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Endocrine-Disrupting Activities and Organic Contaminants Associated with Oil and Gas Operations in Wyoming Groundwater

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Abstract

Unconventional oil and natural gas (UOG) operations couple horizontal drilling with hydraulic fracturing to access previously inaccessible fossil fuel deposits. Hydraulic fracturing, a common form of stimulation, involves the high-pressure injection of water, chemicals, and sand to fracture the target layer and release trapped natural gas and/or oil. Spills and/or discharges of wastewater have been shown to impact surface, ground, and drinking water. The goals of this study were to characterize the endocrine activities and measure select organic contaminants in groundwater from conventional oil and gas (COG) and UOG production regions of Wyoming. Groundwater samples were collected from each region, solid-phase extracted, and assessed for endocrine activities (estrogen, androgen, progesterone, glucocorticoid, and thyroid receptor agonism and antagonism), using reporter gene assays in human endometrial cells. Water samples from UOG and conventional oil areas exhibited greater ER antagonist activities than water samples from conventional gas areas. Samples from UOG areas tended to exhibit progesterone receptor antagonism more often, suggesting there may be a UOG-related impact on these endocrine activities. We also report UOG-specific contaminants in Pavillion groundwater extracts, and these same chemicals at high concentrations in a local UOG wastewater sample. A unique suite of contaminants was observed in groundwater from a permitted drinking water well at a COG well pad and not at any UOG sites; high levels of endocrine activities (most notably, maximal estrogenic activity) were noted there, suggesting putative impacts on endocrine bioactivities by COG. As such, we report two levels of evidence for groundwater contamination by both UOG and COG operations in Wyoming.

Unconventional oil and natural gas (UOG) operations revolutionized oil and gas production in the United States, coupling horizontal drilling with hydraulic fracturing to access previously inaccessible deposits (shale, sandstone, coalbed methane, etc.). Hydraulic fracturing, a common form of stimulation designed for low permeability geologic formations, involves the high-pressure injection of water, chemicals, and sand or other proppants to fracture the target layer and release trapped natural gas and/or oil (Waxman et al. 2011; Wiseman 2008). More than 1000 different chemicals have been reported to be used for hydraulic fracturing across the United States, although often between 15 and 50 are used in an individual well, dependent on geology and producer (Environmental Protection Agency (EPA) 2015; Waxman et al. 2011). Wastewater is initially produced following injection as "flow back" and continues over the life of the producing well as "produced water" (Deutch et al. 2011; Engle et al. 2014), producing an estimated volume of between 3.18 and 3.97 billion cubic meters of wastewater per year in the United States (Clark and Veil 2009; Harkness et al. 2015). Hydraulic fracturing chemical concentrations decrease in wastewater over time, though the source formation waters often contain naturally occurring radioactive compounds, heavy metals, and other compounds from the

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shale layer (Akob et al. 2015; Rowan et al. 2015), potentially resulting in different chemical profiles.

Recent studies have reported wastewater spill rates occurring at approximately 2–20% of active well pads (Maloney et al. 2017; Patterson et al. 2017), mainly due to storage and transport of wastewater, and suggesting potential widespread environmental contamination, which can subsequently impact surface, ground, and drinking water quality near UOG operations (DiGiulio and Jackson 2016; DiGiulio et al. 2011; Drollette et al. 2015; Rozell and Reaven 2012; Skalak et al. 2014). Our laboratory and others have also previously demonstrated that some commonly used UOG chemicals can act as endocrine disrupting chemicals (EDCs) both in vitro and in vivo (Blewett et al. 2017; He et al. 2017a, b; Kassotis et al. 2015, 2016a). EDCs are exogenous chemicals or mixtures of chemicals that can interfere with any aspect of hormone action (Diamanti-Kandarakis et al. 2009; The Endocrine Disruption Exchange (TEDX) 2017; Zoeller et al. 2012), and EDCs can disrupt development and contribute to disease in both humans and animals (Vandenberg et al. 2012; Welshons et al. 2003). Previous research by our laboratory and colleagues has demonstrated increased endocrine activities in surface and groundwater near UOG spill sites in CO (Kassotis et al. 2014), downstream from an UOG wastewater injection disposal site in WV (Kassotis et al. 2016b), and downstream from an UOG wastewater spill in ND (Cozzarelli et al. 2017).

Pavillion, Wyoming, is a notable case study of groundwater contamination via UOG operations. A small town of approximately 200, the area is home to more than 180 past and present production wells in the shale and sandstone layered Wind River Formation (DiGiulio and Jackson 2016). In 2008, due to landowner concerns, the EPA initiated an investigation into Pavillion water quality, including the drilling and assessment of monitoring wells. A 2011 draft report concluded that hydraulic fracturing occurred throughout the field at depths of only several hundred meters, and cement well casings often did not extend the full distance required to adequately protect groundwater (DiGiulio et al. 2011). Both organic chemistry and geochemistry in monitoring wells revealed several contaminants not known to occur via other sources (naphthalene; benzene, toluene, xylenes, ethylbenzene (BTEX), a range of glycol ethers, 2-butoxyethanol, and others) and suggested a likely impact to groundwater by UOG operations (DiGiulio et al. 2011). This report was never finalized, and the investigation was instead turned over to the Wyoming State Department of Environmental Quality (DEQ), who reported no evidence of UOG-related contamination. Recent research reevaluated all publicly available data and reports; this substantiated the original EPA draft report and provided additional evidence of upward migration of UOG fluids into groundwater (DiGiulio and Jackson 2016). This work also suggested potential impacts to domestic wells by legacy disposal pits, used before the 1990s to dispose of drilling operation waste (DiGiulio and Jackson 2016). Notably, both reported specific UOG contaminants in Pavillion groundwater (DiGiulio and Jackson 2016; DiGiulio et al. 2011) at concentrations that we have previously reported receptor bioactivities (Kassotis et al. 2014, 2015).

