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Endocrine disrupting potential of PAHs and their alkylated analogues associated with oil spills†

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Polycyclic aromatic hydrocarbons (PAHs) and alkylated PAHs are known to be major toxic contaminants in spills of petroleum hydrocarbons (oil). Spilled oil undergoes weathering and over time, PAHs go through a series of compositional changes. PAHs can disrupt endocrine functions, and the type of functions affected and associated potencies vary with the type and alkylation status of PAH. In this study, the potential of five major PAHs of crude oil, *i.e.*, naphthalene, fluorene, dibenzothiophene, phenanthrene, and chrysene, and their alkylated analogues ($n = 25$), to disrupt endocrine functions was evaluated by use of MVLN-*luc* and H295R cell lines. In the MVLN-*luc* bioassay, seven estrogen receptor (ER) agonists were detected among 30 tested PAHs. The greatest ER-mediated potency was observed for 1-methylchrysene (101.4%), followed by phenanthrene and its alkylated analogues (range of %-E2max from 1.6% to 47.3%). In the H295R bioassay, significantly greater syntheses of steroid hormones were observed for 20 PAHs. For major PAHs and their alkylated analogues, disruption of steroidogenesis appeared to be more significant than ER-mediated effects. The number and locations of alkyl-moieties alone could not explain differences in the types or the potencies of toxicities. This observation shows that disruption of endocrine functions by some constituents of oil spills could be underestimated if only parent compounds are considered in assessments of hazard and risk.

Environmental significance

Polycyclic aromatic hydrocarbons (PAHs) and alkylated PAHs are major components of petroleum and can cause endocrine disruption. In order to understand the endocrine disrupting potential of oil spills, it is important to know the effects of various PAHs and their alkylated analogues. We evaluated estrogen receptor binding potency and effects on steroidogenesis *in vitro* for 30 major PAHs frequently detected in oil spilled environments. The endocrine disrupting effects of PAHs are mostly associated with steroidogenic alteration. Alkylation could influence the endocrine disrupting potency of PAHs, but the simple status of alkylation could not easily explain the toxicity changes. Our results provide novel insight for understanding the endocrine disruption potential of petroleum derived PAHs, and demonstrate the importance of bioassay-based assessments of oil spills.

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1. Introduction

Oil spills are a global environmental problem.[1,2] Accidents such as the *Exxon Valdez*, *Prestige* and *Deepwater Horizon* resulted in the discharge of hundreds of thousands of tonnes of crude oil, and have caused serious long-term damage to marine ecosystems.[3–5]

Crude oil is a mixture of a number of different hydrocarbons. Polycyclic aromatic hydrocarbons (PAHs) and alkylated PAHs are common constituents of crude oil, and are regarded as one of the major contributors to the toxic potencies of crude oils.[6–8] In crude oils such as Iranian Heavy, Kuwait Export, and UAE Upper Zakum, the concentrations of alkylated PAHs are greater (about 30 times) than their unalkylated analogues.[9] Meanwhile, when crude oil is released into marine or coastal environments, it undergoes various chemical, biological, and physical weathering processes. These processes include evaporation,

dissolution, emulsification, microbial degradation, photo-oxidation, adsorption to suspended matter, and deposition on sediments.[9,10] During weathering processes, the absolute and relative concentrations of PAHs in crude oil change, and the relative abundances of alkylated PAHs often increase.[6,9,11,12]

In order to understand the potential effects of residual crude oil, it is important to know the effects of various PAHs, especially alkylated PAHs. Recently, several studies focusing on the toxicity of alkylated PAHs have been reported.[6,10,13,14] However, the toxicological characteristics of alkylated PAHs have not yet been clearly understood compared to those of unsubstituted PAHs, especially in terms of endocrine disruption. Some PAHs are known as aryl hydrocarbon receptor (AhR) agonists. The AhR mediated toxicities of various PAHs have been relatively well documented.[6,15–17] While crosstalk between the AhR and estrogen receptors (ERs) is suspected,[18,19] the potential of PAHs to disrupt endocrine functions has been less characterized. Previous studies have shown that several PAHs have estrogenic or anti-estrogenic potencies and effects on steroidogenic pathways.[20–23] Similar effects on the endocrine system were also observed after exposure to oil contaminated sediment or crude oil containing relatively higher concentrations of PAHs.[24–29]

The results of previous studies have shown that potencies for disruption of endocrine functions varied, depending on the type of PAH and type of substitution such as methylation or hydroxylation,[17,21,30] which suggests that alkylation of PAHs alters potencies for disrupting the endocrine system. Therefore, when assessing the effects of crude oil at contaminated sites, it is important to know the characteristics of various alkylated PAHs for disruption of the endocrine system.

In this study, the effects of individual PAHs and their alkylated analogues on sex hormones were assessed by use of two *in vitro* assays based on MVLN-*luc* cells (human breast carcinoma cells transfected with a luciferase reporter gene) and H295R cells (human adrenocortical carcinoma cells).[17,31–33] The assay based on MVLN-*luc* cells detects the estrogen receptor (ER) binding affinity of chemicals, which indicates the estrogenic activity of the chemicals.[17] H295R cells express all of the enzymes involved in steroidogenesis and produce sex steroid hormones such as 17β-estradiol (E2) and testosterone (T).[34–37] These two assays can be used to assess the effects of chemicals on the production of sex steroid hormones[38] and complementarily evaluate the different modes of endocrine disruption of various chemicals.

The objective of the study, results of which are presented here, was to compare the endocrine disrupting potential and characteristics of major unsubstituted PAHs ($n = 5$) and their alkylated analogues ($n = 25$), which are commonly found in crude oil. The results of this study will help characterize and understand the endocrine disrupting effects of the oil spill site.

2. Materials and methods

2.1. Test chemicals

Five parent PAHs that are frequently reported in crude oil and in sediments contaminated with crude oil, including naphthalene, fluorene, phenanthrene, dibenzothiophene, and chrysene,[9,11,39] and their alkylated analogues, including seven alkyl-naphthalenes, four alkyl-fluorenes, three alkyl-dibenzothiophenes, seven alkyl-phenanthrenes, and, four alkyl-chrysenes, were chosen as model chemicals to be studied. The commercial availability of standards was also considered in choosing the PAHs for testing. The test chemicals were purchased from Aldrich (St. Louis, MO, USA), Chiron (Trondheim, Norway), Fluka (Buchs, Switzerland), and Supelco (Bellefonte, PA, USA) (Table 1 and Fig. 1). For use in bioassays, unsubstituted PAHs and their alkylated analogues were dissolved in dimethyl sulfoxide (DMSO) and the concentration of the solvent in culture media was 0.1% v/v. Maximum concentrations of PAHs in media used for *in vitro* bioassays were as great as 1000 μg L^{-1} and differed slightly because of the commercially available maximum concentrations of test chemicals. Chemical properties of the tested PAHs are summarized in Table 1.

