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In vitro assessment of endocrine disrupting potential of organic fractions extracted from hydraulic fracturing flowback and produced water (HF-FPW)

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ABSTRACT

Potential effects of horizontal drilling combined with high-volume hydraulic fracturing (HF) have drawn significant public concern, especially on the handling, treatment, and disposal of HF flowback and produced water (HF-FPW). Previous studies indicated HF-FPW could significantly disrupt biotransformation and expressions of genes related to the endocrine system. This study focused on effects of organic extracts of HF-FPW on receptor binding activity using several transactivation assays. Six HF-FPW samples were collected from 2 wells (Well A and Well B, 3 time points at each well). These were separated by filtration into aqueous (W) and particulate (S) phases, and organics were extracted from all 12 subsamples. Of all the tested fractions, sample B1-S had the greatest Σ_{13}PAH (11,000 ng/L) and B3-S has the greatest $\Sigma_4$alkyl-PAHs (16,000 ng/L). Nuclear receptor binding activity of all the extracts on aryl hydrocarbon receptor (AhR), estrogen receptor (ER), and androgen receptor (AR) were screened using H4IIE-*luc*, MVLN-*luc*, and MDA-kb2 cells, respectively. FPWs from various HF wells exhibited distinct nuclear receptor binding effects. The strongest AhR agonist activity was detected in B3-S, with 450 ± 20 μg BaP/L equivalency at 5 × exposure. The greatest ER agonist activity was detected in A1-W, with 5.3 ± 0.9 nM E2 equivalency at 10× exposures. There is a decreasing trend in ER agonist activity from A1 to A3 in both aqueous and particulate fractions from Well A, while there is an increasing trend in ER agonist activity from B1 to B3 in aqueous fractions from Well B. This study provides novel information on the sources of endocrine disruptive potentials in various HF-FPW considering both temporal and spatial variability. Results suggest that reclamation or remediation and risk assessment of HF-FPW spills likely requires multiple strategies including understanding the properties of each spill with respect to fractured geological formation and physiochemical properties of the injected fluid.

1. Introduction

There is increasing public and scientific concern about potential hazards of leaks or spills of hydraulic fracturing flowback and produced water (HF-FPW) into aquatic environments (Alessi et al., 2017). HF-FPW is defined here as wastewater that returns to the surface after stimulation of oil and gas wells. HF-FPW typically contains salts, metals, natural organics, such as polycyclic aromatic hydrocarbons (PAHs), anthropogenic chemical additives, and associated degradation products from down-hole reactions (He et al., 2017a; Thurman et al., 2017). Accidental and purposeful releases of FPW to the environment have been extensively documented (Alessi et al., 2017; Lauer et al., 2016) and corresponding concerns regarding pollution of both surface and ground water have been voiced (Vengosh et al., 2014). Subsequent accumulations of salts, metals, and organic pollutants observed after releases of HF-FPW can exceed water quality guidelines (ESRD, 2014;

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Vengosh et al., 2014).

Several studies have estimated potential toxicity and effects of chemicals associated with fluids used during hydraulic fracturing that can occur in flowbacks, and produced water on affected environments (Chen et al., 2017; Yost et al., 2016). Recently, exposure to HF-FPW has been reported to cause a range of toxicological effects on fishes and invertebrates, including rainbow trout (Blewett et al., 2017a; He et al., 2017b), zebrafish (Folkerts et al., 2017a, 2017b; He et al., 2017a, 2018), and Daphnia (Blewett et al., 2017b). Some of the most noticeable effects include altered metabolism, cell and tissue damage related to oxidative stress, disruption of cardio-respiratory functions, malformations during developmental and disruption of endocrine functions. It has been demonstrated that exposure to HF-FPW can induce ethoxyresorufin-O-deethylase (EROD) activity associated with upregulated expression of cytochrome p450s in zebrafish and rainbow trout (Blewett et al., 2017a; He et al., 2017a, 2017b). Increased expressions of mRNA for vitellogenin and steroid hormone receptors have also been observed in rainbow trout exposed to HF-FPW (He et al., 2017b). Exposure to diluted HF-FPW was shown to significantly reduce fecundity in *Daphnia magna* (Blewett et al., 2017b). Similarly, sub-lethal exposure of zebrafish embryo to HF-FPW during the critical pre-hatch developmental period resulted in disruption of cardiac and respiratory functions in larvae and decreased swimming performance of juvenile fish, with effects persisting 3 months after being transferred to clean water (Folkerts et al., 2017a, 2017b). A recent study using the organic fractions of HF-FPW samples extracted from different wells demonstrated the organic extracts from HF-FPW significantly interfered with embryo development and the expression of endocrine-related genes in zebrafish (He et al., 2018).

Constituents of HF-FPW responsible for endocrine disrupting effects and their endocrine disrupting mechanism(s) are largely unknown. PAHs (including alkylated PAHs) which are common constituents regarded as one of the major contributors to toxic potencies of crude oil, have significant estrogenic potencies (Lee et al., 2017; Villeneuve et al., 2002). In addition, there are a variety of chemicals applied in HF fluids, which are or have been suspected of being endocrine disruptors. Potential reproductive and developmental toxicity of HF wastewater were predicted based on a systematic evaluation of endocrine disrupting chemicals (EDCs) used in HF fluids (Elliott et al., 2016). More recently, it has also been suggested that EDCs in HF fluids can disrupt endocrine-related nuclear receptor pathways (Kassotis et al., 2014, 2016a), and potentially affect reproduction and development of aquatic animals (Kassotis et al., 2016b). EDCs including polyethylene glycol (PEG) and octylphenol ethoxylate (OPE) have been detected in organic extracts of 2 HF-FPW samples, which caused significant developmental toxic effects in exposed zebrafish embryos (He et al., 2018).

There is a need for further investigation of mechanisms of toxicity to determine whether organic extracts from real-world HF-FPW samples are able to influence endocrine related nuclear receptor binding. In addition, it is also necessary to investigate the nature of endocrine disruptive properties of various samples of HF-FPW, each with various physiochemical characteristics derived from varying target geologic formations, and also considering the time of HF-FPW sampling from the wellhead with respect to the start of flowback. An *in vitro* transactivation, reporter gene assay is an ideal tool for this type of investigation. The objective of this study was to assess the endocrine disrupting potential of organic fractions extracted from 6 samples collected at over 3 time periods from 2 wells. Both the aqueous (filtrate) and particulate (solid residues) fraction organics were isolated using liquid-liquid extraction and accelerated solvent extraction. Alterations of receptor-binding activity involved in reproduction and endocrine system regulation were investigated by exposing organic extracts to aryl-hydrocarbon receptor (AhR), estrogen receptor (ER), and androgen receptor (AR) *in vitro* reporter assays. Furthermore, 16 parent PAHs and 16 alkylated PAHs were analyzed to compare and to contrast profiles of relative concentrations of organics with observed responses.

2. Materials and methods

2.1. Chemicals

Fetal bovine serum (FBS), dextran charcoal coated FBS (DCC-FBS), sodium pyruvate, Benzo[*a*]Pyrene (BaP), 17β-estradiol (E2), testosterone (T), and dichloromethane (DCM) were obtained from Sigma. Sixteen parent PAHs standards (unlabelled) and 16 internal standards (deuterium-labelled) were obtained from Wellington Lab, Canada, 16 alkyl PAHs standards were obtained from Norway Chiron.

2.2. HF-FPW samples

Samples of HF-FPW were collected from 2 stimulated wells located in the Devonian-aged, Duvernay Formation (Fox Creek, Alberta, Canada) by Encana Services Ltd. Stimulation is a technique involving high-pressure injection of fluid into a well to fracture tight rock that comprises the target formation. Time zero was defined as the initial time of pressure release following stimulation, reported here as hours post-stimulation (hps). Samples were temporarily stored in sealed 20 L plastic buckets at room temperature prior to extraction of organics (Fig. S1). Total organic carbon (TOC) and total dissolved solid (TDS) were measured for each sample using the method described in previous study (He et al., 2017a).

2.3. Extraction and fractionation of organic constituents

For each HF-FPW, 1 L was vacuum filtered (Glass Fiber Store, glass fiber, 90 mm diameter, pore size: 0.4 μm) to separate the aqueous phase and particulate phase that was a combination of settled and suspended materials. The corresponding aqueous filtrate was liquid-liquid extracted using DCM. Solid residues on filters were freeze-dried for 2 days (Labconco Crop., Kansas City, MO), then were extracted using accelerated solvent extractor with DCM. A1-W and A1-S refer to the organic fractions extracted from the first sample collected from Well A from aqueous phase (W, water) and particulate phase (S, solid residues), respectively. Parallel blank controls consisting of 1 L of purified water (Optima™ LC/MS grade) were also included in the analysis. Each extract was then reconstituted using 100 μL DCM to an equivalent 10,000× relative concentration compared to the original sample. All organic fractions, including parallel blank control, were further diluted with cell culture media for use in exposing cells. Final concentration of DCM in all exposure media was 0.1%, and cytotoxicity was not observed with this solvent concentration for all 3 cell lines (data not shown). Samples and fractions were then stored in glassware in the dark at 4 °C prior to use.

2.4. Quantification of PAHs

Sub-samples of HF-FPW (50 mL) were filtered prior to identification and quantification of PAHs. Extraction method of PAHs is described in SI. Sixteen parent PAHs and 16 alkyl PAHs were analyzed in both the aqueous and particulate phase samples. PAHs were identified and quantified by comparing retention times and peak areas of native standards, and by use of deuterium-labelled internal standards. A total of 13 parent PAHs and 4 alkyl-PAHs were detected in samples. Results are reported as the total sum of concentrations of the 13 detected parent PAHs (Σ_{13}PAH), and the sum concentration of 4 detected alkyl-substituted PAHs are reported as $\Sigma_4$alkyl-PAH. Finally, the sum of Σ_{13}PAH and $\Sigma_4$alkyl-PAH is referred to as “total PAHs”. Detailed information on PAH standards, internal standard corrected recovery of the 17 detectable PAHs based on liquid-liquid extraction (range from 82.9–179%) and accelerated solvent extraction method (range from 60.0–114%), and the GC–MS instrumental method are presented elsewhere (Zhang et al., 2016). The method detection limit (MDL) for an analyte was defined as the concentration producing a signal-to-noise ratio of 3 when

the PAH was not detected in the blanks. For PAH analytes detected in our blanks, MDL was defined as the mean blank concentration plus three times standard deviation. The blanks for liquid-liquid extraction and accelerated solvent are from same extraction methods in our previous study (Zhang et al., 2016). The MDLs are shown in Table S1. The BaP Equivalency from total PAHs ($EQ_{total\ PAHs}$) for each sample is presented in Table S1, calculated using the concept of concentration addition by the following equation:

$$EQ_{total\ PAHs} = \sum EF_n \blacksquare C_n$$

EF_n is the equivalent factor (EF) of *n*th PAH/PAH analogue compared to BaP, and C_n is the measured concentration of *n*th PAH/PAH analogue in the extract (Larsson et al., 2014; Nisbet and LaGoy, 1992).

