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Chemical and bioassay assessment of waters related to hydraulic fracturing at a tight gas production site



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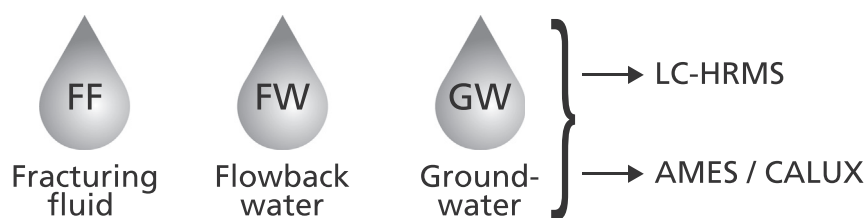
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HIGHLIGHTS

- Lack of European hydraulic fracturing related water assessments
- Chemical and bioassay assessment of Dutch hydraulic fracturing related water samples
- Fracturing/flowback fluids potentially pose human and environmental health concerns.
- Clear genotoxic and oxidative stress responses found for fracturing/flowback fluids
- Measures justified to handle, transport and treat fracturing/flowback fluids.

GRAPHICAL ABSTRACT



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ABSTRACT

Publicly available chemical assessments of hydraulic fracturing related waters are generally based on shale gas practices in the U.S. There is a lack of information on hydraulic fracturing related gas development from EU countries and more generally on other types of extractions. This research fills this knowledge gap by presenting chemical and bioassay assessments of hydraulic fracturing related waters from a tight gas development in the Netherlands. Fracturing fluid, flowback water and groundwater from surrounding aquifers before and after the actual fracturing were analysed by means of high resolution liquid chromatography tandem mass spectrometry, the Ames test and three chemical activated luciferase gene expression bioassays aimed at determining genotoxicity, oxidative stress response and polycyclic aromatic hydrocarbon contamination. After sample enrichment a higher number of peaks can be found in both fracturing fluid and flowback samples. No clear differences in chemical composition were shown in the groundwater samples before and after hydraulic fracturing. Preliminary environmental fate data of the tentatively identified chemicals points towards persistence in water. Clear genotoxic and oxidative stress responses were found in the fracturing fluid and flowback samples. A preliminary suspect screening resulted in 25 and 36 matches in positive and negative ionisation respectively with the 338 possible suspect candidates on the list.

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Extensive measures relating to the handling, transport and treatment of hydraulic fracturing related waters are currently in place within the Dutch context. The results of the present study provide a scientific justification for such measures taken to avoid adverse environmental and human health impacts.

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1. Introduction

The possible use of hydraulic fracturing to retrieve oil and gas from deep underground shale formations, is heavily debated by European governments and in the scientific literature (Hays et al., 2015; Vandecasteele et al., 2015; Thomas et al., 2016;). Different EU member states do however consider shale gas exploration for its competing economic value with other fossil fuels. In Poland and England future explorations and production of shale gas have been planned (Stamford and Azapagic, 2014; Vandecasteele et al., 2015). Other member states postpone developments, in anticipation of in-depth human and environmental health related risk assessments and cost/benefit analyses. Other sources of gas are also being extracted using hydraulic fracturing, such as tight gas (from sandstone or limestone formations) or coal bed methane. Pressures, volumes and characteristics of the fracturing fluids and wastewaters (flowback and produced waters) differ with the geology and the type of gas extracted (King, 2012; Annevelink et al., 2016), nonetheless procedures and activities are comparable (Orem et al., 2014; Tang et al., 2014; Caineng et al., 2015).

Fracturing fluids are roughly made up of 90% water, 9% proppants and 1% chemical additives (Vidic et al., 2013). The additives used include biocides, surfactants, gelling agents and friction reducers among others and are described in greater detail by Vidic et al. (2013) and Faber et al. (2018). Fracturing fluids and wastewaters, including flowback and produced waters, can be highly contaminated by organic and inorganic compounds, salts, acids and possible radioactive material (Stringfellow et al., 2014; Ferrer and Thurman, 2015; Reagan et al., 2015; Butkovskiy et al., 2017). The wastewaters can be stored in large reservoirs, re-used for a new fracturing, injected into the subsoil for disposal, and/or treated by water treatment plants before re-entering the environment (Lutz et al., 2013; Warner et al., 2013; Hladik et al., 2014; Vengosh et al., 2014; Liden et al., 2018). Possible spills or leaks during the different process steps may cause environmental pollution (Gross et al., 2013; Swarthout et al., 2015; Maloney et al., 2017; Patterson et al., 2017), so potential adverse effects of the chemicals involved are relevant (Rahm and Riha, 2012). Most current research on hydraulic fracturing related waters focuses on shale gas in the U.S., but seldom on other types of hydraulic fracturing and are rarely undertaken in European countries (Faber et al., 2018; Santos et al., 2018). However, for hydraulic fracturing related to tight gas or coal bed methane, comparable environmental risks may be at stake as for shale gas. Furthermore, the entire hydraulic fracturing related water cycle including fracturing fluid, flowback and produced waters and surrounding waters is generally not taken into account in the currently available studies, as most studies consider one or two matrices, i.e. surface water and/or groundwater, flowback water or fracturing fluid (Faber et al., 2018). Most studies also focus on target analysis which does not necessarily account for transformation or breakdown products. In addition a lot of the chemicals used in fracturing fluids are proprietary, which would not be detected with target analysis. Reviews of analytical approaches to hydraulic fracturing fluid and flowback samples have been published after this study was undertaken (Oetjen et al., 2017; Santos et al., 2018) and highlight the need for non-targeted approaches to analyse the organic component but do not mention the use of bioassays.

Here, an analysis of fracturing fluids, flowback water and groundwater samples related to tight gas hydraulic fracturing is presented, to yield information on possible chemical exposures within the Dutch context. Due to the large number of compounds involved, we present an *in vitro* bioassay approach providing insight into possible

environmental and human health effects combined with a non-target high resolution mass spectrometry analysis and suspect screening (Brack et al., 2016; Sjerps et al., 2016; Hollender et al., 2017). We focus on polar organic compounds due to their low removal efficiencies during wastewater treatment (Westerhoff et al., 2005; Reemtsma et al., 2016).

Similar approaches have been undertaken by Tang et al. (2014), He et al. (2017), Crosby et al. (2018) and Kassotis et al. (2018) where either groundwater or fracturing fluid and flowback waters related to unconventional oil and gas activities have been assessed chemically and bioanalytically. Tang et al. (2014) and Crosby et al. (2018) assessed toxicological end-points that are comparable to this study and He et al. (2017) undertook a non-targeted HRMS approach similar to the one performed in this study. Oetjen et al. (2018) and Piotrowski et al. (2018) also applied a non-target approach to organically characterize fracturing fluid and/or flowback samples. However the latter focuses on volatile organic compounds, which is not the main focus in the present study.

