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Unconventional oil and gas chemicals and wastewater-impacted water samples promote adipogenesis *via* PPARγ-dependent and independent mechanisms in 3T3-L1 cells

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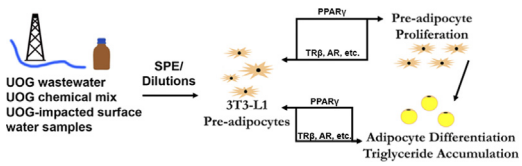
HIGHLIGHTS

- Tested ability of unconventional oil & gas (UOG) chemicals to promote adipogenesis
- UOG wastewater and common chemical mixtures promote adipogenesis.
- UOG-impacted water samples promoted fat cell development at diluted concentrations.
- Fat cell development occurred through PPARγ-dependent and independent mechanisms.
- UOG wastewater may impact metabolic health at environmentally relevant levels.

GRAPHICAL ABSTRACT



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ABSTRACT

Unconventional oil and natural gas (UOG) operations have contributed to a surge in domestic oil and natural gas production in the United States, combining horizontal drilling with hydraulic fracturing to unlock previously inaccessible fossil fuel deposits. >1000 organic chemicals are used in the production process, and wastewater is produced following injection and for the life of the producing well. This wastewater is typically disposed of *via* injecting into disposal wells for long-term storage, treatment and discharge from wastewater treatment plants, and/or storage in open evaporation pits; however, wastewater spill rates are reported at 2–20% of active well sites across regions, increasing concerns about the environmental impacts of these wastewaters. This study assessed adipogenic activity (both triglyceride accumulation and pre-adipocyte proliferation) for a mixture of 23 commonly used UOG chemicals and a small subset of UOG wastewater-impacted surface water extracts from Colorado and West Virginia, using 3T3-L1 cells and a peroxisome proliferator activated receptor gamma (PPARγ) reporter assay. We report potent and efficacious adipogenic activity induced by both a laboratory-created UOG chemical mixture and UOG-impacted water samples at concentrations below environmental levels. We further report activation of PPARγ at similar concentrations for some samples, suggesting a causative molecular pathway for the observed effects, but not for other adipogenic samples, implicating PPARγ-dependent and independent effects from UOG associated chemicals. Taken together, these results suggest that UOG wastewater has the potential to impact metabolic health at environmentally relevant concentrations.

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1. Introduction

Unconventional oil and natural gas (UOG) operations have contributed to a surge in domestic oil and gas production in the United States over the last two decades, combining horizontal drilling with hydraulic fracturing to unlock previously inaccessible fossil fuel deposits from nonporous geologic formations (Waxman et al., 2011; Wiseman, 2008). Fluids are recovered over the first two weeks as "flow back", and mainly contain injected water and chemicals, with a gradual transition to "produced water" that continues over the life of the producing well (Deutch et al., 2011; Engle et al., 2014) and contains increasing concentrations of naturally occurring radioactive compounds, heavy metals, and other compounds from the shale layer (Rowan et al., 2015; Akob et al., 2015). UOG wells produce estimated wastewater volumes of up to 4 billion m^3 per year (Clark and Veil, 2009; Harkness et al., 2015), which are increasingly injected into disposal wells, treated and discharged from wastewater treatment plants, and/or pumped into open evaporation pits for disposal (Wiseman, 2008; Deutch et al., 2011; Lee et al., 2011; Lester et al., 2015). Recent studies have reported UOG fluid spills rates of between 2 and 20% of active well sites (Patterson, 2017; Maloney et al., 2017), and spills and/or discharges have been demonstrated to impact surface, ground, and drinking water quality near UOG operations (Harkness et al., 2015; DiGiulio et al., 2011; Rozell and Reaven, 2012; Skalak et al., 2014; DiGiulio and Jackson, 2016; Drollette et al., 2015; Osborn et al., 2011; Jackson et al., 2013; Fontenot et al., 2013; Warner et al., 2013; Hladik et al., 2014).

>1000 different chemicals are used for hydraulic fracturing across the US (Waxman et al., 2011; Environmental Protection Agency (EPA), 2015), many of which have been demonstrated to act as endocrine disrupting chemicals (EDCs) both *in vitro* and *in vivo* (Kassotis et al., 2014; Webb et al., 2014; Bolden et al., 2015; Kassotis et al., 2016a; Kassotis et al., 2015; He et al., 2017a; He et al., 2017b; Blewett et al., 2017a). EDCs are exogenous chemicals or mixtures of chemicals that can interfere with any aspect of hormone action (Zoeller et al., 2012; The Endocrine Disruption Exchange (TEDX), 2017; Diamanti-Kandarakis et al., 2009) and may disrupt development and contribute to disease in both humans and animals, particularly during critical windows of development (Vandenberg et al., 2012; Welshons et al., 2003). Previous research by our laboratory and colleagues has demonstrated significant impacts on nuclear receptor activity from samples collected in surface and groundwater near UOG spill sites in Colorado, USA (CO) (Kassotis et al., 2014), downstream from an UOG wastewater injection disposal site in West Virginia, USA (WV) (Kassotis et al., 2016b), and downstream from an UOG wastewater spill in North Dakota, USA (ND) (Cozzarelli et al., 2017). Notably, antagonism of thyroid receptor beta (TRβ) and the androgen receptor (AR) are both pathways that can influence adipogenesis (Kassotis et al., 2017a; Kassotis et al., 2017b; Niemelä et al., 2008) suggesting a potential for metabolic disruption by UOG chemicals.

Both *in vitro* and *in vivo* studies have highlighted potential metabolic disruption by UOG operations. *In vitro*, one recent study described peroxisome proliferator activated receptor gamma (PPARγ) agonism and adipogenic activity for several oil sands process-affected water samples (Peng et al., 2016). In addition, several laboratories have recently reported alcohol and/or alkylphenol polyethoxylates in UOG wastewater (Lester et al., 2015; Thurman et al., 2014; Getzinger et al., 2015). Getzinger et al. (2015) reported ng/L to μg/L retention of these compounds (C_6–C_{10} alkyl chain length and 2–12 ethoxymer chain number) in wastewater effluent discharged into a receiving stream (Getzinger et al., 2015). We have recently described both potent and efficacious adipogenic responses (triglyceride accumulation and pre-adipocyte proliferation) for various alkylphenol and alcohol ethoxylates (Kassotis et al., 2018a), suggesting that these UOG chemicals may be capable of promoting adipogenesis. *In vivo*, research from our laboratory reported increased male and female birth and body weights following gestational exposure to a common UOG chemical mixture (Kassotis et al., 2016a; Kassotis et al., 2015). Disparate outcomes have also been reported in epidemiological studies. UOG development has been associated with increased prevalence of low birth weight and small for gestational age births (Stacy et al., 2015) as well as preterm births and physician-recorded high-risk pregnancies (Casey et al., 2016) in the Marcellus Shale region. A separate study in CO reported that UOG development density was associated with decreased prevalence of low birth weights and increased risk of higher birth weight babies (McKenzie et al., 2014). Importantly, both low (Jornayvaz et al., 2016; Curhan et al., 1996) and high (Hirschler et al., 2008; Danielzik et al., 2004) birth weights are associated with greater risks for obesity later in life.

Obesity and metabolic disease impose severe economic and adverse health burdens globally (Heindel et al., 2015; Janesick and Blumberg, 2016), with 18.5% of youth (2–19 year olds) and 39.8% of adults (20 and older) classified as obese in the US (Hales et al., 2017). Obesity contributes to >$215 billion in annual US health care costs, and also promotes increased risks of type II diabetes, cardiovascular disease, hypertension, and other adverse health effects (Hammond and Levine, 2010). Legler et al. estimated the economic costs attributable to five EDCs with the strongest epidemiological evidence from obesity, diabetes, and associated costs at €18–29 billion per year in the European Union (Legler et al., 2015). Reports of potential metabolic disruption caused by environmental chemicals have been increasing, as the means to sensitively and inexpensively assess these outcomes have proliferated (Kassotis et al., 2017a; Heindel et al., 2015; Auerbach et al., 2016; Heindel et al., 2017). One of these tools, 3T3-L1 mouse pre-adipocytes, is commonly used to assess putative metabolic disruptors *in vitro* and has been demonstrated to accurately predict metabolic disruption *in vivo* (Angle et al., 2013; Chamorro-Garcia et al., 2013; Li et al., 2011; Masuno et al., 2005). In this assay, adipogenic chemicals promote pre-adipocyte differentiation into adipocytes, drive morphological changes and triglyceride accumulation, and cells come to resemble a mature human white fat cell over time (Green and Kehinde, 1975; Green and Meuth, 1974).

As such, the goals of this study were to characterize the potential adipogenic activity (*via* both triglyceride accumulation and pre-adipocyte proliferation; key event) of various UOG-related fluids. Specifically, we selected a mixture of 23 commonly used UOG chemicals that has previously been examined *in vitro* and *in vivo* (Kassotis et al., 2014; Kassotis et al., 2016a; Kassotis et al., 2015), a subset of surface water (n = 4) extracts near a UOG wastewater-impacted site in WV, USA (Kassotis et al., 2016b), a subset of surface water samples (n = 4) near UOG spill sites in CO, USA (Kassotis et al., 2018b), and two UOG wastewater samples from CO that we have previously analytically characterized (Kassotis et al., 2015). All samples were screened for potential adipogenic activity in 3T3-L1 cells, and PPARγ activity was interrogated using a FRET reporter gene assay to further delineate mechanism (likely molecular initiating event). We hypothesized that UOG chemicals, UOG-impacted water, and UOG wastewater could promote metabolic disruption *via* PPARγ-dependent and independent mechanisms.