A 2010 report, commissioned by various community groups in Pavillion, found that 94% of respondents experienced adverse health effects that were known to be associated with contaminants that EPA had reported in area drinking water (Subra 2010). This is mirrored by a growing number of laboratory animal and epidemiological studies suggesting adverse health near UOG operations. Mechanistic animal studies in our laboratory have reported adverse developmental and reproductive effects in mice (Kassotis et al. 2015, 2016a), and aquatic models (Folkerts et al. 2017a, b; Blewett et al. 2017), following exposure to UOG chemicals and/or wastewater. Many of these effects are mirrored in epidemiological studies, reporting associations between UOG operations and human health (reviewed in Kassotis et al. 2014, 2016c; Webb et al. 2014); these include but are not limited to congenital heart defects (McKenzie et al. 2014), low birth weight and small for gestational age births (Stacy et al. 2015), and preterm births and physician-recorded high-risk pregnancies (Casey et al. 2016).

As such, it is critical to better characterize endocrine impacts on groundwater near UOG operations, the effects of which have never been compared or contrasted to conventional oil and gas (COG) operations. As such, the goals of this pilot study were to determine the endocrine-disrupting activities of groundwater samples collected from a notable UOG production region (Pavillion, WY) and to compare these with samples from COG regions in Clark, WY. We hypothesized that the UOG production region would exhibit a distinct endocrine activity profile and greater receptor bioactivities than nearby COG regions. Samples from Clark were collected from distinct conventional oil versus gas drilling fields to evaluate potential differences. Groundwater samples were collected at each site, solid-phase extracted, assessed for endocrine activities (estrogen, androgen, progesterone, glucocorticoid, and thyroid receptor agonist and antagonist activities), and analyzed for a range of UOG/COG-associated volatile organic contaminants (VOCs) to attempt to determine causative links between measured bioactivities and these operations.

Materials and Methods

Chemicals

17β-estradiol (E2; estrogen agonist, 98% pure), ICI 182,780 (estrogen antagonist, 98% pure), 4,5α-dihydrotestosterone (DHT; androgen agonist, ≥ 97.5% pure), flutamide (androgen

antagonist, 100% pure), 3,3′,5-triiodo-L-thyronine (T3; thyroid agonist, ≥ 95% pure), progesterone (P4; progesterone agonist, ≥ 99% pure), mifepristone (glucocorticoid/progesterone antagonists, ≥ 98% pure), and dexamethasone (DEX; glucocorticoid agonist, 99.5% pure) were purchased from Sigma-Aldrich Co. (St. Louis, MO). 1–850 (thyroid antagonist, ≥ 95% pure) was purchased from EMD Millipore (Billerica, MA). A stock solution of each chemical was prepared at 10 mM in HPLC-grade methanol and stored at − 20 °C; T3 and 1–850 were prepared in dimethylsulfoxide (DMSO). Stock solutions were then diluted in respective solvents to required working solution concentrations.

Description of Groundwater Sampling Regions

Water samples ($n = 22$ unique sites, various replicates) were collected from groundwater sites in August 2014 through June 2015 (Fig. 1; Supplemental Table 1) from sites within Fremont County (Pavillion; UOG) and Park County (Clark; COG), Wyoming. Sites included groundwater sources collected from private land, selected by a local contact who knew the regions and landowners as well as by a word-of-mouth campaign, and were sources used for drinking water whenever possible. Several sites were municipal water sourced from groundwater wells in the region and transported to homes for cistern storage; these samples were included as groundwater in these analyses. Following sampling, Wyoming Oil and Gas Conservation Commission (WOGCC) data (available at: http://wogccms.state.wy.us/flexviewers/unitmap) were used to determine approximate number of natural gas or oil wells near each sample collection region. The UOG region (Pavillion) was selected due to previous reports of UOG-influenced contamination of area groundwater supplies (DiGiulio and Jackson 2016; DiGiulio et al. 2011). This field consists of approximately 180 active and plugged conventional and unconventional wells, targeting mainly natural gas at approximately 3000–6000 feet in depth (DiGiulio and Jackson 2016; DiGiulio et al. 2011). Sampling sites were within 1 mile of approximately 50 UOG wells and within 5 miles of approximately 200 (Table 1). The conventional oil region (Clark) was selected as a putative reference region, as there were no oil or gas wells within one mile of sampling sites, though sites were within 5 miles of approximately 30 wells in the conventional oil development region of Badger Basin. The conventional gas region (Clark), in contrast, overlaps a conventional natural gas field. Sampling sites were within one mile of approximately ten wells and within five miles of approximately 20. Both conventional production regions targeted geologic layers at approximately 8000–11,000 feet in depth.

State spill data, compiled by the Center for Western Priorities from data provided by the Wyoming Oil and Gas Conservation Commission (available at: http://www.westernprioriti
es.org/wp-content/uploads/2016/10/Wyoming-Spill-Repor

