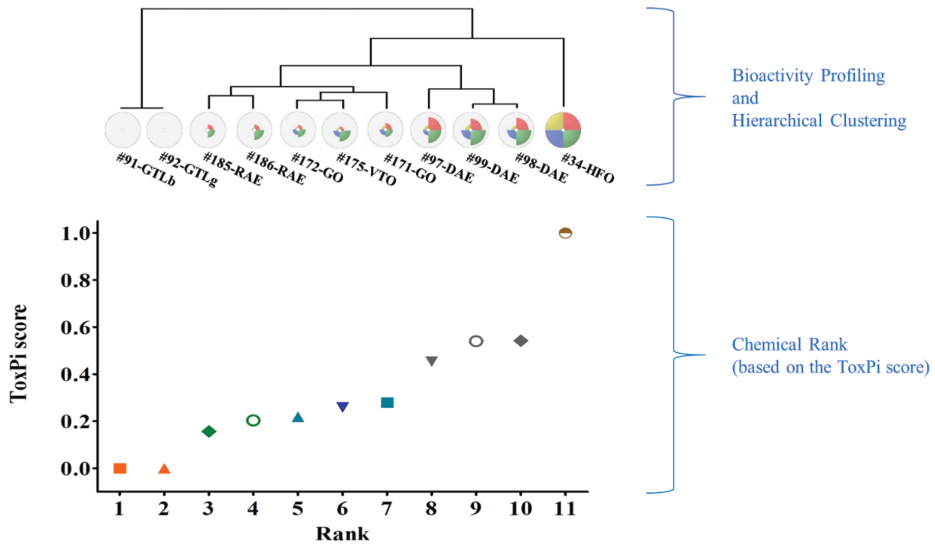


Figure 6 Bioactivity profiling, hierarchical clustering, and chemical ranking using ToxPi GUI 2.0. Bioactivity profiling, illustrated by the pie-charts, represents data from combinatorial integration of in vitro potencies of the DMSO-extracts of 9 PS and 2 GTL products (expressed either as BMC50s or EC50s) from three different in vitro assays: ZET (blue quadrant), EST (red quadrant), and AhR CALUX assay (green quadrant); and data

from the chemical analysis for PAH content (yellow quadrant) present in each of these substances. The same data were used as data input for hierarchical clustering and global chemical rank in ToxPi.

Abbreviations. HFO, heavy fuel oil; DAE, distillate aromatic extract; GO, gas oil; VTO, vacuum tower overhead oil; RAE, residual aromatic extract; GTLb, gas-to-liquid base oil; GTLg, gas-to-liquid gas oil; AhR, aryl hydrocarbon; CALUX, chemical activated luciferase gene expression.

4. Discussion and conclusions

Published in vivo studies show that some PS are able to cause PDT in pregnant rats, and it is suspected that this adverse effect is induced by specific constituents present in these substances, mainly 3- to 7-ring PAHs (Feuston et al., 1994; Murray et al., 2013). The present study evaluates the applicability of the ZET to assess PDT potency of the DMSO-extracts of 9 PS and 2 GTL products. Our results indicate that all PS extracts, varying in PAH level and content, were able to inhibit the development of zebrafish embryos in a concentration-dependent manner and this potency is associated with their 3-5 ring PAH content. On the contrary, DMSO-extracts of both GTL products, with no aromatics, showed no effects in the ZET. This points to a major role of PAHs present in the PS extracts used in the study in causing the PDT effects observed in the ZET.

Prominent developmental aberrations, specifically pericardial and yolk sac edemas, dorsal curvature (only in the hatched embryo at 96 hpf), and cumulative mortality, were observed in the zebrafish embryo exposed to PS extracts in addition to the effects scored using the extended GMS. This particular observation is believed to be compound-specific, or only induced by PAHs and PAH-containing PS. These findings are in line with what has been reported in the literature, where exposure to individual PAHs and mixtures of PAHs caused similar developmental effects (Goodale et al., 2013; Gu et al., 2010; Huang et al., 2012; Zhang et al., 2012; Wincent et al., 2015; Geier et al., 2018). For example, studies by Incardona et al. (2004, 2006, 2011) showed that some 3-ring (fluorene, phenanthrene, dibenzothiophene), 4-ring (pyrene, benz[a]anthracene), and 5-ring PAHs (benzo[a]pyrene, benzo[k]fluoranthene), and mixtures of PAHs cause severe pericardial and yolk sac edemas, dorsal curvature of the trunk and tail, and growth retardation in the exposed-zebrafish embryo at 48-96 hpf. In addition, these authors also concluded that the relative amount of specific PAH constituents is more important than the total PAH content in a PAH mixture for the observed developmental effects in the exposed zebrafish embryo.

In the present study, it seems like both specific and total PAH content play an important role in mediating the PDT induced by some PS.

The ZET, which was performed in this study, is the third assay of the test battery that we have applied to study the PDT potency (and modes-of-action) of a similar series of PS extracts. Hence, it is now possible to compare the results in the ZET with the results obtained from the other in vitro assays, i.e. the EST and the AhR CALUX assay. The main goal of this cross-model comparison is to see whether each of these assays could be used either as a stand-alone assay or be combined to predict the PDT potency of PS extracts. The comparison revealed that PDT potencies of the DMSO-extracts of the 9 PS and 2 GTL products in the ZET and EST were moderately correlated (R^2: 0.61). This is in line with results reported by de Jong et al. (2011), who founda moderate correlation (R^2: 0.57) of potencies in the ZET and EST when assessing the embryotoxicity potency of azoles.

A moderate correlation also exists between potencies in the ZET and the AhR-mediated activity of the same substances (R^2: 0.66), pointing at a possible role of the AhR in mediating the observed PDT in the ZET. It is generally accepted that the PDT induced by PAHs is partly mediated via activation of the AhR (Puga et al., 2005; Goodale et al., 2013). Similar observations of developmental effects, such as pericardial and yolk sac edemas, dorsal curvature, and increased mortality rates, have previously been found to occur upon exposure of zebrafish embryos to PAHs and PAH-containing extracts, which supports these effects to be typical phenotypic effects of AhR-mediated embryotoxicity in zebrafish embryos (Billiard et al., 2006; Incardona, 2004, 2006; Wincent et al., 2015). A lower correlation between potencies in the ZET and AhR-mediated activity (R^2: 0.66), compared to one obtained between the EST and AhR-mediated activity (R^2: 0.80; Kamelia et al., 2018), could be explained by the fact that not all individual PAHs induce PDT via the AhR pathway. For instance, pyrene, a 4-ring PAH, is known to cause developmental defects in zebrafish embryos via ion transportation and homeostasis pathways, not the AhR, while benzo[a]pyrene (5-ring PAHs) induces embryotoxicity via the AhR pathway (Huang et al., 2012; Goodale et al., 2013).

Interestingly, a different potency ranking is seen when comparing the relative potencies obtained in the ZET (results from the current study) with the EST (Kamelia et al., 2017). One of the prominent disparities is that in vitro PDT potencies among the classes of PS in the EST differed by at least 1-order of magnitude (10 times), where this is not the case for

the ZET. In the ZET, it is evident that all PS extracts were able to induce concentration-dependent developmental effects, however, there is no wide separation of potency among some classes of PS extracts, for example between the GOs and the DAEs. In the ZET, the relative PDT potency of GOs differed only 3-3.5-fold from that of the DAEs, while in the EST, their BMCd50s varied at least 25-fold. Also, the RAEs appear to be the least potent substances to induce PDT in the ZET whilst they were ranked as the second last in the EST. This discrepancy could be attributed to the fact that the observed effects in both assays might be induced by a different group of PAH constituents, involving different molecular pathways for the observed PDT. Furthermore, the disparity of PDT potencies between ZET and EST might also be explained by the difference in complexity between the two assays, the compound kinetics, and/or the experimental set-up of the assay itself. For example, a saltwater-based medium is used for the ZET and a serum-based medium is used for the EST, which may affect the solubility and bioavailability of test compounds in the assay medium (Fischer et al., 2017) throughout the test. In spite of these differences in potency ranking, the overall correlation between the assays was still moderate with an R^2: 0.61. Another reason for the differences between the ZET and EST might be related to the fact that zebrafish embryos are metabolically active, due to the cytochrome P450 (CYP)-related activity during organogenesis (Verbueken et al., 2017), and to some extent able to transform the PAHs present in PS extracts into their reactive metabolites. This suggests that the potency obtained in the ZET reflects the PDT effects in the presence of a biotransformation system, where this is not the case for the EST. Such a role of metabolic activation in the PDT of PAHs and PAH-containing mixtures remains to be established/ investigated in further detail.

Comparing the BMC50s, it appears that the BMC50 values obtained for the EST span a wider range than those of the ZET. The PDT potency of heavy PS, i.e. HFO and two of the most similar DAEs, #098 and #099, is higher in the EST (lower BMC50) than in the ZET, while in the ZET the obtained BMC50s of light PS, such as GOs and VTO, are lower than those quantified in the EST (see Table 1). This result may be associated with the role of hydrophobicity of particular PAHs present in the PS extracts under study, which eventually affect their bioavailability in the assay medium throughout the test. The hydrophobicity of PAHs rises with the molecular mass (Sverdrup et al., 2002), which means the LMW PAHs are more soluble in aqueous medium then the HMW PAHs. Heavy PS of the present study comprise mainly HMW PAHs (4-7 ring PAHs), while the light PS

contain more LMW PAHs (2-3 ring PAHs). Hence, it is suspected that the effects induced by light PS of the present study in the ZET are due to the role of these LMW PAHs. This is supported by the fact that sample #097-DAE does not result in a proportionate potency in the ZET, compared to the other 2 DAEs (#098 and #099), which could partly be explained by the difference in aromatic ring class (ARC) profile and PAH content among these samples, but not by their total PAH content (Figure 1). Sample #097-DAE mainly consists of 5-7 ring PAHs, where the other two DAEs contain 3-5 ring PAHs. This supports the above-mentioned postulation, where PS extracts that contain a high amount of 3-5 ring PAHs, including samples #034- HFO, #098-DAE, #099-DAE, result in lower BMC50s compared with those containing only 5-7 ring PAHs, like the RAEs. So, the fact that the RAEs are low in total PAH content (1.5-3.3 wt.%) and consist of mainly 5-7 ring PAHs explains why both of them are the least potent substances in the ZET.

The discrepancy between potencies in the ZET and EST may also be related to the presence of a protective envelope, called the chorion, prior to hatching of the zebrafish embryo in the ZET. The chorion of the zebrafish embryo acts as a permeability barrier during development, which could limit the uptake of certain toxicants by the embryo (Henn and Braunbeck, 2011). A recent publication by Geier et al. (2018), using the dechorionated zebrafish embryo model, demonstrated that HMW PAHs are significantly more developmentally toxic than LMW PAHs. Hence, the role of the chorion for assessing the PDT potency of PAH-containing PS and whether that will result in different outcomes remains to be investigated further.

PDT potencies obtained in the ZET were also compared to in vivo data. The correlations with potencies reported in in vivo studies were lower for the ZET (R^2: 0.69) than previously reported for the EST (R^2: 0.85; Kamelia et al., 2017) or the AhR CALUX (R^2: 0.80) (Kamelia et al., 2018). It may be considered that the EST (using mouse ES cells) and the AhR CALUX assay (using rat H4IIE.luc cells) are ontogenetically closer to rats (in vivo data) than to fish, which may be the reason for a better in vivo-in vitro correlation. It should be noted that the better correlation between the mammalian-based in vitro and in vivo assays, as seen here, refers to PAH-containing PS. Whether this observation would also hold for other types of developmental toxicants remains to be established. The ZET uses a complete embryo and covers a wider series of events during embryogenesis, but, as previously mentioned, the hydrophobicity of a specific group of PAHs, mainly the HMW

PAHs, in the egg water may limit the uptake of the PS extracts by the zebrafish embryo throughout the test. As a consequence, the ZET might not quantitatively cover the whole range of PDT potencies among PS extracts, thereby, correlating less with the in vivo data, in which the BMD10 values between different classes of PS extracts varied by at least 1-order of magnitude (see Table 1). Lastly, the differences between potencies in the ZET and in vivo findings may also be explained by the differences in toxicokinetics, developmental stage of the embryos used in the assay, and complexity of the respective assay itself. Taking possible differences in toxicokinetics between in vivo and in vitro models into account may further improve the in vitro-in vivo correlation.

Results of the present study show that PDT potency of the PS extracts in the ZET is well-correlated with their level of 3-5 ring PAHs. Available evidence from the published literature confirms our findings that only some 3- to 5-ring PAHs, including phenanthrene, pyrene, benz[a]anthracene, benzo[a]pyrene, may cause developmental effects in the zebrafish embryo (Incardona et al., 2004, 2006, 2011; Goodale et al., 2013). Hence, it is possible that the observed developmental retardations, as seen in the ZET in the present study, were induced by a specific group of PAH constituents, and not all of them. Another possible explanation why potencies in the ZET are well-correlated with especially the 3-5 ring PAH content may be related to the hydrophobicity feature of these and >5 ring PAHs influencing their solubility in the egg water medium used for the ZET. It is known that individual PAHs with a log K_{ow} > 5 (Sverdrup et al., 2002; Lu et al., 2008), including 5- to 7-ring PAHs like BaP, benzo[g,h,i]perylene, benzo[b]fluoranthene, indeno[1,2,3-c,d]pyrene, and coronene have solubility limitations in aqueous based medium. This means, the solubility of these 5- to 7-ring PAH constituents, present in the PS extracts under study, in the egg water medium may be limited, limiting the bioavailability and uptake and thus the developmental toxicity of these PAHs in the ZET.

Combining the results of the ZET with those reported for the EST (Kamelia et al., 2017), AhR CALUX assay (Kamelia et al., 2018), and the PAH content, ranked and clustered the test compounds in line with their in vitro potencies and chemical characteristics. Hierarchical clustering grouped PS that belong to the same class of substances together. Moreover, there is a clear separation of cluster between the PS and GTL products, showing an opposite bioactivity of substances with and without PAHs. Altogether, ToxPi analysis successfully integrated and visualized the multivariate data obtained from distinct

informative domains, thereby clustering and differentiating between the PS, within and among classes, and also between PS and GTL extracts, in a way that is in line with the differences in their PAH composition and their bioactivity in several in vitro assays for PDT testing. This result suggests the applicability of the present assay battery to group similar substances.

The use of the BMD approach to estimate and determine in vitro concentrations (BMC) that reflect the potencies obtained from different in vitro assays for developmental toxicity testing, including the EST, WEC, and ZET, has been widely accepted (Piersma et al., 2008, 2013; Hermsen et al., 2011; de Jong et al., 2011). In the present study, we applied the BMD approach to determine both in vivo (BMDs) and in vitro (BMCs) PDT potencies of the test compounds under study, thereby allowing us to compare both potencies derived using the same approach. Nonetheless, additional analysis of the in vitro data using GraphPad Prism software resulted in EC50s in the ZET that were comparable and correlate well with the calculated BMC50s (R^2: 0.99), resulting in similar results and conclusions (data are provided in Appendix).

Several published studies (Hermsen et al., 2011; Selderslaghs et al., 2012) used the so-called teratogenicity index (TI) for classifying teratogenic compounds using the ZET. The TI, within the ZET, is defined as a ratio between the 50% lethal concentration (LC50; based on the GMS) and the 50% effect concentration (EC50; based on teratogenic endpoints) (Selderslaghs et al., 2012; Beekhuijzen et al., 2015). According to the publications by Beekhuijzen et al. (2015) and Selderslaghs et al. (2009, 2012), 6 teratogenic endpoints/effects are scored from 96 hpf to 144 hpf to define the EC50. PAH-containing PS tested in the present study induced mainly pericardial and yolk sac edemas when scored at 96 hpf. However, given that most of the zebrafish exposed to PS did not hatch at 72-96 hpf, full scoring of these teratogenic endpoints was seriously hampered, although deformed body shape and malformation of the tail (i.e. kinked tail) were observed in several of the zebrafish embryos that hatched at 72-96 hpf. The other 2 endpoints, including malformation of the head and malformation of the otoliths, were not observed upon exposure to PAH-containing PS extracts for up to 96 hpf in the ZET, suggesting that they may not represent effects following exposure to PAH-containing substances. As a result, for the PS samples tested, the EC50 for teratogenic endpoints could not be derived and thus also the TI could not be established. These observed effects

are in line with those of previous studies where prominent developmental aberrations upon exposure to PAHs and PAH-mixture are limited to pericardial edema, yolk sac edema, and cumulative mortality (Goodale et al., 2013; Gu et al., 2010; Huang et al., 2012; Zhang et al., 2012; Wincent et al., 2015; Geier et al., 2018). In view of this, one may argue about the suitability of the ZET performed in the present study to evaluate possible PDT potency of PAH-containing PS and also about how the morphological effects relate to mortality upon exposure to these substances. Based on Wilson's Principles of Teratology (Finnel, 1992), embryolethality (i.e. cumulative mortality), growth retardation (i.e. unhatched zebrafish embryos), and functional deficit (i.e. pericardial and yolk sac edemas), as seen in the ZET results of the present study, are all manifestations of developmental toxicity. Further, the in vivo developmental effects induced by some PAH-containing PS, especially those tested in the present study, are mainly associated with increased incidence of re-sorptions (prenatal loss) and decreased fetal body weight, and not so much with malformations of the fetuses (ARCO, 1993; Feuston et al., 1989, 1994, 1996; Feuston and Mackerer, 1996; Hoberman et al., 1995; Mackerer et al., 2003). In this case, given that a good correlation exists between the obtained PDT potency in the ZET and the in vivo BMD10 values (particularly for live fetuses/litter and increased resorptions endpoints; Figure 5A) for developmental toxicity, this validates the ZET for detection of developmental toxicity of the PAH-containing PS under study.

To conclude, here we show the applicability of the ZET as an in vitro alternative assay to predict the relative in vivo PDT potency of DMSO-extracts of PS and GTL products. Our findings confirm the hypothesis that PAH are the major inducers of PDT in PS; for PS it seems that the EST is a better predictive model for in vivo PDT, but it should be taken into account that this may not be the case for other classes of compounds. In addition, the ZET does not outperform the EST as a stand-alone assay for testing PDT of PS, but the ZET is a useful addition to the battery of in vitro tests able to predict the in vivo PDT of PS. Zebrafish embryos are able to bioactivate some parent compounds thanks to the expression of cytochrome P450 enzymes, allowing evaluation of PDT potency of PS extracts in the presence of a biotransformation system. PAHs and PAH-containing PS require metabolic activation to exert their genotoxicity effect, but it is still unknown whether bioactivation is essential to induce PDT. PS used in the present study are categorized as UVCBs or substances of unknown and variable composition, hence the observed PDT in both in vivo and in vitro studies may be caused by a wide range of

compounds and underlying mechanisms. Having said that, it can be foreseen that it is almost impossible to assess the PDT potency of these substances by using just one single assay. The use of a battery of in vitro assays that focuses on the relevant modes-of-action of PDT by some PS will answer the challenge for PDT testing of these substances. Upon addition of the ZET, our testing battery of PDT consists of three in vitro assays: the EST, ZET, and AhR CALUX assay. The results of the present study revealed that such a battery enables prediction of the PDT potency and possible underlying mechanisms of PS. Future studies will focus on gene expression studies to see whether these may provide additional value to the current testing battery, and if so to what extent. It would also be interesting to study the PDT potency of individual PAHs present as major constituents in the DMSO-extracts of PS samples to fully understand and unravel the association between PAHs in PS and PDT.

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Supplementary data

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Chapter 6

The role of metabolism in the developmental toxicity of polycyclic aromatic hydrocarbons-containing petroleum substances

Lenny Kamelia, Laura de Haan, Bert Spenkelink, Ben Bruyneel, Hans B. Ketelslegers, Peter J. Boogaard, Ivonne. M.C.M. Rietjens

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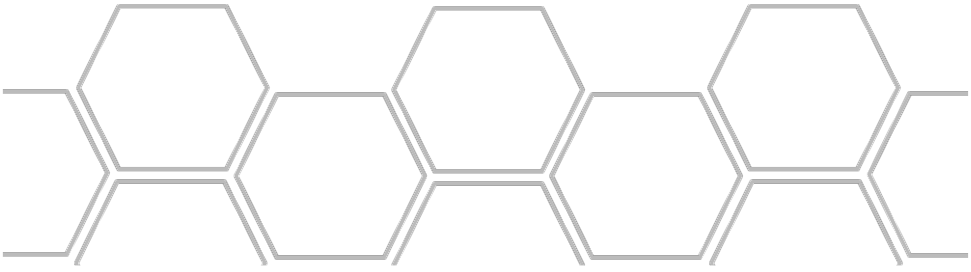

Abstract

In vitro assays presently used for prenatal developmental toxicity (PDT) testing only assesses the embyrotoxic potential of parent substances and not that of potentially embryotoxic metabolites. Here we combine a biotransformation system, using hamster liver microsomes, with the embryonic stem cell test (EST) to compare the in vitro PDT potency of two 5-ring PAHs, benzo[a]pyrene (BaP) and dibenz[a,h]anthracene (DBA), and DMSO-extracts from five PAH-containing petroleum substances (PS) and a gas-to-liquid base oil (GTLb), with and without bioactivation. For the PS in vitro PDT has been linked to their 3- to 7-ring PAH content. In the absence of bioactivation, DBA, but not BaP, inhibited the differentiation of ES-D3 cells into beating cardiomyocytes in a concentration-dependent manner. Upon bioactivation, BaP induced in vitro PDT, while its major metabolite 3-hydroxybenzo[a]pyrene was shown to be active in the EST as well. This means BaP needs to be transformed into its reactive metabolite to exert its embryotoxic effects. GTLb tested negative in the EST, with and without bioactivation. The PS-induced PDT in the EST was not substantially changed following bioactivation, implying that metabolism may not play a crucial role for PS to exert their in vitro PDT effects. Altogether, these results indicate that although some PAH require bioactivation to induce PDT, some do not and this latter also appears to hold for the (majority of) the PS constituents responsible for the in vitro PDT of these complex substances.

1. Introduction

REACH is the first chemical legislation in the world that requires prenatal developmental toxicity (PDT) testing on all chemical substances produced at a volume of ≥100 tonnes/year (ECHA, 2009). Petroleum substances (PS) are UVCBs (substances of Unknown or Variable composition, Complex reaction products or Biological materials) and regulated as such under the REACH legislation. Most PS are produced at a volume far greater than 100 tonnes annually, hence, they need to be assessed for PDT. Testing for PDT is one of the most animal- (~2500 animals/test/substance) and resource-intensive (>1M€ testing cost/substance) regulatory requirement in the field of toxicology (OECD, 2001; van der Jagt et al., 2004). Given the large number of experimental animals and a considerable amount of resources potentially connected to PDT testing of all currently active registered PS (n: ±186 EINECS numbers) (Concawe, 2019), the application of robust and reliable in vitro alternative testing strategies to assess the PDT potency of PS is highly relevant. This will ultimately reduce the required animal tests and resources to study the PDT potency of PS, and support the application of the 3Rs (Reduction, Replacement, and Refinement) principle of animal use in toxicological research.

To date, there are several in vitro alternative assays that have been scientifically validated and are widely used for PDT testing of chemical substances. One of these assays is the mouse embryonic stem cell test (EST) (Genschow et al., 2002, 2004). The EST is a completely animal-free test system, since it uses the permanent embryonic ES-D3 stem cell line. ES-D3 cells are able to spontaneously differentiate into any derivatives of all three primary germ layers (endoderm, ectoderm and mesoderm), including cardiomyocytes in the absence of the cytokine, leukemia inhibitory factor (LIF), in the growth medium (Maltsev et al., 1993). In the EST, the ability of test substances to inhibit the differentiation process of ES-D3 cells is used as the endpoint to study PDT potency of test substances in vitro (Genschow et al., 2002, 2004; Seiler and Spielmann, 2011). Recent studies in our lab showed the ability of the ES-D3 cell differentiation assay of the EST to assess the PDT potencies of DMSO-extracts of PS (within and across categories with variable PAH content) and GTL products (containing no PAHs) (Kamelia et al., 2017, 2019). PDT potencies obtained for the different PS in the EST appeared to be in line with their potencies observed in vivo (Kamelia et al., 2017, 2019). GTL (which is totally devoid of aromatic compounds) extracts tested negative in the EST, while all PS extracts inhibited

the differentiation of ES-D3 cells into beating cardiomyocytes in a concentration-dependent manner at non-cytotoxic concentrations with a potency that was proportional to their 3- to 7-ring PAH content (Kamelia et al., 2017, 2019). This result was obtained without including a metabolic system in the ES-D3 cell differentiation assay, a result that is of interest given that it is generally assumed that PAHs require metabolic activation to exert their toxicity. It has been reported that some PAHs and PS need to be bioactivated in order to show their carcinogenicity and mutagenicity (Blackburn et al., 1986, Clonfero et al., 1996). However, it is unclear whether bioactivation would also be required for PAH-containing PS to exert PDT. Initial studies suggest bioactivation may not be required since PS tested positive in the EST, without the inclusion of a mammalian metabolic activation system in this in vitro PDT assay (Kamelia et al., 2017, 2019). In view of this, and in particular to answer the question of whether the PDT potency of PS would be modified upon biotransformation, and to better mimic the in vivo situation, the aim of the present study was to evaluate the consequences of biotransformation for the in vitro PDT of some selected model PAHs and PS by incorporating a metabolic activation system in the EST. To this purpose, two 5-ring PAH model compounds: benzo[a]pyrene and dibenz[a,h]anthracene, the DMSO-extracts of five PS from different product categories (varying in their PAH content); and of one gas-to-liquid base oil (GTLb; totally devoid of PAHs) were tested in the EST, with and without preliminary metabolic activation. The metabolite cocktails of the parent-substances were generated using hamster liver microsomes since these have been shown superior for in vitro bioactivation of PAHs and PAH-containing PS, in comparison to the rat or mouse liver microsomes (Blackburn et al, 1986; Hermann et al., 1980).

