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Effects on Biotransformation, Oxidative Stress, and Endocrine Disruption in Rainbow Trout (*Oncorhynchus mykiss*) Exposed to Hydraulic Fracturing Flowback and Produced Water

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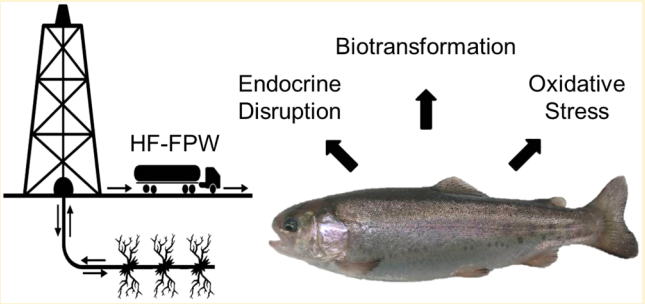
Supporting Information

ABSTRACT: The effects of hydraulic fracturing (HF) flowback and produced water (HF-FPW), a complex saline mixture of injected HF fluids and deep formation water that return to the surface, was examined in rainbow trout (*Oncorhynchus mykiss*). Exposure to HF-FPWs resulted in significant induction of ethoxyresorufin-O-deethylase (EROD) activity in both liver and gill tissues. Increased lipid peroxidation via oxidative stress was also detected by thiobarbituric acid reactive substances (TBARS) assay. The mRNA expressions of a battery of genes related to biotransformation, oxidative stress, and endocrine disruption were also measured using quantitative real-time polymerase chain reaction (Q-RT-PCR). The increased expression of *cyp1a* (2.49 ± 0.28-fold), *udpgt* (2.01 ± 0.31-fold), *sod* (1.67 ± 0.09-fold), and *gpx* (1.58 ± 0.10-fold) in raw sample exposure group (7.5%) indicated elevated metabolic enzyme activity, likely through the aryl hydrocarbon receptor pathway, and generation of reactive oxygen species. In addition, the elevated *vtg* and *era2* expression demonstrated endocrine disrupting potential exerted by HF-FPW in rainbow trout. The overall results suggested HF-FPW could cause significant adverse effects on fish, and the organic contents might play the major role in its toxicity. Future studies are needed to help fully determine the toxic mechanism(s) of HF-FPW on freshwater fish, and aid in establishing monitoring, treatment, and remediation protocols for HF-FPW.



INTRODUCTION

Horizontal drilling with high-volume hydraulic fracturing (HF) is a practice being used in Alberta/Canada for improving the extraction of oil and gas from tight reservoirs. Energy production from these resources in North America is expected to continue, with estimated increases of 45% and 25% above current production levels for the US and Canada, respectively, in the next 25 years.[1,2] The rapid expansion of HF practices, together with its large quantity of water usage and process affected water production, poses potential environmental hazards to the environment, including contamination of surface and shallow groundwater aquifers via discharges and spills,[3−6] as well as subsurface gas migration.[7−11]

However, there remain significant knowledge deficits on the environmental impacts and risks of the flowback and produced water (HF-FPW) to aquatic ecosystems.[12] HF-FPW is a complex, tripartite mixture of injected HF fluid components, deep formation water, and secondary byproducts of downhole reactions with the formation environment.[11,13−15] HF-FPW brine may contain numerous inorganic and organic constituents, including high levels of metals (e.g., barium, strontium, chromium, cadmium, lead), radionuclides (e.g., radium and uranium), and a complex profile of organic compounds, theorized to be additive components in HF fluid, natural organics related to *in situ* formation hydrocarbons (e.g., polycyclic aromatic hydrocarbons (PAHs)), and even secondary chemical products from the interaction between the fracturing environment in the well (elevated temperature and/or pressure) and the deep saline groundwater.[16−18] Correspondingly, the concentrations of metals, radionuclides, and PAHs detected in FPW are often well above the maximum contamination level for water quality guidelines.[5,6,19] Together, these chemicals return to the surface with released oil and gas, and the HF-FPW is separated for treatment/reuse, or disposed in deep, subsurface injection wells.[20] Risks of ground and/or surface fresh water contamination are principally associated with on-site fluid handling, transportation of HF-FPW to disposal wells, and well integrity issues.[5] Accidental release of HF-FPW in certain regions is well documented, with more than 2500 spills in Alberta from 2011 to 2014.[21] It has been suggested that the presence of endocrine disrupting chemicals

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in HF wastewater may be linked to reproductive and developmental impairment in laboratory animals based on the systematic evaluation of chemicals used in HF fluids.[22] While the potential biological risk and impacts of chemicals used during the fracturing process have been predicted and documented in several reviews,[23,24] there is very limited information regarding the toxicity of any real HF-FPW samples and the potential toxicological impacts of HF-FPW spills on freshwater organisms.

The lack of available hazard assessment for HF-FPW spills in Canada and the United States hinders environmental impact and risk assessment of hydraulic fracturing activities.[25] Mandatory disclosure of the chemical constituents of fracturing fluids for example, through the chemical disclosure registry, FracFocus, has somewhat improved our understanding but the toxicity data of many chemicals is often missing.[23,25] The environmental fates of those chemicals are further complicated by potential down-hole reactions and generation of secondary products.[18] Therefore, there exists an obvious need to investigate the toxicity on aquatic organisms. In this study, juvenile rainbow trout (*Oncorhynchus mykiss*), commonly used as a biologically relevant freshwater model for regulatory science, were used to determine responses to potential spills and leaks of HF-FPW in the aquatic environment. Acute exposures (48 h) were conducted followed by measurements of a variety of endpoints including hepatic and branchial ethoxyresorufin-O-deethylase (EROD) activity, thiobarbituric acid reactive substance (TBARS) formation in various tissues, and mRNA abundance of a battery of genes related to biotransformation, oxidative stress, and endocrine disruption by quantitative real-time polymerase chain reaction (Q-RT-PCR). This is one of the first studies to investigate the physiological responses to HF-FPW exposure in a whole organism. Our study will help address the ecotoxicological hazards associated with HF-FPW, and provide potential biomarkers for water quality monitoring in areas affected by hydraulic fracturing activities.

