Estrogen and Androgen Receptor Activities of Hydraulic Fracturing Chemicals and Surface and Ground Water in a Drilling-Dense Region

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The rapid rise in natural gas extraction utilizing hydraulic fracturing increases the potential for contamination of surface and ground water from chemicals used throughout the process. Hundreds of products containing more than 750 chemicals and components are potentially used throughout the extraction process, including over one hundred known or suspected endocrine disrupting chemicals. We hypothesized that a selected subset of chemicals used in natural gas drilling operations and also surface and ground water samples collected in a drilling-dense region of Garfield County, CO would exhibit estrogen and androgen receptor activities. Water samples were collected, solid-phase extracted, and measured for estrogen and androgen receptor activities using reporter gene assays in human cell lines. Of the 39 unique water samples, 89%, 41%, 12%, and 46% exhibited estrogenic, anti-estrogenic, androgenic, and anti-androgenic activities, respectively. Testing of a subset of natural gas drilling chemicals revealed novel anti-estrogenic, novel anti-androgenic, and limited estrogenic activities. The Colorado River, the drainage basin for this region, exhibited moderate levels of estrogenic, anti-estrogenic, and anti-androgenic activities, suggesting that higher localized activity at sites with known natural gas related spills surrounding the river might be contributing to the multiple receptor activities observed in this water source. The majority of water samples collected from sites in a drilling-dense region of Colorado exhibited more estrogenic, anti-estrogenic, or anti-androgenic activities than reference sites with limited nearby drilling operations. Our data suggest that natural gas drilling operations may result in elevated EDC activity in surface and ground water.

Hundreds of synthetic and naturally occurring chemicals have the ability to disrupt normal hormone action and have been termed endocrine disrupting chemicals (EDCs). EDCs can act through multiple mechanisms: direct interaction with hormone receptors (1, 2), indirect enhancement or suppression of a receptor’s ability to respond to endogenous hormones (3, 4), or modulation of endogenous hormone levels (4, 5). EDCs are unique from toxicants in that they have been shown to exhibit non-monotonic dose response curves, resulting in quantitatively and qualitatively different health outcomes at low vs high doses. Laboratory experiments have shown a wide range of effects at environmentally relevant, low concentrations that were not predicted by traditional risk assessments from high-dose testing (6–9). EDCs may be of particular concern during critical windows of development when exposure can alter normal development and has been linked to adult disease (6, 9).

EDCs have been measured in humans and other animals and exposure has been linked to a number of negative health effects (9–11). While EDCs have been described to disrupt many hormone systems, chemicals that disrupt
of wastewater can be heavily laden with naturally occurring chemicals from the shale layer (22). Produced water is composed of naturally occurring compounds ranging from increasing the viscosity to serving as anti-androgenic activities of twelve suspected or known EDCs used in natural gas operations. Second, we measured the same activities in surface and ground water from a natural gas drilling-dense region in Garfield County, Colorado (Figure 1), an area with approximately 10,444 active wells (33). Of particular concern with exposure to EDCs is the potential for additive effects of mixtures of chemicals that act through a common biological pathway, even when each chemical in the mixture is present at levels below an observed effect threshold (17, 34, 35). Due to this, several researchers have taken the approach of measuring the total bioactivity of chemicals with a common mechanism of action in water samples (36, 37). This approach leads to a greater sensitivity of detection as multiple chemicals with the same mechanism of action have additive effects, very relevant when detecting potential contamination of water with hundreds of chemicals at low

A potential novel source of EDCs is through their use in hydraulic fracturing operations for natural gas and/or oil extraction processes. Hydraulic fracturing involves the underground injection of several million gallons of water combined with chemicals and suspended solids (proppants) into each well under high pressure. More than 750 chemicals are reportedly used throughout this process. Of these, more than 100 are known or suspected endocrine disrupting chemicals and still others are toxicants and/or carcinogens (19, 20). The rapid expansion in drilling operations utilizing hydraulic fracturing increases the potential for environmental contamination with the hundreds of hazardous chemicals used (20, 21). Importantly, hydraulic fracturing was exempted from multiple federal regulatory acts in 2005 including the Safe Drinking Water Act, the Clean Water Act, and the Clean Air Act (21).

