Environmental Science & Technology

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Sublethal and Reproductive Effects of Acute and Chronic Exposure to Flowback and Produced Water from Hydraulic Fracturing on the Water Flea *Daphnia magna*

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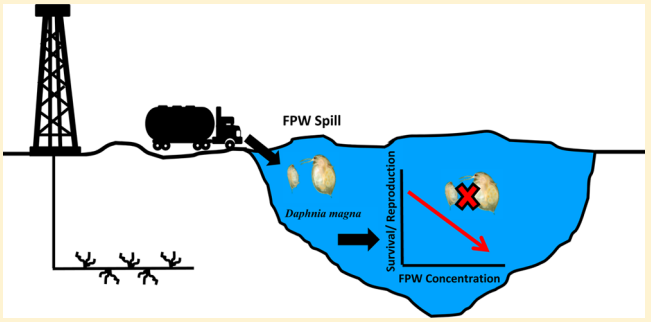
Supporting Information

ABSTRACT: Hydraulic fracturing is an industrial process allowing for the extraction of gas or oil. To fracture the rocks, a proprietary mix of chemicals is injected under high pressure, which later returns to the surface as flowback and produced water (FPW). FPW is a complex chemical mixture consisting of trace metals, organic compounds, and often, high levels of salts. FPW toxicity to the model freshwater crustacean *Daphnia magna* was characterized utilizing acute (48 h median lethal concentrations; LC_{50}) and chronic (21 day) exposures. A decrease in reproduction was observed, with a mean value of 18.5 neonates produced per replicate over a 21 day chronic exposure to 0.04% FPW, which was a significant decrease from the average of 64 neonates produced in the controls. The time to first brood was delayed in the highest FPW (0.04%) treatment. Neonates exhibited an LC_{50} of 0.19% of full-strength FPW, making them more sensitive than adults, which displayed an LC_{50} value of 0.75%. Quantitative PCR highlighted significant changes in expression of genes encoding xenobiotic metabolism (*cyp4*) and moulting (*cut*). This study is the first to characterize chronic FPW toxicity and will help with the development of environmental monitoring and risk assessment of FPW spills.



INTRODUCTION

Hydraulic fracturing is an industrial process that allows for the extraction of oil and gas resources trapped in low permeability formations that are unable to be recovered by conventional extraction techniques.[1] The hydraulic fracturing process itself often requires large quantities of water (10 000−100 000 m^3), to which are added a number of components, including proppants such as ceramic beads and sand (to prevent the fracture reclosure), biocides, gelling and foaming agents, pH adjustors, clay stabilizers, and surfactants.[2] This mixture, pumped into wells at high pressures (up to 69 000 kPa),[3] eventually returns to the well head where it is classified as flowback and produced water (FPW). The distinction between flowback and produced waters is not well-defined but depends on the time spent in the formation and the chemical characteristics of the water.[3] Generally, flowback refers to the injected fluid that returns within the first few days, while produced water spends a longer time in the well and is more characteristic of the formation. However, mixing of flowback and produced waters does occur.[2,4,5] FPW is often separated for treatment and subsequent reuse. However, when the quality for reuse is compromised, FPW is then transported (via truck or pipeline) for disposal in deep (approximately 2−4 km in depth), subsurface injection wells.[6−8]

Compositions of FPW are known to be highly variable because of heterogeneity within geologic formations and their variable chemical contribution of the formation to returning FPW.[3] Furthermore, fracturing fluids vary by operator and formation, and thus, the exact composition and relative concentration of components of hydraulic fracturing fluids used in each well varies widely. However, most FPW is high in salts (e.g., sodium, calcium, magnesium, chloride) with trace metals (e.g., arsenic), naturally occurring radioactive materials, and petrogenic organics (e.g., polycyclic aromatic hydrocarbons and other organic compounds).[9−12] Owing to the composition of FPW, there is concern regarding the toxicity of these complex mixtures.[13] In 2015 alone, there were more than 113 documented spills of FPW fluids into the environment in the Canadian province of Alberta, and most were associated with transportation to disposal wells.[13,14] These spills can pollute

Received: October 17, 2016
Revised: January 9, 2017
Accepted: January 31, 2017
Published: January 31, 2017

DOI: 10.1021/acs.est.6b05179
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

both surface and groundwater reserves. However, little to no data exist on the potential environmental impacts of these spills and the consequences of metals, radioactive materials, and organics detected in FPW, that often greatly exceed the maximum contamination level guidelines for drinking water.[15]

FPW spills into lakes and river systems may affect freshwater invertebrate and fish species. Of the species that may be affected, the water flea, *Daphnia magna*, represents an ideal model for examining FPW toxicity. Water fleas are small crustaceans that inhabit almost all freshwater lakes and shallow ponds and play a critical role in the aquatic food chain.[16] Furthermore, it is an important model for ecotoxicological studies due to the ease of culturing it in the laboratory,[17] its short life cycle, and its high sensitivity to many chemicals.[18]

Very few studies have reported the toxicological effects of FPW on aquatic species. Previous research from our laboratory has shown that zebrafish embryos exposed to FPW displayed developmental toxicity as a result of the high salinity and organic toxicants present. FPW exposure also resulted in induction of cytochrome P450 activity, a key pathway for organic contaminant metabolism.[19] In studies conducted on freshwater rainbow trout, it was shown that, after acute 48 h exposure, FPW caused oxidative stress and changes in gill morphology.[20,21] However, to date, no data exist regarding effects on any invertebrate species.