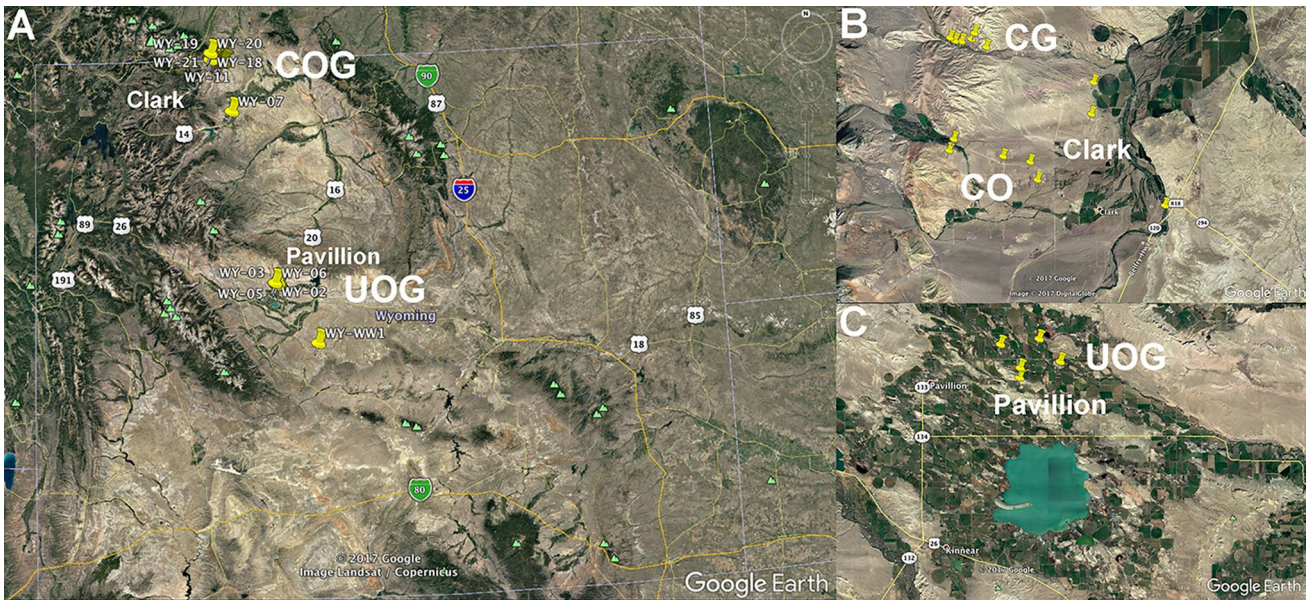


Fig. 1 Map of approximate sampling locations (yellow pins) within Wyoming (**a**) and subsampling regions within the Clark, WY area (COG = conventional oil and natural gas production) of Park County (**b**) and within the Pavillion, WY (UOG = unconventional oil and natural gas production) area of Fremont County (**c**). Panels B and C provide closer views of main sampling locations for the conventional oil (CO), conventional natural gas (CG), and UOG production regions within the Park and Fremont County areas, with approximate locations deidentified to protect landowner anonymity. Credit for map data to Google, Image Landsat. Green triangles represent topographical features only and do not denote oil and gas or sampling sites

Table 1 Description of sampling regions

Region	Sites (*n*)	Samples (*n*)	Average well # (1 mile)	Average well # (5 mile)
Controls	N/A	6	N/A	N/A
UOG (Pavillion)	6	13	> 50	> 200
Conventional oil (Clark)	8	10	~ 0	> 30
Conventional gas (Clark)	7	7	> 10	> 20

Description of sampling regions assessed in this study. Controls included $n = 1$ field blank, $n = 5$ process controls, performed alongside experimental samples for quality control purposes. Average well numbers are general well number estimates from the majority of the sampling sites from that collection region. Replicate samples were collected at certain sites at various time points, leading to more samples than sample collection sites for most regions. Wyoming Oil and Gas Conservation Commission (WOGCC) data (available at: http://wogccms.state.wy.us/flexviewers/unitmap) was used to determine approximate number of natural gas or oil wells near each sample collection region

ts-2014-2015-WOGCC.xlsx), reports approximately 1.7 spills per day in 2014 and 2015, comprised of > 400 gallons of crude oil and > 12,000 gallons of produced water spilled per day across Wyoming.

Grab Sample Collection

Samples for endocrine assays were collected as follows and as described previously (Kassotis et al. 2014, 2016b), using 1-L amber glass bottles (Thermo Scientific catalog # 05-719-91). Ground water samples were filled at frost-free pump outdoor spigots, when possible, or at indoor taps if not otherwise available, making every effort to avoid any potential source of plastic contamination when possible. All samples were immediately stored on ice, overnight-shipped in coolers to our lab, stored at 4 °C on arrival, and extracted within 2 weeks of collection. All processing, extractions, analytical assessments, and analyses were performed blinded to sample identification using nonidentifiable coded IDs.

A field blank was collected at one site and consisted of 1 L of laboratory HPLC-grade control water (Fisher Scientific, cat # WFSK-4), opened and exposed to the air during site sampling, and then preserved and processed in the same manner as all other experimental samples. Process controls were prepared as per experimental samples, using 1 L of HPLC water. These controls were included within each assay to assess any receptor activities contributed by the laboratory processing.

Extraction of Water Samples for Bioassays

Water samples for endocrine assays were solid-phase extracted (SPE) as described in detail previously (Kassotis et al. 2014, 2016b). Briefly, samples were prefiltered using a glass-fiber filter to remove suspended solids, and then SPE using Oasis HLB glass cartridges. Elution was performed with 100% methanol, and a DMSO "keeper" was used during evaporation and reconstitution. 4000× stocks were created in 80:20 methanol:DMSO and were stored at − 20 °C, protected from light, until tested. To be applied to cells, stock samples were diluted 100 and/or 1000-fold in tissue culture medium, creating final concentrations, in contact with cells, of 40×/4× the original water concentration.

Sample Toxicity

Before assessing endocrine activities, all samples were assessed for toxicity at 40×/4× to ensure that no cytotoxic concentrations were included in receptor bioassays, using the CellTiter 96 nonradioactive cell proliferation assay (Promega cat# G4000) as described previously (Kassotis et al. 2015). Briefly, Ishikawa cells (Sigma cat# 99040201) were seeded into 96-well plates at approximately 30,000 cells/well and allowed to settle. Cells were induced with test chemicals and water sample extracts, diluted in assay media using a 1% methanol vehicle, induced for 18–20 h, dye solution added, incubated for a further hour, and absorbance then read at 570 nm. Toxicity was defined as a significant decrease (as per paired *t* test) of ≥ 15% of the vehicle control levels, though no samples exhibited significant toxicity. As such, all water samples were included at the 40× and 4× concentrations for all reporter gene assays.

Mammalian Hormone Receptor Activity Assays

Ishikawa cells were maintained and transiently transfected with plasmids as described previously (Kassotis et al. 2014, 2015) for estrogen receptor alpha (ERα), androgen receptor (AR), progesterone receptor B (PR B), glucocorticoid receptor (GR), and thyroid receptor beta (TRβ) reporter gene assay assessment. Transfected cells were induced with dilution series of the positive/negative controls (Supplemental Figure 1) or of the water sample extracts, diluted in medium using a 1% methanol vehicle. Each sample test concentration was performed in quadruplicate within each assay and each assay was repeated three times with varying cell passage.

Further confirmatory cytotoxicity testing was included in reporter gene assays, using CMV-β-Gal activity as a marker of cell number, and as a surrogate marker for sample toxicity as described previously (Kassotis et al. 2014). These assays confirmed that no cytotoxic samples were included in bioassays.