2.2. MVLN-*luc* cell culture and assay

MVLN-*luc* cells were cultured in a hormone-free DMEM/F12 nutrient mixture, 1 mM sodium pyruvate, 1 mg L^{-1} of insulin (Sigma Aldrich, St. Louis, MO, USA), and 10% dextran–charcoal stripped fetal bovine serum (Hyclone, Logan, UT, USA). The methodological details of this reporter gene-based assay have been previously published.[33,40,41] Cells for bioassay were seeded into the 60 interior wells of 96-well plates (250 μL per well) at a density of approximately 1.25×10^5 cells per mL. After 24 h, cells were dosed with 0.25 μL of the standard (E2), test chemicals or solvent control (DMSO). After 72 h of incubation, ER-induced luciferase activity was quantified by use of the Steady-Glo-Luciferase Assay System (Promega Corp., Madison, WI, USA) with a microplate reader (Tecan, Infinite 200®, Mannedorf, Switzerland). Viabilities of cells exposed to individual PAHs were checked by use of the WST-1 cell proliferation assay (Roche, Indianapolis, IN, USA). The maximum exposure concentration of each tested PAH that was determined based on cell viability (>80%) is shown in Table 1.

Responses of the MVLN-*luc* bioassay (expressed as average relative luminescence units) were converted to percentages of the maximum response (%-E2max) caused by 41.1 pM of E2 (100%-E2max). The %-E2max was calculated from the maximum response among multiple doses of each PAH. Relative potencies (RePs) were calculated directly from dose–response relationships for each tested PAH and a standard curve was generated from the varying doses of E2, *i.e.*, 1.5, 4.6, 13.7, and 41.1 pM (Fig. S1†). ReP_{20}, ReP_{50}, and ReP_{80} were determined at doses of a given chemical for which responses are equivalent to 20, 50, and 80% of responses by 41.1 pM E2 (100%-E2max), respectively. In cases where the observed maximum response for the sample was less than 80%-E2-max, extrapolation beyond the range of the empirical results was made to estimate RePs based on the slope ($R^2 \geq 0.95$) of each.[15] All MVLN-*luc* assays were conducted in triplicate.

2.3. H295R cell culture and assay

H295R human adrenocortical carcinoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured at 37 °C in a 5% CO_2 atmosphere in 75 cm^2 culture flasks containing 12 mL DMEM/F12 culture

Table 1 List of unsubstituted and alkylated PAHs tested in the present study

Chemical	CAS RN.	Molecular weight (g mol^{-1})	Maximum concentration (μg L^{-1})		Manufacturer
			H295R[a]	MVLN-*luc*[b]	
Naphthalene	91-20-3	128.17	1000	1000	Fluka
1-Methylnaphthalene	90-12-0	142.20	1000	1000	Fluka
2-Methylnaphthalene	91-57-6	142.20	1000	1000	Fluka
1,3-Dimethylnaphthalene	575-41-7	156.23	1000	1000	Chiron
2,3-Dimethylnaphthalene	581-40-8	156.23	1000	1000	Aldrich
1,4,5-Trimethylnaphthalene	2131-41-1	170.25	500	500	Chiron
2,3,5-Trimethylnaphthalene	2245-38-7	170.25	500	500	Chiron
1,2,5,6-Tetramethylnaphthalene	2131-43-3	184.28	500	500	Chiron
Fluorene	86-73-7	166.22	1000	1000	Aldrich
1-Methylfluorene	1730-37-6	180.25	1000	1000	Chiron
9-Methylfluorene	2523-37-7	180.25	1000	1000	Chiron
1,7-Dimethylfluorene	442-66-0	194.27	500	500	Chiron
9-*n*-Propylfluorene	4037-45-0	208.30	1000	1000	Chiron
Dibenzothiophene	132-65-0	184.26	1000	1000	Aldrich
2-Methyldibenzothiophene	20928-02-3	198.28	500	500	Chiron
2,4-Dimethyldibenzothiophene	31317-18-7	212.31	500	500	Chiron
2,4,7-Trimethyldibenzothiophene	216983-03-8	226.34	500	500	Chiron
Phenanthrene	85-01-8	178.23	1000	1000	Aldrich
2-Methylphenanthrene	2531-84-2	192.26	1000	1000	Chiron
3-Methylphenanthrene	832-71-3	192.26	1000	1000	Chiron
1,2-Dimethylphenanthrene	20291-72-9	206.28	500	500	Chiron
1,6-Dimethylphenanthrene	20291-74-1	206.28	500	500	Chiron
1,2,6-Trimethylphenanthrene	30436-55-6	220.31	500	500	Chiron
1,2,9-Trimethylphenanthrene	146448-88-6	220.31	500	500	Chiron
1,2,6,9-Tetramethylphenanthrene	204256-39-3	234.34	500	500	Chiron
Chrysene	218-01-9	228.28	1000	1000	Supelco
1-Methylchrysene	3351-28-8	242.31	200	200	Chiron
3-Methylchrysene	3351-31-3	242.31	40	200	Chiron
6-Ethylchrysene	2732-58-3	256.34	200	1000	Chiron
1,3,6-Trimethylchrysene	NA	270.37	200	1000	Chiron

NA: not available. [a] Dilution factor for H295R was 5. [b] Dilution factor for MVLN-*luc* was 3.

medium supplemented with 1.2 g L^{-1} $NaHCO_3$, 1% ITS-premix, and 2.5% Nu-serum as described previously.[31,32,42] H295R cells were seeded into 24-well plates at a density of 3×10^5 cells per mL in 1 mL of medium per well. After 24 h, cells were exposed to unsubstituted and alkylated PAHs for 48 h. After exposure, sex steroid hormones (E2 and T) were extracted from H295R cell culture media by use of diethyl ether and then quantified by competitive enzyme-linked immunosorbent assay (ELISA). Commercially available kits (Cayman chemical, Ann Arbor, MI, USA) for E2 [Cat # 582251] and T [Cat # 582701] were employed following the manufacturer's instructions. Absorbance of extracts was measured by use of a microplate reader (Tecan Group Ltd., Mannedorf, Switzerland) at a wavelength of 415 nm. Each measurement was made in triplicate. To determine non-cytotoxic doses (>80% of survival) and exposure concentrations, viabilities of cells exposed to individual PAHs were determined by use of the WST-1 cell proliferation assay (Roche, Indianapolis, IN, USA). Exposure concentrations for each tested PAH for H295R cell line assay are presented in Table S1.† Forskolin (0.1, 1, and 10 μM) was employed in the H295R assay as a positive control (Fig. S2(a) and (b)†).