The aim of the current study was to characterize the effects of both acute and chronic exposures to FPW with both lethal and sublethal end points evaluated. The expression of target genes involved in metabolism (carboxypeptidase A1 precursor), reproduction (vitellogenin 1 and 2, ecdysone receptor a and b, ultraspiracle, cuticle 12, doublesex-Mab related 93B), homeostasis (hemoglobin, cuticle 12), toxicant metabolism (cytochrome 4, 314, and 18a1), and oxidative stress response (glutathione-*S*-transferase and catalase) was examined. This information will provide a needed step toward an understanding of the biological effects of FPW spills and will facilitate development of postspill environmental effects monitoring, remediation, and risk assessment policies.

METHODS

Animals. *Daphnia magna* were obtained (January, 2015) from a colony cultured at the University of Saskatchewan and housed in the Department of Biological Sciences at the University of Alberta. *Daphnia* were maintained following Organization for Economic Cooperation and Development guidelines,[17] with some adjustments. Briefly, *Daphnia* were held at 20 ± 1 °C in 8 L glass aquaria with dechlorinated city of Edmonton tap water (moderately hard: $[Na^+]$ = 14.6 mg/L, $[Ca^{2+}]$ = 55.9 mg/L, $[Mg^{2+}]$ = 15.3 mg/L, $[K^+]$ = 2.5 mg/L, titration alkalinity ≈ 119 mg/L as $CaCO_3$, pH ≈ 7.6, hardness ≈ 180 mg/L as $CaCO_3$, conductivity ≈ 385 μS/cm). Water was changed every 2−3 days. Daphnids were fed once daily to satiation, on a diet of Roti-Rich invertebrate food (VWR, Edmonton, Alberta, Canada). A 12 h light/12h dark photoperiod was maintained.

Chemical Analysis. Flowback and produced water was obtained from a hydraulically fractured well in the Devonian-aged Duvernay Formation (Fox Creek, Alberta, Canada; Encana Corporation). This supplied FPW, collected 10 days into the flowback period, was termed 100% FPW, and a chemical characterization of this mixture is displayed in Table S1. To generate an activated charcoal control (AC) for the removal of organics[19] (leaving salts), 2 L of FPW sample was filtered through a 0.22 μm Corning bottle top disposable membrane (Fisher Scientific, USA). Once the fluid had been filtered, 10 g of activated charcoal was added and this combination was stirred and mixed for 24 h before filtration occurred again. To generate a salinity-matched control (SW; no added metals or organics), salts (NaCl, $CaCl_2$, $MgCl_2$, KCl; Sigma-Aldrich, Oakville, Ontario, Canada) were added to nanopure water (generated by PURELAB Flex, ELGA LabWater) in order to replicate the salt composition present in the 100% FPW sample (Table S1). The measured concentrations of ions for each treatment are recorded in Table S2. All samples were stored in the dark at room temperature until experimentation.

Elemental analysis on all samples (FPW, AC, and SW) was performed using Agilent 8800 Inductively Coupled Plasma-Double Mass Spectrometry (ICP-MS/MS). Briefly, samples were acidified by the addition of 6 μL of 16 M trace metal grade nitric acid per 10 mL of sample (Sigma-Aldrich). The ICP-MS/MS was operated with a microMist nebulizer and nickel/copper cones. Concentrations of organic contaminants of the FPW sample were previously described by He et al.[19]

Acute Exposure. *Daphnia magna* acute toxicity assessment was performed according to the standard OECD guidelines.[17] Acute 48 h median lethal concentrations (LC_{50}) were determined for both adult (7 day old) and neonate (<24 h) *D. magna*. Exposures were conducted in FPW, AC, and SW media, and each concentration series was run four times (with $N = 5$ for each concentration). Before testing, all glass beakers used in exposures were prerinsed in 10% trace metal grade nitric acid (Sigma-Aldrich, Oakville, Ontario, Canada) and then with dechlorinated Edmonton tap water.