MATERIALS AND METHODS

HF-FPW Collection. The HF-FPW sample analyzed in this study was collected at 7 days post-stimulation from a horizontal, hydraulically fractured well in the Devonian-aged Duvernay Formation (Fox Creek, Alberta, Canada) by Encana Services Company Ltd. In this study, HF-FPW-S (abbreviated as S in figures, the same below) refers to the original, raw sample containing sediment and/or suspended particles. A summary of key compositional information on this sample is presented in Table 1. Detailed geological and chemical information on this sample is reported in a companion study.[18] All tests were conducted within 60 days of sample acquisition and samples were stored at room temperature to best reflect on-storage conditions. Sediment-free (HF-FPW-SF, or SF) was prepared by vacuum filtration of the raw sample through a 0.22 μm membrane, which also greatly reduced the levels of total organic contaminants in the sample. An activated-charcoal treated (HF-FPW-AC, or AC) sample was also prepared by treating the raw sample with activated charcoal, followed by vacuum filtration through a 0.22 μm membrane. This resulted in most of the organic contaminants being removed. We acknowledge that vacuum filtration may also have caused a loss of volatile dissolved components in SF and AC subsamples but this was necessary to remove the sediment fraction. Once we aliquoted a raw sample for either treatment or direct exposure (HF-FPW, HF-FPW-SF, and HF-FPW-AC), these aliquots were stored at 4 °C until the start of the exposure period. Details of the sample preparation are described in a companion study.[18]

Table 1. A Summary of Key Composition of a HF-FPW Sample. Data Are Obtained from a Companion Study.[18]

HF-FPW sample	
time (post-stimulation)	7 days
pH	4.78
total dissolved solid	242 624 mg/L
total nitrogen	498 mg/L
total organic carbon	211 mg/L

Chemicals. All the chemicals were purchased from Sigma-Aldrich (USA). Details are provided in the Supporting Information.

Fish. Rainbow trout embryos were obtained from the Raven Brood Trout Station (Caroline, AB, Canada) and grown to the appropriate size (24.9 ± 10.1 g) as juveniles for experimentation. Juvenile fish were maintained indoors in flow-through 450 L tanks supplied with aerated and dechlorinated facilitate water (hardness as $CaCO_3$, 1.6 mmol/L; alkalinity, 120 mg/L; NaCl, 0.5 mmol/L; pH 8.2, temp, 10 ± 1 °C). Fish were fed ground dry commercial trout pellets (Purina trout chow) once daily and kept on a 14 h/10 h day/night photoperiod. All animal use was approved by the University of Alberta Animal Care Committee under Protocol AUP00001334.

Exposure Design. Exposure was conducted in 8 L glass tanks filled with 4 L of aerated control/treatment water. Water temperature was maintained at 10 ± 1 °C by partial immersion of tanks in a water bath with constant facility water flow; 50% control/treatment water changes were made in each tank every 24 h. Fish were fasted 3 days prior to and during experimentation. Fish were exposed to control/treatment waters in triplicate tanks, with each tank containing two individuals. The concentrations of low dose (2.5%) and high dose (7.5%) were selected based on the results of preliminary range finding test of HF-FPW-S using finger-length juvenile rainbow trout (Figure S1). Fish were exposed to facility water as a control (Ctl), HF-FPW-AC (2.5% and 7.5% dilutions in facility water, the same below), HF-FPW-SF (2.5% and 7.5%) and HF-FPW-S (2.5% and 7.5%), as well as benzo[a]pyrene (BaP) (0.33 and 1 μM) as a positive control for 24 and 48 h. At the end of exposure, fish were euthanized by cephalic blow and decapitated. Gill filament and liver samples were collected and immediately assayed for EROD activity. Subsamples (0.25 g) of gill, liver, and kidney in all 48 h exposure groups were placed into 1.5 mL Eppendorf tubes containing 500 μL of phosphate buffer (100 mM KH_2PO_4, 5 mM EDTA, pH = 7.5), frozen in liquid nitrogen and stored at −80 °C for TBARS assay. Subsamples of liver in all 48 h exposure groups were frozen and stored at −80 °C for Q-RT-PCR assay. The exposure water from each treatment was sampled and stored in the dark at 4 °C prior to PAHs analysis.

PAH Analysis. Subsamples of exposure water at 24 and 48 h (500 mL), including control, AC (2.5% and 7.5%), SF (2.5% and 7.5%), and S (2.5% and 7.5%), were collected for polycyclic aromatic compound analysis by the liquid−liquid extraction method as described previously.[26] Detailed methodology is provided in the Supporting Information and in Table S1.

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Hepatic and Branchial EROD Assays. The hepatic EROD activity was determined via a modified method from Hudson et al. (1991).[27] The gill-filament-based EROD assay was performed followed the method described by Jönsson et al. (2002).[28] Details are provided in the Supporting Information.

TBARS Assay. TBARS assay using fish tissues was performed following a previous study with minor modification.[29] Briefly, the supernatant of the homogenized samples were treated with thiobarbituric acid and TBARS formation was quantified by fluorescence measurement at 531 and 572 nm (excitation and emission, respectively). In addition, the intrinsic oxidative potentials of HF-FPW fractions alone were also determined via a modified protocol from Biaglow et al. (1997).[30] HF-FPW samples were incubated with a final concentration of 4 mM 2-deoxy-d-ribose (2-DR) for 1 h at 25 °C under ambient room light, followed by fluorescence measurement of TBARS formation. Details are provided in the Supporting Information.

Quantitative Real-Time PCR assay. Total RNA was extracted from liver samples and cDNA was prepared for quantitative real-time PCR (Q-RT-PCR) measurement using SYBR Green master mix system (Applied Biosystems, CA). Details of RNA extraction, cDNA synthesis, and Q-RT-PCR reactions were provided in the Supporting Information. Eleven genes representing biotransformation, oxidative stress, and endocrine disruption in rainbow trout were selected for screening. Changes in abundances of transcripts of target genes were quantified by normalizing to *elongation factor 1a* (*elf1a*). There was no difference in the expression of *elf1a* among all the exposure groups (Figure S2). Gene name, abbreviation, sequences of primers, efficiency, and GeneBank reference number are listed in Table S2.