2. Materials and methods

2.1. Chemicals

Rosiglitazone (adipogenesis positive control; >98%; cat# R2408) and all UOG production chemicals (Table S1) were purchased from Sigma-Aldrich Co. The twenty-three UOG chemicals selected were previously assessed for bioactivities independently and in combination (Fig. S1) (Kassotis et al., 2014; Kassotis et al., 2015), and were selected on the basis of being EDCs and common-use chemicals in the UOG extraction process (Kassotis et al., 2014; Kassotis et al., 2015). An equimolar mixture of these 23 chemicals was created at 10 mM. Stock solutions were prepared in 100% DMSO (Sigma cat # D2650) and stored at −20 °C between uses.

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2.2. Selection of UOG wastewater and wastewater-impacted samples

UOG wastewater-impacted surface water samples were collected from Garfield County, CO (n = 4) and Fayette County, WV (n = 4) (Kassotis et al., 2016b) in August 2014 and June 2014, respectively (Table 1). Grab sample collection procedures, handling, and processing have previously been described in detail (Kassotis et al., 2014; Kassotis et al., 2016b), and subsets of samples from each study region were selected to span a spectrum of putative UOG wastewater impacts. Briefly, one-liter surface water samples were collected after rinsing the bottle and cap three times with source water, samples were stored on ice, shipped overnight to the laboratory, refrigerated until analysis, and solid-phase extracted within one week.

WV samples (Table 1 and previous (Kassotis et al., 2016b; Akob et al., 2016)) included four sites: one sample collected from a reference stream in a drainage area with no known UOG inputs (WV-02), one sample collected near the site of the UOG wastewater disposal injection well (WV-06), one sample collected downstream of the UOG injection well site (WV-03), and one field blank (WV-03BLK).

CO samples (Table 1) included: one sample collected from a UOG wastewater retention pond outside a well pad (CO-15), one sample collected from a small pond on private property in a drilling-dense area (CO-23), one sample collected directly from an UOG produced water storage tank on a well pad (CO-65), and one process control (CO-PC). In addition, one produced water sample was collected at the site of a ruptured pipeline in Garfield County, CO that was actively leaking (Kassotis et al., 2015); the sample naturally separated into organic (WW1-org) and aqueous (WW1-aq) layers after collection, and we have previously reported organic contaminants in each (Kassotis et al., 2015). Multiple process controls and field blanks were collected and analyzed in both regions for endocrine bioactivities and organic chemical analyses; each was found to induce no significant bioactivity and contain no organic contaminants associated with UOG operations (Kassotis et al., 2016b; Kassotis et al., 2018b). For this study, one process control (from CO) and one field blank (from WV) were selected to serve as controls for adipogenic testing, as representative controls from the larger experiments. These samples were prepared using 1 L of Fisher HPLC-grade water (Fisher Scientific catalog # WFSK-4) and followed the same processing and analysis procedures used for all experimental samples. The process control was poured and processed during the solid-phase extraction of CO samples in order to determine whether there was any impact of the sample extraction and processing on the outcomes measured herein. The field blank was prepared with Fisher HPLC-grade water during WV sample collection, un-capping a one-liter bottle of control water during the collection of experimental samples, then closing, storing and shipping alongside other samples; this was then processed with all other WV samples, in order to ensure no contribution of the sampling procedures to the outcomes measured herein.

2.3. Extraction of water samples

The solid-phase extraction procedure utilized for the WV and CO water extracts listed above were performed according to protocols published previously (Kassotis et al., 2014; Kassotis et al., 2016b). Briefly, Oasis HLB glass cartridges were conditioned with 100% HPLC-grade methanol and HPLC-grade water, then 1 L water samples were loaded onto the cartridge, washed with 5% methanol in HPLC water, and three one-mL 100% methanol elutions were performed and combined. A DMSO "keeper" (50 μL) was added before drying samples under nitrogen gas, and concentrated samples were subsequently reconstituted with 200 μL methanol, creating stock concentrations of 4000× the original water concentration (80:20 methanol:DMSO). Samples were further diluted 10-fold in methanol to create final working concentrations, used in this study, of 400× (98:2 methanol:DMSO). Reconstituted samples were stored at −20 °C, protected from light, until tested. In order to be applied to cells, stock samples were diluted 100-fold in tissue culture medium; this created test concentrations, in contact with cells, of 4× the original water concentration (and subsequent 10-fold serial dilutions).

2.4. Cell care

3T3-L1 cells were obtained from Zenbio, Inc. at passage 8 (cat# SP-L1-F, lot# 3T3062104; Research Triangle Park, NC) and were maintained, differentiated, and maintained as described in detail previously (Kassotis et al., 2017a; Kassotis et al., 2017b). Cells were cultured in Dulbecco's Modified Eagle Medium–High Glucose (DMEM-HG; Gibco cat# 11995) supplemented with 10% bovine calf serum and 1% penicillin and streptomycin, and were maintained in a sub-confluent state until differentiation; each thaw was differentiated within 5 passages (p9–13), with no apparent changes in control chemical response in this time.

HEK 293H cells utilized for the GeneBlazer™ PPARγ assay (Invitrogen cat# K1094) were cultured according to manufacturer's directions (Invitrogen, 2010) and as described previously (Kassotis et al., 2018a). Briefly, cells were cultured in DMEM-HG supplemented with 10% dialyzed fetal bovine serum (Invitrogen cat# 26400-036), 1% non-

Table 1
Description of UOG-impacted environmental samples.

Sample ID	Approximate location	Date collected	Conc. tested	Description
WV-03BLK	Lochgelly, WV	6/2014	4×, 0.4×, 0.04×, 0.004×	Field blank; HPLC water opened and exposed to the site air during sampling
WV-02	Lochgelly, WV	6/2014	4×, 0.4×, 0.04×, 0.004×	Surface water from reference stream, different drainage with no UOG impacts
WV-06	Lochgelly, WV	6/2014	4×, 0.4×, 0.04×, 0.004×	Surface water collected from UOG injection well pad, adjacent to the injection well shed
WV-03	Lochgelly, WV	6/2014	4×, 0.4×, 0.04×, 0.004×	Surface water collected downstream from UOG injection well pad and facility
CO-PC	N/A	9/2014	4×, 0.4×, 0.04×, 0.004×	Process control; HPLC water subjected to laboratory processing with experimental samples
CO-15	Garfield County, CO	9/2014	4×, 0.4×, 0.04×, 0.004×	Surface water from an apparent UOG wastewater retention pond outside of a UOG well pad
CO-23	Garfield County, CO	9/2014	0.4×, 0.04×, 0.004×, 0.0004×	Surface water from pond on private property; historical UOG fluid spill at residence several years prior
CO-65	Garfield County, CO	9/2014	4×, 0.4×, 0.04×, 0.004×	Putative UOG wastewater collection from a produced water storage tank directly from UOG well pad
WW1-org	Garfield County, CO	9/2010	0.01×, 0.001×, 0.0001×, 0.00001×	Putative UOG wastewater collected at site of UOG wastewater pipeline rupture; organic fraction
WW1-aq	Garfield County, CO	9/2010	0.01×, 0.001×, 0.0001×, 0.00001×	Putative UOG wastewater collected at site of UOG wastewater pipeline rupture; aqueous fraction

Sampling and testing information for selected unconventional oil and gas (UOG)-impacted environmental samples assessed in this study. Concentrations tested were dependent on toxicity tested performed prior to bioassays to prevent potential erroneous interpretation of receptor antagonism due to toxicity; all concentrations tested exhibited no toxicity in MTT assay. Results for WV samples published previously; Kassotis et al., 2016, Endocrine disrupting activities of surface water associated with a West Virginia oil and gas industry wastewater disposal well; Science of the Total Environment.

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essential amino acids, 1% sodium pyruvate, 1% penicillin-streptomycin, 25 mM HEPES, 80 μg/mL hygromycin, 2% glutamax, and 500 μg/mL Geneticin. Each thaw was maintained in a sub-confluent state and utilized for assays within ten passages.

2.5. 3T3-L1 cell differentiation and induction

Cells were induced to differentiate as follows: cells were seeded at approximately 30,000 cells per well into 96-well tissue culture plates (Greiner cat # 655090). Once confluent, cells were cultured for an additional 48 h to undergo growth arrest and revert to clonal expansion. Media was then replaced with test chemicals, samples, and/or controls using a 0.1% DMSO or 0.98% methanol:0.02% DMSO vehicle (based on experimental samples on plate) diluted in differentiation media (DMEM-HG with 10% fetal bovine serum, 1% penicillin/streptomycin, 1.0 μg/mL human insulin, and 0.5 mM 3-isobutyl-1-methylxanthine, IBMX). Approximately 48 h later, media was replaced with test chemicals diluted in adipocyte maintenance media (differentiation media without IBMX) and refreshed every 2–3 days until plates were assayed ten days after addition of test chemicals.

2.6. 3T3-L1 lipid and DNA staining protocols

Plates were assayed for triglyceride accumulation, DNA content, and cell viability (ATP production and lactate dehydrogenase (LDH) release) ten days after induction of differentiation, as described previously (Kassotis et al., 2017b; Kassotis et al., 2018a). Briefly, cells were rinsed with phosphate-buffered saline (PBS) and then replaced with 200 μL dye mixture (18.5 mL PBS, 20 drops NucBlue (Thermo# R37605), and 500 μL AdipoRed (Lonza# PT-7009)). Plates were wrapped to protect from light and incubated for approximately 40 min, then read on fluorimeter with excitation 485 nm/emission 572 nm for AdipoRed and 360/460 nm for NucBlue.

