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ENVIRONMENTAL SIGNIFICANCE STATEMENT

Given the frequency of surface spills of produced fluids from unconventional oil and gas operations, there is a need to better characterize resulting groundwater contamination. Produced fluids are known have complex and variable chemical and microbial composition that could influence contaminant fate and transport in groundwater; however, studies on the behavior of compounds measured in produced water under environmentally-relevant conditions are limited. This study investigates degradation pathways and kinetics of the frequently-used ethoxylated surfactants polyethylene glycol and polypropylene glycol under conditions simulating a release to shallow groundwater of produced water from two hydraulically-fractured oil and gas wells at varying production times. These results may be utilized to better characterize shallow groundwater contamination following a release of produced water.

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ABSTRACT

Polyethylene glycols (PEG) and polypropylene glycols (PPG) are frequently used in hydraulic fracturing fluids and have been detected in water returning to the surface from hydraulically-fractured oil and gas wells in multiple basins. We identified degradation pathways and kinetics for PEGs and PPGs under conditions simulating a spill of produced water to shallow groundwater. Sediment-groundwater microcosm experiments were conducted using four produced water samples from two Denver-Julesburg Basin wells at early and late production. High-resolution mass spectrometry was used to identify the formation of mono- and di-carboxylated PEGs and mono-carboxylated PPGs, which are products of PEG and PPG biodegradation, respectively. Under oxic conditions, first-order half-lives were more rapid for PEG (<0.4-1.1 d) compared to PPG (2.5-14 d). PEG and PPG degradation corresponded to increased relative abundance of primary alcohol dehydrogenase genes predicted from metagenome analysis of the 16S rRNA gene. Further degradation was not observed under anoxic conditions. Our results provide insight to the differences between degradation rates and pathways of PEGs and PPGs, which may be utilized to better characterize shallow groundwater contamination following a release of produced water.

INTRODUCTION

The combined technologies of horizontal drilling and hydraulic fracturing have facilitated rapid expansion of unconventional oil and gas development, raising a number of public concerns including the introduction of chemicals used in hydraulic fracturing fluids to the environment.[1-3] Hydraulic fracturing fluids are pumped into low-permeability hydrocarbon-bearing formations at high pressures to enhance well production. A typical fracturing fluid is composed of about 90% water, 9% sand, and 0.5–3% chemical additives.[4-6] Following well stimulation, the fracturing fluids along with formation brines are co-produced with the hydrocarbons, referenced herein as "produced water." Recent studies and advances in analytical methods have improved knowledge of the composition of produced water,[7-9] which has been shown to vary between different wells, formations, and production ages.[8-11]

Several studies have reported groundwater contamination linked to known or suspected releases of oil- and gas-related fluids.[12-14] In the Denver-Julesburg Basin in northeastern Colorado, the number of reported surface spills that contaminated shallow groundwater with one or more of benzene, toluene, ethylbenzene, and xylenes increased from about 60 to 100 spills annually between 2007 and 2014.[15] Produced water has been reported as one the most frequently-released materials in most major United States unconventional resource plays.[16, 17] Given the frequency of accidental surface spills, there is a need to understand the natural attenuation rates and pathways of organic constituents identified in the produced fluids.

Homologous series of polyethylene glycols (PEG) and polypropylene glycols (PPG) have been detected in produced water from multiple basins.[18-22] PEGs and PPGs are frequently reported as constituents in fracturing fluid additives including surfactants, emulsifiers, and crosslinkers.[5, 6, 23] A recent study of the temporal evolution of produced water composition in the

Denver-Julesburg Basin showed that PEGs and PPGs were present after 400 days of production.[11] PEGs and PPGs have been suggested as potential environmental tracers because they have been frequently reported in produced water and are present from the injected fluids as opposed to being natural constituents of the formation brines.[11, 19]