Statistical Analysis. Juvenile rainbow trout were exposed to the control/treatment solutions in triplicate tanks, with each tank containing two fish. No differences in responses to the same treatment between fish in the same tanks were observed. Therefore, each individual fish is considered an experimental unit. Statistical analyses were conducted by use of SPSS16.0 (SPSS, Chicago, IL). All data are expressed as mean ± standard error mean. Log transformation was performed if necessary to meet the assumptions. Statistical differences were evaluated by one-way ANOVA followed by posthoc Tukey test. Differences were considered significant at $\rho < 0.05$.

RESULTS AND DISCUSSION

PAH Analysis. Nominal and measured exposure concentrations of parent PAHs and total PAHs (parent + alkylated PAHs) are presented in Table 2. Detailed results of individual analytes are presented in Table S3. Generally, the measured PAH concentrations in the sediment free fraction exposure water (HF-FPW-SF dilutions) were lower or equivalent compared to nominal levels; however, PAH concentrations in the raw exposure water (HF-FPW-S dilutions) were higher than nominal (Table 2), suggesting the possibility of chemical desorption from the sediment particles present in the HF-FPW sample during the exposure period. Since HF-FPW is a complex mixture of HF fluid and formation water, it is likely that not only PAHs but a variety of other contaminants could desorb from sediment particles, resulting in more adverse effects on exposed fish compared to those exposed to a sediment-free fraction.

EROD Assays. Exposure to HF-FPWs caused significant induction of EROD activity in both hepatic and branchial tissues in rainbow trout. The hepatic and branchial EROD assays were modified from previous studies using S-9 fraction[27] and gill filament,[28] respectively. Assay validation was performed by using a parallel BaP positive control (Figure 1). The relative fold of induction in hepatic and branchial EROD activity in 24 and 48 h exposure groups are presented in Figure 1.

Significant induction of EROD activity has been widely used as a biomarker of exposure to aryl hydrocarbon receptor (AhR) agonistic contaminants, including, PAHs, dioxins, polychlorinated biphenyls (PCBs), and various petroleum-related extracts.[31] BaP is a prototype and well documented PAH widely used as positive control in EROD activity assay.[32] Previously, significant EROD induction was demonstrated in zebrafish larvae exposed to HF-FPWs using a nondestructive assay,[33] indicating that the presence of PAHs and possibly other contaminants acting as AhR agonists may play a significant role in HF-FPW toxicity.[18] In the current study, hepatic and branchial EROD activities were measured in juvenile rainbow trout exposed to HF-FPWs by using liver S-9 fractions and gill filaments as indicator tissues. The results clearly demonstrate that exposure to HF-FPWs significantly induces EROD activity in both liver and gill tissues in juvenile rainbow trout, which is consistent with the companion study.[18] In addition, on the basis of induction fold data, branchial tissues seem to display more sensitive EROD activity responses than hepatic tissue after HF-FPW exposure (Figure 1A,B). Hepatic EROD inductions greater than 2.5-fold were not observed, and significant induction only occurred in tissue exposed to HF-FPW-S. Conversely, significant branchial EROD induction was detected in the 2.5% dilution of the HF-FPW-SF group, and in general, induction of EROD activity in gill tissues was greater than those in hepatic tissues. For example, exposure to 7.5% of HF-FPW-S for 48 h resulted in an EROD induction of 9.30 ± 0.31-fold in branchial tissue while a 2.41 ± 0.25-fold increase was found in hepatic tissue. Since the gill is an organ characterized by high surface areas, a thin membrane, concentrated vascularization, and is directly exposed to water-born contaminants, it is likely gill filaments receive higher levels of exposure compared to liver tissue, resulting in a more

Table 2. Parent PAHs and Total PAHs (Parent + Alkylated) in HF-FPW-AC, HF-FPW-SF, and HF-FPW-S Exposure Waters at 24 and 48 h[a]

sample		dilutions	nominal concn (ng/L)	measured concn (ng/L)	
				at 24 h	at 48 h
HF-FPW-AC	parent	2.5%	N.D.	N.D.	N.D.
		7.5%	N.D.	N.D.	N.D.
	total	2.5%	N.D.	N.D.	N.D.
		7.5%	N.D.	N.D.	N.D.
HF-FPW-SF	parent	2.5%	13	30	19
		7.5%	39	31	17
	total	2.5%	29	34	20
		7.5%	87	39	19
HF-FPW-S	parent	2.5%	24	89	64
		7.5%	73	220	160
	total	2.5%	51	150	110
		7.5%	150	390	300

[a]Nominal concentrations are calculated based on the results from a companion study.[18] All the PAHs in control and HF-FPW-AC samples were below the detection limit. N.D. means not detected.

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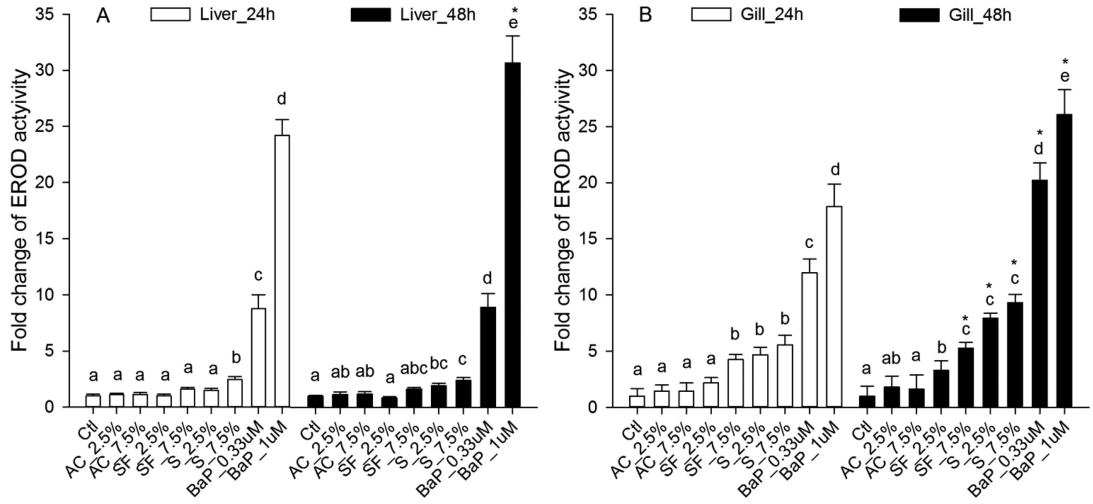

Figure 1. Induction of (A) liver and (B) gill EROD activity in rainbow trout exposed to HF-FPWs at 2.5% and 7.5% dilutions for 24 and 48 h. BaP (0.33 and 1 μM) was used as positive control. Different letters indicate significant differences within the same treatment time in liver or gill tissues (n = 6, ρ < 0.05,). Asterisks indicate significant differences between 24 and 48 h treatments (n = 6, ρ < 0.05).