2. Materials and methods

Fracturing fluids, produced waters and groundwaters, obtained in January 2016 from a Dutch tight gas hydraulic fracturing location, were analysed for their chemical composition and toxicological effects. Using both direct injection and concentrated extracted samples, the chemical composition was analysed using liquid chromatography-high resolution mass spectrometry (LC-HRMS) and suspect screening. Different *in vitro* bioassays were performed to detect possible adverse health effects of the chemical mixture present in the water samples. First, the Ames fluctuation test was carried out, which uses bacteria to determine mutagenic effects, with and without the addition of liver enzymes to detect substances which need metabolic activation before they become mutagenic as well (Mortelmans and Zeiger, 2000; Reifferscheid et al., 2012). Second, the chemical-activated luciferase gene expression (CALUX) assay was performed to determine three endpoints: genotoxicity response (p53-dependent pathway activation), oxidative stress response (activation of the Nrf2 pathway) and dioxin receptor activation, which is an indicator of polycyclic aromatic hydrocarbon (PAH) contamination (Murk et al., 1996).

2.1. Site, sample collection and sample preparation

The Dutch tight gas producing site from which water samples were taken has been producing gas since 1995 and the well has been fractured twice recently. The samples were taken before, during and after the second fracture. The previous fracture may have influenced the results of the flowback water samples. Chemicals from the first fracturing event might have remained in the formation and may be present in the flowback samples. This could affect the number of peaks and response intensities for the chemical analyses and the responses for the bioassay assessments. The well was drilled in an S-shape: starting off vertically followed by an increasing deviation up to roughly 50 degrees then back to vertical to a depth of approximately 3.7 km. The fracturing was applied to a sandstone formation belonging to the carboniferous formation that is roughly located between 3000 and 4500 m below surface. The topmost formation extending to roughly 120 m below the surface corresponds to the Upper North Sea Group of the Neogene and Quaternary systems (DGM diep v4.0; Dinoloket, n.d.), which is made up of a succession of sandy layers and two clay layers that are located

at roughly –15 and –100 m below the surface (DGM v.2.2, REGIS II v.2.2; Dinoloket, 2019). The soil type at the extraction site is sandy. Here we studied in-depth the fracturing fluids, flowback waters and groundwater related to a fracturing event at a single location.

Samples and controls (1.5 L) were taken in clean plastic bottles, frozen and transported to the laboratory. The samples consisted of the two different fracturing fluids which were injected separately at the start of the hydraulic fracturing process, seven flowback water samples, taken at day 1 to 8 (excluding day 4) after the hydraulic fracturing started, and 8 groundwater samples taken at a depth of 10 m, at 2 and 20 m distance from the borehole, on two separate dates before and after the hydraulic fracturing process (Fig. 1). The samples were immediately frozen after sampling and were defrosted shortly before analysis. Each sample type (fracturing fluid, flowback water and groundwater samples) was delivered in different types of plastic bottles. A control was prepared for each sample type by adding Evian water to the same type of plastic bottles and storing them in the same manner than the samples. The aqueous phase of every sample was analysed. The fracturing fluids and flowback samples were all analysed by direct injection (without concentration or dilution). The groundwater samples, the first fracturing fluid phase (FF1), and three flowback samples (FWD1, FWD6 and FWD7) were concentrated using solid phase extraction. The rest of the fracturing and flowback samples could not be concentrated due to the limited volume of the aqueous phase that could be extracted from these highly viscous samples.

For sample preparation of fracturing fluid and flowback samples, the top oil and gel layer was removed using a 20 mL plastic disposable syringe. The remaining sample was centrifuged in 250 mL polypropylene centrifuge tubes for 10 min under 3000 g, subsequently the remaining top oil and formed pellet were removed. The remaining aqueous phase was filtered using low vacuum and a 500 mL rapid flow filter unit over a 0.2 µm aPES membrane with a 90 mm diameter (ThermoScientific – Nalgene) covered by a 1 µm cellulose filter. Groundwater samples were filtered using ignited sea sand. The sea sand was conditioned twice with 20% (v/v) methanol (99.9%) in acetonitrile (99.9%), and then three times with Evian water at pH 2.3. Flowback samples and fracturing fluids were concentrated up to a factor of 5000, groundwater samples were concentrated up to a factor of 7500. 200 mg OASIS® HLB 5 cc LP glass cartridges were used, conditioned twice with 20% (v/v) methanol (99.9%) in acetonitrile (99.9%), subsequently conditioned once with methanol (99.9%), and finally conditioned three times with Evian water pH 2.3. Samples were acidified to pH 2.3 with 15% ultrapure HCL. The acidified samples of 1.5 L each were connected to the inlet of the SPE cartridge and run over the SPE column at 10 mL/min under low vacuum. Then the samples were eluted three times with 2.5 mL 20% (v/v) methanol (99.9%) in acetonitrile (99.9%). The 7.5 mL eluate was collected in test tubes with screw cap and Teflon inlay and stored at –18 °C. Before analysis the samples

were allowed to evaporate at a temperature of 60 °C to 0.5 mL, after which 0.5 mL methanol (99.9%) was added and the samples were further evaporated to 100 µL under 60 °C. Then 100 µL DMSO was added and the sample was evaporated for 10 min. DMSO was added to reach a final volume of 200 µL. Procedure blanks, prepared using Evian, were subjected to all of the previously mentioned processes relevant for their samples, except for the centrifugation. They were also stored in the freezer in the same bottles that the samples were taken. For both the chemical analysis and the bioassays, the samples were put in transparent glass vials of 1.5 mL. These vials were cleaned with a solution of 10% (v/v) of a 40% (m/m) sodium hydroxide (97%) solution in ultrapure water diluted in absolute ethanol (99.9%). The vials were then rinsed with hot water and a solution of 1% (v/v) hydrochloric acid (37–38%) in ultrapure water before being rinsed again with ultrapure water, acetone (99.7%) and petroleum ether (ultrapure grade), and left to dry at room temperature.

2.2. Chemical analysis using LC-HRMS

The resulting sample extracts were analysed using Liquid Chromatography coupled to a linear ion trap (LTQ) Orbitrap high resolution mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), in positive and negative ionisation mode (Sjerps et al., 2016). The liquid chromatography setup includes an Accela UHPLC system and autosampler, a 150 mm × 2.1 mm i.d. Xbridge C18-column with 3.5 µm particles and a 4.0 mm × 2.0 mm i.d. C18 Guard column (Phenomenex). The columns were kept at a temperature of 21 °C and the injection volume was 20 µL. The analytes were separated using a linear gradient at a flow rate of 0.3 mL/min: starting at 95% water, 5% acetonitrile, 0.05% formic acid (v/v/v), increased to 100% acetonitrile with 0.05% formic acid at 40 min. Between consecutive runs, the analytical column was re-equilibrated for 5 min. Detection limits for compounds were set between 115 and 1300 g/mol in order to avoid as much background noise as possible whilst still covering a large mass range. Product ions measured by the Orbitrap were generated in the LTQ trap at a normalized collision energy setting of 35%, using an isolation width of 2 Da. Electrospray ionisation (ESI) source conditions were; capillary voltage 3.0 kV, heated capillary temperature 350 °C, capillary voltage 24 V, tube lens 70 V. In order to estimate the semi-quantitative concentrations of the detected masses, an internal standard (IS) containing atrazine-d₅ (99.8%; J.H. Ritmeester B.V., Nieuwegein, The Netherlands) for positive ionisation mode and bentazon-d₆ (98.5%; Dr. Ehrenstorfer GmbH, Augsburg, Germany) for negative ionisation mode was used. These internal standards were selected due to the stable ionisation response in various sample matrices. An IS equivalent (IS eq) concentration does not represent the actual concentration of a given compound and may vary between 2 and 4 orders of magnitude from the actual concentration depending on the specific compound (Sjerps et al., 2016).