2.5. In vitro bioassays justification

Nuclear receptor binding plays a crucial role in cell signaling pathways (Zhao et al., 2015). It has been suggested that the induction of CYP1A enzymes through the AhR binding is one of the major toxic mechanism of HF-FPW in fish (He et al., 2017a, 2017b, 2018). In addition, hormone mimics and steroid receptor binding are also common endocrine disrupting pathways of crude oil in wastewater. The current study used several widely-used reporter gene assays, including H4IIE-*luc*, MVLN-*luc*, and MDA-kb2 assays, to directly quantify the AhR, ER, and AR binding by xenobiotics in HF-FPW extracts (He et al., 2011; Leclair et al., 2015; Snyder et al., 2001). All cell lines were obtained from the Toxicology Centre, University of Saskatchewan. Assay for protein content using the Bradford reagent (BioRad, #500-0006) was performed following the manufacture's protocol to determine the organic extracts concentrations that did not affect normal cell proliferation. For each cell line, the protein content assay was conducted in a separate 96-well plate under the exact same conditions as the corresponding receptor assay. Concentrations of 5× for H4IIE-*luc* cells and 10× for MVLN-*luc* and MDA-kb2 cells causing no overt effects on cell proliferation were chosen as the highest exposure concentrations in cell assays. Parallel blank control (in 0.1% DCM), included in each cell assay, did not show any significant difference from medium control (no DCM as delivering solvent), and was used as the baseline of binding activity calculation in this study. For each cell assay, stock standard solutions of positive controls (BaP, E2 and T) were prepared in 100% DCM, and a standard curve was included on each plate by applying serially diluted BaP, E2 or T solutions in assay medium. Based on the results from our previous studies and results from preliminary tests (data not shown), agonist activity in all 3 cell lines together with antagonist effect in MDA-kb2 were tested in all 12 organic extracts.

2.5.1. H4IIE-luc assay

H4IIE-*luc* (AhR activity) assay was conducted as previously described with minor modification (Leclair et al., 2015). H4IIE-*luc* cells (ATCC®CRL-1548™) were maintained in DMEM containing 10% FBS at 37 °C with 5% CO_2. Cells were plated with the concentration of 125,000 cells/mL in 96-well luminometer plate (Perkin-Elmer, Woodbridge, ON, Canada). After 24 h incubation, the medium was replaced with exposure medium (spiked with parallel blank control, BaP standards, or FPW organic extracts) for 24 h exposure. Final concentrations of organic extracts in media used for the H4IIE-*luc* assay were 5×, 2.5×, and 1×. A BaP standard curve was included on each plate (Fig. S2). Stock standard solutions of BaP were prepared in 100% DCM, and serial dilutions were prepared in assay medium, with final concentrations ranging from 1 to 500 μg/L. At the end of exposure, luciferase activity assay was conducted by use of the Steadylite Plus Kit (PerkinElmer, MA, USA), and luminescence was read by use of a VICTOR3V 1420 Multilabel Counter (PerkinElmer, MA, USA). Dose-response curves generated from serially diluted positive BaP controls were used to determine the BaP equivalency (EQ_{BaP}) of each organic extract tested on the same plate (Nisbet and LaGoy, 1992).

2.5.2. MVLN-luc and MDA-kb2 cell assays

MVLN-*luc* (ER activity) and MDA-kb2 (AR activity) assays were conducted following previous studies with minor modifications (He et al., 2011; Snyder et al., 2001). MVLN-*luc* cells are MCF-7 cells (ATCC® HTB-22™) stably transfected with estrogen-response element with luciferase reporter gene (Demirpence et al., 1993). MVLN-*luc* cells were maintained in DMEM with Ham's F-12 supplemented with 10% FBS and 1 mM sodium pyruvate at 37 °C with 5% CO_2. MDA-kb2 cells (ATCC® CRL-2713™) were maintained in L15 medium supplemented with 10% FBS at 37 °C without CO_2. Additional assay media for MVLN-*luc* and MDA-kb2 were also prepared by supplementation with 10% DCC-FBS instead of 10% normal FBS. To minimize the baseline reporter gene activation, MVLN-*luc* and MDA-kb2 cells were plated in their respective assay media at 125,000 cells/mL in a 96-well luminometer plate with 250 μL of assay media per well. After 24 h incubation, assay media were replaced with exposure media, which were prepared fresh on the day of exposure by spiking the parallel blank control, chemicals, or extract fractions into assay media, for 72 h exposure. Final concentrations in media used for MVLN-*luc* and MDA-kb2 assays were 5× and 10×. Antagonist effect in MDA-kb2 was tested by co-exposing the organic extracts with low and high levels of T (0.3 and 1 nM, respectively). E2 or T standard curves were included on each plate to calculate the equivalency of E2 (EQ_{E2}) or T (EQ_T) for agonist activity, respectively (Figs. S3 and S4). Stock standard solutions of E2 and T were prepared in 100% DCM, and serial dilutions were prepared in assay medium, with final concentrations ranging from 0.005 to 50 nM and 0.12 to 10 nM for E2 and T, respectively. Luciferase activities were determined by the same method described above.

2.6. Data analyses

All luminescence cell assay experiments were performed in quadruple independent plates (n = 4), and on each plate the average luminescence of duplicate wells was considered a single measurement. All data are presented as mean ± standard error mean. Statistical analyses were conducted by use of SPSS 16 (SPSS Inc., Chicago, IL, USA). Normality was confirmed by the Kolmogorov-Smirnov test and homogeneity of variance was confirmed by use of Levine's test. Log transformation was performed if necessary to meet assumptions. Significant differences were evaluated with a one-way ANOVA with Tukey's posthoc test. Differences with $p < 0.05$ were considered significant.

3. Results and discussion

3.1. HF-FPW samples

Samples of HF-FPW were collected from 2 stimulated wells (Well A and Well B) (Fig. S1). The sampling time, TOC and TDS for each sample are listed in Table 1. Generally, for both wells, the concentrations of TOC in HF-FPWs decrease and the concentrations of TDS in HF-FPWs increase during the period of flowback. Particularly, for Well A, the initial sample (A1) collected at 1 hps contained extremely high TOC and low TDS compared to all the other samples. It is likely that initial HF-

Table 1
Key information of HF-FPW samples collected for the current study.

Parameters	Well A			Well B		
	A1	A2	A3	B1	B2	B3
Collection time (hps)	1	48	144	1	48	136
TOC (mg/L)	11,800	271	212	4710	282	240
TDS (mg/L)	5310	164,000	192,000	145,000	162,000	175,000

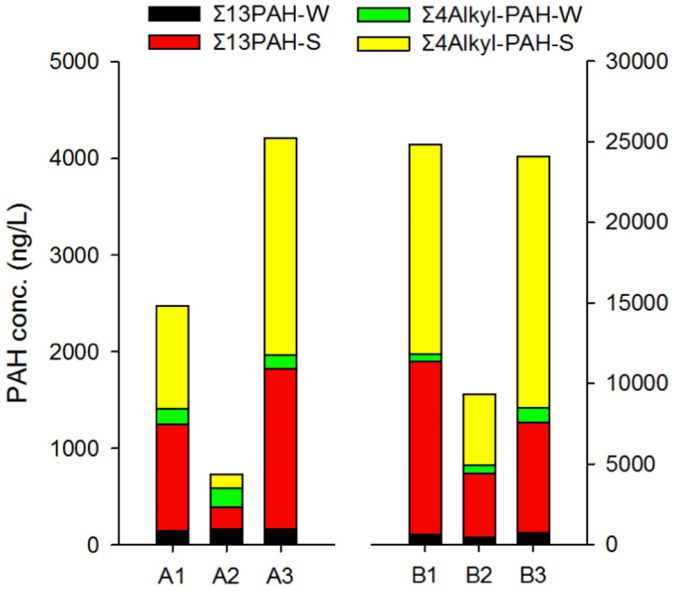

Fig. 1. Concentrations of Σ_{13}PAH and $\Sigma_4$alkyl-PAHs in organic extracts of HF-FPW in aqueous (W) and particulate phases (S). The left 3 bars are PAHs in HF-FPW samples collected from Well A. The right 3 bars are PAHs in HF-FPW samples collected from Well B.

FPW contains higher concentrations of the organics found in fracturing fluid additives, and their concentrations quickly decrease with increase of flowback time, being replaced with formation water containing higher TDS levels (unpublished data).

3.2. PAHs in FPW

HF-FPWs from both wells had greater concentrations of total PAHs at the initial (1 hps) and later time points (144/136 hps), with slightly lesser concentrations of total PAHs at intermediate time points (48 hps) (Fig. 1A & B). Of all the aqueous fractions, B3-W contained the greatest concentration of Σ_{13}PAH (720 ng/L) and of $\Sigma_4$alkyl-PAHs (930 ng/L), while in all particulate fractions, B1-S had the greatest Σ_{13}PAH (11,000 ng/L) and B3-S has the greatest $\Sigma_4$alkyl-PAHs (16,000 ng/L) (Table S1). Generally, the fractions from Well B had greater concentrations of PAHs, compared to those from Well A (Fig. 1). For example, at 1 hps total PAHs in A1-W and A1-S were 320 and 2300 ng/L, respectively, while the total PAHs in B1-W and B1-S were 1100 and 24,000 ng/L, respectively (Table S1). The $EQ_{total\ PAH}$ for each extract followed the same trend as total PAH concentrations, with the highest $EQ_{total\ PAH}$ for B3-W (260 ng BaP/L) (Table S1). This result, together with the different TDS profiles between two wells (unpublished data), suggest that chemical compositions of HF-FPWs vary from well to well, likely due to different geological formations of drilling sites. In addition, although total PAHs in B1 was 3–10 times higher than in A1, the TOC detected in A1 was 2.5× higher compared to B1. An explanation for this apparent discrepancy is that the organic compounds in flowback at very early stages contained a large proportion of fracturing fluids injected into the wellbore, which could reasonably be expected to be highly variable from well to well.

For both wells, samples collected at 48 hps (A2 and B2) contained relatively less total PAHs compared to other time periods. This was mainly due to the lower levels of Σ_{13}PAH and $\Sigma_4$alkyl-PAHs in extracts of particulate phases (Fig. 1). For most of the samples tested, concentrations of PAHs in particulate fractions were greater than those in aqueous fractions. The only exception was A2, in which concentrations of PAHs in aqueous and particulate fractions were approximately the same (Fig. 1, Table S1). PAHs of lower mass (3-ring and 4-ring species) were dominant in all fractions. Three-ring species accounted for approximately 45% to 80% of total PAHs, while 4-ring species accounted for 19% to 27% (Fig. 2). PAHs of greater molecular masses (5-ring and 6-ring) were predominantly detected in particulate fractions, and were non-detectable in most of the aqueous fractions, except for B3-W (Fig. 2). These results indicate temporal and spatial variations in absolute and relative concentrations of PAHs. Other organic contaminants in HF-FPWs could also vary, and their distribution could also be affected by the properties and amount of particles in HF-FPW samples.