2. Materials and Methods

2.1 Test substances

Benzo[a]pyrene (BaP; CAS no. 50-32-8), dibenz[a,h]anthracene (DBA; CAS no. 53-70-3), and 5-fluorouracil (5-FU; CAS no. 51-21-8) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands), and 3-hydroxybenzo[a]pyrene (3OH-BaP; CAS no. 13345-21-6) was obtained from BIOZOL Diagnostica Vertrieb GmbH (Eching, Germany).

All compounds were of high purity (≥ 95%) and stock solutions and dilutions were prepared in dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany).

Moreover, the DMSO-extracts of 5 PS, varying in the types and levels of PAH, and 1 gas-to-liquid base oil (GTLb; CAS no. 848301-69-9) that was totally devoid of PAHs, were also tested in the present study. DMSO extraction and chemical analysis of the PS and GTL products tested was performed essentially as described before by Roy et al. (1988), and was carried out at Port Royal Research Laboratory (Hilton Head, South Carolina, USA). An overview of the types and levels of PAH present in each PS tested, grouped by the number of aromatic rings, is provided in Supplementary Materials (Appendix A). Of the 5 PS, 1 was a heavy fuel oil (HFO; CAS no. 64741-62-4), 1 was a gas oil (GO; CAS no. 68915-96-8), 1 was a vacuum tower overhead oil (VTO; CAS no. 64741-49-7), 1 was a distillate aromatic extract (DAE; CAS no. 64742-04-7), and 1 was a residual aromatic extract (RAE; CAS no. 91995-70-9). The raw material of all PS and GTLb used to generate the aforementioned DMSO-extracts were kindly provided by Concawe (Brussels, Belgium) and Shell International B.V. (The Hague, The Netherlands), respectively.

2.2 Microsomal incubations of parent substances for generation of metabolites

Since liver microsomes are toxic for embryonic stem cells (Wobus and Löser, 2011), metabolite cocktails of parent substances were generated by pre-incubation of the compounds with pooled-male Golden Syrian Hamster liver microsomes (Xenotech, Lenexa, USA). Incubations were performed in 0.1 M K_2HPO_4 buffer (pH 7.4) (VWR International, Darmstadt, Germany), containing 5 mM $MgCl_2$ (VWR International), NADPH (final concentration 15 mM) (Roche Diagnostic Gmbh, Mannheim, Germany), and microsomal protein (final concentration 3 mg/ml), in glass reaction tubes (VWR International). Prior studies suggested that a high concentration of NADPH as a cofactor is needed when generating metabolite mixtures of PAHs and PS extracts in the microsomal incubations (Blackburn et al., 1986; Haugen and Peak, 1983). Taking these findings into consideration, the present study applied hamster liver microsomes at a final concentration of 3 mg/ml microsomal protein with the final concentration of NADPH at 15 mM, which is a 1:5 ratio between microsomes:NAPDH. This ratio was obtained by doing an optimization study using BaP as the model compound (data not shown), and the

chosen ratio of microsomes and NADPH, in combination with an incubation time of 4 hours at 37°C shows a reasonable compromise between the efficiency of activation and costs, and time of incubation. The total volume of the incubation mixtures was 3 ml and the reaction was initiated by adding the substrate, being the parent substances under study, after the pre-incubation at 37°C for 1 min. Incubation mixtures without the cofactor NADPH were used as controls (without bioactivation). In the microsomal incubations (3 ml total volume), BaP and DBA were added at a final concentration of 50 μg/ml (200 μM) and 5.6 μg/ml (20 μM), respectively. For all DMSO-extracts of PS and GTLb, they were added at a final concentration of 800 μg raw material/ml, except for sample #034-HFO that was tested at a concentration of 80 μg raw material/ml. It is worth mentioning that all of the aforementioned concentrations of parent-substances were chosen based on their highest possible solubility in phosphate buffer used for the microsomal incubations. In addition, those concentrations were previously shown not to induce any cytotoxicity effect to ES-D3 cells of the EST (Kamelia et al., 2017). The microsomal incubation reactions were terminated after 4 hours of incubation at 37°C (tubes were vortexed every 30 minutes) by the addition of 300 μl (10% out of the total volume) of ice-cold 10% v/v perchloric acid ($HClO_4$) (Merck). The incubation mixtures were extracted three times using diisopropyl ether (DIPE) (Acros, Geel, Belgium) and combined DIPE-extracts were evaporated to dryness under a stream of nitrogen. Samples (with and without bioactivation) were re-dissolved in 180 μl DMSO and stored at -20°C until further UPLC analysis and testing in the EST. Ascorbic acid at a final concentration of 0.5 mg/ml was added to each DMSO-extract (from microsomal incubations) to prevent further oxidation during storage.

2.3 Ultra-high performance liquid chromatography (UPLC) analysis

All re-dissolved extracts from microsomal incubations of parent-substances, with and without bioactivation, were analysed by UPLC to characterise the level of metabolic conversion. The UPLC system used consisted of a Waters Acquity (Milford, MA) binary solvent manager, sample manager, and photodiode array (PDA) detector, equipped with a Waters Acquity UPLC® BEH C18 column (1.7 μm, 2.1 x 50 mm) and Waters Xbridge UPLC® BEH C18 pre-column (2.5 μm, 2.1 x 5 mm). The temperature of the column was set at 40°C and the auto-sampler at 10°C during the UPLC analysis. The mobile phase used

for the analysis was (A) nanopure water with 0.1% trifluoroacetic acid (TFA) and (B) acetonitrile with 0.1% TFA. The flow rate was set to 0.6 ml/min and the total run time of the UPLC analysis for each sample was 23 minutes. The gradient elution conditions were set as follows: isocratic 90:10 (A:B) from 0 to 12 min, changing to 10:90 (A:B) from 12 to 20 min, then to 90:10 (A:B) from 20 to 23 min, followed by 1 min at beginning conditions. Before injection, samples were 100-times diluted in phosphate buffer. The injection volume for each diluted sample was 10 µl and test substances were detected at a wavelength of 270 nm. Calibration curves of PAH model compounds, being BaP, DBA, and 3OH-BaP, were also included in the UPLC analysis for quantification.

2.4 Liquid chromatography-mass spectrometry (LC-MS/MS) analysis

In brief, LC-MS/MS analysis was performed on a Shimadzu Nexera HPLC system coupled to a Shimadzu LCMS-8045 mass spectrometer. A volume of 10 µl incubation mixture was loaded onto a reverse phase C18 column (Kinetex® 1.7 µm C18 100 Å, LC Column 50 x 2.1 mm). The flow rate was 0.3 ml/min and the eluents were water (H_2O) and methanol (MeOH). Analysis started at 5% methanol which was linearly increased to 100 % MeOH in 15 min, after which the percentage kept at 100% for 5 min. This was followed by a decrease to 5% MeOH for 1 min and re-equilibration for 9 min. For mass spectrometric analysis, a Shimadzu LCMS-8045 triple quadrupole with electrospray ionization (ESI) interface was used. The instrument was operated in both positive and negative mode in full scan mode (m/z 50-650).

2.5 Mouse embryonic stem cell test (EST)

2.5.1 ES-D3 cell culture

The pluripotent mouse embryonic stem cell line ES-D3 (ES-D3; CRL-1934™) was purchased from ATCC (Wesel, Germany) and was grown in HyClone AdvanceSTEM Low Osmo Dulbecco's Modified Eagle Medium (DMEM) (GE Healthcare, Eindhoven, The Netherlands), supplemented with 15% fetal bovine serum (FBS) ES cell qualified (ATCC, USA), 2 mM L-glutamine (Invitrogen, Thermo Fisher Scientific, Breda, The Netherlands), 50 U/ml penicillin (Invitrogen), and 50 µg/ml streptomycin (Invitrogen). ES-D3 cells were

maintained in 25 cm^2 polystyrene cell culture flasks (Greiner Bio-One, Alphen a/d Rijn, The Netherlands), pre-coated with 0.1% gelatine for at least 1 hour prior to use. Cells were grown at 37°C, 5% CO_2 and routinely subcultured every 2-3 days using non-enzymatic cell dissociation solution (Sigma-Aldrich). To maintain the pluripotency of ES-D3 cells in the culture, murine Leukemia Inhibiting Factor (mLIF; Sigma-Aldrich) at a final concentration of 1000 U/ml was added into the growth medium.

2.5.2 ES-D3 cell viability and ES-D3 cell differentiation assays of the EST

ES-D3 cell viability and ES-D3 cell differentiation assays of the EST were performed essentially as described by Kamelia et al. (2017, 2019). The bioactivated-BaP and -DBA were tested in the EST at the highest concentration of 2.1 µg/ml (8.4 µM) and 0.23 µg/ml (0.84 µM), respectively. PS and GTLb extracts were tested at the highest concentration of 33.5 µg raw material/ml, except for sample #034-HFO that was tested at the highest concentration of 3.3 µg raw material/ml, because of limited solubility at higher concentrations.

For the ES-D3 cell viability assay, cells were seeded in 96-well plates (100 µl cell suspension/well) at concentrations of $20x10^4$ cells/ml (1-day exposure) or 10^4 cells/ml (5-days exposure) and incubated at 37°C, 5% CO_2 for one day to facilitate cell adherence. After 24 hours of cell seeding, cells were exposed to increasing concentrations of test substance in triplicate by the addition of 100 µl exposure medium (containing the test compound at 2x the final concentration) to each well. The exposure media were prepared by mixing 400 times concentrated DMSO-stock solutions of the test substances to the growth medium (without mLIF). The final concentration of solvent, DMSO, was kept at 0.25% (v/v). After 1-day or 5-days incubation at 37°C, 5% CO_2, the WST-1 assay was performed to assess the effects on ES-D3 cell viability. For this purpose, WST-1 reagent (Roche Diagnostics, Mannheim, Germany) was added to each well according to the protocol provided by the supplier and cells were incubated for 3 hours at 37°C, 5% CO_2. WST-1, a stable tetrazolium salt, will be cleaved into a soluble formazan by the succinate-tetrazolium reductase enzyme that is only active in viable cells. Hence, the amount of formed formazan directly correlates to the number of metabolically active cells in the culture. The absorbance of the formed formazan was measured at 440 nm (background

at 620 nm) using a SpectraMax M2 (Molecular Devices, Sunnyvale, USA). The cell viability of each well was expressed as the percentage cell viability compared to that of the solvent control 0.25% DMSO (set at 100%). At least three independent experiments were done for the ES-D3 cell viability assay of each test substance.

The ES-D3 cell differentiation assay of the EST lasts for 10 days. On day 0, droplets of 20 µl of a cell suspension ($3.75x10^4$ cells/ml), with or without test substances, were placed between the well borders on the inner side of the lid of a 96-well plate to initiate the formation of embryoid bodies (EBs) via hanging drop culture. All wells of the 96-well plate were filled with 250 µl of phosphate buffer saline (PBS; Invitrogen) to create an optimal humidity and to prevent evaporation of the hanging drops during 3-days incubation at 37°C, 5% CO_2. Sterile caps of Eppendorf tubes were placed in the corner of the 96-well plates to prevent direct contact of the hanging drops with the plate and the plate was then sealed with Micropore tape (3M, Neuss, Germany). On day 3, the resulting EBs were collected and transferred to bacteriological petri dishes (60x15mm; Greiner Bio-One) containing 5 ml exposure medium (with or without test substances). EBs were incubated for another 2 days. On day 5, the EBs were transferred to a 24-well plate (Greiner Bio-One) (1 EB/well) containing 1 ml exposure medium/well, and incubated at 37°C, 5% CO_2. On day 10, the number of wells (of the 24-well plate) containing beating cardiomyocytes was scored by visual inspection using a light microscope. The ES-D3 cell differentiation assay was considered valid if the solvent control had at least 21 out of 24 wells that contained beating cardiomyocytes. For each 24-well plate, the fraction of the 24 wells containing beating cardiomyocytes was determined. The inhibition of differentiation by the test substances was presented as this fraction of total. Thus, the fraction of 1 indicates that all plated-EBs have differentiated into beating cardiomyocytes. 0.25% DMSO (v/v) and 0.065 µg/ml 5-FU were used as solvent and positive control of this assay, respectively, and were included in each independent experiment. At least three independent experiments were done for the ES-D3 cell differentiation assay of each test substance.

2.6 Data analysis

Figures of concentration-response curves obtained from the EST were made using GraphPad Prism software version 5.0 (California, USA). To this end, results from ES-D3

cell viability (1-day and 5-days exposure) and differentiation assays of the EST were fitted to a sigmoid dose-response curve with 3 parameters in the GraphPad Prism 5.0. In parallel, Benchmark Dose (BMD) software version 2.6.1 from US-EPA was used to calculate the benchmark concentration (BMC) that corresponds to 50% decrease of ES-D3 cell viability (BMCv50) or 50% inhibition of ES-D3 cell differentiation into beating cardiomyocytes (BMCd50). For the BMCv50 determination, concentration-response curves from the ES-D3 cell viability assay were fitted to the continuous models available in the BMD software (hill, exponential, linear, polynomial, and power models). For the BMCd50 determination, concentration-response curves from ES-D3 cell differentiation assay were fitted to all dichotomous concentration-response models (gamma, logistic, log-logistic, probit, log-probit, weibull, multistage-cancer, multistage, and the quantal-linear models) available in the BMD software. The benchmark response (BMR) was set at 50%, representing either a 50% decrease in cell viability (BMCv50) or 50% inhibition of cardiomyocyte differentiation (BMCd50). The performance of each fitted model was evaluated based on the goodness-of-fit, the scaled residuals, and the visual inspection of the model fit. The BMC values were selected from the accepted model with the lowest Akaike's Information Criterion (AIC) (Haber et al., 2018; Kamelia et al., 2017).

3. Results

3.1 Effects of BaP and DBA in the EST without metabolic activation

Figure 1 presents the effect of the two 5-ring PAH model compounds, BaP and DBA, in the ES-D3 cell viability and differentiation assays of the EST. As shown in Figure 1A, BaP did not affect cell viability or inhibit ES-D3 differentiation up to the highest concentration tested (12.6 μg/ml or 50 μM). Interestingly, DBA, another 5-ring PAHs, inhibited the differentiation of ES-D3 cells into beating cardiomyocytes in a concentration-dependent manner (BMCd50: 3 μg/ml equal to 10.8 μM) at non-cytotoxic concentrations (Figure 1B).

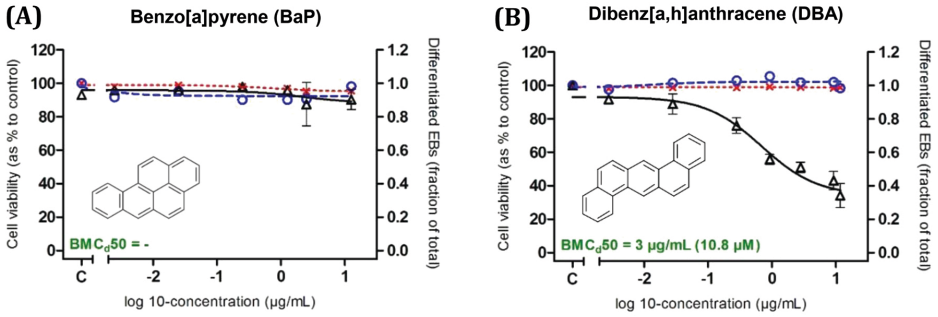

Figure 1 Concentration-dependent effects of (A) BaP and (B) DBA on ES-D3 cell viability upon one-day (x and red-dotted line) and five-days (○ and blue-dashed line) exposure and on ES-D3 cell differentiation into beating cardiomyocytes (Δ and black-continuous line). Results represent data from at least three independent experiments and are presented as mean ± standard error of the mean (SEM).

3.2 Microsomal conversion of BaP and DBA using hamster liver microsomes

Figure 2 presents the UPLC chromatograms of the re-dissolved extracts from incubations of BaP and DBA with and without bioactivation. These results reveal that the optimized biotransformation system using hamster liver microsomes has effectively transformed the parent PAH substances into metabolites. For bioactivated-BaP, the formation of 3OH-BaP (retention time/RT: 7.8 minutes) as the major metabolite formed from BaP (RT: 9.5 minutes) is readily observed (Figure 2). The concentration of 3OH-BaP upon bioactivation is ± 200 µg/ml (± 760 µM). Similarly, DBA has successfully been converted, as reflected by the appearance of metabolite peaks at an earlier retention time (~6 - 9.5 minutes) than that of DBA itself (RT: 9.9 minutes). Also for DBA the formation of one major metabolite is observed (RT: 8.4 minutes) which is, based on available literature data on metabolism of DBA (Boyland et al., 1964; 1965; Platt et al., 1990) tentatively identified as dihydroxy-dibenz[a,h]anthracene. LC-MS/MS analysis confirmed the presence of this metabolite, with m/z 309.3, in this incubation mixture (Appendix C).

3.3 Microsomal conversion of PS and GTLb extracts using hamster liver microsomes

Figure 3 presents the UPLC chromatograms obtained for extracts of the microsomal incubations with PS and GTLb, performed using the method shown valid for metabolic conversion of the model PAHs BaP and DBA. Results obtained reveal conversion of various PS constituents into metabolites, which, given the complex nature of these samples were not further identified, but likely reflect CYP450-mediated formation of metabolites since they are more polar than the parent compounds, as shown by a higher peak area in the earlier retention time of the UPLC chromatogram as compared to the corresponding control without bioactivation (Figure 3). For the GTLb extract, the differences between the re-dissolved extracts obtained with and without metabolic activation were limited (bottom right graph of Figure 3).

3.4 Effects of BaP and DBA extracts with and without preliminary metabolic activation in the EST

Figure 4 compares the in vitro PDT potency of BaP and DBA with and without metabolic activation as observed in the differentiation assay of the EST. Cytotoxicity at both day 1 and 5 was not observed for any of the samples (data not shown). Figure 4A shows that while BaP tested negative without bioactivation, BaP was able to inhibit the differentiation of ES-D3 cells after bioactivation using hamster liver microsomes (BMCd50: 1 µg/ml; Appendix D). Given the fact that 3OH-BaP was the major metabolite formed (Figure 2), this metabolite was also tested itself in the EST. The concentration of 3OH-BaP in the bioactivated-BaP extract is ± 200 µg/ml (± 760 µM), which was determined by fitting the area under the curve (AUC) from UPLC measurement to the calibration curve of 3OH-BaP (from DMSO-stock solutions). As can be seen in Figure 5, 3OH-BaP caused a concentration-dependent inhibition of ES-D3 cell differentiation at concentrations that do not affect cell viability, giving a BMCd50 value of 1 µg/ml (3.7 µM; Appendix D). For DBA, the ability of this 5-ring PAH model compound to inhibit the differentiation of ES-D3 cell differentiation in the EST was not notably different with and without bioactivation (Figure 4B).

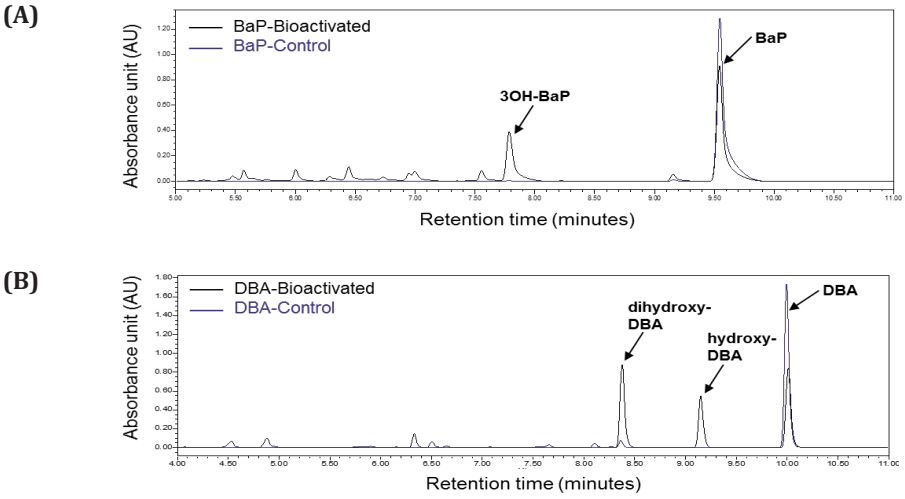

Figure 2 Relevant parts of the UPLC chromatograms of the re-dissolved extracts from incubations with and without bioactivation of 5-ring PAH model compounds (A) BaP and (B) DBA.

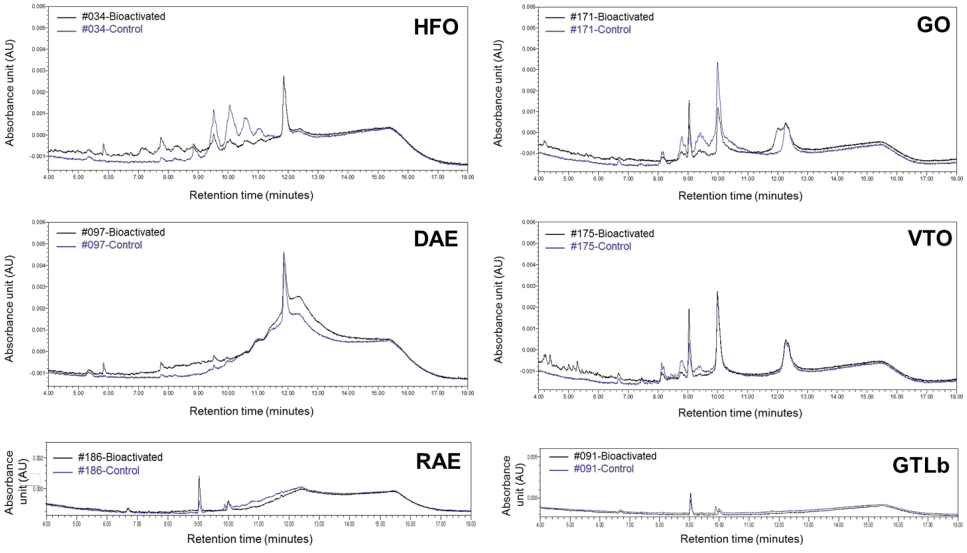

Figure 3 Relevant parts of the UPLC chromatograms of the re-dissolved extracts from incubations with and without bioactivation of selected PS and GTLb extracts.

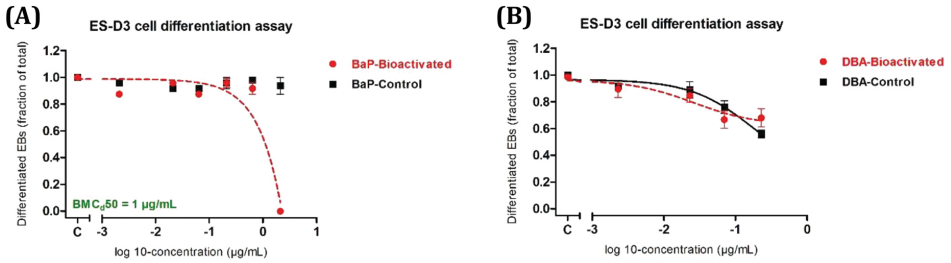

Figure 4 Comparison of effects of (A) BaP and (B) DBA in the ES-D3 cell differentiation assay of the EST, with (● and red-dashed line) and without (■ and black-continuous line) bioactivation using hamster liver microsomes. The concentration ranges tested here are known not to induce any cytotoxicity effects to ES-D3 cells used for the EST (data not shown).

