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,[*,1] Jochem Louisse,[*] Laura de Haan,[*] Anna Maslowska-Gornicz,[*] Hans B. Ketelslegers,[†] Abraham Brouwer,[‡] Ivonne M.C.M. Rietjens,[*] and Peter J. Boogaard[*,§]

[*]Division of Toxicology, Wageningen University and Research, 6708 WE Wageningen, The Netherlands; [†]European Petroleum Refiners Association, Concawe Division, 1160 Brussels, Belgium; [‡]BioDetection Systems, 1098XH Amsterdam, The Netherlands; and [§]Shell Health, Shell International B.V., 2596HR The Hague, The Netherlands

[1]To whom correspondence should be addressed at Division of Toxicology, Wageningen University and Research, PO Box 8000, 6700 EA Wageningen, The Netherlands. Fax: +31 (0) 317-484-931, E-mail: lenny.kamelia@wur.nl.

ABSTRACT

Recent evidence suggests that the interaction of polycyclic aromatic hydrocarbons (PAHs), 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, ie, sample No. 34, 98, and 99, 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.

Key words: polycyclic aromatic hydrocarbons; petroleum substances; reporter gene assays; endocrine activity; dioxin-like activity; prenatal developmental toxicity.

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 (GTLs) 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.

MATERIALS AND METHODS

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), 17β-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.

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
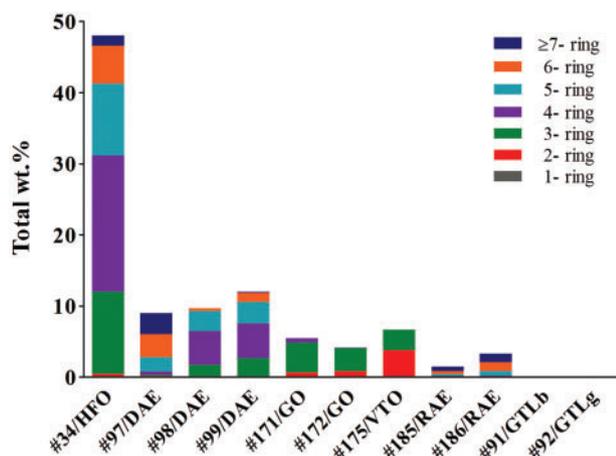
Figure 1. ARC profiles[a] of PS and GTL products tested in this study. [a]The weight percent of the DMSO-soluble 1- to ≥7 aromatic-ring compounds present in each sample, from the starting material of 4.0 g, as determined by Method II chemical characterization procedure (Kamelia et al., 2017; Roy et al. 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.

(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 μg/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.

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 μl 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 (2.9×10^{-5} μg/ml DHT for the U2OS AR antagonist assay; 3.1×10^{-4} μg/ml PGT for U2OS PR antagonist assay; 2.7×10^{-6} μg/ml E2 for U2OS ERα antagonist assay; 1.3×10^{-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 3×10^5 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 $(MgCO_3)_4$ $Mg(OH)_2 \cdot 5H_2O$ (Sigma-Aldrich), 2.67 mM magnesium sulfate ($MgSO_4$, 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 (2.9×10^{-7}–2.2×10^{-3} µg/ml) for the AR agonist assay; flutamide (1.4×10^{-4}—1.4 µg/ml) for the AR antagonist assay; E2 (2.7×10^{-8}–1.4×10^{-4} µg/ml) for the ERα agonist assay; fulvestrant (3.0×10^{-10}–3.0×10^{-4} µg/ml) for the ERα antagonist assay; PGT (3.1×10^{-7}–3.1×10^{-3} µg/ml) for the PR agonist assay; RU468 (2.1×10^{-8}–2.1×10^{-3} µg/ml) for the PR antagonist assay; T3 (6.5×10^{-7}–6.5×10^{-3} µg/ml) for the TRβ agonist assay; 1-850 (2.3×10^{-3}–5.8 µg/ml) for the TRβ antagonist assay; and BaP (1.3×10^{-5}–1.3×10^{-1} µg/ml) for the AhR agonist assay.

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 ($n \geq 4$). 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 2.7×10^{-6} µg/ml (10 pM) E2-induced estrogenic response, not from the 100% of maximal estrogenic activity of E2.

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 BMC_d50 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 $< .05$.

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.