Cell Viability was examined using two separate assays (lactate dehydrogenase (LDH) release and ATP content). LDH release was assessed using the CytoTox-ONE™ assay (Promega cat # G7890), as described previously (Kassotis et al., 2018a). Briefly, before lipid and DNA measurements, 25 μL of treatment media from plates was aliquoted into separate 96-well plates and mixed with 25 μL CytoTox-ONE™ reagent. Plates were incubated for 10 min protected from light, and fluorescence was measured at 560/590 nm for LDH release quantification.

ATP content was assessed using the CellTiter-Glo™ assay (Promega cat# G7572), as described previously (Kassotis et al., 2018a). Following lipid and DNA measurements, all but 30 μL of DPBS/dye mixture was removed from plates and remaining volume was mixed with 30 μL of CellTiter-Glo™ reagent, incubated for 10 min, and luminescence measured for ATP quantification.

Efficacies (percent activities) across the examined dose responses were calculated relative to the intra-assay average rosiglitazone-induced maximal fold induction over intra-assay differentiated vehicle controls (0.1% DMSO or 0.98% methanol:0.02% DMSO), after correcting for background fluorescence by subtracting raw fluorescence units from cell-free wells (intra-assay Z'-factor: 0.86; signal/noise: 9.1-fold change). DNA content was calculated as percent change from vehicle controls for each chemical at each concentration, and DNA content was then used to normalize total triglyceride values in each well to obtain triglyceride content per unit DNA (as a proxy for triglyceride content per cell). CytoTox-ONE™ viability (LDH release) was calculated as percent viability loss relative to maximal lysed control (Triton X-100), and CellTiter-Glo™ viability (ATP content) was calculated as percent deviation from vehicle controls, with >15% considered significant. Potencies were calculated relative to maximal response, with EC_{10} concentrations representing the concentrations at which a chemical exhibited 10% of its or the positive control's maximal activity. Cytotoxicity (DNA content) was only observed at concentrations above maximal bioactivities, so responses for cytotoxic concentrations were not included in potency or maximal efficacy calculations.

2.7. PPARγ GeneBLAzer™ reporter assay

PPARγ agonism were measured according to manufacturer's instructions (Invitrogen, 2010) and as described previously (Fang et al., 2015). Briefly, cells were seeded at 30,000 cells per well into duplicate 384-well black clear-bottom plates (Corning cat# 354663) and allowed to settle for 2–4 h. Cells were then induced with test chemicals, samples, and control doses responses (0.1 nm–1 μM rosiglitazone), using a 0.1% DMSO vehicle. Cells were incubated for approximately 16 h, and then duplicate plates were assayed for receptor activity and cell viability. Receptor activity was assessed using the LiveBLAzer™-FRET B/G Loading Kit (Invitrogen cat # K1095). Reagents were added according to manufacturer's instructions (Invitrogen, 2010), and plates were incubated at room temperature, protected from light, for 2 h. Following incubation, fluorescence was measured at 409/460 nm and 409/530 nm. Percent activity was assessed as a background-corrected fold induction of test chemical response relative to maximal rosiglitazone-induced response (intra-assay Z'-factor: 0.81; signal/noise: 3.1-fold change). Cell viability was assessed as previous (Fang et al., 2015), by adding 0.025 mg/mL resazurin solution to cells, incubating at 37 °C for 2 h, and measuring fluorescence at 560/590 nm; cell viability loss of >15% was considered significant. Inhibited cell viability (reduced resorufin fluorescence) was only observed at concentrations above maximal bioactivities, so responses for concentrations with inhibited cell viability were not included in potency or maximal efficacy calculations.

2.8. Transient transfection reporter assays

Prior to assessing ER, AR, PR, GR, and TR bioactivities, cell viability was assessed 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, induced with positive/negative controls and water sample extracts for 18–20 h, and then dye solution was added. Plates were incubated for an hour and absorbance was read at 570 nm. Inhibited cell viability was defined as a decrease (paired *t*-test) of ≥15% of the vehicle control response. Mammalian hormone receptor activity assays were then performed with extracts pre-diluted to below toxic concentrations to prevent non-specific confounding in bioassays. For bioassays, Ishikawa cells were transiently transfected with plasmids as described previously (Kassotis et al., 2014; Kassotis et al., 2015) for ERα, AR, PR B, GR, and TRβ. Transfected cells were induced with dilution series of controls or water sample extracts, using a 1% methanol vehicle. Samples were assessed in quadruplicate within each assay and each assay was repeated three times. Further confirmatory cytotoxicity testing was assessed in tandem, using CMV-β-Gal activity as a surrogate marker for cytotoxicity as described previously (Kassotis et al., 2014), confirming no cytotoxic samples were included in bioassays. No samples reported herein exhibited a significant inhibition of cell viability relative to intra-assay vehicle controls. Percent receptor activities were calculated as described above. Antagonist activities were calculated as a percent suppression or enhancement of the co-treated positive control at its EC50 (concentration required to induce half of maximal activity: 20 pM estradiol, ER; 300 pM dihydrotestosterone, AR; 30 pM progesterone, PR; 2 nM triiodothyronine, TR; and 5 nM dexamethasone, GR). Results from these bioassays were reported previously (Kassotis et al., 2016b; Kassotis et al., 2018b) and are reported in Supplemental Information.

2.9. Statistical analysis

Data are presented as means ± SE from four technical replicates of three independent experiments. Dose response curves and $EC/IC_{10's}$

were estimated using curves generated using a 4-parameter variable-slope Hill model in GraphPad Prism 7.0, and were calculated using two distinct methods. Chemical EC_{10} values were calculated as the concentration at which a chemical exhibited 10% of its own maximal response, whereas Relative EC_{10} values represented the concentration at which a chemical exhibited 10% of the positive control's maximal activity. Percent activity results were log-transformed to achieve normality. Statistical analysis was performed using a one-way analysis of variance (ANOVA) with post-test comparisons between test chemicals at each examined concentration and vehicle controls using a Dunnett's multiple comparison test, using GraphPad Prism 7.0, with differences considered statistically significant at $p < 0.05$.

3. Results

A mixture of 23 commonly-used UOG chemicals (Table S1) and ten UOG-impacted water samples (Table 1) were assessed for pre-adipocyte proliferation (DNA content, relative to vehicle control), triglyceride accumulation (total per well and per cell, normalized to DNA content; both relative to maximal rosiglitazone response), and cell viability (ATP production and lactate dehydrogenase (LDH) release) using 3T3-L1 cells. Rosiglitazone (positive control) induced a typical, robust response for triglyceride accumulation and pre-adipocyte proliferation (Fig. 1A–C), no impact on ATP production independent of increased cell number (Fig. 1D), and a large release of LDH (Fig. 1E), consistent with previous reports (Kassotis et al., 2018a; Zeng et al., 2012; Staiger and Loffler, 1998).

3.1. Adipogenic activity of UOG laboratory chemical mixture

The 23-mix of UOG chemicals promoted significant robust triglyceride accumulation at 10 μM (60% relative to rosiglitazone control when each constituent chemical was present at 10 μM; Fig. 1A, Table S2) and significant accumulation at 1 μM. Substantial pre-adipocyte proliferation was also observed, with the 23-mix inducing approximately 75% increased DNA content relative to the differentiated vehicle control at 10 μM (Fig. 1B), which tempered the triglyceride accumulation on a per cell basis (Fig. 1C). Some inhibition of cell viability was also observed at concentrations which promoted adipogenic activity. Notably, the 10 μM concentration caused complete loss of ATP content relative to vehicle control (Fig. 1D), and approximately 40% LDH release relative to lysed control (Fig. 1E). Cell imaging confirmed classical triglyceride accumulation for the 23-mix (Fig. 1F).

3.2. Adipogenic activity of UOG-impacted environmental samples

The environmental samples promoted diverse adipogenic responses across concentrations (Fig. 2, Table S2). Notably, the organic fraction of the CO wastewater sample (WW1-org) induced approximately 80% triglyceride accumulation relative to the rosiglitazone control and approximately 60% increase in DNA content at 0.01× concentration (Fig. 2A–C), while inducing no adverse effects on cell viability as measured by ATP content (Fig. 2D) or LDH release (Fig. 2E). The aqueous fraction of this sample promoted significant cell proliferation only at 0.01× (Fig. 2B), and no significant triglyceride accumulation. The environmental samples induced varied activity. CO-65, 23, and 15 all promoted significant triglyceride accumulation at 0.4× and/or 4× (Fig. 2A, C), and significant cell proliferation at 0.04× and/or 0.4× (Fig. 2B). CO-65 and 23 promoted significant cytotoxicity at 4× (Fig. 2B) and inhibited cell viability at 0.4 and/or 4× (Fig. 2D–E), while CO-15 was neither cytotoxic nor negatively impacted cell viability. WV-03 induced significant triglyceride accumulation after accounting for cytotoxicity at 4× (Fig. 2B–C), and WV-06 promoted some cell proliferation at 4× (Fig. 2B) but no triglyceride accumulation. The field and laboratory blanks, CO-PC and WV-03BLK, as well as the WV reference stream, WV-02, induced neither triglyceride accumulation nor altered DNA content.