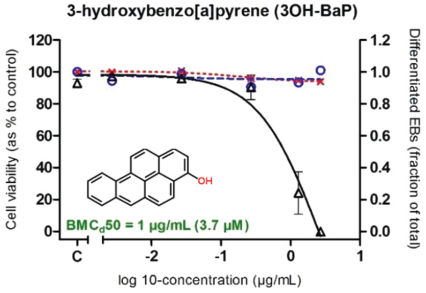

Figure 5 Concentration-dependent effects of 3OH-BaP, identified as the major metabolite formed upon microsomal incubations of BaP (see Figure 2A), on ES-D3 cell viability upon one-day (x and red-dotted line) and five-days (○ and blue-dashed line) exposure and on ES-D3 cell differentiation into beating cardiomyocytes (Δ and black-continuous line). Results represent data from at least three independent experiments and are presented as mean ± standard error of the mean (SEM).

3.5 Effects of PS and GTLb extracts with and without preliminary metabolic activation in the EST

Also for the PS and GTLb extracts under study, their potencies in the EST with and without metabolism were compared (Figure 6). The results obtained show that the DMSO-extracts

of the PS tested (#034-HFO, #097-DAE, #171-GO, #175-VTO, #186-RAE) were able to inhibit the differentiation of ES-D3 cells in the EST, as reported before (Kamelia et al., 2017). Interestingly, their PDT potency was not remarkably changed when ES-D3 cells were exposed to the metabolite mixtures of the corresponding PS (Figure 6A-E). The GTLb extract, remained unable to induce any effect in the EST regardless of the presence or absence of preliminary metabolic activation (Figure 6F).

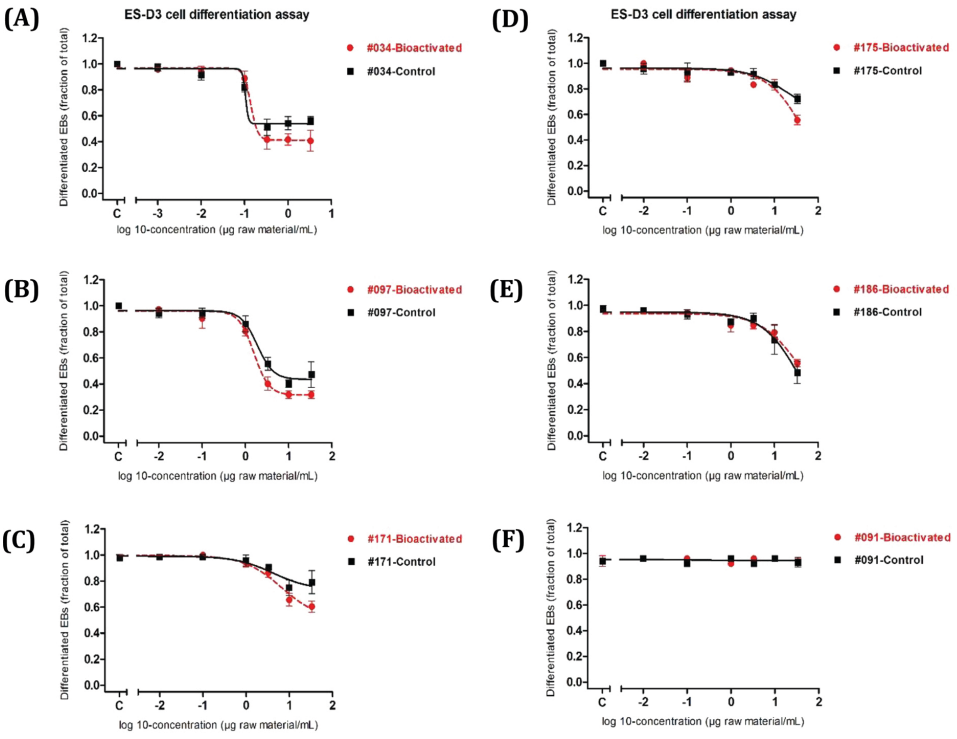

Figure 6 Effects of PS: (A) #034-HFO, (B) #097-DAE, (C) #171-GO, (D) #175-VTO, (E) #186-RAE, and (F) GTLb extracts on inhibition of ES-D3 cell differentiation into beating cardiomyocytes in the EST, with (● and red-dashed line) and without (■ and black-continuous line) bioactivation using hamster liver microsomes. It should be noted that the concentrations tested here are known not to induce any cytotoxicity effects to ES-D3 cells used for the EST (Kamelia et al., 2017). Results represent data from at least three independent experiments and are presented as mean ± standard error of the mean (SEM).

4. Discussion and conclusions

Recently we reported on the use of the EST to study PDT potency of PS and GTL products (Kamelia et al., 2017, 2019). A highly significant correlation was found between the EST results and in vivo data (R^2: 0.97; Kamelia et al., 2017). Additionally, the results obtained in the EST correlated well with the 3- to 7-ring PAH content in the tested substances. This is of interest since for several of the adverse effects of PAHs, including especially their genotoxicity and carcinogenicity, they require bioactivation (Blackburn et al., 1986; Hermann et al., 1990). Whether this also holds for their PDT potency remains to be elucidated. In this respect it is important to note that the ES-D3 cells used for the EST do not contain substantial xenobiotic metabolising capacity, and will therefore in principle assess the embryotoxicity potential of the parent substances without taking possible bioactivation into account. The present study aimed to evaluate the consequences of biotransformation for the in vitro PDT of some selected model PAHs and PS. To this end, the EST was combined with an exogenous biotransformation system. Two 5-ring PAH model compounds, BaP and DBA, together with five PS and one GTLb extract were tested in the EST, with and without preliminary metabolic activation. Preliminary metabolic activation was achieved by incubating the parent substances with hamster liver microsomes in the presence of NADPH. BaP and DBA were selected as parent PAH model compounds as they represent 5-ring PAH constituents present in the PS extracts under study while both of them are known to induce embryotoxicity in vivo (Bui et al., 1986; D'Mello et al., 2003). The selected PS samples represented different categories of PS (DAE, GO, HFO, RAE, VTO) with a systematic variation in their PAH content (from 3.3 to 48% wt. PAHs; Appendix A) and were shown before to induce concentration- and PAH content-dependent in vitro PDT in the EST (Kamelia et al., 2017).

Results obtained using BaP and DBA as the PAH model compounds show that biotransformation may modify the PDT potency of the test substances, although it appeared not essential for PDT induction by all model substances. BaP tested positive for the ES-D3 cell differentiation assay of the EST, only upon bioactivation (Figure 4A). These results indicate that bioactivation to 3OH-BaP likely plays a role in the PDT of BaP (Figure 2A). In contrast, DBA, also a 5-ring PAHs, showed a concentration-dependent inhibition of ES-D3 cell differentiation by the parent compound, and this potency was not affected upon its microsomal conversion. Furthermore, when ES-D3 cells were exposed to the

extracts of a series of model PS, including #034-HFO, #097-DAE, #171-GO, #175-VTO, and #186-RAE, the PS-induced PDT in the EST was not remarkably changed following bioactivation (Figure 6). This suggests that bioactivation may play just a minor role in the in vitro PDT of these PS model compounds. GTLb extracts remained unable to induce any effect in the EST regardless of the presence or absence of preliminary metabolic activation, which is in line with the absence of PAHs in these substances (Kamelia et al., 2017, 2019; Boogaard et al., 2017). Altogether, the results obtained indicate that some PAH constituents may require bioactivation to become able to induce PDT, but some do not and this latter also appears to hold for the (majority of) the PS constituents responsible for the in-vitro PDT of these complex substances.

This conclusion is corroborated by the observation reported before that the EST, without combining it with a metabolic activation system, appeared to already adequately assess the in vitro PDT potency of PS UVCBs, as compared to their potencies observed in vivo (Kamelia et al., 2017, 2019). The fact that microsomal bioactivation of PAH-containing PS may not be a prerequisite for their PDT may be linked to the fact that the underlying modes of action may require non-covalent receptor or enzymes interactions rather than the chemical (DNA) reactivity that is necessary to exert the mutagenic and carcinogenicity potency of these substances (Blackburn et al., 1986; Siddens et al., 2012). Corroborating this notion, our recent observation showed that the in vitro PDT of the PS extracts tested in the present study was at least in part mediated via the aryl hydrocarbon receptor (AhR) (Kamelia et al., 2019). It is generally accepted that 3- to 7-ring PAHs are able to activate the AhR (Pieterse et al., 2013; Puga et al., 2005), and the AhR-mediated activity of PAH-containing PS has been linked to the developmental toxicity potency of these group of substances (Billiard et al., 2006; Goodale et al., 2013; Incardona et al., 2011). The ability of chemical substances to interact with certain nuclear receptors is affected by their molecular size and chemical structure (Balaguer et al., 2017). Comparing the chemical structure of DBA and BaP, the molecular arrangement of the aromatic rings is more angular in DBA as compared to BaP. Regarding their molecular size, BaP (MW: 252.3) has a lower molecular weight compared to the DBA (MW: 278.3). With these differences in molecular size and structure, DBA is able to bind to the AhR with a higher affinity than BaP, as measured in the AhR CALUX assay (Appendix B). This may also imply that DBA displays a higher intrinsic potential for interaction with other receptors present in the ES-D3 cells, including the retinoic acid receptor (RAR) and the estrogen receptor (ER) that

are known to be potentially relevant in PDT (Adam et al., 2019; Dimopoulou et al., 2018; Louisse et al., 2011). Hence, future studies may consider the influence of structural features and size of PAHs, especially those present in PS, on their ability to activate receptors relevant for their embryotoxicity potency.

For bioactivation of the PAHs and PAH-containing PS, the present study made use of hamster liver microsomes since it has been shown that they are more effective for bioactivation of these group of substances in comparison to rat or mouse liver microsomes (Blackburn et al., 1986; Haugen and Peak, 1983; Hermann et al., 1980; Hermann, 1981). For instance, Blackburn et al. (1986) modified the standard Ames test, later known as the modified-Ames test, for mutagenicity testing of PS by i) using hamster rather than rat liver S9-mix for generating metabolite mixtures of PS, and ii) using DMSO-extracts of PS instead of neat/bulk materials, which improved the interaction of potentially toxic constituents present in PS with the aqueous in vitro test system. Using the above-mentioned modifications, Blackburn and co-workers were able to correctly assess the mutagenicity potency of 18 PS samples from different product categories (Blackburn et al., 1986). Furthermore, hamster liver microsomes, and not hamster S9 fractions, were used in the present study because the activity of cytochrome P450 enzymes in hamster liver microsomes (expressed in nmol/mg protein) is 3-times higher than in the hamster S9 fractions (Appendix E), while it was assumed that especially cytochrome P450 mediated biotransformation to hydroxylated metabolites would bioactivate the PAHs (Diamante et al., 2017; Geier et al., 2018). The results obtained for 3OH-BaP support this assumption, while DBA did not need bioactivation. Thus, to what extent bioactivation by cytochromes P450 to hydroxylated metabolites is required for the PDT of other PAHs constituents remains to be evaluated, although the data obtained with the PAH containing PS seem to indicate that this role may be not essential.

In addition, it is of interest to note that integrating an exogenous biotransformation system with an in vitro test system can be done by either direct addition of the liver S9/microsomes to the cells or via pre-incubation of parent substances with liver S9/microsomes followed by incubations of cells with the extracted activated parent substances. Since it is known that liver S9/microsomes are toxic for embryonic stem cells (Wobus and Löser, 2011), in the present study pre-incubation was applied and the extracted bioactivated metabolite mixtures were incubated with the ES-D3 cells of the

EST. The other reason to use the extraction of pre-incubation mixtures was that this enabled concentration of the samples and testing of biologically active concentrations (Langsch and Nau, 2005).

In conclusion, the present study has successfully combined an exogenous biotransformation system, using hamster liver microsomes, with the EST to compare in vitro PDT potency of two 5-ring PAHs: BaP and DBA, and the DMSO-extracts of PS and GTLb, with and without bioactivation. BaP is able to inhibit the ES-D3 cell differentiation only upon bioactivation where DBA does not need to be bioactivated to exert its embryotoxicity potency in the EST. Furthermore, PS-induced PDT in the EST does not substantially change with and without bioactivation, suggesting that metabolism may not play a crucial role in mediating their in vitro embryotoxicity potency. In contrast, GTLb extracts remain unable to induce any effects in the EST regardless of the presence or absence of metabolic activation. By combining a biotransformation system with the EST, the assay may better reflect the in vivo situation. Our findings may serve as a basis for future research to fully unravel the role of metabolism in the developmental toxicity of PAHs-containing PS.

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Supplementary data

Appendix A

Aromatic ring class (ARC) profiles of PS and GTL products that are tested in the present study, listed from highest to the lowest total weight percentage (wt.%) of PAH content.

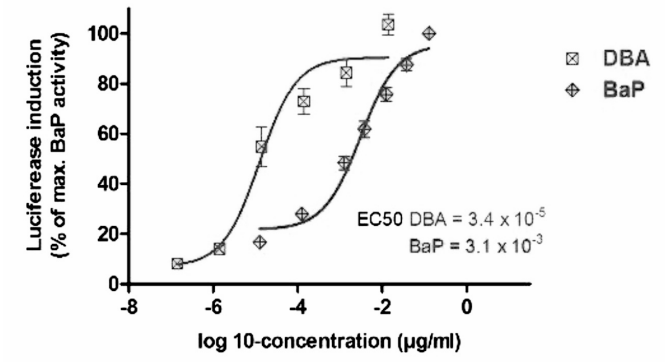
Compound	ARC profile[a] (wt.%)							Total (wt.%)
	1-ring	2-ring	3-ring	4-ring	5-ring	6-ring	≥ 7-ring	
#034-HFO	0	1	24	40	21	11	3	48
#097-DAE	0	2	1	6	22	36	33	9
#175-VTO	1	56	43	0	0	0	0	6.7
#171-GO	0	12	76	12	0	0	0	5.5
#186-RAE	0	0	1	6	18	38	36	3.3
#091-GTLb	0	0	0	0	0	0	0	0

[a]The weight percent of the DMSO-soluble 1- to ≥7 aromatic-ring compounds present in each substance, from the starting raw material of 4 gram sample, as determined by Method II extraction and chemical characterization procedure (Roy et al., 1988).

Abbreviations. HFO: heavy fuel oil; DAE: distillate aromatic extract; VTO: vacuum tower overhead oil; GO: gas oil; RAE: residual aromatic extract; GTLb: gas-to-liquid base oil.

Appendix B

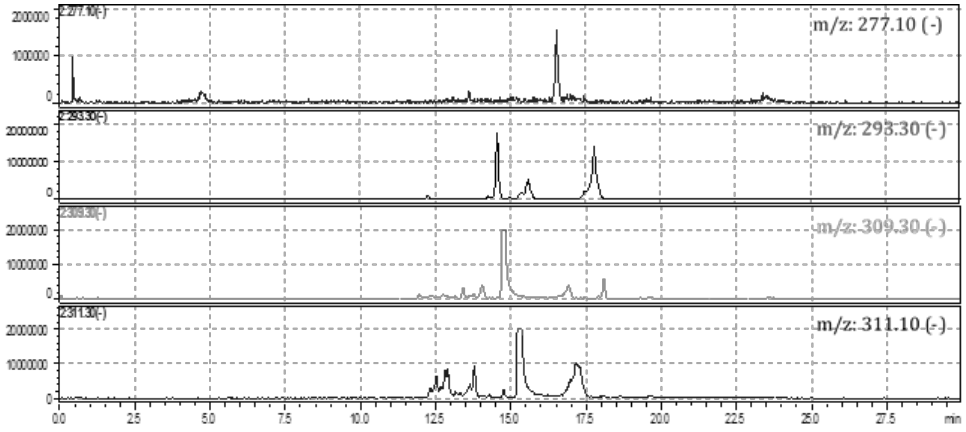
AhR-mediated activity of two 5-ring PAH model compounds, BaP and DBA, in the AhR CALUX assay.

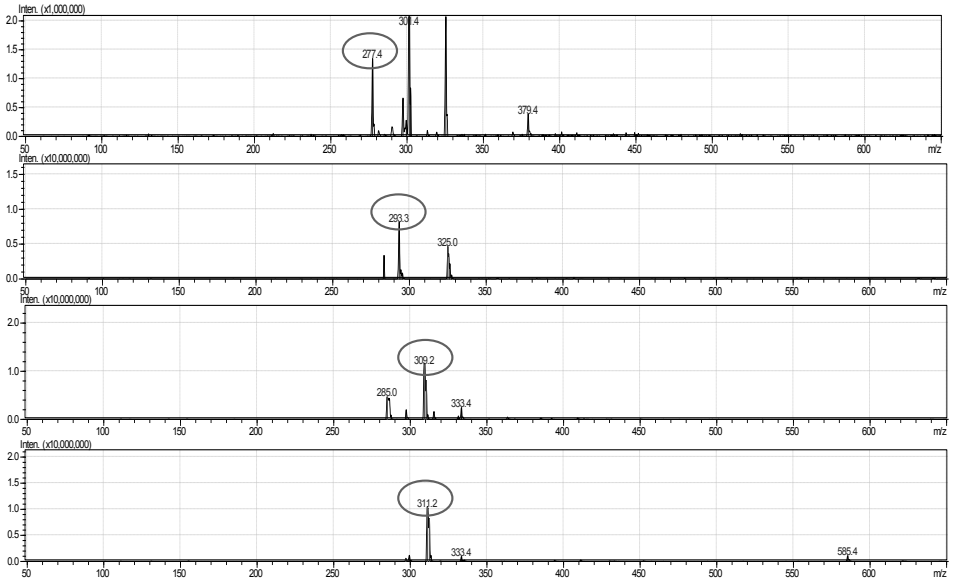


Appendix C

LC-MS analysis to identify the metabolites of DBA upon microsomal incubations using hamster liver microsomes.

Compound	Formula	Mass spectrum on the negative ion mode (M-H)
Dibenz[a,h]anthracene (DBA)	C22H14	277.1
Hydroxy-DBA	C22H14O	293.3
Dihydroxy-DBA	C22H14O2	309.3
Dihydrodiol-DBA	C22H16O2	311.3





Appendix D

Results from concentration-response modeling of the data on ES-D3 cell differentiation assay of EST for DBA, 3OH-BaP, and bioactivated-BaP.

Table D1 Results from a BMD analysis of the data on ES-D3 cell differentiation assay of **DBA**. The table presents the benchmark concentration for 50% effect on differentiation (BMCd50) and the 95% benchmark dose lower confidence limit (BMCdL50) values for a BMR of 10% extra risk with characteristics of the model fit. The selected model (BMD/BMDL < 3, lowest AIC) is given in bold.

Model type	Risk Type	BMFR	Restricted model	No of para-meters	p-value (goodness of fit)	AIC	BMCd50 (µg raw material/ml)	BMCdL50 (µg raw material/ml)	BMD/ BMDL
Gamma	Extra	0.1	yes	2	0.0122	209.42	7.14	5.12	1.39
Logistic	Extra	0.1	no	2	0.0025	214.81	9.25	7.41	1.25
LogLogistic	Extra	0.1	yes	3	0.0638	204.39	4.69	2.84	1.65
LogProbit	**Extra**	**0.1**	**no**	**2**	**0.9697**	**192.20**	**3.03**	**1.40**	**2.17**
Multistage	Extra	0.1	no	3	0.0122	209.42	7.14	5.12	1.39
Multistage Cancer	Extra	0.1	yes	2	0.0122	209.42	7.14	5.12	1.39
Probit	Extra	0.1	yes	2	0.0027	214.47	9.21	7.43	1.24
Weibull	Extra	0.1	yes	2	0.0122	209.42	7.14	5.12	1.39
Quantal Linear	Extra	0.1	na	2	0.0122	209.42	7.14	5.12	1.39

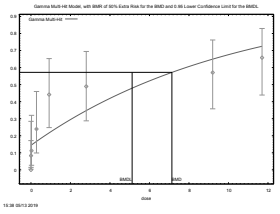
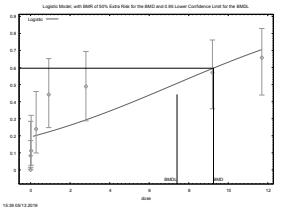
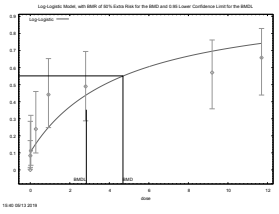
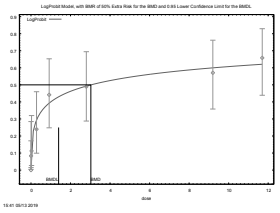
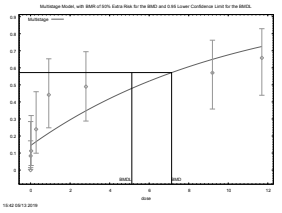
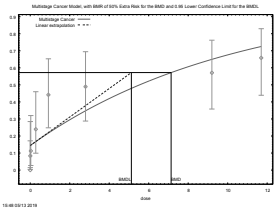
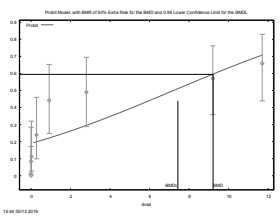
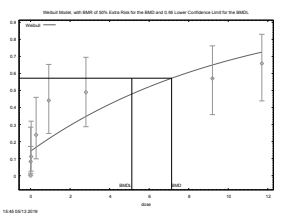
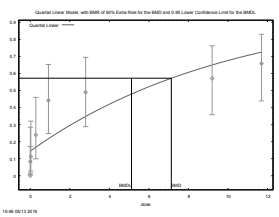










Table D2 Results from a BMD analysis of the data on ES-D3 cell differentiation assay of **3OH-BaP**. The table presents the benchmark concentration for 50% effect on differentiation (BMCd50) and the 95% benchmark dose lower confidence limit (BMCdL50) values for a BMR of 10% extra risk with characteristics of the model fit. The selected model (BMD/BMDL < 3, lowest AIC) is given in bold.

Model type	Risk Type	BMFR	Restricted model	No of para-meters	p-value (goodness of fit)	AIC	BMCd50 (µg raw material/ml)	BMCdL50 (µg raw material/ml)	BMD/ BMDL
Gamma	Extra	0.1	yes	2	0.8151	75.60	0.91	0.66	1.38
Logistic	Extra	0.1	no	2	0.951	73.19	0.99	0.82	1.21
LogLogistic	Extra	0.1	yes	3	0.8761	73.62	1.26	0.66	1.91
LogProbit	**Extra**	**0.1**	**no**	**2**	**0.9575**	**73.02**	**0.98**	**0.82**	**1.20**
Multistage	Extra	0.1	no	3	n.a	n.a	n.a	n.a	n.a
Multistage Cancer	Extra	0.1	yes	2	n.a	n.a	n.a	n.a	n.a
Probit	Extra	0.1	yes	2	0.7501	75.62	1.23	0.63	1.95
Weibull	Extra	0.1	yes	2	0.8966	75.06	0.97	0.70	1.39
Quantal Linear	Extra	0.1	na	2	0.1815	80.72	0.66	0.49	1.35

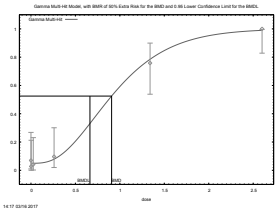
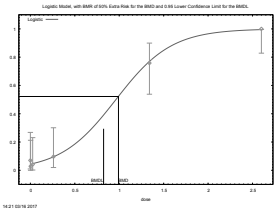
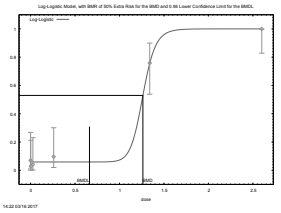
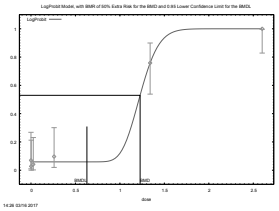
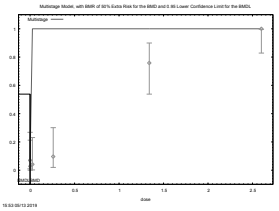
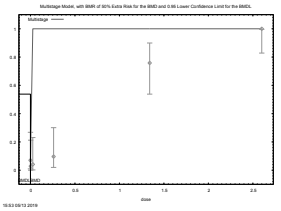
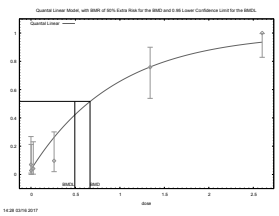
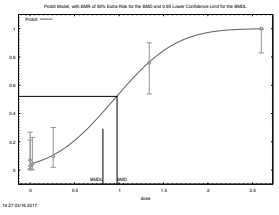
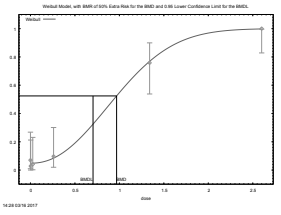
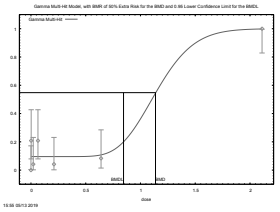
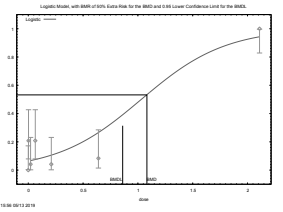
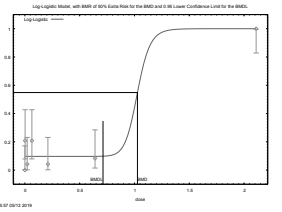










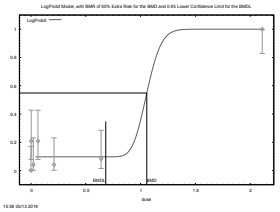
Table D3 Results from a BMD analysis of the data on ES-D3 cell differentiation assay of **bioactivated-BaP**. The table presents the benchmark concentration for 50% effect on differentiation (BMCd50) and the 95% benchmark dose lower confidence limit (BMCdL50) values for a BMR of 10% extra risk with characteristics of the model fit. The selected model (BMD/BMDL < 3, lowest AIC) is given in bold.