For adult exposures, each replicate (4 replicates total) consisted of five adult daphnids (24 h starved) that were removed from the colony and placed into 50 mL glass beakers containing Edmonton tap water at room temperature (20 °C). Six concentrations of FPW were used to determine the FPW lethal concentration where 50% of the population was impacted (LC_{50}), 0% (no added FPW), 0.008%, 0.04%, 0.2%, 1%, and 5%, where the percentage is a dilution (using city of Edmonton dechlorinated tap water) from the supplied full-strength FPW. For neonates, a similar procedure was followed. Five individuals were placed into 50 mL glass beakers and dosed with FPW concentrations of 0%, 0.004%, 0.008%, 0.016%, 0.032%, 0.04%, and 1%. Mortality was recorded at the end of the 48 h exposure.

In parallel to FPW treatments, LC_{50}'s were also determined in AC and SW solutions. These were run identically to those above: 5 individuals, 4 replicates, and the following dilutions from stock solutions on adult (0% (no added AC/SW), 0.008%, 0.04%, 0.4%, 0.8%, 1%, 1.6%, 2.8%, 3.2%, 5%) and neonate daphnids (0% (no added AC/SW), 0.008%, 0.032%, 0.13%, 0.5%, 2, 4%, 8%).

Chronic Exposure. A chronic 21 day exposure was performed on <24 h old second brood neonates of *Daphnia* following OECD guidelines.[17] Concentrations were chosen on the basis of acute LC_{50} results. Ten neonates were placed individually into a 50 mL glass beaker and exposed to one of the following treatments: control (no added FPW, AC, or SW) or 0.004%, 0.008%, or 0.04% FPW, AC, or SW. Exposures were maintained at 20 ± 1 °C and pH 7 with a 12:12 h light/dark photoperiod. Every 2 days, the animals were moved into clean beakers, redosed, and fed with 80 μL of Roti-Rich food (diluted to half-strength with nanopure water; VWR, Edmonton,

DOI: 10.1021/acs.est.6b05179
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Alberta, Canada). Survival was observed and offspring production was counted daily.

At the end of the 21 day exposure, respiration rate (measured as oxygen consumption; MO_2) was tested. Two adult daphnids from the same treatment were placed in a glass microplate cell with oxygen sensor spots (Loligo Systems, Denmark) containing 750 μL of fresh Edmonton tap water. The total partial pressure of oxygen in each cell was measured over the course of 2 h, before data were processed by the software PreSens SDR (SensorDish Reader) version 508 (Regensburg, Germany). The respiration rate (MO_2) was calculated using the following equation:

$$MO_2 = \frac{[PO_2]_i - [PO_2]_f}{\Delta t} \times V$$

In this equation, $[PO_2]_i$ and $[PO_2]_f$ represent the initial and final partial pressures of oxygen (μmol/L), respectively, Δt is the time period of the exposure (h), and V is the volume of the respirometer (L). To achieve a final measurement of nmol/daphnid/h, these values were divided by 2 (per daphnid in each container) and by 1000 to achieve nmol.

Quantitative Real-Time PCR Assay. Adult daphnids (three replicates of 5 to 7 adults) were exposed to one of five different solutions (control and 0.004%, 0.008%, and 0.04% FPW and 0.04% AC). Exposures were conducted for 24 h before *Daphnia* were removed and sampled for analysis.

Total RNA was extracted from pooled samples (5 to 7 individuals) using a NucleoSpin RNA Midi Kit according to the manufacturer's protocol (Macheret-Nagel, Düren, Germany). Purified RNA was quantified by placing a 1 μL sample of RNA on a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, Delaware, USA). First-strand cDNA was synthesized from 500 ng of total RNA using SuperScript III First Strand cDNA Synthesis Kit (Thermo Scientific, Ontario, Canada) according to the manufacturer's protocol. The cDNA was stored at −80 °C until further analysis.

Quantitative real-time PCR was performed on an ABI 7500 Real-Time PCR System in 96-well PCR plates (Applied Biosystems, Canada). A PCR reaction mixture for one reaction contained 5 μL of SYBR Green master mix (Applied Biosystems, Canada), 2.5 μL of sense/antisense gene-specific primers (Integrated DNA Technologies, IA), and 2.5 μL of cDNA that was diluted in RNase-free water (Qiagen, Venlo, Netherlands). The PCR reaction mix was denatured at 95 °C for 2 min followed by 40 thermal cycles with denaturization for 15 s at 95 °C and annealing and extension for 1 min at 60 °C. Dissociation curve analysis was performed after amplification reactions to ensure a single product. Primer efficiency, uniformity, and linear dynamic range of each qPCR assay were assessed by construction of standard curves using serially diluted cDNA standards. All primers were designed on the basis of sequences available in the NCBI GenBank database. Changes in abundances of transcripts of target genes were quantified by normalizing to *prohibitin 2* (*phb2*). Primer sequences and efficiency are given in Table S3.