Chemicals are added throughout the drilling and fracturing processes for a variety of reasons. For example, during drilling they are used to reduce friction and shorten drilling time (21, 22). In horizontal or directional wells, drilling starts vertically and then turns and proceeds for up to a mile or more. Following stabilization, several million gallons of water, chemicals, and proppants are injected into the well under high pressure to form and maintain fractures throughout the shale or coal bed layer to liberate natural gas and/or oil. Chemicals are injected for reasons ranging from increasing the viscosity to serving as anti-bacterial agents (22, 23). Once the water mixture has been forced into the well under high pressure, up to 40% may be immediately recovered as flow back and contains the chemicals used for fracturing as well as some naturally occurring chemicals from the shale layer (22). Produced water is composed of naturally occurring compounds from the shale formation as well as remaining hydraulic fracturing fluids that come to the surface over the life of a producing well. It should be noted that both of these types of wastewater can be heavily laden with naturally occurring radioactive compounds, heavy metals from the shale layer, and chemicals used in fracturing operations (22, 24) and may be injected into disposal wells, reused in drilling operations, or pumped into open evaporation pits (21, 22).

There have been many reports of changes in surface, ground, and drinking water quality near natural gas drilling operations, particularly in drilling-dense regions, with some specifically linked to natural gas extraction (21, 25, 26). For example, in 2011, the EPA concluded that chemicals used in natural gas operations had contaminated ground water and domestic water supply in Pavillion, Wyoming (25).

There are many pathways for chemicals used in natural gas operations to contaminate surface and ground waters: spills during transport before and after extraction, the drilling and fracturing processes, disposal of wastewater, failure of well casings, and from structural issues surrounding abandoned wells (27, 28). Multiple researchers have demonstrated that levels of stray gases and heavy metals in drinking water increased with proximity to natural gas wells, suggesting the possibility of underground migration of fluids associated with hydraulic fracturing (29–31). Vengosh and colleagues further reported natural connectivity between shallow drinking water aquifers and formations deep underground in areas of the Marcellus Shale (32), suggesting a route for the potential migration of natural gas drilling fluids into ground water. These studies support the hypothesis that fracturing fluids remaining underground have the potential to migrate into shallow ground water sources over time. Taken together, there is the potential for surface and ground water contamination throughout the entire extraction process.

The goals of this study were two fold. First, we measured the estrogenic, antiestrogenic, androgenic, and antiandrogenic activities of twelve suspected or known EDCs used in natural gas operations. Second, we measured the same activities in surface and ground water from a natural gas drilling-dense region in Garfield County, Colorado (Figure 1), an area with approximately 10,444 active wells (33). Of particular concern with exposure to EDCs is the potential for additive effects of mixtures of chemicals that act through a common biological pathway, even when each chemical in the mixture is present at levels below an observed effect threshold (17, 34, 35). Due to this, several researchers have taken the approach of measuring the total bioactivity of chemicals with a common mechanism of action in water samples (36, 37). This approach leads to a greater sensitivity of detection as multiple chemicals with the same mechanism of action have additive effects, very relevant when detecting potential contamination of water with hundreds of chemicals at low
concentrations. We hypothesized that 1) a subset of chemicals used in natural gas operations would exhibit estrogen and/or androgen receptor activity and 2) surface and ground water in this natural gas drilling-dense area, impacted by drilling-related spills, would exhibit greater estrogen and androgen receptor activities than reference sites with no or limited drilling activities.

Materials and Methods

Chemicals. 17β-estradiol (98% pure), ICI 182–780 (Fulvestrant, 98% pure), testosterone (98% pure), flutamide (100% pure), and all other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO). Stock solutions were prepared in HPLC-grade methanol (Fisher Scientific catalog # A452–1) at 10 mM and stored at 4°C. The twelve chemicals used in natural gas operations that were selected (Supplemental Table 1) were chosen from lists of all known chemicals used in natural gas operations (19, 20), narrowed by selecting only chemicals that were known or suspected endocrine disrupting chemicals (20), those reportedly used in Colorado, and preference given to chemicals used in multiple chemical products.

Sample Collection. All samples were collected in one-liter amber glass bottles (Fisher Scientific catalog # 12–100–130) and certified to meet the EPA standards for metals, pesticides, volatiles, and nonvolatile. Surface water samples were taken from water that had collected on the ground such as rivers, creeks, and ponds, and were collected by submerging bottles approximately ten inches. Ground water samples were taken from water that had collected underground, typically accessed via drinking or monitoring wells. Artesian water samples were defined as ground water sources that had flowed to the surface under pressure and were collected where they met the surface. Samples were collected by filling bottles two times from the source prior to keeping the third collection. Samples were stored on ice in the field, stored at 4°C in the laboratory, and processed within two months of collection. All analyses were performed blinded to sample identification using a unique 6-digit bottle ID.