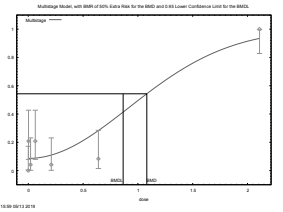
Model type	Risk Type	BMFR	Restricted model	No of para-meters	p-value (goodness of fit)	AIC	BMCd50 (µg raw material/ml)	BMCdL50 (µg raw material/ml)	BMD/ BMDL
Gamma	Extra	0.1	yes	2	0.0442	96.05	1.14	0.85	1.35
Logistic	Extra	0.1	no	2	0.0003	105.95	1.08	0.86	1.26
LogLogistic	**Extra**	**0.1**	**yes**	**3**	**0.0498**	**95.85**	**1.03**	**0.71**	**1.44**
LogProbit	Extra	0.1	no	2	0.0257	97.85	1.06	0.68	1.55
Multistage	Extra	0.1	no	3	0.0017	105.79	1.08	0.87	1.25
Multistage Cancer	Extra	0.1	yes	2	0.0017	105.79	1.08	0.87	1.25
Probit	Extra	0.1	yes	2	0.0003	106.68	1.07	0.86	1.24
Weibull	Extra	0.1	yes	2	0.0257	97.85	1.67	0.70	2.38
Quantal Linear	Extra	0.1	na	2	0	123.41	1.04	0.73	1.42

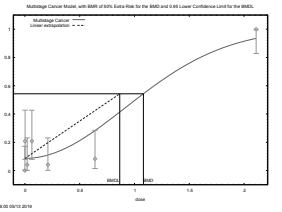


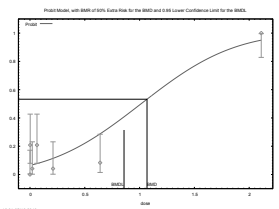


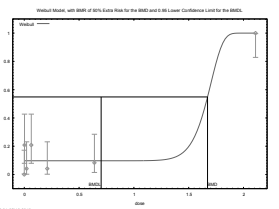


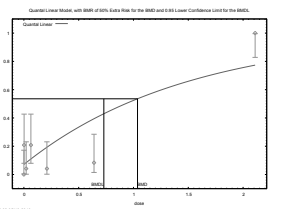












Appendix E

Comparison of cytochrome P450 enzymes activity present in hamster liver microsomes and hamster liver S9 fractions, purchased from Xenotech.

XENOTECH
A DIVISION OF SEKISUI

Data Sheet

S1000 Lot No. 1210379

Golden Syrian Hamster Liver Microsomes
Untreated, Male, Pool of 97
0.5 mL at 20 mg protein / mL
Suspension medium: 250 mM sucrose

Specific Content and Enzyme Activities		*Content / Rate*
Cytochrome P450 content	(nmol/mg protein)	0.881
Cytochrome b_5 content	(nmol/mg protein)	0.598
NADPH-cytochrome *c* reductase	(nmol/mg protein/min)	250 ± 15
7-Ethoxycoumarin *O*-dealkylation	(pmol/mg protein/min)	7840 ± 250

Characterization is performed when the first lot of a product from a given subcellular fraction (e.g., S9) is prepared. Subsequent lots are subject to a verification test only. Values for enzyme activities were determined at a single substrate concentration and are mean ± standard deviation of three or more determinations.

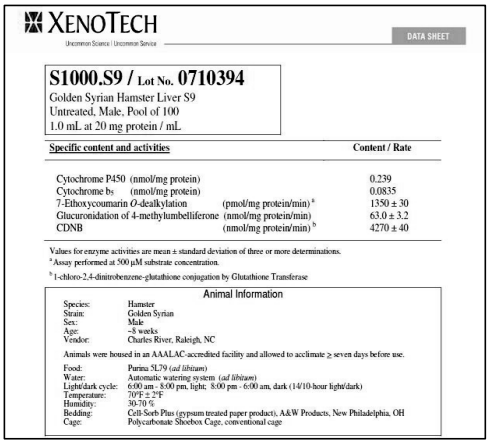
To measure cytochrome P450 (CYP) activity, liver microsomes (50 µg/mL) were incubated in triplicate at 37 ± 1°C for 10 minutes in potassium phosphate buffer (50 mM, pH 7.4), containing $MgCl_2$ (3.0 mM), EDTA (1.0 mM), NADP (1.0 mM), glucose-6-phosphate (5.0 mM), glucose-6-phosphate dehydrogenase (1 Unit/mL) and 7-ethoxycoumarin (500 µM), at the final concentrations indicated. Metabolite formation was determined by validated LC-MS/MS methods with deuterated metabolites as internal standards.

Animal Information

Species:	Hamster
Strain:	Golden Syrian
Sex:	Male
Age:	~ 8-11 weeks
Vendor:	Charles River, Kingston, NY

Animals were housed in an AAALAC-accredited facility and allowed to acclimate ≥ seven days before use.

Food:	Purina 5L79 (*ad libitum*)
Water:	Automatic watering system (*ad libitum*)
Light/dark cycle:	14 hours light / 10 hours dark
Temperature:	70°F ± 2°F
Humidity:	30-70 %
Bedding:	Beta Chip (hardwood), NEPCO, Warrensburg, NY
Cage:	Polycarbonate Shoebox Cage, conventional cage



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DATA SHEET

S1000.S9 / Lot No. 0710394

Golden Syrian Hamster Liver S9
Untreated, Male, Pool of 100
1.0 mL at 20 mg protein / mL

Specific content and activities		Content / Rate
Cytochrome P450	(nmol/mg protein)	0.239
Cytochrome b_5	(nmol/mg protein)	0.0835
7-Ethoxycoumarin *O*-dealkylation	(pmol/mg protein/min) [a]	1350 ± 30
Glucuronidation of 4-methylumbelliferone	(nmol/mg protein/min)	63.0 ± 3.2
CDNB	(nmol/mg protein/min) [b]	4270 ± 40

Values for enzyme activities are mean ± standard deviation of three or more determinations.
[a] Assay performed at 500 µM substrate concentration.
[b] 1-chloro-2,4-dinitrobenzene-glutathione conjugation by Glutathione Transferase

Animal Information

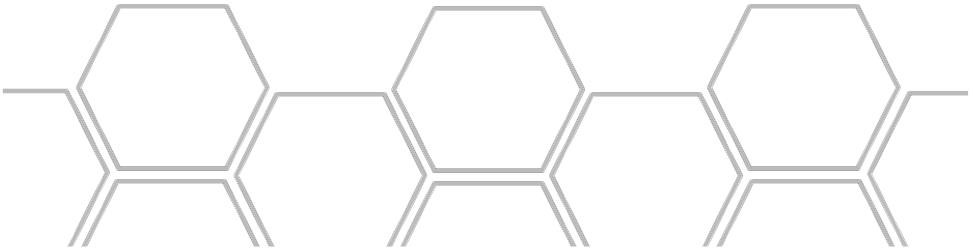
Species:	Hamster
Strain:	Golden Syrian
Sex:	Male
Age:	~8 weeks
Vendor:	Charles River, Raleigh, NC

Animals were housed in an AAALAC-accredited facility and allowed to acclimate ≥ seven days before use.

Food:	Purina 5L79 (*ad libitum*)
Water:	Automatic watering system (*ad libitum*)
Light/dark cycle:	6:00 am - 8:00 pm, light; 8:00 pm - 6:00 am, dark (14/10-hour light/dark)
Temperature:	70°F ± 2°F
Humidity:	30-70 %
Bedding:	Cell-Sorb Plus (gypsum treated paper product), A&W Products, New Philadelphia, OH
Cage:	Polycarbonate Shoebox Cage, conventional cage

Chapter 7

General discussion and future perspectives



Overview of the Results and Main Findings

The REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) legislation requires prenatal developmental toxicity (PDT) testing for all chemical substances that are produced in the European Union (EU) at a volume of ≥100 tonnes per year (ECHA, 2009). Petroleum substances (PS) are UVCBs; substances of Unknown or Variable composition, Complex reaction products and Biological materials, and regulated as such under REACH. REACH requirements for PDT testing applies to PS since most of them are produced at a volume far greater than 100 tonnes annually. Theoretically, this requires a huge number of experimental animals and a considerable amount of resources if the REACH data gaps are fulfilled following the current OECD 414 testing guidelines (OECD, 2001; van der Jagt et al., 2004). Hence, the application of intelligent alternative testing strategies to assess PDT potency of PS is of high relevance. In addition, previous in vivo studies showed that some PS, especially those containing high concentrations of polycyclic aromatic hydrocarbons (PAHs), may induce PDT whilst their gas-to-liquid (GTL) analogues, the modern synthetic products of PS that are completely devoid of PAHs, do not induce PDT (Hoberman et al., 1995; Boogaard et al., 2017; Murray et al., 2013). This led to the hypothesis that PDT as observed with some PS is caused by certain types of PAHs present in these products. Based on these considerations, the present thesis aimed to test the hypothesis that PAHs, which are present in some PS, are responsible for the observed PDT by these substances, and that this potential can be demonstrated using a battery of in vitro alternative testing strategies that consisted of the mouse embryonic stem cell test (EST; **Chapter 2, 4, and 6**), CALUX (Chemical Activated Luciferase Gene Expression) reporter gene assays (**Chapter 3 and 4**), and the zebrafish embryotoxicity test (ZET; **Chapter 5**).

Chapter 2 presented the results obtained using the EST to evaluate the in vitro PDT potency of a range of PS (varying in their PAH content) and GTL products (containing no PAHs), as compared to their potency observed in vivo in the rat. To this end, DMSO-extracts of 9 PS, from 5 different product categories being heavy fuel oil (HFO), distillate aromatic extract (DAE), residual aromatic extract (RAE), gas oil (GO), and vacuum tower overhead oil (VTO); and 2 GTL products, from 2 different product categories namely GTL base oil (GTLb) and GTL gas oil (GTLg), were tested in the ES-D3 cell viability and differentiation assays of the EST. The selected samples included extremes regarding their

PAH content: HFO (total weight percent PAHs = 48% wt. PAHs) and GTL products (0% wt. PAHs), and 8 other PS extracts with PAH contents in-between these extremes, ranging from 1.5 to 12% wt. PAHs. The results in the EST showed that all DMSO-extracts of PS inhibited ES-D3 cell differentiation into beating cardiomyocytes in a concentration-dependent manner at non-cytotoxic concentrations. On the contrary, both GTL extracts did not affect either cell viability or differentiation of ES-D3 cells in the EST. Among all PS extracts tested, HFO was the most potent test substance in inhibiting the differentiation of ES-D3 cells, followed by DAE, VTO, RAE, and GO, and it was also shown that their potency in the EST was proportional to their 3- to 7-ring PAH content. This finding provides a first line of evidence for the role of a specific group of PAHs present in PS extracts in their observed PDT in the EST. Furthermore, in vitro potencies obtained in the EST were compared to results from available in vivo PDT studies, and a good correlation was found between in vitro and in vivo results (R^2: 0.97). Altogether, the results obtained in **Chapter 2**, showed that the EST holds a great promise as one of the alternative assays to evaluate the in vitro embryotoxic potency of PS, within and across product categories, and the obtained results also corroborated the hypothesis that PAHs are the main inducers of PDT for some PS.

To date, the underlying modes-of-action of PAHs and PAH-containing PS in causing developmental toxicity is poorly understood. Shreds of evidence have suggested that it may involve the interaction between PAHs and particular nuclear-hormone-receptors (NHRs) and/or the aryl hydrocarbon receptor (AhR), which eventually leads to the observed PDT by these highly complex PS. Hence, to get a better insight in possible modes-of-action underlying the PDT by some PS, and to evaluate the potential of NHR based in vitro assays for the battery of tests to detect PDT of PS, **Chapter 3** applied a panel of CALUX reporter gene assays to investigate the role of endocrine- and AhR-mediated activity of extracts of PS in their developmental toxicity. The receptors selected included the androgen receptor (AR), the estrogen receptor alpha (ERa), the progesterone receptor (PR), the thyroid receptor beta (TRb), and the AhR. DMSO-extracts of PS and GTL products, the same set of samples tested in **Chapter 2**, were tested in the U2OS AR, ERα, PR, TR CALUX assays and the AhR CALUX assay (also known as the DR CALUX assay) for their ability to (de)activate the aforementioned receptors. Upon cell exposure, all DMSO-extracts of PS showed a strong AhR agonist activity and rather weak antiprogesterone, antiandrogenic, and estrogenic activities. Moreover, only minor effects on thyroid-related

and antiestrogenic activities were seen following exposure to the PS extracts under study. Another prominent finding was that the HFO sample, containing the highest amount of PAHs (48% wt. PAHs), was the most potent (ant)agonist in all assays where activity was observed. On the contrary, none of the DMSO-extracts of GTL products showed an activity or interaction with any of the selected receptors including the AR, ERα, PR, TR, and AhR. Of all CALUX reporter gene assays applied in **Chapter 3**, the AhR CALUX assay appeared to be the most useful for determining receptor-mediated activities of PS UVCBs that are relevant for their developmental toxicity potency, because there was a good correlation between the AhR-mediated activity of the PS and their in vivo PDT (R^2: 0.80) and between the AhR-mediated activity and the in vitro PDT potency of the same PS tested previously in the EST (R^2: 0.80) **(Chapter 2)**. In a subsequent step, principle component analysis (PCA) and hierarchical clustering were conducted using the combined CALUX data, and the results showed that every PS was clustered in line with its chemical characteristics, suggesting a PS category-specific effect signature in the various CALUX assays, which was shown to be mainly driven by their PAH profiles. Overall, the DMSO-extracts of 9 PS (from 5 different PS categories) evaluated in **Chapter 3** showed diverse in vitro endocrine- and AhR-mediated activities, as quantified in the CALUX reporter gene assays applied, and this potency was shown to be associated with the types and levels of a specific group of PAHs present in these substances. To add, of the NHRs tested the AhR-mediated activity of the PS extracts appeared to be the most relevant mode-of-action in relation to their in vitro PDT potency.

Chapter 4 challenges the applicability of both the EST and AhR CALUX assay once more by assessing the PDT potency and AhR-mediated activity of a series of PS extracts mainly from the same product category, HFO, which contained a systematic increment of their level of 3- to 7-ring PAHs, and one highly refined base oil (HRBO) extract that contained no PAHs. The results showed that all DMSO-extracts of HFO, but not of the HRBO, inhibited the ES-D3 cell differentiation into beating cardiomyocytes in a concentration-dependent manner and that their potency was proportional to the amount of 3- to 7-ring PAHs they contained. In addition, HFO extracts also showed AhR-mediated activities, as quantified in the AhR CALUX assay, where HRBO-extract tested negative in this assay. To study the possible role of AhR activation in mediating the observed in vitro PDT effects, ES-D3 cells of the EST or H4IIE.luc cells of the AhR CALUX assay were co-exposed with the DMSO-extracts of various PS and a GTL product, in the absence or presence of the AhR antagonist

trimethoxyflavone (TMF). The selected DMSO-extracts of PS and GTL products included sample #034-HFO, #097-DAE, #172-GO, #175-VTO, #186-RAE, #145-HRBO, and #091-GTLb. This range of samples was selected because they represent both the different categories of PS and GTL product that we tested in the EST and AhR CALUX assay, and display a representative range of concentrations of PAHs. It was shown that co-exposure of ES-D3 cells (EST) or H4IIE.luc cells (AhR CALUX assay) with the selected DMSO-extracts of PS or GTLb and TMF, successfully neutralized the PS-induced inhibition of ES-D3 cell differentiation into cardiomyocytes as well as the AhR-mediated induction of gene expression by these substances. This pointed at the role of AhR activation in the modes-of-action underlying both endpoints. Taken together, the results of **Chapter 4** corroborate the hypothesis that the PS-induced PDT is caused by 3- to 7-ring PAHs present in these substances. Also, the PDT as observed with some PS is (at least) partially mediated via the AhR.

Chapter 5 applied the ZET to assess the relative PDT potency of the same set of samples as tested in **Chapter 2 and 3**, including the DMSO-extracts of 9 PS (varying in their PAH content from 5 PS categories) and 2 GTL products (devoid of PAHs), as compared to their in vivo PDT potency. The potency obtained in the ZET was compared to the previously obtained data in the EST **(Chapter 2)** and AhR CALUX assay **(Chapter 3)** for cross-model comparison and to see whether all of these assays could be combined as a battery of assays to assess the PDT potency of PS UVCBs. The obtained results in the ZET show that the DMSO-extracts of PS induced concentration-dependent developmental retardation in the zebrafish embryos (scored at 96 hours post fertilization (hpf)). HFO was the most potent test substance for the observed embryotoxicity in the ZET, followed by DAEs, GOs and VTO, and lastly RAEs. In contrast, GTL products did not induce any effect at all in the ZET. Further data analysis showed that the potency obtained in the ZET was proportional to the amount of 3- to 5-ring PAHs present in the corresponding PS sample (R^2: 0.96). In addition, the ZET-results were moderately correlated with those obtained in the EST (R^2: 0.61), while the correlation with potencies reported in in vivo studies was higher for the EST (R^2: 0.85) than for the ZET (R^2: 0.69). A moderate correlation also existed between potencies in the ZET and the AhR-mediated activity of the same test substances as measured in the AhR CALUX assay (R^2: 0.66). Combined data, using results obtained in the EST, AhR CALUX assay, ZET, and PAH content, ranked and clustered the test substances in line with their in vivo potencies and chemical characteristics. Hierarchical

clustering revealed that PS belonging to the same product category were grouped together, and so were the GTL. Moreover, there was a clear separation of clusters between the PS and GTL products, showing a clearly different bioactivity of substances, with and without PAH constituents. The combined data suggested the applicability of the assay battery that consisted of the EST, the ZET, and the AhR CALUX assay to be used in combination with the analytical data (i.e. PAH content) for grouping and prioritization of PDT testing of these highly complex PS UVCBs. Finally, it was concluded from **Chapter 5** that i) the ZET is able to predict the in vivo PDT of PS but does not outperform the EST as a stand-alone assay for testing PDT of these substances, ii) the ZET is a useful addition to a battery of alternative assays able to predict the in vivo PDT of PS UVCBs, and iii) 3- to 5-ring PAHs present were the major inducers of PS-induced PDT in the ZET.

Despite a good concordance between in vitro PDT potency of the PS and GTL extracts under study (as shown in **Chapter 2, 4, and 5**) and their potencies observed in vivo, it was assumed that the aforementioned alternative assays would only assess the embyrotoxicity potential of parent substances and not that of potentially embryotoxic metabolites. This because of the limited or even absent metabolic capacity in the respective cell models. In view of this, in **Chapter 6** we combined a biotransformation system, using hamster liver microsomes, with the EST to compare the in vitro PDT potency of two 5-ring PAHs, benzo[a]pyrene (BaP) and dibenz[a,h]anthracene (DBA), and DMSO-extracts from five PAH containing PS and GTLb, with and without bioactivation. The results obtained showed that in the absence of bioactivation, DBA, but not BaP, inhibited the differentiation of ES-D3 cells into beating cardiomyocytes in a concentration-dependent manner. Upon bioactivation, BaP tested positive in the EST, which showed that BaP needed to be transformed into its reactive metabolites to exert its embryotoxic effect. This metabolite includes 3-hydroxybenzo[a]pyrene that was shown to be active in the EST itself. GTLb extracts tested negative in the EST, with or without bioactivation. Interestingly, the ability of the selected PS extracts to inhibit the ES-D3 cell differentiation into beating cardiomyocytes were not substantially changed when ES-D3 cells were exposed to the metabolite mixtures of the corresponding PS. This suggested that metabolism may not play a crucial role for PS to exert their in vitro PDT effects. Altogether, results obtained in **Chapter 6** indicate that although some PAH constituents require bioactivation to become able to induce PDT, some do not and this latter also

appears to hold for the (majority of) the PS constituents responsible for the in vitro PDT of these complex substances.

To conclude, the present thesis generated a proof-of-principle for the use of a battery of mode-of-action-based alternative testing strategies to predict in vivo PDT of PS. This battery consists of the EST, the ZET, and the AhR CALUX assay, and enables evaluation of the PDT potency of highly complex PS UVCBs. The obtained results corroborate the hypothesis on the role 3- to 7-ring PAHs present in the PS extracts for the observed in vivo PDT. The data generated in the present thesis may assist in the grouping of PS into a limited number of categories and/or in facilitating the selection of worst-case representatives per PS category for in vivo testing to fulfil the REACH data requirements with regard to PDT. Such intelligent alternative testing strategies will significantly minimize the required number of animals and resources needed to study the PDT potency of highly complex PS.

General Discussion and Future Perspectives

Although the present thesis extends the understanding and qualitative prediction of potential PDT by various PS (within and across product categories), as compared to their potency observed in vivo, several issues for further improvements remain to be elucidated to i) fully unravel the role of different types of PAHs for the observed PDT by these highly complex substances and ii) further apply intelligent and integrative testing strategies for PDT testing of PS UVCBs. Thus, in the following sections, an in-depth discussion of the obtained results, including study limitations, their implications (e.g. for REACH compliance), and the future perspectives is provided. The topics discussed include:

1. Characterization of PAH constituents present in PS extracts tested
2. The in vitro alternative assays used for developmental toxicity testing of PS and other assays to be considered
3. Comparison of in vitro and in vivo findings
4. Relation of in vitro developmental toxicity potency to PAH content
5. Quantitative in vitro to in vivo extrapolation (QIVIVE)
6. The implications of results obtained for the REACH roadmap for PS and the 3Rs
7. Future recommendations/challenges and concluding remarks

1. Characterization of PAH constituents present in PS extracts tested

The Method II extraction and chemical characterization procedures as described by Roy et al. (1988) and McKee et al. (2013) were applied to generate the DMSO-extracts of PS and GTL products tested in the present thesis. The use of DMSO-extracts, which contain most of the more polar constituents of the PS (e.g. 3- to 7-ring PAHs), is a common and standard approach for in vitro testing of these group of PS. This method is widely applied and routinely used for example for in vitro mutagenicity testing of PS since the '80s (Blackburn et al., 1986; Carrillo et al., 2019; Concawe 1994; Hermann et al., 1980). In the present thesis, the application of the DMSO-extracts of PS and GTL products for in vitro dosing proved to adequately capture the desirable and expected developmental toxicity potency of these substances. This indicates that the DMSO-extracts contain and adequate representation of the biologically active fractions of the PS relevant for their embryotoxicity potency.

Two relevant questions emerge when considering the use of PS extracts for in vitro testing of these substances, including i) what kind of constituents are actually present following the Method II extraction procedure (DMSO-extraction) of neat/bulk materials of PS, and ii) how relevant is it to use the DMSO-extracts for the hazard assessment of these substances? DMSO selectively extracts and concentrates the polycyclic aromatic compounds, including 2- to 7-ring PAHs, present in PS that are either unsubstituted (naked) or contain short chain alkyl substituents (Carrillo et al., 2019; Roy et al., 1988). This selectivity is due to the affinity of the outer electrons of DMSO's sulphur moiety towards the π electron-rich sites of the poly-aromatic ring molecules, including naked and lowly-alkylated 3- to 7-ring PAHs (Natusch and Tomkins, 1978; Carrillo et al., 2019). An increase in the carbon number in the side-chain of alkylated PAHs results in steric hindrance and resulting decreased affinity of DMSO towards the aromatic molecules with a long carbon side-chain substituent, such as for example the highly-alkylated 3-ring PAH dodecylphenanthrene. Consequently, the non-extracted fraction from the DMSO-extraction procedure, known as the raffinate fraction, contains saturated hydrocarbons and lower aromatics PAHs (i.e. 2-ring PAHs) that are highly-alkylated (from 6 to 13 carbon atoms of the alkyl chain length). For heteroatom (S, N, or O)-containing PAHs, their abundance in both the DMSO-extracts and raffinate fractions are very low/minor (Carrillo

et al., 2019). These characteristics make the DMSO extracts suitable to test the hypothesis for the role of 3- to 7-ring PAHs in the PDT of the PS.