3.3. Absence of PPARγ activation by UOG laboratory chemical mixture

Activation of PPARγ was subsequently interrogated using FRET-based reporter assay to assess a likely molecular mechanism for observed effects. Rosiglitazone induced a classical, robust response in the PPARγ FRET assay (~1.5 nM EC_{50}, ~100 nM maximal response; Fig. 3A, Table S2), with no inhibited cell viability observed (Fig. 3B). The mixture of 23 commonly used UOG chemicals (23-mix) promoted no significant activation of PPARγ relative to the positive control at any concentration tested (Fig. 3A). Inhibited cell viability was observed at 10 μM (Fig. 3B), suggesting some potential cytotoxicity.

3.4. PPARγ activity and absence by UOG-impacted environmental samples

The organic fraction of the Colorado wastewater sample, WW1-org, promoted significant PPARγ activity at 0.001× and 0.01×, reaching approximately 45% activity relative to the rosiglitazone positive control (Fig. 4A, Table S2). In contrast, the aqueous fraction induced no PPARγ activity (Fig. 4A). CO-65 promoted significant PPARγ activity as low as 0.04× (~35%), CO-23 as low as 0.4× (~10%), and WV-03 only at the 4× concentration (~30%; Fig. 4A). CO-15, WV-06, WV-02, and the laboratory and field blanks induced no significant PPARγ activity at any concentration. Inhibited cell viability was observed only at the 4× concentrations for CO-65 and WV-06 (Fig. 4B), suggesting that cell stress and subsequent non-specific activation was not the cause of reported activation by these samples.

4. Discussion

Herein, we report potent adipogenic activity by both a laboratory-created UOG chemical mixture and UOG-impacted environmental samples. The UOG wastewater promoted nearly as efficacious (percent activity relative to positive control) a response for triglyceride accumulation as the positive control, rosiglitazone, at ≥100-fold diluted concentrations. Several UOG-impacted surface water extracts collected from drilling-dense regions also promoted significant triglyceride accumulation at diluted concentrations (<1× relative to pure water). Notably, the wastewater sample and most of the UOG-impacted water extracts promoted equal or greater stimulation of pre-adipocyte proliferation than the rosiglitazone control, many at <1× relative water concentrations. The activity of the UOG-impacted environmental samples mirrored the activity induced by the UOG chemical mixture, which also stimulated efficacious triglyceride accumulation and a high degree of pre-adipocyte proliferation. This was similar to activity we reported previously for a range of nonionic ethoxylated surfactants (Kassotis et al., 2018a), which promoted both potent and efficacious triglyceride accumulation and pre-adipocyte proliferation, and which have been reported at ng/L to μg/L concentrations in UOG wastewater and effluent discharged into a receiving stream (Lester et al., 2015; Thurman et al., 2014; Getzinger et al., 2015). Two similar alkylphenol ethoxylates were included in the 23-mix of common UOG chemicals (nonylphenol and octylphenol ethoxylate), suggesting that these compounds may be the causative constituent driving some of the observed adipogenic activity. Future research should work to better characterize the specific causative chemicals in these mixtures and environmental samples. Due to sample processing, many of the common (semi)volatile contaminants present may have been lost, limiting chemical analysis. A split-processing design will be utilized in future analysis to allow both analytical characterization and bioassays.

Activation of PPARγ likely contributed to the adipogenic activity promoted by certain UOG-impacted samples (likely molecular initiating event), though others appear to be active through different mechanisms. A few specific UOG-impacted samples promoted significant PPARγ activation: WW1-org at 0.001× and 0.01×, CO-65 at 0.04× and 0.4×, CO-23 at 0.4× and 4×, and WV-03 at 4× relative water concentrations. These activities matched the adipogenic activities (triglyceride

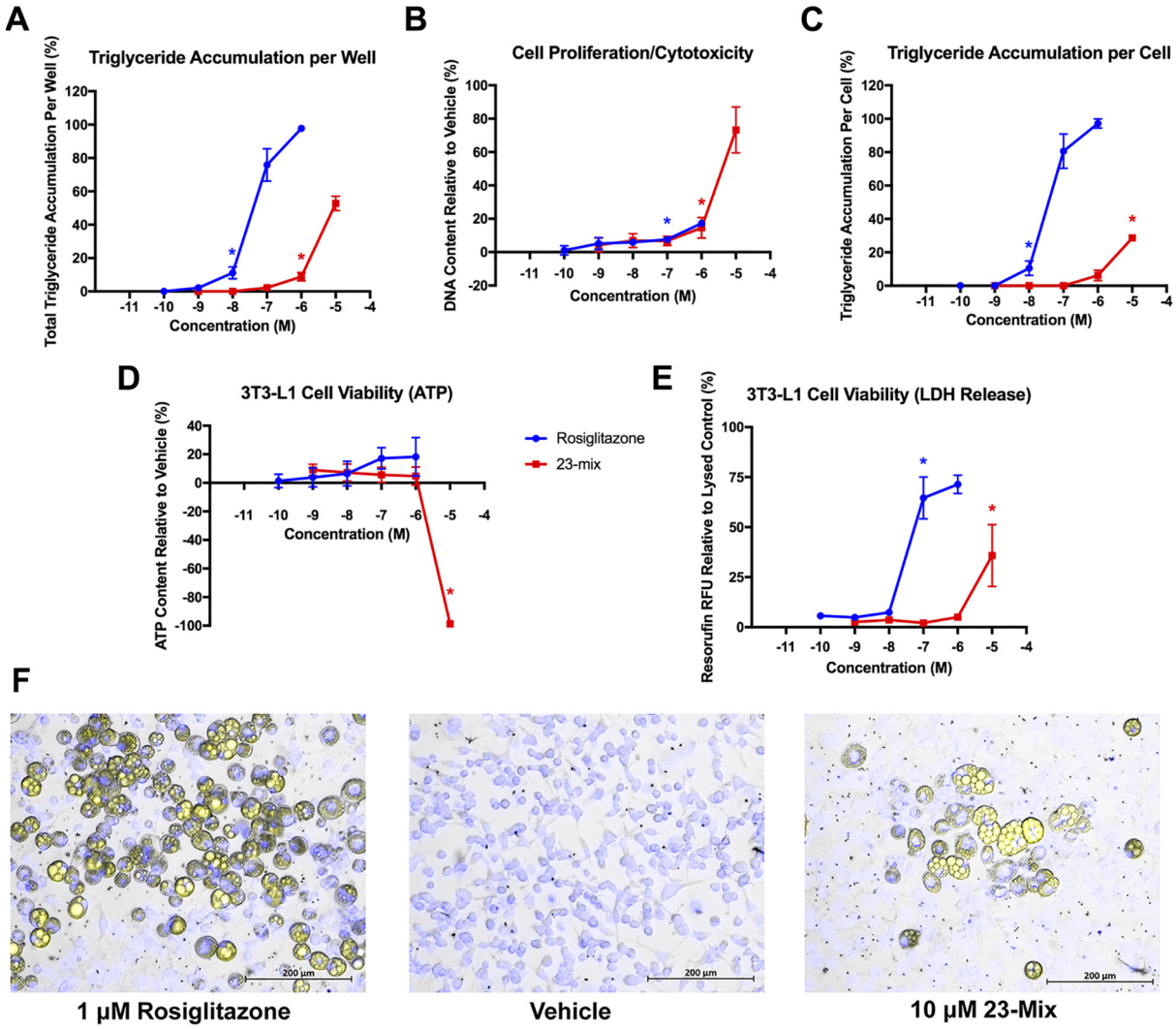

Fig. 1. Mixture of common UOG chemicals promotes adipogenesis. 3T3-L1 cells were induced to differentiate as described in Materials and methods and assessed for degree of adipocyte differentiation (Nile Red staining of intracellular lipids) and pre-adipocyte proliferation (Hoechst nuclear DNA staining) after ten days of differentiation while exposed to a mixture of 23 commonly-used hydraulic fracturing/unconventional oil and gas (UOG) operation chemicals, with each constituent chemical present at equimolar concentrations from 1 nM to 10 μM. Percent raw triglyceride accumulation per well relative to maximal intra-assay response for rosiglitazone (A), increase (pre-adipocyte proliferation) or decrease (potential cytotoxicity) in DNA content relative to vehicle control (B), percent normalized triglyceride accumulation relative to maximal intra-assay rosiglitazone response (normalized to DNA content) (C), increase or decrease in cell viability (ATP content) relative to vehicle control (D), and increase in lactate dehydrogenate release (cell viability measure) relative to lysed control (E). Data presented as mean ± SEM from three independent experiments. Representative images of differentiated cells induced in a 24-well plate following ten days of exposure to 1 μM rosiglitazone, 0.1% DMSO (vehicle), or 10 μM 23-mix (F), using a Zeiss Axio Observer microscope and Photometrics CoolSNAP ES2 CCD camera with ZEN Pro 2.3. Imaging merged a phase contrast field with yellow fluorescent protein (Nile Red lipid staining) and DAPI (NucBlue DNA content) filters to visualize the cells. Bars are provided for scale. * indicates lowest concentration with significant change from vehicle control, $p < 0.05$, as per one-way ANOVA in GraphPad Prism 7.0.

accumulation and/or pre-adipocyte proliferation) at similar or greater concentrations for WW1-org at 0.01×, CO-65 at 0.04× (pre-adipocyte proliferation) and 0.4× (proliferation and triglyceride accumulation), CO-23 at 0.4× (proliferation and triglyceride accumulation), and WV-03 at 0.4× (pre-adipocyte proliferation). Notably, we have demonstrated that there appear to be overlapping but distinct mechanisms that promote triglyceride accumulation *vs.* pre-adipocyte proliferation (Kassotis et al., 2017a; Kassotis et al., 2017b; Kassotis et al., 2018a), so despite the reported PPARγ activities, some of these may be occurring through distinct co-occurring molecular pathways. For example, WW1-org, CO-65, CO-23, and WV-03 all induced anti-androgenic activities (Fig. S2B), and WV-03 also induced anti-thyroid activity, other mechanisms that can promote triglyceride accumulation in these cells (Kassotis et al., 2017a).