Recent studies have shown biodegradation to be an important removal mechanism for some organic compounds identified in fracturing fluids and produced waters under environmentally-relevant conditions.[24-28] PEGs have been reported as rapidly biodegraded under aerobic conditions.[28-31] While PPGs have also been found to be biodegradable under aerobic conditions,[29, 31-33] they have been shown to be more persistent than PEGs.[29, 31] Under aerobic conditions, both PEGs and PPGs are biotransformed through stepwise shortening of terminal primary alcohol groups via alcohol and aldehyde dehydrogenase enzymatic pathways in succession, producing carboxylated intermediates.[34, 35] Anaerobically, PEGs are biodegraded via the diol dehydratase pathway,[36] while PPGs have been shown to be more recalcitrant under anoxic conditions.[36-38] Given their relatively hydrophilic nature, PEGs and PPGs are expected to be fairly mobile in groundwater.

Our objective was to measure degradation pathways and kinetics for PEGs and PPGs under conditions simulating a release of produced waters to shallow groundwater. Produced water chemical and microbiological composition varies between different wells and production ages;[10, 11] thus, sediment-groundwater microcosm experiments were conducted using four produced water samples from two Denver-Julesburg Basin wells at early and late production. High resolution mass spectrometry was complemented by analysis of microbial community dynamics and predictive metagenomics analysis to investigate PEG and PPG degradation pathways and kinetics.

METHODS

Produced water samples

Four produced water samples were collected from two horizontal wells (referenced as wells "A" and "B") targeting the Niobrara Formation in the Denver-Julesburg Basin in Weld County, Colorado. Both wells were hydraulically fractured with gel-based treatments (Tables S1 and S2). Samples were collected from each well at early and late production times. Early time samples were collected 22 d after production began from well A ("A-22") and 14 d after production began from well B ("B-14"). Late time samples were collected 611 d after production began from well A ("A-611") and 161 d after production began from well B ("B-161"). All samples were collected from the gas/oil/water separator in pre-baked 4 L amber glass jugs with no headspace, and stored at 4ºC.

Aquifer material and groundwater composition

Sediments were collected via hand auger between the depths of 0.9 m (depth of the water table) and 2.5 m from an alluvial aquifer adjacent to the South Platte River in the Denver-Julesburg Basin. The formation is characterized as unconsolidated, coarse-grain sand and gravel with interbedded clays in some areas.[39] Collected sediments were homogenized, sieved through a 2 mm mesh, and stored saturated with native groundwater at 4°C (details in Supporting Information). Sieved sediments were composed of well-graded sand, with an organic carbon content of 0.37% w/w (Table S3).

During a release, spilled fluids mix with shallow groundwater; thus, the produced water was diluted with a synthetic groundwater representative of Denver-Julesburg Basin surficial aquifers with respect to major ions and pH (details in SI). The synthetic groundwater was

dominated by sodium and sulfate with a pH of 7.5 (Table S3). Dilution factors ranged from 7-12× and were determined by normalizing the initial benzene concentration in the microcosms to 1 mg L^{-1}, which was representative of groundwater concentrations measured immediately following surface spills of produced water in the Denver-Julesburg Basin (details on spills analysis provided in SI).

Microcosm experiments

Microcosm experiments were conducted using sacrificial sampling. Individual microcosms were constructed by diluting the produced water with synthetic groundwater (7-12×) and adding 100 mL of the mixture to 125 mL pre-baked borosilicate glass serum bottles with 25 g of saturated sediments (1:5 solids-to-liquids ratio). Abiotic control microcosms were prepared for each produced water by adding 5.0 g L^{-1} sodium azide to the synthetic groundwater (NaN_3, ≥99%, Amresco). For each produced water treatment, triplicates (biotic) or duplicates (abiotic controls) were sacrificed at every time point. The serum bottles were sealed with PTFE-lined septa with a 10% v/v regular atmosphere headspace, which allowed the microcosms to progress from initially oxic to more reducing conditions. Bottles were mixed continuously on an orbital shaker (100 rpm) in the dark at 21±1°C.