RESULTS

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 2.9×10^{-5} µg/ml (0.1 nM). In the U2OS AR antagonist assay, all DMSO-extracts of PS, except for sample No. 185-RAE, showed a concentration-dependent antiandrogenic effect (Figure 2B). Sample No. 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 No. 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 No. 34-HFO was the most potent antagonist of
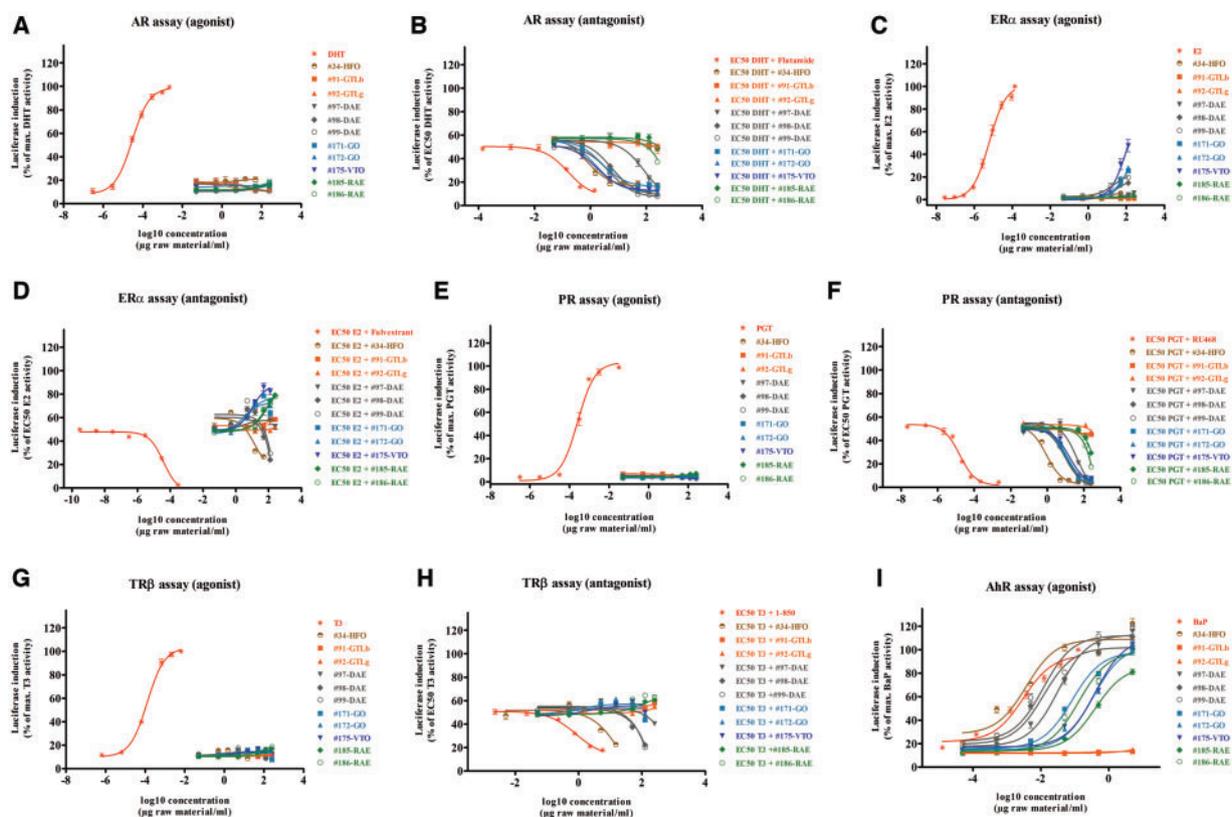
Figure 2. Effects of the DMSO-extracts of PS and GTL products in the selected CALUX reporter gene assays: (A) U2OS AR agonist assay; (B) U2OS AR antagonist assay; (C) U2OS ERα agonist assay; (D) U2OS ERα antagonist assay; (E) U2OS PR agonist assay; (F) U2OS PR antagonist assay; (G) U2OS TRβ agonist assay; (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.

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 No. 97, 98, and 99 were 70.6, 5.7, and 4.8 μg/ml, respectively. This means that sample No. 98 and 99 are more potent antagonists of the AR (>14-fold) than sample No. 97, 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 noncytotoxic concentrations of the test compounds were used (data not shown), hence, the observed antagonism effects were not due to cytotoxicity. Furthermore, both GTL extracts, No. 91-GTLb and 92-GTLg, showed neither agonist, nor antagonist activity in the U2OS AR CALUX assay (up to 250 μg/ml).

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 (No. 98 and 99), 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 No. 98-DAE and 99-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 No. 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, 2.7×10^{-6} μg/ml (10 pM). Fulvestrant, a known antagonist of the ERα, was used

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 of This Study

Substance/ Compound	AR Antagonist Assay IC$_{50}$ (μg/ml)	AR Antagonist Assay Min.-Max. Response (% Mean ± SEM)	ERα Antagonist Assay IC$_{25}$ (μg/ml)	ERα Antagonist Assay Min.-Max. response (% Mean ± SEM)	PR Antagonist Assay IC$_{50}$ (μg/ml)	PR Antagonist Assay Min.-Max. Response (% Mean ± SEM)	TRβ Antagonist Assay IC$_{50}$ (μg/ml)	TRβ Antagonist Assay Min.-Max. Response (% Mean ± SEM)	ERα Agonist Assay EC$_{25}$ (μg/ml)	ERα Agonist Assay Min.-Max. Response (% Mean ± SEM)	AhR Agonist Assay EC$_{50}$ (μg/ml)	AhR Agonist Assay 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 x 10^{-5}	5 ± 0.9–53 ± 0.6	—	—	—	—	—	—
TR antagonist (1-850)	—	—	—	—	—	—	1.9	18 ± 0.5–50 ± 2.2	—	—	—	—
Estradiol (E2)	—	—	—	—	—	—	—	—	5.9 × 10^{-6}	2 ± 0.5 –100 ± 0.0	—	—
BaP	—	—	—	—	—	—	—	—	—	—	3.1 × 10^{-3}	17 ± 0.8–100.0 ± 0.0
PS 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
GTL 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.

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: No. 34-HFO (IC25: 9.7 μg/ml), No. 98-DAE (IC25: 40.7 μg/ml), and No. 99-DAE (IC25: 54.1 μg/ml). The antagonistic effects were considered weak because the luminescence activity was reduced just to $\leq \pm 30\%$ (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 No. 34-HFO and 250 μg/ml for sample Nos. 98 and 99-DAE). Again, no agonist or antagonist responses were observed for the DMSO-extract of GTL products.

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 No. 98 and 99), GOs and VTO, sample No. 97-DAE, and RAEs. As expected, no PR activity was detected for both GTL extracts (No. 91-GTLb and 92-GTLg) at any of the concentrations tested (Figs. 2E and F).

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 No. 34-HFO and 2 of the DAE samples (No. 98 and 99) 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 (Figs. 2G and H).