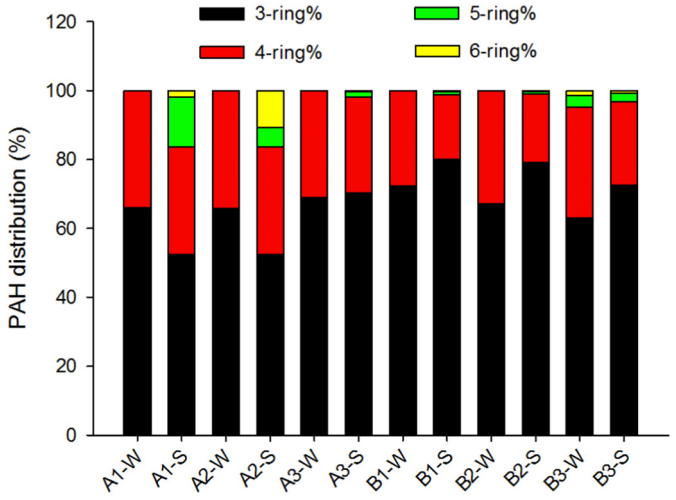

Fig. 2. Relative proportions of PAH species by number of rings (3–6) in aqueous (W) and particulate (S) phases of organic extracts of HF-FPW samples collected from Well A and Well B.

3.3. AhR agonist activity

All organic extracts tested exhibited significant AhR agonist activity. The strongest activity was detected in B3-S fraction, with EQ_{BaP} of 57 ± 8.0, 160 ± 20, and 450 ± 20 μg BaP/L for 1×, 2.5× and 5× exposure, respectively (Fig. 3). For particulate phase extracts from Well A, A3-S displayed the strongest AhR agonist potency (27 ± 3.0 μg BaP/L, at 5× EQ_{BaP}, same below), followed by A2-S, and then A1-S (Fig. 3A). For water extracts from Well A, A1-W (8.0 ± 1.0 μg BaP/L) showed stronger AhR agonistic effect than A2-W and A3-W (Fig. 3B). A3-S exhibited significantly stronger AhR agonist potency than A3-W, and so did A2-S compare to A2-W (Fig. 3C). For Well B, all extracts exhibited similar AhR agonist potencies except for B3-S. At 5× exposure, B3-S showed significantly stronger AhR agonist potency than B3-W, while no significant differences were found in earlier samples (Fig. 3D).

AhR is a ligand-activated transcription factor regulating Phase I and Phase II xenobiotic-metabolizing enzymes such as cytochrome P450 family 1As (CYP1As), uridine diphosphate glucuronosyltransferase (UGT) and glutathione S-transferase (GST) (Nebert et al., 2000). Exposure to exogenous AhR agonists, including PAHs and dioxin-like compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polychlorinated biphenyls (PCBs), can result in blue sac disease associated with oxidative stress and DNA damage (Cantrell et al., 1998). Activation of AhR binding not only causes the metabolism and bio-activation of PAHs to toxic metabolites through induction of CYP1A, but also disorders the steroids metabolism by alteration of global changes in gene expression, and in particular, cross-talk between AhR and nuclear receptor pathways leading to endocrine disruption (Swedenborg et al., 2009).

The AhR agonistic activity and corresponding CYP1A1 and induction of ethoxyresorufin-*O*-deethylase (EROD) have been previously reported in various oil spills scenarios and/or crude oil contaminated

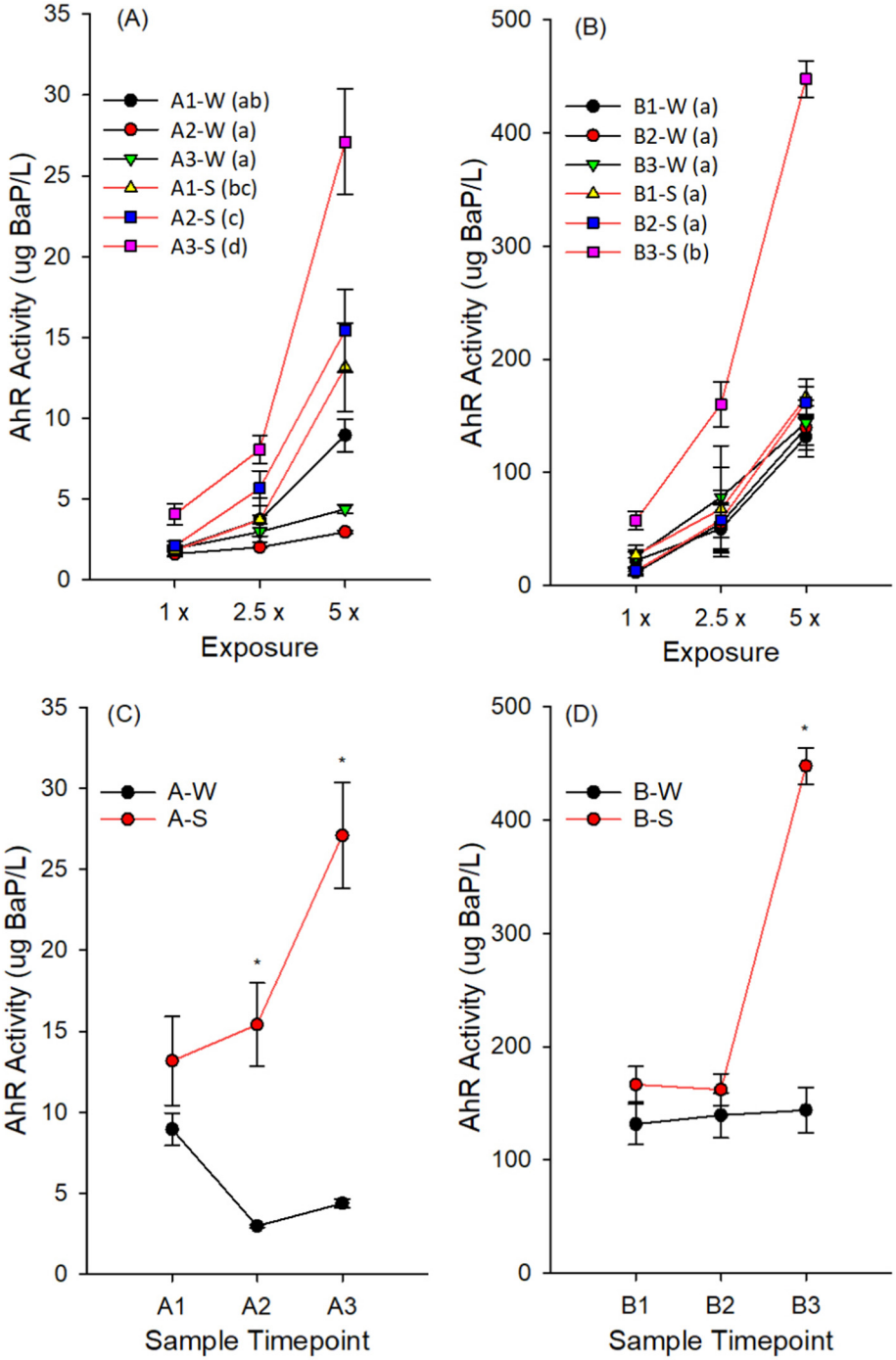

Fig. 3. AhR activities in the organic extracts of HF-FPW samples. In the legend, A and B represent Well A and B, respectively. W and S represent aqueous (water) phase and particulate (solid) phase, respectively. Panel (A) shows organic fractions from Well A. Panel (B) shows organic fractions from Well B. Panel (C) shows the flowback time trend of AhR activity in Well A samples (tested in 5× exposure concentration, the same below). Panel (D) shows the flowback time trend in Well B. Different letters in brackets in panel (A) and (B) indicate significant difference between sample treatments. Asterisk (*) indicates significant difference within the same sample time point. Differences with $p < 0.05$ were considered significant.

water. *In vitro* AhR agonist potency has been reported in offshore produced water effluents collected from the North Sea (Hurst et al., 2005) and in water soluble fraction of weather spilled oil from the Prestige Oil Spill (Navas et al., 2006). Significant AhR-active PAH and AhR mediated activity were determined in extracts of contaminated sediments collected 2 years after Hebei Spirit Oil Spill site (Hong et al., 2012). Increases in CYP1A mRNA transcript and protein abundances in liver and intestinal tissues were also found in Gulf Killifish collected from the sites with various degrees of oiling in the Deepwater Horizon Oil Spills (Dubansky et al., 2017). In our previous studies, exposure to raw HF-FPW samples induced EROD activity in zebrafish and rainbow trout (Blewett et al., 2017a; He et al., 2017a, 2017b). In addition, elevated expression of *ahr*, *cyp1a* and *cyp1b* in exposed fish also indicated the activation of AhR binding towards HF-FPW exposure (He et al., 2017b, 2018).

In the current study, all organic extracts displayed dose-dependent AhR activation in H4IIE-*luc* cells (Fig. 3). Overall, extracts of HF-FPW from Well B exhibited stronger AhR activation than extracts from Well A. This result is consistent with the fact that Well B contained greater concentrations of PAHs than Well A. For both wells, extracts of the particulate phase exhibited greater AhR potencies than the corresponding aqueous phase extracts. This is attributed to PAHs species with greater molecular masses. PAHs with higher ring numbers and molecular masses were often the species with greater AhR potencies (Nisbet and LaGoy, 1992). In addition, both wells exhibited greater AhR potencies in extracts of particulate phases as a function of flowback times, while there wasn't a significant relationship for extracts of aqueous phases. However, this correlation was not perfectly in line with

total PAHs levels, given that samples collected at intermediate time points (A2 and B2) contained lesser concentrations of total PAHs than during other time periods. Similarly, concentrations of EQ were not congruent since both A2 and B2 have relatively lesser BaP equivalency compared to other samples taken at time points (Table S1). Moreover, for both wells, the particulate phase of last time points (A3-S and B3-S) displayed significantly stronger AhR potencies compared to the initial time points (A1-S and B1-S), while only A3-S contained higher Σ_{13}PAH and $\Sigma_4$alkyl-PAHs than A1-S, and in fact B3-S contained similar levels of Σ_{13}PAH and $\Sigma_4$alkyl-PAHs compared to B1-S. However, when considering BaP equivalency factors (Table S1), A3-S had similar BaP equivalency with A1-S, but B3-S had much higher BaP equivalency compared to all the other extracts, being consistent that B3-S displayed the strongest AhR agonist activity in this study.

These results suggest that the differences in AhR activity among these fractions cannot be simply explained by and attributed to either total PAHs, or total BaP equivalencies of those PAH species determined in the current study. One possible explanation for this complexity is there are significant amounts of other AhR agonists present in HF-FPW and concentrations of these compounds are increasing in HF-FPW as a function of flowback time. This might also explain why AhR potencies of extracts of HF-FPW measured by use of the H4IIE-*luc* assay are in μg BaP/L, while EQ calculated based on concentrations of PAH and their respective relative potency values are in ng BaP/L scale (Table S1). Further study is needed to better characterize the AhR active components in HF-FPW samples.

