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ARTICLE

Toxicity of Hydraulic Fracturing Wastewater from Black Shale Natural-Gas Wells Influenced by Well Maturity and Chemical Additives

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Hydraulic fracturing of deep shale formations generates large volumes of wastewater that must be managed through treatment, reuse, or disposal. Produced wastewater liberates formation-derived radionuclides and contains previously uncharacterized organohalides thought to be generated within the shale well, both posing unknown toxicity to human and ecological health. Here, we assess the toxicity of 42 input media and produced fluid samples collected from four wells in the Utica Formation and Marcellus Shale using two distinct endpoint screening assays. Broad spectrum acute toxicity was assessed using a BioLuminescence Inhibition Assay employing the halotolerant bacterium *Aliivibrio fischeri,* while predictive mammalian cytotoxicity was evaluated using a N-acetylcysteine (NAC) thiol reactivity assay. The acute toxicity and thiol reactivity of early-stage flowback was higher than later produced fluids, with levels diminishing through time as the natural gas wells matured. Acute toxicity of early stage flowback and drilling muds were on par with the positive control, 3,5-dichlorophenol (6.8 mg/L). Differences in both acute toxicity and thiol reactivity between paired natural gas well samples were associated with specific chemical additives. Samples from wells containing a larger diversity and concentration of organic additives resulted in higher acute toxicity, while samples from a well applying a higher composition of ammonium persulfate, a strong oxidizer, showed greater thiol reactivity, predictive of higher mammalian toxicity. Both acute toxicity and thiol reactivity are consistently detected in produced waters, in some cases present up to nine months after hydraulic fracturing. These results support that specific chemical additives, the reactions generated by the additives, or the constituents liberated from the formation by the additives contribute to the toxicity of hydraulic fracturing produced waters and reinforces the need for careful consideration of early produced fluid management.

Environmental Significance

Hydraulic fracturing stimulates the release of natural gas and other hydrocarbons from low permeability shale formations. We identified temporal shifts from higher to lower acute toxicity and predictive mammalian cytotoxicity as four shale gas wells matured. Our findings add to the growing body of work quantifying the toxic effects of hydraulic fracturing waste fluids, and links higher toxicities by these end points with chemical composition. As the volume of hydraulic fracturing waste fluids produced increases year after year, it is imperative to utilize information regarding their composition and toxicity to inform safe and effective wastewater management.

1. Introduction

The process of extracting hydrocarbon resources from low permeability formations, such as black shales using horizontal drilling and hydraulic fracturing techniques, remains a growing practice in the oil and gas industry, especially in North America.[1] During hydraulic fracturing, a large-volume (up to 20 million L or more[2,3]) of a water-based fluid is injected into the well at a high pressure (up to 69 MPa[4]), generating new fractures in the formation to enhance the release of hydrocarbons. Injected fluids are primarily water (~90%) combined with proppants such as ceramic beads or sand (~9%), and a short list of chemical modifiers (~1%), commonly including biocides, gelling or foaming agents, pH adjustors, clay stabilizers, and surfactants,[5-7] with the fluid chemistry designed specifically for the conditions of each well. Additionally, the composition of the flowback fluid (generated during the first few weeks after well completion) and produced water (generated once the well transitions into production), collectively abbreviated as FPW, are highly variable and depend on the fracture fluid chemistry, formation-specific geogenic constituents, chemicals formed *in situ* by industry design, or those generated through unanticipated subsurface reactions.[7-11] Well age exerts the greatest influence over major ion concentrations in FPW.[12]

Despite the wide-spread application of these technologies in unconventional oil and gas formations, there remain substantial environmental concerns surrounding water resource use,

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management, and pollution.[2,13-16] One major water resource challenge is the management of large volumes of wastewater containing elevated levels of dissolved solids,[17] salts, radionuclides, [13,18] bromide,[12] and iodide,[19,20] which are difficult to treat using conventional treatment processes.[21] Treatment of FPW fluids has also been associated with the generation of toxins resulting from disinfection byproducts, including organoiodides and brominated sulfonates.[20] Additionally, accidental release of hydraulic fracturing fluids, flowback fluids, and produced waters during well integrity issues, on-site fluid handling and transportation to disposal wells has resulted in contamination of surface water and groundwater.[22] Spills have been shown to negatively affect freshwater invertebrate and fish species, highlighting the potential for both short and long-term environmental impacts to downstream aquatic ecosystems[23,24] and water resource users.[25]

As the practice of hydraulic fracturing in oil and gas extraction continues to grow, the need for research evaluating FPW toxicity also increases. In a recent review, Danforth and coauthors summarized toxicity information for individual produced water constituents, finding ecological or human health risk values for only 14% of identified chemicals.[26] Moreover, interaction effects between fracture fluid chemicals and the FPW itself on toxicity remains poorly understood. The salt content of FPW samples (up to 400 g/L)[27] can be far beyond the salinity of seawater (~33 g/L),[27] representing a significant physiological challenge to freshwater and estuarine organisms. As a result, the high salt content alone of FPW can disrupt organism ionic balance, leading to toxicity; [23,28] aquatic invertebrate acute toxicity has primarily attributed to the high salinity from Duvernay Basin and Appalachian Basin produced waters.[23,29,30] FPW toxicity beyond acute salinity effects have been observed in several model aquatic organisms,[23,27,28,31] with some toxicity endpoints linked to higher organic compound concentrations[32] as well as the solid phase, which may concentrate some toxicants (e.g., polycyclic aromatic hydrocarbons) relative to the dissolved phase.[33]