Microcosm samples were collected using sterile needles and glass syringes. Dissolved oxygen (DO) and pH were measured immediately using a luminescent dissolved oxygen probe (LDO101, Hach) and a pH electrode (PHC301, Hach). DO was considered depleted at 1.5 mg L^{-1} due to the sampling procedure; it was not possible to eliminate all opportunities for re-oxygenation of the sample. Samples for major anions and cations analysis were syringe-filtered (0.2 μm, polyethersulfone membrane, Pall Corporation). Cation samples were preserved

with 1% nitric acid and analyzed using inductively coupled plasma-optical emission spectroscopy (model 3410+, Applied Research Laboratories). Anions were analyzed by ion chromatography (model 4500I, Dionex). Suspended and attached adenosine triphosphate (ATP) was measured using a luminescence assay and luminometer (PhotonMaster, LuminUltra). Method details for ion and ATP analysis are provided in the SI. Total dissolved solids (TDS) were analyzed using Standard Method 2540C.[40] Samples collected for PEG and PPG analysis were filtered through surfactant-free syringe filters[18] (0.2 μm, PTFE membrane, Pall Corporation) and stored at 4ºC in 2 mL amber glass vials. Sediment samples from a subset of the A-22 and A-611 (d 0, 1, 3, 21, 49, 86) and B-14 (d 0, 1, 4, 20, 47, 90) microcosms in addition to samples of the aquifer sediments prior to exposure to the produced water were collected and stored in 15 mL sterile, RNase- and DNase-free centrifuge tubes at -80°C for microbial community analysis.

PEG and PPG identification and analysis

Identification of PEGs, PPGs, and degradation products was conducted by accurate mass analysis using ultra high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC/qTOF-MS).[19] Analytes were separated using an UHPLC system (Series 1290, Agilent Technologies) equipped with a reversed-phase C_8 analytical column (150 mm x 4.6 mm, 3.5 μm particle size; Zorbax Eclipse XDB-C8, Agilent Technologies), sample volume of 20 μL, and mobile phases A and B of 0.1% formic acid in water and acetonitrile, respectively, at a flow rate of 0.6 mL min^{-1}. The initial mobile phase composition (10% B) was held constant for 5 min, followed by a linear gradient to 100% B after 30 min. A 10 min post-run time was used after each analysis. The UHPLC system was connected to a

qTOF-MS (Model 6545, Agilent Technologies) operating in positive ion mode. Accurate mass spectra were recorded across 50-1000 *m/z* at 2GHz in full-spectrum mode, and data were processed with MassHunter software. MS-MS was used to identify selected compounds (isolation width of ~4 *m/z* and collision energies of 10, 20, and 40 eV). Analytical standards are only available for mixtures of PEG and PPG homologous and not individual compounds, and ionization efficiencies may not be the same for all homologues.[8, 11] Thus, concentrations in day 0 microcosm samples were estimated from 1 mg L^{-1} PEG-400 and PPG-425 standards (polydispersal mixtures of PEG and PPG with average molecular weights of 400 and 425, respectively; Sigma-Aldrich), using the total response of all homologues. PEGs, PPGs, and identified products were semi-quantified by comparing the integrated response of each homologue detected in the microcosm samples to the corresponding homologues measured in the day 0 microcosm sample.

Following identification of PEGs, PPGs, and products by accurate mass analysis, high-performance liquid chromatography mass spectrometry (HPLC/MS) was used to semi-quantify PEG and PPG concentrations to estimate removal kinetics. Analytes were separated at 25ºC using an HPLC system (Series 1100, Agilent Technologies) equipped with an analytical column as described above. The mobile phase composition was identical to the UHPLC/qTOF-MS method with a flow rate of 0.4 mL min^{-1} and an increased sample injection volume of 40 µL. The HPLC system was connected to an ion trap MS (LC-MSD Trap XCT Plus, Agilent Technologies) using electrospray ionization (ESI) operating in full-scan, positive ion mode (*m/z* scan range 50-1000). Pseudo-first-order removal rate coefficients were estimated by linear regression using OriginPro 2016 for the period of observed rapid removal (e.g., within the linear range).