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 No. 185-RAE) to 122% (sample No. 34-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.

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 No. 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 3 CALUX assays where substantial agonist or antagonist effects were observed (ie, 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).

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 BMC_d50s EST, with an R^2 of 0.80 (Figure 4C). In contrast, there were no meaningful correlations found when plotting the BMC_d50s 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.

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
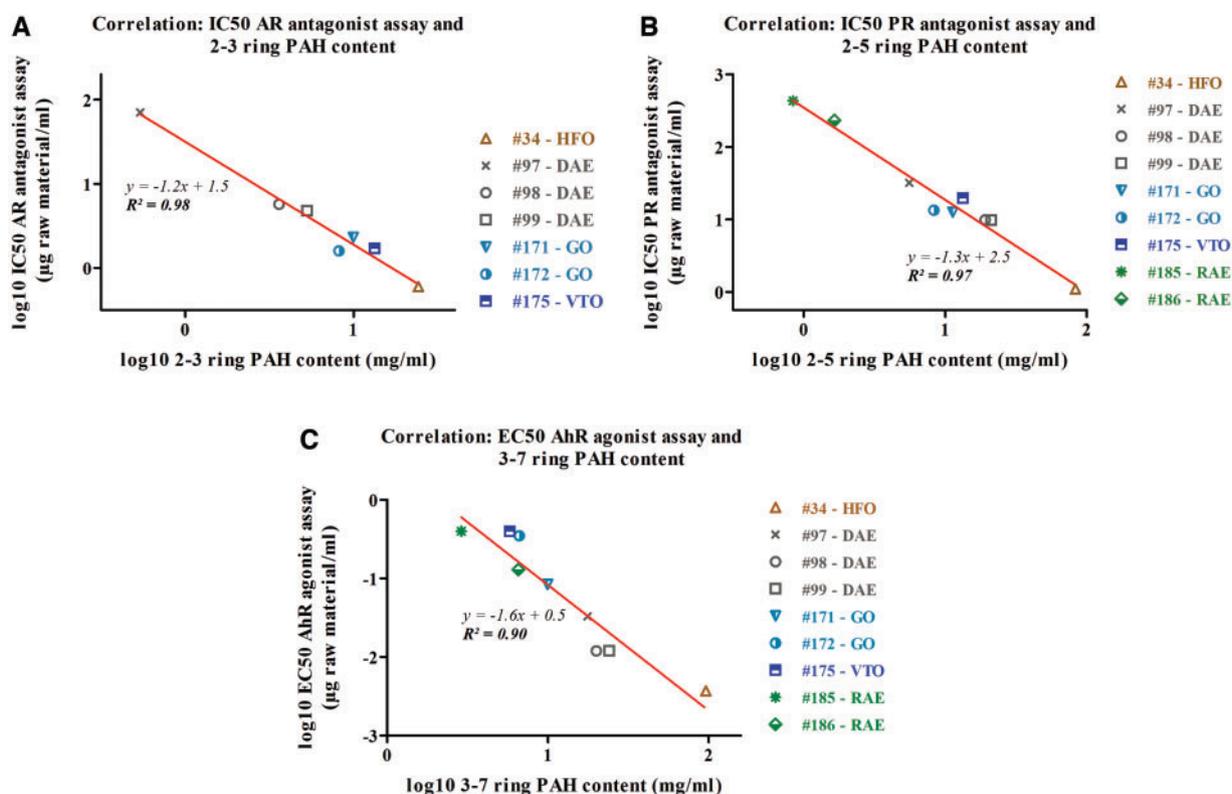
Figure 3. Correlation between *in vitro* agonist/antagonist potencies (EC50s/IC50s), obtained from (A) U2OS AR antagonist assay; (B) U2OS PR antagonist assay; (C) H4IIE AhR agonist assay; and specific PAH content in PS samples. Abbreviations: HFO, heavy fuel oil; DAE, distillate aromatic extract; GO, gas oil; VTO, vacuum tower overhead oil; RAE, residual aromatic extract.

(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, eg, 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 No. 34-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 (No. 98 and 99) were clustered together while the other DAE, sample No. 97, 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.