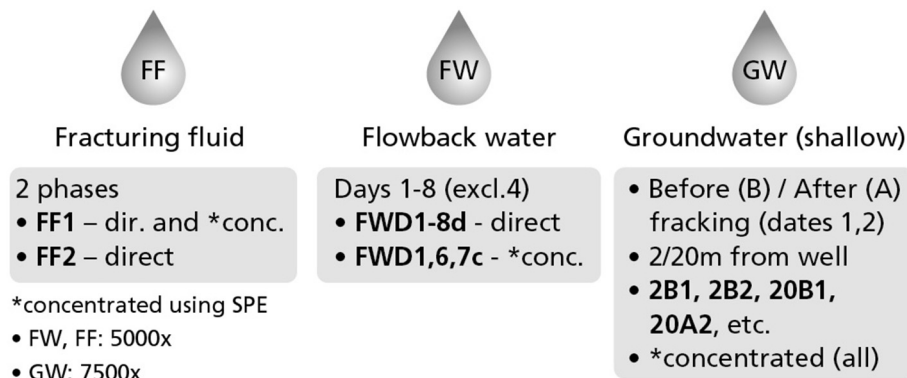


Fig. 1. Overview of analysed samples: two separate fracturing fluid (FF) phases FF1 and FF2, seven flowback (FW) samples from day 1 to day 8 after hydraulic fracturing occurred FWD1–8 (excluding day 4), and eight groundwater (GR) samples before (B) and after (A) hydraulic fracturing took place, 2 and 20 m from the well. SPE = Solid Phase Extraction.

Interpretation of the raw data was performed using Sieve 2.2 for peak integration in combination with Xcalibur Software version 2.1 (Thermo Fisher Scientific, Breda, The Netherlands) for molecular formula identification. For peak integration the following parameters were used: a threshold peak of 250,000 and an MZ tolerance of 5 ppm. Peaks with an absolute threshold of at least 100,000 counts and with at least a factor 5 difference with the blank response were considered. In addition, the detected masses were compared to the accurate masses of chemicals on the suspect list relevant for UO&G related waters (Faber et al., 2018) using the Compound Discoverer 2.0 software (Thermo Fischer Scientific, San Jose, CA) with a mass range of \pm 5 ppm. Confidence levels () regarding the identification of compounds was reported according to Schymanski et al. (2014). Level 5 is of low confidence and level 1 is of high confidence. The identification to higher levels of confidence was performed on suspects and peaks with the highest concentration sums over all the samples. Identification to confidence levels 3 or 2 were only possible if MS2 data was available. Metfrag (Ruttkies et al., 2016; Metfrag, n.d.) and CSI fingerID (Dührkop et al., 2015; CSI FingerID, n.d.) was used for MS2 in silico fragmentation to further identify the peaks. The following parameters were used for Metfrag: Chemspider database, 5 ppm m/z deviation, molecular formula (if possible), Mzppm of 5, Mzabs of 0.001 and a tree depth of 2, MONA spectral similarity (50%), MONA exact spectral similarity (100%) and Chemspider reference count (50%). If available, experimental MS2 spectrums were compared to theoretical MS2 spectrums from mzCloud (mzCloud, n.d.) and massbank (Horai et al., 2008; Massbank, n.d.) libraries. The retention time of the identified peaks were compared to logP or logKow values of the candidates in order to further validate the identifications. A high logP or logKow is indicative of a longer retention time and vice versa (Bade et al., 2015). This was only used as an additional verification step for identification and was only applied to non-ionisable candidates. n-octanol water partition coefficients (KOWWIN™ v.168; US EPA a, 2019), volatilisation half-lives (WVOLWIN™; US EPA a, 2019), wastewater treatment removal (STPWIN™; US EPA a, 2019), reference doses and weight of evidence for cancer (EPA-IRIS; US EPA b, 2019) data was gathered for all of the peaks that were successfully identified to a level 3 or 2 confidence.

The used suspect list consists of fracturing fluid additives from the US fracfocus database (US Fracfocus, n.d.), the Polish and Dutch registries, and additional chemical compounds based on reviewed literature mobilised from the subsurface. The list and background of it can be found in Faber et al. (2018). Mixtures, inorganic chemicals, metalloids and non-ionisable chemicals were excluded. Only compounds with at least one heteroatom were considered ionisable with ESI (e.g. N, S, O, and P) and only compounds with a molecular weight between 115 and 1300 g/mol are targeted. The suspect list consists of 1386 chemicals that might be expected in oil and gas related water samples, of which 338 suspects are expected to be detected with our analytical LC-HRMS method. The analysable suspect list is made up of 64% fracturing fluid additives and 36% subsurface contaminants.

In order to relate the results of the chemical analyses to possible risks for drinking water, the internal standard equivalent concentrations in groundwater destined for the use of drinking water were compared to the generic threshold of toxicological concern (TTC) of 0.1 µg/L for chemicals that are not genotoxic in drinking water (Schriks et al., 2010; Mons et al., 2013). This concentration is also used as a signalling value for anthropogenic substances in drinking water in the Netherlands (Dutch Ministry of Infrastructure and Water, 2009) and as a target value in surface water according to the Memorandum regarding the protection of European rivers and watercourses for the protection of drinking water (RIWA, n.d.). Drinking water concentrations below the TTC values are considered to be of negligible human health risk. The screening-level TTC values have been introduced due to the lack of relevant toxicological data for many organic compounds and is based on toxicological data from a representative number of organic compounds (Baken et al., 2018).