Reference Control Sites. Ground water reference samples were collected from one drilling absent location in Boone County, Missouri (MO Ref) in 2011 and two drilling sparse (<2 wells within one mile) locations in Garfield County, Colorado (CO Ref) in February 2013 within the bounds of the Piceance Shale Basin (Figure 1, Table 1, Supplemental Table 2). Surface water reference samples were collected from two drilling absent locations in Boone County, Missouri (MO Ref) in 2011. Surface water reference samples from drilling sparse locations in Garfield County were not obtained due to the scarcity of surface water sources not impacted by nearby drilling operations.

Sample Sites. Water samples were collected from ground, surface, and artesian (n = 9, 19, and 1, respectively) water sources in September 2010 in drilling-dense areas of Garfield County, CO from five distinct sites with unique characteristics (Figure 1, Table 1, Supplemental Table 2). All sites were located within the Colorado River Drainage Basin and the Piceance Shale Basin, had been directionally fractured to extract natural gas, contained from 43 to 136 natural gas wells within one mile (Table 1), and a spill or incident related to natural gas drilling processes had occurred within the past six years. Five surface water samples were also collected from the Colorado River, the drainage basin for this drilling dense region.

Process Controls. Process controls were prepared using one liter of Fisher HPLC-grade water (Fisher Scientific catalog # WFSK-4) following the same procedure used for all experimental samples. These controls were included in all assays to measure any background hormonal activity contributed by the solid phase extraction process.

Extraction of Water Samples. Water samples (1-L) were filtered through a ceramic Buchner funnel using Whatman Filter Paper #54, 90 mm, to remove suspended solids and were then subjected to solid-phase extraction using Oasis HLB glass car-

Figure 1. Map of Garfield County sample collection area. Pictoral representation of the sample collection area in Garfield County, CO. White rectangle denotes the zone from which all high-density sample collection sites (Sites 1–5) were collected. X marks denote high-density drill sites that had also experienced a drilling-related spill and R marks denote local reference sites outside of the high-density drilling area. Red, orange, and yellow circles denote natural gas drilling wells in various stages of operation as of Jun 2008. This represents an underestimation of the wells present when our samples were collected in Sep 2010. Credit for map data to Google, Image Landsat. Credit for well data to SkyTruth for tabulating and mapping Colorado Oil & Gas Conservation Commission data on wells active as of Jun 2008.
tridges (Waters catalog # 186000683) (38). All additions to the cartridges were made using disposable borosilicate glass pipets. Cartridges were attached to a vacuum manifold and conditioned with 100% HPLC-grade methanol and 100% HPLC-grade H2O. Water samples were loaded onto the cartridge and washed with 5 mL of 5% methanol. They were then removed from the manifold and seated on amber glass vials, where elution was performed with three 1-mL additions of 100% methanol. Eluted samples were then dried under nitrogen and reconstituted in 250 µL methanol (100%), creating stock concentrations of 4,000x the original water concentration. Reconstituted samples were stored at 4°C, protected from light, until tested. In order to be applied to cells, stock samples were diluted 100 and 1,000-fold in tissue culture medium, creating final concentrations, in contact with the cells, of 40x and 4x the original water concentration.

**Extraction Method Recovery Efficiencies.** Extraction method recovery efficiencies were determined using 3H-17β-estradiol (100 Ci/mmol; Perkin Elmer, Waltham, MA), 3H-testosterone (70 Ci/mmol; Perkin Elmer, Waltham, MA), and 3H-bisphenol A (7.3 Ci/mmol; Moravek Biochemicals, Brea, CA). Trinitiated chemicals were spiked at an activity of 1 µCi each in one liter of water and processed in the manner described above. Final concentrations of test chemicals used included 1.4 pM testosterone, 1.4 pM 17β-estradiol, and 140 pM bisphenol A. Recovery was 71.5% ± 3.5% for 3H-17β-estradiol, 79.0% ± 3.6% for 3H-testosterone, and 71.1% ± 4.1% for 3H-bisphenol A.

**Cell Culture.** HepG-2 cells (ATCC # HB-8065) were maintained in Gibco Minimum Essential Medium (MEM) supplemented with 8% fetal bovine serum (Thermo Hyclone cat # SH30396.03), 2 mM glutamnax, 0.1 mM nonessential amino acids, and 6 ng/mL bovine insulin. Water sample and chemical dilutions were performed in respective media as described above with the following exceptions: medium used was phenol-red free and sera were charcoal-stripped to remove endogenous steroids. Cell lines were transferred to this modified medium two days prior to the start of assays.