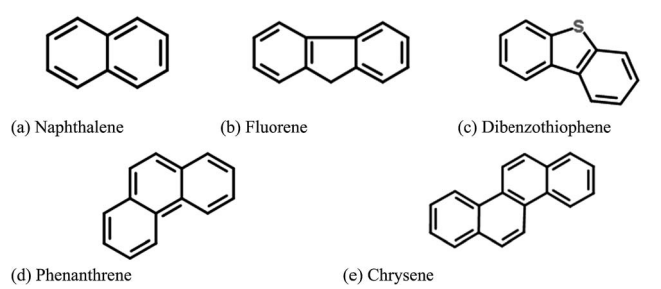

Fig. 1 Chemical structures of tested unsubstituted PAHs.

To compare the endocrine disrupting potential among PAHs, a qualitative estrogenic index (qEI) and androgenic index (qAI) were calculated (eqn (1)).[32]

$$\text{qEI (or qAI)} = \frac{\text{NOEC of cell survival}}{\text{NOEC of changes in E2 (or T) concentration}} \quad (1)$$

2.4. Statistical analyses

Significance of %-E2max was defined as a change greater than three times (3×) the standard deviation (expressed in % standard max.) of the mean solvent control response (0% standard

max.) according to a previous study.[15] Normality and homogeneity of each variance were tested by use of the Shapiro–Wilk test and Levene's test, respectively. To determine significant differences of hormone production between the control and PAH-exposed group in H295R bioassay, one-way analysis of variance (ANOVA) followed by Dunnett's test or the T3 test was carried out using SPSS 20 for Windows® (SPSS, Chicago, IL, USA). For data that did not meet the assumptions for use of parametric tests, Kruskal–Wallis tests were conducted. Differences at which p values less than 0.05 were considered to be statistically significant.

3. Results & discussion

3.1. ER-mediated potencies of unsubstituted and alkylated PAHs

Potencies as ER agonists varied among PAHs. %-E2max values of PAHs ranged from negligible (less than the value of solvent control, <0%) to 101.4% (Table 2). Seven of 30 PAHs exhibited significant potency for ER-mediated responses that were greater than that of the solvent control (>0%). Dose-dependencies of ER-mediated responses observed for PAHs with >10% ER-mediated potency are shown in Fig. S3.† ER-mediated potencies varied by PAHs. Among tested PAHs, phenanthrene and its alkylated analogues generally showed greater %-E2max (range from 1.6% to 47.3%) than other PAHs. Six of eight phenanthrenes showed significant E2-mediated potency (Table 2). Alternatively, most of the PAHs based on naphthalene, fluorine or chrysene, such as unsubstituted naphthalene (3.8%), 1-methylnaphthalene (0.4%), and 1-methylfluorene (2.6%), exhibited negligible %-E2max except 1-methylchrysene. In addition, all the dibenzothiophenes exhibited negligible ER-mediated potency.

Alkylation influenced ER-mediated potencies of some PAHs. The greatest potency as an ER agonist was observed for 1-methylchrysene (101.4%), although those of unsubstituted chrysene and other alkylated chrysenes were negligible. Number of alkyl-moieties alone did not explain ER-mediated potencies. Importance of the structural modification of PAHs on their E2-like potencies has been reported elsewhere. Hydroxylated analogues of certain PAHs, such as 2-OH-chrysene, 2-OH-5-methylchrysene, 8-OH-5-methylchrysene, 9,10-OH-benzo[a]pyrene, 9-OH-benzo[a]pyrene, and 3-OH-benzo[a]pyrene, elicited different ER-mediated potencies[21,30] and were more potent than analogous unsubstituted PAHs. The results of the present study demonstrate that the status of alkylation, such as the number or the position of alkyl-moieties, is an important determinant of ER-mediated potencies of PAHs. Further computational modelling, such as ligand–receptor docking, might provide more accurate mechanistic explanations of ER-mediated responses caused by various alkylations.

Table 2 ER-mediated potencies (%-E2max and RePs) of unsubstituted and alkylated PAHs measured using MVLN-*luc* bioassay

Chemicals	%-E2max	Sig.	ReP_{20}	ReP_{50}	ReP_{80}
Naphthalene	3.8	N	7.92×10^{-9}	1.65×10^{-10}	3.44×10^{-12}
1-Methylnaphthalene	0.4	N	3.90×10^{-8}	9.58×10^{-9}	2.36×10^{-9}
2-Methylnaphthalene	<0[a]	N		NA	
1,3-Dimethylnaphthalene	<0[a]	N		NA	
2,3-Dimethylnaphthalene	<0[a]	N		NA	
1,4,5-Trimethylnaphthalene	<0[a]	N		NA	
2,3,5-Trimethylnaphthalene	<0[a]	N		NA	
1,2,5,6-Tetramethylnaphthalene	<0[a]	N		NA	
Fluorene	<0[a]	N		NA	
1-Methylfluorene	2.6	N	7.64×10^{-16}	3.73×10^{-22}	1.82×10^{-28}
9-Methylfluorene	<0[a]	N		NA	
1,7-Dimethylfluorene	<0[a]	N		NA	
9-*n*-Propylfluorene	<0[a]	N		NA	
Dibenzothiophene	<0[a]	N		NA	
2-Methyldibenzothiophene	<0[a]	N		NA	
2,4-Dimethyldibenzothiophene	<0[a]	N		NA	
2,4,7-Trimethyldibenzothiophene	<0[a]	N		NA	
Phenanthrene	47.3	Y	3.34×10^{-7}	4.65×10^{-7}	6.49×10^{-7}
2-Methylphenanthrene	11.2	Y	6.56×10^{-8}	9.68×10^{-9}	1.43×10^{-9}
3-Methylphenanthrene	1.6	N	1.11×10^{-8}	3.75×10^{-10}	1.26×10^{-11}
1,2-Dimethylphenanthrene	18.8	Y	3.84×10^{-7}	1.45×10^{-7}	5.44×10^{-8}
1,6-Dimethylphenanthrene	7.9	Y	7.72×10^{-8}	5.07×10^{-9}	3.33×10^{-10}
1,2,6-Trimethylphenanthrene	15.2	Y	2.78×10^{-7}	9.77×10^{-8}	3.43×10^{-8}
1,2,9-Trimethylphenanthrene	13.0	Y	2.60×10^{-7}	7.53×10^{-8}	2.18×10^{-8}
1,2,6,9-Tetramethylphenanthrene	<0[a]	N		NA	
Chrysene	<0[a]	N		NA	
1-Methylchrysene	101.4	Y	3.97×10^{-5}	4.05×10^{-5}	4.13×10^{-5}
3-Methylchrysene	<0[a]	N		NA	
6-Ethylchrysene	<0[a]	N		NA	
1,3,6-Trimethylchrysene	<0[a]	N		NA	