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 (No. 98 and 99), 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
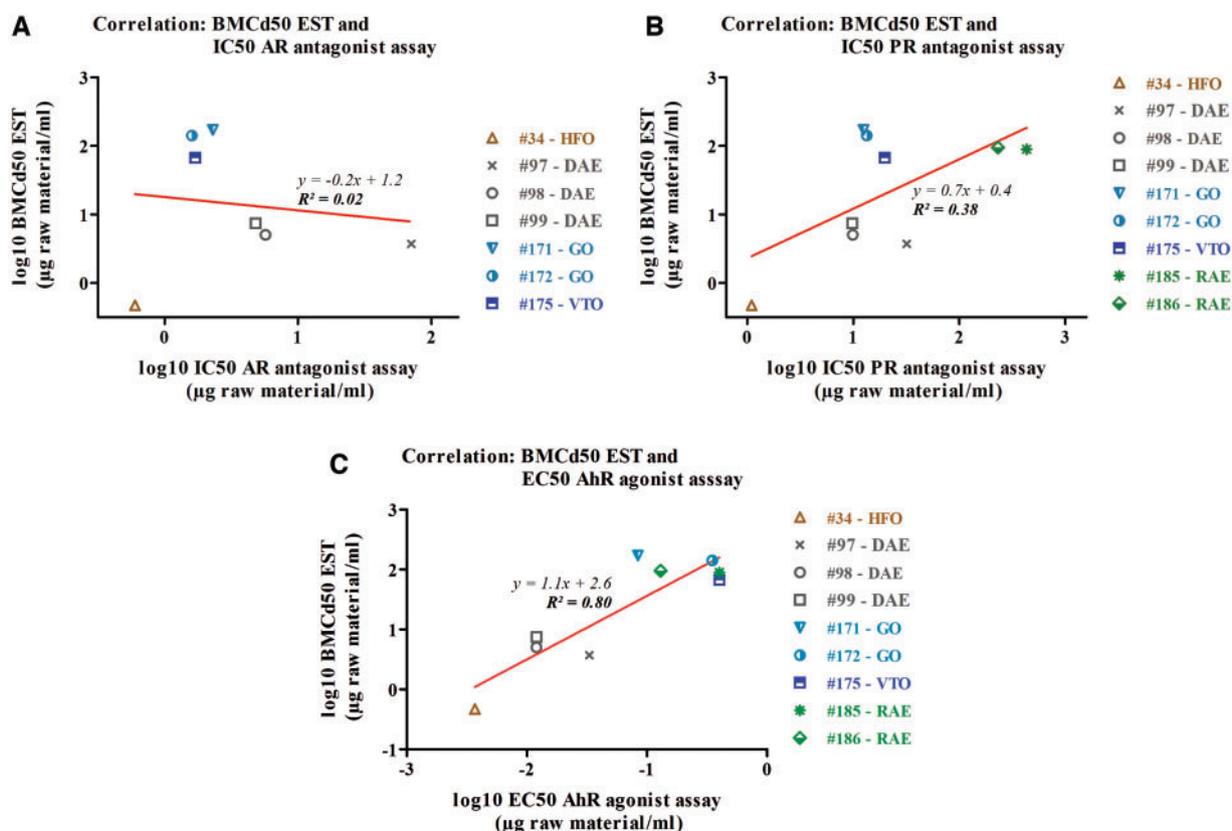
Figure 4. Correlation between *in vitro* developmental toxicity of PS in the EST (expressed as BMC_d50s), obtained from our previous study (Kamelia et al., 2017), and *in vitro* agonist/antagonist potencies (expressed as EC50s/IC50s), obtained from (A) U2OS AR antagonist assay; (B) U2OS PR antagonist assay; (C) H4IIE AhR agonist assay of this study. Abbreviations: HFO, heavy fuel oil; DAE, distillate aromatic extract; GO, gas oil; VTO, vacuum tower overhead oil; RAE, residual aromatic extract; BMC_d50, benchmark concentration for 50% inhibition of cell differentiation in the EST.

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 No. 34-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
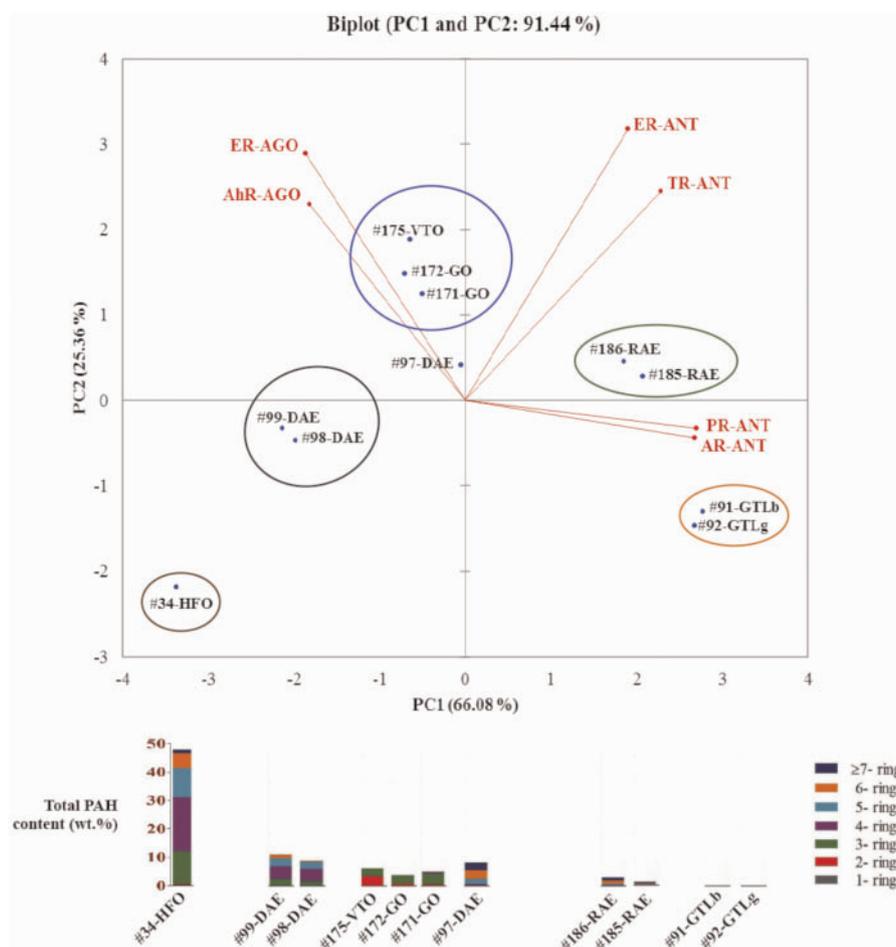
Figure 5. PCA based on luciferase expression profiles induced by the DMSO-extracts of PS and GTL products in 6 different CALUX assays: AhR; ERα agonist assays and ERα; TRβ; PR; AR antagonist assays. The average luciferase induction factor at the highest tested concentration of each test compound, from 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; RAE, residual aromatic extract.

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, No. 98 and 99, 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.