Statistics. The LC_{50} values and confidence intervals (C.I.) were calculated using Toxicity Relationship Analysis Program (TRAP) version 1.30a (EPA, Washington, DC, USA). To determine if LC_{50} values were significantly different from each other, standard Environment Canada protocols using the Litchfield-Wilcoxon methodology were applied.[22]

For chronic exposure end points and qPCR data, a one-way ANOVA, followed by Tukey's post hoc test were performed, using either SigmaPlot version 11.0 with SigmaStat version 3.5 integration (chronic end points; Systat Software Ind. San Jose CA, USA) or Statistical Package for the Social Sciences (SPSS) version 16.0 (qPCR; SPSS, Chicago, IL). All data have been expressed as means ± SEM (standard error of the mean). Significance for all tests was accepted at $\alpha = 0.05$.

RESULTS

Water Chemistry. Chloride (Cl) was present at the highest concentration (109 000 mg/L), while sodium (Na; 59 500 mg/L) was also highly concentrated. Although present at lower concentrations than Na, calcium (Ca), potassium (K), and magnesium (Mg) were all elevated with concentrations ranging from 6500 to 706 mg/L. A number of metal contaminants were also reported. For example, zinc (Zn) was present at a concentration of 1.24 mg/L (Table S1). An analysis of water sampled from the exposures showed that ion concentrations were consistent between the different treatments (i.e., SW 0.04%, AC 0.04%, and FPW 0.04%; Table S2).

Acute Exposures. The 48 h LC_{50} values for neonate and adult daphnids were significantly different from each other ($p < 0.05$, Figure 1). The lowest LC_{50} values for both adults and neonates occurred in the FPW treatment group (0.75% adult, 0.19% neonate), and these were 4-fold lower than the LC_{50} values for both SW and AC treatments. Adults were less sensitive to SW and AC waters than were neonates, with LC_{50} values of 2.6% and 2.7%, respectively (Figure 1).

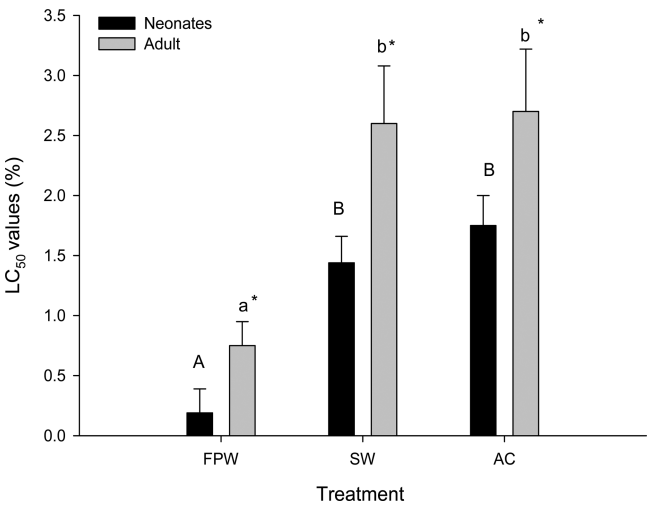

Figure 1. LC_{50} values (% of undiluted exposure solution) for adult and neonate daphnia ($N = 5$, 4 replicates). Error bars represent upper confidence intervals. Asterisks represent differences within a treatment group, while lowercase letters denote significant differences in adult daphnids across treatment groups. Uppercase letters denote significant differences in neonate daphnids across treatment groups.

Gene Expression. Only two genes showed significant differences in expression from control values after 24 h of exposure (Figure 2). Expression of the cytochrome P450 4 (*cyp4*) gene was down-regulated (0.5-fold from control) when the daphnids were exposed to either AC or FPW treatments ($p < 0.05$; Figure 2) but not SW. Expression of the gene cuticle 12 (*cut*) was upregulated in all FPW treatment exposures, 0.004%, 0.008%, and 0.04% of FPW (20- to 30-fold from control), but

DOI: 10.1021/acs.est.6b05179
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

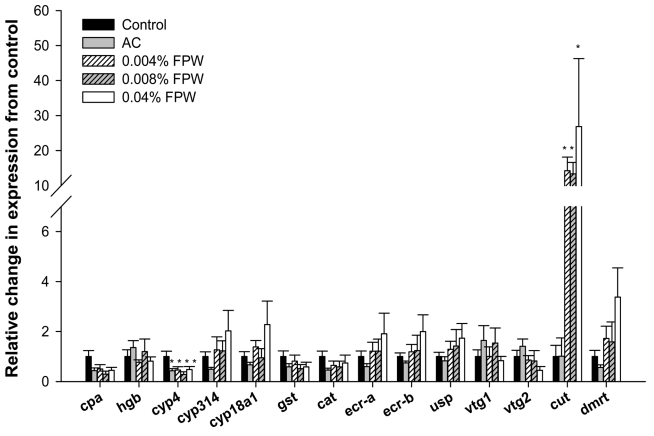

Figure 2. Relative change in mRNA expression of examined genes in adult *Daphnia* after a 24 h exposure to control, 0.04% AC or 0.004%, 0.008%, or 0.04% FPW. Asterisks denote significant differences from control treatment only.

was unchanged in the AC water ($p < 0.05$). All other genes (*cpa, hbg, dmrt93b, vtg 1, vtg 2, ecr-a, ecr-b, usp, cyp314, cyp18a1, gst,* and *cat*) were unchanged in treatments relative to controls.