[a] Values are less than that of solvent control. Sig.: statistical significance. NA: not available.

RePs estimated for PAHs (Table 2) can be applied to calculate estrogen equivalents (EEQ) quantitatively. Generally, chemicals exhibiting greater %-E2max have greater RePs. In this study, 1-methylchrysene, for which %-E2max was higher than others, also showed the greatest ReP among the PAHs studied. The ReP_{20-80} of 1-methylchrysene ranged between 3.97×10^{-5} and 4.13×10^{-5}. This indicates that the estrogenic potency of this compound is approximately 10^5-fold less than that of E2. The ReP_{50}s of phenanthrenes, except for 1,2,6,9-tetramethylphenanthrene, were in the range of 4.65×10^{-7} to 3.75×10^{-10}. However, since the response of the assay was not sufficient (<10% E2max), reliability of RePs derived through extrapolation might be poor.[10,15]

Previous studies that reported ER agonistic potencies of PAHs are limited to unsubstituted PAHs.[17,21,22,30,43] Reported potencies were often inconsistent among the tested PAHs. Some reports have indicated that chrysene, and benzo[*a*]pyrene exhibited negligible ER-mediated potencies,[17,21,43] while other studies documented that chrysene, and benzo[*a*]pyrene exhibited ER-mediated potencies.[22,30,44] The results from previous studies were consistent with the response of naphthalene, fluorine and chrysene in this present study.[17,22,43,44] Unlike previous reports, phenanthrene exhibited ER-mediated potency among the unsubstituted PAHs tested in this study.[17,43,44] Discrepant responses among studies might be partly explained by different assay systems which were used to detect E2 binding[43] and different assay conditions, *e.g.*, exposure duration (6 or 24 h).[44] Nevertheless, MVLN-*luc* assay is still regarded as a valuable tool for detecting ER-mediated responses of target compounds in a rapid, efficient and convenient way, and therefore has been used in studies evaluating endocrine disruption of many chemicals and environmental samples.[17,32,40,41]

In this study, we focused only on the agonistic effects induced by target PAHs, which may provide novel information regarding the endocrine disruption modes of action of alkylated PAHs. However, in order to comprehend the overall toxic potential of these alkylated PAHs, other modes of endocrine disruption, *e.g.*, the anti-estrogenic or anti-androgenic effect, should also be considered for PAHs. In fact, the anti-estrogenic effects of some unsubstituted PAHs (*e.g.*, chrysene, benzo[*a*]pyrene and benzo[*e*]pyrene) were reported, which could be explained by metabolism of E2 mediated by up-regulation of cytochrome P450 1A1 enzyme activity.[43] On the other hand, in a human androgen receptor gene assay, the parent PAHs containing four or five aromatic rings, such as benz[*a*]anthracene, benzo[*a*]pyrene and chrysene, exhibited anti-androgenic activities.[45] These antagonistic effects of PAHs could compound our estimation of the overall estrogenic or androgenic effects of crude oil contamination, warranting further studies on anti-estrogenicity and anti-androgenicity of crude oil contamination.

3.2. Effects of PAHs on the production of steroid sex hormones

Significantly greater production of steroid, sex hormones were observed in H295R cells exposed to 20 of the 30 PAHs examined (Fig. 2 and 3): 17 PAHs resulted in greater production of E2, while 12 PAHs resulted in greater synthesis of T. Nine PAHs (1-methylnaphthalene, 1,3-dimethylnaphthalene, 9-methylfluorene, 1,7-dimethylfluorene, dibenzothiophene, 2,4-dimethyldibenzothiophene, 1,2,9-trimethylphenanthrene, 1,2,6,9-tetramethylphenanthrene, and chrysene) resulted in