The adipogenic activities induced by the other half of the active samples (4/8) were not explained by PPARγ activity, suggesting a role of other adipogenic mechanisms. Specifically, the 23-mix promoted efficacious triglyceride accumulation and pre-adipocyte proliferation, which might be explained by the efficacious anti-androgenic and anti-thyroid activities reported at adipogenic concentrations. WV-06 promoted significant pre-adipocyte proliferation at 4×, the same concentration that anti-androgenic and anti-thyroid activities were observed. WW1-aq promoted significant pre-adipocyte proliferation at 0.01×, the same concentration at which glucocorticoid receptor agonist activity was reported (Fig. S2A). In contrast, CO-15 promoted significant pre-adipocyte proliferation at 0.04× and triglyceride accumulation at 4×, though no measured bioactivities explain this, highlighting the diversity of receptor pathways that can contribute to adipogenesis. To further probe the potential PPARγ activity of the 23-mix, we utilized the ToxCast™ database (available at: https://actor.epa.gov/dashboard): results for 20 of 23 constituent chemicals (23-mix) were reported in this database, and none of them promoted PPARγ activity as measured by the same bioassay utilized herein, confirming our finding of no PPARγ activity for this mixture. This highlights a need to examine disruption of other receptor pathways by experimental samples, and specifically, UOG water sample extracts, to gain a better understanding of the molecular mechanisms

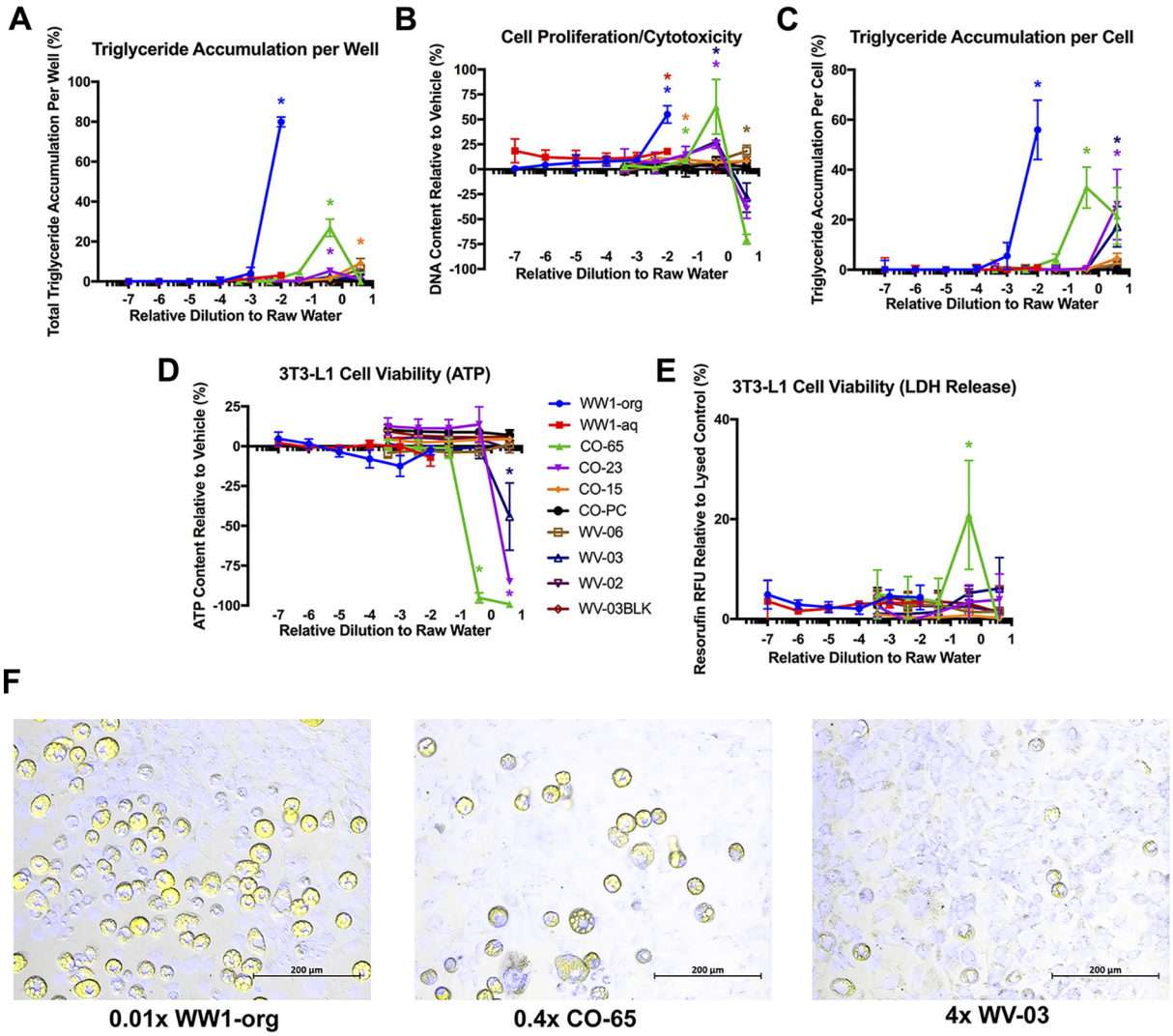

Fig. 2. UOG-impacted environmental samples promote adipogenesis at environmental concentrations. 3T3-L1 cells were induced to differentiate as described in Materials and methods and assessed for degree of adipocyte differentiation (Nile Red staining of intracellular lipids) and pre-adipocyte proliferation (Hoechst nuclear DNA staining) after ten days of differentiation while exposed to various unconventional oil and gas (UOG) wastewater or UOG-impacted surface water samples. X axis values describe test concentration relative to undiluted water: 1 = 10× concentrated water, 0 = raw water (neither diluted nor concentrated), −1 = 10-fold water dilution (0.1× concentration), etc. Percent raw triglyceride accumulation per well relative to maximal intra-assay response for rosiglitazone (A), increase (pre-adipocyte proliferation) or decrease (potential cytotoxicity) in DNA content relative to vehicle control (B), percent normalized triglyceride accumulation relative to maximal intra-assay rosiglitazone response (normalized to DNA content) (C), increase or decrease in cell viability (ATP content) relative to vehicle control (D), and increase in lactate dehydrogenate release (cell viability measure) relative to lysed control (E). Data presented as mean ± SEM from three independent experiments. Representative images of differentiated cells induced in a 24-well plate following ten days of exposure to 0.01× WW1-org, 0.4× CO-65, or 4× WV-03 (F), using a Zeiss Axio Observer microscope and Photometrics CoolSNAP ES2 CCD camera with ZEN Pro 2.3. Imaging merged a phase contrast field with yellow fluorescent protein (Nile Red lipid staining) and DAPI (NucBlue DNA content) filters to visualize the cells. Bars are provided for scale. * indicates lowest concentration with significant change from vehicle control, $p < 0.05$, as per one-way ANOVA in GraphPad Prism 7.0.

driving adipogenesis by complex environmental samples that are putatively more meaningful for human and animal health.

As noted above, several samples appeared to promote pre-adipocyte proliferation (as measured *via* increased DNA content) and also inhibited cell viability (as measured *via* decreased ATP content or increased LDH release). Induction of oxidative stress and other mitochondrial toxicity mechanisms have been demonstrated previously to result in ATP depletion (Tiwari et al., 2002; Brookes et al., 2004), suggesting a potential mechanism. In these cases, chemicals inhibit ATP production without promoting cytotoxicity, which could explain the disparate findings between assays. We noted this previously for several strobilurin pesticides that promoted increased triglyceride accumulation, increased DNA content, and inhibited ATP production (Kassotis et al., 2017a). Further assessment of one of these, pyraclostrobin, found that it promoted triglyceride accumulation *via* differentiation and PPARγ-independent mitochondrial toxicity, inhibiting complex III of the electron transport chain and disrupting lipid homeostasis *via* cAMP responsive element binding protein signaling (Luz et al., 2018). Given robust proliferation data (as measured *via* DNA content), clear absence of dramatic cytotoxicity as assessed *via* a ten day assay and microscope visualization, and lack of putative cell viability loss in a secondary cell viability assay (for reporter gene assays) it suggests a secondary mechanism is driving the altered cell viability measures, rather than overt toxicity.