2.3. Bioassay testing

The sample extracts were tested in a selection of in vitro bioassays. The Ames-fluctuation test, which uses genetically modified *Salmonella typhimurium* bacteria to investigate whether a given sample can cause DNA mutations, was used to determine potential genotoxic effects (Heringa et al., 2011; Reifferscheid et al., 2012). The Ames fluctuation test was performed as reported previously (Heringa et al., 2011) with minor modifications, using strain TA98 for the detection of frame-shift mutations and TA100, which is sensitive to base-pair substitution, instead of TAmix. All samples and procedure controls were tested in triplicate with and without S9 enzyme mix, as well as a solvent control (DMSO) and the following positive controls: 20 µg/mL of 4-nitroquinoline N-oxide (4-NQO) in DMSO and 500 µg/mL of 4-nitro-*o*-phenylenediamine (4-NOPD) in DMSO for TA98-S9, 5 µg/L of 2-aminoanthracene (2-AA) in DMSO for TA98 + S9, 12.5 µg/mL of nitrofurantoin (NF) in DMSO for TA100-S9, and 20 µg/mL of 2-AA in DMSO for TA100 + S9. Ames fluctuation test bacterial strains, culture media, and S9 liver enzymes from phenobarbital/β-naphthoflavone-exposed rats were purchased from Xenometrix GmbH (Allschwil, Switzerland). Histidine, nutrient broth no. 2 oxioid, 2-AA, MgCl₂·6H₂O, NaH₂PO₄·H₂O, and Na₂HPO₄·2H₂O were obtained in analytical grade from Boom (Meppel, the Netherlands). NaCl and KCl were purchased from Avantor Performance Materials B.V. (Deventer, the Netherlands). 4-NOPD, 4-NQO, NF, D-glucose-6-phosphate, nicotinamide adenine dinucleotide phosphate, and ampicillin were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). The 24- and 96-well plates were obtained from Greiner Bio-one (Alphen a/d/Rijn, the Netherlands) and the Corning 384-well plates from Sigma-Aldrich. Results are expressed as the number of cell culture wells in which the pH indicator in the culture medium has turned yellow. The average of the triplicate solvent control should show ≤10 yellow wells and for the positive controls ≥25 yellow wells need to be counted for the test to be valid. The Ames fluctuation test gives a binomial response, therefore a χ^2 -test with $p < 0.05$ was performed to determine if the response significantly differs from the solvent control. When mutagenicity is detected in the procedure control, statistical significance can also be calculated compared to the procedure control in order to prevent false positive results caused by contamination introduced during sample preparation. When a sample shows a statistically significant response in at least one of the test conditions (TA 98 or TA100 \pm S9), the sample is considered to be mutagenic. Samples that test negative for genotoxicity but show cytotoxicity may be false negatives.

The CALUX test makes use of modified mammalian cell lines to investigate different toxicological end points. In this study the P53 CALUX® (with and without metabolic activation), Nrf2 CALUX® and PAH CALUX® were performed by Biodetection Systems in Amsterdam, the Netherlands (Pieterse et al., 2013; van der Linden et al., 2014; BDS, n.d.). A first selection of five CALUX assays was performed on one representative sample of each water matrix and their procedure blank in order to determine the most suitable end-points for these types of samples. An end-point was considered suitable when a positive response for one or more of the test samples was obtained. Anti-androgenic activity and estrogenic response were not considered as end-points for further testing. DMSO was used as a solvent control for all of the selected assays. For the positive controls actinomycin D, cyclophosphamide, curcumin and benzo[*a*]pyrene were used for the P53 + S9, the P53-S9, the Nrf2 and the PAH assays respectively. The results from the CALUX assays can either be positive or below the limit of quantification (LOQ) and were corrected for the different concentration factors. It can be assumed that for the samples where the response falls below the LOQ value, no activation on the specific pathway has occurred and the response can be interpreted as negative. The LOQ is based on the induction ratio relative to the reference chemical used for each assay, the volume of material processed and the percentage of DMSO used in each CALUX assay. The differences in LOQ values are due to the differences in volumes

and concentration factors used for the samples. Samples that test positive but for which the corresponding procedure blanks showed a positive response may be false positives. Furthermore, PAHs cannot be detected with the chemical analytical method used in this study. Therefore PAH CALUX® was included in order to account for these compounds.

3. Results and discussion

3.1. Chemical analysis using LC-HRMS

An overview of the total IS-eq concentrations and the number of detected peaks in fracturing fluid, flowback and surrounding groundwater is given in Fig. 2. A detailed overview of the non-target results including detected peaks, retention time and corresponding concentrations for all the samples in positive and negative ionisation can be found in tables A1 and A2. Table A3 provides an overview of the loss and gain of detected peaks after sample concentration. Fig. 3 provides a fingerprint of the detected peaks measured at concentrations below and above the TTC value of 0.1 µg/L IS-eq. The corresponding numerical results for the fracturing fluid and flowback samples can be found in Table 2.

The number of detected peaks does not necessarily represent the number of compounds present in these samples, but may include fragments or adducts other than hydrogen, for example sodium or ammonium adducts. Unstable chemicals may be prone to in-source fragmentation, which generally does not affect the chemical response since the fragmentation pattern for a specific compound remains the same throughout the analysis. More peaks have been detected in positive ionisation than in negative ionisation mode (Figs. 2, 3 and Table 2). Roughly 75% and 25% of the detected peaks in positive ionisation mode relate to fracturing additive and subsurface contaminant peaks respectively, whereas in positive ionisation mode only slightly more fracturing additive peaks are detected than subsurface contaminant peaks (Fig. 3 and Table 2). A significant decline in additive

peaks is observed in the fracturing fluid samples after day 1 in positive ionisation (Table 2). After concentration a much higher number of peaks can be found for both fracturing fluid and flowback samples, eg. for the concentrated fracturing fluid compared to the directly injected sample 2322 vs. 88 (positive ionisation mode) or 1468 vs. 39 (negative ionisation mode). This could also be due to the matrix effect and the high salt content of the flowback samples. Very few peaks are lost during the concentration procedure (8–23) while a high number of peaks is gained (1269–2251) (table A3). Both the loss and the gain primarily involve compounds with a low molecular weight (MW 100–400), which include the more polar and mobile chemicals that are relevant in the context of drinking water production (Reemtsma et al., 2016). The direct fracturing fluid and flowback samples and groundwater samples have similar number of detected peaks.

Most of the peaks found in the fracturing fluid and the flowback water samples are detected at concentrations higher than the TTC value of 0.1 µg/L IS-eq (Fig. 3 and Table 2). The highest total IS-eq concentrations are found for the fracturing fluid and flowback samples. In the first 8 days after the hydraulic fracture occurred, no significant decline in concentrations or detected peaks is observed in the flowback samples. For the directly injected fracturing fluids, total concentrations of 6.1 to 217 or 2.8 to 46 mg/L IS-eq are found for positive or negative ionisation modes respectively. For the direct flowback samples, total concentrations are in the same range as the fracturing fluids with 1.6 to 372 and 0.23 to 29 mg/L IS-eq for positive and negative ionisation respectively. The directly injected flowback sample of day 1 has the highest total IS-eq concentration. The corresponding concentrated sample has a lower total concentration but a higher number of detected peaks compared to the directly injected sample; the same observation is made for flowback samples taken later. In the concentrated groundwater samples, total concentration ranges are multiple orders of magnitude lower with 9 to 159 and 15 to 63 µg/L IS-eq for positive and negative ionisation respectively. No clear differences are found between groundwater samples before and after the current hydraulic fracturing