Percent receptor activities were calculated as follows: test chemical fold induction at each concentration was calculated relative to intra-assay 1% methanol or 0.1% DMSO vehicle controls. Agonist activities were then calculated as a percent activity relative to the maximal positive control fold induction (200 pM E2, 3 nM DHT, 100 pM P4, 100 nM T3, or 100 nM DEX for ERα, AR, PR B, TRβ, and GR receptor assays, respectively). Antagonist activities were calculated as a percent suppression or enhancement of the co-treated positive control at its EC_{50} (concentration required to exhibit half of its own maximal activity; 20 pM E2, 300 pM DHT, 30 pM P4, 2 nM T3, and 5 nM DEX, respectively). The EC_{50} concentration was chosen specifically, because it is the most sensitive concentration to assess receptor antagonism, based on the slope of the dose–response curve (Welshons et al. 2003). Significant activity was determined by comparing raw luminescence values from specific test chemical concentration replicates to the vehicle control using a one-way analysis of variance (ANOVA) with Dunnett's post-test (GraphPad Prism 7.0). At least two of the three biological replicates had to exhibit significant modulation from baseline to consider the sample active at a given concentration.

Analytical Measurement of UOG Contaminants

The volatile organic EDCs in water were extracted by headspace solid-phase microextraction (SPME) followed by GC–MS. Toluene-d_8 was spiked into each sample as the internal standard. The VOCs were extracted by SPME using an 85-µm carboxen/polydimethylsiloxane fiber. In brief, samples were diluted in 20 mL of deionized water with 8 g of NaCl in 40-mL vials. The vials were sealed and the temperature increased to 30 °C. The SPME fiber was inserted into the headspace of the vigorously stirred sample and left for 15 min until the extraction process was complete. Following extraction, the fiber was removed from the vial, then immediately inserted into the injection port of a Varian CP-3400CX GC (Walnut Creek, CA) interfaced to an ion-trap Varian Saturn 2000 mass spectrometer at 250 °C for 3 min to desorb the VOCs. The VOCs were separated by a Hewlett Packard cross-linked methylsiloxane DB-5 capillary column (30 m × 0.25 mm I.D.). The GC temperature program was 35 °C for 10 min, ramped to 200 °C at 10 °C/min, and then to 230 °C at 3 °C/min and held for 6 min. Injector temperature was held at 250 °C. The transfer line between the GC and mass spectrometer was held at 150 °C and the MS source was set to 230 °C. The mass spectra of each peak identified on chromatograms was characterized by comparison with the mass spectra of commercially available reference standards and mass spectral libraries developed by National Institute for Standard and Technology (NIST/EPA/NIH). Selection of diagnostic and quantitative ions was optimized, and the calibration equations were developed following the procedure described previously (Lin et al. 2007, 2008). For development of the calibration equations, standard solutions were prepared at seven concentration levels (0.05, 0.1, 0.5, 1, 2, 5, 10 µg/L). The limit of detection (LOD) and limit of quantification (LOQ) were determined by signal-to-noise ratios of three and ten, respectively. Based on pre- and post-SPE analysis for 13 VOCs evaluated in both samples, estimated recovery rates of ≤ 0.1% were calculated.

Statistical Analyses

Data are presented as means ± SE from four technical replicates of three independent experiments. Dose response curves and EC/IC_{50s} were estimated using curves generated from raw fluorescence data using a four-parameter variable-slope Hill model in GraphPad Prism 7.0. Percent activity results were log-transformed, when necessary, to achieve normality. Statistical analysis was performed using a one-way ANOVA with post-test comparisons between reference samples and other sampling regions using a Dunnett's multiple comparison test, with differences considered statistically significant at $p < 0.05$, using GraphPad Prism 7.0.

Results

Water samples were collected from groundwater sites near UOG (Pavillion, WY), conventional oil (Clark, WY), and conventional gas (Clark, WY) production regions. Samples were solid-phase extracted and assessed for agonist and antagonist receptor bioactivities for the estrogen, androgen, progesterone, glucocorticoid, and thyroid receptors, as well as for select VOCs known to be associated with oil and gas operations.

Receptor Agonist Activities of Water Extracts

SPE extracts of water samples exhibited a range of agonist activities in reporter gene assays. Agonist activities for individual receptors of 40× extracts in UOG and COG regions were significantly greater than controls (Fig. 2a and S2) and not significantly different between oil and gas production regions. The magnitude of combined agonist activity for all receptors was also significantly greater in each sampling region relative to controls ($p < 0.01$ for each region). Notably, WY-20 was collected at a well pad on state land from a permitted state potable drinking water well in the

conventional gas region. This sample exhibited approximately 120% estrogenic activity relative to the maximal E2 response at the 40× concentration, and approximately 100% activity at 4× (Figure S2), suggesting either high levels or particularly potent estrogenic contaminants in the groundwater at this site. When excluding this sample from analysis, due to the unique well pad location, no differences were observed between groups. WY-06, 15, 22, and WY-WW1 exhibited approximately 20% ER agonist activity, whereas WY-02 and WY-11 exhibited approximately 15% GR agonist activity (Figure S2). The wastewater sample, WY-WW1, exhibited low levels of agonist activity for ER, AR, PR, and GR but none for TR. Additive agonist activities (agonist activity measured above an added EC_{50} assay baseline) paralleled agonist activities for ER and TR ($p < 0.05$; Figure S3). Low added agonist activities were observed across nearly all samples for AR, GR, and TR. Agonist activities in 4× extracts did not significantly differ between groups (Figure S2).