Chronic Exposure. After 21 days of exposure, total neonates produced across all *Daphnia* within a treatment, and the average number of neonates produced per daphnid was monitored (Figure 3A,B). There was a systematic decline in brood size as FPW, SW, or AC increased in concentration, with the lowest values for total neonates for all *Daphnia* (148), and the lowest average neonates per daphnid (18.5), occurring in the highest FPW concentration (0.04%). These values were one-third of those in the control (Edmonton tap water). Average neonates per *Daphnia* were not significantly impacted by AC and SW waters at exposure levels of 0.004% ($p < 0.05$), but at 0.004% FPW, there was a significantly reduced brood size relative to the control (Figure 3). Overall, time to first brood was relatively constant across all treatments. The exception was in 0.04% AC (11.3 ± 0.3 day) and 0.04% FPW (11.8 ± 0.5 day), which both displayed first broods that were significantly later than controls (9.7 ± 0.2 day; $p < 0.05$; Table 1).

Oxygen consumption (MO_2) decreased as exposure concentration increased for all treatments (AC, SW, and FPW). Control values of 0.017 nmol/daphnid/h were significantly higher than the MO_2 for the highest 0.04% FPW concentration (0.009 nmol/daphnid/h; Figure 4).

DISCUSSION

The current study shows that exposure to FPW induces perturbations of reproduction in *Daphnia magna*. Assessment of reproduction as a toxicity end point in *Daphnia* is a standard approach.[23,24] This is an end point that represents the summed impacts at the molecular, biochemical, and physiological levels and, given that reproduction is highly energy dependent, is thought to reflect increased costs of toxicant exposure. Furthermore, reproductive output is a measure of fitness and is of direct relevance to survival of the species in the natural environment. In the current study, exposure to FPW reduced neonate production (Figure 3) and delayed time to maturation (e.g., time to first brood) (Table 1), but effects were only observed in the most concentrated FPW samples (Figure 3A, B). These results are consistent with previous studies exposing *Daphnia* to organic contaminants. For example, decreased

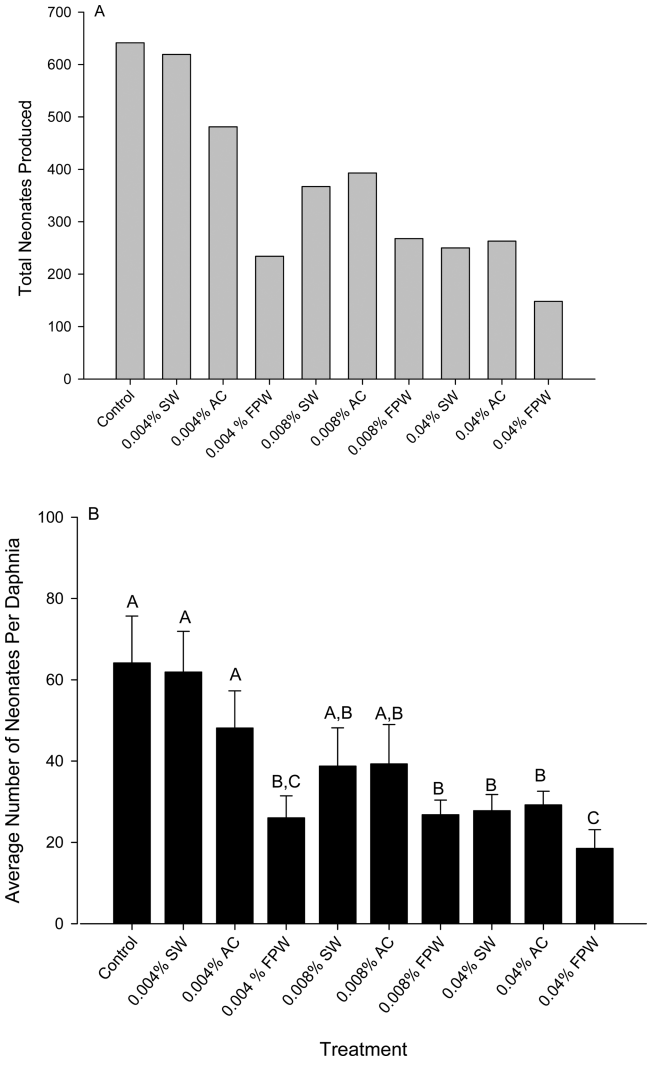

Figure 3. Total (A) and average (B) brood size per daphnia per treatment. Bars represent means ± SEM (N = 9–10). Bars sharing letters are not significantly different.