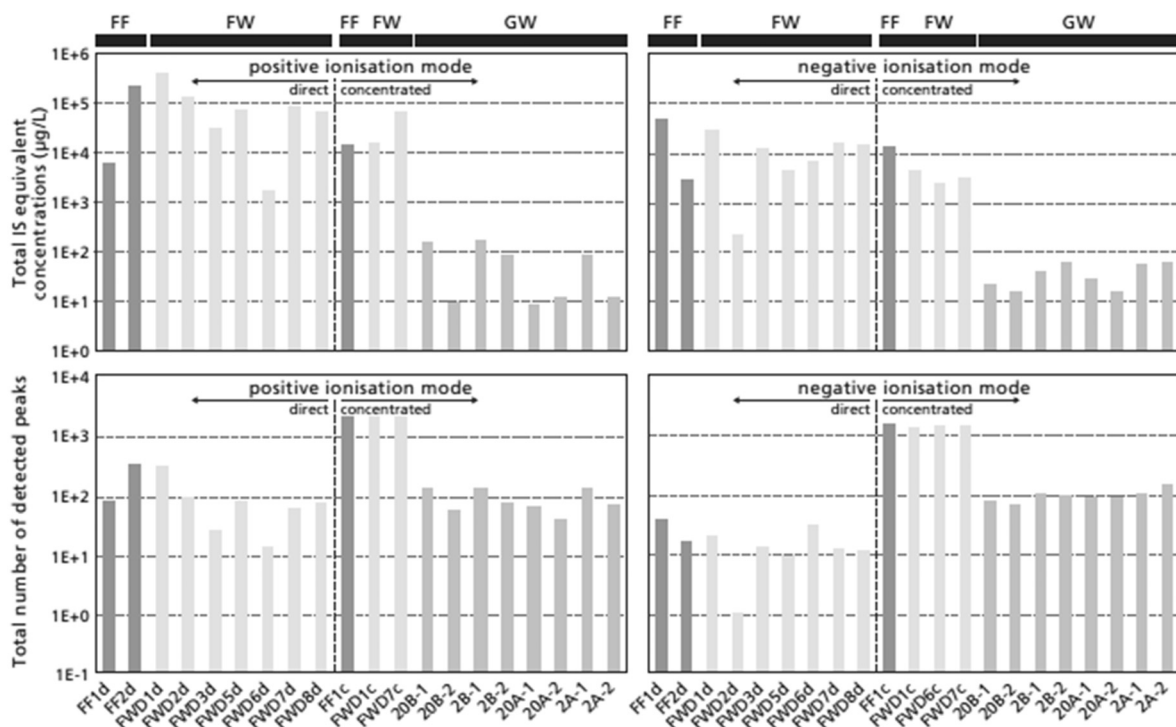


Fig. 2. : (top): Total IS equivalent concentrations in positive ionisation mode (left) and negative ionisation mode (right) given in atrazine- d_5 (positive) equivalent and in bentazone- d_6 (negative) equivalent concentrations (µg/L) for the fracturing fluid, flowback and groundwater samples. (bottom): Number of peaks detected in positive ionisation mode (left) and negative ionisation mode (right) for the fracturing fluid, flowback and groundwater samples. FF=Fracturing Fluid (dark grey), 2 phases; FW=Flowback Water (light grey), days D1–8 (excluding 4). GW = Groundwater (medium grey), taken 2 m (2) and 20 m (20) from the well, before (B) and after (A) hydraulic fracturing took place on 2 separate dates (1, 2).