Whole organism toxicity tests are invaluable in linking adverse outcomes of chemicals on organisms and their receptors, and can be used to assess both individual and synergistic effects. However, whole organism studies are frequently time consuming to perform, may require large fluid volumes, and FPW salinity may mask a range of additional adverse effects. Toxicity screening assays provide a complimentary tool to whole organism tests to quickly assess toxic bioactivity across a large number of environmental samples. The *Aliivibrio fischeri* (*A. fischeri*) bioluminescence assay[34-36] is a widely employed acute toxicity screening test that is sensitive across a wide range of organic and inorganic contaminants and effective in an array of complex waste matrices.[37,38] Hull and coauthors[34] applied this approach to track acute toxicity over 220 days in produced fluids from a Denver-Julesberg Basin (Colorado) shale well, finding BioLuminescence Inhibition Assay (BLIA) inhibition stabilized in the 4 months after hydraulic fracturing. Biological assays have also identified the induction of mutagenicity,[34] estrogenicity,[39] and specific cellular pathways indicative of xenobiotic toxicity response (including pregnane-X receptor, aryl hydrocarbon receptor[30,39]) to compounds concentrated in the organic fraction of shale gas flowback and produced waters. Halogenated organic compounds identified in the organic fraction have the potential to cause a biological response by inducing cysteine thiol in glutathione as a reductant, which can lead to adverse effects including an immunotoxicity reaction.[40,41] The NAC thiol reactivity assay has recently been applied to environmental samples to detect interactions between organic chemical constituents and biological thiols,[42,43] and can be utilized on FPW extracts previously characterized to include a diverse array of halogenated organic compounds.[44]. Although these two screening assays are not capable of characterizing specific toxicity effects at the whole organism level, they provide a rapid, high throughput measure of broad acute toxicity (BLIA) to thiol reactive toxicity (NAC thiol) that can be applied to complex and variable FPW samples.

Although the health effects of some disclosed chemicals used in hydraulic fracturing are known,[21] many gaps remain,[45] and toxicity studies evaluating synergetic effects between compounds is relatively unexplored. With the exception of the aforementioned temporal study for a shale well in the Denver-Julesberg Basin,[34] long term assessment of FPW temporal toxicity also remains limited. Barriers to such studies include gaining continuing access and coordinating sampling with operators at well pads, obtaining and transporting sufficient sample volume for toxicity studies, and sample matrix shifts that occur during well maturation, possibly influencing toxicity results. Among the existing studies, toxicity of flowback collected from a shale well in the Canadian Duvernay formation was highest among most aquatic species at the earliest time[32] while a second study from this same formation noted an inverse relationships through time in toxicity of FPW organic extracts for two different endocrine disrupting assays.[39]

Given the continued need to better understand the character and possible environmental impacts from unconventional oil and gas wastewaters, the goal of this work was to compare and track temporal changes in toxicity for input media and produced fluid samples from multiple hydraulically fractured natural-gas wells in the northern Appalachian Basin using two high throughput screening assays.[46,47] We hypothesized that toxicity would decrease through time after fracturing and that both geological formation and chemical additive components would play an important role in toxicity differences. We were also interested in assessing the toxicity of specific sample fractions to better understand the source of toxicity through time. Results from this study improve our understanding of the toxicity of hydraulic fracture wastewaters and its variability through time in the Appalachian Basin to better inform monitoring and risk assessment efforts in these and other formations slated for future hydraulic fracturing development.

2. Material and Methods

2.1 Fluid Sampling and Processing

Hydraulic fracturing fluids were collected and processed as previously described.[44,48-50] Toxicity analyses were conducted on input media and produced fluid samples from four hydraulically fractured natural-gas wells in the northern Appalachian Basin: two from the Marcellus Shale (M-4 and M-5) and two from the Utica-Point Pleasant Formation (U-6 and U-7). Field samples were collected as part of several large scale, multi-university collaborative efforts to characterize a diverse array of geological, chemical, and biological parameters; access to fluid samples was contingent upon operator and collaborative research plans. Although not all wells were sampled at the same time intervals, the wide array of related data available for analyzed samples is a unique advantage of these multiyear datasets. A total of 42 samples (see Table S2) were collected from drilling equipment, holding tanks, drill muds, or from gas-fluid separators, including: M-4 (n=10 flowback and produced water (FPW), 1 "kill" fluid (a high density fluid pumped into a well to temporarily stop gas flow for maintenance purposes), 1 drill mud, and 1 sidewall mud), M-5 (n= 8 FPW), U-6 (n= 9 FPW, 2 freshwater tank, 1 produced water additive, and 1 recycled produced water additive (make-up water from recycled produced water)), and U-7 (n= 8 FPW) for acute toxicity (bioluminescence assay) and were immediately stored at -20ºC until analysis (see Figure S1 for field experimental details).

Samples for predictive mammalian cell cytotoxicity (NAC thiol assay) were pre-processed using solid phase extraction (SPE) as previously described,[44] then stored at -20ºC until analysis (see Table S3). SPE is important for reducing interferences for the NAC thiol assay and enables increased sensitivity and detection of thiol reactivity by concentrating larger volume samples. Briefly, a 200 mL sample was filtered (0.7 mm glass fiber filter (Whatman GF/F)) and acidified to pH 2 with concentrated HCl to increase extraction efficiency for organic acids and phenols.[51] SPE cartridges (Agilent Bond Elut PPL SPE cartridges (1 g, 3 mL)) were pre-conditioned with methanol (HPLC grade, Fisher Scientific) then rinsed with 0.1% formic acid (Acros Organics). Samples were applied to the SPE resin at a flow rate of ~5-10 mL/min before rinsing the resin with 10 mL 0.1% formic acid and cleaning the cartridge exterior with Milli-Q water to remove sample impurities. SPE cartridges were dried under vacuum before eluting the concentrated and de-salted sample using 10 mL methanol.

2.2 Bioluminescence Inhibition Assay

Acute toxicity for 42 field samples (35 FPW and 7 drill muds/input fluids) was assessed using an *A. fischeri* (NRRL B11177) bioluminescence inhibition microassay (BLIA) (Lumoplate Ultimate Matrix kit, EBPI, Mississauga, ON, Canada). Briefly, lyophilized bacteria were reconstituted into a background diluent (supplied by the manufacturer) for 30 minutes at 4ºC, followed by 30 minutes at 15ºC to activate the *A. fischeri* reagent. Next, samples (200 µL) were added to the plate and serially diluted (1:2) throughout the microplate column. The *A. fischeri* reagent (100 µL) was then dispensed into microplate wells. Luminescence was measured at 1, 5, 15, and 30 minutes of sample exposure using a Synergy HTX microplate reader (BioTek, Winooski, VT, USA) located in an environmental growth chamber held at 15ºC. Orbital shaking of the plates occurred every one second between measurements.