Furthermore, there are two other reasons to apply DMSO-extracts rather than the neat/bulk materials, of PS for in vitro testing of these substances. Firstly, the neat/bulk materials of PS and GTL products are hydrophobic substances and extremely poorly soluble in aqueous-based media. Thus, it is almost impossible to dose them into the vast majority of in vitro test systems, as their bioavailability would be very limited if not absent. Secondly, the DMSO-extraction procedure of PS was designed to improve the interaction of potentially toxic constituents present in PS with aqueous in vitro test systems (Blackburn et al., 1986). In this respect, it is also relevant to mention that prior studies have noted a poor correlation between results obtained in an in vitro mutagenicity assay (i.e. the standard Ames test) and in vivo dermal carcinogenicity studies when using the neat/bulk materials of PS for in vitro dosing (Blackburn et al, 1986; Cragg et al., 1985). The authors concluded that the poor correlation was due to the inadequate interaction between PS and the test organism used, and this issue was solved by using the DMSO-extracts of the corresponding PS for the in vitro mutagenicity assay. When using the DMSO-extracts of the corresponding PS, a good correlation was obtained between in vitro mutagenic activity and in vivo dermal carcinogenicity (R^2: 0.92) (Blackburn et al., 1986). Therefore, to overcome the solubility issue and to ensure that the test substances are introduced in such a way that they are bioavailable during the test, the Method II DMSO-extraction procedure was applied and the generated DMSO-extracts were used for in vitro dosing of PS and GTL products in the present thesis. The results obtained in the present thesis confirmed the suitability of the DMSO extracts thus obtained for obtaining data on in vitro PDT of PS that correlated well with their in vivo potency **(Chapter 2, 4, and 5)**.

The chemical composition of the DMSO extracts was characterised in terms of their 2-, 3-, 4-, 5-, 6- and 7-ring PAH content using gas chromatography with mass selective detection (GC/MS). Naphthalene, phenanthrene, pyrene, benzo[a]pyrene, benzo[ghi]perylene, and coronene were used as standards to define the boundaries of retention times for (unsubstituted) 2- to 7-ring PAHs present in the corresponding PS (McKee et al., 2013). Results were reported as the aromatic ring class (ARC) profile, representing the weight percent of 2- to 7-ring PAHs from the starting materials of 4.0 gram sample. This implies that the chemical characterization method applied was not able to provide information

on the presence of other types of PAH that may be present in PS extracts under study, which may include alkylated PAHs and heteroatom (S, N, or O)-containing PAHs. Hence, it should be well-noted that the correlation analysis between in vitro findings and PAH content as presented in **Chapter 2, 3, and 5** was done using weight percent of (unsubstituted) 2- to 7-ring PAHs present in each PS sample, without knowing or considering the amount of the other types of PAHs, if present, yet. A recent publication by Carrillo et al. (2019), using the same DMSO-extraction procedure as applied in the present thesis, but in combination with an NMR method, showed that the DMSO-extracts of HVI160, from the DAE category, also contained 3-5 ring low-alkylated PAHs (with 4-5 carbon atoms in the alkyl chain length) in addition to the presence of 3-5 ring unsubstituted PAHs. In view of this, more analytical characterization of the PS samples tested may be required to fully characterize the types and amount of both unsubstituted and substituted PAHs (i.e. alkylated PAHs) present in these substances. Chemical characterization of PS can be quite a challenge due to the inherent complexity of these substances. It is suggested to apply a structured analytical approach tailored to the properties of each PS category to know (at least) the vast majority of constituents present, including PAHs (Concawe, 2012). For instance, the GC/MS method is suitable for characterization and identification of constituents present in light PS, such as naphthas/gasoline. For heavy PS (e.g. HFO, DAE, RAE), a combination of several analytical methods, including LC/MS, GC/MS, and NMR spectroscopy, may be needed to provide complementary information for characterization of these substances. Such data and information will obviously help to further unravel the role of specific groups of PAH constituents for the observed PDT induced by these substances.

In total, 17 PS samples were tested in the present thesis and their PAH profiles varied, not only across product categories but also within one category possessing the same CAS number (i.e. the DAEs). In short, light PS like GO and VTO categories, containing mainly 2- and 3-ring PAHs were tested and heavy PS, such as HFO and DAE, containing primarily 3- to 7-ring PAHs. The tested PS samples represent 6 categories: HFO, DAE, RAE, SRGO (also known as GO, gas oil category), VTO (member of the vacuum gas oils, hydrocracked gas oil and distillates fuel (VHGO) category), and HRBO; out of 22 PS categories (Petroleum HPV, 2013) that have to be evaluated for their potential effects on prenatal development under EU REACH legislation (Figure 1). The 17 PS samples tested are quite representative for the 6 PS categories now investigated, in particular with regard to the types, total

amount, and the diversity of PAH constituents they contain. Therefore it can be concluded that within these 6 PS categories, the potential PDT effects of an untested sample can be predicted, if its PAH content and product category is known, using the data obtained in the present thesis from the 17 model PS tested.

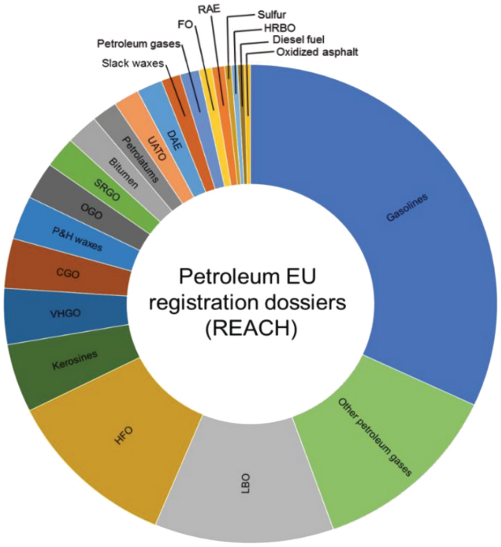


Figure 1 Overview of the 22 PS categories of petroleum EU REACH registration dossiers (Petroleum HPV, 2013).

Abbreviations. LBO: lubricating base oil; HFO: heavy fuel oil; VHGO: vacuum gas oils, hydrocracked gas oil and distillate fuels; CRO: cracked gas oils; P&H waxes: paraffinic and hydrocarbon waxes; OGO: other gas oils; SRGO: straight run gas oil; UATO: unrefined/acid treated oil; DAE: distillate aromatic extract; FO: foot oil; RAE: residual aromatic extract; HRBO: highly refined base oil.

2. The in vitro alternative assays used for developmental toxicity testing of PS and other assays to be considered

PS are highly complex substances (UVCBs), thus, the PDT as observed with some of these substances may be caused by a wide range of constituents and corresponding underlying modes-of-action. Therefore, a battery of alternative assays, instead of a stand-alone assay, that focuses on various potentially relevant modes-of-action of PDT induced by PS may be required to answer the challenge of in vitro PDT testing for these group of substances. Of many available and validated in vitro assays, the present thesis applied the EST and ZET to evaluate the PDT potency of the DMSO-extracts of PS, within and across product categories, as compared to their PDT potency in vivo. Both test systems are considered animal-free, and they were shown previously to have good predictability and accuracy for

assessing the embryotoxicity potency of different chemical classes possessing different modes-of-action in inducing their PDT (Brannen et al., 2016; de Jong et al., 2009; Dimopoulou et al., 2018; Geier et al., 2018; Goodale et al., 2013; Li et al., 2015; Louisse et al., 2011; Piersma et al., 2013; Strikwold et al., 2012). In the EST, the inhibition of ES-D3 cell differentiation into beating cardiomyocytes is used as the endpoint to assess relative developmental toxicity potency of test substances (Genschow et al., 2002, 2004). Whereas, lethality and interference of morphological developments of zebrafish embryos upon chemical exposure (up to 144 hpf) are used to determine the embryotoxicity potency of the corresponding test substances in the ZET (Beekhuijzen et al., 2015; Hill et al., 2011; OECD, 2011a, b). Using both assays with given pre-defined endpoints, the present thesis assessed the developmental toxicity potency of PS UVCBs and GTL products and showed that results obtained in these in vitro assays are in line with their PDT potency in vivo **(Chapter 2, 4, and 5)**.

A moderate correlation exists (R^2: 0.61, **Chapter 5**) when the relative PDT potency obtained in the EST and ZET are compared. This is in agreement with results of a study reported by de Jong et al. (2011) who found a moderate correlation between the EST and ZET results for the PDT potency of a series of azoles. The moderate concordance between the EST and ZET is not unexpected considering the different complexity and setup, the different assessment endpoints, and the potentially different compound kinetics, within each assay. As discussed in **Chapter 5**, the EST used a serum-based medium where the ZET used a saltwater-based medium, which consequently affects the solubility and thus free-concentration of test substances during the test (Fischer et al., 2017). This can be seen when comparing the results obtained in **Chapter 2 and 5**. The potency obtained in the EST is best correlated to the amount of 3-7 ring PAHs present in each PS sample and for the ZET, it is mainly affected by the group of 3-5 ring PAHs. The disparity in this correlation may be due to the solubility limit of some 5-7 ring PAHs with log K_{ow} >5 in the ZET medium (Sverdrup et al., 2002; Lu et al., 2008), and as a consequence, limiting the bioavailability and uptake and thus the PDT effects of these group of PAHs in the ZET. Moreover, the starting time of exposure and the exposure time-window are quite different between the EST and ZET. The EST lasts for 10 days, in which exposure medium is refreshed on day 3 and 5 of the assay. For the ZET, the dosing is done once, at the beginning of the assay and zebrafish embryos are subsequently exposed for 5 days until the end of the test.

In addition to the EST and ZET, the present thesis also applied a panel of CALUX reporter gene assays, to evaluate the possible role of endocrine- and AhR-mediated activity of PS extracts, tested in association with their developmental toxicity potency **(Chapter 3)**. It was concluded that the AhR-mediated activity appeared to be the most relevant receptor-mediated activity when compared to PS-induced PDT. This is also the reason why only the AhR CALUX assay, and not the whole panel of CALUX assays, was included in the test battery applied in **Chapter 4**, when testing a series of PS from the HFO category. The role of the AhR to partially mediate the PS-induced PDT was confirmed by results obtained in **Chapter 4**. Co-exposure of cells with PS extracts and the AhR antagonist trimethoxyflavone (TMF) counteracted the PS-induced PDT and AhR-mediated gene expression induced by these substances. It should be noted that the present thesis does not intend to use the AhR CALUX assay in isolation as a predictive assay for assessing PDT potency of PS UVCBs. Instead, it should be used in conjunction with the EST and/or ZET, as shown in **Chapter 4**, to study the PDT potency and potential underlying mode-of-action for the observed PDT by PS extracts. Extending this notion for receptor-mediated PDT by PS extracts, the results in **Chapter 6** demonstrate that metabolism may not play an essential role in the PS-induced PDT in the EST. This implies that an interaction between the PAH constituents present in the PS and certain receptor-ligand complexes, rather than bioactivation, is needed to exert their PDT potency. In view of this, the contribution of other receptors to mediate the PS-induced PDT should be investigated further, as they may play a role in addition to the AhR activation.

The use of a combination of in vitro assays (or an assay battery) to predict relative in vivo embryotoxicity of chemical substances has been used before proving its capability to assess the PDT potency of different classes of compounds (Piersma et al., 2013). Using a combination of assays is based on the notion that different in vitro test systems reflect different complexity and molecular pathways and thus provide together a better way to capture the various underlying modes-of-action for the observed embryotoxicity potency of test substances. A particular assay may be designed to specifically study the possible interaction of test substances with a specific pathway/receptor, such as for example the U2OS CALUX reporter gene assays, while other assays may capture different mode(s)-of-action. Hence, having a battery of tests that complement each other and cover relevant modes-of-action underlying potential PDT would be the best approach to evaluate PDT of chemical substances in vitro. For example, Li et al. (2015, 2016) used the EST, in

combination with the BeWo transport model, and an ex ovo assay using chicken embryos to predict the PDT of antifungal compounds, and Dimopoulou et al. (2017, 2018) applied the rat whole embryo culture (WEC) and the EST, both in combination with omics approaches, for assessing the in vivo embryotoxicity of azoles. Thus, the selection of alternative assays as integrative testing strategies should include consideration of whether the relevant modes-of-action (or relevant pathway) of PDT caused by the test substances of interest would be covered by the chosen assays or not.

Considering the complexity of the mechanisms underlying PDT itself, the application of suitable (or fit-for-purpose) alternative assays that adequately mimic the in vivo situation can be quite a challenge. The selected alternative assays to define the battery of tests for in vitro PDT testing of PS may represent only part of the complexity of the in vivo situation. Hence, the usefulness of adding other model organisms or additional endpoints to the current test systems should also be considered. Here we choose to discuss three emerging alternative assays or approaches that may prove useful additions to our battery of tests and have been proposed in recent years able to predict PDT of chemical substances, namely: Stemina devTOX *quick*Predict (devTOX^{qP}), *Caenorhabditis elegans* (*C. elegans)* model, and gene expression in selected relevant in vitro models.

- **Stemina devTOX quickPredict (devTOX^{qP})**

The devTOX *quick*Predict assay (devTOX^{qP}) was developed by Stemina Biomarker Discovery, Inc. (Madison, Wisconsin, USA) for predicting the developmental toxicity potential of chemicals based on changes in human induced-pluripotent stem (hiPS) cell metabolism. The devTOX^{qP} assay measures changes in the abundance of the metabolic biomarkers ornithine and cystine following exposure to a broad concentration range of test substances (Palmer et al., 2017). These two metabolites were identified as a metabolic signature of hiPS cells relevant for detecting developmental toxicity potential since both are actively involved and essential for normal cell proliferation and differentiation during embryonic development (Palmer et al., 2013; West et al., 2010). Hence, the imbalance of measured ornithine/cystine ratio (o/c ratio) in the medium of hES cells upon the final 24 hours treatment period is the predictive indicator used in the devTOX^{qP} assay for determining developmental toxicity potential in human (Palmer et al., 2013). Using the o/c ratio, the Stemina devTOX^{qP} assay is able to show 85% accuracy with

89% specificity and 82% sensitivity when applied to predict the developmental toxicity potency of 80 chemical substances, regardless their classification as weak or strong embryotoxicants (Zhu et al., 2016; Palmer et al., 2017). In the past years, the US EPA has also evaluated the applicability of the Stemina devTOX^{qP} platform and the preliminary results show an 82% balanced accuracy (with 71% sensitivity and 100% specificity) of this assay to evaluate developmental toxicity potency of chemical substances listed in the ToxCast phase-I and -II library (~1065 samples) (Knudsen et al., 2015, 2016).

In addition to the high predictive value of the devTOX^{qP} platform, this assay allows high-throughput automation while reducing false-positive risk due to the use of human embryonic cells that are assumed to have a higher sensitivity to embryotoxicants than the rodent models, e.g. mouse ES cells (Palmer et al., 2013). The use of human cell lines for in vitro testing is favored nowadays to narrow the interspecies differences based on the notion that every species may react differently when exposed to certain toxicants, including embryotoxicants. Although the devTOX^{qP} platform is undergoing continuous development and optimization, its usefulness to evaluate the developmental toxicity potencies of chemical mixtures and complex substances, such as PS UVCBs under study, should be explored.

- ***C. elegans*** **model**

C. elegans is another alternative model that is getting increasingly popular for predictive toxicity testing (Boyd et al., 2012; Xiong et al., 2017). *C. elegans* is a free-living small nematode (~1 mm) with a fast reproductive and developmental cycle (± 3 days at 20 ^{o}C), thus making the organism a suitable model to study the potential developmental-related effects at the whole-organism level (Boyd et al., 2012; Hunt et al., 2016). Besides its application to evaluate general toxicity of chemicals, recent publications show the usefulness of *C. elegans* for developmental toxicity testing (Boyd et al., 2009, 2012, 2016; Hunt et al., 2016, 2018). Boyd and co-workers at the NIEHS-USA have developed the so-called *C. elegans* growth and larval development assay able to provide an indication of a chemical's effect on the growth and larval development of *C. elegans*. The growth of *C. elegans* is not a continuous process; rather, it occurs through four distinct molts (L1-L4) with differing sizes at different life stages, starting from stage Larval 1 (L1) to an adult nematode (Byerly et al., 1976). Hence, the delayed or inhibition of the growth

development from the L1 stage to adult *C. elegans* upon chemical exposure is used as the endpoint and regarded as an indication of inhibition of the developmental process. Using the *C. elegans* growth and larval development assay, Boyd et al. (2016) have successfully screened the developmental toxicity effects of the ToxCast Phase I and Phase II libraries (968 chemicals in total), and compared the obtained results with those of zebrafish, rat, and rabbit PDT studies. Of these chemicals 62% tested positive in the *C. elegans* assay and the best concordance was found between the potency in *C. elegans* and the results from the ZET with a balanced accuracy (the average value of the sensitivity and specificity for an assay) of 69%. Moreover, the balanced accuracy between results from the *C. elegans* assay and from rat or rabbit PDT studies was slightly lower, ranging from 43% to 53% (Boyd et al., 2016).

Not many published articles on the usefulness of *C. elegans* as a model organism to evaluate the developmental toxicity potency of PAHs and PAH-containing PS are available up to date. Sese et al. (2009) evaluated the toxicity of PAHs, including phenanthrene, pyrene, and BaP, and the authors concluded that the disturbances of growth and reproduction processes are the most sensitive adverse parameters and preferred over mortality when assessing PAHs toxicity using *C. elegans.* In line with this, Boyd et al. (2016) reported the concentration-dependent inhibition of *C. elegans* development by different PAHs, such as phenanthrene, anthracene, pyrene, benz[a]anthracene, and benzo[b]fluoranthene. Surprisingly, when the *C. elegans* assay was challenged to investigate the potential developmental toxicity effects of the DMSO-extracts of PS and GTL products (Hughes and Kirkels, 2018), no prominent developmental-related effects were observed. A minor disturbance of the development and reproduction of *C. elegans* upon exposure to PS and GTL extracts was indeed observed, but no conclusive result could be obtained when correlating the obtained data with available in vivo PDT data on the same substances, also in association with their PAH content (Hughes and Kirkels, 2018). One of the noticeable findings was that the supposed-to-be negative control substances, one of the GTLs, tested positive in the *C. elegans* assay for developmental and reproductive toxicity, hence, generating potentially false-positive results. This may indicate a lower predictive capability of the *C. elegans* assay for assessing the PDT potency of the DMSO-extracts of PS and GTL extracts, in comparison to the other assays conducted in the present thesis like the EST or ZET. The better performance of the EST and ZET for detecting PDT of PS could partly be explained by the absence or nature of certain

receptors in *C. elegans*, such as the AhR, that is known to mediate the developmental toxicity of some PAHs and PAH-containing substances in mammals (Billiard et al., 2006; Puga et al., 2005; Ketelslegers et al., 2018). *C. elegans* has an AhR that shows only 38% amino acid identity but similar biochemical properties as the human AhR (Powell-Coffman et al., 1998), although the spectrum of ligands that activate the C. *elegans*-AhR is thought to be different than those that activate the mammalian AhR. The AhR in C. *elegans* is expressed in neurons to direct their differentiation and to regulate the social feeding behavior of these organisms (Qin and Powell-Coffman, 2004; Qin et al., 2006). This means that the AhR in invertebrates like *C. elegans* is not regulated in the same way as it is in mammals. Even so, the usefulness of *C. elegans* as an alternative model for PDT testing of PS UVCBs should be investigated and remains open for future studies.

- **Gene expression in selected relevant in vitro models**

As technology quickly advances, it is now possible to investigate the underlying mechanism of developmental toxicity using gene expression profiling, i.e. transcriptomics, in addition to the classical readouts derived from different in vitro alternative assays like the EST, WEC, or ZET. Transcriptomics can be used as a tool to investigate exposure-response relationships (time-dependent effects), classification of test substances based on common/unique gene signatures related to the specific modes-of-action in causing the observed developmental toxicity, for cross-species comparisons and/or for in vitro vs in vivo comparisons) (Robinson et al., 2012). Phenotypic changes, used as the readout parameters in different in vitro assays to evaluate the PDT potency, may occur as a result of changes in gene expression during embryonic development. Hence, transcriptomics-based studies may result in the identification of perturbation of relevant biological pathway underlying the observed PDT and might be used as an early biomarker to identify developmental toxicity induced by a specific group of substances.

Integrating a transcriptomics-based endpoint with an in vitro test system able to evaluate the PDT potency of chemical substances can be done using either non-targeted (i.e. total RNA-sequencing) or targeted RNA-sequencing (e.g. Tempo-seq or Illumina targeted RNA-seq platforms). Total RNA-sequencing provides a more comprehensive understanding of phenotypic effects of interest and allows biomarker identification across the broader range of transcripts (including both coding and non-coding RNA transcripts) (Ozsolak and

Milos, 2011). Targeted RNA-sequencing allows the selection and sequencing of specific transcripts of interest or in other words, it focuses on a set of genes of interest, including genes related to the developmental toxicity of the embryotoxicants under study.

As an example, van Dartel et al. (2009a,b, 2010a,b, 2011, a,b,c) have reported studies using a combination of the EST with transcriptomics-based identification (Microarray Affimetrix 21997-mer oligonucleotides) to evaluate gene expression changes (biomarkers) during embryonic stem cell differentiation into cardiomyocytes following exposure to different types of known embryotoxicants, including 5-fluorouracil (5-FU), retinoic acid, and phthalates. In short, using an RNA-seq platform, the authors identified differentially expressed genes (DEGs) that were commonly expressed among the embryotoxicants tested and these genes were then assembled into a minimal subset of genes called Van_Dartel_heartdiff_24h and EST_BIOMARKER_GENES. The van_Dartel_heartdiff_24h gene set contains 38 genes that are enriched for embryonic development and morphogenesis where the EST_BIOMARKER_GENES gene set consists of 26 genes with functions related to development, transcription and cellular proliferation (van Dartel and Piersma, 2011). Additionally, the authors also defined a so-called "PCA-derived differentiation track" on the basis of the genes identified to be involved in the continuous development of ES-D3 cells from 0 to 24/48 hours after initiation of cell differentiation. The prediction of developmental toxicity using the "differentiation track" is based on the significance of deviation or changes in gene expression of exposed cells in comparison to their time-matched control. Moreover, using the aforementioned two gene sets, the accuracy of van_Dartel_heartdiff_24h and EST_BIOMARKER_GENES to predict relative developmental toxicity potency of a series of 12 compounds (tested at a single concentration) was 83% and 67%, respectively (van Dartel et al., 2011b). However, the control negative compound (i.e. saccharin) was misclassified using the EST_BIOMARKER_GENES and false-negative results was also found when using the two gene subsets for predicting PDT potency. This may be caused by the large variation of gene changes in ES-D3 cell differentiation, which may overwhelm the effects induced by the test compounds. Cell proliferation and differentiation are indeed very dynamic processes involving upregulation and downregulation of many genes, hence, it might indeed be challenging to visualize the effects of compounds related to their PDT potency using a limited number of predefined gene subsets.

Also, unpublished results of the current PhD study revealed a variation in gene changes during ES-D3 cell differentiation when a targeted RNA-seq platform, Tempo-seq assay, was used to study global changes in gene expression following exposure to 0.25% DMSO (solvent control) and culture medium of the EST. In short, ES-D3 cells were exposed for either 48 or 120 hours and the resulting embryoid bodies were collected, lysed, and used for the Tempo-seq assay. Preliminary results of this experiment show quite a diverse response of DEGs, in particular among the replicates of either the solvent control (0.25% DMSO) or just the culture medium (data not shown). In other words, significant DEG responses already occur due to the developmental process as such, providing a substantially changing background even within the controls without compound exposure. As a consequence, it was indeed challenging to identify the compound-related DEGs, relative to the controls applied, since the variation of gene changes i the controls seemed to overwhelm the effects induced by the test compounds.