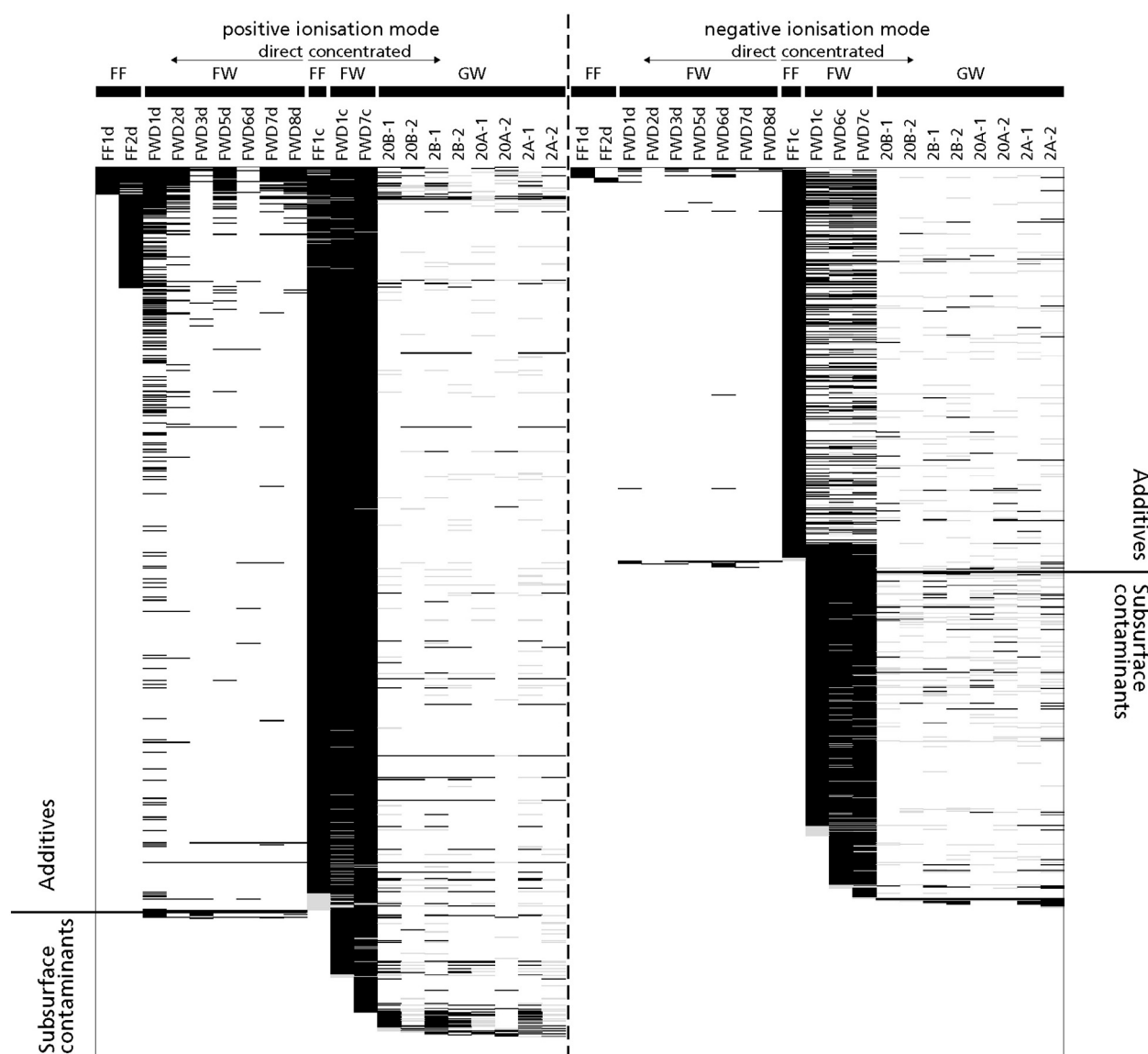


Fig. 3. Fingerprint of detected peaks for all fracturing fluid, flowback and groundwater samples in positive (left) and negative (right) ionisation mode. FF=Fracturing Fluid, 2 phases; FW=Flowback Water, days D1–8 (excluding 4); GW = Groundwater, 2 and 20 m from the well taken before (B) and after (A) the hydraulic fracture, on 2 separate dates (1, 2); c = concentrated sample; d = direct injected sample. Concentration ranges: grey <0.1 µg/L IS-eq, black ≥0.1 µg/L IS-eq.

at this site. It was noted that hydraulic fracturing also took place before the currently studied event, so it cannot be ruled out that this may have influenced the current study.

The analyses of the directly injected and the concentrated samples in both positive and negative ionisation are necessary in providing a complete overview of polar organic compounds in hydraulic fracturing related waters. However, the fracturing fluid and flowback samples have a high matrix load, hampering identification of peaks and limiting their confidence levels. Analyzing diluted samples would minimize the number of detected peaks but would also reduce the observed matrix effects.

The 20 peaks with the highest concentration sums over all the samples were tentatively identified. These are relevant because they are either present in very high concentrations and/or are present in many of the samples. However, most of these peaks could only be identified to level 5 confidence level (Schymanski et al., 2014) due to inconclusive MS1 data or level 4 confidence due to a lack or inconclusive MS2 data. A total of 5 peaks could be identified to a level 3 or 2 confidence in positive ionisation (Table 1). Several chemicals can be candidates for an

identified peak. Five out of the sixteen candidates are polyethylene glycols, of which four are present in high amounts. High responses for polyethylene glycols have also been found by He et al. (2017) and by Oetjen et al. (2018) in flowback and produced water samples from Canada and the US, respectively. Diethylene glycol n-butyl ether (112-34-5) was found in the fracturing fluid and flowback samples and is used as a surfactant. It is also included in the used suspect list and is therefore relevant to the hydraulic fracturing process. Bis(2-ethylhexyl) adipate (103-23-1), dioctyl adipate (123-79-5), pentaethylene glycol (4792-15-8), hexaethylene glycol (2615-15-8) and heptaethylene glycol (5617-32-3) were all found in the fracturing fluid, flowback and groundwater samples. These candidates may therefore have been used as additives in the fracturing fluids. Both bis(2-ethylhexyl) adipate and dioctyl adipate are two possible candidates for one peak which is present in the order of 1 IS eq µg/L in the groundwater samples. These candidates are both used as plasticizers (Musser 2005). Pentaethylene glycol, hexaethylene glycol and heptaethylene glycol were present in low amounts in the order of 0.01–0.1 IS eq µg/L in the groundwater samples. Pentaethylene glycol is used in brake fluids and as an aid in cement

Table 1
Identified peaks with 3 or 2 confidence level in positive and negative ionisation mode and their logKow, volatilisation half-life, wastewater treatment removal percentage, reference dose and weight of evidence for cancer. Ref# refers to the number given to this peak in appendix B. The presence in fracturing fluid (FF), flowback water (FW) and groundwater (GW) samples provides the number of samples in which the peak has been detected. Top 20ΣC refers to the peaks that are among the 20 highest concentration sums.

Ionisation mode	Ref#	Compound (CAS#)	Molecular formula	Confidence level	Measured MZ neutral (g/mol)	Corrected MZ neutral (g/mol)	Reason for correction	Retention time (min)	Suspect	Presence in samples			Top 20 ΣC	LogKow (KOWWIN™ v.168)	Volatilisation half-lives (WVOWIN™)		Removal WWTP % (STPWINTM)	Reference Dose (IRIS) mg/kg bw/day	Weight of Evidence for Cancer (IRIS)
										FF	FW	GW			River (hrs)	Lake (hrs)			
Positive	1	Bis(2-ethylhexyl) adipate (103-23-1)	C22H42O4	3	392.29014	370.30849	[M + Na] +	42.96444	No	3	7	4	Yes	8.12	2.60E+03	2.85E+04	94.02	6.00E-01	C
	2	Diethylene glycol n-butyl ether (112-34-5)	C8H18O3	3	184.10724	162.12569	[M + Na] +	10.14951	Yes	2	7	0	Yes	8.26 0.29	2.60E+03 1.04E+05	2.85E+04 1.13E+06	94.02 1.86		
	3	Pentaethylene glycol monodecyl ether (3055-95-6)	C20H42O6	3	378.29838			25.55277	No	2	3	0	No	3.40	1.71E+04	1.87E+15	10.95		
	4	Pentaethylene glycol (4792-15-8)	C10H22O6	2	260.12327	238.14162	[M + Na] +	4.88227	No	2	7	7	Yes	-2.30	1.19E+11	1.29E+12	1.85		
	5	Hexaethylene glycol (2615-15-8)	C12H26O7	2	304.14955	282.16789	[M + Na] +	5.69744	No	2	7	8	Yes	-2.57	8.34E+12	9.10E+13	1.85		
	6	Heptaethylene glycol (5617-32-3)	C14H30O8	2	348.17561	326.19428	[M + Na] +	6.33562	No	2	5	8	Yes	-2.85	5.75E+14	6.27E+15	1.85		
Negative	7	Oleic acid (112-80-1)	C18H34O2	2	282.25572			30.65627	Yes	1	2	0	No	7.73	5.24E+01	7.13E+02	93.99		
	8	Monoisobutyl phthalic acid (30833-53-5)	C12H14O4	3	222.08902			8.84134	No	0	2	1	No	2.77	1.75E+06	1.91E+07	4.15		
		Diethyl phthalate (84-66-2)							Yes					2.65	1.43E+03	1.58E+04	2.92	8.00E-01	D
	9	2-Dodecylbenzenesulfonic acid (27176-87-0)	C18H30O3S	3	326.19084			32.59177	Yes	0	3	4	No	4.78	1.69E+04	1.84E+05	69.53		
		4-Dodecylbenzenesulfonic acid (121-65-3)							No					4.78	1.69E+04	1.84E+05	69.53		
	10	Adipic acid (124-04-9) 2- and 3-Methylglutaric acid (617-62-9/626-51-7)	C6H10O4	3	146.05788			6.21017	Yes No	0	3	0	No	0.23 0.15/0.15	1.50E+08 5.66E +08/7.27E +08	1.64E+09 6.18E +09/7.93E +09	1.85 1.85/1.85		
	11	2,2-Dimethylsuccinic acid (597-43-3) Dibutyl phthalate (84-74-2)	C16H22O4	2	278.15138			27.04687	No Yes	0	3	0	No	0.08 4.61	3.65E+08 5.41E+02	3.98E+09 6.05E+03	1.85	1.00E-01	D

grinding (DOW 2009). Hexaethylene glycol is used as an antioxidant and antimicrobial agent (Chemical Book 2019). No uses could be found for heptaethylene glycol.