We have previously reported receptor agonist and/or antagonist equivalent activities for the environmental samples examined herein at 0.4× and 4× concentrations, levels associated with adverse health outcomes in aquatic species (Kassotis et al., 2014; Kassotis et al., 2016b; Kassotis et al., 2018b). In this study, we report effects on adipogenic outcomes at 100-fold dilutions of the wastewater sample and 0.04× and 0.4× concentrations of the UOG-impacted surface water extracts. Significant PPARγ activity was also observed at parallel concentrations (as low as 0.001× relative water concentration);

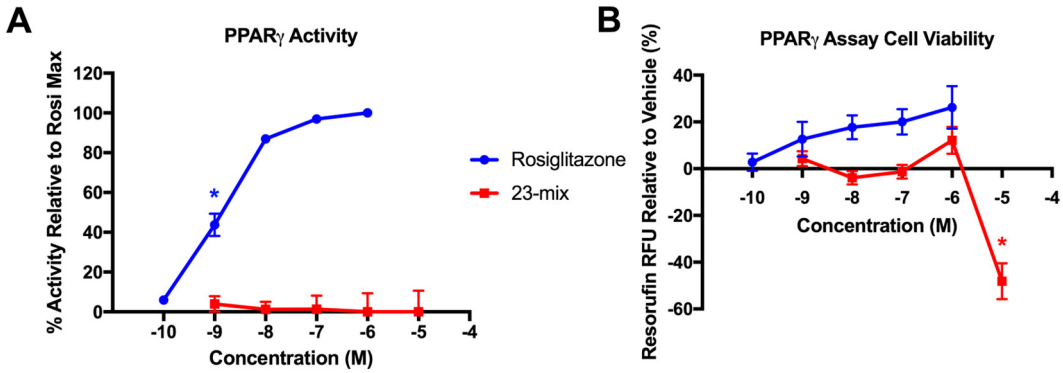

Fig. 3. Mixture of common UOG chemicals does not induce PPARγ agonism. Agonist activity for the peroxisome proliferator activated receptor gamma (PPARγ) as measured via FRET reporter gene assay using the PPARγ GeneBLAzer™ Reporter Assay as described in Materials and methods. Percent agonist PPARγ activity for the rosiglitazone positive control and a mixture of 23 commonly-used hydraulic fracturing/unconventional oil and gas (UOG) operation chemicals, with each constituent chemical present at equimolar concentrations from 1 nM to 10 μM, all relative to the maximum response of rosiglitazone (A). Inhibition or enhancement of cell viability relative to vehicle control (as measured by resazurin to resorufin conversion) for rosiglitazone and the 23-mix of common UOG chemicals (B). Data presented as mean ± SEM from three independent experiments. * indicates lowest concentration with significant change from vehicle control, $p < 0.05$, as per one-way ANOVA in GraphPad Prism 7.0.

importantly, several of these samples promoted more potent PPARγ activation than the other receptor pathways we've examined previously (Table S2), suggesting that it is the likely causative adipogenic activity pathway for certain samples. This suggests that even at considerable dilution, UOG-impacted environmental samples may be capable of disrupting metabolic health for aquatic organisms and/or other wildlife. Few studies have assessed PPARγ activity of water samples, though recent work reported significant PPARγ activity at concentrations as low as 0.025× relative water concentrations for oil sands process-affected water (Peng et al., 2016), and significant triglyceride accumulation by 1× fractions. Other studies have recently reported PPARγ activity of water samples impacted by wastewater treatment plants, reporting relative potencies (EC_{10}) of 0.2–5.7× (Nivala et al., 2018) and 8–215 relative enrichment factors (Konig et al., 2017). Herein, we reported significant PPARγ activity at 0.001× of the CO UOG wastewater sample, and at 0.04× in one UOG-impacted water sample, with relative potencies ranging from 0.00025 to 0.005× (EC_{10}, Table S2), considerably more potent than those reported in other studies. Other wastewater-impacted samples exhibited relative potencies (EC_{10}) of 0.25–0.45, similar to or lower than those reported in these other studies (Nivala et al., 2018; Konig et al., 2017). Given the small samples sizes in all of these studies, more comprehensive assessment of water for PPARγ and adipogenic activity is needed. Future studies should also more comprehensively evaluate metabolic function using *in vivo* models, and should more concretely evaluate the environmental fate of these chemicals in surface water and the extent to which they might influence drinking water in local communities.

Some *in vivo* research has begun to delineate potential metabolic health impacts following exposure to UOG chemicals in animal models. Previous research from our laboratory reported increased male and female birth and body weights in C57Bl/6J mice following gestational exposure to a common UOG chemical mixture (Kassotis et al., 2016a; Kassotis et al., 2015). Recent work from our laboratory reported decreased birth weights in C57 mice after perinatal exposure to a similar UOG chemical mixture (Balise et al., 2018). These mice exhibited lower total and resting energy expenditure and lower spontaneous activity, but did not exhibit altered body weight, composition, or glucose tolerance by seven months of age (Balise et al., 2018). Related work has reported decreased oxygen consumption in *Daphnia magna* following exposure to UOG wastewater (Blewett et al., 2017b), as well as decreased heart rate and reduced metabolic rate in zebrafish larvae (Folkerts et al., 2017). These laboratories mirror many of the effects observed in epidemiological studies, including increased (Stacy et al., 2015; Walker Whitworth et al., 2018; Hill, 2014) or decreased (McKenzie et al., 2014) prevalence of low birth weights and physician-recorded high-risk pregnancies (Casey et al., 2016). Other work has reported increased atmospheric carbon disulfide near UOG operations, which has the ability to dysregulate normal glucose metabolism and may be a causative pathway through which UOG operations may influence metabolic health (Rich et al., 2016).

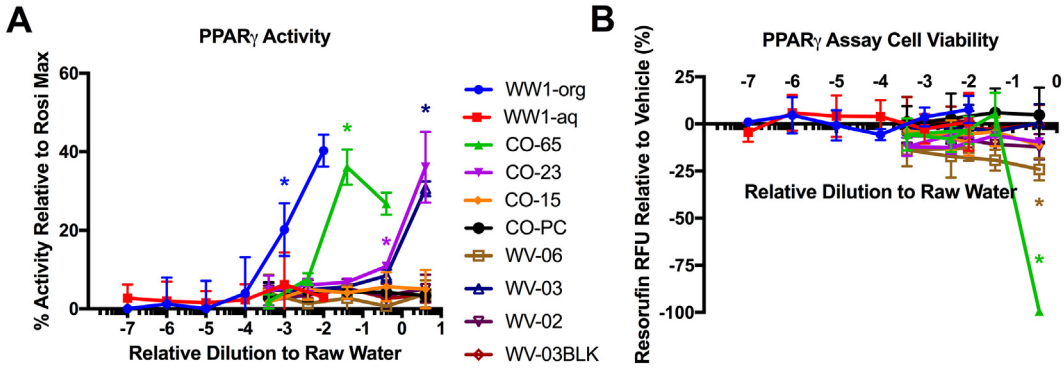

Fig. 4. UOG-impacted environmental samples promote mixed activity for PPARγ. Agonist activity for the peroxisome proliferator activated receptor gamma (PPARγ) as measured via FRET reporter gene assay using the PPARγ GeneBLAzer™ Reporter Assay as described in Materials and methods. Percent agonist PPARγ activity for various unconventional oil and gas (UOG) wastewater or UOG-impacted surface water samples relative to the maximum response of rosiglitazone (A). Inhibition or enhancement of cell viability relative to vehicle control (as measured by resazurin to resorufin conversion) for these same UOG-impacted environmental samples (B). X axis values describe test concentration relative to undiluted water: 1 = 10× concentrated water, 0 = raw water (neither diluted nor concentrated), −1 = 10-fold water dilution (0.1× concentration), etc. * indicates lowest concentration with significant change from vehicle control, $p < 0.05$, as per one-way ANOVA in GraphPad Prism 7.0.

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5. Conclusions

In conclusion, we report potent PPARγ and subsequent adipogenic activities (both triglyceride accumulation and pre-adipocyte proliferation) at considerable dilutions of UOG wastewater and UOG-impacted environmental water extracts. Given these bioactivities at <1× relative water concentrations, this suggests potential metabolic health impacts for wildlife near dense UOG operations. Importantly, while PPARγ explained the adipogenic activity for certain samples, it did not explain each, suggesting that the metabolic disruption potential induced by UOG-impacted water may stem from disruption of several nuclear receptor pathways. Additional work is needed to clarify whether these chemicals persist through wastewater treatment and may contribute to elevated exposure for humans living in these regions. Recent work has suggested elevated body burdens of UOG chemicals in humans living near these sites (Crowe et al., 2016; Caron-Beaudoin et al., 2018), though working out the potential oral, dermal, and inhalation exposure routes will take more directed work in future studies. Given the extremely high societal costs of obesity and associated metabolic health disease (Hammond and Levine, 2010; Legler et al., 2015), as well as the growing understanding of environmental metabolic disruptors (Kassotis et al., 2017a; Heindel et al., 2015; Auerbach et al., 2016; Heindel et al., 2017), future studies should more specifically assess potential metabolic health concerns near UOG operations.

Competing financial interests declaration

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.05.030.

References

Akob, D.M., Cozzarelli, I.M., Dunlap, D.S., Rowan, E.L., Lorah, M., 2015. M. Organic and inorganic composition and microbiology of produced waters from Pennsylvania shale gas wells. Appl. Geochem. 60, 116–125.

Akob, D.M., et al., 2016. Wastewater disposal from unconventional oil and gas development degrades stream quality at a West Virginia injection facility. Environ. Sci. Technol. 50, 5517–5525.

Angle, B.M., et al., 2013. Metabolic disruption in male mice due to fetal exposure to low but not high doses of bisphenol A (BPA): evidence for effects on body weight, food intake, adipocytes, leptin, adiponectin, insulin and glucose regulation. Reprod. Toxicol. 42:13. https://doi.org/10.1016/j.reprotox.2013.07.017.

Auerbach, S., et al., 2016. Prioritizing environmental chemicals for obesity and diabetes outcomes research: a screening approach using toxcast high-throughput data. Environ. Health Perspect. 124:1141–1154. https://doi.org/10.1289/ehp.1510456.

Balise, V. D. et al. Perinatal exposure to hydraulic fracturing chemical mixture alters energy expenditure in female mice. (2018) (in preparation).