Receptor Antagonist Activities of Water Extracts

SPE extracts of water samples exhibited a range of antagonist activities in reporter gene assays and diverged from each other more than was observed for agonist activities. Antagonist activities of 40× groundwater extracts from UOG sites exhibited significantly greater ER antagonism than controls ($p < 0.05$) and tended to exhibit greater PR

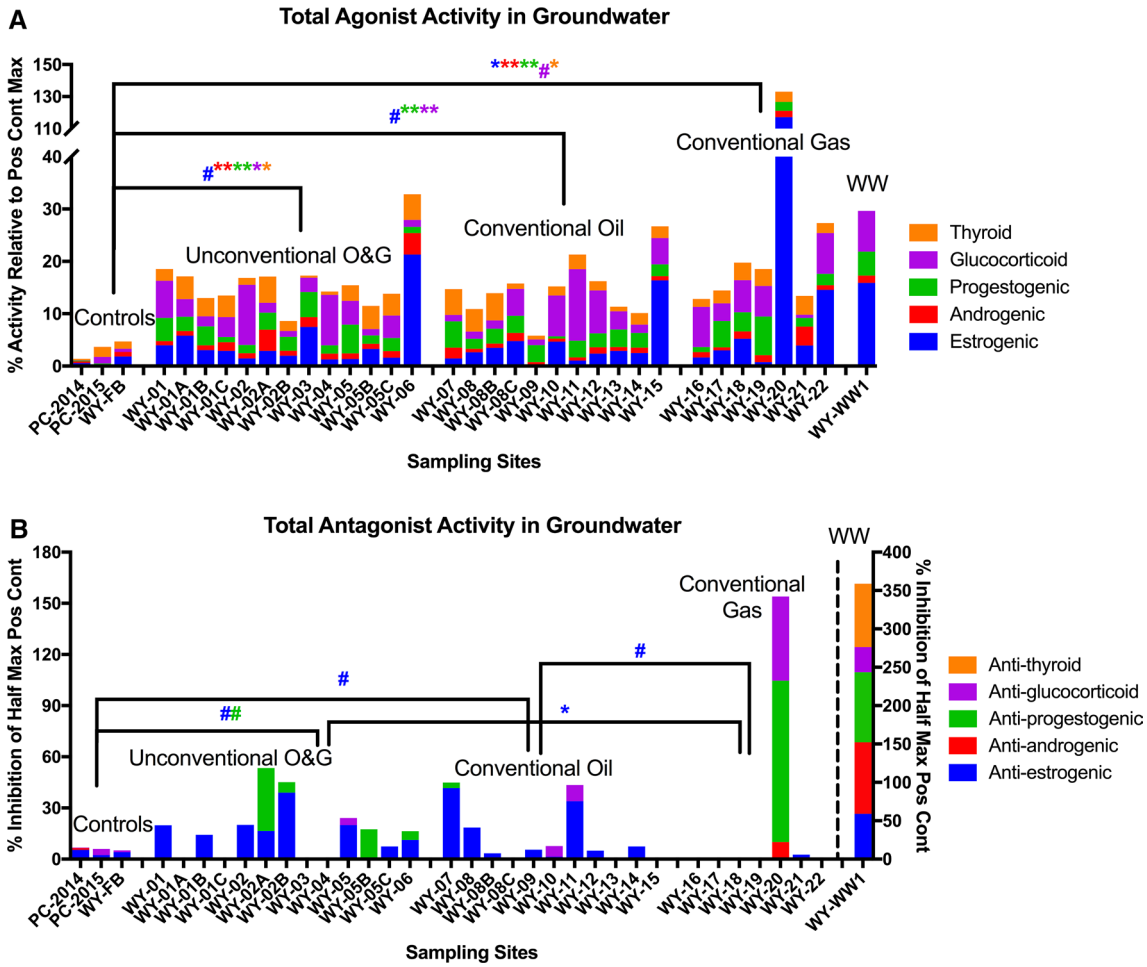


Fig. 2 Agonist and antagonist combined receptor activities of Wyoming groundwater samples. Combined total mean receptor activities for each water sample at 40× concentration, determined using a transiently transfected human receptor reporter gene assay in Ishikawa cells. Combined total agonist activities (**a**) as percent activity relative to the mean intra-assay maximal positive control fold induction for each receptor: 200 pM 17β-estradiol (E2), 3 nM dihydrotestosterone (DHT), 100 pM progesterone (P4), 100 nM tri-iodothyronine (T3), or 100 nM dexamethasone (DEX) for ERα, AR, PR B, TRβ, and GR receptor assays, respectively. Combined total antagonist activities (**b**) as percent suppression of half maximal intraplate positive control response for each receptor: 20 pM E2, 300 pM DHT, 30 pM P4, 2 nM T3, and 5 nM DEX, respectively. Results from replicate process controls are presented as mean values for each analysis period. Right *y* axis applies only to the WY-WW1 SPE sample, as denoted by the dashed *x* axis line. #$p < 0.10$, *$p < 0.05$, as per one-way ANOVA in GraphPad Prism 7.0

antagonism ($p < 0.10$; Fig. 1b). Conventional oil sites tended to exhibit greater ER antagonism than controls ($p < 0.10$), whereas the conventional gas sites were equivalent to controls for all activities (Fig. 1b). Removal of WY-20 from these analyses did not significantly modify any of these relationships. Samples from UOG and conventional oil regions exhibited greater ER antagonist activities than conventional gas sites ($p < 0.05$ and $p < 0.10$, respectively) and exhibited equivalent activities to each other (Fig. 2b). Greater than 20% of samples (3/13) in the UOG region exhibited antagonist activity for PR, whereas none of the conventional oil and one of the conventional gas samples (WY-20) had PR antagonist activity. Conventional gas samples lacked antagonist activities except for the WY-20 well pad site, which exhibited 10, 95, and 50% antagonism for AR, PR, and GR, respectively (Fig. 2b; Supplemental Figure 3). Similarly, the UOG wastewater extract, WY-WW1, exhibited antagonism for AR, PR, and GR, though unlike the well pad site also exhibited antagonism for ER and TR. Antagonist activities in 4× extracts did not significantly differ between groups (Figure S2).