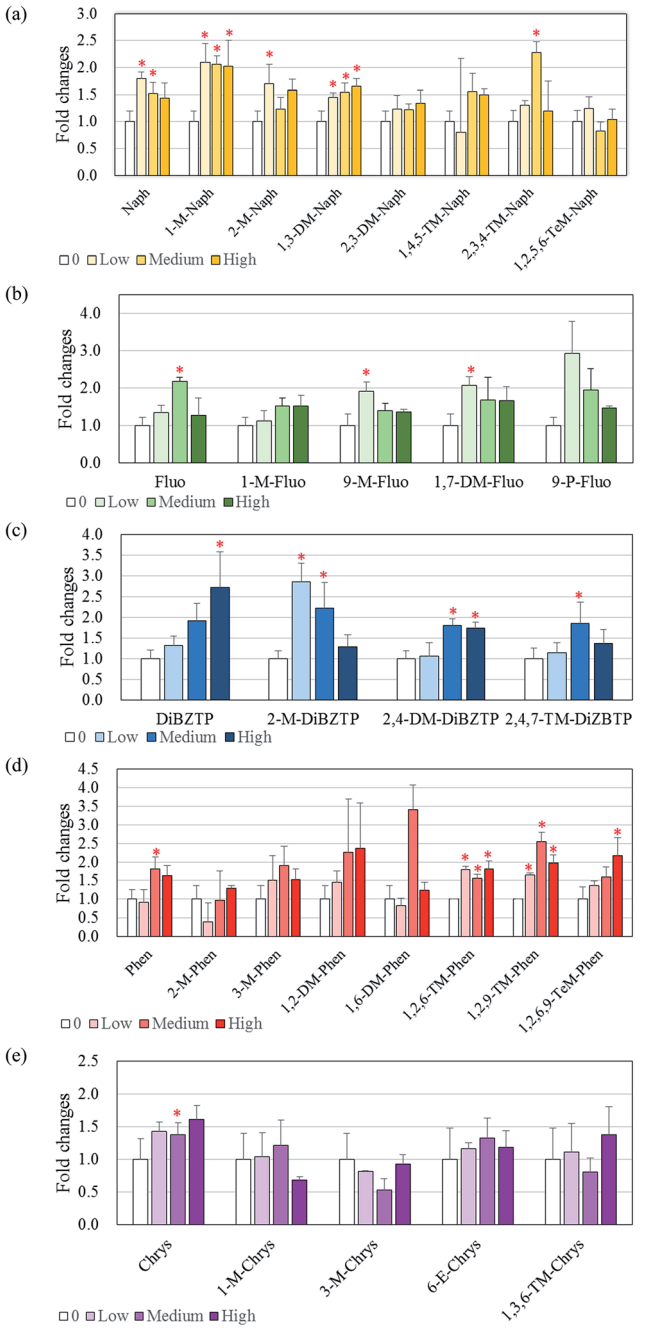

Fig. 2 Effects of unsubstituted and alkylated PAHs on production of E2 by H295R cells. (a) Naphthalene and alkylated naphthalene, (b) fluorene and alkylated fluorene, (c) dibenzothiophene and alkylated dibenzothiophene, (d) phenanthrene and alkylated phenanthrene, and (e) chrysene and alkylated chrysene. Data are expressed as fold changes compared to appropriate solvent control (means ± standard deviations ($n = 3$)). Asterisk (*) indicates a significant difference from the solvent control ($p < 0.05$).

significantly greater production of E2 and T in H295R adrenocortical carcinoma cells.

Alkylation affected potencies of PAHs affect the production of steroid sex hormones, but in different directions depending on the type of PAH. Among naphthalenes, less alkylated (1-methylnaphthalene, 2-methylnaphthalene, 1,3-dimethylnaphthalene) and unsubstituted naphthalene generally caused greater production of E2. However, among phenanthrenes, greater production of E2 was generally observed from unsubstituted phenanthrene and its higher alkylated analogues, *e.g.*, tri- or tetra-methylated analogues. All dibenzothiophenes and three out of five fluorenes showed a significant increase of E2 production at least at one or more exposure concentrations by as much as 3-fold. For chrysene, only unsubstituted chrysene resulted in greater production of E2 at the medium level of exposure concentration by as much as 1.6-fold. None of the tested alkylated analogues of chrysene significantly affected the production of E2.

Several PAHs resulted in greater production of T by H295R cells (Fig. 3), including 1-methylnaphthalene, 1,3-dimethylnaphthalene, and 1,2,5,6-tetramethylnaphthalene among naphthalenes and unsubstituted dibenzothiophene and 2,4-dimethyldibenzothiophene among dibenzothiophenes. Greater production of T was caused by exposure to alkylated fluorenes and phenanthrenes, whereas among chrysenes, only unsubstituted chrysene caused an increase in production of T by 4.1-fold.

The results of the study reported here indicate that alkylation of PAHs is responsible for changes in steroidogenesis, compared to unsubstituted analogues, although a monotonic direction of change was not observed. The fact that alkylation of PAHs can change their endocrine disrupting effects can explain changes of the estrogenic effects of the constituents of oil spills over time. Greater production of E2 was reported for weathered than unweathered, Iranian heavy, crude oil, although the possibility of concentration due to evaporation cannot be excluded.[25]

PAHs have previously been reported to alter steroidogenesis and affect endocrine function. In gold fish testis, exposure to naphthalene, β-naphthoflavone, and retene resulted in greater production of T.[46] β-Naphthoflavone also stimulated productions of cAMP and T in rainbow trout.[46] Alternatively, phenanthrene, benzo[*a*]pyrene, and chrysene resulted in lower production of androstenedione and E2 by the ovary of flounders, *Platichthys flesus*.[23] Significantly lower concentrations of T in both serum and intra-testicular fluid were observed in rats after exposure to benzo[*a*]pyrene,[20] which was associated with down-regulation of steroidogenic acute regulatory protein (StAR) and 3β-hydroxysteroid dehydrogenase (3β-HSD).

Crude oil, oil contaminated samples, or industrial wastes containing large concentrations of PAHs also affected steroidogenesis and resulted in an imbalance of sex hormones or related expressions of steroidogenic genes.[28,32,47] Most of these studies reported greater production of E2, but lesser production of T. Principal component 1 (PC1) for concentrations of PAHs exhibited a marginal, positive relationship with concentrations of E2.[32] Those results might also be linked to our results of significantly greater production of E2 caused by various PAHs.

Because of different cytotoxicity of test PAHs, test doses varied and therefore direct and quantitative comparison of the alteration of steroidogenesis among PAHs is not possible. The qEI and qAI that were used to compare cytotoxicity adjusted