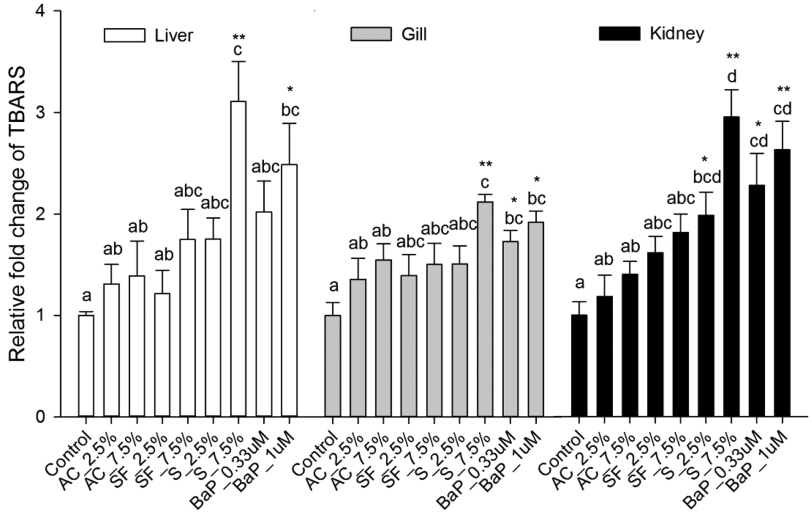

Figure 2. TBARS in the liver, gill, and kidney tissue of rainbow trout exposed to control, HF-FPW-AC, HF-FPW-SF, and HF-FPW-S at 2.5% and 7.5% dilutions for 48 h. Asterisks (n = 6, *ρ < 0.05; ** ρ < 0.01) indicate significant differences from control. Different letters indicate significant differences within the same treatment time in liver or gill tissues (n = 6, ρ < 0.05).

significant and sensitive EROD activity response. In addition, since most of the organic content has been removed by activated charcoal treatment,[18] the absence of significant effects on EROD activity in all AC exposure groups clearly supports the hypothesis that the organic contaminants were the major components responsible for EROD induction. Considering the dilution factors and the low total PAH levels detected in the sample, the results observed in the current study confirmed that EROD activity in fish is sensitive to highly diluted HF-FPW exposures, and could be used as efficient biomarker for an HF-FPW spill or in a post-spill monitoring program.

TBARS Assay. HF-FPW exposure resulted in elevated TBARS formation in juvenile rainbow trout tissues indicating oxidative stress. The highest TBARS tissue levels were observed in fish exposed to 7.5% HF-FPW-S (2.95 ± 0.66, 2.12 ± 0.18, and 3.11 ± 0.97-fold for kidney, gill, and liver tissues, respectively). Exposure to 2.5% of HF-FPW-S also significantly increased TBARS formation in rainbow trout kidney tissue (1.99 ± 0.55-fold). There were no additional significant oxidative stress effects observed in either high or low exposure of HF-FPW-SF and HF-FPW-AC groups. Significant increases in TBARS formation were also seen in our positive control (BaP) treatments, validating our methodologies (Figure 2). Overall, it was observed that HF-FPW-S displayed increased TBARS formation, with highest oxidative stress responses seen in higher HF-FPW concentrations.

Interestingly, the results of the TBARS assay demonstrated significant intrinsic oxidative potential of HF-FPW samples (Figure 3). Incubation of 2.5% and 7.5% of HF-FPW-S with 4 mM 2-DR at 25 °C for 1 h resulted in significantly elevated TBARS formation by 8.68 ± 1.41, and 13.74 ± 0.59-fold, respectively, compared to control water. Similarly, 2.5% and 7.5% of HF-FPW-SF (incubation with 4 mM 2-DR at 25 °C for 1 h,) also resulted in significantly elevated TBARS formations (9.01 ± 1.12 and 13.13 ± 1.32-fold, respectively). No TBARS formation was observed in HF-FPW-AC (Figure 3). Both HF-FPW-SF and HF-FPW-S contain various organic contaminants including PAHs, while organic contaminants, including PAHs,

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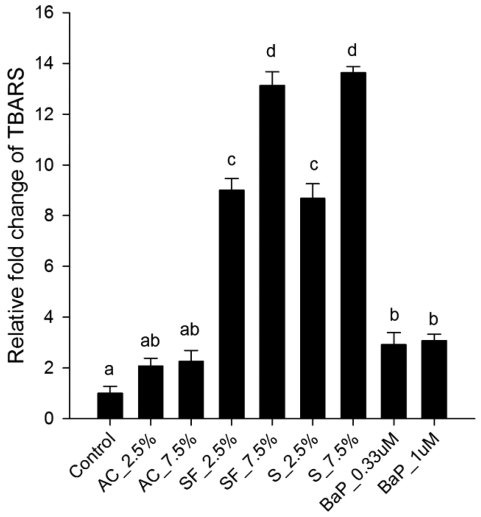

Figure 3. TBARS formation in control as well as HF-FPW-AC, HF-FPW-SF, HF-FPW-S at 2.5% and 7.5% dilutions spiked with 4 mM 2-deoxy-d-ribose and samples taken for TBARS measurement after 1 h incubation under ambient light at 25 °C. Different letters indicate significant differences between treatment groups (n = 6, ρ < 0.05).

in HF-FPW-AC are below detection limits (Table 2).[18] Our results suggest that significantly increased oxidative potential is inherent to both HF-FPW-S and HF-FPW-SF, but not to HF-FPW-AC, presumably due to organic compounds present in HF-FPWs.