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 No. 97-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 No. 97 in comparison to the other 2 PS in this class, No. 98-DAE and 99-DAE, which is
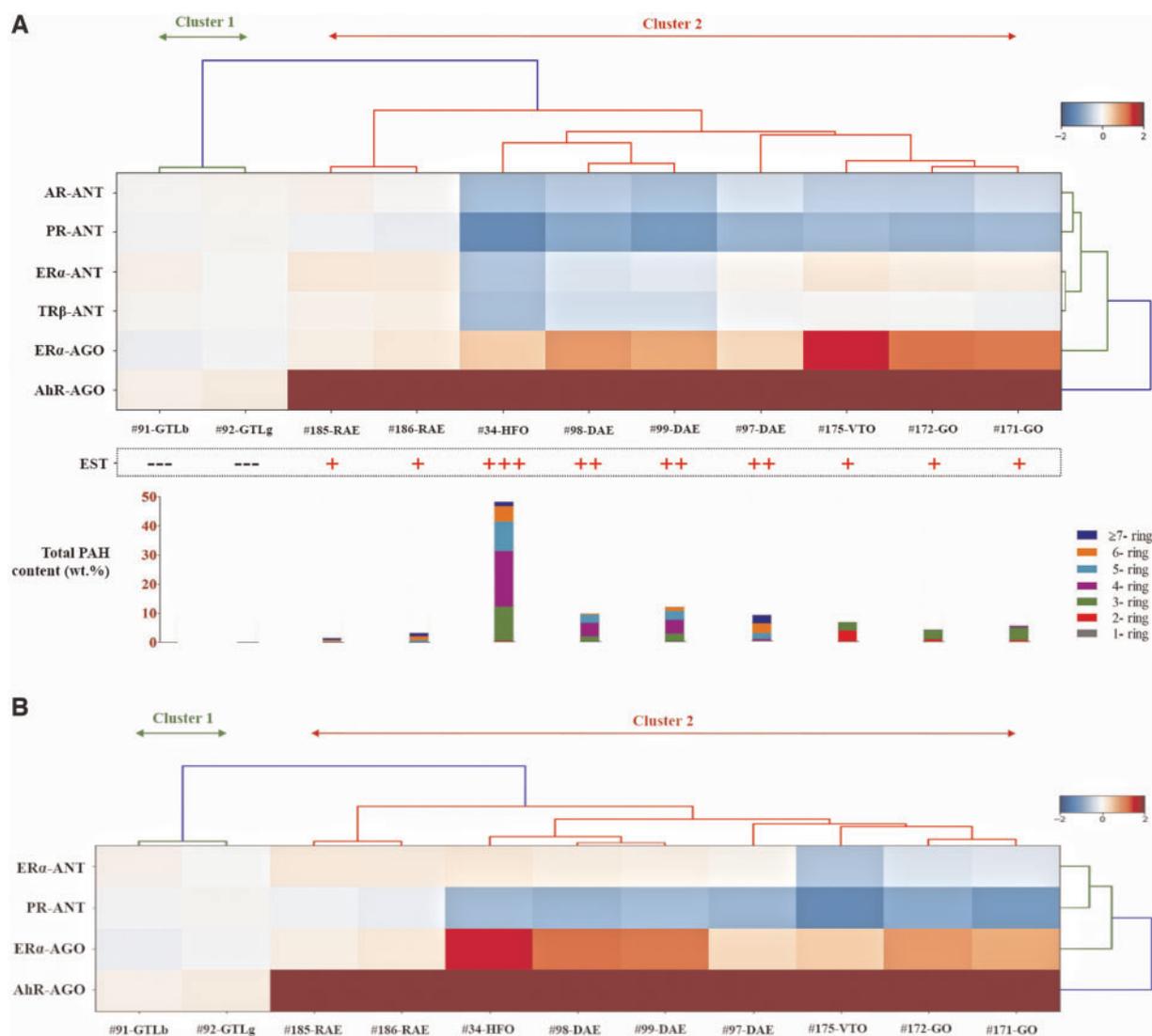
Figure 6. Hierarchic clustering, illustrated by heatmap summarizing luciferase expression profiles of the DMSO-extract of PS and GTL products in (A) 6 different CALUX assays: AhR; ERα agonist assays and ERα; TRβ; PR; AR antagonist assays; (B) 4 different CALUX assays: AhR; ERα agonist assays and ERα; 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 BMC$_d$50s, 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). 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; 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.

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

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 This Study Are In Line With the Following Published Literature?
Vinggaard et al. (2000)	Phenanthrene Anthracene Fluoranthene Chrysene Pyrene Benz[a]anthracene BaP 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 No. 98 and 99, also show AR antagonist effect.
Kizu et al. (2003)	Anthracene Pyrene Chrysene Benzo[k]fluoranthene BaP	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 BaP, 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)	BaP (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- to 5-ring PAHs: DAEs, demonstrate (weak) estrogen activity in the U2OS ERα agonist assay (Figure 2C).
Kummer et al. (2008)	Fluoranthene Benz[a]anthracene BaP 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) BaP (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 2 (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.

Table 2. (continued)

References	Substances	Receptor and Model/Method	Exposure	Effects	Remarks	Results of This Study Are In Line With the Following Published Literature?
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 No. 34-HFO, No. 98-DAE, No. 99-DAE, induce antiestrogenic 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 antiestrogenic potency increases with increasing concentration of polycyclic aromatic compounds.	
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- to 6-ring PAHs)	AhR-H4IIE cells	0–10 µM	AhR agonist	4- to 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- to 5-ring PAHs)	AhR-H4IIE cells	0–100 µM	AhR agonist	3- to 5-ring PAHs efficiently bind and activate the AhR but not 2-ring PAHs, such as naphthalene.	
Vondráček et al. (2017)	19 individual PAHs (3- to 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.
Abbreviations: 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.