Another possible drawback of using omics-based studies is the limitation for evaluating a biologically-relevant response, e.g. developmental toxicity, in a dose or concentration-dependent manner, which in contrast can easily be assessed using the apical readout of the in vitro test system, such as for example the effect on beating cardiomycocytes as used in the EST. In most cases, only one concentration or low vs high doses of test substance are applied for gene expression studies, mainly because of budget limitations. The limited dosing regimen in combination with the relatively large background changes resulting from the developmental process as such make definition of suitable concentration response curves challenging. Even when testing a full dose response curve, the variability in the omics read out appeared larger than in the classical read out as shown by the results of van Dartel et al. (2011a). Furthermore, some published studies indicate that alteration in gene expression provides a more sensitive parameter than the apical readout of the classic in vitro developmental toxicity test system (Dimopoulou et al., 2017; Hermsen et al., 2012). The basis is that changes in gene expression generally occur at lower concentrations than those causing for example the morphological embryotoxicity effects. However, the interpretation of toxicogenomics data should be made carefully since these early changes do not necessarily reflect adverse effects, but could also indicate homeostatic or adaptative responses towards chemical exposure (Levorato et al., 2019). Altogether, toxicogenomics may serve as a supportive tool to get a better insight into a chemical mode-of-action and define the affected molecular pathways related to the

observed in vivo or in vitro effects. Knowing the expected target organs and possible mode-of-action from omics-based studies, will also help in choosing the relevant mechanistic-based in vitro assay for hazard assessment of the chemical under study.

Available literature on the application of omics for studying the underlying modes-of-action of PDT by PAHs and PS are limited. Several studies using zebrafish embryos as the model organism showed that exposure to several PAHs like pyrene and BaP affect the expression of particular genes like HOX and FOX (related to skeletal malformations), and pathways related to xenobiotic metabolism and embryonic development, such as the AhR and hedgehog signalling pathway (Goodale et al., 2013; He et al., 2011; Huang et al. 2012). For PS UVCBs, an omics-based study was done only for the purpose of grouping or categorization using iPSC cardiomyocytes and hepatocytes in combination with a targeted gene-expression profiling assay called Tempo-seq (Grimm et al., 2016). Furthermore, the Cat-App project, initiated by Concawe in 2016, integrates in vitro testing using apical endpoints, high-throughput genomics, and integrative data analyses and visualisation into a workflow for read-across assessment of UVCBs in regulatory programmes (Concawe, 2018). Using this approach, the Cat-App project of Concawe was able to group the DMSO-extracts of 141 PS UVCBs according to their inherent manufacturing process-based categories. In addition, the in vitro bioactivity profiles (using 15 cell-based assays: 4 iPS-induced cells, 8 human cancer cell lines, and 2 human primary cell lines) of the above-mentioned PS was shown to have a strong positive correlation with their 3- to 7-ring PAH content (Concawe, 2018).

It is important to note that PS UVCBs that belong to the same product category might induce similar toxicological apical effects in in vitro studies, for example inhibition of differentiation into beating cardiomyocytes in the EST **(Chapter 2)**, or similar developmental toxicity in vivo, while the underlying modes-of-action and thus gene transcription profiles might differ. In other words, PS that belong to the same or different categories might induce similar phenotypic effects reflecting PDT as measured in the EST or ZET, but may induce this PDT via different molecular modes-of-action. Therefore it is of interest to evaluate the applicability of omics technology in combination with classical in vitro assays designed for PDT testing of PS UVCBs. Given the inherent complexity of PS UVCBs, the observed PDT by some of these substances may be caused by a wide range underlying modes-of-action. Thus, the application of whole RNA-seq, but not targeted

RNA-seq, seems promising and could be applied for defining modes-of-action underlying PDT by some of these highly complex substances.

3. Comparison of in vitro and in vivo findings

Two validated alternative assays for PDT testing, being the EST and ZET, were applied in the present thesis to evaluate the embryotoxic potency of the DMSO-extracts of PS (within and across product categories) and GTL products. As discussed in **Chapter 2**, a good in vitro-in vivo correlation was found when the EST results were compared to in vivo PDT data of the corresponding PS, giving an average R^2 for the in vitro-in vivo correlation of 0.85. Additionally, the potencies obtained in the ZET also correlated well with PDT potency observed in vivo (R^2: 0.69, **Chapter 5**). The PS-induced PDT in the ZET correlated best with the live fetuses/litter endpoint (R^2: 0.91), where the potency in the EST correlated best with the endpoint increased incidence of resorptions (R^2: 0.95) (Figure 2 below). Overall, both in vitro alternative assays applied in the present thesis adequately assessed the relative in vitro embryotoxicity potency of PS (from 6 product categories) and GTL extracts providing results in line with their potency observed in vivo. Increased incidence of resorptions and/or the number of live fetuses/litter appeared to be a better in vivo endpoint for comparison with the results from the EST and ZET, than the fetal body weight endpoint. Corroborating this finding, Murray et al. (2013) also found a stronger correlation when the predicted PDT potency of various high-boiling PS was compared with the increased resorptions or number of live fetuses/litter data than with the fetal bodyweight endpoint.

In vivo PDT by some PS tested was observed only at doses that cause maternal toxicity, indicating the developmental effects on fetuses may reflect an effect secondary to maternal toxicity (Murray et al., 2013). For example, a decrease in fetal body weight is observed only at the dose affecting the maternal body weight as well. However, both of the in vitro test systems applied, the EST and ZET, do not have the ability to detect or model this maternal-fetal interaction. This could be one of the reasons why the results obtained in the EST and ZET are less well-correlated with the fetal body weight endpoint as compared to the increased resorptions or number of live fetuses/litter endpoint (see Figure 2).

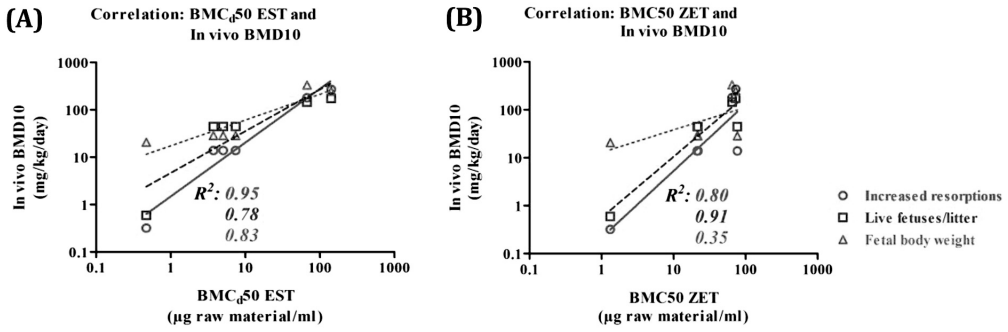


Figure 2 Correlation between in vitro potencies obtained in (A) the EST and (B) the ZET assay and in vivo developmental toxicity endpoints that include increased incidence of resorptions, number of live fetuses/litter, and fetal body weight (for further details see Chapter 2 and 5).

The mode-of-action underlying the developmental toxicity induced by some PAHs and PAH-containing PS is not fully known, while the existing evidence from prior studies suggests a role of the AhR in mediating this toxicity (Billiard et al., 2006; Goodale et al., 2013; Incardona et al., 2004; Ketelslegers et al., 2018; Puga et al., 2005). Based on these previous findings, the AhR-mediated activity and the role of the AhR in mediating the PS-induced PDT of the selected PS model substances were investigated in **Chapter 3 and 4**. Of all PS-induced receptor-mediated activities, especially the AhR-mediated activity appeared relevant for the observed PDT by these substances, as shown by a good correlation between the PS induced AhR-mediated activity and their PDT potency obtained in either the EST (R^2: 0.80; **Chapter 3**) or ZET (R^2: 0.66; **Chapter 5**). Moreover, in **Chapter 4**, co-exposure of ES-D3 (EST) or H4IIE.luc cells (AhR CALUX) with the DMSO-extracts of PS and the AhR antagonist trimethoxyflavone (TMF) neutralized the PS-induced PDT as well as the AhR-mediated gene expression by these substances. This confirms that the PDT as observed with some PS is, at least partially, mediated via the AhR. Supporting this notion, exposure of pregnant AhR knock-out mice ($AhR^{-/-}$, AhR-KO) to BaP did not cause any embryotoxicity in comparison to the PDT observed in the offspring of pregnant wild-type rats exposed to BaP (WT, Sprague-Dawley) (Ketelslegers et al., 2018). In these studies, a single oral gavage BaP dose of 300 mg/kg bw/day to pregnant WT and $AhR^{-/-}$ rats (from gestation day 6 through 20) induced various developmental toxicity effects in especially the WT rats, including increased incidence of early resorptions (56.4%

of fetuses versus 3.7% in WT control) and reduced number of live fetuses/litter (43.6% versus 96.3% in WT control). In contrast, no developmental toxicity was seen in the BaP exposed AhR$^{-/-}$ rats (Ketelslegers et al., 2018). In light of this, it would be of interest to study the PS in the same pregnant WT and AhR-KO rat model. Overall, it can be concluded from our and the above-mentioned findings that PAHs- and PS-induced PDT is (at least partially) mediated via the AhR.

Despite good in vitro-in vivo concordance, as shown in the present thesis, there are some limitations to the in vitro models used that should be discussed in more detail. This includes the absence of a maternal-fetal component (i.e. placenta) and also of a prominent metabolism capacity as compared to the in vivo situation. The role of metabolism in PS-induced PDT, was addressed to some extent in **Chapter 6** where it was shown that some PAH constituents require bioactivation to become able to induce PDT, while some do not and this latter also appeared to hold for the (majority of) the PS constituents responsible for the in vitro PDT of these complex substances. In other words, metabolism does not seem to be a prerequisite for the observed PDT induced by the PS extracts under study. As discussed in **Chapter 6**, one of the possible explanations for this is that the PS-induced PDT may require non-covalent receptor or enzyme interactions rather than the chemical (DNA) reactivity that is necessary to exert the mutagenic and carcinogenic effects of these substances (Blackburn et al., 1986; Siddens et al., 2012). This is corroborated by the results obtained in **Chapter 4** showing the PS-induced PDT to be partially AhR-mediated. Surely, the interference of the AhR-related pathway may not be the only mode-of-action underlying the observed developmental toxicity potency of the PS under study, but the results of **Chapter 4** indicate that the AhR plays an important role in mediating this adverse effect, in particular for this group of heavier PS. However, the contribution of other receptors known to be relevant in developmental processes, such as for example the RAR or PPAR, for the PS-induced PDT remains to be investigated in the future.

Furthermore, both EST and ZET lack a placental transport system. The placenta regulates nutrients and oxygen exchange between the mother and the fetus during fetal growth and development (Gude et al., 2004; Murphy et al., 2006). When the mother is affected by chemical exposure this will have direct consequences to the developing fetus (Coussons-Read, 2013; Dimasuay et al. 2016). Prior studies have combined the BeWo placental transport model with the EST or WEC to model the placental transport system in the in

vitro test system applied, thereby improving the predictions made by the in vitro approach (Li et al., 2015, 2016, Dimopoulou et al., 2018). In these studies the accuracy and concordance between in vitro and in vivo findings increased significantly upon correcting in vitro potencies of the test substances with their relative placental transport rate (Papp value). Learning from these examples and since the present thesis has not considered the role of placental transport rate for PAH- or PS-induced PDT, the addition of a BeWo placental transport model into the current assay battery should be investigated.

4. Relation of in vitro developmental toxicity potency to PAH content

With respect to the importance of the type and amount of PAHs present in each PS for their PDT, it is of interest that a strong relationship between the observed PDT by some PS and the presence of polycyclic aromatic compounds, in particular, 3- to 7-ring PAHs, in these highly complex substances was noted in in vivo studies. At the same time, the abundance of low-molecular-weight (LMW) PAHs, i.e. 2-ring PAHs, is more correlated to skin irritation effects in experimental animals (Feuston et al., 1994; Murray et al., 2013). Corroborating this finding, the present thesis showed that the PS-induced PDT in the EST was highly correlated with the total amount of 3- to 7-ring PAHs they contain (R^2: 0.85, **Chapter 4**), while the potency obtained in the ZET correlated best to their 3- to 5-ring PAH content (R^2: 0.96, **Chapter 5**). Overall, both in vivo and in vitro findings support the hypothesis that PAHs are the primary inducers of PDT for the heavier PS.

To further support this conclusion, we have combined the data obtained in the EST, AhR CALUX, and chemical analysis (i.e. PAH content) of all samples tested in the present thesis for PCA analysis. This visualizes the importance of both the type and amount of PAHs present in each PS for their relative PDT potency and AhR-mediated activity. To add, the manufacturing of PS is such a way that only certain types of PAH constituents will be present in a specific PS category, due to the particular processing conditions applied (e.g. boiling points) during the distillation process of the crude oil. For example, light PS comprise mainly LMW PAHs and heavy PS contain more high-molecular-weight (HMW) PAHs. As depicted in the PCA biplot below (Figure 3), all samples that are virtually devoid of PAHs: #091-GTLb, #092-GTLg, and #145-HRBO, are clustered together and completely separated from the other samples that tested positive in both the EST and AhR CALUX

assay. For these PS samples, 3 main clusters are seen. The first cluster consists of the GO, VTO, RAE, and HFO samples that mainly contain either just LMW PAHs, such as GO and VTO categories, or a low amount of total PAHs like the RAE (1.5-3.3% wt. PAHs) and 3 of the HFOs (0.62-4.4% wt. PAHs). The second cluster is occupied by the DAE (#097, #098, and #099) and 3 HFO samples with sample code #091-HFO, #021-HFO, #129-HFO. These samples represent heavy distillates of PS that contain mainly HMW PAHs. The amounts of total PAHs of the samples that belong to this cluster range from 9 to 12% for DAEs and from 8 to17% for HFOs. Lastly, 2 HFO samples that contain a relatively high amount of both HMW and total PAHs, being sample #008-HFO (27% wt. PAHs) and #034-HFO (48% wt. PAHs) are grouped together in the PCA biplot (Figure 3). Altogether, the type (i.e. LMW vs HMW) and amount of PAHs present in each PS is determined by the manufacturing process, thus PS belonging to the same category should have comparable PAH constituents, otherwise, they will be off-specs products.

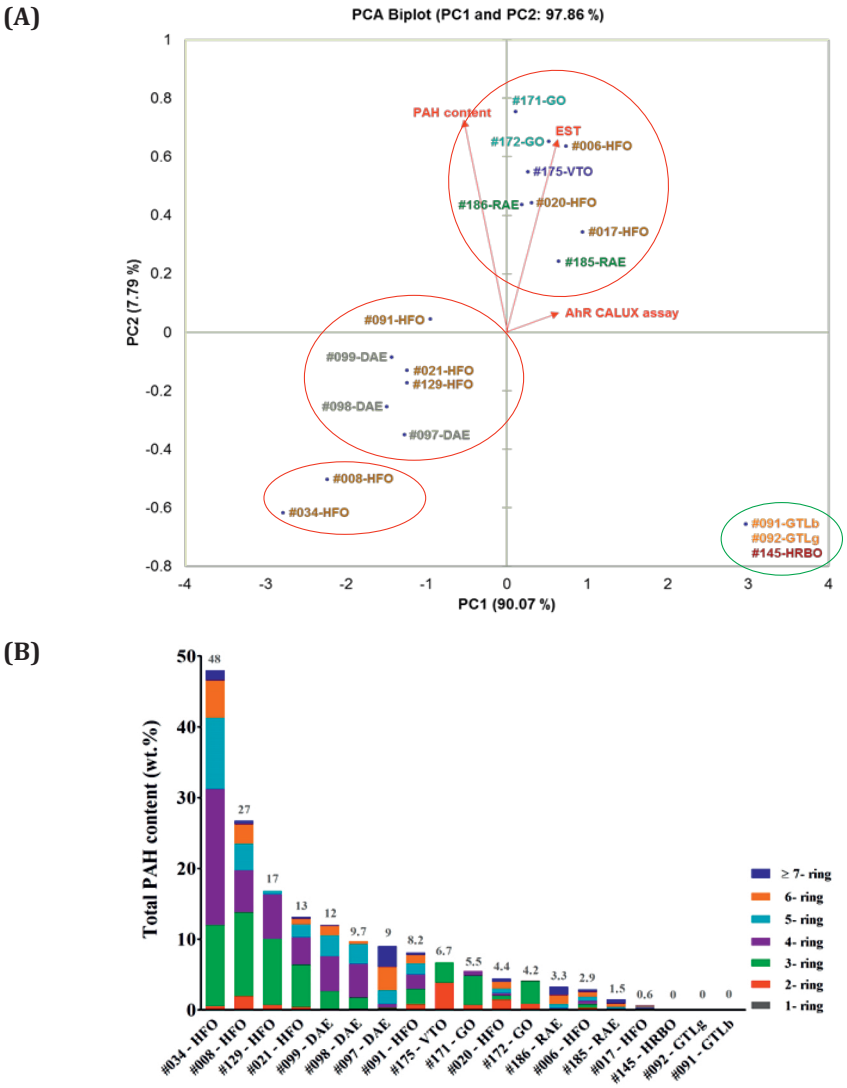


Figure 3 (A) Principal component analysis (PCA) based on the results obtained in the EST and AhR CALUX assays of all samples tested in the present thesis: 8 HFOs, 2 GOs, 1 VTO, 2 RAEs, 3 DAEs, 1 HRBO, and 2 GTL products. (B) The weight percent (wt.%) of the DMSO-soluble 1- to ≥7-ring PAHs present in PS and GTL products tested in the present study.

5. Quantitative in vitro to in vivo extrapolation (QIVIVE)

It is worth noting that the present thesis was able to provide a qualitative, and not a quantitative, prediction towards the endpoint studied (i.e. PDT) by comparing the obtained in vitro potency/ranking relative to available in vivo PDT data of the corresponding PS. This qualitative prediction can be used for read-across and prioritization for the selection of a worst-case representative per PS category, but does not provide quantitative point of departure (PODs) for risk assessment (yet). This latter would require insight in not only actual exposure levels but also in the relevance of the concentrations shown active in the in vitro PDT models for the in vivo situation. Proofs of principle for such a quantitative in vitro to in vivo extrapolation (QIVIVE) have been demonstrated for individual compounds causing developmental toxicity using physiologically-based kinetic (PBK) modeling facilitated reverse dosimetry (Abdullah et al., 2016; Adam et al., 2019; Boonpawa et al., 2017a,b; Chen et al., 2018; Li et al., 2017; Louisse et al., 2011, 2015; Ning et al., 2019; Strikwold et al., 2013, 2017; Zhang et al., 2018). However, to apply such an approach for highly complex substances like PS would be very challenging since modelling of all constituents would be virtually impossible. In an attempt to overcome this challenge, another approach to predict PODs was evaluated, testing the usefulness of quantitative structure-activity relationship (QSAR) based predictions, using the correlation equations obtained in **Chapter 2, 4 and 5**. These equations are summarised in Table 1 and describe the relationship between: 1) 3- to 7-ring PAH content and BMCd50 in the EST, 2) 3- to 5-ring PAH content and BMC50 in the ZET, 3) the BMCd50 in the EST and the BMDL10 for in vivo PDT detected by the increased incidence of resorptions and 4) the BMC50 in the ZET and the BMDL10 for in vivo PDT detected by the decreased number of live fetuses/litter. By combining QSAR 1 and 3, the BMDL10 for in vivo PDT reflected by the increased incidence of resorptions could be predicted based on the 3- to 7-ring PAH content, while using QSAR 2 and 4, the BMDL10 for in vivo PDT reflected by the decreased number of live fetuses/litter could be predicted based on the 3- to 5-ring PAH content of the PS. In this way, based on the PAH content, the BMDL10 for in vivo PDT was predicted for other PS from the same categories for which the QSARs were defined, but that were not yet tested in vivo. *(Note: The list and PAH content of the untested PS evaluated was obtained from Concawe through personal communication).*

Table 1 Equations used for the QSAR-based predictions in the present thesis.

QSAR	QSAR equation	Number of samples included for each QSAR model
[1] EST	$y = -1.63x + 3.04$ $R^2 = 0.85$	16 PS samples → 8 HFOs, 2 GOs, 1 VTO, 3 DAEs, 2 RAEs
[2] ZET	$y = -1.23x + 2.80$ $R^2 = 0.96$	9 PS samples → 1 HFO, 2 GOs, 1 VTO, 3 DAEs, 2 RAEs.
[3] EST – In vivo PDT (increased incidence of resorptions endpoint)	$y = 1.15x + 0.04$ $R^2 = 0.96$	6 PS samples → 1 HFO, 1 GO, 1 VTO, 3 DAEs.
[4] ZET – In vivo PDT (decreased number of live fetuses/litter endpoint)	$y = 1.32x - 0.44$ $R^2 = 0.91$	6 PS samples → 1 HFO, 1 GO, 1 VTO, 3 DAEs.

Figure 4 below presents an overview of the QSARs used to predict the BMDL10s of untested PS for two PDT endpoints, namely increased incidence of resorptions and decreased number of live fetuses/litter. As above-mentioned, these QSAR models were established using equations obtained from the correlation analysis done in **Chapter 2, 4 and 5** between PAH content and in vitro PDT potency in the EST and ZET, and between in vitro PDT potency and in vivo PDT data. First, the PAH content of untested PS was used as data input for predicting their BMC50s in the EST or ZET. Afterward, the predicted-BMC50s were subsequently used to predict the BMDL10s-PDT of the corresponding untested PS. From **Chapter 2,** it is known that in vitro PDT potency of PS in the EST is best correlated with the increased incidence of resorptions endpoint (R^2: 0.97), where in **Chapter 5**, the PS-induced PDT in the ZET correlates best to the number of live fetuses/litter endpoint (R^2: 0.91). Thus, only those specific endpoints were used to build the EST- and ZET-QSAR model to predict the BMDL10s-PDT of the untested PS.

The aforementioned QSAR models were applied to the PS tested in the present thesis to evaluate the approach by comparing the experimental values of the BMDL10 obtained in available in vivo studies to the BMDL10 values predicted by the QSAR approach based on their PAH content. Table 2 and 3 presents the comparison between the experimentally observed BMDL10 values derived from in vivo PDT studies and the BMDL10 values predicted for the 6 PS samples (#172-GO, #175-VTO, #097-DAE, #098-DAE, #099-DAE, and #034-HFO) for which in vivo PDT data are available. In brief, the predicted- BMDL10s of the PS listed in Table 2 and 3 are highly comparable to their experimental in vivo

BMDL10s, with these values varying less than ± 3-4 fold. Also the in vitro BMCd50 values were well predicted by the first QSAR applied. For example, using the first QSAR model, the predicted BMCd50 of sample #034-HFO in the EST was 0.66 µg raw material/ml where its experimental BMCd50 value was 0.47 µg raw material/ml, indicating a 1.4 fold difference between the predicted and the obtained experimental BMCd50. Similarly, the predicted-BMDL10s of sample #034-HFO for either the increased incidence of resorptions or live fetuses/litter differ only 2.8 and 3.7 fold, respectively, from the observed BMDL10s for these PDT endpoints (Table 2 and 3).

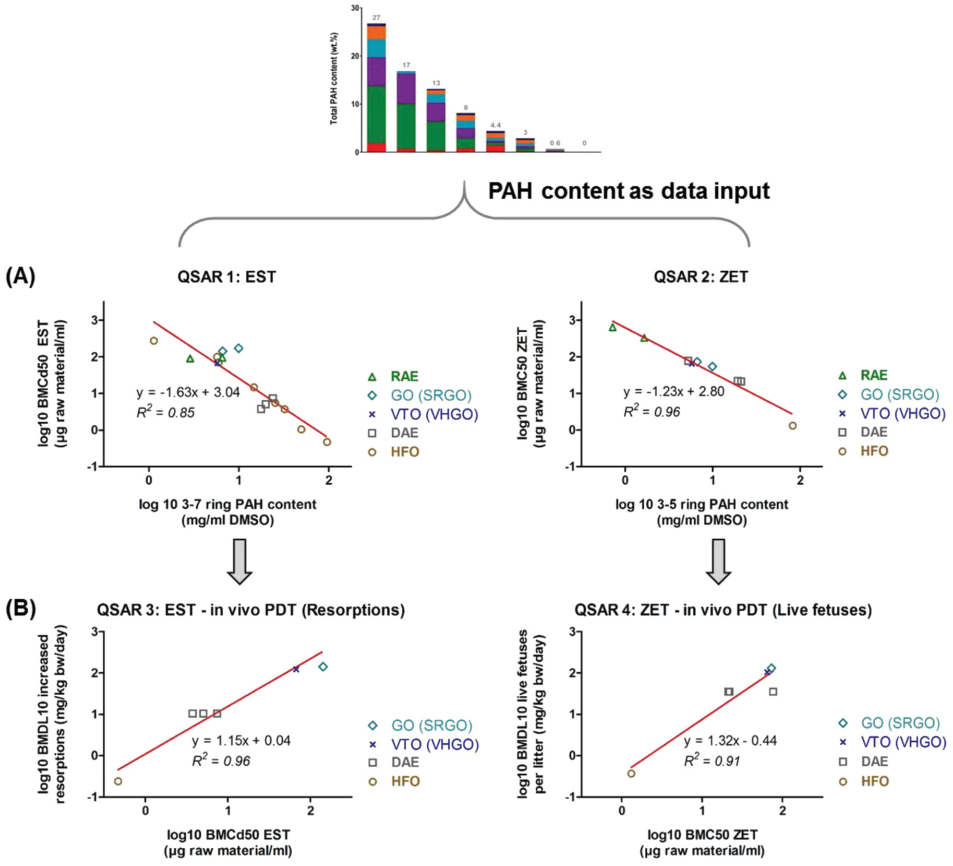


Figure 4 Graphic presentation of (A) the QSAR models used to predict the relative in vitro PDT potency (BMC50) of PS in the EST and ZET using their PAH content as data input, and (B) the QSARs used subsequently to predict the in vivo PDT potency of the corresponding PS based on their predicted in vitro BMC50s.