DNA extraction, sequencing, and bioinformatics analysis

Total nucleic acids were extracted in replicate from 0.25 g of homogenized sediment slurry using the DNeasy PowerSoil Kit (Qiagen). Triplicate replicates were extracted for all A-22, A-611, and B-14 microcosm sediment samples except day 0 (extracted in singlet) and aquifer sediment samples (extracted in duplicate). Sediment samples from the B-161 microcosms were not analyzed for microbial community analysis. DNA was quantified and quality checked using a NanoDrop 2000 (Thermo Fisher Scientific), and the polymerase chain reaction (PCR) amplification of the appropriate sized template was checked prior to sequencing. Library prep and amplification of the V4 region of the 16S rRNA gene was performed at Argonne National Lab according to previously established protocol[41] using primers 515F-806R. The 16S rRNA iTag sequence data were obtained by an Illumina MiSeq at Argonne National Lab. Sequences were processed using the QIIME 1.9.1 pipeline and Ohio Supercomputer[42, 43] using closed reference operational taxonomic unit (OTU) picking against the GreenGenes database (v.13.8)[44] with BLASTn and QIIME parameters set at 97% similarity.[42] An OTU table was generated using taxonomic assignments from BLAST[45] for all statistical analyses. Rarefaction curves were generated to evaluate sequencing depth (Fig. S1 and S2) and alpha diversity indices were generated in QIIME. After removal of singlets, the OTU table was submitted to the Galaxy portal of the Langille Lab (v.1.1.1) for PICRUSt analysis for predictive metagenome analysis from the 16S rRNA gene.[46] Gene abundances of primary alcohol dehydrogenase genes (PA-DH, KEGG Orthology identifier K00114)[47] and anaerobic diol dehydratase genes (*pduC*, K01699)[36] were normalized to copies of a housekeeping gene, *recA* (K03553), to account for fluctuations in the number of predicted metagenomes through time and genomes present but lacking functional genes of interest (Fig. S3).[36, 48] PICRUSt has been

shown to yield accurate predictions based on small numbers of 16S rRNA gene sequences across a breadth of environmental systems.[46] Statistical testing was performed using R (v.3.4.3). The 16S rRNA gene sequences were submitted to the NCBI database and are available under BioProject ID PRJNA445449.

RESULTS AND DISCUSSION

Microcosm water chemistry

The microcosms were initially oxic and progressed to more reducing conditions. The oxic/anoxic transition was rapid for the A-22 and B-14 microcosms (3 and 4 d, respectively), and slower for the A-611 and B-161 microcosms (21 and 28 d, respectively; Fig. 1). In all biologically-active microcosms, oxygen and nitrate were simultaneously depleted; thus, the oxic period consisted of mixed oxygen- and nitrate-reducing conditions (Figs. S4-S7). Following the oxic/anoxic transition, the different microcosms progressed to more reducing conditions, with final conditions ranging from mixed manganese- and iron-reducing (A-611 and B-161 microcosms) to sulfate-reducing (A-22 and B-14). In the abiotic control microcosms, dissolved oxygen remained approximately saturated throughout the experiment, and no significant changes were observed in the concentrations of any redox-active species (Figs. S4-S7). TDS concentrations in the microcosms were relatively low (1,920-3,260 mg L^{-1}, Table S7). In all microcosms, the pH quickly dropped from approximately 7.5 to 7.0, and subsequently remained steady throughout the experiment (Fig. S8).

In all biotic microcosms, ATP production rapidly increased during the oxic period (Fig. S9). Under oxic conditions, a sharp increase to 10^{10}-10^{11} pg total ATP (suspended and attached) occurred within the first 2 d for all waters. Following the oxic/anoxic transition, total

ATP in the A-22 and B-14 microcosms rapidly declined by two orders of magnitude, after which ATP concentrations remained steady throughout the remainder of the experiment. In both the A-611 and B-161 microcosms, the initial ATP peak was followed by a steady decline to approximately 10^7 pg total ATP. For all produced water treatments, ATP concentrations in the abiotic control microcosms were at least two orders of magnitude lower than in the corresponding biotic microcosms. Rapid ATP production which corresponded to distinct redox transitions indicate significant microbial activity in the biotic microcosms.