Samples were analyzed in duplicate and fractionated as described below, and positive (3,5-dichlorophenol (Sigma-Aldrich)) and negative controls (background diluent) were included on every plate. A salt control (SW) was analyzed containing Na, Mg, K, Ca and Cl, typical of Appalachian shale FPW (Table S1). To separate toxicity effects between solid (particles) and aqueous (dissolved) phases, field samples were fractioned into a sediment-containing sample (S) and a sediment-free (SF) portion. Sediment-containing fractions were raw and unprocessed fluids or muds, while sediment-free fractions contained the supernatant after centrifugation (9.6x*g* for 10 minutes) that also passed through a 0.2 µm pore size polyethersulfone (PES) filter. When samples were outside of a circumneutral range, the pH was adjusted to 7.0±0.2 using 1.0 M NaOH or HCl solutions for the samples shown in Table S2. Considering the high concentration of chloride (g/L) present in flowback and produced water samples, hydrochloric acid addition to adjust sample pH is not expected to produce halogenated toxicants. The total dissolved solids (TDS) concentration was adjusted to 20 g/L with MilliQ water for all samples per standard procedure for optimal *A. fischeri* growth (ISO 21338:2010 standard); dilution factors ranged from 3 to 7 for FPW samples (see Table S2).

The percentage of bioluminescence inhibition (INH%) was determined by comparing sample bioluminescence (I_s) and background control (I_c) as follows (Eq. 1):

$$INH\% = 100\text{-}100 \times (I_{S30} / KF) \times I_{S1}) \quad (1)$$

where KF is the correction factor ($KF = I_{C30}/ I_{C1}$), I_{C1} is the control luminescence intensity at one minute, I_{C30} is the control luminescence intensity after 30 minutes, I_{S1} is the sample luminescence intensity at one minute, and I_{S30} is the sample luminescence intensity after 30 minutes. The concentration that inhibited 50% of the population (EC_{50}) was determined from bioluminescence results using EBPI toxicity calculation software following ISO standard 21338. A Toxicity Unit (TU_{50}) was then calculated as $100/EC_{50}$. Based on the TU_{50} values, samples were classified as non-toxic ($TU_{50}= 0$), slightly toxic ($0 < TU_{50} < 1$), toxic ($1 < TU_{50} < 10$), very toxic ($11 < TU_{50} <100$), and extremely toxic ($TU_{50} >100$).[52]

2.3 N-Acetylcysteine Thiol Reactivity Assay

The toxicity of SPE extracts for 18 FPW and 4 input fluid samples from M-4 and M-5 were analyzed using the NAC thiol reactivity assay (Table S3), which is a predictor of reactive cytotoxicity for mammalian cells.[43,46,47] The NAC thiol assay quantifies the availability of cysteine thiol groups on N-acetylcysteine as a surrogate for glutathione, a biomolecule that defends against reactive toxins in

biological systems.[53] In this assay, 2-nitro-5-thiobenzoate (NTB) is produced and measured from a reaction between Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB)[54] with unreacted thiol groups on NAC after sample exposure. A dilution series of four replicate sample extracts in Tris buffer (1M, pH 8.0, Thermofisher) (40 µL volume) was incubated with 4 mM NAC (10 µL) at room temperature in the dark for 20 min with linear shaking. Next, 50 µL of Ellman's reagent (1 mM DTNB in 100 mM potassium phosphate buffer and 0.1 mM EDTA at pH 8.0) was added to the assay to react with any remaining NAC during 10 seconds of linear shaking. The produced NTB was immediately quantified by measuring absorbance at 412 nm using the Synergy HTX microplate reader. In addition to sample extracts and their corresponding blanks (extract, Tris buffer, and Ellman's reagent), each plate contained a negative control (NAC, Tris buffer and Ellman's reagent), and a positive control (NAC, Tris buffer, Ellman's reagent, and 10 mM maleimide (2,5-Pyrroledione, Sigma-Aldrich)). The percent of concurrent negative control was defined by averaging blank-corrected negative control data and dividing these estimates into individual absorbance value measured at 412 nm for each FPW sample. The data were reported as the percentage of the concurrent negative control.

2.4 Geochemical and Organic Analyses

Total dissolved solids (TDS) were estimated based on measured electrical conductivity on unfiltered samples using Orion star field probes (ThermoFisher Scientific, Waltham, MA). Samples for geochemical and organic analysis were collected in high density polyethylene or glass containers with no headspace and stored at 4°C until analysis within 48 hours. Samples for total dissolved carbon (DOC) were filtered (0.22 µm pore size PES filters, EMD Millipore, Burlington, MA) and measured as non-purgeable organic carbon by combustion using a TOC/TN analyzer equipped with autosampler (TOC-V CSN/TNM-1/ASI-V, Shimadzu, Kyoto, Japan). During the M-4 and M-5 sampling campaigns, samples were also collected for BTEX (inc. benzene, toluene, ethylbenzene, total xylene, m,p-xylene, o-xylene) and surfactants (methylene blue active substances [MBAS]). Samples for BTEX were preserved using hydrochloric acid (pH <2) and analyzed using GC-MS using EPA SW-846 8260B while the methylene blue active substances assay was performed per EPA SM5540C. Comparisons are also made against a previously published chemical dataset of iodinated organic ions detected in the same produced fluid solid phase extracts utilized for the thiol reactivity assay.[44]

2.5 Statistical analyses

All statistical analyses were conducted using SigmaPlot version 14.0 (Systat Software Inc., San Jose, CA, USA). Data were first tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test). Data that failed these tests were transformed or analyzed using nonparametric statistical approaches (e.g. Wilcoxon Signed Ranked test). Positive bioluminescence inhibition of the sediment-containing and sediment-free samples were compared using a Kruskal-Wallis One Way Analysis of Variance on Ranks, while interactions between BLIA, NAC-thiol reactivity, geochemical parameters, organic chemical parameters, and halogenated organic compounds were investigated by regression analyses. Differences were considered statistically significant at $p \leq 0.05$.