**Plasmids.** For androgenic activity testing, HepG-2 cells were transfected with androgen receptor, pSG5-AR (39), androgen response element linked to the firefly luciferase gene, 2XC3ARETKLuc (lab of Donald P. McDonnell), and CMV-β-Gal (40). For antiandrogenic activity testing, HepG-2 cells were transfected with androgen receptor, CMV-AR1 (41), androgen response element linked to the firefly luciferase gene, PSA-Enh E4TATA-luc (42), and CMV-β-Gal (40). For estrogenic and antiestrogenic activity testing, MCF-7 cells were transfected with estrogen response element linked to the firefly luciferase gene, 3XERETKLuc (43), and CMV-β-Gal (40).

**Estrogen and Androgen Receptor Reporter Gene Assays.** Activities were measured using reporter gene assays containing a hormone response element linked to luciferase. Each treatment concentration for each sample was performed in quadruplicate within each assay and each assay was repeated three times. Cells were cotransfected with the vectors listed above using MEM with reduced serum (Invitrogen catalog # 31985). Cells were transfected in T25 or T75 flasks for approximately 5 hours using Lipofectamine LTX and Plus Reagent (Invitrogen catalog # 15338–100) and then allowed to recover overnight. Transfected cells were then trypsinized, seeded into 96-well tissue culture plates at approximately 70,000 cells per well, and allowed to settle for four hours before induction. Cells were induced with dilution series of the positive/negative controls, the reconstituted water samples at 4x and 40x concentrations, or a dilution series of the selected subset of chemicals from 10 µM – 10 nM, diluted in medium as described above using a 1% methanol vehicle for all concentrations tested. Androgen assays used a dose response

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**Table 1. Description of Sample Collection Sites**

<table>
<thead>
<tr>
<th>Site Number</th>
<th>Samples Collected (n = j)</th>
<th># NGD wells within 1 mile(1)</th>
<th>Distance to CO River (miles)</th>
<th>Approximate Well Depth (ft)(2)</th>
<th>Approximate Frack Fluid Vol (gal)(2)</th>
<th>Description of Incident</th>
<th>Date of incident(3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO Ref</td>
<td>3</td>
<td>0</td>
<td>N/A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CO Ref</td>
<td>2</td>
<td>≤2</td>
<td>4.75–6.5</td>
<td>Unknown</td>
<td>Unknown</td>
<td>-</td>
<td>-</td>
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<tr>
<td>1</td>
<td>8</td>
<td>43</td>
<td>5.25</td>
<td>5,500</td>
<td>4,000,000</td>
<td>Natural gas upwelling</td>
<td>May-08(3)</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>78</td>
<td>0.75</td>
<td>8,000</td>
<td>1,500,000</td>
<td>Fluid spill into creek</td>
<td>Dec-09(3)</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>69</td>
<td>8.75</td>
<td>9,500</td>
<td>1,000,000</td>
<td>Spill at nearby drill pad</td>
<td>May-08(3)</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>136</td>
<td>6.00</td>
<td>9,000</td>
<td>4,000,000</td>
<td>Produced water tank leak</td>
<td>Nov-04(3)</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>95</td>
<td>0.50</td>
<td>7,500</td>
<td>3,000,000</td>
<td>Produced water line leak</td>
<td>Jul-10(3)</td>
</tr>
<tr>
<td>CO Riv</td>
<td>5</td>
<td>Varied</td>
<td>N/A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

NGD = natural gas drilling  
(1) Uses a radius of one mile from the sampling location. Number is approximate based on data obtained from the Colorado Oil & Gas Conservation Commission, accessed at http://dnrweb.coma useDispatch.state.co.us/mg2010app/ on April 19, 2012.  
(2) Information on well depth and typical fracturing fluid volume obtained from Fracfocus based on wells after January 1, 2011 for the same radius as used for well number determination. All samples were collected in September 2010.  
(3) Documented benzene levels exceeding acceptable limits detected in water tests conducted on or around this date.
of testosterone as a positive control (EC50 ~ 40 nM) and fluoxetine as a negative control (10 μM; IC50 ~ 200 nM, concentration required to suppress half the positive control activity), while estrogen assays used a dose response of 17β-estradiol as a positive control (EC50 ~ 5 pM) and ICI 182,780 (ICI) as a negative control (100 nM; IC50 ~ 250 pM) (Supplemental Figure 1). The estrogen and androgen reporter gene assays have sensitivities within the ranges of other published studies, as reviewed previously (44). After induction for 18–24 hours, cells were incubated in a cell lysis solution for twenty minutes at 37°C before using lysate for a luciferase reporter gene assay and β-galactosidase assay.