Targeted Analytical Measurement of Oil and Gas Chemicals in Groundwater Extracts

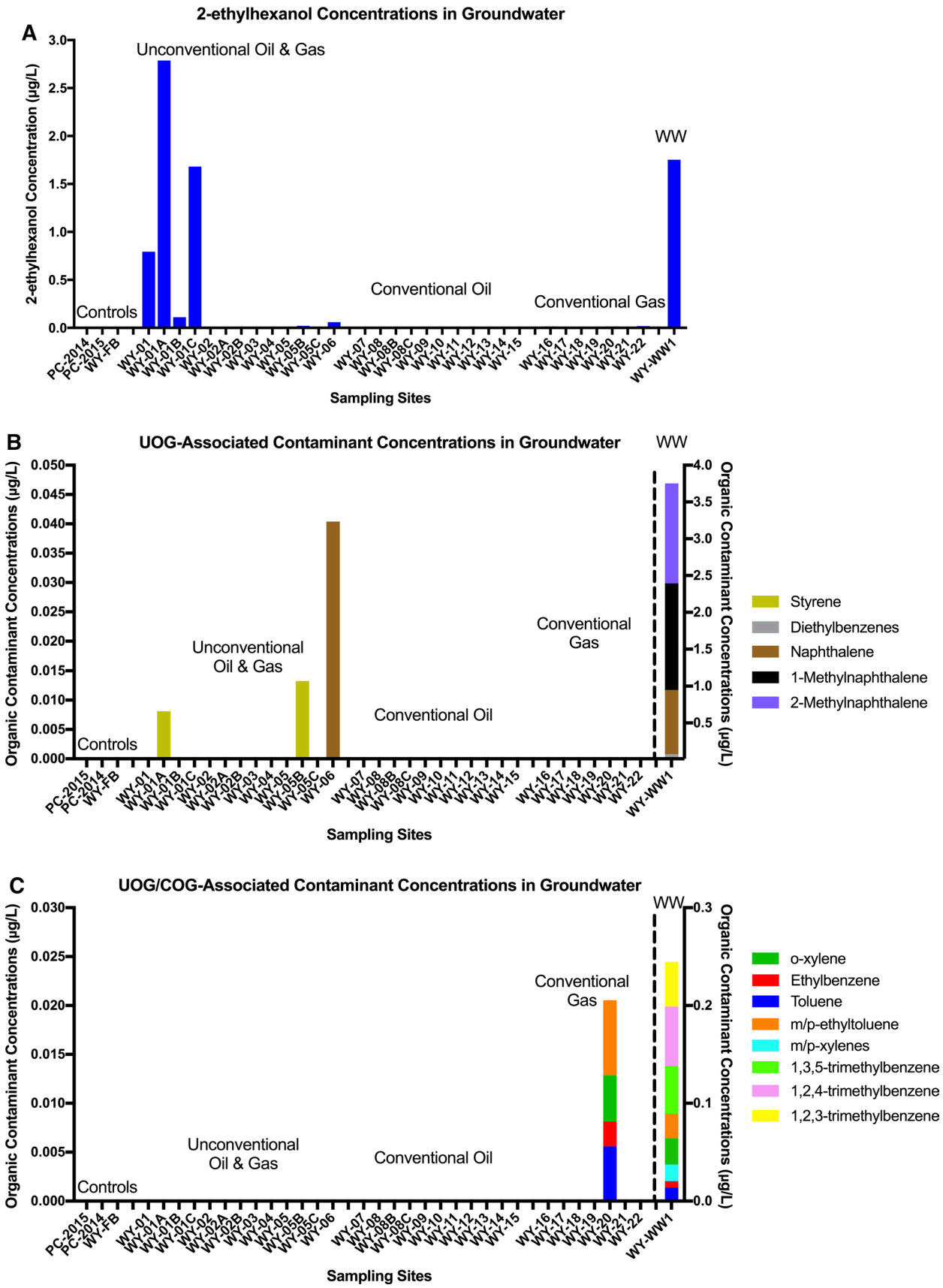
A suite of VOCs, known to be associated with oil and gas operations, was measured in SPE extracts of groundwater samples from each region as well as in one UOG wastewater sample that was assessed both before and after SPE (WY-WW1). Notably, contaminants were measured at several UOG sites and at low concentrations at several conventional gas sites, whereas no contaminants were detected in any conventional oil sites (Fig. 3, Supplemental Table 2). In particular, 2-ethylhexanol was measured at approximately 3 µg/L in WY-01A and was detected in six UOG samples at three sites (Fig. 3a). This chemical was also present in the wastewater sample, WY-WW1 (at 105 µg/L pre-SPE, and at 1.75 µg/L post-SPE; Table 2, Supplemental Table 2). This chemical was also detected at three conventional gas sites (WY-16, 20, and 22) at < 0.01 µg/L. Five VOCs are not known to occur naturally in petroleum, so were herein considered specific to UOG (Fig. 3b). Styrene and naphthalene were detected only at three UOG sites, whereas diethylbenzenes, 1-methylnaphthalene, and 2-methylnaphthalene were not detected at any sites (Fig. 3b); these contaminants were all reported in the UOG wastewater sample (WY-WW1) at concentrations ranging from 4 to 400 µg/L. Eight other VOCs that were assessed are used in UOG operations but also occur naturally in petroleum, so may be associated with either UOG or COG (Fig. 3c). Notably, none of these contaminants were reported at any UOG sites, although they were all reported in the UOG wastewater sample (Table 2). The COG well pad site, WY-20, was the only sample from any region that contained concentrations of these contaminants, with low levels of *o*-xylene, toluene, ethylbenzene, and *m*/*p*-ethyltoluene observed (Fig. 3c; Supplemental Table 2).

Interestingly, the 2014 sample from WY-01 (WY-01A) exhibited approximately three times higher 2-ethylhexanol than the resampling from the same source in 2015 (WY-01), and styrene was only detected in the 2014 sample. This could suggest an abatement of contaminant concentrations over time; this was mirrored in endocrine bioactivity at WY-02, with a decrease in antagonist activities for ER, AR, TR, and PR between 2014 sampling (WY-02A) and 2015 sampling (WY-02) from the same source.