Oxidative stress is one of the major adverse effects on aquatic organisms of many contaminants.[34] Our study determined that raw HF-FPW at concentrations of 7.5% significantly increased TBARS formation (lipid peroxidation) in kidney, gill, and liver tissue after acute exposures to HF-FPW (Figure. 2). While endogenous production of reactive oxygen species (ROS) is essential to many cellular functions, increased ROS production by exogenous influences may overwhelm cell oxidative homeostatic and antioxidant defense systems, negatively impacting cell and organism physiological health.[34] The TBARS assay detects malondialdehyde (MDA), an aldehyde produced via lipid membrane oxidation and damage.[35] However, it is important to note that many other reactive lipid peroxide products, such as alkoxyls, alkanes, and lipid epoxides, which are known to be toxic and mutagenic,[36] may be produced. Many components of HF-FPW are theorized to induce oxidative stress upon exposure. Added oxidant chemicals in the fracturing fluid may elicit oxidative responses, while other chemicals secondarily formed in the subsurface may additionally increase oxidative stress to organisms exposed to HF-FPW. The intrinsic ROS potential of both the SF and S fractions were significantly greater than control as well as AC fraction, suggesting that the organic constituents are responsible for the majority of the ROS activity in HF-FPWs (Figure 3). However, we observed significant TBARS formation only in tissues of fish exposed to sediment containing fractions. This indicates that sediment constituents (including all the suspended particles and the chemicals potentially sorbed to them) further contribute to physiological oxidative stress and observed adverse effects in fish exposed to HF-FPWs (Figure 2). Our companion study suggested the suspended particles present in FPW might play a synergistic role in causing adverse effects by adhering on the fish body surface acting as a delivering vehicle to enhance exposure rate.[18] Another possibility is that the concentrations of contaminants were better maintained by desorption of chemicals from sediment particles, thus resulting in greater response in adverse effects in HF-FPW fish exposure. This hypothesis is supported by the fact that in HF-FPW samples, PAHs levels in sediment-containing samples were higher than nominal levels potentially due to desorption, while PAH levels in sediment-free samples were lower than nominal levels potentially due to dissipation. This response supports our EROD analyses showing higher EROD activity in gill and liver tissue of fish exposed to sediment-containing HF-FPW fractions over sediment-free fractions (Figure 1A and B). Nephritis, characterized by increased lipid peroxidation products, is a well-studied physiological inflammatory response observed in kidney tissue under oxidative stress.[37] Correspondingly, significant TBARS formation in kidney tissue was observed after 48 h exposures to 2.5% and 7.5% HF-FPW-S (1.99 ± 0.55 and 2.95 ± 0.66-fold, respectively). Liver tissues are responsible for many detoxification and antioxidant defense responses to xenobiotics,[38] and our results similarly displayed elevated liver TBARS formation after exposure to HF-FPW-S (7.5% after 48 h; 3.11 ± 0.97-fold). Gill tissue also displayed increased TBARS formation (2.12 ± 0.18-fold). Although the magnitudes of fold induction of TBARS formation in fish

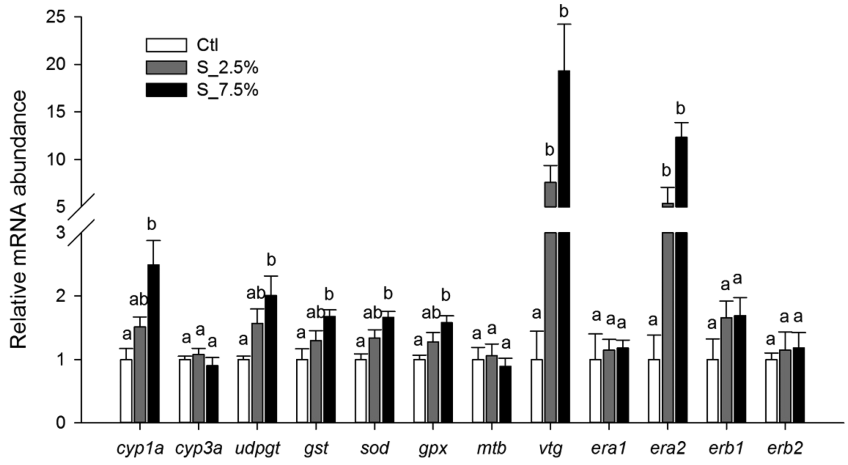

Figure 4. Hepatic mRNA expression in rainbow trout exposed to control and HF-FPW-S (2.5% and 7.5%) for 48 h. Different letters indicate significant differences within the group (n = 6, ρ < 0.05).

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tissues were less significant than the intrinsic oxidative potentials observed in direct measurement of HF-FPWs, this does not necessarily imply that the TBARS formation observed in exposed fish tissues was less biologically significant. In fact, the elevated TBARS formation indicated that the oxidative stress generated by HF-FPWs exposure had already overwhelmed the ROS defense system. Rainbow trout tissue oxidative stress responses after HF-FPW exposure were consistent with various other tissue expression profiles observed in fish exposed to oil-contaminated wastewater effluent and spills,[39] suggesting oxidative stress likely contributes to the overall adverse effects observed in the current study.

Quantitative Real-Time PCR Assay. Compared to the control, exposure to HF-FPW-S (2.5% and 7.5%) for 48 h significantly induced several tested genes in juvenile rainbow trout liver (Figure 4). In 7.5% of HF-FPW-S group, as compared to control, the expressions of *cyp1a* and *udpgt* were significantly elevated by 2.49 ± 0.38 and 2.01 ± 0.31-fold, respectively, indicating the activation of Phase I and Phase II biotransformation genes. In addition, in the 7.5% of the HF-FPW-S group, the expression of *sod* and *gpx* were also significantly elevated by 1.67 ± 0.09 and 1.58 ± 0.10-fold, respectively, indicating the activation of oxidative stress response genes. Moreover, the expression of *vtg* and *erα2* were significantly elevated by 7.60 ± 1.77 and 5.37 ± 1.70-fold, respectively, in the 2.5% of HF-FPW-S group, and 19.33 ± 4.89 and 12.33 ± 1.56-fold, respectively in the 7.5% of HF-FPW-S group, suggesting exposure to HF-FPW-S has the potential to alter endocrine metabolism in fish. There were no significant changes of mRNA abundance in the genes tested in the groups of AC and SF (2.5% and 7.5%) (Figure S3). Low and high doses of BaP (0.33 and 1 μM) were also applied as positive controls to confirm the dose-dependent response of *cyp1a* in tested fish (Figure S4).