Table 2 Comparison between the predicted in vitro PDT potency in the EST (expressed as BMCd50s) or the predicted in vivo PDT potency (expressed as BMDL10s; increased resorptions endpoint) obtained from the QSAR models, and the experimental values of the 6 PS samples (#172-GO, #175-VTO, #097-DAE, #098-DAE, #099-DAE, and #034-HFO) for which in vivo PDT data are available.

Substances	BMCd50 EST (µg raw material/ml)	Predicted BMCd50-EST (µg raw material/ml)	BMDL10 increased resorptions (mg/kg bw/day)	Predicted BMDL10 increased resorptions (mg/kg bw/day)	Ratio between the observed BMDL10 : predicted BMDL10
#172-GO	142	50.41	250.03	100.43	2.5
#175-VTO	67.2	63.46	122.11	130.97	1.1
#097-DAE	3.74	10.24	10.42	15.98	1.5
#098-DAE	5.05	8.77	10.42	13.37	1.3
#099-DAE	7.42	6.20	10.42	8.96	1.2
#034-HFO	0.47	0.66	0.24	0.67	2.8

Note. The experimental BMDL10s were calculated using the BMD US-EPA software version 2.6.1., whereas, the predicted values were obtained using the established QSARs.

Table 3 Comparison between the predicted in vitro PDT potency in the ZET (expressed as BMC50s) or the predicted in vivo PDT potency (expressed as BMDL10s; decreased number of live fetuses/litter endpoint) obtained from the QSAR models, and the experimental values of the 6 PS samples (#172-GO, #175-VTO, #097-DAE, #098-DAE, #099-DAE, and #034-HFO) for which in vivo PDT data are available.

Substances	BMC50 ZET (µg raw material/ml)	Predicted BMC50-ZET (µg raw material/ml)	BMDL10 Live fetuses/litter (mg/kg bw/day)	Predicted BMDL10 Live fetuses/litter (mg/kg bw/day)	Ratio between the observed BMDL10 : predicted BMDL10
#172-GO	73.13	60.35	131.14	80.27	1.6
#175-VTO	64.77	71.85	104.02	100.98	1.0
#097-DAE	76.71	81.16	35.4	118.56	3.4
#098-DAE	21.82	16.89	35.4	15.01	2.4
#099-DAE	21.23	14.46	35.4	12.23	2.9
#034-HFO	1.32	2.73	0.37	1.36	3.7

Note. The experimental BMDL10s were calculated using the BMD US-EPA software version 2.6.1., whereas, the predicted values were obtained using the established QSARs.

In a next step, the established QSAR models were used to predict the relative in vitro and in vivo PDT potency for a series of PS that were not yet tested in vivo. This was done only for PS from categories that were part of the training set used for the definition of the QSAR models, including GO, VTO, DAE, RAE, HRBO, and HFO, thus defining the applicability domain of the QSARs. The predicted BMC(d)50 and BMDL10 values for both tested and untested PS, obtained from this double QSAR model-based simulation, are listed in Table 4 and 5. When comparing the predicted-BMDL10s, the HFO category appears to contain the most potent PS (sample #034-HFO) (Figure 5). Moreover, the range of predicted-BMDL10s of HFO samples appeared to be quite broad in comparison to that of the other PS categories tested, which may be mainly due to the high systematic variation in their PAH level that could range from 0.62 to 48% wt. PAHs. HRBO tested negative for PDT both in vitro and in vivo **(Chapter 4)** and as a consequence, its BMC(d)50s or BMDL10s could not be defined, and this category was not included in the QSARs defined. For the RAE category, two samples (i.e. #185-RAE and #86-RAE) were tested in the present thesis but no in vivo PDT data are available for validation of this category, hence, this category was included for making only the QSAR1 and QSAR2-based predictions (see Figure 4).

Table 4 Predicted BMCd50s in the EST, and predicted in vivo BMDL10s for increased incidence of resorptions, for PS with known 3-7 PAH constituent level that belong to the applicability domain of the QSARs defined in the present thesis. Only increased incidence of resorptions is predicted using the QSAR-EST since this endpoint gave the best in vitro-in vivo correlation, as shown in **Chapter 2**.

Substances	3-7 ring PAHs (mg/ml)	Predicted BMCd50 – EST (μg raw material/ml)	Predicted BMDL10 increased resorptions endpoint (mg kg bw/day)
131/HFO	2.00	356.3	957.40
079/HFO	2.96	188.2	458.73
007/HFO	3.46	146.0	342.39
166/HFO	3.88	120.9	275.44
025/HFO	4.99	80.3	171.77
018/HFO	6.86	47.8	94.35
078/HFO	5.11	77.2	164.14
197/HFO	6.58	51.1	102.04
024/HFO	5.98	59.8	122.29
164/HFO	9.02	30.6	56.44
071/HFO	8.84	31.6	58.63
028/HFO	9.31	29.0	53.12
050/HFO	10.00	25.8	46.46
031/HFO	10.30	24.6	43.98
080/HFO	8.50	33.7	63.11
097/HFO	15.11	13.2	21.39
058/HFO	12.38	18.2	31.12
134/HFO	43.74	2.3	2.90
155/HFO	3.36	152.9	361.01
170/ SRGO	2.57	236.1	595.78
168 /SRGO	6.66	50.1	99.75
188/ SRGO	3.33	155.0	366.73
169 /SRGO	4.64	90.2	196.46
096B/DAE	12.00	19.2	32.98
096A/DAE	18.20	9.7	15.07
089/DAE	31.36	4.0	5.42
195/DAE	35.64	3.3	4.26
181/ VHGO	0.84	(-)	(-)
180 /VHGO	1.51	(-)	(-)
179 /VHGO	1.41	(-)	(-)
177 /VHGO	1.70	464.4	1299.52
178/ VHGO	3.44	147.1	345.39
182 /VHGO	6.72	49.4	98.19
184 /VHGO	8.29	35.1	66.12
176 /VHGO	12.67	17.6	29.76
183 /VHGO	5.63	66.0	136.98

Table 5 Predicted BMC50s in the ZET, and predicted in vivo BMDL10s for decreased number of live fetuses/litter, for PS with known 3-5 PAH constituent level that belong to the applicability domain of the QSARs defined in the present thesis. Only increased incidence of resorptions is predicted using the QSAR-ZET since this endpoint gave the best in vitro-in vivo correlation, as shown in **Chapter 5**.

Substances	3-5 ring PAHs (mg/ml)	Predicted BMC50 – ZET (μg raw material/ml)	Predicted BMDL10 decreased number of live fetuses/litter endpoint (mg kg bw/day)
017/HFO	0.81	(-)	(-)
131/HFO	0.58	(-)	(-)
079/HFO	2.41	210.2	504.5
007/HFO	0.47	(-)	(-)
166/HFO	3.48	133.9	284.2
006/HFO	3.31	142.6	308.0
025/HFO	3.94	114.7	233.5
018/HFO	4.76	90.9	173.9
078/HFO	4.41	99.9	196.0
197/HFO	6.02	68.1	120.3
024/HFO	5.26	80.5	148.8
020/HFO	3.17	150.3	329.3
164/HFO	7.54	51.5	84.4
071/HFO	7.43	52.5	86.5
028/HFO	8.35	45.4	72.0
050/HFO	9.10	40.9	62.9
031/HFO	7.07	55.8	93.4
080/HFO	7.32	53.5	88.6
091/HFO	11.64	30.2	42.7
097/HFO	12.28	28.2	39.3
058/HFO	10.74	33.3	48.5
021/HFO	23.40	12.7	14.3
129/HFO	32.30	8.6	8.6
008/HFO	43.20	6.0	5.5
134/HFO	43.74	5.9	5.4
155/HFO	3.36	139.8	300.3
170/ SRGO	2.57	194.2	456.2
168 /SRGO	6.66	60.1	102.7
188/ SRGO	3.33	141.2	304.3
169 /SRGO	4.64	93.8	180.7
096B/DAE	4.56	95.9	186.0
096A/DAE	18.02	17.6	21.5
089/DAE	31.36	8.9	9.0
195/DAE	32.76	8.4	8.4
181/ VHGO	0.84	(-)	(-)
180 /VHGO	1.51	(-)	(-)
179 /VHGO	1.41	(-)	(-)
177 /VHGO	1.70	(-)	(-)
178/ VHGO	3.44	135.8	289.4
182 /VHGO	6.72	59.5	101.3
184 /VHGO	8.29	45.9	72.8
176 /VHGO	12.53	27.5	38.1
183 /VHGO	5.63	74.0	133.8

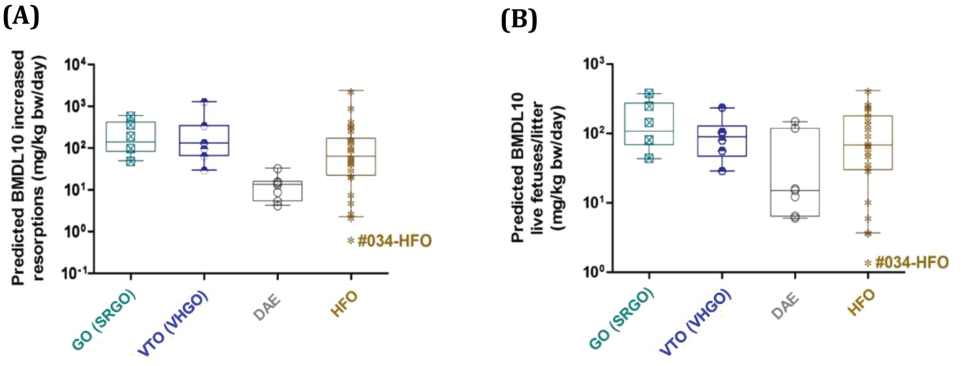


Figure 5 Whisker plot illustrating the range of predicted BMDL10 values of (A) increased incidence of resorptions endpoint and (B) decreased number of live fetuses/litter endpoint for the PS categories GO (SRGO), VTO (VHGO), DAE, and HFO, based on the QSAR predictions (Table 4 and 5).

Altogether, here we show the feasibility of using a QSAR approach, using equations obtained in the present thesis **(Chapter 2, 4 and 5)**, for i) read-across from PS for which in vivo PDT data are available to other PS for which in vivo data are lacking, and for ii) QIVIVE of data on PS UVBCs to define a POD (i.e. BMDL10s), which can possibly be used for risk assessment practice or regulatory purposes of these highly complex substances. In other words, the results obtained provide the proof that in vivo PDT potency of some PS (i.e. increased resorptions and decreased number of live fetuses/litter endpoints) can adequately be predicted by using the combination of analytical (i.e. PAH content) and in vitro PDT data obtained from the EST and ZET. This may also indicate that both the EST and ZET adequately capture the relevant modes-of-action for PS-induced PDT, at least for the product categories tested in the present thesis. Ultimately, this work may therefore contribute to future implementation of the use of in vitro PDT data to support not only the regulatory compliance (e.g. REACH legislation) but also the risk assessment of these highly complex substances.

Despite its promising application, some drawbacks and information gaps from the above-mentioned QIVIVE framework should also be addressed. For instance, it should be emphasized that the dataset used to build the presented QSAR models in Figure 4 is still somewhat limited number of samples from 5 PS categories were used as the training set to define the correlations. To add, it is shown in **Chapter 4** that the correlation between

3- to 7-ring PAH content and in vitro PDT potency in the EST is higher when comparing the data within 1 PS category HFO (R^2: 0.95) than all PS samples tested in the present study combined (R^2: 0.85). In view of this and to broaden the applicability domain of the established QSAR models for predicting the PDT potency of PS UVCBs, future studies should consider testing more PS extracts in the current assay battery, not only for PS that belong to the product categories tested in the present thesis but also for those from different PS categories. By this, a QSAR model for each PS category could be established. Such an approach could be used to strengthen the basis to identify the worst-case representative(s) for each PS category for further in vivo testing where needed as a last resort and/or facilitate the definition of PODs for in vivo PDT of PS without a further need for in vivo testing.

6. The implications of results obtained for the REACH roadmap for PS and the 3Rs

The initial Registration phase of REACH dossiers for PS has been completed and now the Evaluation phase is taking place. This means the registrants are required to fill the missing data gaps, including the information on potential PDT effects by PS produced at a volume of ≥100 tonnes/annually and submit the updated dossiers to the regulators. Given the large number of experimental animals and the considerable amount of resources potentially connected to PDT testing of all currently registered PS (n: ±186 EINECS numbers) under REACH (Concawe, 2019), the application of intelligent alternative testing strategies to assess the PDT potency of PS for the required data gaps is of high relevance. To this end, the present thesis applied a battery of in vitro alternative assays that included the EST **(Chapter 2, 4, and 6)**, a series of CALUX reporter gene assays (in particular AhR CALUX assay) **(Chapter 3)**, and the ZET **(Chapter 5)**, to evaluate the PDT potency (and modes-of-action) of highly complex PS. This turned out to be successful in spite of the inherent complex nature of the PS UVCBs and the complex PDT endpoints to be detected. Briefly, the obtained results in the present thesis showed that the current assay battery is able to predict the PDT potency of PS, within and across product categories, and this potency is proportional to the type and amount of PAHs they contain.

Given that very few of the currently registered PS (n: ±186 EINECS numbers) have a complete PDT dataset, read-across from PS for which PDT data are already available to

other PS for which these data are lacking could be applied to fill the missing data gaps. This may result in the prioritization and identification of the worst-case representative(s) for each PS category for further in vivo testing where needed as a last resort. A hypothetical example can be taken from the results obtained in the present thesis and the QSAR prediction for the HFO category. For instance, sample #034-HFO is the most potent substance, among all HFO samples presented in Figure 5C, thus, is the worst-case sample representative for the HFO category. Alternatively, the QSAR models may be used to described the in vivo PDT potential of all PS that match the applicability domain of the QSARs.

The present thesis has tested 6 out of 22 PS categories that have to be evaluated for their effects on prenatal development under REACH legislation. Of these 6 PS categories, 4 are known to induce PDT in vivo (i.e. HFO, GO/SRGO, VTO/VHGO, DAE), 1 has no available in vivo PDT data (i.e., RAE), and 1 category (i.e. HRBO) does not cause any embryotoxicity effects in experimental animals. For the other 16 PS categories, not all of them have historical in vivo PDT data, but limited evidence (Murray et al., 2013) suggest that light PS that mainly contain 1- to 2-ring aromatics, for example gasoline and kerosine, do not induce PDT where heavy PS and residues that contain a significant amount of 3- to 7-ring PAHs will likely cause PDT. Hence, since there is a direct relationship between each PS category and a specific group of PAH constituents present, it is tempting to speculate that knowledge on the type and total amount of PAHs present in each PS may help to predict their embryotoxicity potency. This concept was supported by the QSAR analysis presented. Provided the PS belongs to the applicability domain of the QSAR models presented in Figure 4, the QSAR equations can be used to predict the relative in vitro PDT potency of the untested PS, for two PDT endpoints: increased incidence of resorptions and decreased number of live fetuses/litter endpoints. Based on the QSAR predictions, it can be expected that some PS, especially those containing low or no PAHs (e.g. HRBO category), may not induce any in vitro and in vivo PDT effects. Whereas, PS with a relatively high amount of 3- to 5-ring PAHs or 3- to 7-ring PAHs, such as those from the HFO category, may induce PDT.

To conclude, the present thesis shows proof-of-principle for use of a battery of mode-of-action-based alternative testing strategies to support reduction and replacement (2Rs out of the 3RS) of in vivo PDT of complex PS using predicted relative potencies and read-

across from the already available in vitro and in vivo PDT data. One could envisage that when the proposed assay battery and also the proposed QSAR concept would be applied and extended to include all 186 EINECS numbers of currently registered PS, a huge number of experimental animals and of testing costs would be saved. Given the complexity of the endpoint studied, the application of reliable in vitro alternative assays for PDT testing remains a challenge, while at the same time providing a huge contribution to the 3Rs given that so many chemical substances are scheduled for PDT testing in the coming years. Looking at the results obtained in the present thesis showing very good in vitro-in vivo correlations, this could help convincing the regulatory bodies to consider this approach to reduce the number of PS for full PDT evaluation according to the current OECD testing guidelines. Also, more focused testing on particularly PS categories that are of high PDT potential should be prioritized. In a long-term vision, this will ultimately reduce the required animal tests and resources to assess the PDT potency of PS, and support the application of the 3Rs (Reduction, Replacement, and Refinement) principle of animal use in toxicological research.

7. Future recommendations/challenges and concluding remarks

Besides the great applicability of the current battery of assays that includes the EST, ZET, and AhR CALUX assays for PDT testing of PS UVCBs, the shortcomings and limitations of the in vitro alternative assays applied should also be acknowledged. Thus, the following paragraphs present some study limitations and considerations for improvements of the in vitro alternative testing strategies applied. This may then broaden the applicability of the current battery of assays for PDT testing of not only PS UVCBs that have not been included in the present thesis but also of other groups of chemical substances.

- **Embryonic stem cell test (EST): limitations and future promises**

The classic EST, as performed in the present thesis, uses the inhibition of ES-D3 cell differentiation into beating cardiomyocytes as a readout to evaluate the PDT potency of test substances. This readout proved to adequately predict the relative in vivo PDT of PS UVCBs under study, as shown by a good in vivo-in vitro correlation (R^2: 0.97, **Chapter 2**). In other words, the given endpoint of the classic EST is suitable to assess the potential

PDT induced by these group of substances, within and across product categories. Yet, what is considered a sensitive endpoint may not be suitable or sensitive enough to assess PDT potency for other classes of chemical substances or other UVCBs of diverse nature. In such cases, the evaluation of endpoints other than cardiac differentiation, including skeletal, neuronal, and endothelial differentiation, is possible since ES-D3 cells of the EST are able to differentiate into any cell type of the three germ layers (endoderm, mesoderm, and ectoderm), and this approach has been used in the last decade to assess potential toxicity of chemical substances on different lineages of embryonic development in vitro (de Jong et al., 2014; Theunissen et al., 2010, 2011).

In addition to the importance of choosing the most sensitive lineages and suitable endpoint for the differentiation assay of the EST, the duration of exposure also plays a role in the assessment of PDT using the EST. The classic EST protocol lasts for 10 days, starting from the embryoid bodies (EBs) formation via hanging drop cultures on day 0 until the scoring of beating cardiomyocytes on day 10. A publication by van Dartel et al. (2009b) demonstrated that the sensitivity of the 10-days cardiac differentiation assay of the EST decreases with a delay of the onset of exposure, as shown when a side-by-side comparison was made between day 0-10, day 3-10, day 4-10, day 5-10, and day 7-10 exposure scenarios. This means the later start of exposure resulted in a decreased inhibition of ES-D3 cell differentiation into beating cardiomyocytes (van Dartel et al., 2009b). It was concluded that exposure scenarios of day 0-10 and day 3-10, depending on cytotoxicity effect and class of substances, are the best options to adequately capture the PDT potency of test substances using the cardiac differentiation assay of the EST. As presented in **Chapter 2 and 4**, day 0-10 was chosen as the exposure scenario to study in vitro PDT potency of the DMSO-extracts of PS and GTL products under study. This is because the results from the ES-D3 cell viability assay showed that the range of concentrations tested (0-500 μg raw material/ml) did not induce any cytotoxicity effects to the ES-D3 cells. Proliferation (growth) and differentiation of ES-D3 cells in the EST are co-occurring processes, thus effects of test substances on both processes are relevant for inclusion in one assay as effects on cell viability would surely affect the embryonic development (van Dartel et al., 2009b). Altogether, as also stated by van Dartel et al. (2009b), the selection of the exposure scenario in the ES-D3 cell differentiation assay of the EST is of importance and appears to be chemical-dependent.

Another possible drawback of the EST is that the readout parameter of the ES-D3 cell differentiation assay of the EST, is the microscopic scoring of differentiated-EBs on day 10 of the assay, which is a time-consuming and to some extent subjective approach requiring experienced and well-trained persons to identify the specific beating areas of cardiomyocytes. Thus, a few attempts have been made to improve the performance and endpoint evaluation of the classic EST, by including the addition of quantitative flow cytometry (Seiler et al., 2006) or of transcriptomics-based read-outs (van Dartel and Piersma, 2011; Dimopoulou et al. 2018). These molecular-based additional parameters are expected to enhance the predictability and applicability domain of the classic EST for assessing developmental toxicity potency of chemical substances. However, as discussed already above one should be careful when combining the classic EST with a toxicogenomic approach since changes in gene expression do not necessarily reflect adverse effects, but could equally well reflect the homeostasis of relevant biological pathways responsible for the normal developmental processes. When successful, compound-specific gene-expression patterns based on the phenotypic observation of induced-PDT in the EST might be established, as shown by van Dartel et al. (2009a,b, 2010a,b) and Dimopoulou et al. (2018) for other test compounds than the PS of the present thesis.

Furthermore, ES-D3 cell differentiation assays of the EST covers only the early stage events in developmental toxicity, hence, the PDT potency of chemical substances that affect the prenatal development at the late stages may not be captured adequately using this assay (Adam et al., 2019). This is partly overcome by the ZET that covers a longer period of the development of the organism, from the late blastula stage until the full development of zebrafish embryo. The existing pieces of evidence show that the applicability domain of the EST to evaluate in vitro PDT potency is limited to compounds with certain modes-of-action, in particular for receptor-mediated PDT like PDT that is ER-mediated (Adam et al., 2019), RAR-mediated (Louisse et al., 2011; Dimopoulou et al., 2018), and/or AhR-mediated (Hiben et al., 2019). Other potential modes-of-action, including inhibition of angiogenesis and epigenetic mechanisms, may not be captured by the ES-D3 cell differentiation assay (Adam et al., 2019). Hence, the selection of the in vitro test system applied is essential and should cover the relevant mode-of-action of the tested substances.

- **Zebrafish embryotoxicity test (ZET): limitations and future promises**

The selection of morphological and teratogenicity endpoints is important when using the ZET to evaluate the PDT potency of test substances. Here we used the pre-defined general morphology scoring system (GMS) listed by Beekhuijzen et al. (2015), which is an extended version of GMS proposed by Hermsen et al. (2011). Using this GMS, the zebrafish embryos are scored daily for effects on general developmental delay and developmental toxicity to up to 96 hpf. The daily assessment is done to provide crucial information on the development of certain endpoints over time in a concentration-dependent manner. Our preliminary study demonstrated that 72 and 96 hpf are more sensitive time points, than 24 and 48 hpf, to differentiate developmental retardation of zebrafish embryos following exposure to the PS extracts under study. Furthermore, the listed morphological endpoints by Beekhuijzen et al. (2015) proved to adequately assess the PDT potency of the test substances under study, although the effects were dominated by the event of unhatched embryos at 72-96 hpf, and also the absence of movement and circulation at the higher exposure concentration. For teratogenicity endpoints, malformations of heart and yolk-sac are the most affected endpoints by the PS extracts tested. As discussed in **Chapter 5**, the scoring for teratogenicity endpoints was seriously hampered by the fact that most of the embryos did not hatch (the chorion still being intact) at 72-96 hpf. Hence, the possible PS-induced teratogenicity effects, including deformed body shape (i.e. dorsal curvature) and shorter body-length (growth retardation) cannot be scored for all exposed-embryos. This could be solved by the removal of the chorion with enzymatic (i.e. protease) treatment after the morphological assessment at 96 hpf. Subsequently, these embryos can be kept for some extra hours to properly assess the potential teratogenicity effects of test substances. By this, the teratogenicity index (TI) could be determined, enabling definition of the teratogenicity potency in addition to the general developmental aberrations now evaluated.