Discussion

We report the presence of putative UOG VOCs (2-ethylhexanol, naphthalene, and styrene) in UOG groundwater extracts from Pavillion, WY, as well as increased endocrine bioactivities in this area relative to controls. We also report the presence of BTEX contaminants in a potable drinking water well (WY-20) from a well pad in the conventional gas region, chemicals that are associated with COG. Sample sites in UOG and conventional gas regions were nearest development, with an average of 50 and 10 wells within 1 mile, respectively. Samples from the conventional oil region had no oil or gas wells within 1 mile, but all three areas had UOG or COG wells within 5 miles. Notably, 2-ethylhexanol was detected in the UOG wastewater sample and in six UOG groundwater extracts from three of six sites (~ 50% detection rate across sites/samples). Considering the low recovery during SPE, this chemical should be evaluated further as a potential candidate organic tracer of UOG contamination. Interestingly, this chemical also was detected at 10- to 100-fold lower concentrations at three sites in the conventional gas region. This suggests potential utilization of this chemical for COG operations. This chemical has an application in drilling muds (used to aid in the drilling of boreholes), which could explain the observed presence in the COG region, or could suggest reworking of COG wells to increase production using UOG technologies, or contamination from another source (it also is used as a plasticizer and in some sunscreens).

Antagonist activities in groundwater extracts appeared varied across regions, with the conventional gas region exhibiting no significant antagonist activities, other than at WY-20. Greater ER antagonism was measured in UOG and conventional oil groundwater relative to conventional gas ($p < 0.05$ and $p < 0.10$, respectively). PR antagonism occurred more frequently in the UOG sites than elsewhere; however, this study was not sufficiently powered to detect significant differences between groups. Agonist receptor activities were similar across drilling regions and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2-ethylhexanol Concentration (µg/L)
Unconventional Oil & Gas
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UOG-Associated Contaminant Concentrations in Groundwater
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Conventional Gas
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Styrene
Diethylbenzenes
Naphthalene
1-Methylnaphthalene
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1,2,3-trimethylbenzene
Sampling Sites

◂**Fig. 3** Organic contaminant concentrations in Wyoming groundwater samples (μg/L). Estimated concentrations (μg/L) for various organic contaminants in Wyoming groundwater extracts. Estimated concentrations for chemicals that are suspected to be specific to unconventional oil and gas (UOG) production (**a**); these have no known natural occurrence in conventional oil and gas (COG) operations. Estimated concentrations for 2-ethylhexanol (**b**), an UOG-specific chemical with no known COG applications or occurrence but that was detected at three COG sites. Estimated concentrations for chemicals that are used in and occur naturally at both UOG and COG operations (**c**); whereas these are all reported to be used in UOG operations, they also naturally occur in petroleum and thus are not specific to UOG extraction. Concentrations are corrected for laboratory concentration due to solid-phase extraction and reflect the calculated concentration that would be present in μg/L of pure water. Due to the techniques employed in the solid-phase extraction process for endocrine bioassays (sample dry-down under nitrogen gas), many of these semivolatile organic contaminants may have been aerosolized, and thus, these concentrations should be considered underestimations of the concentrations likely present in actual samples. As such, nondetects should not infer that the chemicals are not present, only that they are below the level of detection given our laboratory processing techniques and analytical capabilities. Right *y* axis for panels **a** and **c** applies only to the WY-WW1 SPE sample, as denoted by the dashed vertical line

significantly greater than laboratory controls and similar to the agonist activity that we measured in unaffected West Virginia surface water controls (Kassotis et al. 2016b), suggesting potential background activity in Wyoming groundwater. The wastewater sample exhibited minimal agonist but significant antagonist receptor activities across all five receptors, which we have reported for wastewater from WV and CO previously (Kassotis et al. 2014, 2015, 2016b), demonstrating a consistent endocrine activity pattern for UOG wastewater. Interestingly, a groundwater sample collected from the site of a COG well pad (WY-20) exhibited significant antagonist activity for AR, GR, and PR and significant agonist activity for ER. This endocrine activity pattern is distinct from the UOG samples herein as well as previously assessed UOG-impacted samples but is similar to EDC activity reported previously for COG-impacted samples. For example, several studies assessing produced water from North Sea oil production platforms have described high levels of ER agonist and AR antagonist activities (Thomas et al. 2004, 2009; Tollefsen et al. 2007). Given that this was the only sample collected from a well pad, further study should assess the potential source of these chemicals (chemicals used for drilling, maintenance, cleaning, etc.) and prevalence of these activities across well pad sites.

Several chemicals were detected in groundwater extracts from UOG and conventional gas regions that we have previously assessed for bioactivity (Kassotis et al. 2014, 2015). Specifically, 2-ethylhexanol was detected in both regions, naphthalene and styrene were detected in UOG sites exclusively, and the conventional gas well pad site (WY-20) contained toluene, ethylbenzene, and xylene. While BTEX constituents also are associated with petroleum and could be present due to UOG or COG operations, 2-ethylhexanol, naphthalene, and styrene are not and could suggest UOG-sourced contamination in Pavillion. Importantly, these chemicals are all utilized for non-oil and gas operation purposes as well, including gasoline, detergents, cosmetics and personal care products, chemical precursors, and other purposes, and thus could be present at these sites due to non-UOG/COG operations. Future research should attempt to link more directly the identified chemicals to specific anthropogenic environmental inputs. These putative UOG chemicals also are more bioactive than those associated with petroleum more broadly and those detected at WY-20 more specifically. For example, at 10 μM using a reporter gene assay in human cells, 2-ethylhexanol exhibited 30–60% antagonism for ER, AR, PR, and GR; naphthalene exhibited 35–40% antagonism for ER, AR, PR, and TR; and styrene exhibited 25–65% antagonism for ER, GR, and TR (Kassotis et al. 2015). The broader BTEX chemicals (COG and UOG-associated) exhibited less bioactivity. At 10 μM, toluene exhibited 10% antagonism for ER and AR; ethylbenzene exhibited 14–30% antagonism for ER and AR, and xylenes exhibited 13–38% antagonism for ER, AR, and PR (Kassotis et al. 2015). Given the low ng/L concentrations of most contaminants, they individually contributed a minimal amount to the total endocrine activities measured herein. Although as we have demonstrated previously, mixtures containing many of these same chemicals can act additively, antagonistically, and in some cases synergistically in vitro (Kassotis et al. 2015), and given the more than 1000 chemicals used in UOG operations, mixture effects or (yet to be assessed) contaminants are likely promoting most of the activity herein.