Hormonal activity was measured using a firefly luciferase reporter gene assay, as described previously (45). CMV-β-Gal activity was measured using a chlorophenolred-β-d-galactopyranoside substrate diluted to a concentration of 500 µg/mL in a buffer consisting of 60 mmol/L sodium phosphate dibasic, 40 mmol/L sodium phosphate monobasic, 10 mmol/L potassium chloride, 1 mmol/L magnesium sulfate, and 50 mmol/L β-mercaptoethanol. The above mixture (200 µL) was added to 20 µL of cell lysate in a 96-well microtiter plate. Color was allowed to develop before reading the absorbance on a plate reader at a 570 nm wavelength.

CMV-β-Gal activity was used to normalize estrogen receptor assays but not used for androgen receptor assays. We found androgens to regulate CMV-β-Gal expression so did not use this to normalize the androgenic luciferase data. However, transfections were performed in flasks and then seeded into tissue culture plates, controlling for changes in transfection efficiency between wells. As such, comparing the coefficient of variation (CV; standard deviation/mean) of normalized samples to un-normalized samples resulted in minimal change.

Sample Toxicity. In MCF-7 cells, we used CMV-β-Gal activity as a marker of cell number. A serial 10-fold dilution of transfected cells was used to assess the reliability of using CMV-β-Gal activity as a marker of cell number (r² = 0.996). As a result, we used this as a surrogate marker for sample toxicity, as estrogens were not found to regulate CMV-β-Gal expression. Thus, any sample found to have deviated significantly from the activity of the vehicle was deemed toxic and excluded from analysis. The following samples were excluded from analysis at the 40x concentration only: 1E, 3D, 5B, 5C, and 5E, while sample 3B was excluded at both the 4x and 40x concentrations for all assays. All samples were excluded from analysis at the 40x concentration within the androgenic assays due to observed cell-specific toxicity in the HepG-2 cell line. No evidence of toxicity was observed at the 4x concentration.

Calculation of Estrogen/Androgen Receptor Activities. Agonist activities were calculated as percent activity relative to the maximal positive control response of 100 pM 17β-estradiol and 1 μM testosterone for estrogen and androgen receptor assays, respectively. Antagonist activities were calculated as a percent suppression or enhancement of 10 pM E2 or 100 nM testosterone, based on the EC50s of the positive controls. Positive values denote additive agonist activities and negative values denote antagonist activities.

Statistical Analysis. Linear mixed models (hierarchical linear models) were used to analyze the results from all three assays (estrogenic, antiestrogenic, and antiandrogenic), and incorporated random effects to account for among measurements arising from the same sampling source within a site (Supplemental Figure 3–5). Fixed effects considered included site (Sites 1–5, Colorado River, Colorado Reference, and Missouri Reference), water type (ground/surface), concentration (40x/4x) and a covariate for the negative control of the assay plate, which was conceived as a baseline response for the assay. The Kenward-Roger method was used for estimating the degrees of freedom. Least-squares (LS) means, based on the final models, were used for planned contrasts and to compute 95% confidence intervals for differences of interest. A model selection criterion, corrected Akaike information, was used to evaluate relative goodness of fit of the models, and therefore helped determine the final form of the model for the estrogenic assay. For ease of comparison and to avoid averaging over effects which may interact based on statistical results from the estrogenic assay, the same model form was used for the other assays when possible. Diagnostic plots were used to assess model fit and check distributional assumptions. PROC GLIMMIX in SAS 9.3 (SAS Inc., Cary, NC) was used for the data analysis.