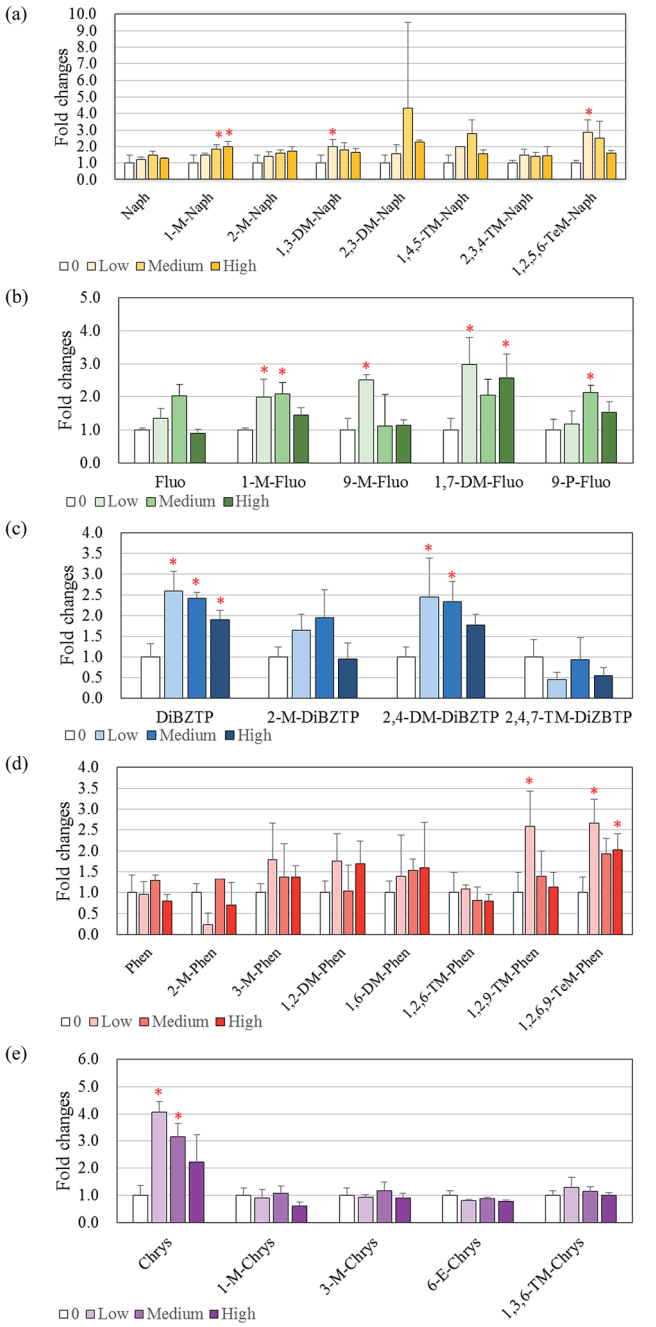

Fig. 3 Effects of unsubstituted and alkylated PAHs on T levels in H295R cells. (a) Naphthalene and alkylated naphthalene, (b) fluorene and alkylated fluorene, (c) dibenzothiophene and alkylated dibenzothiophene, (d) phenanthrene and alkylated phenanthrene, and (e) chrysene and alkylated chrysene. Data are expressed as fold changes compared to appropriate solvent control (means ± standard deviations ($n = 3$)). Asterisk (*) indicates a significant difference from the solvent control ($p < 0.05$).

endocrine disruption potencies of the tested PAHs showed that alkylation affects potencies of PAHs to influence productions of E2 and T (Fig. 4 and Table S2†). However, similar to ER-mediated affinity, potency and the direction of the steroidogenic alteration could not be predicted solely from the number or positions of alkyl-moieties on PAHs.

3.3. Implications

The disruption of endocrine functions by several PAHs and their alkylated analogues was identified in the present study using MVLN-*luc* and H295R cells. Steroidogenic alteration by PAHs, *i.e.*, effects on the production of E2 and T seemed to be more potent than ER-mediated effects (Fig. 4 and Table S2†). Significant changes in the production of sex hormones were observed for 20 of 30 tested PAHs. Endocrine disruption mediated by the ER binding was observed for only 1-methyl chrysene and phenanthrenes. Endocrine disruption through steroidogenic pathways should be considered in evaluating endocrine disrupting potential of oil spill or PAH contaminated sites.

The results of the present study also showed that alkylation could influence the effects of PAHs on endocrine functions (Fig. 4). Since PAHs in the environment can undergo weathering, various alkylated PAHs can be produced. Hence, assessments of hazard and risk, based solely on parent PAHs measured in the environment might result in incorrect estimation of endocrine disruption. Further investigations on determinants of toxicities for alkylated PAHs are warranted, because the number and locations of alkyl-moieties alone cannot explain the potencies of PAHs for effects on endocrine functions. The results of our study provide novel information about sex endocrine disruption potencies of major alkylated PAHs that are present in oil spills, and could be used for developing *in silico* predicting models for PAHs related to oil spills.

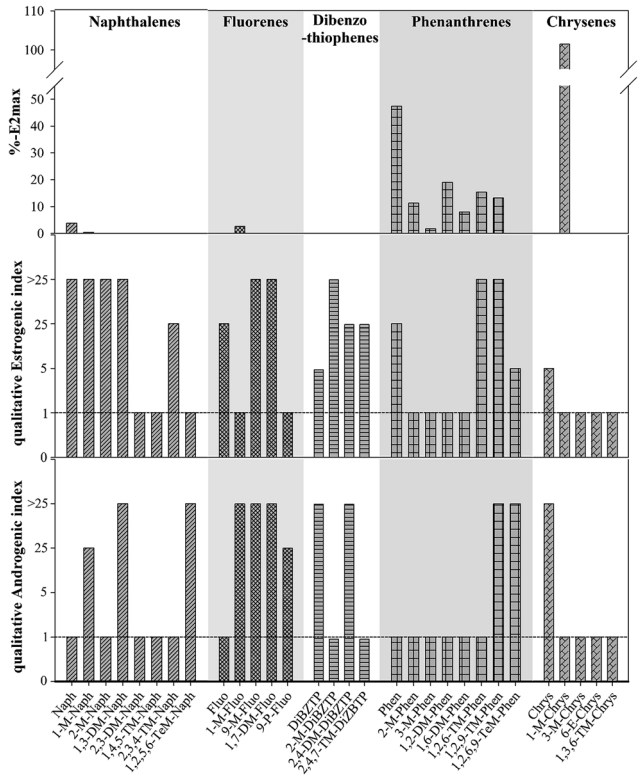

Fig. 4 % E2-max, qEI (qEI = NOEC of cell survival/NOEC of changes in E2 concentration), and qAI (qEI = NOEC of cell survival/NOEC of changes in T concentration) of tested PAHs. qEIs or qAIs are used to compare relative estrogenic or androgenic potential among PAHs.

Conflicts of interest

There are no conflicts of interest to declare.

List of abbreviations

Naph	Naphthalene
1-M-Naph	1-Methylnaphthalene
2-M-Naph	2-Methylnaphthalene
1,3-DM-Naph	1,3-Dimethylnaphthalene
2,3-DM-Naph	2,3-Dimethylnaphthalene
1,4,5-TM-Naph	1,4,5-Trimethylnaphthalene
2,3,4-TM-Naph	2,3,4-Trimethylnaphthalene
1,2,5,6-TeM-Naph	1,2,5,6-Tetramethylnaphthalene
Fluo	Fluorene
1-M-Fluo	1-Methylfluorene
9-M-Fluo	9-Methylfluorene
1,7-DM-Fluo	1,7-Dimethylfluorene
9-P-Fluo	1-Propylfluorene
DiBZTP	Dibenzothiophene
2-M-DiBZTP	2-Methyldibenzothiophene
2,4-DM-DiBZTP	2,4-Dimethyldibenzothiophene
2,4,7-TM-DiBZTP	2,4,7-Trimethyldibenzothiophene
Phen	Phenanthrene
2-M-Phen	2-Methylphenanthrene
3-M-Phen	3-Methylphenanthrene
1,2-DM-Phen	1,2-Dimethylphenanthrene
1,6-DM-Phen	1,6-Dimethylphenanthrene
1,2,6-TM-Phen	1,2,6-Trimethylphenanthrene
1,2,9-TM-Phen	1,2,9-Trimethylphenanthrene
1,2,6,9-TeM-Phen	1,2,6,9-Tetramethylphenanthrene
Chrys	Chrysene
1-M-Chrys	1-Methylchrysene
3-M-Chrys	3-Methylchrysene
6-E-Chrys	6-Ethylchrysene
1,3,6-TM-Chrys	1,3,6-Trimethylchrysene