A comparison of the peaks found with a suspect list, made specifically for oil and gas related hydraulic fracturing activities (Faber et al., 2018), resulted in 25 out of a total of 2814 detected peaks and 36 out of a total of 2803 detected peaks matches in positive and negative ionisation respectively with one of the 338 possible suspect candidates on the list. The total number of detected peaks relates to the fracturing fluid, flowback and groundwater samples. Two suspects could be identified in positive ionisation and four suspects in negative ionisation with a confidence level of 3 or 2 (Table 1). The evidence of the identification including experimental and theoretical mass spectra can be found in appendix Appendix B. Diethylene glycol n-butyl ether has been previously discussed. Oleic acid (112-80-1), 2-dodecyl benzene sulfonic acid (27176-87-0) and adipic acid (124-04-9) are all used as corrosion inhibitors. Oleic acid was found in the fracturing fluid and flowback samples, as expected. However, the other two corrosion inhibitors were only found in the flowback samples and 2-dodecyl benzene sulfonic acid was also found in low amounts in the groundwater samples (order of 0.1 IS eq µg/L). These chemicals may have been used as fracturing fluid additives during the first hydraulic fracture and remained in the subsurface formation during well production until the second fracture took place. Diethyl phthalate (84-66-2) and dibutyl phthalate (84-74-2) are subsurface contaminants and both were found in the flowback samples and diethyl phthalate was also found in one groundwater sample taken after the hydraulic fracture in very low concentrations (4.02E-2 IS eq µg/L). Some of the tentatively identified chemicals are present in the groundwater samples but generally in low amounts. The related groundwater contaminations cannot be directly linked the hydraulic fracture and may be due to surrounding activities from agriculture and/or industry.

Except for diethylene glycol n-butyl ether, the tentatively identified top 20 summed concentration candidates are not on the hydraulic fracturing related suspect list. The suspect list is mainly based on chemical additives used for extraction of shale gas and chemicals originating from shale gas formations within the U.S. The samples in the current study are from Dutch tight sand gas formation sites. Considering the general uses of the candidates, they may be used for hydraulic fracturing purposes in the European context. Very few databases for the European situation are currently available (Faber et al., 2018). Both variations in geological conditions and in chemical legislation may however result in differences in chemical use within the European context compared to the U.S., possibly explaining the relatively low number of matches with the suspect list. Another reason could be that many chemicals are transformed during the hydraulic fracturing process and possible transformation products are currently not included in the suspect list. Analysing and identifying European hydraulic fracturing related samples and including all known transformation products may provide better insight into the chemicals used within the European framework. This would also render the suspect list more relevant for European samples and would facilitate identification of compounds during analysis. Eleven of the seventeen candidates have n-octanol water partition coefficients below 4.50 with relatively low molecular weights. These candidates are relevant for the water sector due to their relatively high solubility and mobility in water (Westerhoff et al., 2005). Volatilisation half-lives range from roughly 2 days to >7 billion years. Many of the compounds have relatively high persistence in water (i.e. longer than 10 years). Moreover, removal percentages during wastewater treatment are generally low (i.e. 1–10%), with only four candidates with a removal efficiency of >90% and three candidates with a medium removal capacity (50–70%). The persistence in water may be problematic to human and environmental health depending on the toxicity. However, due to the limited number of peaks identified to level 2 or 3 confidence, this preliminary environmental fate data is not representative of the samples in their entirety. Reference doses and weight of evidence for cancer

was only available for three of the candidates. The gathered reference doses are relatively low (0.1–0.8 mg/kg bw/day), and there is no evidence of carcinogenicity in humans. Due to the limited toxicity data, it is difficult to conclude on the toxicity of the tentatively identified peaks.

3.2. Ames fluctuation test and CALUX assay

Table 2 provides an overview of the chemical analyses and the bioassay testing results. The results of the chemical analyses focus on the fracturing additive and the subsurface contaminant peaks in the fracturing fluid and the flowback water samples. The peaks that were found in the groundwater samples cannot be directly related to the fracturing additives and subsurface contaminants related to the hydraulic fracturing activity and are therefore not shown in this overview. The graphs corresponding to the Ames fluctuation test and the CALUX test results for all samples can be found in appendix C. Results that were deemed untrustworthy due to cytotoxicity, which indicates a possible false negative or due to a positive response in the control sample, which indicates a possible false positive are shown in grey. Looking at the AMES test results for TA98–S9, small responses are obtained for fracturing fluid 1 and the flowback sample from day 2 compared to their respective procedure blanks. None of the samples showed a positive response for TA98 + S9. The groundwater samples 2A-2 and 20A-2 show clear genotoxic responses for TA100-S9 and TA100 + S9 respectively. These samples were taken at 2 and 20 m distance from the well after the (second) hydraulic fracturing event had taken place. However, it is not possible to designate the hydraulic fracture as a cause of the groundwater contamination. Surrounding activities, such as agriculture, may have contributed to these results. All concentrated fracturing fluid and flowback samples (FF1c, FWD1c, FWD6c and FWD7c) tested positive for cytotoxicity. This indicates that the concentrated samples hampered cell growth and test results for these four samples could be false negatives. Payne et al. (2015) found cytotoxic responses for fracturing fluid samples related to coal seam gas activities in Australia, which correlates to the cytotoxic activity found in the concentrated fracturing fluid sample FF1c. A lower concentration factor and/or a further dilution of the original fracturing fluid and flowback samples would improve these results. Furthermore, duplicate testing would indicate which responses are reproducible, providing more trust in the significance of the observed effects.

Regarding the CALUX test results for p53 pathway activation without metabolic activation of S9 liver enzymes, the responses for all samples fall below the limit of quantification, so the response is considered negative. For genotoxicity with metabolic activation of S9 liver enzymes, the results show clear positive responses for the direct fracturing fluids, for the direct flowback sample of day 6, and for the three concentrated flowback samples. There are no clear positive responses for the PAH tests. For oxidative stress, only the three concentrated flowback samples showed clear positive responses. Similar results were found by Tang et al. (2014) for groundwater samples related to coal seam gas activities, where low levels of PAH and no genotoxic responses were detected. Moreover, Crosby et al. (2018) also found positive responses for cytotoxicity and oxidative stress responses in fracturing fluid and flowback samples. Although the bioassay results pose some challenges as to their significance due to the presence of possible false negatives and false positives, there are clear positive responses for genotoxicity and/or oxidative stress for the fracturing fluid, flowback and some groundwater samples.