The present thesis did not include a correction for the so-called body burden analysis when determining the concentration of the test substances that caused developmental aberrations in the exposed-zebrafish embryos at 96 hpf. Such a body burden analysis is usually done to quantify the internal exposure concentration of a test compound to which the zebrafish embryo is actually exposed. This is important considering the ability of some compounds to bind to the plastic or to be degraded over time during the exposure period. For example, a known teratogenic compound fluconazole was misclassified as non-

teratogenic in the ZET due to a very low uptake by the zebrafish embryo during the exposure period (5.1% uptake of the nominal well concentration) (Gustafson et al., 2012) and this became apparent only after the body burden analysis was incorporated. Hence, body burden analysis may help to determine the true compound uptake by the embryo during a specific time period.

The development of the zebrafish embryo is similar to the embryogenesis in higher vertebrates, including humans, thus, making them a suitable model to study the effects of chemical substances on prenatal development. However, the compounds' kinetics may be different since the biotransformation system in a zebrafish embryo is far more limited in comparison to the in vivo situation in humans. This could partly be solved by incorporating an exogenous biotransformation system to the in vitro test applied to study the consequences of metabolism for the observed PDT, as it was done in **Chapter 6** for the EST. Such an approach could also be combined with the ZET to assess the embryotoxicity potential of both parent substances and that of potentially embryotoxic metabolites. Although it was concluded in **Chapter 6** that metabolism may not play an important role for the observed in vitro PDT by the PS extracts tested, the correction for absorption and distribution of the compound into the text organism should be considered. As above-mentioned, this could be done by performing body burden analysis to quantify the internal exposure concentration to provide a better insight on true compound bioavailability in the zebrafish embryo. For the EST, correction for protein binding (e.g. by serum components in the medium) may be necessary to determine the free concentration to which cells are exposed.

- **CALUX reporter gene assays: limitations and future promises**

Besides strong AhR-mediated activities, a diverse endocrine-like activity was also noticed following exposure to the DMSO-extracts of different PS extracts under study. Specifically, the DMSO-extracts of heavy PS like HFO and DAE also acted to some extent as antiandrogens, antiprogesterones, and antiestrogens, while the light PS (GO and VTO) showed rather weak antiandrogenicity, antiprogesteronicity, and estrogenicity effects. This may raise questions towards the relevance of the obtained data in the CALUX reporter gene assays to PS-induced PDT in vivo. Before presenting a further explanation, one should keep in mind that the CALUX reporter gene assays are simply in vitro

screening assays that provide information on receptor (in)activation, and do not necessarily reflect what happens in vivo. This is because the feedback regulation mechanisms and metabolism are lacking in these in vitro test systems. In vivo, the endocrine system functions within positive and negative feedback loops to help maintain homeostasis, so hormone production and metabolism are kept in balance. A homeostatic imbalance may result in an imbalanced state of normal body functions. Thus, prenatal exposure to substances that are known to interfere with the endocrine system, in this vulnerable time window, might affect the homeostasis of endogenous hormones, and as a consequence, may interfere with embryonic development, in particular tissue and organ differentiation.

Nevertheless, CALUX reporter gene assays could provide the information on the affinity of the test substance for a certain nuclear receptor. Stably transfected cell lines used for the CALUX assays of the present study are made by fusing multiple copies of highly specific hormone responsive element (HRE) to a minimal promoter containing the TATA box linked to luciferase genes in one plasmid construct, followed by transfection of this vector to a suitable cell line (Aarts et al., 1995; Sonneveld et al., 2005). For example, to create the U2OS ERα cells for the ERα CALUX assay, an ER-specific 3x ERE-TATA-Luciferase construct was made and transfected to the human osteocarcinoma U2OS cell line (Sonneveld et al., 2005). It is worth noting that the U2OS is one of the suitable cell lines for creating robust and selective reporter gene assays since they are relatively devoid of endogenous steroid receptors, or in other words, no endogenous steroid receptor activity is detected in this cell line (Escande et al., 2006; Sedlak et al., 2011). Hence, the stably transfected U2OS ERα CALUX cells possess a selective response towards ERα interacting ligands, only. Moreover, the interpretation of results obtained from a panel of CALUX reporter gene assays should be made carefully as it does not directly reflect the relative abundance of endocrine receptors present in vivo. However, when in vitro findings, using CALUX reporter gene assays, match those of the in vivo studies, this may reflect involvement in the mode(s)-of-action underlying the PDT or reproductive toxicity induced by certain chemical substances. For example, from the results obtained in **Chapter 4** it is demonstrated that the PS-induced PDT is partially mediated via the AhR. Some published studies reported that sustained AhR activation may result in adverse toxicity, including developmental toxicity, where transient AhR activation may reflect the adaptive responses towards xenobiotic exposure. In the case of the PS extracts under

study, their AhR-mediated activity was not remarkably eliminated when the exposure time was prolonged to 24 hours (as compared to 6 hours like how it was done in **Chapter 3 and 4**). This may indicate that the PS-induced PDT is due to the persistent AhR-activation by these substances, although more studies are required to confirm this hypothesis further. Furthermore, looking at the results of the other CALUX assays applied **(Chapter 3)**, a role for the other receptors for some specific classes of PS in relation to their PDT potency cannot be excluded. This could be further investigated in the future by testing the PDT of these PS in the presence of selected receptor-antagonists as done for the AhR in **Chapter 4**.

- **Future priority: the role of substituted (i.e. alkylated) PAHs in developmental toxicity potency of some PS**

It is suspected that some PS may contain alkyl-substituted PAHs and heteroatom (S, N, or O)-containing PAHs, in addition to the presence of unsubstituted (naked) PAHs. This information is of importance since the present study assumed that the PS-induced PDT is mainly caused by the 3- to 7-ring PAHs present in these substances without differentiation with regard to the substitution patterns. In other words, the relative contribution of substituted or hetero-PAHs, if present, to the PS-induced PDT is not yet considered. This may be relevant since previous studies using the zebrafish embryo model reported that some substituted PAHs, including oxygenated- and nitrated-PAHs, are more embryotoxic than their unsubstituted/naked congeners (Chlebowski et al., 2017; Geier et al., 2018; Wincent et al., 2015). Given the limited knowledge available especially on the role of alkyl-substituted PAHs in developmental toxicity, testing on PDT for this group of PAHs may be a higher priority than testing other types of substituted PAHs. Hence, future studies should focus on i) evaluating the PDT potency of naked versus alkylated PAHs, and ii) if the results indeed confirm that the alkylated PAHs are embryotoxic, then the effect of the degree of alkylation on PDT (e.g., naked-BaP versus methylated-, dimethylated-, hexylated-, and dodecylated-BaP) should be studied. Additionally, more detailed methods for chemical analysis and characterization (e.g. GC/MS, LC/MS, and NMR) of the DMSO-extracts of PS tested would be needed to get the information on the relative abundance of the alkylated PAHs present in these substances.

The resulting data are highly relevant to fully unravel the role of specific PAHs in the developmental toxicity of some PS.

Concluding remarks

In conclusion, the present thesis shows the applicability of a battery of alternative assays to study the developmental toxicity of highly complex PS UVCBs, within and across product categories, and the role of role of PAHs in this toxicity. The work presented in this thesis strengthens the hypothesis that the types and total amount of specific group of PAHs, mainly 3- to 7-ring PAHs, in PS UVCBs do play an important role in determining the PDT potency of these substances. In vitro PDT testing using the current assay battery for sample from the other PS categories than tested here should be done to fill the missing data gaps and to facilitate read-across for PS under REACH compliance. This approach taken in the present thesis may contribute to i) the grouping of PS into a limited number of categories based on their analytical characterization (e.g. PAH content) and bioactivity-profiling, ii) facilitating the selection of worst case representatives per PS category where in vivo PDT testing is needed as a last resort, iii) facilitating read-across in REACH submissions for petroleum products, from PS for which in vivo data are available to other PS for which in vivo data are lacking. This will ultimately significantly minimize the required animal testing for PDT testing of PS UVCBs thereby supporting the application of the 3Rs principle of animal use in toxicological research.

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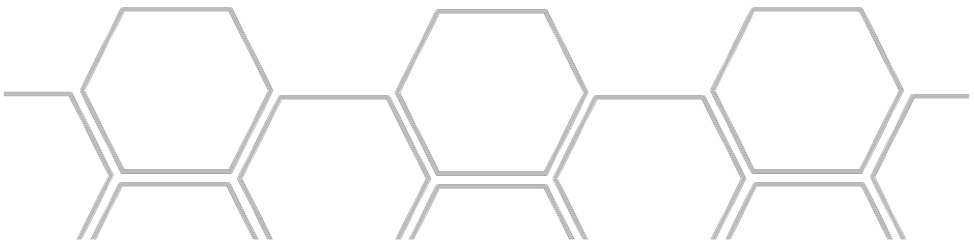
Chapter 8

Summary



Summary

REACH requires prenatal developmental toxicity (PDT) testing for substances registered in the EU at a volume of ≥100 tonnes/year. One of the consequences is that many petroleum substances (PS) will need to be tested for their potential adverse effect on prenatal development according to the current OECD 414 testing guidelines. This will involve a huge number of experimental animals and a considerable amount of resources. Therefore, the application of in vitro alternative testing strategies may reduce the animal experimentation and resources needed to study PDT potencies of PS. Furthermore, since some PS with high concentrations of polycyclic aromatic hydrocarbons (PAH) may induce PDT whilst their gas-to-liquid (GTL) analogues, which are synthetic products completely devoid of aromatics, do not induce PDT, it was hypothesized that PDT observed for some PS is caused by certain types of PAH in these products. This hypothesis was tested in the present thesis using a battery of in vitro alternative assays.

Chapter 1 provided background information and presented the aim of the thesis. In addition, the selected test substances and in vitro alternative assays used in the present thesis were also introduced. In total, 19 samples derived from 6 PS and 2 GTL product categories were tested. These samples were selected because i) they represent a series with a systematic variation in PAH content, being substances containing a range of 3- to 7-ring PAHs including extremes regarding their PAH content (with and without PAHs) and ii) in vivo PDT data for these product categories were available, enabling in vitro-in vivo comparisons. The selected in vitro alternative assays were presented, including the embryonic stem cell test (EST), the zebrafish embryotoxicity test (ZET), and a panel of CALUX reporter gene assays. Finally, the general outline of the thesis was also provided.

Chapter 2 assessed the applicability of the EST to evaluate in vitro embryotoxic potencies of the DMSO extracts of 9 PS (varying in their PAH content, from 5 PS categories) and 2 GTL products (containing no PAHs) as compared to their in vivo potencies. All DMSO-extracts of PS induced a concentration-dependent inhibition of ES-D3 cell differentiation into beating cardiomyocytes at non-cytotoxic concentrations, and their potency was proportional to their 3- to 7-ring PAH content. In contrast, both GTL extracts, which are completely devoid of PAHs, tested negative in the EST. When the EST results were compared to in vivo PDT data of the corresponding PS, a good correlation was found between in vitro and in vivo results (R^2: 0.97). Overall, the EST showed able to evaluate

the in vitro embryotoxicity of PS, within and across categories, a result for the in vitro assay that was in line with the in vivo PDT data. The results also supported the hypothesis that PAHs are the primary inducers of the PDT resulting from PS exposure.

In **Chapter 3**, the role of endocrine- and dioxin-like activity in the developmental toxicity of PS extracts was investigated using a panel of Chemical Activated LUciferase gene eXpression (CALUX) assays. The same set of samples as in **Chapter 2** was tested in the panel of CALUX assays that included agonist and antagonist assays for the androgen, estrogen-α, progesterone, and thyroid-β receptor, and also for the aryl hydrocarbon receptor (AhR). All DMSO-extracts of the PS showed strong AhR agonist activity and weak antiprogesterone, antiandrogen, and estrogenic activities. Only minor effects were seen for thyroid-related and antiestrogenic activity with some products. PS that are grouped in the same class induced similar luciferase expression profiles, suggesting a class specific signature of effects. None of the GTL products showed a meaningful interaction with the selected receptors, thus testing negative in all CALUX assays applied. The AhR-mediated activity of the PS correlated best (R^2: 0.80) with the in vitro PDT potency of the corresponding PS as quantified previously in the EST, suggesting an important role of the AhR in mediating this effect. In conclusion, a high potential for endocrine and dioxin-like activity of some PS extracts was elucidated, which correlated with their in vitro PDT, and was driven by the type and level of PAHs present in the PS extracts. The prominent AhR-mediated activity as induced by the PS extracts tested could be one of the underlying mechanisms of PDT by these substances.

Chapter 4 investigated the usefulness of both the EST and the AhR CALUX assay to evaluate the in vitro PDT potency of an additional series of DMSO-extracts of HFOs, heavy PS containing mainly 3- to 7-ring PAHs, and one HRBO, a highly refined mineral oil that contains no aromatics and no PAHs. All DMSO-extracts of HFOs, but not of the HRBO, resulted in inhibition of ES-D3 cell differentiation in the EST and induced AhR-mediated activity in the AhR CALUX assay, and these potencies were was shown to be proportional to the amount of 3- to 7-ring PAHs they contain. Co-exposure of ES-D3 cells (EST) or H4IIE.luc cells (AhR CALUX assay) with the selected DMSO-extracts of PS and the AhR antagonist trimethoxyflavone (TMF), successfully counteracted the PS-induced inhibition of ES-D3 cell differentiation into cardiomyocytes as well as the AhR-mediated induction of gene expression by these substances. Moreover, also for this series of PS a good

concordance was obtained when comparing the EST results with available in vivo PDT data. Altogether, the resulting data corroborate the hypothesis that PS-induced PDT is induced mainly by their 3- to 7-ring PAH content and that the observed PDT is partially mediated via the AhR.

In **Chapter 5**, the applicability of the ZET to evaluate developmental toxicity potency of the same set of samples as tested in **Chapter 2 and 3** (DMSO-extracts of 9 PS and 2 GTL products) was investigated. All PS extracts, varying in PAH level and content, were able to inhibit the development of zebrafish embryos in a concentration-dependent manner and this potency could be associated with the amount of 3-5 ring PAHs they contain. On the contrary, DMSO-extracts of both GTL products, with no aromatics, showed no effect at all in the ZET. The potencies obtained in the ZET moderately correlated with those previously reported for the EST (R2: 0.61) and the AhR CALUX assay (R^2: 0.66), while the correlation with potencies reported in in vivo studies were higher for the EST (R^2: 0.85) than the ZET (R^2: 0.69). Combining the results obtained from the EST **(Chapter 2)**, AhR CALUX assay **(Chapter 3)**, and ZET **(Chapter 5)** ranked and clustered the test substances in line with their in vivo potencies and chemical characteristics. It was concluded that the ZET did not outperform the EST as a stand-alone assay for testing PDT of PS but confirms the hypothesis that PAHs are the major inducers of PDT by some PS, and that the ZET is a useful addition to a battery of in vitro tests able to predict the in vivo PDT of PS.

In **Chapter 6** we combined an exogenous biotransformation system, using hamster liver microsomes, with the EST to compare the in vitro PDT potency with and without bioactivation of two model 5-ring PAHs, benzo[a]pyrene (BaP) and dibenz[a,h]anthracene (DBA), and of PAH containing PS and GTL base oil (GTLb) extracts. In the absence of bioactivation, DBA, but not BaP, inhibited the differentiation of ES-D3 cells into beating cardiomyocytes. Upon bioactivation, BaP induced in vitro PDT, while its major metabolite 3-hydroxybenzo[a]pyrene was shown to be active in the EST as well. This indicates that BaP needs metabolic activation to exert its in vitro embryotoxic effect. The PS-induced PDT in the EST was not substantially changed following bioactivation, implying that metabolism may not play a crucial role for PS to exert their in vitro PDT effects. GTL extracts tested negative in the EST, with and without bioactivation. Altogether, although some PAH constituents require metabolic activation to be able to induce PDT,

some do not and this latter also appeared to hold for the (majority of) the PS constituents responsible for the in vitro PDT of these complex substances.

Chapter 7, first presented an overview of the results and main findings, which was combined with a general discussion of the data obtained and with future perspectives for follow-up studies to be performed in the near future. It was concluded that PAHs present in PS are the major inducers of PDT caused by these substances and that this was successfully and adequately assessed using several in vitro alternative assays, including the EST, ZET, and AhR CALUX assay. The results obtained in **Chapter 2, 4, and 5** of the thesis were used in a QSAR (quantitative structure activity relationship) approach to predict the in vivo PDT of a series of PS based on their PAH content. More PS extracts, ideally from different PS categories than those tested in the present thesis, should be tested to broaden the applicability domain of the proposed assay battery and the related QSAR approach for PDT testing of PS UVCBs in the future.

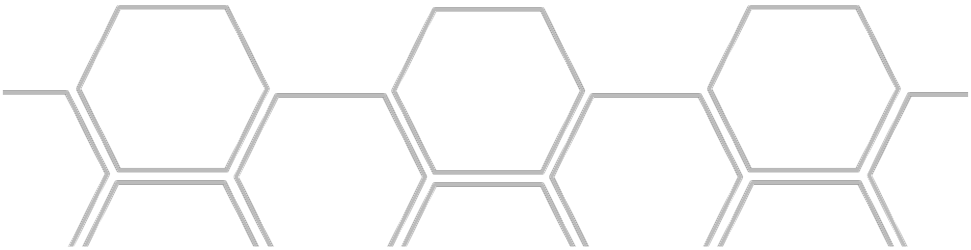
Appendix

Acknowledgements

List of publications

Curriculum vitae

Overview of completed training activities



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Lenny Kamelia

List of publications

Peer-reviewed papers:

- **Kamelia, L.,** de Haan, L., Ketelslegers, H.B., Rietjens, I.M.C.M., Boogaard, P.J. (2019). In vitro prenatal developmental toxicity induced by some petroleum substances is mediated by their 3- to 7-ring PAH constituent with a potential role for the aryl hydrocarbon receptor (AhR). *Toxicology Letters (in press).*
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- **Kamelia, L.,** Brugman, S., de Haan, L., Ketelslegers, H.B., Rietjens, I.M.C.M., Boogaard, P.J. (2019). Prenatal developmental toxicity testing of petroleum substances using the zebrafish embryotoxicity test. *ALTEX, 36*(2), 245-260.
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- Wang, D., Bruyneel, B., **Kamelia, L.,** Wesseling, S., Rietjens, I.M.C.M., Boogaard, P.J. (2019). In vitro metabolism of naphthalene and its alkylated congeners by human and rat liver microsomes via alkyl side chain or aromatic oxidation. *(submitted).*
- Jonathan, N., Bovee, T.F.H., **Kamelia, L.,** Rietjens, I.M.C.M., Hendriksen, P.J.M. (2015). Exploration of new functional endpoints in neuro-2a cells for the detection of the marine biotoxins saxitoxin, palytoxin and tetrodotoxin. *Toxicology in Vitro, 30*(1), 341-347.

Conference abstracts and others:

- **Kamelia, L.,** de Haan, L., Ketelslegers, H.B., Rietjens, I.M.C.M., Boogaard, P.J. (2019). A battery of animal-free in vitro assays for evaluating prenatal developmental toxicity potency of highly complex petroleum substances. *Toxicology Letters (in press).*
- **Kamelia, L.,** Ketelslegers, H.B., Rusyn, I. (2017). Review: Special Symposium Edition, 12th Concawe Symposium (Antwerp, Belgium), Using new methodologies for a better understanding of the health impacts of petroleum substances. *Concawe Review, Special Symposium Edition,* May 2017 page 15.
- **Kamelia, L.,** Louisse, J., Rietjens, I.M.C.M., Boogaard, P.J. (2017). The role of metabolism in the prenatal developmental toxicity (PDT) of polycyclic aromatic hydrocarbons (PAHs) in petroleum substances. *Reproductive Toxicology, 72,* 24-25.
- **Kamelia, L.,** Louisse, J., Rietjens, I.M.C.M., Boogaard, P.J. (2016). In vitro developmental toxicity potencies of petroleum substances in the embryonic stem cell test as compared to their in vivo developmental toxicity potencies. *Reproductive Toxicology, 64,* 22-23.

Curriculum Vitae

Lenny Kamelia was born on August 22nd, 1989 in Pangkalan Baru, Bangka Belitung, Indonesia. After receiving her Bachelor of Science (Biotechnology) in 2011 from Atma Jaya Catholic University Indonesia, she worked for 2 years as a researcher at P.T. Gosyen Medika, Jakarta and Department of Neurosurgery, Beijing Xishan Institute for Neuroregeneration and Functional Recovery, Beijing. During that period, she got herself acquainted with human stem cell research and therapy. In August 2013, Lenny moved to the Netherlands to follow a master program in Molecular Life Sciences (specialty: Biomedical Sciences) at Wageningen University and Research (WUR), with the support from Wageningen University Fellowship Program (WUFP). During her MSc study, Lenny did a thesis entitled *"Towards the implementation of functional endpoints for the screening of marine neurotoxins"* at TOX-WUR, and an internship also at TOX-WUR with a project entitled "*Optimizing the incubation options of petroleum substances for embryonic stem cell test (EST)"*, which is a collaboration project between WUR and Concawe (Brussels, Belgium). After her MSc study, she decided to continue her career path as a PhD student at TOX-WUR, moving further with the collaboration project between WUR and Concawe with the research topic *"The role of PAHs in developmental toxicity of petroleum substances"*. Lenny has followed the postgraduate education in toxicology that will allow her to register as European Registered Toxicologist (ERT) after the completion of her PhD study. In 2017, Lenny was one of the organizing committee members for the Annual Meeting of the Netherlands Society of Toxicology (NVT) in Doorn, the Netherlands, and in 2018, she was involved in organizing the TOX-WUR PhD trip to Japan. During her PhD study, Lenny had a chance to go to Texas for a short research visit to Rusyn's lab at Texas A&M. Lenny was greatly honored for the opportunity to receive two prizes during her PhD study, namely, the 1st prize-Graduate Student Poster, Reproductive and Developmental Toxicology Specialty Section at the SOT meeting in San Antonio, Texas, in 2018, and she was awarded the 2019 Robert L. Dixon International Award, which she received during the 15th ICT-IUTOX meeting in Honolulu, Hawaii. Currently, Lenny is working as a postdoctoral researcher at TOX-WUR, Wageningen, The Netherlands.

Overview of completed training activities

Discipline specific courses

- Molecular Toxicology, Postdoctoral Education in Toxicology (PET), Amsterdam, 2016
- Epidemiology, PET, Utrecht, 2016
- Reproductive Toxicology, PET, Utrecht, 2016
- Laboratory Animal Sciences, PET, Utrecht, 2016
- Organ Toxicology, PET, Nijmegen, 2017
- Mutagenesis and Carcinogenesis, PET, Leiden, 2017
- Toxicogenomics, PET, Maastricht, 2017

Conferences and Meetings

- 44th Annual Meeting European Teratology Society (oral presentation), Dublin, Ireland, 2016
- 12th Concawe Symposium (speaker; oral presentation), Antwerp, Belgium, 2017
- Annual Meeting of the Netherlands Society of Toxicology (poster presentation), Doorn, The Netherlands, 2017
- 45th Annual Meeting European Teratology Society (oral presentation), Budapest, Hungary, 2017
- Section Meeting, Teratology and Reproductive Toxicology of the Netherlands Society of Toxicology (speaker; oral presentation), RIVM, Bilthoven, The Netherlands, 2017
- 57th Annual Meeting Society of Toxicology (poster presentation), San Antonio, Texas, USA, 2018
- 45th Annual Meeting of the Japanese Society of Toxicology (poster and oral presentation), Osaka, Japan, 2018
- 58th Annual Meeting Society of Toxicology, Baltimore, Maryland, USA, 2019
- Annual Meeting of the Netherlands Society of Toxicology (platform presentation), Ede, The Netherlands, 2019
- 15th International Congress of Toxicology (ICT) – International Union of Toxicology (IUTOX) (platform presentation), Honolulu, Hawaii, 2019
- 55th Congress of the European Societies of Toxicology (EUROTOX) (poster presentation), Helsinki, Finland, 2019

General courses

- VLAG PhD Week, VLAG, Soest, 2016
- Brain Training, Wageningen Graduate Schools (WGS), Wageningen, 2016
- Scientific Publishing, WGS, Wageningen, 2016
- The Essentials of Writing and Presenting, WGS, Wageningen, 2017
- Risk Assessment, PET, Wageningen, 2017
- PhD Peer Consultation, WGS, Wageningen, 2018-2019

Optional courses and other activities

- Thesis Proposal
- Scientific Presentations at TOX-WUR
- General Toxicology, WUR, Wageningen, 2016
- Environmental Toxicology, Wageningen, 2017
- PhD trip Japan, TOX-WUR, 2018

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