3.4. Estrogenicity

Some of the organic extracts tested exhibited significant ER agonist activity in the MVLN assay (Fig. 4). Final concentrations in media used for MVLN assays were 5× and 10× and no cytotoxicity was observed. Among exposures, the greatest ER agonist activity was detected in fraction A1-W, with a concentration of EQ_{E2} of 2.6 ± 0.3 and 5.3 ± 0.9 nM for 5× and 10× exposures, respectively (Fig. 4). For extracts of both aqueous and particulate fractions from Well A, there is a decreasing trend in ER agonist activity from A1 to A3 (Fig. 4A). Comparing aqueous and particulate fractions, A1-W exhibited greater ER agonist potency than A1-S, but A2-S exhibited greater ER agonist potency than A2-W. For Well B, there is an increasing trend in ER agonist potency from of extracts of HF-FPW from B1 to B3, where aqueous fractions displayed significantly greater ER agonist potency than particulate fractions at all the time points, while there was no significant difference among ER agonist potencies in extracts of particulates (Fig. 4B). Extracts A2-W, A3-W, A3-S, and all extracts of particulate phase from Well B did not show significant ER activity.

Estrogenic potency has been reported in various contaminated environments, including crude oil related wastewater, such as offshore effluent, oil sands process-affected water (OSPW), and spilled oil and gas wastewater (He et al., 2011; Kassotis et al., 2014; Thomas et al., 2004). Both natural and anthropogenic compounds thought to possess estrogenic potency have been found in the abovementioned samples. For example, *in vitro* ER agonist potency was determined in North Sea offshore produced water, and mixtures of isomers of alkylphenols were identified as major contributors of ER agonist potency (Thomas et al., 2004). Similarly, steroidal aromatic naphthenic acids, which are naturally formed estrogenic compounds, have been identified in OSPW (Rowland et al., 2011) and are suspected of being the primary endocrine disrupting chemicals causing altered expressions of genes involved in the endocrine system and genes differentially expressed during development of embryos of fishes (He et al., 2012a, 2012b). Certain chemicals applied in oil and gas operations, such as ethylene glycol, naphthalene, and bronopol, have significant EDC potency and could potentially cause adverse outcomes in aquatic organisms (Kassotis et al., 2014, 2016b). Octylphenol ethoxylates (OPE), commercially referred as Triton-X, that are applied as surfactants in hydraulic fracturing fluids have been identified in HF-FPW samples (He et al., 2018). This group of chemicals could be degraded by microbes to a weak ER agonist, octylphenol (Orem et al., 2014). Some 4- and 5-ring PAHs and their derivatives can also interact with both isoforms of the ER (Fertuck et al., 2001). In the current study, several PAHs with high molecular weight (HMW), including Benz[*a*]anthracene, and Dibenz[*a*,*h*]anthracene, as well as several alkylated PAHs, including 1-Methylphenanthrene, and 3,6-Dimethylephenanthrene, that have been reported to have significant estrogenic potency (Lee et al., 2017; Villeneuve et al., 2002) were detected.

Temporal and spatial analyses demonstrated several trends in potencies of ER agonists between Wells A and B. For Well A, potencies of extracts of aqueous phases were inversely proportional to durations of flowback. A similar but more moderate pattern was observed for extracts of the particulate phase. However, for Well B, the aqueous phase extracts displayed an increasing trend of ER potency as the flowback

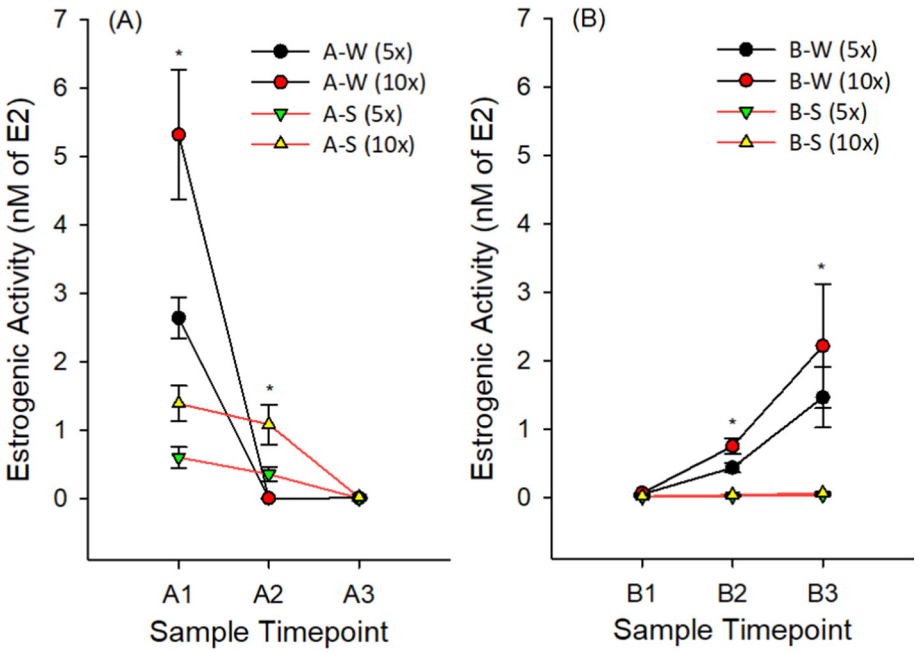

Fig. 4. Estrogenic activities in organic extracts of HF-FPW samples. In the legend, A and B stand for Well A and Well B, respectively. W and S stand for aqueous (water) phase and particulate (solid) phase, respectively. 5× and 10× in the brackets indicate the exposure concentrations. Panel (A) shows organic fractions from Well A. Panel (B) shows organic fractions from Well B. Asterisk (*) indicates significant difference between the treatments of aqueous (W) and particulate (S) phases within the same sample time point and exposure concentration. Differences with $p < 0.05$ were considered significant.

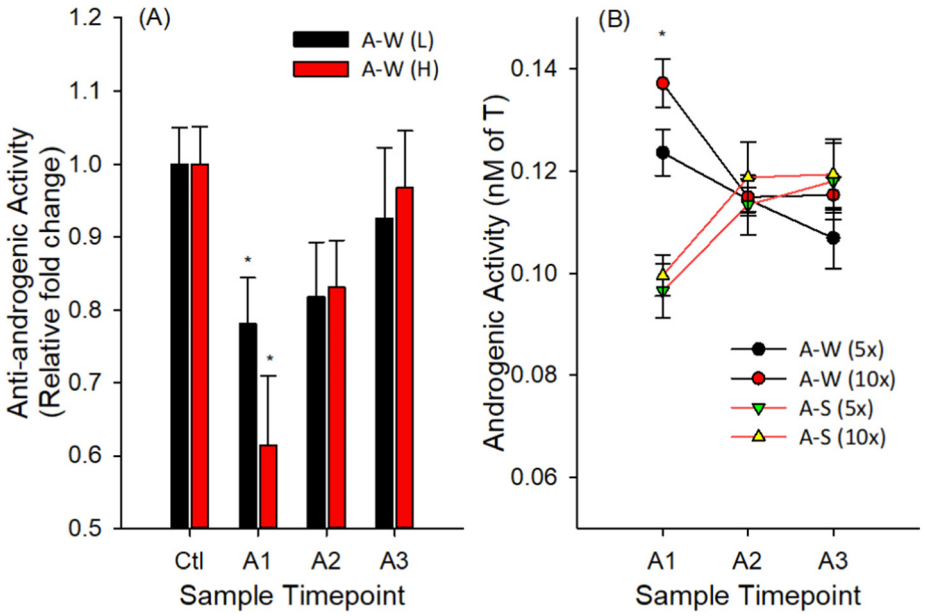

Fig. 5. Anti-androgenic and androgenic activities in organic extracts of HF-FPW samples. In the legend, A stands for Well A. W and S stand for aqueous (water) phase and particulate (solid) phase, respectively. Panel (A) shows anti-androgenic activity of samples collected from Well A. L and H in brackets indicate low (0.3 nM) and high (1 nM) levels of co-exposed T, respectively. Asterisk (*) indicates significant difference from control group. Panel (B) shows androgenic activity in the organic extracts of HF-FPW from Well A. 5× and 10× in the brackets indicate the exposure concentrations. Asterisk (*) indicates significant difference within the same sample time point between different phases. Differences with $p < 0.05$ were considered significant.

time increases, while there was no significant ER potency observed in extracts of the particulate phase at all the time points. Although most environmental estrogens have relatively small solubilities in water, for both wells, the majority of the estrogenicity was observed in aqueous extracts rather than the particulate extracts. These results suggest that estrogenic compounds present in both Wells A and B exhibit varying properties and possibly have different origins.\

3.5. Androgenicity

Among all extracts, only A1-W at 10× equivalents significantly decreased androgenic potency to 0.78 ± 0.06 and 0.61 ± 0.10-fold when co-exposed with low and high levels of T (0.3 and 1 nM, respectively) (Fig. 5A). However, A1-W also exhibited androgenic potency. At 10× exposure A1-W alone displayed statistically significant AR agonist potency in the MDA-kb2 assay. All other extracts from both wells did not show any significant AR agonist potency (Fig. S6).

The fact that in this study, greater concentrations of A1-W exhibited both androgenic and anti-androgenic potency is difficult to explain. Since A1-W was the one with greatest estrogenic potency among all the extracts, it is not surprising that A1-W is the only extract exhibiting anti-androgenic effect when co-exposed with T. Many studies have demonstrated that environmental estrogens such as Dichloro-diphenyl-trichloroethane (DDT) and its metabolites, Bisphenol A, phthalates and alkyphenols could inhibit AR activity (Fang et al., 2003; Kelce et al., 1995; Xu et al., 2005). However, when exposed alone, extracts of HF-FPW from A1-W also resulted in limited but significant AR agonist potency at greater concentrations. It has been reported that AR transcriptional potency can be induced by binding of E2 to the AR and AR coactivators (Yeh et al., 1998). Therefore, the AR agonist potency found in A1-W is not unexpected since A1-W is also the extract with strongest ER agonist potency.

4. Conclusions

The most important conclusion of this study is that organic extracts of HF-FPW can disrupt several nuclear receptors binding activities at environmentally relevant concentrations. In an accidental release scenario, the FPW would locally deposit FPW associated particles, resulting in a local aquatic environment (*e.g.* lake or streambed for embryos) with contaminant concentrations above those in the wastewater itself. In the current study, exposure with 1× extracts (*i.e.* relevant concentrations) was sufficient to cause significant AhR-mediated activation. An additional novel contribution of this study is the demonstration of temporal and spatial variation of HF-FPWs with respect to their effects on endocrine related pathways. Our results demonstrate a trend of increasing AhR activation for both wells as a function of flowback time, and from both wells the extracts demonstrated stronger AhR agonist potency in the particulate phase compared to aqueous phase. However, extracts of HF-FPW from Well B exhibited greater AhR agonist potency, compared with that of Well A. In addition, it also suggests that organic compounds responsible for biotransformation and oxidative stress (associated with AhR activation) and endocrine disruption (associated with steroid hormone receptor activation) tend to partition into specific fractions of the HF-FPW. Most notably, ER agonist potency decreases in extracts of Well A flowback fluids as a function of flowback time, while ER agonist potency increases in Well B aqueous extracts as a function of flowback time. These results suggest that the properties and origins of EDCs in FPWs from Wells A and B are different, complicating our understanding of potential environmental effects of releases of FPW. A study of a range of HF-FPWs is needed to circumscribe the range of potential effects and potencies of wells during fracturing operations.