Identification and transformation products

Polyethylene glycols (PEGs). PEGs were identified by accurate mass analysis. The average measured mass difference between PEG homologues was 44.0262 u, which represents the addition of one ethylene oxide unit (-CH_2CH_2-O-).[19] Series of PEG homologues with three to fourteen ethylene oxide units were detected in all four produced water samples (Fig. 2; Table S8).

The relative abundance of shorter chain PEGs was greater in the initial distribution of the PEG series detected in the A-22 day 0 microcosm relative to the B-14 day 0 microcosm (Fig. 2a,c). The initial distribution of PEGs was nearly identical in A-22 and A-611 (Fig. 2a,b); however, there was a shift towards shorter chains in B-161 compared to B-14 (Fig 2c,d). The estimated total concentration of all PEG homologues in the A-22 day 0 microcosm was in the mg L^{-1} range and was approximately an order of magnitude greater than the B-14 day 0 microcosm. Differences in initial PEG distributions and concentrations between the two wells were likely due to varying fracturing fluid composition. The FracFocus Chemical Disclosure Registry[49] report for well A listed PEG as an ingredient. Well B's FracFocus report listed a

proprietary "surfactant blend" and did not specifically report the use of PEGs (Tables S1 and S2). Downhole reactions could explain the distribution differences between the two production ages in well B. Based on hydrophobicity, sorption to the hydrocarbon formation would not be a significant downhole removal mechanism for the relatively short-chained PEGs returning to the surface.[18] ATP concentrations indicated biological activity in the produced water collected from B-14 (8,010 pg L^{-1} suspended ATP), while ATP concentrations were approximately 3 orders of magnitude lower in the A-22 produced water sample (10 pg L^{-1} suspended ATP). Thus, downhole biodegradation could have occurred in well B, but low biomass activity suggests that downhole biodegradation was unlikely in well A. Shifts to smaller homologues have been reported during PEG biodegradation,[29, 30] which could explain the distribution differences between B-14 and B-161.

After day 1 of the microcosm experiment, chromatographic peaks separated by 44.0262 u (one ethylene oxide group) were observed eluting approximately 0.5 min later than the detected PEGs (Fig. 3). The peak at 11.4 min had a measured mass of 460.2396 with companion adducts at *m/z* 465.1947 and 443.2126 (Fig. S10). Based on mass differences, the ions were identified as the ammonium, sodium, and proton adducts, respectively. Figure S10 also shows ions at *m/z* 487.1764 and 509.1582, which are 21.9815 and 43.9635 greater than the single sodium adduct, respectively. The mass difference equates to the addition of a sodium ion minus a proton, indicating the presence of double (*m/z* 487.1764) and triple (*m/z* 509.1582) sodium adducts. Accurate mass putatively identified a formula of $C_{18}H_{34}O_{12}Na^{+}$ (single sodium adduct) with a calculated exact mass of *m/z* 465.1942, which is within 1.1 ppm of the measured mass. The compound was putatively identified as a di-carboxylated PEG (PEG-diCOOH). The double and triple sodium adducts occur when one or both carboxyl groups, respectively, are in the form