Results

Estrogen and Androgen Receptor Activities of Chemicals Used in Natural Gas Operations. Antiestrogenic, antiandrogenic, and limited estrogenic activities were observed in the 12 natural gas drilling chemicals tested (Figure 2, Supplemental Table 1), while no androgenic activity was observed. At 10 μM, antiestrogenic activities ranged from 24% to 65% suppression of 10 pM 17β-estradiol (E2) and antiandrogenic activities ranged from 0% to 63% suppression of 100 nM testosterone. The chemicals exhibited IC10s (concentrations required to suppress 10% of the maximal activity of the positive control) ranging from 0.15–6.33 μM (Figure 2). Of note, 2-ethyl-1-hexanol (IC10 = 0.60 μM) and ethylene glycol (IC10 = 0.15 μM) exhibited the greatest potencies for antiestrogenic activities and ethylene glycol (IC10 = 0.50 μM), n,n-dimethylformamide (IC10 = 0.50 μM), and cumene (IC10 = 0.62 μM) exhibited the greatest potencies for antiandrogenic activities. Estrogenic activity was observed for bisphenol A, which exhibited supra-agonistic activity and an EC50 of 2.00 μM (concentration required to exhibit half of its maximal activity). To our knowledge this is the first report of antiestrogenic activity of ethylene glycol monobutyl ether, 2-ethylhexanol, ethylene glycol, diethanolamine, diethyleneglycol methyl ether, sodium tetraborate decahydrate, 1,2-bromo-2-nitropropane-1,3-diol, n,n-dimethylformamide, cumene, and styrene; and novel anti-androgenic activity of 2-ethylhexanol, naphthalene, diethanolamine, sodium tetraborate decahydrate, 1,2-bromo-2-nitropropane-1,3-diol, and cumene.
Overall Estrogen and Androgen Receptor Activities of Water Samples. Surface and ground water samples were collected from Sites 1–5 (sites in Garfield County with known natural gas drilling spills in high-density natural gas drilling region), several locations along the Colorado River (the drainage basin for the entire drilling region), local reference sites in Garfield County with limited drilling activities nearby, and reference sites in Boone County, Missouri, an area devoid of natural gas drilling (Figure 1, Table 1, Supplemental Table 2). Estrogenic, antiestrogenic, androgenic, and antiandrogenic activities were observed in 89%, 41%, 12%, and 46% of all water samples, respectively (Supplemental Figures 2, 3). The type of activities observed differed widely between sites (Figure 3, Supplemental Figure 2, 3). Ground water at Sites 1, 2, and 3 exhibited near maximal estrogenic activities and low to moderate androgenic activities, while both Garfield County and Missouri reference sites exhibited low levels of estrogenic activities only (Figure 3A). Surface water at Sites 1–5 varied greatly; Sites 1 and 4 exhibited low estrogenic, high antiestrogenic, and low to moderate antiandrogenic activities, Sites 3 and 5 exhibited higher estrogenic and lower antiestrogenic activities, and Site 2 exhibited only estrogenic activities (Figure 3B). Colorado River samples exhibited activities at moderate levels, while Missouri reference sites exhibited low estrogenic, very low antiestrogenic, and no antiandrogenic activities.

The results from all three assays

Figure 2. Estrogen and Androgen Receptor Activities of Selected Chemicals Used in Natural Gas Operations. Representative dose responses of selected hydraulic fracturing chemicals tested for antiestrogenic (A) and antiandrogenic (B) activities. Antiestrogenic activity presented as the percent suppression of 10 pM 17β-estradiol (set to 100%) for each chemical from 0.1–100 μM. Antiandrogenic activity presented as the percent suppression of 100 nM testosterone (set to 100%) for each chemical from 0.1–100 μM.

Figure 3. Combined estrogen and androgen receptor activities of ground and surface water by site. Combined estimated marginal means of estrogenic (blue), antiestrogenic (red), and antiandrogenic activities (green) at each sample collection site for ground water (A) and surface water (B). Estrogenic activities expressed as a percent of the activity of 100 pM 17β-estradiol at 40x concentration, antiestrogenic activities expressed as percent suppression of 10 pM 17β-estradiol at 40x concentration, and antiandrogenic activities expressed as percent suppression of 100 nM testosterone at 4x concentration. Antagonist activities expressed as positive values; additive agonist activities not expressed on this figure. The absence of a sample group for a particular figure panel is due to no samples present at that site for that particular water type. See Supplemental Table 2 for more details on each group.
were modeled using a mixed model framework (Supplementary Figures 4, 5, 6), with final model forms for the estrogenic and antiestrogenic assays consisting of a three-way interaction (and all lower order terms) among the fixed effects (site, water type, and concentration), along with the baseline covariate (vehicle control). For the antiandrogenic assay, there was only one level of concentration used (4x), so three-way interactions were not applicable. The antiandrogenic model consisted of a site-by-water type interaction term, main effect terms for site and water, and the baseline covariate (vehicle control).

Estrogenic Activities of Water Samples from Natural Gas Drilling-Dense vs Sparse Sites. Estrogenic activities were observed in both ground and surface water at Sites 1–5 and in Colorado River samples. Low estrogenic activities were also observed in Garfield County and Missouri reference sites. Ground water samples collected from Sites 1–3 exhibited higher estrogenic activities than both Garfield County and Missouri reference samples ($P < .0001$; Figure 4A, Supplemental Tables 3, 4). Interestingly, ground water samples collected from Garfield County reference sites exhibited higher estrogenic activities than Missouri.