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Disclosure

The authors declare no competing financial interest.

Appendix A. Supplementary data

The Supplementary Information, including methods, supplementary figures and table is available online at https://doi.org/10.1016/j.envint.2018.10.014.

References

Alberta Environment & Sustainable Resource Development (ESRD), 2014. Environmental Quality Guidelines for Alberta Surface Waters (24–48; 625). Water Policy Branch, Policy Division, Edmonton.

Alessi, D.S., Zolfaghari, A., Kletke, S., Gehman, J., Allen, D.M., Goss, G.G., 2017. Comparative analysis of hydraulic fracturing wastewater practices in unconventional shale development: water sourcing, treatment, and disposal practices. Can. Water Res. J. 42, 105–121.

Blewett, T.A., Weinrauch, A.M., Delompré, P.L., Goss, G.G., 2017a. The effect of hydraulic flowback and produced water on gill morphology, oxidative stress and antioxidant response in rainbow trout (*Oncorhynchus mykiss*). Sci. Rep. 7, 46582.

Blewett, T.A., Delompré, P.L., He, Y., Folkerts, E.J., Flynn, S.L., Alessi, D.S., Goss, G.G., 2017b. Sublethal and reproductive effects of acute and chronic exposure to flowback and produced water from hydraulic fracturing on the water flea *Daphnia magna*. Environ. Sci. Technol. 51, 3032–3039.

Cantrell, S.M., Joy-Schlezinger, J., Stegeman, J.J., Tillitt, D.E., Hannink, M., 1998. Correlation of 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced apoptotic cell death in the embryonic vasculature with embryotoxicity. Toxicol. Appl. Pharmacol. 148, 24–34.

Chen, S.S., Sun, Y., Tsang, D.C., Graham, N.J., Ok, Y.S., Feng, Y., Li, X.D., 2017. Potential impact of flowback water from hydraulic fracturing on agricultural soil quality: metal/metalloid bioaccessibility, Microtox bioassay, and enzyme activities. Sci. Total Environ. 579, 1419–1426.

Demirpence, E., Duchesne, M.J., Badia, E., Gagne, D., Pons, M., 1993. MVLN cells: a bioluminescent MCF-7-derived cell line to study the modulation of estrogenic activity. J. Steroid Biochem. Mol. Biol. 46, 355–364.

Dubansky, B., Rice, C.D., Barrois, L.F., Galvez, F., 2017. Biomarkers of aryl-hydrocarbon receptor activity in Gulf Killifish (*Fundulus grandis*) from Northern Gulf of Mexico Marshes following the Deepwater Horizon Oil Spill. Arch. Environ. Contam. Toxicol. 73, 63–75.

Elliott, E.G., Ettinger, A.S., Leaderer, B.P., Bracken, M.B., Deziel, N.C., 2016. A systematic evaluation of chemicals in hydraulic-fracturing fluids and wastewater for reproductive and developmental toxicity. J. Expo. Sci. Environ. Epidemiol. 27, 90–99.

Fang, H., Tong, W., Branham, W.S., Moland, C.L., Dial, S.L., Hong, H., Xie, Q., Perkins, R., Owens, W., Sheehan, D.M., 2003. Study of 202 natural, synthetic, and environmental chemicals for binding to the androgen receptor. Chem. Res. Toxicol. 16, 1338–1358.

Fertuck, K.C., Kumar, S., Sikka, H.C., Matthews, J.B., Zacharewski, T.R., 2001. Interaction of PAH-related compounds with the alpha and beta isoforms of the estrogen receptor. Toxicol. Lett. 121, 167–177.

Folkerts, E.J., Blewett, T.A., He, Y., Goss, G.G., 2017a. Alterations to juvenile zebrafish (*Danio rerio*) swim performance after acute embryonic exposure to sub-lethal exposures of hydraulic fracturing flowback and produced water. Aquat. Toxicol. 193, 50–59.

Folkerts, E.J., Blewett, T.A., He, Y., Goss, G.G., 2017b. Cardio-respirometry disruption in zebrafish (*Danio rerio*) embryos exposed to hydraulic fracturing flowback and produced water. Environ. Pollut. 231, 1477–1487.

He, Y., Wiseman, S.B., Hecker, M., Zhang, X., Wang, N., Perez, L.A., Jones, P.D., El-Din, M.G., Martin, J.W., Giesy, J.P., 2011. Effect of ozonation on the estrogenicity and androgenicity of oil sands process-affected water. Environ. Sci. Technol. 45, 6268–6274.

He, Y., Wiseman, S.B., Wang, N., Perez-Estrada, L.A., El-Din, M.G., Martin, J.W., Giesy, J.P., 2012a. Transcriptional responses of the brain-gonad-liver axis of fathead minnows exposed to untreated and ozone-treated oil sands process-affected water. Environ. Sci. Technol. 46, 9701–9718.

He, Y., Patterson, S., Wang, N., Hecker, M., Martin, J.W., El-Din, M.G., Giesy, J.P., Wiseman, S.B., 2012b. Toxicity of untreated and ozone-treated oil sands process-affected water (OSPW) to early life stages of the fathead minnow (*Pimephales promelas*). Water Res. 46, 6359–6368.

He, Y., Flynn, S.L., Folkerts, E.J., Zhang, Y., Ruan, D., Alessi, D.S., Martin, J.W., Goss, G.G., 2017a. Chemical and toxicological characterizations of hydraulic fracturing flowback and produced water. Water Res. 114, 78–87.

He, Y., Folkerts, E.J., Zhang, Y., Martin, J.W., Alessi, D.S., Goss, G.G., 2017b. Effects on biotransformation, oxidative stress, and endocrine disruption in rainbow trout (*Oncorhynchus mykiss*) exposed to hydraulic fracturing flowback and produced water. Environ. Sci. Technol. 51, 940–947.

He, Y., Sun, C., Zhang, Y., Folkerts, E.J., Martin, J.W., Goss, G.G., 2018. Developmental toxicity of the organic fractions of hydraulic fracturing flowback and produced water to early life stages of zebrafish (*Danio rerio*). Environ. Sci. Technol. 52, 3820–3830.

Hong, S., Khim, J.S., Ryu, J., Park, J., Song, S.J., Kwon, B.O., Choi, K., Ji, K., Seo, J., Lee, S., Park, J., Lee, W., Choi, Y., Lee, K.T., Kim, C.K., Shim, W.J., Naile, J.E., Giesy, J.P., 2012. Two years after the Hebei Spirit oil spill: residual crude-derived hydrocarbons and potential AhR-mediated activities in coastal sediments. Environ. Sci. Technol. 46, 1406–1414.

Hurst, M.R., Chan-Man, Y.L., Balaam, J., Thain, J.E., Thomas, K.V., 2005. The stable aryl hydrocarbon receptor agonist potency of United Kingdom Continental Shelf (UKCS) offshore produced water effluents. Mar. Pollut. Bull. 50, 1694–1698.

Kassotis, C.D., Tillitt, D.E., Davis, J.W., Hormann, A.M., Nagel, S.C., 2014. Estrogen and androgen receptor activities of hydraulic fracturing chemicals and surface and ground water in a drilling-dense region. Endocrinology 155, 897–907.

Kassotis, C.D., Iwanowicz, L.R., Akob, D.M., Cozzarelli, I.M., Mumford, A.C., Orem, W.H., Nagel, S.C., 2016a. Endocrine disrupting activities of surface water associated with a West Virginia oil and gas industry wastewater disposal site. Sci. Total Environ. 557–558, 901–910.

Kassotis, C.D., Tillitt, D.E., Lin, C.H., McElroy, J.A., Nagel, S.C., 2016b. Endocrine disrupting chemicals and oil and natural gas operations: potential environmental contamination and recommendations to assess complex environmental mixtures. Environ. Health Perspect. 124, 256–264.

Kelce, W., Stone, C., Laws, S., Gray, L., Kemppainen, J., Wilson, E., 1995. Persistent DDT metabolite p,p′-DDE is a potent androgen receptor antagonist. Nature 375, 581–585.

Larsson, M., Giesy, J.P., Engwall, M., 2014. AhR-mediated activities of polycyclic aromatic compound (PAC) mixtures are predictable by the concept of concentration addition. Environ. Int. 73, 94–103.

Lauer, N.E., Harkness, J.S., Vengosh, A., 2016. Brine spills associated with unconventional oil development in North Dakota. Environ. Sci. Technol. 50, 5389–5397.

Leclair, L.A., Pohler, L., Wiseman, S.B., He, Y., Arens, C.J., Giesy, J.P., Scully, S., Wagner, B.D., van den Heuvel, M.R., Hogan, N.S., 2015. In vitro assessment of endocrine disrupting potential of naphthenic acid fractions derived from oil sands-influenced water. Environ. Sci. Technol. 49, 5743–5752.

Lee, S., Hong, S., Liu, X., Kim, C., Jung, D., Yim, U.H., Shim, W.J., Khim, J.S., Giesy, J.P., Choi, K., 2017. Endocrine disrupting potential of PAHs and their alkylated analogues associated with oil spills. Environ. Sci. Process. Impacts 19, 1117–1125.

Navas, J.M., Babín, M., Casado, S., Fernández, C., Tarazona, J.V., 2006. The Prestige oil spill: a laboratory study about the toxicity of the water-soluble fraction of the fuel oil. Mar. Environ. Res. 62, S352–S355 (Suppl).

Nebert, D.W., Roe, A.L., Dieter, M.Z., Solis, W.A., Yang, Y., Dalton, T.P., 2000. Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis. Biochem. Pharmacol. 59, 65–85.

Nisbet, I.C., LaGoy, P.K., 1992. Toxic equivalency factors (TEFs) for polycyclic aromatic hydrocarbons (PAHs). Regul. Toxicol. Pharmacol. 16, 290–300.

Orem, W., Tatu, C., Varonka, M., Lerch, H., Bates, A., Engle, M., Crosby, L., McIntosh, J., 2014. Organic substances in produced and formation water from unconventional natural gas extraction in coal and shale. Int. J. Coal Geol. 126, 20–31.

Rowland, S.J., West, C.E., Jones, D., Scarlett, A.G., Frank, R.A., Hewitt, L.M., 2011. Steroidal aromatic 'naphthenic acids' in oil sands process-affected water: structural comparisons with environmental estrogens. Environ. Sci. Technol. 45, 9806–9815.

Snyder, S.A., Villeneuve, D.L., Snyder, E.M., Giesy, J.P., 2001. Identification and quantification of estrogen receptor agonists in wastewater effluents. Environ. Sci. Technol. 35, 3620–3625.

Swedenborg, E., Rüegg, J., Mäkelä, S., Pongratz, I., 2009. Endocrine disruptive chemicals: mechanisms of action and involvement in metabolic disorders. J. Mol. Endocrinol. 43, 1–10.

Thomas, K.V., Balaam, J., Hurst, M.R., Thain, J.E., 2004. Identification of in vitro estrogen and androgen receptor agonists in North Sea offshore produced water discharges. Environ. Toxicol. Chem. 23, 1156–1163.