In the current study, mRNA abundance of seven genes related to biotransformation and oxidative stress were determined in juvenile rainbow trout exposed to HF-FPWs for 48 h. Metallothionein β (*mtβ*) is an enzyme important for metal metabolism, and is commonly used as bioindicator of metal exposure.[40] Although there were nominal amounts of heavy metals detected in our sample, with the dilution applied in this study, the concentrations and predicted toxicity would be very limited as compared to other HF samples;[6,18] therefore, it was not surprising that exposure to HF-FPWs did not affect the expression of *mtβ*. Cytochrome p450 1A (*cyp1a*) and 3A (*cyp3a*) are Phase I biotransformation enzymes which are responsible for hydroxylation modification of xenobiotics.[38] UDP-glucoronosyl transferase (*udpgt*) and glutathione transferase (*gst*) are Phase II biotransformation enzymes responsible for detoxifying reactive electrophiles and producing more polar metabolites for active transport.[38] Exposure to HF-FPW-S caused significant induction in *cyp1a*. Together with the observed gill and liver EROD induction, we theorized the presence of AhR agonist(s) in HF-FPW caused activation of Phase I biotransformation. The absence of increased expression of *cyp3a* indicated that there were no or very low concentrations of *cyp3a* agonists (for example, dexamethasone) in the HF-FPW-S sample. However, a significantly increased expression of *udpgt* and *gst* suggests that exposure to HF-FPW-S also triggers Phase II enzymes, which taken together with Phase I enzyme induction results, indicates activation of biotransformation and detoxification processes of xenobiotics in juvenile rainbow trout, which may be used as a biomarker in future monitoring programs. Additionally, mRNA abundance of two oxidative response genes, glutathione peroxidase (*gpx*) and superoxide dismutase (*sod*) was also elevated in the HF-FPW-S exposure group. Oxidative stress in aquatic organisms is one of the most common adverse effects caused by exposure of petroleum-related contaminants in aquatic environments.[34,41] In the current study, ROS may be generated from either activated xenobiotic metabolites generated in the Phase I biotransformation by cytochrome P450 and other oxidation enzymes, or potentially via the oxidizing chemicals originally presented in the fracturing fluid. In this well, ammonium sulfate, a strong oxidizing agent, was added into the fracturing fluid to break the added antioxidant, Irgafos 168, and thus promote the fracturing process. The small but significant elevated expression of *sod* and *gpx* (magnitudes <2-fold), demonstrated the potential activation of ROS defense enzymes in response to HF-FPW exposure. These may be due to oxidative stress from either or both xenobiotic metabolism and oxidizing chemicals originally present in HF-FPW sample. Together with the TBARS results, the activation of *sod* and *gpx* demonstrated the oxidative stress response in juvenile rainbow trout exposed to HF-FPWs.

In this study, mRNA abundance of five endocrine disruptive genes, including *vitellogenin* (*vtg*) and four estrogen receptor isoforms (*erα1*, *erα2*, *erβ1*, and *erβ2*) were also measured. Vitellogenin, the precursor of lipoproteins and phosphoproteins which comprises most of the protein content of fish yolk, has been widely used as a sensitive biomarker on endocrine disrupting chemicals (EDCs) exposure in oviparous species.[42−44] It has been demonstrated that exposure to petroleum related contaminants can induce significant elevation of *vtg* transcripts and protein in fish.[39,42,44] Xenoestrogens can interact with the estrogen receptor (ER) to exert their endocrine disrupting effects in organisms. Rainbow trout has two distinct ER subtypes, ERα and ERβ, each of which has two distinct isoforms.[45,46] In this study, Q-RT-PCR primers of four estrogen receptor isoforms were designed to determine differential receptor isoform responses to potential EDC exposure. Interestingly, exposure to HF-FPW resulted in significantly higher mRNA abundance of *vtg*, as well as *era2*, but not the other three *er* isoforms. In rainbow trout, the full-length estrogen receptor alpha (*era1*) is widely expressed in liver, brain, pituitary, and ovary. However, expression of the alternative splicing isoform resulting in a truncated A domain (*era2* in this paper) is primarily in the liver.[45] These results suggest a role for *era2* in the development and/or maintenance of the vitellogenesis process unique to the liver.[45,46] In this study, the elevated expression of *vtg* and *era2* were consistent in rainbow trout exposed to HF-FPW-S, indicating the presence of EDCs (in particular, xenoestrogens) in HF-FPW that are able to disrupt and/or alter vitellogenesis synthesis in oviparous species. This study is the first research reporting elevation of *vtg* and *era2* expression in fish caused by HF-FPW exposure. Significantly elevated expression of *vtg* and *era2* demonstrate possible endocrine disruptive effects derived from HF-FPW exposure and the potential hazards to the aquatic organisms from a HF-FPW spill. Future research should focus on the potential endocrine disruptive properties of HF-FPW including advanced chemical analyses to determine the potential EDC(s) in HF-FPW.

In conclusion, the current study demonstrates the complexity and diverse mechanisms of adverse effects of HF-FPW using rainbow trout as a model organism. Adverse effects were

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observed at high dilutions in both SF and/or S fractions exposure groups, rather than AC fractions exposure groups. This indicates that the organic contaminants rather than the salts *per se* were the major contributor in acute exposure of diluted HF-FPW in fish. Analysis of multiple biomarkers and gene expression for key markers of adverse effects reveal HF-FPW exposure in a biologically relevant fish elicits responses in a variety of pathways, including biotransformation, oxidative stress, and endocrine disruption. Our results further suggest that sediment found in HF-FPW is an important component in causing adverse effects related to biotransformation and oxidative stress pathways, in agreement with our earlier studies.[18] An alternative hypothesis is that the exposure concentration of various contaminants present in HF-FPW was elevated by chemical desorption from sediment particles, thus enhancing the exposure rate. Future study is needed to address the potential adverse effects derived from sediments of HF-FPW, and special attention should be paid to the sediment residues in spill response and the remediation process. This study is also the first to demonstrate potential endocrine disruptive effects associated with HF-FPW thereby warranting future investigation in this area.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b04695.