The Ames tests and the P53 CALUX tests both evaluate genotoxicity, however they assess different molecular events (DNA mutations vs. cellular response to genotoxicity, respectively). The in vitro bioassay results do not necessarily mean that the samples showing positive responses pose a risk to environmental or human health, but may point to the presence of compounds in these samples at a certain concentration directing adverse effects. Chemical identification, determination of exact concentrations, and thorough toxicological evaluation of

Table 2
(left) Number of peaks related to fracturing additives (add) and subsurface contaminants (subs) detected in the fracturing fluid and flowback samples including the number of peaks detected at a concentration higher than TTC = 0.1 µg/L; (right) Results for the Ames fluctuation test (TA98 and TA100 +/-S9), P53 CALUX +/-S9 (genotoxicity response with and without metabolic activation of liver enzymes), PAH CALUX (dioxin receptor activation) and Nrf2 CALUX (oxidative stress response) for all fracturing fluid, flowback, groundwater and procedure blank samples; + indicates a statistically significant positive response, – a negative response, (+) a possible false positive response and (c) a possible false negative response due to cytotoxicity.

Samples	Chemical analyses results				Bioassay testing results							
	Positive ionisation		Negative ionisation		Ames test				CALUX test			
	#Peaks (add/subs)	>TTC (add/subs)	#Peaks (add/subs)	>TTC (add/subs)	TA98-S9	TA98 + S9	TA100-S9	TA100 + S9	P53-S9	P53 + S9	PAH	Nrf2
FF (total)	2431/0	547/0	1489/0	1480/0								
FF1d	88/0	88/0	39/0	39/0	+	–	(+)	–	–	+	(+)	(+)
FF2d	535/0	535/0	16/0	16/0	–	–	(+)	–	–	+	(+)	–
FF1c	2322/0	324/0	1468/0	1459/0	(c)	(c)	(c)	(c)	–	(+)	(+)	(+)
FW (total)	2297/330	2285/325	374/1279	310/1250								
FWD1d	301/23	301/23	8/11	8/11	–	–	(+)	–	–	–	(+)	(+)
FWD2d	93/3	93/3	0/1	0/1	+	–	(+)	–	–	–	(+)	(+)
FWD3d	14/12	14/12	7/6	7/6	–	–	–	–	–	–	(+)	(+)
FWD5d	80/4	80/4	4/5	4/5	–	–	(+)	–	–	–	(+)	(+)
FWD6d	11/3	11/3	13/17	13/17	–	–	–	–	–	+	(+)	(+)
FWD7d	57/5	57/5	5/7	5/7	–	–	(+)	–	–	–	(+)	(+)
FWD8d	77/5	77/5	6/5	6/5	–	–	(+)	–	–	–	(+)	(+)
FWD1c	2158/207	2116/199	242/1037	222/1002	(c)	(c)	(c)	(c)	–	+	–	+
FWD6c	–	–	282/1092	219/1027	(c)	(c)	(c)	(c)	–	+	–	+
FWD7c	1988/235	1987/235	290/1088	241/1045	(c)	(c)	(c)	(c)	–	+	–	+
CW	nd.											
20B-1					–	–	–	–	–	–	(+)	(+)
20B-2					–	–	–	–	–	(+)	(+)	(+)
2B-1					–	–	–	–	–	(+)	(+)	(+)
2B-2					–	–	–	–	–	–	(+)	(+)
20A-1					–	–	–	–	–	(+)	(+)	(+)
20A-2					–	–	–	+	–	(+)	(+)	(+)
2A-1					–	–	–	–	–	(+)	(+)	(+)
2A-2					–	–	+	–	–	–	(+)	–

these chemicals are required for further risk assessment. Even though the results of the concentrated flowback samples seem to suggest that a high number of detected peaks result in positive responses for the bioassays, this cannot be confirmed by the results of the other samples. The concentrated fracturing fluid showed a high number of peaks but did not give any positive response for the bioassays. Additionally, the direct fracturing fluid sample 1 and the direct flowback samples of day 2 and 6 showed relatively few detected peaks and resulted in positive responses for at least one of the bioassays. The number of detected peaks does not seem to explain the toxicity responses found in the fracturing fluid and flowback samples. The presence of potentially toxic chemicals may only exert adverse effects if exposure at sufficiently high concentrations would occur, against which various precautionary measures are being taken. Fracturing fluids and flowback fluid can pose challenges to drinking water utilities, with regard to removing the polar organic compounds. Proper treatment of the gas extraction related waters is therefore necessary before storage, recycling or release into the environment. Data show that incidents which might lead to contamination may occur (Maloney et al., 2017; Patterson et al., 2017). The associated spills are diluted after entering surface or groundwater. The highest concentration found in both ionisation modes is 37.9 mg/L IS-eq, which corresponds to the direct fracturing fluid 2 sample in positive ionisation. This means that depending on the hazardous properties of the compound in question, a dilution of roughly 4×10^5 may be needed to reach safe drinking water concentration levels based on the generic TTC-level for non-genotoxic substances in case of a contamination with this fracturing fluid. To illustrate this, the Dommel river in the Netherlands is considered as an example of a possible polluted water body by this fracturing fluid with a lowest mean water flow of 3 m³/s (Waterschap de Dommel, n.d.; Butkovskiy et al., 2017). Assuming an acute average spill volume of 24 m³ for surface spills of fracturing fluid (Faber et al., 2018), it would take a maximum of 35 days to reach safe concentration levels of the surface water in the Dommel. The severity of the adverse health effects however would depend on the toxicological properties of the chemical in question. The average surface spill

volume is based on US data because no public data within the Netherlands is available. The associated US spill frequency is 0.02–0.1%/well/year (Faber et al., 2018). The safety regulation related to oil and gas activities is stricter in the Netherlands than in the US and therefore spill frequency will likely be lower in the Netherlands compared to the US.

4. Conclusion

The aim of this study is to provide insight into the possible risk that the hydraulic fracturing related chemicals pose to human and environmental health. The chemical results show high concentrations for fracturing fluid and flowback samples that may potentially be of concern. Moreover, limited environmental fate data points towards persistence in water. The bioassay results show clear genotoxic and oxidative stress responses for fracturing fluid and flowback samples. These results provide a scientific justification for the measures currently in place related to the handling, transport and treatment of hydraulic fracturing related waters, to avoid adverse environmental and human health impacts. This study is however limited to one hydraulic fracture in a single location. More studies on similar hydraulic fracturing related activities in the Netherlands and in the EU in general are needed in order to gain a full overview of the potential risks associated with hydraulic fracturing in the EU.

There is a lack of literature and experimental studies on the analysis of hydraulic fracturing samples within the European context. This research starts to fill this knowledge gap by providing a chemical characterisation and bioassay assessment for tight gas and for the Dutch situation. This may serve as a basis for an environmental risk assessment for Dutch drinking water companies as well as a comparison to shale gas operations and to other geographical locations.

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