3. Results

3.1 Acute toxicity persists in some shale wells up to nine months after hydraulic fracturing

We first investigated changes in acute toxicity on halotolerant bacteria for FPW samples (diluted to 20 g/L TDS) collected up to 764 days after flowback began from two Marcellus shale natural gas wells using the *A. fischeri* assay. We expected a decrease in toxicity with time after hydraulic fracturing as the injected fluid additives reacted with each other and with the shale matrix and were diluted by formation brines before returning to the surface as FPW. Our results support decreased toxicity as Marcellus natural-gas wells mature, with acute toxicity of early-stage flowback measuring higher than that of later FPW for both sediment-containing and sediment-free fractions in both wells (Figure 1). Toxicity of FPW in M-4 remained high for more than 9 months (280 days) after flowback began, then declined in subsequent months for both fractions (**Error! Reference source not found.**a). A similar temporal trend was observed in M-5, although toxicity diminished at an earlier time, by 4 months after flowback began (Figure 1b). Negative INH% was measured in FPW samples from both M-4 and M-5 after nine months production (from 406 to 764 days), indicating the halotolerant bacteria *A. fischeri* was actually stimulated by the geochemistry of later produced fluid samples above that of the negative control, which only contained cells and background diluent (Figure 1a, 1b). Although we cannot pinpoint an explanation for this effect based on our collected data, one possibility is that constituents present in these fluids might serve as additional carbon, nutrient, or energy sources for this taxon, stimulating its growth.

Compared with the two Marcellus wells, FPW acute toxicity from Utica-Point Pleasant Formation natural gas well samples decreased very quickly. Samples from U-6 diminished in toxicity within 30 days, while samples from U-7 showed no acute toxicity regardless of time sampled for both sediment-containing and sediment free fractions (Figure 1c and d). To put these acute toxicity values in perspective, we calculated their half maximal effective concentration (EC_{50}), then applied the classification scheme developed by Persoone et al,[52] which categorically ranks values from non-toxic to extremely toxic. Early flowback samples in M-4, M-5, and U-6 ranged from slightly toxic to toxic, with the highest classified toxicity in days 2, 9 and 119 of flowback in M-5. Using this criteria, FPW samples that were collected from mature wells, or wells producing natural gas for greater than one year, were classified as non-toxic (Figure 1).

Based on previous studies, [23,33,55,56] we expected the removal of sediments and associated metals and/or hydrophobic organic matter to decrease toxicity in FPW samples. However, for samples measuring positive toxicity in the two Marcellus and one Utica well (280 days or less, M-4; 119 days or less, M-5; 22 days or less, U-6), we detected no significant difference between sediment-containing

and sediment-free fractions for individual wells (Figure 2a). We observed samples from one Marcellus well (M-5) contained average higher toxicity than the other on the same well pad (M-4) for both sediment-containing and sediment-free fractions ($p<0.05$). However, average acute toxicities for the Utica well samples (U-6) did not significantly differ from sediment-containing or sediment-free fractions in either M-4 or M-5 indicating that the toxins are primarily dissolved. Altogether, these data suggest acute toxicity of FPW samples from these four natural gas wells as measured using the *A. fischeri* BLIA assay is primarily influenced by sample timing and specific well additives, as opposed to sample fraction or Appalachian shale formation.

3.2 Input media containing additives show high toxicity

In samples showing positive acute toxicity, the average INH% of both sediment-containing fractions (M-4 and M-5) and sediment free fractions (M-4, M-5 and U-6) was less than that of the positive control (6.8 mg L^{-1} 3,5-dichlorophenol, INH%= 65%) (Figure 2b). However, per standard procedure, all FPW samples were diluted to 20 g/L TDS using dilution factors that varied from 3 to 7 (see SI). Considering this, the undiluted toxicity of M-5 and U-6 samples would likely be similar to or considerably higher than the positive control during the first few months of flowback assuming extrapolation of the acute toxicity response within this dilution range. We verified that the assay response was not solely due to major ions by analyzing the toxicity of a salt control. The average INH% of acute toxicity in the salt control was on par with the negative toxicities measured in all four mature wells studied (Figure 2b), which is unsurprising given that *A. fischeri* is a moderate halophile.[57] Among the media tested for toxicity from these sites, input muds and fluids (drill mud, sidewall mud, and kill fluid) exhibited the highest INH%, while produced water and recycled produced water additives exhibited lower INH% (Figure 2c).

Unsurprisingly, negative inhibition was measured in the freshwater used as the source fluid in U-6 and U-7 (Figure 2c). Importantly, the dilution factors for these media varied considerably, ranging from 1 to 7 for all except the drill mud, which required a 70-fold dilution (see Table S2). The Marcellus drill mud and sidewall mud were categorized as toxic while the kill fluid was classified as very toxic based on classification of half maximal effective concentration values (EC_{50}).