Chemicals and consumables used in the experiments, methods, and supplementary tables and figures (PDF)

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Notes

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■ REFERENCES

(1) U.S. Energy Information Administration (USEIA). Annual energy outlook 2015 with projections to 2040. U.S. Department of Energy. 2015. http://www.eia.gov/forecasts/aeo/pdf/0383%282015%29.pdf (accessed 2016-06-30).

(2) Natural Resources Canada (NRCan). Energy Fact book 2015–2016. https://www.nrcan.gc.ca/sites/www.nrcan.gc.ca/files/energy/files/pdf/EnergyFactBook2015-Eng_Web.pdf (accessed 2016-06-30).

(3) Entrekin, S.; Evans-White, M.; Johnson, B.; Hagenbuch, E. Rapid expansion of natural gas development poses a threat to surface waters. *Front. Ecol. Environ.* **2011**, *9* (9), 503–511.

(4) Vidic, R. D.; Brantley, S. L.; Vandenbossche, J. M.; Yoxtheimer, D.; Abad, J. D. Impact of shale gas development on regional water quality. *Science* **2013**, *340* (6134), 1235009.

(5) Vengosh, A.; Jackson, R. B.; Warner, N.; Darrah, T. H.; Kondash, A. A critical review of the risks to water resources from unconventional shale gas development and hydraulic fracturing in the United States. *Environ. Sci. Technol.* **2014**, *48* (15), 8334–8348.

(6) Lauer, N. E.; Harkness, J. S.; Vengosh, A. Brine Spills Associated with unconventional oil development in North Dakota. *Environ. Sci. Technol.* **2016**, *50* (10), 5389–5397.

(7) Kargbo, D. M.; Wilhelm, R. G.; Campbell, D. J. Natural gas plays in the Marcellus shale: challenges and potential opportunities. *Environ. Sci. Technol.* **2010**, *44* (15), 5679–5684.

(8) Osborn, S. G.; Vengosh, A.; Warner, N. R.; Jackson, R. B. Methane contamination of drinking water accompanying gas-well drilling and hydraulic fracturing. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108* (20), 8172–8176.

(9) Wilson, J. M.; Van Briesen, J. M. Oil and gas produced water management and surface drinking water sources in Pennsylvania. *Environ. Pract.* **2012**, *14* (4), 288–300.

(10) Darrah, T. H.; Vengosh, A.; Jackson, R. B.; Warner, N. R.; Poreda, R. J. Noble gases identify the mechanisms of fugitive gas contamination in drinking-water wells overlying the Marcellus and Barnett Shales. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111* (39), 14076–14081.

(11) Llewellyn, G. T.; Dorman, F.; Westland, J. L.; Yoxtheimer, D.; Grieve, P.; Sowers, T.; Humston-Fulmer, E.; Brantley, S. L. Evaluating a groundwater supply contamination incident attributed to Marcellus Shale gas development. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112* (20), 6325–6330.

(12) Harkness, J. S.; Dwyer, G. S.; Warner, N. R.; Parker, K. M.; Mitch, W. A.; Vengosh, A. Iodide, bromide, and ammonium in hydraulic fracturing and oil and gas wastewaters: environmental implications. *Environ. Sci. Technol.* **2015**, *49* (3), 1955–1963.

(13) Drollette, B. D.; Hoelzer, K.; Warner, N. R.; Darrah, T. H.; Karatum, O.; O'Connor, M. P.; Nelson, R. K.; Fernandez, L. A.; Reddy, C. M.; Vengosh, A.; Jackson, R. B.; Elsner, M.; Plata, D. L. Elevated levels of diesel range organic compounds in groundwater near Marcellus gas operations are derived from surface activities. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112* (43), 13184–13189.

(14) Lester, Y.; Ferrer, I.; Thurman, E. M.; Sitterley, K. A.; Korak, J. A.; Aiken, G.; Linden, K. G. Characterization of hydraulic fracturing flowback water in Colorado: implications for water treatment. *Sci. Total Environ.* **2015**, *512–513*, 637–644.

(15) DiGiulio, D. C.; Jackson, R. B. Impact to Underground Sources of Drinking Water and Domestic Wells from Production Well Stimulation and Completion Practices in the Pavillion, Wyoming, Field. *Environ. Sci. Technol.* **2016**, *50* (8), 4524–4536.

(16) Ferrar, K. J.; Michanowicz, D. R.; Christen, C. L.; Mulcahy, N.; Malone, S. L.; Sharma, R. K. Assessment of effluent contaminants from three facilities discharging Marcellus shale wastewater to surface waters in Pennsylvania. *Environ. Sci. Technol.* **2013**, *47* (7), 3472–3481.

(17) Gordalla, B. C.; Ewers, U.; Frimmel, F. H. Hydraulic fracturing: a toxicological threat for groundwater and drinking-water? *Environ. Earth Sci.* **2013**, *70* (8), 3875–3893.

(18) He, Y.; Flynn, S. L.; Folkerts, E.; Zhang, Y.; Ruan, D.; Alessi, D. S.; Martin, J. W.; Goss, G. G. Chemical and toxicological characterizations of hydraulic fracturing flowback and produced water. Submitted to *Water Res.* MS # WR37057.

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

(20) Warner, N. R.; Christie, C. A.; Jackson, R. B.; Vengosh, A. Impacts of shale gas wastewater disposal on water quality in Western Pennsylvania. *Environ. Sci. Technol.* **2013**, *47* (20), 11848–11857.

(21) Goss, G. G. *Unconventional Wastewater Management: A comparative review and analysis of hydraulic fracturing wastewater management practices across four North American basins.* Canadian Water Network, 2015; http://www.cwn-rce.ca/project-library/.