All samples were analyzed for VOC concentrations following sample processing for endocrine bioassays (SPE, dry-down, reconstitution); the wastewater sample, WY-WW1, was analyzed before and after. The strength of this analytical approach is that concentrations of these contaminants can be directly compared with observed endocrine bioactivities, as only the chemicals surviving SPE could contribute to observed receptor agonist/antagonist activities. As noted, a limitation of this approach is the substantial loss of VOCs during the sample dry-down process, as can be observed by comparing pre-SPE WY-WW1 concentrations (Table 2) versus post-SPE (Supplemental Table 2). As such, nondetects may not represent an absence of a particular chemical from the groundwater at a particular site but may instead represent loss during SPE. Thus, calculated water concentrations of detected contaminants are underestimations of the actual concentrations in unadulterated groundwater from these sites. Because defined controls were not included to assess recovery in each sample, they were not corrected based on estimated recoveries. Future research should streamline analytical and bioassay processing to allow for more comprehensive assessment of actual chemical concentrations

Table 2 Concentrations (μg/L) of chemicals in unextracted UOG wastewater sample, WY-WW1w

Compound name	CAS #	RT	Concentration (μg/L)
1,2,3-Trimethylbenzene	526-73-8	16.5	135.3
1,2,4-Trimethylbenzene	95-63-6	15.9	171.4
1,3,5-Trimethylbenzene	108-67-8	15.3	26.2
1,2,4,5-Tetramethylbenzene	95-32-2	18.5	94.4
2-Heptanone	110-43-0	12.9	29.4
4-Heptanone	123-19-3	12.0	5.4
Benzene	71-43-2	3.2	112.9
Butyl cyclohexane	1678-93-9	16.8	46.6
Cumene	98-82-8	13.9	22.5
D-Limonene	5989-27-5	16.7	43.2
p-Diethylbenzene	105-05-5	17.3	106.1
m/*o*-Diethylbenzenes	N/A	17.1	102.1
Ethyl cyclohexane	1678-91-7	9.5	9.9
Ethylbenzene	100-41-4	11.3	79.1
Ethylhexanol-1	104-76-7	16.8	105.5
Heptane	142-82-5	3.9	18.6
m/*p*-Xylenes	N/A	11.8	229.7
o-Xylene	95-47-6	12.8	161.7
Methyl cyclohexane	108-87-2	4.5	24.6
n-Decane	124-18-5	16.1	116.4
n-Dodecane	112-40-3	19.9	364.1
n-Nonane	111-84-2	13.2	39.0
n-Undecane	1120-21-4	18.2	290.1
Naphthalene	91-20-3	19.7	350.6
1-Methylnaphthalene	90-12-0	21.7	340.5
2-Methylnaphthalene	91-57-6	21.4	405.5
Octane	111-65-9	7.5	19.1
Pentadecane	629-62-9	24.0	470.1
Propylbenzene	103-65-1	14.8	97.7
Styrene	100-42-5	12.8	4.0
Tetradecane	629-59-4	22.7	448.6
Toluene	108-88-3	5.9	353.2
m-Ethyltoluene	620-14-4	15.1	108.2
p-Ethyltoluene	622-96-8	15.1	68.6
Tridecane	629-50-5	21.4	367.6

Concentrations in micrograms per liter (μg/L) of select chemicals reportedly used in unconventional oil and gas operations and measured in one wastewater sample, collected from an open wastewater fluid pond in Fremont County, Wyoming

RT retention time; *CAS #* chemical abstract service number

present in unadulterated water samples from these regions. A split-processing design where a portion of samples are retained for VOC analysis and the remainder are concentrated for bioassays may be the best path forward and is certainly a prudent strategy for nonvolatile compounds but also limits the utility of comparisons to extract bioactivities for more volatile contaminants.

During the same period as our water sampling was performed, air quality and human biomonitoring was assessed in Pavillion (Crowe et al. 2016). This project reported high concentrations of airborne BTEX and other VOCs (naphthalene, hexane, cyclohexane, etc.) known to be associated with UOG operations in Pavillion (Crowe et al. 2016). This study also detected metabolites of BTEX and other VOCs reported herein in the urine of residents, for some chemicals at levels exceeding those reported for the general population, suggesting increased exposure near UOG operations (Crowe et al. 2016). Some of the VOCs (and metabolites) measured in water samples also were present in human urine samples from this region (including metabolites of BTEX chemicals, styrene, acrylamide, and others). Recent work found elevated *t*,*t*-MA (a benzene metabolite) in the urine of pregnant women near UOG development in Northeastern British Columbia relative to the general population (Caron-Beaudoin et al. 2018). Further work is needed to establish more comprehensive human biomonitoring data for people and animals living in these regions, as well as to better tease apart putative roles of air versus water exposure routes for residents in these areas.

In conclusion, we report increased UOG-associated VOCs in the Pavillion groundwater extracts and these chemicals at high concentrations in a local UOG wastewater sample. We also observed a tendency for increased ER and PR antagonism at UOG sites relative to controls and greater ER antagonism relative to the conventional gas region, suggesting a putative UOG impact on the endocrine activities at these sites, although future research is needed to identify the specific contaminants promoting the observed bioactivities. While our study was not sufficiently powered to distinguish other differences between UOG and COG regions, PR antagonism occurred more frequently at UOG sites. Because receptor agonism appeared quite homogenous between regions, this suggests that future study should focus on receptor antagonism as a potential unique endocrine tracer to distinguish UOG from COG-associated effects on groundwater. A unique suite of VOCs was observed in a potable drinking water well on a well pad in the conventional gas region and not observed at any UOG sites; these chemicals are reported to occur naturally in petroleum and thus can be associated with COG. Interestingly, high levels of endocrine activities (most notably, maximal ER agonist activity) were noted in the groundwater there, suggesting potential impacts on endocrine bioactivities by COG. As such, we report two levels of evidence for groundwater contamination by both UOG and COG operations in Wyoming. While our data suggest potentially unique endocrine profiles of UOG versus COG contamination (UOG: lower ER agonist activity, increased ER, PR antagonism), further study is needed to define a causative relationship between these bioactivities and respective oil and gas operations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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