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Endocrine disrupting potentials of PAHs and their alkylated analogues associated with oil spills

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Supplementary Information

Tables

Table S1. Exposure concentrations of tested PAHs for H295R assay

Table S2. Qualitative, estrogenic index (qEI) and androgenic index (qAI) in 30 unsubstituted and alkylated PAHs.

Figures

Figure S1. Dose-response curve of estradiol (E2), a positive control used for MVLN-*luc* assay.

Figure S2. Dose-response curve of forskolin, a positive control. (a) estradiol (E2) content and (b) testosterone content in H295R cell assay.

Figure S3. Dose dependent ER-mediated potencies observed from several PAHs with >10% ER-mediated potency.

Table S1. Exposure concentrations of tested PAHs for H295R assay

Chemicals	Concentration represented as Low (μg/L)	Concentration represented as Medium (μg/L)	Concentration represented as High (μg/L)
Naphthalene	40	200	1000
1-Methylnaphthalene	40	200	1000
2-Methylnaphthalene	40	200	1000
1,3-Dimethylnaphthalene	40	200	1000
2,3-Dimethylnaphthalene	40	200	1000
1,4,5-Trimethylnaphthalene	20	100	500
2,3,5-Trimethylnaphthalene	20	100	500
1,2,5,6-Tetramethylnaphthalene	20	100	500
Fluorene	40	200	1000
1-Methylfluorene	40	200	1000
9-Methylfluorene	40	200	1000
1,7-Dimethylfluorene	20	100	500
9-n-Propylfluorene	40	200	1000
Dibenzothiophene	40	200	1000
2-Methyldibenzothiophene	20	100	500
2,4-Dimethyldibenzothiophene	20	100	500
2,4,7-Trimethyldibenzothiophene	20	100	500
Phenanthrene	40	200	1000
2-Methylphenanthrene	40	200	1000
3-Methylphenanthrene	40	200	1000
1,2-Dimethylphenanthrene	20	100	500
1,6-Dimethylphenanthrene	20	100	500
1,2,6-Trimethylphenanthrene	20	100	500
1,2,9-Trimethylphenanthrene	20	100	500
1,2,6,9-Tetramethylphenanthrene	20	100	500
Chrysene	40	200	1000
1-Methylchrysene	8	40	200
3-Methylchrysene	1.6	8	40
6-Ethylchrysene	8	40	200
1,3,6-Trimethylchrysene	8	40	200

Table S2. Qualitative, estrogenic index (qEI) and androgenic index (qAI) in 30 unsubstituted and alkylated PAHs

Chemicals	Cytotoxic NOEC (µg/L)	Estrogen NOEC (µg/L)	Testosterone NOEC (µg/L)	qEI	qAI
Naphthalene	1000	<40*	1000	>25	1
1-Methylnaphthalene	1000	<40*	40	>25	25
2-Methylnaphthalene	1000	<40*	1000	>25	1
1,3-Dimethylnaphthalene	200	<8*	<8*	>25	>25
2,3-Dimethylnaphthalene	200	200	200	1	1
1,4,5-Trimethylnaphthalene	500	500	500	1	1
2,3,5-Trimethylnaphthalene	500	20	500	25	1
1,2,5,6-Tetramethylnaphthalene	500	500	<20*	1	>25
Fluorene	1000	40	1000	25	1
1-Methylfluorene	1000	1000	<40*	1	>25
9-Methylfluorene	1000	<40*	<40*	>25	>25
1,7-Dimethylfluorene	500	<20*	<20*	>25	>25
9-n-Propylfluorene	1000	1000	40	1	25
Dibenzothiophene	1000	200	<40*	5	>25
2-Methyldibenzothiophene	500	<20*	500	>25	1
2,4-Dimethyldibenzothiophene	500	20	<20*	25	>25
2,4,7-Trimethyldibenzothiophene	500	20	500	25	1
Phenanthrene	1000	40	1000	25	1
2-Methylphenanthrene	1000	1000	1000	1	1
3-Methylphenanthrene	1000	1000	1000	1	1
1,2-Dimethylphenanthrene	500	500	500	1	1
1,6-Dimethylphenanthrene	500	500	500	1	1
1,2,6-Trimethylphenanthrene	500	<20*	500	>25	1
1,2,9-Trimethylphenanthrene	500	<20*	<20*	>25	>25
1,2,6,9-Tetramethylphenanthrene	500	100	<20*	5	>25
Chrysene	1000	200	<40*	5	>25
1-Methylchrysene	200	200	200	1	1
3-Methylchrysene	40	40	40	1	1
6-Ethylchrysene	200	200	200	1	1
1,3,6-Trimethylchrysene	200	200	200	1	1

*Significant E2 production was observed at the lowest concentration.