(22) Elliott, E. G.; Ettinger, A. S.; Leaderer, B. P.; Bracken, M. B.; Deziel, N. C. A systematic evaluation of chemicals in hydraulic-fracturing fluids and wastewater for reproductive and developmental toxicity. *J. Exposure Sci. Environ. Epidemiol.* **2017**, *27*, 1−10.
(23) Stringfellow, W. T.; Domen, J. K.; Camarillo, M. K.; Sandelin, W. L.; Borglin, S. Physical, chemical, and biological characteristics of compounds used in hydraulic fracturing. *J. Hazard. Mater.* **2014**, *275*, 37−54.
(24) Kahrilas, G. A.; Blotevogel, J.; Stewart, P. S.; Borch, T. Biocides in hydraulic fracturing fluids: a critical review of their usage, mobility, degradation, and toxicity. *Environ. Sci. Technol.* **2015**, *49* (1), 16−32.
(25) Gagnon, G. A.; Krkosek, W.; Anderson, L.; McBean, E.; Mohseni, M.; Bazri, M.; Mauro, I. Impacts of hydraulic fracturing on water quality: a review of literature, regulatory frameworks and an analysis of information gaps. *Environ. Rev.* **2015**, *24* (2), 122−131.
(26) 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.* **2016**, *50* (4), 1711−1720.
(27) Hudson, P. V.; Kloepper-Sams, P. J.; Munkittrick, K. R.; Lockhart, W. L.; Metner, D. A.; Luxon, P. L.; Smith, I. R.; Gagnon, M. M.; Servos, M.; Payne, J. F. Protocols for measuring mixed function oxygenases of fish liver. *Can. Technol. Rep. Fish. Aquat. Sci.* **1991**, *1829*, 1−49.
(28) Jönsson, E. M.; Brandt, I.; Brunström, B. Gill filament-based EROD assay for monitoring waterborne dioxin-like pollutants in fish. *Environ. Sci. Technol.* **2002**, *36* (15), 3340−33444.
(29) Boyle, D.; Al-Bairuty, G. A.; Ramsden, C. S.; Sloman, K. A.; Henry, T. B.; Handy, R. D. Subtle alterations in swimming speed distributions of rainbow trout exposed to titanium dioxide nanoparticles are associated with gill rather than brain injury. *Aquat. Toxicol.* **2013**, *126*, 116−27.
(30) Biaglow, J. E.; Manevich, Y.; Uckun, F.; Held, K. D. Quantitation of hydroxyl radicals produced by radiation and copper-linked oxidation of ascorbate by 2-deoxy-D-ribose method. *Free Radical Biol. Med.* **1997**, *22* (7), 1129−1138.
(31) Sarkar, A.; Ray, D.; Shrivastava, A. N.; Sarker, S. Molecular Biomarkers: their significance and application in marine pollution monitoring. *Ecotoxicology* **2006**, *15* (4), 333−340.
(32) Agency for Toxic Substances and Disease Registry (ATSDR). *Toxicological Profile for Polycyclic Aromatic Hydrocarbons (PAHs)*; U.S. Department of Health and Human Services, Public Health Service: Atlanta, GA, 1995; retrieved 2016−06−30.
(33) Noury, P.; Geffard, O.; Tutundjian, R.; Garric, J. 2006. Non-destructive in vivo measurement of ethoxyresorufin biotransformation by zebrafish prolarva: development and application. *Environ. Toxicol.* **2006**, *21* (4), 324−331.
(34) Valavanidis, A.; Vlahogianni, T.; Dassenakis, M.; Scoullos, M. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicol. Environ. Saf.* **2006**, *64* (2), 178−189.
(35) Fraga, C. G.; Leibovitz, B. E.; Tappel, A. L. Lipid peroxidation measured as thiobarbituric acid-reactive substances in tissue slices: Characterization and comparison with homogenates and microsomes. *Free Radical Biol. Med.* **1988**, *4* (3), 155−161.
(36) Esterbauer, H.; Eckl, P.; Ortner, A. Possible mutagens derived from lipids and lipid precursors. *Mutat. Res., Rev. Genet. Toxicol.* **1990**, *238* (3), 223−233.
(37) Diamond, J. R.; Bonventre, J. V.; Karnovsky, M. J. A role for oxygen free radicals in aminonucleoside nephrosis. *Kidney Int.* **1986**, *29* (2), 478−483.
(38) Xu, C.; Li, C. Y.; Kong, A. T. Induction of phase I, II, and III drug metabolism/transport by xenobiotics. *Arch. Pharmacal Res.* **2005**, *28* (3), 249−268.
(39) Holth, T. F.; Eidsvoll, D. P.; Farmen, E.; Sanders, M. B.; Martinez-Gomez, C.; Budzinski, H.; Burgeot, T.; Guilhermino, L.; Hylland, K. Effects of water accommodated fractions of crude oils and diesel on a suite of biomarkers in Atlantic cod (*Gadus morhua*). *Aquat. Toxicol.* **2014**, *154*, 240−252.
(40) Cobbett, C.; Goldsbrough, P. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annu. Rev. Plant Biol.* **2002**, *53*, 159−182.
(41) He, Y.; Patterson, S.; Wang, N.; Hecker, M.; Martin, J. W.; El-Din, M. G.; Giesy, J. P.; Wiseman, S. B. Toxicity of untreated and ozone-treated oil sands process-affected water (OSPW) to early life stages of the fathead minnow (*Pimephales promelas*). *Water Res.* **2012**, *46* (19), 6359−6368.
(42) Ankley, G. T.; Johnson, R. D. Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. *ILAR J.* **2004**, *45* (4), 469−483.
(43) Miller, D. H.; Jensen, K. M.; Villeneuve, D. L.; Kahl, M. D.; Makynen, E. A.; Durhan, E. J.; Ankley, G. T. Linkage of biochemical responses to population − level effects: a case study with vitellogenin in the fathead minnow (*Pimephales promelas*). *Environ. Toxicol. Chem.* **2007**, *26* (3), 521−527.
(44) He, Y.; Wiseman, S. B.; Wang, N.; Perez-Estrada, L. A.; El-Din, M. G.; Martin, J. W.; Giesy, J. P. 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.* **2012**, *46* (17), 9701−9708.
(45) Menuet, A.; Anglade, I.; Flouriot, G.; Pakdel, F.; Kah, O. Tissue-specific expression of two structurally different estrogen receptor alpha isoforms along the female reproductive axis of an oviparous species, the rainbow trout. *Biol. Reprod.* **2001**, *65* (5), 1548−1557.
(46) Nagler, J. J.; Cavileer, T.; Sullivan, J.; Cyr, D. G.; Rexroad, C., III. The complete nuclear estrogen receptor family in the rainbow trout: discovery of the novel *ERα2* and both *ERβ* isoforms. *Gene* **2007**, *392* (1−2), 164−173.

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Environ. Sci. Technol. 2017, 51, 940−947