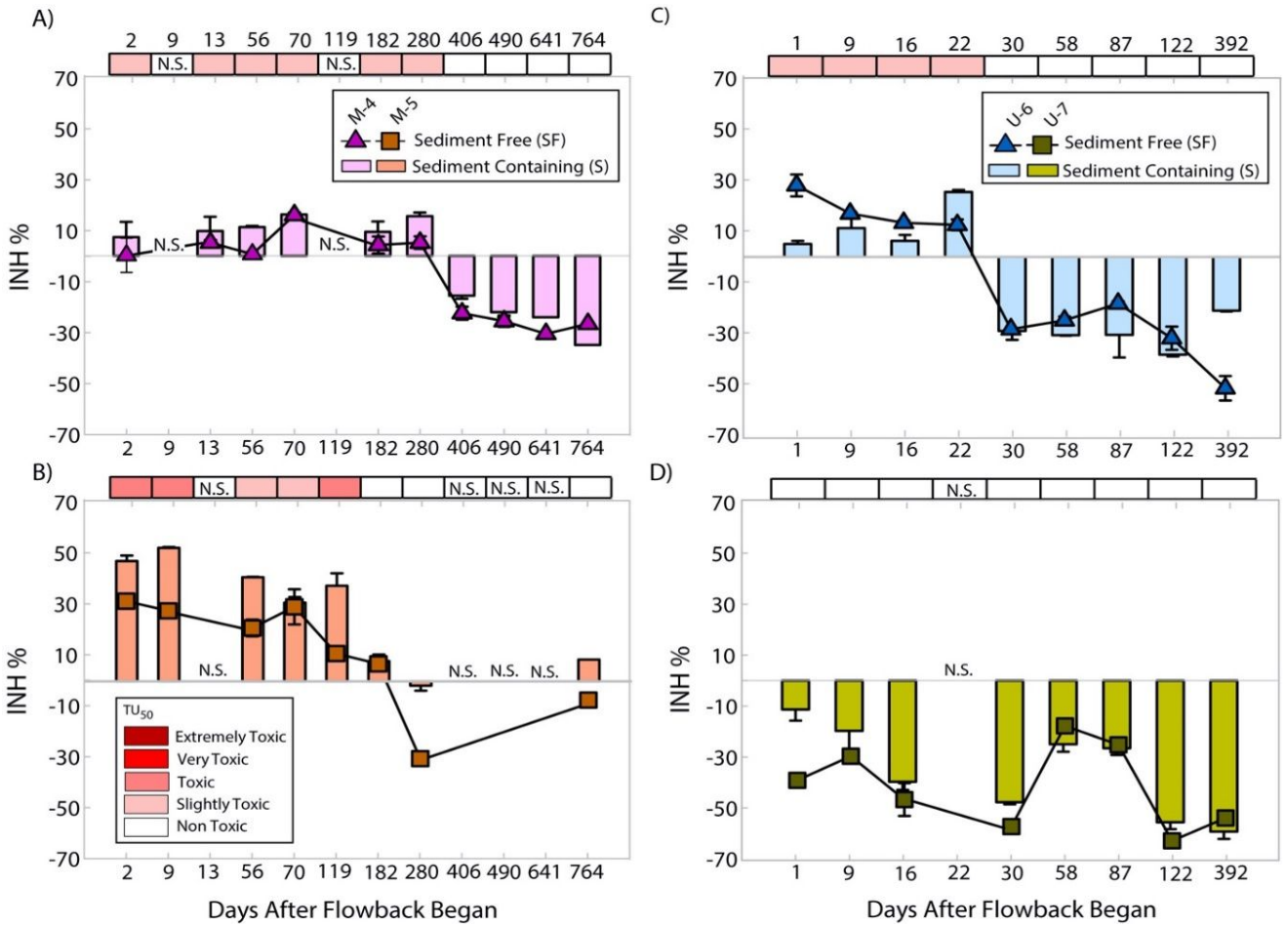

Figure 1 Acute toxicity of flowback and produced water measured using the *A. fischeri* BLIA assay from Marcellus Shale natural-gas wells M-4 (A) and M-5 (B), and Utica natural gas wells U-6 (C) and U-7 (D). Sediment containing fractions are depicted as bars while sediment-free fractions are shown as lines with markers. Boxes above each figure represents toxicity classification (TU_{50}) based on EC_{50} values determined from assay concentrations of sediment containing samples; N.S. indicates no sample was analyzed at that time point.

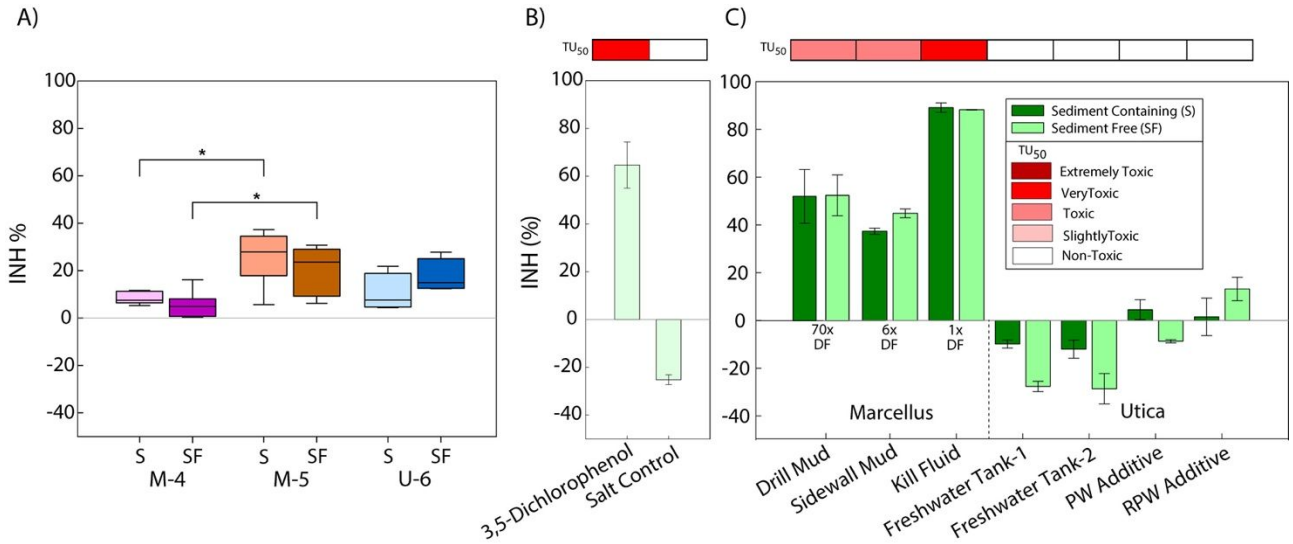

Figure 2. Average acute toxicity as measured using the *A. fischeri* BLIA assay of (A) FPW samples (*indicates statistical significance at p<0.05), (B) positive control (3,5-Dichlorophenol, 6.8 mg/l) and salt control and (C) input media from the Marcellus and Utica well pads.

3.3 NAC thiol reactivity persists in Marcellus wells months after hydraulic fracturing

To further understand the specific pathways that may contribute to the acute toxicity measured using the *A. fischeri* assay, we applied a high-throughput NAC-based thiol reactivity assay that quantified a toxicity pathway predictive of mammalian cells for FPW samples collected up to 764 days post-stimulation from M-4 and M-5. Based on our acute toxicity results, we expected a decrease in thiol reactivity with time after hydraulic fracturing. Our results supported this hypothesis, as the thiol reactivity of early flowback was higher than that of mature produced fluids (Figure 3a). Although M-5 showed higher acute toxicity, its thiol reactivity diminished quickly, within 70 days post-stimulation. In contrast, M-4 had higher sustained NAC thiol reactivity through 182 days of flowback, with values on average 1.3 fold higher than M-5 (Figure 3b). Although we were unable to apply the NAC thiol reactivity to all input media used in the acute toxicity assay, we tested four input media samples that were processed using SPE: two fracture fluids and two input river fluids. The M-4 fracture fluid had higher NAC thiol reactivity response than the fracture fluid used in M-5, which is consistent with early FPW samples from these wells (Figure 3c). Recognizing sample concentration factors (CF) were 8 times higher for FPW samples (200x CF) compared with fracture fluids (25x CF, limited by SPE clogging from fracture fluid additives), measured fracture fluid NAC thiol toxicity was comparable to that of early FPW samples (Figure 3c), indicating injected fluids likely contained considerably higher thiol reactivity if differences in concentration factors were accounted for. Due to the non-linearity of thiol reactivity response, responses of less concentrated injected fluids were not extrapolated. Additionally, the composition of the fracture fluid before and after breaker addition (in well) could also influence the measured toxicity (e.g., possible formation of organohalides following breaker addition[58]). Interestingly, input river samples (200x CF) measured thiol reactivity response similar to that in later time points for both M-4 and M-5 (200x CF). These findings further support fracturing fluid additives as the source of toxicity in early FPW samples, with diminished toxicity as the natural gas well matures.