Thurman, E.M., Ferrer, I., Rosenblum, J., Linden, K., Ryan, J.N., 2017. Identification of polypropylene glycols and polyethylene glycol carboxylates in flowback and produced water from hydraulic fracturing. J. Hazard. Mater. 323, 11–17.

Vengosh, A., Jackson, R.B., Warner, N., Darrah, T.H., Kondash, A., 2014. A critical review of the risks to water resources from unconventional shale gas development and hydraulic fracturing in the United States. Environ. Sci. Technol. 48, 8334–8348.

Villeneuve, D.L., Khim, J.S., Kannan, K., Giesy, J.P., 2002. Relative potencies of individual polycyclic aromatic hydrocarbons to induce dioxin-like and estrogenic responses in three cell lines. Environ. Toxicol. 17, 128–137.

Xu, L.C., Sun, H., Chen, J.F., Bian, Q., Qian, J., Song, L., Wang, X.R., 2005. Evaluation of androgen receptor transcriptional activities of bisphenol A, octylphenol and nonylphenol in vitro. Toxicology 216, 197–203.

Yeh, S., Miyamoto, H., Shima, H., Chang, C., 1998. From estrogen to androgen receptor: a new pathway for sex hormones in prostate. Proc. Natl. Acad. Sci. U. S. A. 95, 5527–5532.

Yost, E.E., Stanek, J., DeWoskin, R.S., Burgoon, L.D., 2016. Estimating the potential toxicity of chemicals associated with hydraulic fracturing operations using quantitative structure-activity relationship modeling. Environ. Sci. Technol. 50, 7732–7742.

Zhang, Y., Shotyk, W., Zaccone, C., Noernberg, T., Pelletier, R., Bicalho, B., Froese, D.G., Davies, L., Martin, J.W., 2016. Airborne petcoke dust is a major source of polycyclic aromatic hydrocarbons in the Athabasca Oil Sands Region. Environ. Sci. Technol. 50, 1711–1720.

Zhao, Y., Zhang, K., Giesy, J.P., Hu, J., 2015. Families of nuclear receptors in vertebrate models: characteristic and comparative toxicological perspective. Sci. Rep. 5, 8554.

Supplementary Data

In Vitro Assessment of Endocrine Disrupting Potential of Organic Fractions extracted from Hydraulic Fracturing Flowback and Produced Water (HF-FPW)

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Table of Content

Methods

Extraction of Organics

One liter of pure water (Optima™, LC-MS grade) was used as a blank sample. For each FPW sample, 1 L of FPW was vacuum filtered using glass fiber membrane filter (90 mm diameter, pore size: 0.4 μm), and the used filters with solid residues were freeze-dried for 48 hours. The corresponding aqueous filtrate was liquid-liquid extracted using dichloromethane (DCM), for example, 50 mL DCM was added and shaken for 3 minutes, then extract was removed and another 50 mL DCM was added and shaken for another 3 minutes again. The extracts were combined (100 mL) and concentrated by nitrogen gas evaporation. Accelerated solvent extraction (ASE) was used for organic extraction from dried solid residues filters. Glass fiber filter was added at the bottom of the extraction cell, then the dried solid residues filters were added. ASE cells were filled DCM (Optima™), pressurized to 14 MPa, and heated to 80 °C within 6 min. Pressure and temperature were held for 5 min (static extraction), followed by rinsing with cold solvent (50% of the cell volume) and purging with nitrogen gas for 90 s. This extraction cycle was repeated 3 times. Approximately 80 mL of total extract was then gently concentrated by nitrogen gas evaporation. Each extract was then reconstituted using 100 μL DCM to an equivalent 10,000× relative concentration compared to the original sample. In the current study, fraction of aqueous filtrate is defined as aqueous phase (W, water), and fraction of solid residues filtrate is defined as particulate phase (S, solid).

PAH Analysis

Fifty milliliters of pure water (Optima™, LC-MS grade) was used as a blank sample. For each FPW sample, 50 mL of FPW was vacuum filtered using glass fiber membrane filter (90 mm diameter, pore size: 0.4 μm), and the used filters with solid residues were freeze-dried for 48 hours. Liquid-liquid extraction method was used for aqueous filtrate. The internal standard mix (10 ng of each) was spiked into the aqueous filtrate and shaken for 3 minutes; then 50 mL DCM was added and shaken for 3 minutes. The DCM extract was removed and another 50 mL DCM was added and shaken for another 3 minutes again. The DCM extracts were combined (100 mL) and was concentrated by nitrogen gas evaporation, finally diluted with 3 mL hexane.

ASE was used for PAH extraction from the dried solid residues. Glass fiber filter was added at the bottom of the extraction cell, and about 1~3 g of florisil powder (precleaned by DCM) was added, followed by dried solid residues filters, to which the internal standard mix was then spiked (10 ng of each). ASE cells were filled with solvent (hexane/DCM 4:1 v:v, Optima™), pressurized to 14 MPa,

and heated to 80 °C within 6 min. Pressure and temperature were held for 5 min (static extraction), followed by rinsing with cold solvent (50% of the cell volume) and purging with nitrogen gas for 90 s. This extraction cycle was repeated once. Approximately 40 mL of total extract was then gently concentrated by nitrogen gas evaporation, finally diluted with 3 mL hexane.

Anhydrous sodium sulfate and copper powder (precleaned by DCM) were added into the 3 mL extract and vortexed. Subsequently, solid phase extraction was used for cleanup of all the sample extracts. The silica cartridge (Waters, 1g/6cc) was conditioned with 5 mL solvent (hexane/DCM 7:3 v:v, Optima™), followed by 5 mL of hexane, then extracts (3 mL) were loaded, and 4 mL of hexane was used to wash the extracts tube. Then, PAHs were eluted with 5 mL of hexane/DCM 7:3 (v/v), and the extract was finally concentrated and diluted with hexane to 200 μL for GC-MS analysis. The details of GC-MS analysis have been described in our previous study (Zhang et al., 2016).

***In Vitro* Bioassays**

The current study used several widely-used reporter gene assays, including H4IIE-*luc*, MVLN-*luc*, and MDA-kb2 assays, to directly quantify the binding activity of chemicals in organic extract of HF-FPW towards several nuclear receptors, including aryl-hydrocarbon receptor (AhR), estrogen receptor (ER), and androgen receptor (AR). (He et al., 2011; Leclair et al., 2015; Snyder et al., 2001). A standard curve of serially diluted positive controls (Benzo[a]Pyrene (BaP), 17β-estradiol (E2), testosterone (T) in H4IIE-luc, MVLN-luc, and MDA-kb2, respectively) was included on each 96-well plate of the corresponding bioassays. The standard curve generated a linear range formula that luminescence readings are proportional to the real concentrations of positive control chemicals. The measured luminescence of each extract was then plotted on the corresponding standard curve to obtain the chemical equivalency. Parallel blank control was also included in each cell assay, and no significant difference was detected between parallel blank control (with 0.1% DCM) and medium control (no DCM as delivering solvent) in each cell assay. Based on the results from our previous studies, agonist activity in all 3 cell lines were tested in all 12 organic extracts. In addition, antagonist effect of all 12 organic extracts were also screened in MDA-kb2 by co-exposure either low or high levels of T (0.3 nM and 1 nM, respectively). The relative fold changes of AR activity of all co-exposure treatment, low/high level of T, as well as parallel blank control were obtained. Antagonist activity against T in each co-exposure treatment was expressed as relative fold change normalized by the corresponding level of T. The examples of standard curve on agonist activity in H4IIE-*luc*, MVLN-*luc*, and MDA-kb2 are presented in Fig S2, S3, and S4. Fig S5 presents the

relative fold changes of AR activity in low and high levels of T used as reference for antagonist activity calculation.

Supplementary Table

	TEF[a]	Aqueous MDL (ng/L)	Particulate MDL (ng/g)	A1-W (ng/L)	A1-S (ng/L)	A2-W (ng/L)	A2-S (ng/L)	A3-W (ng/L)	A3-S (ng/L)	B1-W (ng/L)	B1-S (ng/L)	B2-W (ng/L)	B2-S (ng/L)	B3-W (ng/L)	B3-S (ng/L)
Parent PAHs															
Naphthalene	0.001	3.6	5.3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Acenaphthlene	0.001	0.14	0.36	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Acenaphthene	0.001	2.3	1.4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Fluorene	0.001	2.6	2.3	77	87	53	49	68	690	275	1400	208	897	230	770
Phenanthrene	0.001	4.8	2.9	51	67	71	35	68	410	290	5700	200	2100	230	780
Anthracene	0.01	0.58	0.19	2.3	37	5.6	5.4	2.2	59	4.7	77	2.3	38	7.1	15
Fluoranthene	0.001	1.2	0.78	4.2	82	10	9.7	5.8	71	8.9	400	6.1	92	49	260
Pyrene	0.001	1.5	3.7	7.2	300	17	30	9.3	190	18	2000	14	500	95	1200
Benz[a]anthracene	0.1	0.052	0.13	0.91	53	2.4	11	1.2	23	1.9	68	1.1	30	5.5	1000
Chrysene	0.01	0.072	0.23	5.4	120	8.5	25	8.1	150	19	790	12	240	23	2100
Benzo[b]fluoranthene	0.1	1.5	1.0	ND	310	ND	ND	ND	31	ND	170	ND	50	30	510
Benzo[k+j]fluoranthene	0.1	0.17	0.41	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Benzo[a]pyrene	1	0.17	0.43	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Indeno[1,2,3-cd]pyrene	0.1	0.060	0.18	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Benzo[g,h,i]perylene	0.01	0.061	1.0	ND	39	ND	40	ND	10	ND	89	ND	20	25	150
Dibenz[a,h]anthracene	1	0.094	0.24	ND	5.8	ND	21	ND	30	ND	39	ND	14	24	66
Alkyl PAHs															
1-Methylnaphthalene		0.018	43	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1-Ethylnaphthalene		0.019	1.9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2-n-Propylnaphthalene		0.024	11	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
9-Ethylfluorene		0.033	1.5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
9-n-Butylfluorene		0.065	0.16	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

9-n-Butylphenanthrene		0.088	3.7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1-Ethylpyrene		0.052	0.13	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1-n-Butylpyrene		0.057	0.12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1-Methylbenz[a]anthracene		0.046	0.11	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
6-Ethylchrysene		0.073	0.18	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
6-n-Butylchrysene		0.073	0.18	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5-Methylchrysene		0.097	0.24	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1-Methylfluorene	0.001[b]	0.78	0.78	0.64	95	1.3	5.7	0.85	56	2.9	720	2.7	170	4.7	1800
1-Methylphenanthrene	0.001[b]	0.11	0.37	54	400	78	35	51	990	160	8700	150	2600	400	5000
3,6-Dimethylphenanthrene	0.001[b]	0.070	0.17	17	450	29	65	14	540	40	2400	56	870	170	7900
1-Methylpyrene	0.001[b]	0.050	0.12	87	120	86	40	68	660	250	1200	270	810	360	910
USEPA 13 PAHs				150	1100	170	230	160	1700	620	11000	440	4000	720	6900
USEPA 13 PAHs BaP Equivalency				0.31	45	0.53	23	0.37	39	1.0	82	0.68	29	29	240
4 alkylated PAHs				160	1100	190	150	130	2200	450	13000	480	4400	930	16000
4 alkylated PAHs BaP Equivalency				0.16	1.1	0.19	0.15	0.13	2.3	0.45	13	0.48	4.5	0.93	16
Total PAHs				310	2200	360	380	290	3900	1100	24000	920	8400	1700	23000
Total PAHs BaP Equivalency				0.47	46	0.73	23	0.51	41	1.5	95	1.2	33	30	260

Table S1. Method detection limits (MDLs) in aqueous (W) and particulate (S), and concentrations of US EPA 16 priority parent PAHs, and 16 alkylated PAHs in organic fractions of HF-FPW samples. Concentrations in particulate phase (S) are reported based on the original sample volumne (ng/L).