Blewett, T.A., et al., 2017a. Sublethal and reproductive effects of acute and chronic exposure to flowback and produced water from hydraulic fracturing on the water flea *Daphnia magna*. Environ. Sci. Technol. https://doi.org/10.1021/acs.est.6b05179.

Blewett, et al., 2017b. The effect of hydraulic flowback and produced water on gill morphology, oxidative stress and antioxidant response in rainbow trout (*Oncorhynchus mykiss*). Sci. Rep. 7, 46582.

Bolden, A.L., Kwiatkowski, C.F., Colborn, T., 2015. New look at BTEX: are ambient levels a problem? Environ. Sci. Technol. 49, 5261–5276.

Brookes, P.S., Yoon, Y., Robotham, J.L., Anders, M.W., Sheu, S.S., 2004. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am. J. Phys. Cell Physiol. 287:C817–833. https://doi.org/10.1152/ajpcell.00139.2004.

Caron-Beaudoin, E., et al., 2018. Gestational exposure to volatile organic compounds (VOCs) in Northeastern British Columbia, Canada: a pilot study. Environ. Int. 110: 131–138. https://doi.org/10.1016/j.envint.2017.10.022.

Casey, J.A., et al., 2016. Unconventional natural gas development and birth outcomes in Pennsylvania, USA. Epidemiology 27:163–172. https://doi.org/10.1097/EDE.0000000000000387.

Chamorro-Garcia, R., et al., 2013. Transgenerational inheritance of increased fat depot size, stem cell reprogramming, and hepatic steatosis elicited by prenatal exposure to the obesogen tributyltin in mice. Environ. Health Perspect. 121:359–366. https://doi.org/10.1289/ehp.1205701.

Clark, C., Veil, J., 2009. Produced water volumes and management practices in the United States. Available at:. http://www.ipd.anl.gov/anlpubs/2009/07/64622.pdf, Accessed date: 7 December 2017.

Cozzarelli, I.M., et al., 2017. Environmental signatures and effects of an oil and gas wastewater spill in the Williston Basin, North Dakota. Sci. Total Environ. 579:1781–1793. https://doi.org/10.1016/j.scitotenv.2016.11.157.

Crowe, E., Patton, S., Thomas, D., Thorpe, B., 2016. When the wind blows - tracking toxic chemicals in gas fields and impacted communities. Available at. http://www.comingcleaninc.org/assets/media/documents/When%20the%20Wind%20Blows.pdf, Accessed date: 7 December 2017.

Curhan, G.C., et al., 1996. Birth weight and adult hypertension, diabetes mellitus, and obesity in US men. Circulation 94, 3246–3250.

Danielzik, S., Czerwinski-Mast, M., Langnase, K., Dilba, B., Muller, M.J., 2004. Parental overweight, socioeconomic status and high birth weight are the major determinants of overweight and obesity in 5-7 y-old children: baseline data of the Kiel Obesity Prevention Study (KOPS). Int. J. Obes. Relat. Metab. Disord. 28:1494–1502. https://doi.org/10.1038/sj.ijo.0802756.

Deutch, J., Holditch, S., Krupp, F., McGinty, K., Tierney, S., Yergin, D., Zoback, M., 2011. The Secretary of the Energy Board Shale Gas Production Subcommittee ninety-day report. Available at:. http://www.shalegas.energy.gov/resources/081111_90_day_report.pdf, Accessed date: 7 December 2017.

Diamanti-Kandarakis, E., et al., 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr. Rev. 30:293–342. https://doi.org/10.1210/er.2009-0002.

DiGiulio, D.C., Jackson, R.B., 2016. Impact to underground sources of drinking water and domestic wells from production well stimulation and completion practices in the Pavillion, Wyoming, field. Environ. Sci. Technol. 50:4524–4536. https://doi.org/10.1021/acs.est.5b04970.

DiGiulio, D.C., Wilkin, R.T., Miller, C., Oberley, G., 2011. Investigation of ground water contamination near Pavillion, Wyoming. Available at:. Environmental Protection Agencyhttps://www.epa.gov/sites/production/files/documents/EPA_ReportOnPavillion_Dec-8-2011.pdf, Accessed date: 7 December 2017.

Drollette, B.D., et al., 2015. Elevated levels of diesel range organic compounds in groundwater near Marcellus gas operations are derived from surface activities. Proc. Natl. Acad. Sci. U. S. A. 112:13184–13189. https://doi.org/10.1073/pnas.1511474112.

Engle, M., Cozzarelli, I.M., Smith, B.D., 2014. USGS investigations of water produced during hydrocarbon reservoir development. Available at. https://pubs.usgs.gov/fs/2014/3104/pdf/fs2014-3104.pdf, Accessed date: 7 December 2017.

Environmental Protection Agency (EPA), 2015. Assessment of the Potential Impacts of Hydraulic Fracturing for Oil and Gas on Drinking Water Resources. External Review Draft. US Environmental Protection Agency, Washington, DC Available at:. https://www.epa.gov/sites/production/files/2015-06/documents/hf_es_erd_jun2015.pdf, Accessed date: 7 December 2017.

Fang, M., Webster, T.F., Stapleton, H.M., 2015. Activation of human peroxisome proliferator-activated nuclear receptors (PPARgamma1) by semi-volatile compounds (SVOCs) and chemical mixtures in indoor dust. Environ. Sci. Technol. 49: 10057–10064. https://doi.org/10.1021/acs.est.5b01523.

Folkerts, E.J., Blewett, T.A., He, Y., Goss, G.G., 2017. Cardio-respirometry disruption in zebrafish (*Danio rerio*) embryos exposed to hydraulic fracturing flowback and produced water. Environ. Pollut. https://doi.org/10.1016/j.envpol.2017.09.011.

Fontenot, B.E., et al., 2013. An evaluation of water quality in private drinking water wells near natural gas extraction sites in the Barnett Shale Formation. Environ. Sci. Technol. 47:10032–10040. https://doi.org/10.1021/es4011724.

Getzinger, G.J., et al., 2015. Natural gas residual fluids: sources, endpoints, and organic chemical composition after centralized waste treatment in Pennsylvania. Environ. Sci. Technol. 49:8347–8355. https://doi.org/10.1021/acs.est.5b00471.

Green, H., Kehinde, O., 1975. An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell 5, 19–27.

Green, H., Meuth, M., 1974. An established pre-adipose cell line and its differentiation in culture. Cell 3, 127–133.

Hales, C.M., Carroll, M.D., Fryar, C.D., Ogden, C.L., 2017. Prevalence of Obesity Among Adults and Youth: United States, 2015–2016. National Center for Health Statistics, Hyattsville, MD.

Hammond, R.A., Levine, R., 2010. The economic impact of obesity in the United States. Diabetes Metab. Syndr. Obes. 3:285–295. https://doi.org/10.2147/DMSOTT.S7384.

Harkness, J.S., et al., 2015. Iodide, bromide, and ammonium in hydraulic fracturing and oil and gas wastewaters: environmental implications. Environ. Sci. Technol. 49: 1955–1963. https://doi.org/10.1021/es504654n.

He, Y., et al., 2017a. Chemical and toxicological characterizations of hydraulic fracturing flowback and produced water. Water Res. 114:78–87. https://doi.org/10.1016/j.watres.2017.02.027.

He, Y., et al., 2017b. Effects on biotransformation, oxidative stress, and endocrine disruption in rainbow trout (*Oncorhynchus mykiss*) exposed to hydraulic fracturing flowback and produced water. Environ. Sci. Technol. 51:940–947. https://doi.org/10.1021/acs.est.6b04695.

Heindel, J.J., et al., 2015. Parma consensus statement on metabolic disruptors. Environ. Health 14:54. https://doi.org/10.1186/s12940-015-0042-7.

Heindel, J.J., et al., 2017. Metabolism disrupting chemicals and metabolic disorders. Reprod. Toxicol. 68, 3–33.

Hill, E.L., 2014. Shale gas development and infant health: evidence from Pennsylvania. Available at:. http://www.damascuscitizensforsustainability.org/wp-content/uploads/2014/10/Shale-Gas-Development-and-Infant-Health-Elaine-Hill-Aug-2014.pdf, Accessed date: 13 April 2018.

Hirschler, V., Bugna, J., Roque, M., Gilligan, T., Gonzalez, C., 2008. Does low birth weight predict obesity/overweight and metabolic syndrome in elementary school children? Arch. Med. Res. 39:796–802. https://doi.org/10.1016/j.arcmed.2008.08.003.

Hladik, M.L., Focazio, M.J., Engle, M., 2014. Discharges of produced waters from oil and gas extraction via wastewater treatment plants are sources of disinfection by-products to receiving streams. Sci. Total Environ. 466-467:1085–1093. https://doi.org/10.1016/j.scitotenv.2013.08.008.

Invitrogen, 2010. GeneBLAzer® PPAR gamma 293H DA and PPAR gamma-UAS-bla 293H Cell-based Assay Protocol. Available at:. https://tools.thermofisher.com/content/sfs/manuals/geneblazer_PPARgammaUASbla293H_man.pdf, Accessed date: 17 December 2017.

Jackson, R.B., et al., 2013. Increased stray gas abundance in a subset of drinking water wells near Marcellus shale gas extraction. PNAS 110:11250–11255. https://doi.org/10.1073/pnas.1221635110.

Janesick, A.S., Blumberg, B., 2016. Obesogens: an emerging threat to public health. Am. J. Obstet. Gynecol. 214:559–565. https://doi.org/10.1016/j.ajog.2016.01.182.