**Figure 4.** Average estrogen and androgen receptor activities of ground and surface water samples by site. Estimated marginal means ± SEM of estrogenic activities of each ground water (A) and surface water site (B) relative to 100 pM 17β-estradiol at 40x sample concentration. Estimated marginal means of antiestrogenic activities of each ground water (C) and surface water site (D) as percent suppression or enhancement of 10 pM 17β-estradiol (set to zero) at 40x concentration. Negative values denote suppression of agonist activities and thus antagonist activities. Estimated marginal means of antiandrogenic activities of each ground water (E) and surface water site (F) as percent suppression or enhancement of 100 nM testosterone (set to zero) at 4x concentration. Negative values denote suppression of agonist activities and thus antagonist activities. Superscript letters denote statistical similarities and differences between sample groups within each pane. Groups containing the same letter were found to be the same, while groups with different letters were found to be significantly different. The absence of a sample group for a particular figure panel is due to no samples present at that site for that particular water type. See Supplemental Table 2 for more details on each sample group.
reference sites ($P < .05$). Estrogenic activities tended to be higher in ground water samples than in surface water samples, with Sites 1–5 exhibiting a minimum of 75% of maximal activity compared to a maximum of 60% in surface water samples. Surface water samples at Sites 2, 3, and 5 exhibited greater estrogenic activities than Missouri reference sites ($P < .05$; Figure 4B, Supplemental Tables 3, 4).

**Antiestrogenic Activities of Water Samples from Natural Gas Drilling-Dense vs Sparse Sites.** Antiestrogenic activity was observed in surface water at Sites 1, 3, 4, 5, and in Colorado River samples. Little to no antiestrogenic activity was observed in Garfield County or Missouri reference sites. Ground water samples exhibited little to no antiestrogenic activity, with Sites 1–3 tending to exhibit greater additive agonist activities than reference sites (Figure 4C, Supplemental Tables 3, 4), likely due to the high levels of estrogenic activities exhibited by these samples (Figure 4A). Antiestrogenic activity was almost exclusively exhibited by surface water samples, where more apparent differences were observed between Sites 1–5. Notably, Sites 1 and 4 exhibited greater antiestrogenic activity than Missouri reference sites ($P < .05$; Figure 4D, Supplemental Tables 3, 4). The surface water samples collected from the Colorado River exhibited moderate activity, having less than Site 4, which exhibited the highest antiestrogenic activity ($P < .05$) but no difference from Sites 1, 3, or 5. Site 2 displayed a clear absence of antiestrogenic activity.

**Antiandrogenic Activity of Water Samples from Natural Gas Drilling-Dense vs Sparse Sites.** Antiandrogenic activity was observed in ground and surface water at Sites 1, 3, 4, 5, and in Colorado River samples. No antiandrogenic activity was observed in Garfield County or Missouri reference sites. Water samples collected from Sites 1–3 exhibited higher antiandrogenic activity than Garfield County reference samples that exhibited additive agonist activity ($P < .01$), but did not differ from Missouri reference sites that displayed no androgen receptor activity (Figure 4E, Supplemental Tables 3, 4). Surface water samples collected from Sites 1, 4, and 5 displayed greater antiandrogenic activity than Missouri reference sites ($P < .05$; Figure 4F, Supplemental Tables 3, 4). Surface water samples collected from the Colorado River again displayed intermediate antiandrogenic activity that did not differ from Sites 1–5 but that were significantly greater than the activity exhibited by Missouri reference sites ($P < .05$). Site 2 displayed a clear absence of antiandrogenic activity.

**Discussion**

We report for the first time estrogenic, antiestrogenic, and antiandrogenic activity in a selected subset of chemicals used in natural gas operations and the presence of these activities in ground and surface water from a natural gas drilling-dense area in Garfield County, Colorado. One of twelve chemicals tested exhibited estrogenic activity, eleven had antiestrogenic activity, and ten had antiandrogenic activity. While these chemicals were selected because of their suspected or known EDC activity (19, 20), very few had been shown to have direct receptor activity (44, 46–50). Thus, this is the first demonstration of antiestrogenic or antiandrogenic activity for most these chemicals.

Importantly, we found that water samples from sites with known natural gas drilling incidents had greater estrogen and androgen receptor activities than drilling sparse or absent reference sites. Very little estrogen or androgen receptor activity was measured in drilling-sparse reference water samples, moderate levels were measured in samples collected from the Colorado River (the drainage basin for all Colorado collection sites), and moderate to high activities were measured in water samples from Garfield County spill sites. The Garfield County spill sites were known to have various types of contamination including produced water (wastewater and chemical mixture recovered after hydraulic fracturing) pipe leaks, a produced water tank spill, the improper disposal of produced water into surface water, and a natural gas upwelling (Table 1), which may have resulted in the distinct site-specific patterns of activities observed. At Site 1, several ground water samples exhibited antiestrogenic activities despite the absence of antiestrogenic activities across all other ground water samples (Supplemental Figure 2, 3). However, water quality testing performed at this site in September 2010 revealed high levels of mixing between surface and ground water, possibly explaining the notable differences observed (51). Site 2 exhibited an absence of antiestrogenic and antiandrogenic activities in contrast to the other spill sites. As described in Table 1, the spill at this site occurred into a creek and thus likely traveled away from the spill site more readily than at other sites, suggesting a basis for the different pattern of hormonal activities.