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.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

FUNDING

CONFLICT OF INTEREST

P.J.B. is employed by Shell International, a member company of Concawe, and chairman of the toxicology group of Concawe. H.K. is Science Executive for health at Concawe. Both Prof. P.J.B. and Dr H.K. are totally free (by contract) to freely design and conduct research and express their own scientific opinion without any obligation towards either Shell or Concawe. The current findings are not intended to constitute any product endorsement.

REFERENCES

Aarts, J. M., Denison, M. S., Cox, M. A., Schalk, M. A., Garrison, P. M., Tullis, K., de Haan, L. H., and Brouwer, A. (1995). Species-specific antagonism of Ah receptor action by 2, 2', 5, 5'-tetrachloro- and 2, 2', 3, 3'4, 4'-hexachlorobiphenyl. Eur. J. Pharmacol. 293, 463–474.

Arcaro, K. F., Gierthy, J. F., and Mackerer, C. R. (2001). Antiestrogenicity of clarified slurry oil and two crude oils in a human breast-cancer cell assay. J. Toxicol. Environ. Health, A 62, 505–521.

ARCO. (1993). Developmental toxicity (embryo-fetal toxicity and teratogenic potential) study of F-193 administered percutaneously to Crl: CD®BRK VAF/Plus® presumed pregnant rats. Report ATX-92-0011.

Barlow, S., Kavlock, R. J., Moore, J. A., Schantz, S. L., Sheehan, D. M., Shuey, D. L., and Lary, J. M. (1999). Teratology Society Public Affairs Committee position paper: Developmental toxicity of endocrine disruptors to humans. Teratology 60, 365–375.

Benisek, M., Kubincova, P., Blaha, L., and Hilscherova, K. (2011). The effects of PAHs and N-PAHs on retinoid signaling and Oct-4 expression in vitro. Toxicol. Lett. 200, 169–175.

Bernasconi, S., Sartori, C., Merli, S., Lazzeroni, P., Cesari, S., and Street, M. E. (2015) Thyroid hormones in fetal development. In Thyroid Diseases in Childhood (G. Bona, F. De Luca, and A., Monzani, , Eds.), Springer, Cham.

Billiard, S. M., Timme-Laragy, A. R., Wassenberg, D. M., Cockman, C., and Di Giulio, R. T. (2006). The role of the aryl hydrocarbon receptor pathway in mediating synergistic developmental toxicity of polycyclic aromatic hydrocarbons to zebrafish. Toxicol. Sci. 92, 526–536.

Blackburn, G. R., Deitch, R. A., Schreiner, C. A., and Mackerer, C. R. (1986). Predicting carcinogenicty of petroleum distillation fractions using a modified Salmonella mutagenicity assay. Cell Biol. Toxicol. 2, 63–84.

Bondesson, M., Hao, R., Lin, C.-Y., Williams, C., and Gustafsson, J.-Å. (2015). Estrogen receptor signaling during vertebrate development. Biochim. Biophys. Acta 1849, 142–151.

Boogaard, P. J., Carrillo, J. C., Roberts, L. G., and Whale, G. F. (2017). Toxicological and ecotoxicological properties of gas-to-liquid (GTL) products. 1. Mammalian toxicology. *Crit. Rev. Toxicol.* **47**, 121–144.

Brandenberger, A. W., Tee, M. K., Lee, J. Y., Chao, V., and Jaffe, R. B. (1997). Tissue distribution of estrogen receptors alpha (ER-α) and beta (ER-β) mRNA in the midgestational human fetus. *J. Clin. Endocrinol. Metab.* **82**, 3509–3512.

Charles, G. D., Bartels, M. J., Zacharewski, T. R., Gollapudi, B. B., Freshour, N. L., and Carney, E. W. (2000). Activity of benzo[a]-pyrene and its hydroxylated metabolites in an estrogen receptor-alpha reporter gene assay. *Toxicol. Sci.* **55**, 320–326.

Colborn, T., vom Saal, F. S., and Soto, A. M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect.* **101**, 378–384.

Couse, J. F., and Korach, K. S. (2004). Estrogen receptor-α mediates the detrimental effects of neonatal diethylstilbestrol (DES) exposure in the murine reproductive tract. *Toxicology* **205**, 55–63.

Clonfero, E., Nardini, B., Marchioro, M., Bordin, A., and Gabbani, G. (1996). Mutagenicity and contents of polycyclic aromatic hydrocarbons in used and recycled motor oils. *Mut. Res.* **368**, 283–291.

Concawe. (1994). The use of the dimethyl sulphoxide (DMSO) extract by the IP 346 method as an indicator of the carcinogenicity of lubricant base oils and distillate aromatic extracts. Report no 94/51.

Dunster, J. (2014). GTL diesel (distillates (fischer-tropsch), C8-26 branched and linear): Oral (gavage) pre-natal development toxicity study in the rat. Report 41301744.

Feuston, M. H., Hamilton, C. E., and Mackerer, C. R. (1996). Systemic and developmental toxicity of dermally applied distillate aromatic extract in rats. *Fundam. Appl. Toxicol.* **30**, 276–284.

Feuston, M. H., Kerstetter, S. L., Singer, E. J., and Mehlman, M. A. (1989). Developmental toxicity of Clarified Slurry Oil applied dermally to rats. *Toxicol. Ind. Health* **5**, 587–599.

Feuston, M. H., Low, L. K., Hamilton, C. E., and Mackerer, C. R. (1994). Correlation of systemic and developmental toxicities with chemical component classes of refinery streams. *Fundam. Appl. Toxicol.* **22**, 622–630.