To further explore how results from the whole effluent acute toxicity assay (BLIA) related to the NAC thiol assay, which targeted electrophilic and redox reactive compounds in organic extracts, we compared their responses (Figure 4). These two toxicity assays were significantly inversely correlated to each other (p<0.0001), indicating high acute toxicity was associated with higher thiol reactivity. Moreover, the NAC thiol reactivity explained only about half the acute toxicity measured via the *A. fischeri* assay (Figure 4), which is not surprising, considering the BLIA is sensitive to a broad range of chemical compounds while NAC thiol is measuring a specific reaction between DNTB and NAC thiol groups. The NAC thiol reaction involves the nucleophilic attack of the thiolate on an electrophile[59] and therefore its response would not include other forms of toxicity derived from the diverse chemical additives, products, and radionuclides commonly present in FPW samples.

3.4 Differences in toxicity are associated with variations in fracture fluid chemical additives

Since the samples from these four natural gas wells represent two well pairs on two different pads, we had the opportunity to examine differences in geochemistry and specific chemical additives that might influence the toxicity of input media and resulting FPW. Interestingly, the fracture fluid composition in M-5 had 18 additives added at 1.1 to 2.7 times higher concentration than M-4, while only 5 additives were higher in M-4 (Table S4 & S5). Moreover, a larger overall fraction of organic chemical additives were present in M-5, including the biocide C_{12}-C_{16} alkyl dimethylbenzyl ammonium chloride, breaker ethylene glycol, and the corrosion inhibitor methanol. These compounds are associated with oral acute toxicity[60] identified by the EPA's Human Health Benchmarks for Pesticides and the EPA's Integrated Risk Information System database, which may in part explain the higher acute toxicity measured in M-5 through time. In contrast, M-4 fracture fluid contained 2.7 times higher concentration of guar gum (a gelling agent), 6.5 times higher

Table 1. Hydraulic fracturing fluid additives of (A) Marcellus Shale natural-gas wells M-4 and M-5, and (B) Utica Point Pleasant Formation natural gas wells U-6 and U-7, as disclosed in the FracFocus database. Disclosed information was used to calculate fold differences in wells on the same pad. The intensity of box shading represents the fold difference in mass fractions for hydraulic fracturing fluid additives for wells from the same well pad and formation. Specific mass fractions for additives listed here are provided in Tables S4-S7. Dark grey shading indicates additive absent/fold difference not calculated due to the lack of information.

a)

CAS RNs	Ingredient	Fold difference in max concentration	
		M-4	M-5
14808-60-7	Quartz, Crystalline silica		
7647-01-0	Hydrochloric acid		
7783-20-2	Ammonium sulfate		
9000-30-0	Guar gum		
38193-60-1	Acrylamide[a]		
111-30-8	Glutaraldehyde		
68171-29-9	Sodium salt[b]		
7727-54-0	Diammonium peroxidisulphate		
136793-29-8	Methyl acrylate[c]		
68424-85-1	Alkyl (c12-16) DAC[d]		
6381-77-7	Sodium erythorbate		
7601-54-9	Trisodium ortho phosphate		
57-13-6	Urea		
25322-69-4	Polypropylene glycol		
67-56-1	Methanol		
61790-12-3	Fatty acids, tall-oil		
68527-49-1	Thiourea, polymer[e]		
107-21-1	Ethylene Glycol		
7631-86-9	Non-crystalline silica		
25038-72-6	Halogenated polymers[f]		
7757-82-6	Sodium sulfate		
68951-67-7	Alcohols, alkoxylated[g]		
64-17-5	Ethanol		
107-19-7	Propargyl alcohol		
79-06-1	2-Propenamid (impurity)		
629-73-2	Hexadec-1-ene		
112-88-9	1-Octadecene (C18)		
63148-62-9	Silicones[h]		
64-02-8	TSEDT		
67762-90-7	Silica[i]		
556-67-2	Octamethylcyclotetrasiloxane		
9002-84-0	Poly (tetrafluoroethylene)		
50-00-0	Formaldehyde		
541-02-6	Decamethyl cyclopentasiloxane		
14807-96-6	Magnesium silicate hydrate		
9003-06-9	Polyacrylamide-co-acrylic acid		
7647-14-5	Sodium Chloride		
Trade	Alcohol Ethoxylate Surfactants		
64742-47-8	Petroleum Distillate		

[a]Acrylamide, 2-acrylamido-2-methylpropanesulfonic acid, sodium salt polymer
[b]Ethanol, 2,2',2"-nitrilotris-, 1,1',1"-tris (dihydrogen phosphate), sodium salt
[c] Polymer of 2-acrylamido-2-methylpropanesulfonic acid sodium salt and methyl acrylate
[d] Alkyl (c12-16) dimethylbenzyl ammonium chloride
[e] Thiourea, polymer with formaldehyde and 1-phenylethanone
[f]Halogenated polymers: Vinylidene chloride/methylacrylate copolymer
[g] Alcohols, C14-15, ethoxylated (7EO)
[h] Dimethyl siloxanes and silicones
[i] Siloxanes and silicones, dimethyl, reaction products with silica

b)

CAS RNs	Ingredient	Fold difference in max concentration	
		U-6	U-7
14808-60-7	Sand		
7647-01-0	Hydrogen chloride		
69418-26-4	Acrylamide[a]		
Proprietary	Proprietary		
64742-47-8	Petroleum Distillate		
84133-50-6	Alcohol – alkoxylated[b]		
124-04-9	Adipic acid		
111-30-8	Glutaraldehyde		
7173-51-5	DD ammonium chloride[c]		
68424-85-1	n-Alkyl dimethyl BAC[d]		
64-17-5	Ethanol		
7722-84-1	Hydrogen Peroxide		
Proprietary	Sodium Polyacrylate		
9000-30-0	Guar Gum		
64742-47-8	Light petroleum distillates[e]		
Proprietary	Organophylic Clay		
34398-01-1	Alcohol ethoxylate		
14808-60-7	Crystalline Silica		
67-56-1	Methanol		

[a]Ethanaminium, N,N,N-trimethyl-2-[(1-oxo-2-propenyl)oxy]-, -chloride, polymer with 2-propenamide
[b] Alcohols, C12-14-secondary, ethoxylated
[c] Didecyl dimethyl ammonium chloride
[d] n-Alkyl dimethyl benzyl ammonium chloride
[e] Distillate (petroleum), hydrotreated light

Legend
10-100
5-10
1-5
0-1
No Data

polyacrylamide-co-acrylic acid and alcohol ethoxylates (friction reducers), and 74 times higher ammonium persulfate (an oxidative radical initiator) than M-5.