Table 1. Time to First Brood (Days) Over the Course of 21 Days[a]

treatment	time to first brood
control	9.7 ± 0.2 a
0.004% SW	10.0 ± 0.2 a,b
0.004% AC	10.6 ± 0.2 a,b
0.004% FPW	10.5 ± 0.3 a,b
0.008% SW	10.1 ± 0.3 a,b
0.008% AC	10.6 ± 0.2 a,b
0.008% FPW	10.6 ± 0.3 a,b
0.04% SW	10.3 ± 0.4 a,b
0.04% AC	11.3 ± 0.3 b
0.04% FPW	11.8 ± 0.5 b

[a]All means are ±S.E.M. (N = 9–10 per treatment). SW and AC stand for saltwater and activated charcoal matched controls, and FPW refers to flow back and produced waters. Letters that are the same denote no significant differences from each other while different letters denote significant differences from each other.

fecundity and time to first brood were shown during PAH and bisphenol A exposure.[25,26] This finding is consistent with the composition of FPWs. A previous analysis of FPW collected

DOI: 10.1021/acs.est.6b05179
Environ. Sci. Technol. XXXX, XXX, XXX–XXX

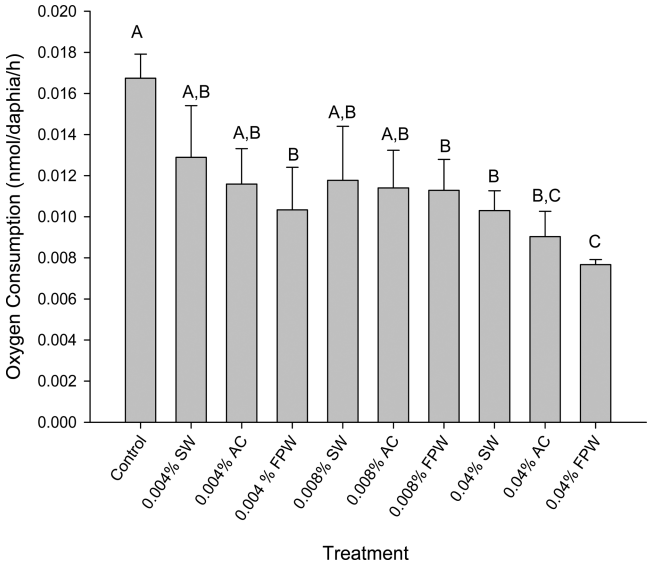

Figure 4. Oxygen consumption in *Daphnia* after 21 days of exposure to FPW, AC, or SW; all means are ± SEM (N = 9–10).

from the same well as that used in the current study showed that these waters contain a complex assortment of polycyclic aromatic hydrocarbons (PAHs). The organic contaminants present at the highest concentration were fluorene (294 ng/L), and 3,6-dimethylphenanthrene (224.5 ng/L), respectively.[19] PAHs are formed by combustion coal, oil, wood, and other organic substrates.[27] They are considered to be carcinogenic and are often highly concentrated in polluted sediments and organic matter owing to their high lipophilicity.[27] Furthermore, mixtures of organic chemicals, such as those which occur in FPW, are higher in toxicity than individual chemicals,[28] potentially explaining the magnitude of the impacts observed.

While organic constituents of FPW are the most likely to be responsible for toxicity, it is important to highlight that a control matching only the salt concentration of 0.04% FPW (SW) also caused significant impairment of reproduction at 0.04% (Figure 3), albeit an effect of lower magnitude than that generated by the full FPW (i.e., containing salts and organics). It has been shown that salt has a negative impact on *Daphnia magna* reproduction, with a decrease in total progeny and average number of clutches per female when they are exposed to 0.7%[29] and 0.48%[30] sodium chloride (NaCl), levels approximately 10-fold higher than those of the current study. The finding of salt as an important constituent of FPW is not unique,[19,21] but the discovery that this is an FPW component that can cause toxicity to freshwater invertebrates is novel.

It is notable, however, that in the current study ion concentrations did not double from 0.004% to 0.008% as might be predicted. It is possible that some evaporation of treatment waters occurred throughout the exposure, such that small differences in evaporation could have altered measured concentrations in solutions.

Metals also inhibit reproduction in *Daphnia* and do so in a synergistic manner. Biesinger et al. observed that combined exposure of zinc, mercury, and cadmium cause enhanced reproductive effects over singular metal exposures.[31] However, it is important to note that metals are unlikely to cause the effects seen in the current study. In AC treatments, where organics are removed and some metals remain, there were relatively minor effects compared to FPW treatments (metals and organics). While metals were present in the full strength FPW, the dilutions applied decreased most metals to below detection limits in the final exposure waters (Table S2). Owing to this, it is unlikely that metals contributed to toxicity in the current study.