In the present study, we identified EDC activity of several individual chemical components used in natural gas operations that may contribute to the activity that we measured in water. Independent analyses identified these or similar chemicals at several of the sites we collected water from, despite the fact that our study did not pursue analytical identification of chemicals present in our water samples. At Site 1, researchers at the University of Colo-
rado collected water samples in September 2010 and performed analytical identification of chemicals present. Their testing revealed five polyethylene glycols used in natural gas drilling operations to be present in ground water from a monitoring well at this site (51). Our analysis of three ethylene glycols revealed antiestrogenic and antiandrogenic activities for ethylene glycol, ethylene glycol butyl ether, and diethylene glycol methyl ether. At Site 5, an analytical laboratory found that water samples contained elevated levels of several BTEX (benzene, toluene, ethylbenzene, and xylenes) chemicals, which are reported to be associated with fracturing fluids (19–21). Naphthalene, which exhibited both antiestrogenic and antiandrogenic activity in the current study, was detected in soil samples collected from Site 5 (52). Further, it was only detected at the site of the spill and not in the surrounding area, strongly suggesting that the source was the produced water leak.

Both naturally occurring chemicals and synthetic chemicals from other sources could contribute to the activity observed in the water samples collected in this study (53–56). While agricultural and animal care operations could potentially contribute to the measured activity in Garfield County, all sample sites were on land devoid of any recent animal care or agricultural use so these sources are likely to have minimal contributions. Wastewater contamination is another potential source of EDCs and we acknowledge that Missouri Reference samples were collected in a more urban area than Colorado samples (Boone County population approximately three times greater than Garfield County). However, as Garfield County samples were all collected in more rural areas, we expect that any potential contribution through wastewater contamination would be lower in these samples. Further, the more urban samples were found to exhibit the lowest levels of hormonal activity in the current study. Taken together with independent analytical identification of drilling-related chemicals at sites we sampled from, this provides further support for a link to the source of the activity observed.

Exposure to EDCs has been linked to a number of negative health outcomes in laboratory animals, wildlife and humans (2, 12–17). Despite an understanding of adverse health outcomes associated with exposure to EDCs, research on the potential health implications of exposure to chemicals used in hydraulic fracturing is lacking. Bamberger and Oswald analyzed the health consequences associated with exposure to chemicals used in natural gas operations and found respiratory, gastrointestinal (GI), dermatologic, neurologic, immunologic, endocrine, reproductive, and other negative health outcomes in humans, pets, livestock, and wildlife species (26). Of note, Site 4 in the current study was used as a small-scale ranch prior to the produced water spill in 2004. This use had to be discontinued because the animals no longer produced live offspring, perhaps due to the high antiestrogenic activity observed at this site. There is evidence that hydraulic fracturing fluids are associated with negative health outcomes, and there is a critical need to quickly and thoroughly evaluate the overall human and environmental health impact of this process. It should be noted that although this study focused on only estrogen and androgen receptors, there is a need for evaluation of other hormone receptor activities to provide a more complete endocrine disrupting profile associated with natural gas drilling.

In conclusion, most water samples from sites with known drilling-related incidents in a drilling-dense region of Colorado exhibited more estrogenic, antiestrogenic, and/or antiandrogenic activities than the water samples collected from reference sites and twelve chemicals used in drilling operations exhibited similar activities. Taken together the following support an association between natural gas drilling operations and EDC activity in surface and ground water: hormonal activities in Garfield County spill sites and the Colorado River are higher than reference sites in Garfield County and in Missouri, selected drilling chemicals displayed similar activities to those measured in water samples collected from a drilling-dense region, several of these chemicals and similar compounds were detected by other researchers at our sample collection sites, and known spills of natural gas fluids occurred at these spill sites. Taken together, this suggests that natural gas drilling operations may result in elevated EDC activity in ground and surface water.

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References


34. Silva E, Rajapakse N, Kortenkamp A. Something from “nothing”– eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environmental Science, Technology. 2002;36(8):1751–1756.


48. Creasy D. 1985 A quantitative study of stage-specific spermatocyte damage following administration of ethylene glycol monomethyl ether in the rat. Experimental and Molecular Pathology 43(3).