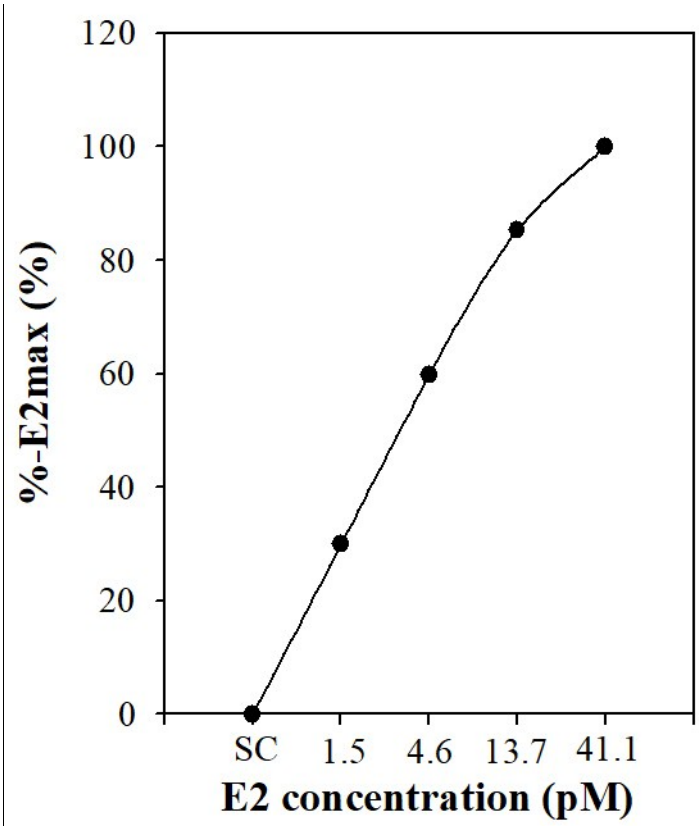
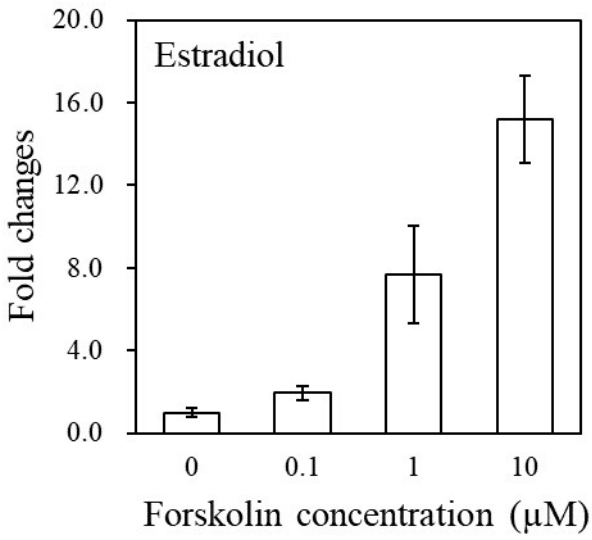


Figure S1. Dose-response curve of estradiol (E2), a positive control used for MVLN-*luc* assay.

(a)



(b)

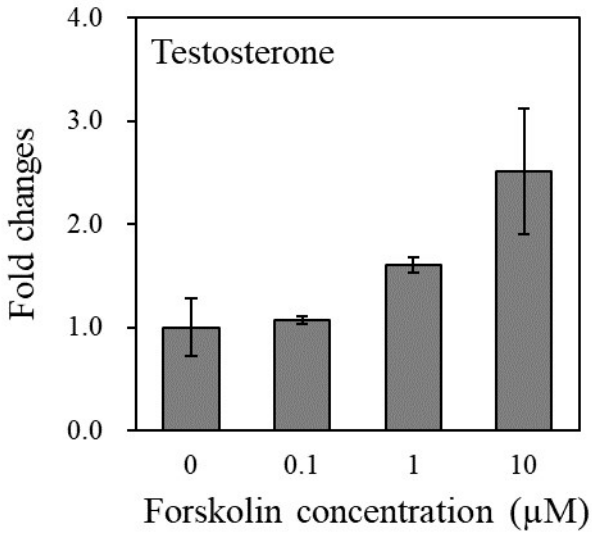


Figure S2. Dose-response curve of forskolin, a positive control. (a) estradiol (E2) content and (b) testosterone content in H295R cell assay.

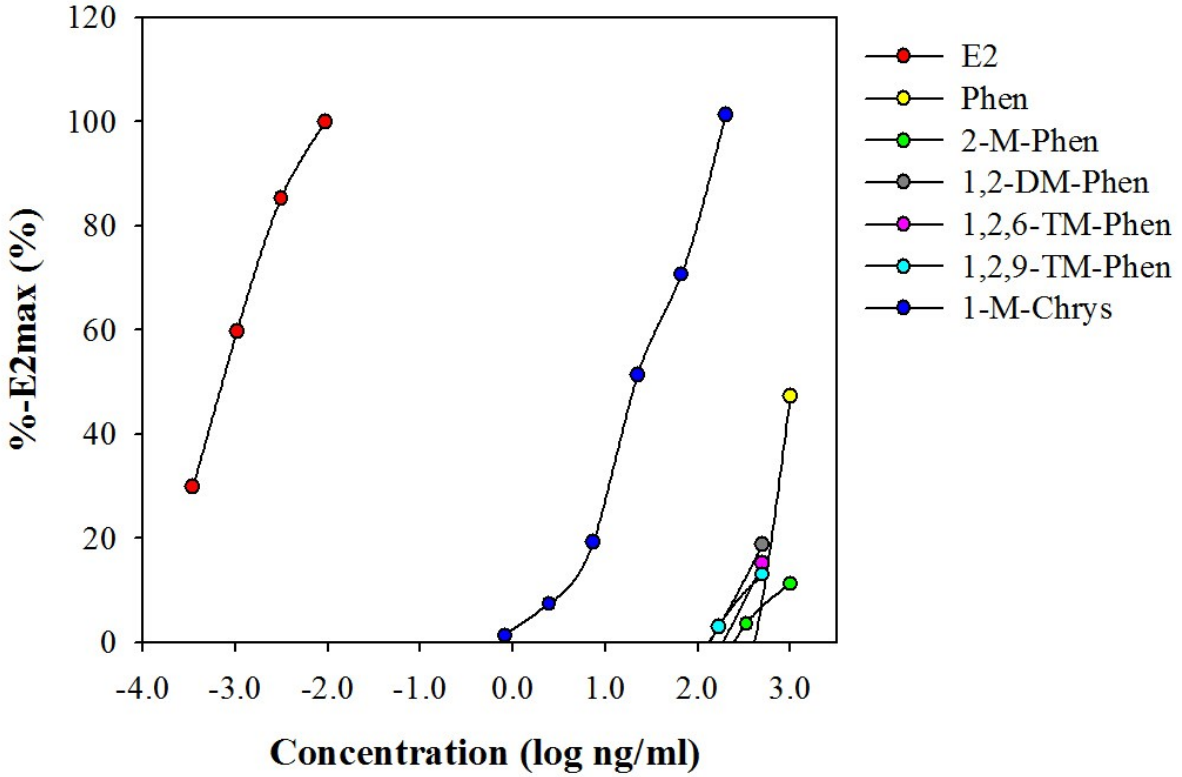


Figure S3. Dose dependent ER-mediated potencies observed from several PAHs with >10% ER-mediated potency.