N.D. = not detected. Data in grey are below the MDL.

[a] Toxic Equivalency Factor (TEF) obtained from Larson et al. (2014) and Nisbet and LaGoy (1992).

[b] TEFs of alkylated PAHs are estimated with similar forms of parent PAHs.

Supplementary Figure

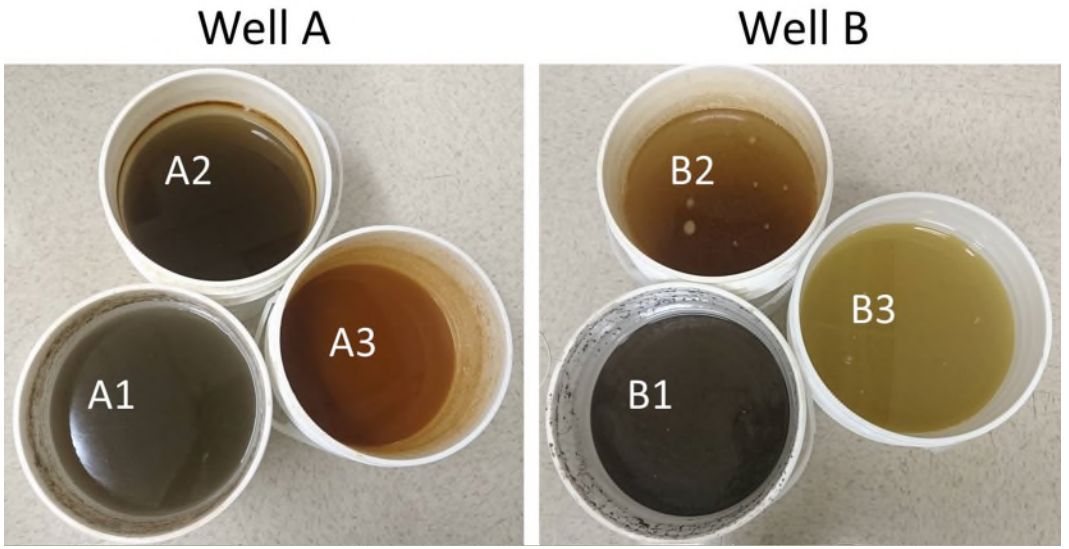

Fig. S1. Six Samples of HF-FPW collected from 2 stimulated wells (Well A and Well B).

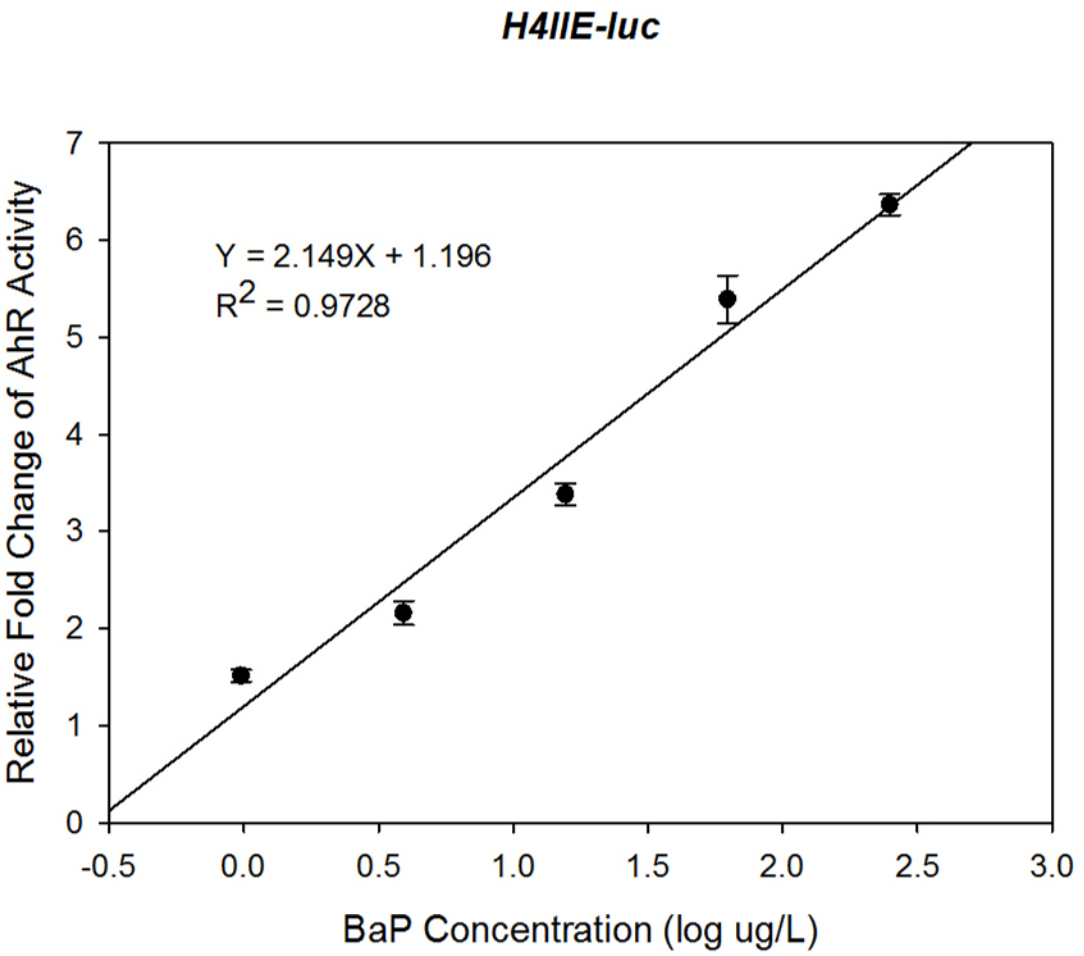

Fig. S2. Example of standard curve of Benzo[a]Pyrene (BaP) in H4IIE-*luc* agonist cell assay.

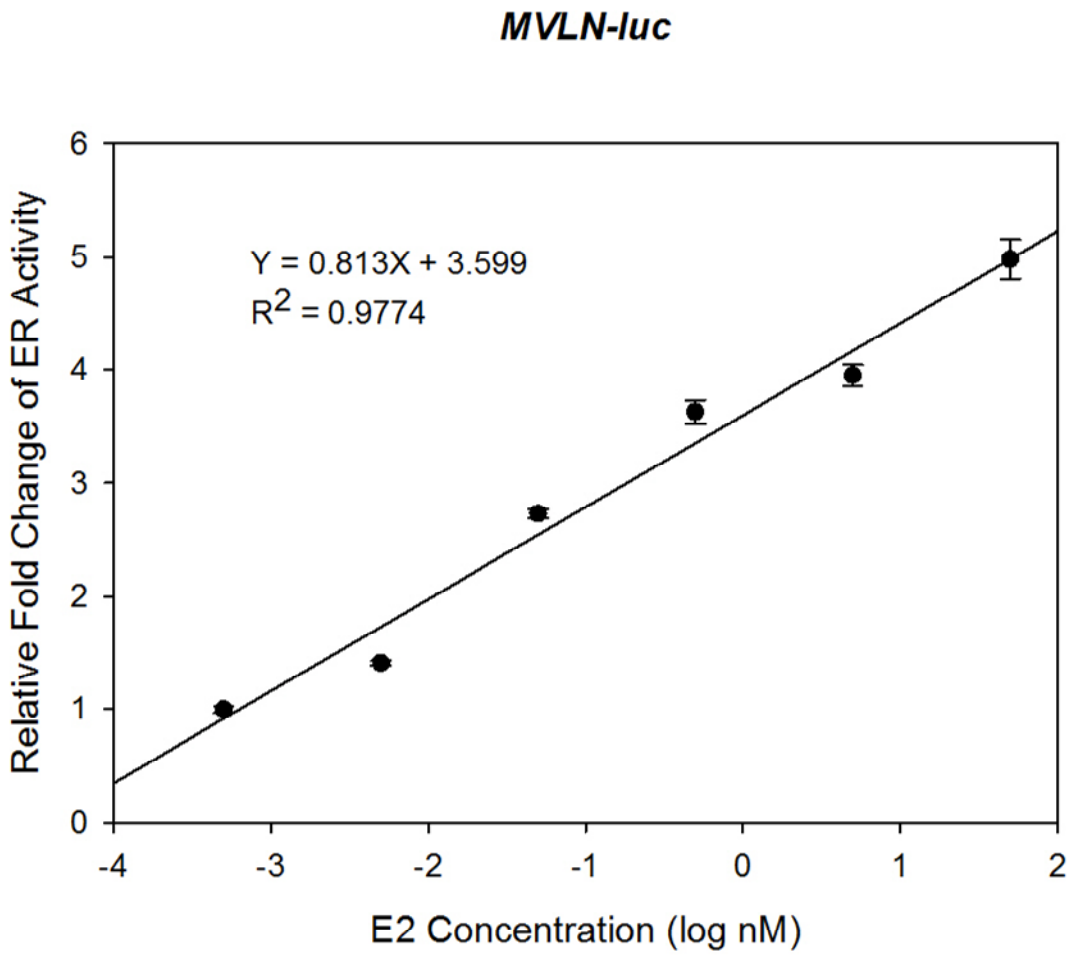

Fig. S3. Example of standard curve of 17β-estradiol (E2) in MVLN-*luc* agonist cell assay.