Jornayvaz, F.R., et al., 2016. Low birth weight leads to obesity, diabetes and increased leptin levels in adults: the CoLaus study. Cardiovasc. Diabetol. 15:73. https://doi.org/10.1186/s12933-016-0389-2.

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

Kassotis, C.D., et al., 2015. Endocrine-disrupting activity of hydraulic fracturing chemicals and adverse health outcomes after prenatal exposure in male mice. Endocrinology 156:4458–4473. https://doi.org/10.1210/en.2015-1375.

Kassotis, C.D., et al., 2016a. Adverse reproductive and developmental health outcomes following prenatal exposure to a hydraulic fracturing chemical mixture in female C57Bl/6 mice. Endocrinology 157:3469–3481. https://doi.org/10.1210/en.2016-1242.

Kassotis, C.D., et al., 2016b. Endocrine disrupting activities of surface water associated with a West Virginia oil and gas industry wastewater disposal site. Sci. Total Environ. 557-558, 901–910.

Kassotis, C.D., Hoffman, K., Stapleton, H.M., 2017a. Characterization of adipogenic activity of semi-volatile indoor contaminants and house dust. Environ. Sci. Technol. 51, 8735–8745.

Kassotis, C.D., et al., 2017b. Characterization of adipogenic chemicals in three different cell culture systems: implications for reproducibility based on cell source and handling. Sci. Rep. 7, 42104.

Kassotis, C.D., Kollitz, E.M., Ferguson, P.L., Stapleton, H.M., 2018a. Nonionic ethoxylated surfactants induce adipogenesis in 3T3-L1 cells. Toxicol. Sci. 162, 124–136.

Kassotis, C. D. et al. Endocrine disrupting activities and geochemistry of water resources associated with unconventional oil and gas activity. (2018b) (in preparation).

Konig, M., et al., 2017. Impact of untreated wastewater on a major European river evaluated with a combination of in vitro bioassays and chemical analysis. Environ. Pollut. 220:1220–1230. https://doi.org/10.1016/j.envpol.2016.11.011.

Lee, D.S., Herman, J.D., Elsworth, D., Kim, H.T., Lee, H.S., 2011. A critical evaluation of unconventional gas recovery from the Marcellus Shale, Northeastern United States. KSCE J. Civ. Eng. 15:679–687. https://doi.org/10.1007/s12205-011-0008-4.

Legler, J., et al., 2015. Obesity, diabetes, and associated costs of exposure to endocrine-disrupting chemicals in the European Union. J. Clin. Endocrinol. Metab. 100: 1278–1288. https://doi.org/10.1210/jc.2014-4326.

Lester, Y., et al., 2015. Characterization of hydraulic fracturing flowback water in Colorado: implications for water treatment. Sci. Total Environ. 512-513C:637–644. https://doi.org/10.1016/j.scitotenv.2015.01.043.

Li, X., Ycaza, J., Blumberg, B., 2011. The environmental obesogen tributyltin chloride acts via peroxisome proliferator activated receptor gamma to induce adipogenesis in murine 3T3-L1 preadipocytes. J. Steroid Biochem. Mol. Biol. 127:9–15. https://doi.org/10.1016/j.jsbmb.2011.03.012.

Luz, A.L., Kassotis, C.D., Stapleton, H.M., Meyer, J.N., 2018. The high-production volume fungicide pyraclostrobin induces triglyceride accumulation associated with mitochondrial dysfunction, and promotes adipocyte differentiation independent of PPARgamma activation, in 3T3-L1 cells. Toxicology 393:150–159. https://doi.org/10.1016/j.tox.2017.11.010.

Maloney, K.O., et al., 2017. Unconventional oil and gas spills: materials, volumes, and risks to surface waters in four states of the U.S. Sci. Total Environ. 581–582:369–377. https://doi.org/10.1016/j.scitotenv.2016.12.142.

Masuno, H., Iwanami, J., Kidani, T., Sakayama, K., Honda, K., 2005. Bisphenol a accelerates terminal differentiation of 3T3-L1 cells into adipocytes through the phosphatidylinositol 3-kinase pathway. Toxicol. Sci. 84:319–327. https://doi.org/10.1093/toxsci/kfi088.

McKenzie, L.M., et al., 2014. Birth outcomes and maternal residential proximity to natural gas development in rural Colorado. Environ. Health Perspect. 122:412–417. https://doi.org/10.1289/ehp.1306722.

Niemelä, S., Miettinen, S., Sarkanen, J.R., Ashammakhi, N., 2008. Topics in Tissue Engineering. vol. 4 pp. 1–26 (Ch. 4).

Nivala, J., et al., 2018. Application of cell-based bioassays to evaluate treatment efficacy of conventional and intensified treatment wetlands. Environ. Sci. Water Res. Technol. 4, 206–217.

Osborn, S.G., Vengosh, A., Warner, N.R., Jackson, R.B., 2011. Methane contamination of drinking water accompanying gas-well drilling and hydraulic fracturing. Proc. Natl. Acad. Sci. 108:5. https://doi.org/10.1073/pnas.1100682108/-/DCSupplemental.

Patterson, L.A., et al., 2017. Unconventional oil and gas spills: risks, mitigation priorities, and state reporting requirements. Environ. Sci. Technol. https://doi.org/10.1021/acs.est.6b05749.

Peng, H., et al., 2016. Peroxisome proliferator-activated receptor γ is a sensitive target for oil sands process-affected water: effects on adipogenesis and identification of ligands. Environ. Sci. Technol. 50, 7816–7824.

Rich, A.L., Patel, J.T., Al-Angari, S.S., 2016. Carbon disulfide (CS2) interference in glucose metabolism from unconventional oil and gas extraction and processing emissions. Environ. Health Insights 10:51–57. https://doi.org/10.4137/EHI.S31906.

Rowan, E.L., et al., 2015. Geochemical and isotopic evolution of water produced from Middle Devonian Marcellus Shale gas wells, Appalachian Basin, Pennsylvania. Am. Assoc. Pet. Geol. Bull. 99, 181–206.

Rozell, D.J., Reaven, S.J., 2012. Water pollution risk associated with natural gas extraction from the Marcellus Shale. Risk Anal. 32:1382–1393. https://doi.org/10.1111/j.1539-6924.2011.01757.x.

Skalak, K.J., et al., 2014. Surface disposal of produced waters in Western and Southwestern Pennsylvania: potential for accumulation of alkali-earth elements in sediments. Int. J. Coal Geol. 126, 162–170.

Stacy, S.L., et al., 2015. Perinatal outcomes and unconventional natural gas operations in Southwest Pennsylvania. PLoS One 10:e0126425. https://doi.org/10.1371/journal.pone.0126425.

Staiger, H., Loffler, G., 1998. The role of PDGF-dependent suppression of apoptosis in differentiating 3T3-L1 preadipocytes. Eur. J. Cell Biol. 77:220–227. https://doi.org/10.1016/S0171-9335(98)80110-6.

The Endocrine Disruption Exchange (TEDX), 2017. TEDX list of potential endocrine disruptors. Available at:. https://endocrinedisruption.org/interactive-tools/tedx-list-of-potential-endocrine-disruptors/search-the-tedx-list, Accessed date: 7 December 2017.

Thurman, E.M., Ferrer, I., Blotevogel, J., Borch, T., 2014. Analysis of hydraulic fracturing flowback and produced waters using accurate mass: identification of ethoxylated surfactants. Anal. Chem. 86, 9653–9661.

Tiwari, B.S., Belenghi, B., Levine, A., 2002. Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition, and programmed cell death. Plant Physiol. 128: 1271–1281. https://doi.org/10.1104/pp.010999.

Vandenberg, L.N., et al., 2012. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr. Rev. 33:378–455. https://doi.org/10.1210/er.2011-1050 (er.2011-1050 [pii]).

Walker Whitworth, K., Kaye Marshall, A., Symanski, E., 2018. Drilling and production activity related to unconventional gas development and severity of preterm birth. Environ. Health Perspect. 126:037006. https://doi.org/10.1289/EHP2622.

Warner, N.R., Christie, C.A., Jackson, R.B., Vengosh, A., 2013. Impacts of shale gas wastewater disposal on water quality in western Pennsylvania. Environ. Sci. Technol. 47: 11849–11857. https://doi.org/10.1021/es402165b.

Waxman, H.A., Markey, E.J., DeGette, D., 2011. Chemicals used in hydraulic fracturing. Available at:. https://conservationco.org/admin/wp-content/uploads/2013/02/Final-Rebuttal-Exhibits.pdf-Adobe-Acrobat-Pro.pdf, Accessed date: 7 December 2017.

Webb, E., et al., 2014. Developmental and reproductive effects of chemicals associated with unconventional oil and natural gas operations. Rev. Environ. Health 29: 307–318. https://doi.org/10.1515/reveh-2014-0057.

Welshons, W.V., et al., 2003. Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. Environ. Health Perspect. 111, 994–1006.

Wiseman, H.J., 2008. Untested waters: the rise of hydraulic fracturing in oil and gas production and the need to revisit regulation. Fordham Environ. Law Rev. 20, 115–169.

Zeng, X.Y., et al., 2012. Screening for the efficacy on lipid accumulation in 3T3-L1 cells is an effective tool for the identification of new anti-diabetic compounds. Biochem. Pharmacol. 84:830–837. https://doi.org/10.1016/j.bcp.2012.07.003.

Zoeller, R.T., et al., 2012. Endocrine-disrupting chemicals and public health protection: a statement of principles from The Endocrine Society. Endocrinology 153:4097–4110. https://doi.org/10.1210/en.2012-1422.