Feuston, M. H., and Mackerer, C. R. (1996). Developmental toxicity of clarified slurry oil, syntower bottoms, and distillate aromatic extract administered as a single oral dose to pregnant rats. *J. Toxicol. Environ. Health* **49**, 45–66.

Fowles, J. R., Banton, M. I., Boogaard, P. J., Ketelslegers, H. B., and Rohde, A. M. (2016). Assessment of petroleum streams for thyroid toxicity. *Toxicol. Lett.* **254**, 52–62.

Goodale, B. C., Tilton, S. C., Wilson, G., Corvi, M. M., Janszen, D. B., Anderson, K. A., Waters, K. M., and Tanguay, R. L. (2013). Structurally distinct polycyclic aromatic hydrocarbons induce differential transcriptional responses in developing zebrafish. *Toxicol. Appl. Pharmacol.* **272**, 656–670.

Haddow, J. E., Palomaki, G. E., Allan, W. C., Williams, J. R., Knight, G. J., Gagnon, J., O'Heir, C. E., Mitchell, M. L., Hermos, R. J., Waisbren, S. E., *et al.* (1999). Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. *N. Engl. J. Med.* **341**, 549–555.

Hilscherova, K., Machala, M., Kannan, K., Blankenship, A. L., and Giesy, J. P. (2000). Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. *Environ. Sci. Pollut. Res.* **7**, 159–171.

Hoberman, A. M., Christian, M. S., Lovre, S., Roth, R., and Koschier, F. (1995). Developmental toxicity study of clarified slurry oil (CSO) in the rat. *Fundam. Appl. Toxicol.* **28**, 34–40.

Jomaa, B., Hermsen, S. A., Kessels, M. Y., van den Berg, J. H., Peijnenburg, A. A., Aarts, J. M., Piersma, A. H., and Rietjens, I. M. (2014). Developmental toxicity of thyroid-active compounds in a zebrafish embryotoxicity test. *Altex* **31**, 303–317.

Kamelia, L., Louisse, J., de Haan, L., Rietjens, I., and Boogaard, P. J. (2017). 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. *Toxicol. In Vitro* **44**, 303–312.

Kim, J. H., Yamaguchi, K., Lee, S. H., Tithof, P. K., Sayler, G. S., Yoon, J. H., and Baek, S. J. (2005). Evaluation of polycyclic aromatic hydrocarbons in the activation of early growth response-1 and peroxisome proliferator activated receptors. *Toxicol. Sci.* **85**, 585–593.

Kizu, R., Ishii, K., Kobayashi, J., Hashimoto, T., Koh, E., Namiki, M., and Hayakawa, K. (2000). Antiandrogenic effect of crude extract of C-heavy oil. *Mater. Sci. Eng. C* **12**, 97–102.

Kizu, R., Kato, S., Usui, O., and Hayakawa, K. (1999). Estrogenic activity of heavy oil and its assay method. *Bunseki Kagaku* **48**, 617–622.

Kizu, R., Okamura, K., Toriba, A., Kakishima, H., Mizokami, A., Burnstein, K. L., and Hayakawa, K. (2003). A role of aryl hydrocarbon receptor in the antiandrogenic effects of polycyclic aromatic hydrocarbons in LNCaP human prostate carcinoma cells. *Arch. Toxicol.* **77**, 335–343.

Kummer, V., Maskova, J., Zraly, Z., Neca, J., Simeckova, P., Vondracek, J., and Machala, M. (2008). Estrogenic activity of environmental polycyclic aromatic hydrocarbons in uterus of immature Wistar rats. *Toxicol. Lett.* **180**, 212–221.

Lammer, E. J., Chen, D. T., Hoar, R. M., Agnish, N. D., Benke, P. J., Braun, J. T., Curry, C. J., Fernhoff, P. M., Grix, A. W., Lott, I. T., *et al.* (1985). Retinoic acid embryopathy. *N. Engl. J. Med.* **313**, 837–841.

Legler, J., van den Brink, C. E., Brouwer, A., Murk, A. J., van der Saag, P. T., Vethaak, A. D., and van der Burg, B. (1999). Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. *Toxicol. Sci.* **48**, 55–66.

Lemmen, J. G., Broekhof, J. L. M., Kuiper, G. G. J. M., Gustafsson, J., van der Saag, P. T., and van der Burg, B. (1999). Expression of estrogen receptor alpha and beta during mouse embryogenesis. *Mech. Dev.* **81**, 163–167.

Ma, Q. (2001). Induction of CYP1A1. The AhR/DRE paradigm: Transcription, receptor regulation, and expanding biological roles. *Curr. Drug Metab.* **2**, 149–164.

Machala, M., Vondracek, J., Blaha, L., Ciganek, M., and Neca, J. V. (2001). Aryl hydrocarbon receptor-mediated activity of mutagenic polycyclic aromatic hydrocarbons determined using in vitro reporter gene assay. *Mutat. Res.* **497**, 49–62.

Mackerer, C. R., Griffis, L. C., Grabowski, J. S., Jr., and Reitman, F. A. (2003). Petroleum mineral oil refining and evaluation of cancer hazard. *Appl. Occup. Environ. Hyg.* **18**, 890–901.

Mimura, J., and Fujii-Kuriyama, Y. (2003). Functional role of AhR in the expression of toxic effects by TCDD. *Biochim. Biophys. Acta* **1619**, 263–268.

Morreale de Escobar, G. (2001). The role of thyroid hormone in fetal neurodevelopment. *J. Pediatr. Endocrinol. Metab.* **14**, 1453–1462.