Although Hull et. al suggested labile organics (e.g., organic acids, guar gum) may mask toxicity of other additives,[34] our results show that M-4, which contained 2.7 times higher concentrations of guar gum than M-5, had lower acute toxicity but higher thiol reactivity. The combination of organic additives in M-5 likely contributed to its higher acute toxicity, while specific reactions initiated by ammonium persulfate[58,61] may explain the higher thiol reactivity in M-4 as compared to M-5. Halides compete with the intended radical reaction initiated by ammonium persulfate[62] and unsurprisingly ammonium persulfate has been shown to initiate halogenation of fracturing chemicals including cinnamaldehyde.[58,61] Although these samples were desalted prior to analysis, organohalides generated through halogenation reactions would still be present in the extract, as evidenced by Luek et al.[44] In a similar capacity, we compared the disclosed chemical additives in U-6 and U-7, finding that six constituents were added at 1.1 to 1.5 higher concentration in U-6, including petroleum distillates, n-Alkyl dimethyl benzyl ammonium chloride and methanol (Table S6 & S7). These minor differences in overall chemical additive composition could partially explain differences in acute toxicity for U-6 well as compared to U-7. The n-Alkyl dimethyl benzyl ammonium chloride and methanol are associated with oral acute toxicity[60,63] while certain petroleum distillates may not be readily biodegraded by the bacteria used in this assay.

3.5 Toxicity correlated with organic chemical concentrations

Based on these disclosed differences in organic chemical compositions, we further explored the relationship between BLIA acute and NAC thiol toxicity results and organic chemical measurements. A general trend in hydraulically fractured Appalachian Basin shales is a decrease in dissolved organic carbon concentration (DOC) with time after hydraulic fracturing.[32,64] Consistent with studies of other well sites, DOC decreased through time in each of these four wells. Toxicity also decreased with time, therefore we identified a significant positive correlation between DOC and acute toxicity in the sediment free fraction (SF) of M-5 and sediment containing fraction (S) of M-4 ($p<0.05$). In addition, a weak correlation between DOC and BLIA acute toxicity was observed for U-6 ($R^2=0.5$) and U-7 ($R^2=0.54$). No significant correlation was found between DOC concentrations and thiol reactivity for either M-4 or M-5. Samples from M-4 and M-5 were also analyzed for several organic constituents (BTEX, MBAS, oil and grease (O&G), Table S8) frequently detected in produced waters.[10,12,65-68] Like bulk DOC, the concentration of BTEX, MBAS, and O&G generally decreased in M-4 and M-5 natural-gas wells as they matured (Figure S2A-Figure S2C). Benzene and toluene were found in all FPW samples, with the highest benzene (14 µg/L, day 8) and toluene (53 µg/L, 42 days) measured in M-5 (Figure S2A). Similarly, MBAS and O&G were present in all FPW samples, with M-5 containing higher MBAS (3mg/L, 402 days) and O&G concentrations (500 mg/L, 56 days) (Figure S2A and S2B). MBAS concentrations were positively correlated with acute toxicity ($p<0.05$) for both S and SF fractions from M-4 and for SF fractions from M-5. Ethylbenzene, total xylenes, m,p-xylene, and o-xylene varied through time from non-detect to 31 µg/L in the two Marcellus wells (Figure S2B and S2C). Both benzene and toluene were positively correlated to acute toxicity ($p<0.05$) in M-4 for both S and SF fractions, as well as the SF fractions in M-5.

The M-4 and M-5 samples analyzed here were also assessed for halogenated organic ions characterized in a previous study using the non-target mass spectrometry approach FT-ICR-MS.[44] Luek and coauthers[44] identified dozens of previously uncharacterized iodinated organic ions (Table S9) and suggested the compounds could be associated with toxicological effects based on previously characterized species (e.g. disinfection byproducts).[42,43,69,70] A recent study demonstrated enhanced mammalian cell cytotoxicity due to the generation of iodinated organic compounds that formed during chloramination of oil and gas wastewaters.[20] Here, we found a significant correlation between acute toxicity measurements and the