The exact mechanism underlying the effects of FPW on *Daphnia* reproduction are unknown. However, reproduction will be impacted by a reduced availability of energy. Typically, following exposure to toxicants, this is explained by increased costs of homeostasis and repair.[32] However, in the current study, it is possible that the reduction in reproduction relates to the impaired MO_2. There was a strong correlation between decreased MO_2 and reproductive impairment (R^2 = 0.76, p = 0.001; Figure S1). Treatments with the lowest MO_2 were also the treatments where reproduction was most significantly affected (e.g., 0.04% FPW). The most likely explanation for this is that chemicals in FPW (most likely organics, given the lesser responses of AC and SW treatments) impaired either metabolism or uptake of oxygen across the gills. A decrease in oxygen consumption in *Daphnia* in response to acute organic toxicant exposure has been noted previously and has been proposed as a simple and effective biomarker of toxicant exposure.[33] The decline in *Daphnia* MO_2 in FPW could be manifested by a direct effect on respiratory tissues. For example, exposure to organic contaminants has been shown to generate histopathological changes in gills of aquatic crustaceans,[34] while exposure to oil decreases blood oxygen in fish, likely through changes in gill structure.[35,36] Decreases in oxygen uptake could reflect biochemical inhibition of oxidative metabolism (see below). Alternatively, it has been hypothesized that crustaceans may reduce oxygen consumption upon toxicant exposure as a survival strategy,[37] and thus, the decline is a specific biological response to the stressor. Studies of gill histology, feeding rates, and respiratory enzymes would be needed to appropriately ascribe a mechanistic basis for the decline in metabolism that appears to underlie the decrease in reproductive output in FPW-exposed *Daphnia*.

It is known that animals reduce feeding rate when the diet is contaminated.[38] Although exposure in the current study was waterborne, organic toxicants could have adhered to food, making this a potential pathway of exposure. A decrease in energy inputs may have compromised the energy resources necessary for reproduction, an effect that could be related to altered food detection. Previous work showed that oil-sands process water (a chemical mixture similar in composition to FPW) decreased grazing behavior, food consumption, and total activity in *Daphnia magna*.[16] Observations of *Daphnia* in the current study noted that animals in 0.04% FPW were more lethargic than in other exposures. *Daphnia* rely upon chemoreception to locate food and avoid predators,[16,39,40] suggesting that, as well as impacting energy reserves for fueling reproduction, in natural settings FPW could also influence survival.

The acute LC_{50} data showed that there were large differences in sensitivities between neonate and adult daphnids. At the end of 48 h, neonates were three times as sensitive as adults to FPW (LC_{50} of 0.19% and 0.75%, respectively, Figure 2). Generally, invertebrates at early developmental stages are more sensitive to environmental stressors than their adult counterparts.[41] There are several explanations for this. The first is that neonates have a relatively larger surface area to body volume ratio. This means that there is relatively greater surface area across which toxicants may be absorbed.[42] Thus, for toxicants such as high

DOI: 10.1021/acs.est.6b05179
Environ. Sci. Technol. XXXX, XXX, XXX–XXX

salts and organics that are known to impact osmoregulation, there is a relatively greater toxicological impact. This is demonstrated by the strong inverse relationship between body size and sensitivity of aquatic biota to sodium uptake-disrupting toxicants such as copper and silver.[43] The second is that metabolic rate is higher in neonate daphnids.[44] This is proposed to facilitate uptake of chemicals into the body by increasing epithelial exposure to waterborne toxicants as flow rate over the gills is increased to meet oxygen demand.[45] Muyssen and Janssen showed that 24 h old neonates were significantly more sensitive to copper and zinc than 7 day old daphnids, an effect attributed to increased metabolic rate.[46] Third, it is generally considered that early life stages have reduced mechanisms for protection against toxicants. For example, neonate *Daphnia* have significantly lower transcription levels of DNA repair genes, making them more susceptible to genotoxicants than adults.[47]

As for effects on reproduction, PAHs are likely to be key constituents contributing to acute mortality.[19,21] Previous research has shown that the LC_{50} values of PAHs to *Daphnia* are at concentrations above those in the curent study (0.25−15 μM).[25] This suggests that there is additive toxicity whereby a combination of the organic constituents, and/or their combination with the elevated salts, enhances FPW toxicity.