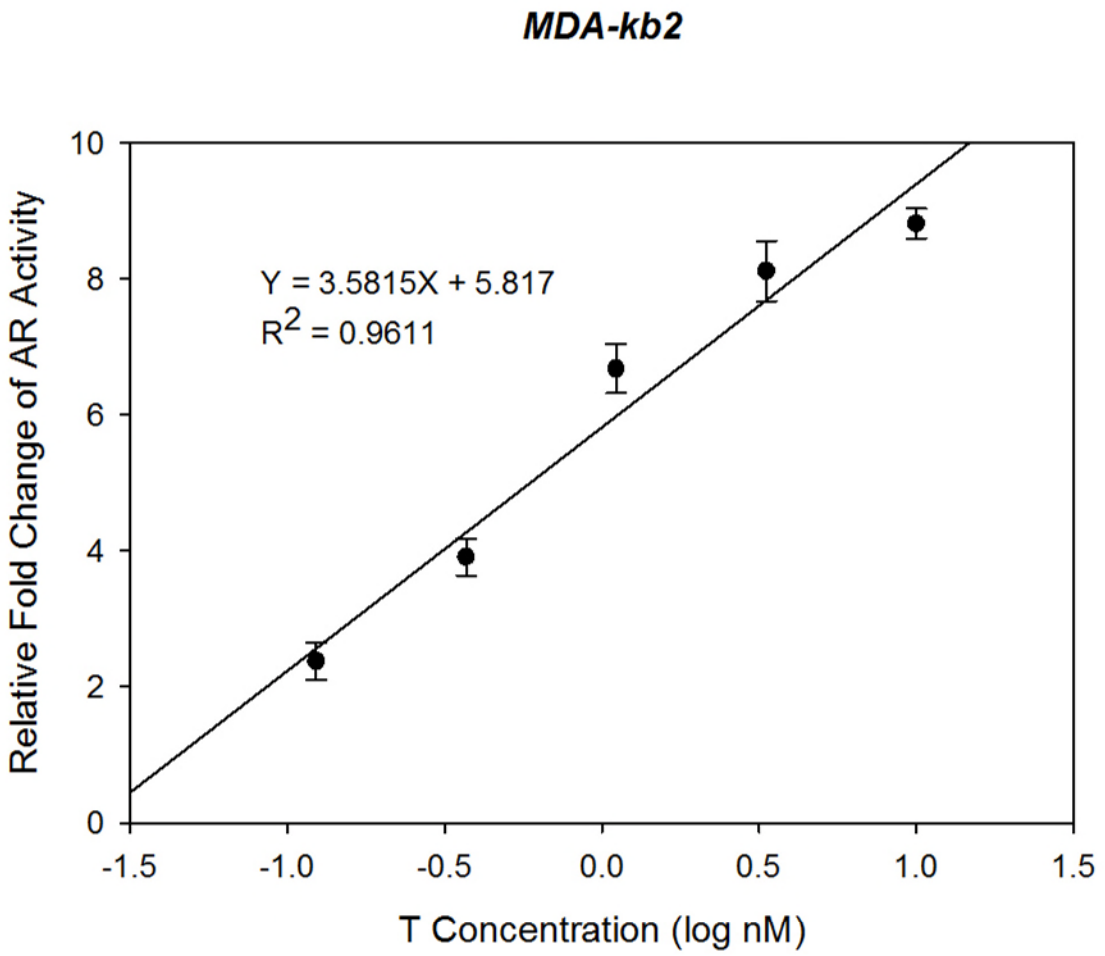

Fig. S4. Example of standard curve of Testosterone (T) in MDA-kb2 agonist cell assay.

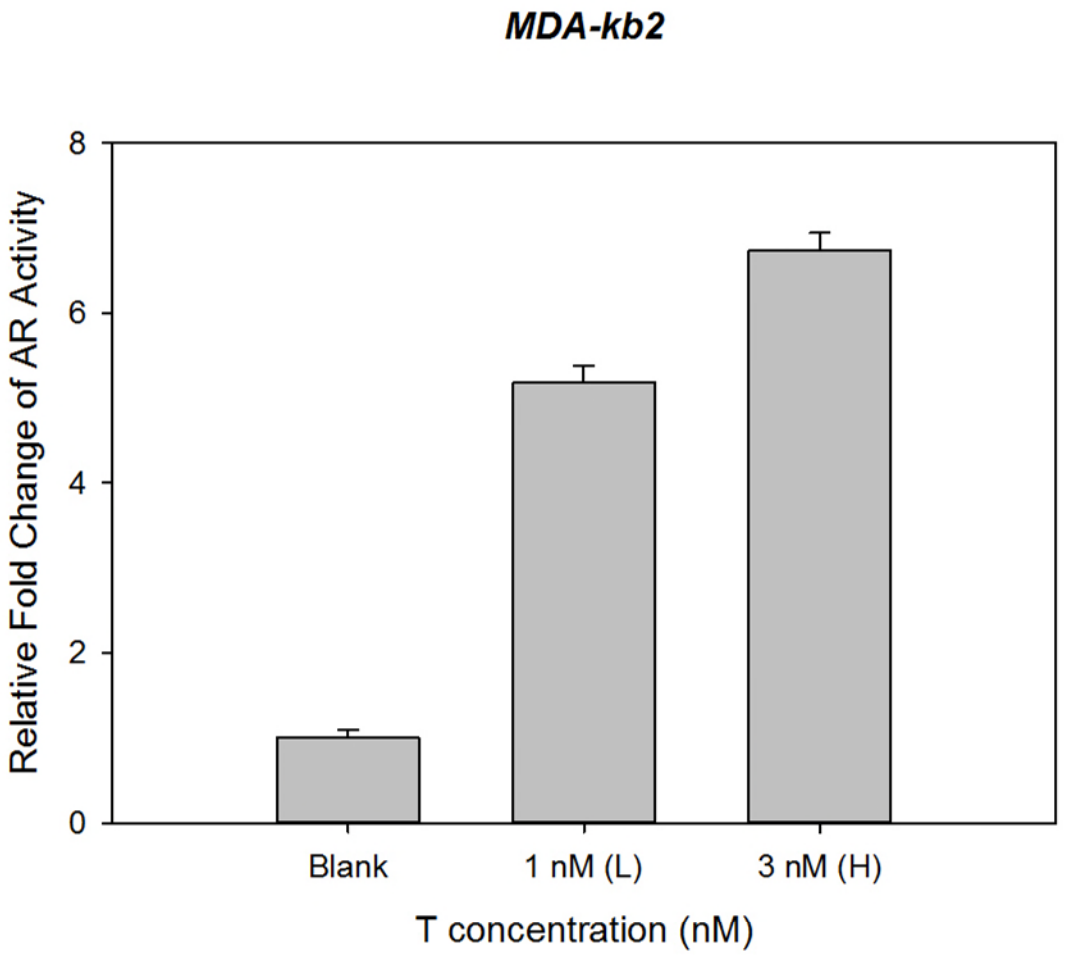

Fig. S5. Relative fold changes of positive ligand (low and high levels of T) in MDA-kb2 antagonist cell assay.

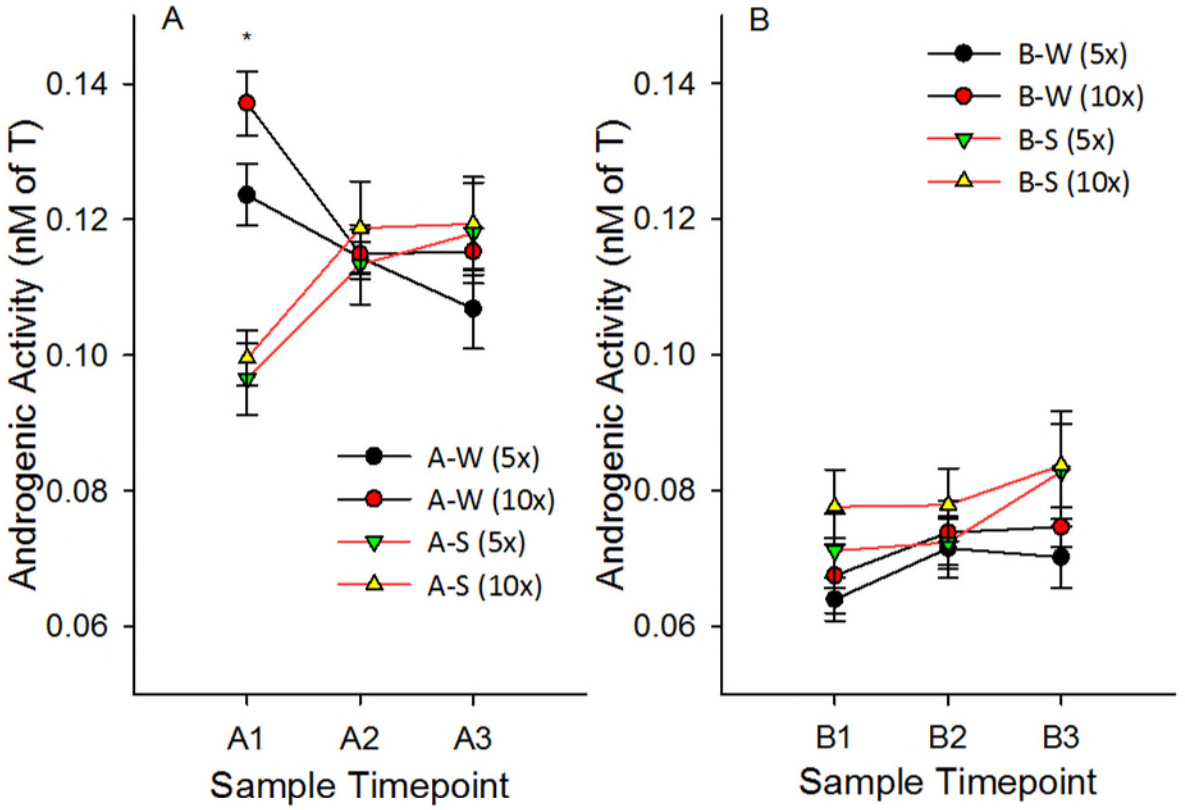

Fig. S6. Androgenic activity in the organic extracts of HF-FPW in MDA-kb2 antagonist cell assay. (A) Well A. (B) Well B. A1-W at 10× exposure was the only one that displayed statistically significant AR agonist activity (indicated by asterisk) ($p < 0.05$).

Reference

He, Y., Wiseman, S.B., Hecker, M., Zhang, X., Wang, N., Perez, L.A., Jones, P.D., El-Din, M.G., Martin, J.W., Giesy, J.P., 2011. Effect of ozonation on the estrogenicity and androgenicity of oil sands process-affected water. Environ. Sci. Technol. 45, 6268-6274.

Larsson, M., J.P. Giesy and M. Engwall., 2014. AhR-mediated activities of polycyclic aromatic compound (PAC) mixtures are predictable by the concept of concentration addition. Environ. Internat. 73, 94–103.

Leclair, L.A., Pohler, L., Wiseman, S.B., He, Y., Arens, C.J., Giesy, J.P., Scully, S., Wagner, B.D., van den Heuvel, M.R., Hogan, N.S., 2015. *In vitro* assessment of endocrine disrupting potential of naphthenic Acid fractions derived from oil sands-influenced water. Environ. Sci. Technol. 49, 5743-5752.

Nisbet, I.C., LaGoy, P.K., 1992. Toxic equivalency factors (TEFs) for polycyclic aromatic hydrocarbons (PAHs). Regul. Toxicol. Pharmacol. 16, 290-300.

Snyder, S.A., Villeneuve, D.L., Snyder, E.M., Giesy, J.P., 2001. Identification and quantification of estrogen receptor agonists in wastewater effluents. Environ. Sci. Technol. 35, 3620-3265.

Zhang, Y., Shotyk, W., Zaccone, C., Noernberg, T., Pelletier, R., Bicalho, B., Froese, D., Davies, L.J., Martin, J.W., 2016. Airborne petcoke dust is a major source of polycyclic aromatic hydrocarbons in the Athabasca Oil Sands Region. Environ. Sci. Technol. 50, 1711-1720.