Piersma, A. H., Bosgra, S., van Duursen, M. B., Hermsen, S. A., Jonker, L. R., Kroese, E. D., van der Linden, S. C., Man, H., Roelofs, M. J., Schulpen, S. H., *et al.* (2013). Evaluation of an

alternative in vitro test battery for detecting reproductive toxicants. *Reprod. Toxicol.* **38**, 53–64.

Pieterse, B., Felzel, E., Winter, R., van der Burg, B., and Brouwer, A. (2013). PAH-CALUX, an optimized bioassay for AhR-mediated hazard identification of polycyclic aromatic hydrocarbons (PAHs) as individual compounds and in complex mixtures. *Environ. Sci. Technol.* **47**, 11651–11659.

Puga, A., Tomlinson, C. R., and Xia, Y. (2005). Ah receptor signals cross-talk with multiple developmental pathways. *Biochem. Pharmacol.* **69**, 199–207.

Roy, T. A., Johnson, S. W., Blackburn, G. R., and Mackerer, C. R. (1988). Correlation of mutagenic and dermal carcinogenic activities of mineral oils with polycyclic aromatic compound content. *Fundam. Appl. Toxicol.* **10**, 466–476.

Senn, C. (2014). GTL base oil distillate (distillates (fischer-tropsch), heavy, C18-50-branched, cyclic and linear): Prenatal developmental toxicity study in the Han Wistar rat. Report D80072.

Shimada, T., Inoue, K., Suzuki, Y., Kawai, T., Azuma, E., Nakajima, T., Shindo, M., Kurose, K., Sugie, A., Yamagishi, Y., et al. (2002). Arylhydrocarbon receptor-dependent induction of liver and lung cytochromes P450 1A1, 1A2, and 1B1 by polycyclic aromatic hydrocarbons and polychlorinated biphenyls in genetically engineered C57BL/6J mice. *Carcinogenesis* **23**, 1199–1207.

Sonneveld, E., Jansen, H. J., Riteco, J. A., Brouwer, A., and van der Burg, B. (2004). Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid-responsive bioassays. *Toxicol. Sci.* **83**, 136–148.

Speight, J. G. (2006). *The Chemistry and Technology of Petroleum*, 4th ed. CRC Press, Boca Raton.

Ssempebwa, J., Carpenter, D., Yilmaz, B., DeCaprio, A., O'Hehir, D., and Arcaro, K. (2004). Waste crankcase oil: An environmental contaminant with potential to modulate estrogenic responses. *J. Toxicol. Environ. Health A* **67**, 1081–1094.

Tsitou, P., Heneweer, M., and Boogaard, P. J. (2015). Toxicogenomics in vitro as an alternative tool for safety evaluation of petroleum substances and PAHs with regard to prenatal developmental toxicity. *Toxicol. In Vitro* **29**, 299–307.

van der Linden, S. C., von Bergh, A. R. M., van Vught-Lussenburg, B. M. A., Jonker, L. R. A., Teunis, M., Krul, C. A. M., and van der Burg, B. (2014). Development of a panel of high-throughput reporter-gene assays to detect genotoxicity and oxidative stress. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* **760**, 23–32.

van Lipzig, M. M., Vermeulen, N. P., Gusinu, R., Legler, J., Frank, H., Seidel, A., and Meerman, J. H. (2005). Formation of estrogenic metabolites of benzo[a]pyrene and chrysene by cytochrome P450 activity and their combined and supra-maximal estrogenic activity. *Environ. Toxicol. Pharmacol.* **19**, 41–55.

Vinggaard, A. M., Hnida, C., and Larsen, J. C. (2000). Environmental polycyclic aromatic hydrocarbons affect androgen receptor activation in vitro. *Toxicology* **145**, 173–183.

Vondráček, J., Pěnčíková, K., Neča, J., Ciganek, M., Grycová, A., Dvořák, Z., and Machala, M. (2017). Assessment of the aryl hydrocarbon receptor-mediated activities of polycyclic aromatic hydrocarbons in a human cell-based reporter gene assay. *Environ. Pollut.* **220**, 307–316.

Vrabie, C. M., Candido, A., van den Berg, H., Murk, A. J., van Duursen, M. B., and Jonker, M. T. (2011). Specific in vitro toxicity of crude and refined petroleum products: 3. Estrogenic responses in mammalian assays. *Environ. Toxicol. Chem.* **30**, 973–980.

Vrabie, C. M., Candido, A., van Duursen, M. B., and Jonker, M. T. (2010). Specific in vitro toxicity of crude and refined petroleum products: II. Estrogen (alpha and beta) and androgen receptor-mediated responses in yeast assays. *Environ Toxicol. Chem.* **29**, 1529–1536.

Vrabie, C. M., Jonker, M. T., and Murk, A. J. (2009). Specific in vitro toxicity of crude and refined petroleum products. 1. Aryl hydrocarbon receptor-mediated responses. *Environ. Toxicol. Chem.* **28**, 1995–2003.

Williams, G. R., and Franklyn, J. A. (1994). 1 Physiology of the steroid-thyroid hormone nuclear receptor superfamily. *Bailliere's Clin. Endocrinol. Metab.* **8**, 241–266.

Wincent, E., Jonsson, M. E., Bottai, M., Lundstedt, S., and Dreij, K. (2015). Aryl hydrocarbon receptor activation and developmental toxicity in zebrafish in response to soil extracts containing unsubstituted and oxygenated PAHs. *Environ. Sci. Technol.* **49**, 3869–3877.

Ziccardi, M. H., Gardner, I. A., Mazet, J. A., and Denison, M. S. (2002). Application of the luciferase cell culture bioassay for the detection of refined petroleum products. *Mar. Pollut. Bull.* **44**, 983–991.