It was notable that the SW treatment, containing only salts, had a similar LC_{50} to the AC treatment (containing salts and metals). This suggests an acute effect of high salinity on *Daphnia* mortality, consistent with the role for salinity in reproductive effects as described above. Teschner showed that *D. magna* exposed to 5 g/L salinity displayed elevated mortality and concluded that this concentration was beyond the tolerance range for this species.[48] This conclusion was consistent with the findings of Cowgill and Milazzo, who showed that population growth, reproduction, and survival decreased over a sodium range of 0.08 to 6000 mg/L.[49] Daphnids are known to be especially susceptible to toxicants that disrupt osmoregulation, owing to their high diffusive loss and need for robust active uptake pathways.[43,50] Consequently, any factor that disrupts osmoregulation, such as acute environmental salinization, is likely to lead to death.

The current study shows that, while organic components of FPW drive toxicity, salts also play a role. One potential mechanism by which these components may interact is via the generation of oxidative stress. Both salinity and PAHs increase reactive oxygen species (ROS) and interfere with antioxidant defense systems in aquatic organisms.[25,51,52] An increase in damaging free radicals, coupled with impaired defense mechanisms, would exacerbate oxidative stress. For example, PAHs decrease levels of glutathione, a key biomolecule involved in reducing oxidative stress in *Daphnia*.[25] Previous work has shown that FPWs induce lipid peroxidation in fish,[21] suggesting that salt/organic interactions could be a mechanism underlying toxicity of FPW in *Daphnia*. Furthermore, in a recent study in rainbow trout, similar impacts of SW and FPW treatments were noted on superoxide dismutase and catalase activity suggesting a conserved pathway of effect.[20]

In order to delineate molecular-scale mechanism of FPW toxicity to *Daphnia*, gene expression was measured by qPCR (Figure 2). Of the genes tested, only two showed expression profiles that were significantly altered by 24 h FPW and/or AC exposure: *cyp4* and *cut*. The gene *cyp4* encodes for a member of the cytochrome P450 family, of principal importance as a detoxification enzyme in Phase I metabolism of organics.[53] Specifically, *cyp4* has endogenous roles in fatty acids and steroid metabolism[54] and, as such, is under hormonal regulation.[55] The expression of this gene was down-regulated in daphnids exposed to AC and FPW (0.004%, 0.008%, and 0.04%). This could indicate a direct toxic inhibition of *cyp4*. Le et al. studied the impact of two organophosphate pesticides (glyphosate and methidathion) on the expression of *cyp4* in *Daphnia magna*.[56] They highlighted a down-regulation of *cyp4* expression in the presence of glyphosate, suggesting an effect of the pesticide on fatty acids and steroid metabolism. It is also possible that, because CYPs are oxidative enzymes, downregulation could be a consequence of perturbed metabolism. This is supported by the decrease in MO_2 values observed after 21 days of exposure and may suggest a decreased availability of oxygen required to fuel oxidative metabolism.

In contrast to *cyp4* expression, an up-regulation of the gene cuticle 12 (*cut*) was observed after 24 h of exposure to FPW (0.004%, 0.008%, and 0.04%) but not to AC. This gene is implicated in the molting process in crustaceans,[57] whereby the cuticle protein is produced by the epidermis during embryogenesis and renewed during molting. Cuticle protein contributes toward the exoskeleton of *Daphnia* by maintaining support and acting as a barrier to environmental contaminants.[26] Molting is under hormonal control, regulated directly by the hormone 20-hydroxyecdysone, a major biologically active ecdysteroid.[58] Changes in steroid metabolism, as highlighted above with respect to *cyp4* expression, could therefore be contributing toward altered *cut* expression. Because the effect on *cut* was observed in FPW, but not AC treatments, it is strongly suggested that the effect was mediated by the organic constituents of the FPW.

As *Daphnia* moult, they release the developing neonates, and thus, the effect on *cut* expression may be related to the enhanced mortality seen in acute FPW treatments over 48 h. Essentially, the increase in *cut* could be part of a "spawn and die" approach employed by many invertebrates in response to a toxicant exposure.[59]

Overall, the current study showed impacts of FPW on reproduction and mortality in the important model species *Daphnia magna*, a species that is also likely to be found in environments subjected to FPW spills. On the basis of an approach that involved exposing daphnids to both whole FPW and combinations of key FPW components, the organic fraction and to a lesser extent the salt components were identified as key mediators of toxicity. On the basis of an analysis of sublethal end points, FPW likely causes effects through altered oxygen uptake, with impacts on organic toxicant metabolism and moulting identified at a molecular level.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b05179.

Methodology on ICP QA/QC, tables on water chemistry, and forward and reverse primers for assessed genes (PDF)

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DOI: 10.1021/acs.est.6b05179
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The project was funded by Natural Sciences and Engineering Research Council of Canada (NSERC) Collaborative Research and Development (CRD) grant CRDPJ 469308-14 and support from the Encana Corporation to D.S.A. and G.G.G. We would like to thank both Arthur Qi and Dr. Chris Glover for their assistance and technical help with the *Daphnia* colony.

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DOI: 10.1021/acs.est.6b05179
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