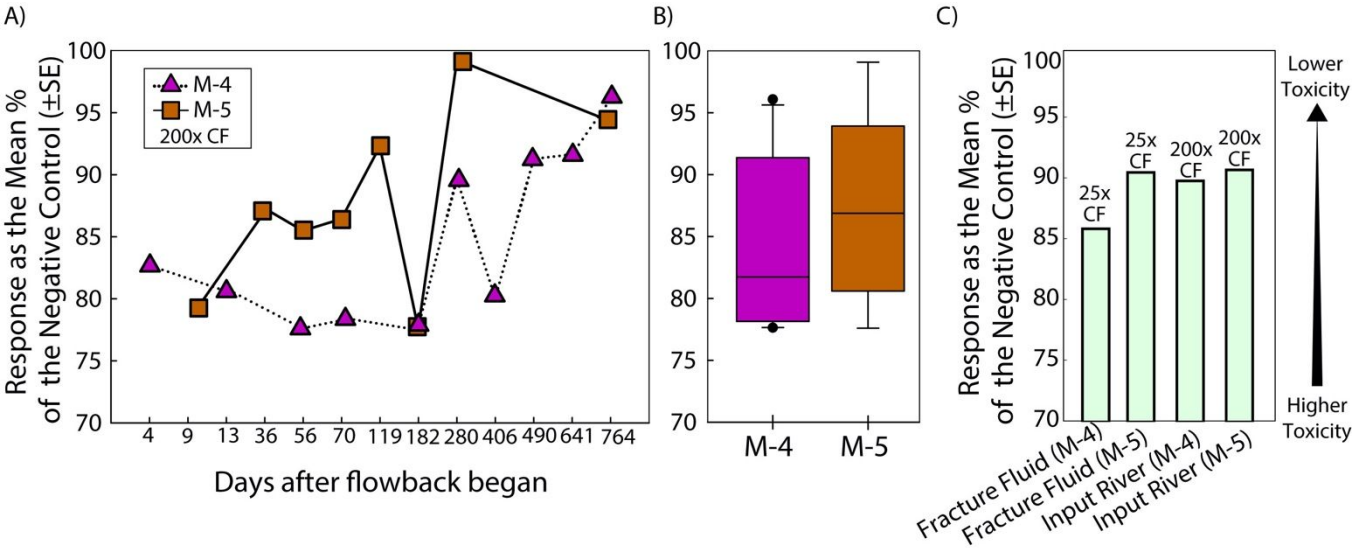

Figure 3. (A) NAC thiol reactivity for flowback and produced water from Marcellus Shale natural-gas wells M-4 and M-5. Average NAC thiol reactivity of (B) FPW samples and (C) input media from the Marcellus well pad samples.

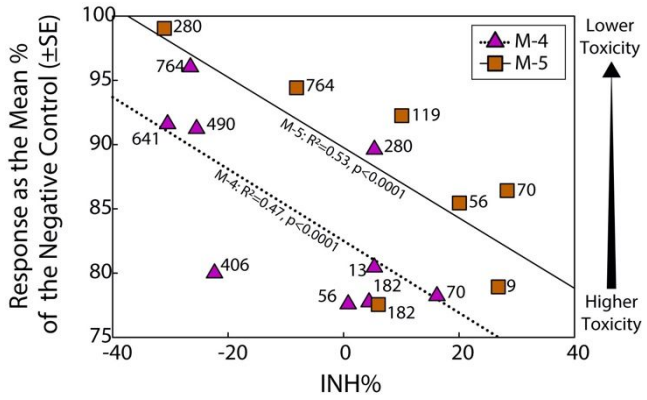

Figure 4. Comparison between the *A. fischeri* acute toxicity assay and the NAC thiol reactivity response for all FPW samples from M-4 and M-5.

number of detected iodinated ions ($p<0.05$) for both S and SF fractions from M-5. However, a similar correlation was not seen in M-4 samples, potentially due to the overall lower acute toxicity level measured therein. Lastly, we investigated the relationship between NAC thiol reactivity and specific organic chemical concentrations (*inc.*, BTEX, MBAS, O&G, and iodinated organic ions). Only benzene and toluene were positively correlated to NAC thiol reactivity in M-5 ($p<0.05$) while the concentration of all other constituents were statistically unrelated.

4. Conclusion

In this work, we sought to investigate how toxicity of hydraulic fracturing media differed across formations and through time. We also aimed to separate toxicity effects from inorganic salts versus organic constituents, and constituents associated with solids as opposed to those in the dissolved phase. The high salinity of hydraulic fracturing wastewater is a primary mechanism of toxicity for freshwater organisms, but other potentially harmful chemical compounds are present.[23,24,28] Here, our application of a BLIA screening assay employing a halotolerant bacterium identified acute toxicity of FPW beyond salinity that is broadly associated with changes in dissolved constituents. Although distinguishing between toxicity effects for specific chemical additives, for compounds generated by these chemical additives within the formation, or for other constituents liberated by these additives outside of salinity (e.g., radionuclides) was beyond the scope of this study, the results from our acute screening assay is in agreement with recent whole organism toxicity studies reporting toxicity and other adverse effects derived from organic compounds in FPW.[71,24,27] In contrast to previous work that identified toxicity in higher order organisms is associated with solid fractions,[27,33] here we found no significant difference in acute toxicity for sediment-containing versus filtered fractions of wastewater, indicating the halotolerant bacteria used in the BLIA assay are more sensitive to dissolved constituents.

Our work shows the acute toxicity and NAC thiol reactivity of FPW is highest during initial flowback and diminishes as the natural gas well matures. Importantly, toxicity as measured by these assays was associated with the dissolved sample fraction and was correlated to bulk organic carbon concentrations, specific organic constituents (MBAS, benzene, toluene), and the number of iodinated compounds in these wells. Higher toxicity in early flowback samples is in agreement with a study tracking acute toxicity of FPW samples in the Denver–Julesburg Basin[34] although our data provides a broader assessment of these trends through time, utilizes replicate wells on a pad, and encompasses two shale formations that differ considerably in depth and geological characteristics from Denver-Julesburg Basin. Moreover, our work is consistent with that of Folkerts et al,[32] who show earlier FPW samples to be more toxic to *Daphnia magna*, *Lumbriculus variegatus*, *Danio rerio*, and *Oncorhynchus mykiss*. The chemical additives highlighted here could pose toxicity in their original form, via the compounds they generate through abiotic or biotic mechanisms before or after injection, or through the chemicals they liberate from the formation.[58,61,72] Although difficult to quantify, our work suggests toxicity for the measured endpoints is higher for FPW samples when larger quantities of specific chemical additives are applied, including oxidative radical initiators, biocides and surfactants. Moreover, we also observed that samples with the highest NAC thiol reactivity derived from well samples utilizing injected fluids that specifically contained a higher amount of oxidative radical chemicals. This finding is consistent with related studies quantifying NAC thiol reactivity in water and wastewater samples receiving higher oxidative inputs from disinfection processes,[43,70,73] which are thought to initiate the biological thiol-specific detoxification mechanism and suggests that similar reactions may occur in the fractured shale system.

Our results support the need for careful management of wastewater from hydraulically fractured shale wells, particularly during the handling of injection fluids and fluids initially returning from the well after hydraulic fracturing in order to reduce their release to the environment. Higher acute toxicity in early flowback is particularly problematic for wastewater management in "tight" hydraulically fractured formations, such as shale gas, as the largest volume of water is produced during initial flowback. Our findings further highlight the need for additional testing of multiple toxicity end points to better characterize differences in FPW toxicity that vary through time and geologic formation as well as assess potential interactions between chemical additives.

Conflicts of interest

The authors have no conflicts of interest to disclose.

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