of a sodium salt. MS-MS was conducted (details in S2.1; Fig. S11); however, a standard of the putative PEG-diCOOH product was not available, thus the identification remains unconfirmed. Mono-carboxylated PEGs (PEG-COOH) were also identified by accurate mass analysis (e.g., peak at 11.1 min apparent in Fig. 3b). Single carboxylated PEGs have been previously reported in produced waters.[19] The small increase in retention time for the carboxylated products (Fig. 3b) can be explained by the slightly greater hydrophobicity of protonated PEG-COOH and PEG-diCOOH compared to the parent PEG in the acidic mobile phase in addition to increasing molecular weights (+14 u) of the carboxylated products. Thurman et al.[18, 19] demonstrated via the Kendrick mass scale that for homologous series of ethoxylates it is only necessary to identify the structure of one homologue in the series, and that the remaining compounds represent the addition of ethylene oxide units. Thus, accurate-mass analysis presented for the di-carboxylated product of example homologue PEG-9 (Fig. S10) and the MS-MS analysis conducted for the example homologue PEG-6-diCOOH (e.g., Fig. S11) apply to the identification of all PEG homologues in the series. Both PEG-COOH and PEG-diCOOH with four to fourteen ethylene oxide units were detected in all of the microcosms (Table S8).

PEG-COOH was detected in the day 0 microcosm samples with an integrated response that was 4-22% of the corresponding PEG; however, PEG-diCOOH was not observed until after day 1 of the microcosm experiments (Fig. 3c). Both compounds are known PEG biodegradation intermediates under aerobic conditions.[34] In the presence of oxygen, PEGs are enzymatically altered through oxidation of the terminal primary alcohol group (Fig. 3d), followed by cleavage of the terminal ether bonds to produce a shorter-chained PEG with two fewer ethylene oxide units.[34, 35] The detection of PEG-COOH in the day 0 microcosm sample suggests it may be present initially as an impurity in the industrial surfactant mixture used in the fracturing fluid or

formed under downhole conditions.[19] Nevertheless, the increasing response of PEG-COOH with time and the emergence of PEG-diCOOH (not present at day 0) in conjunction with the removal of the corresponding PEG provides strong evidence of further formation of the carboxylated products in the microcosms (Fig. 3). Mass spectrometry analysis was not conducted for abiotic control samples due to high sodium azide concentrations used to inhibit microbial activity; thus, information needed to distinguish abiotic and biotic removal mechanisms was not obtained. Significant abiotic removal of PEGs has not been reported;[28] therefore, PEG transformation and removal observed in the microcosms was almost surely a result of biodegradation.

Polypropylene glycols (PPGs). PPGs were also identified by accurate mass analysis. The average measured mass difference between PPG homologues was 58.0419 u, which represents the addition of one propylene oxide unit (-$CH_2CH(CH_3)$-O-).[19] Series of PPG homologues ranging from two to ten propylene oxide units were detected in all four produced water samples (Fig. 4; Table S9).

There was greater relative abundance of shorter chain PPGs in the initial distribution of the PPG series present the A-22 day 0 microcosm compared to the B-14 day 0 microcosm (Fig. 4a,c). The initial PPG distribution was shifted towards shorter chains in the A-611 and B-161 microcosms (Fig. 4b,d) compared to A-22 and B-14, respectively. The estimated total PPG concentration was in the range of hundreds of μg L^{-1} and was approximately an order of magnitude lower in the A-22 day 0 microcosm compared to B-14. The differences in initial PPG distributions and concentrations between the two wells and production times could be due to both varying fracturing fluid composition and downhole reactions including shale interactions. FracFocus reports from both wells cited proprietary “surfactant blends,” but PPG or blends of

ethoxylated surfactants were not specifically identified on either report. PPGs are more hydrophobic than PEGs;[11] thus, sorption of longer-chained PPGs to the hydrocarbon formation or partitioning to the oil phase could contribute to the shift towards shorter-chained homologues observed at later production times. Sorption of longer-chained PPGs could explain the similar initial distributions observed in both the A-611 and B-161 day 0 microcosms.

After day 1 of the microcosm experiment, chromatographic peaks separated by 58.0419 u (one propylene oxide group) were observed approximately 0.2 min after the detected PPGs. With the same approach used to identify PEG-diCOOH, sodium (*m/z* 403.2302), proton (*m/z* 381.2483), and ammonium (*m/z* 398.2749) adducts were measured for the peak from 16.0-16.6 min (Fig. S12). Accurate mass putatively identified a formula of $C_{18}H_{36}O_8Na^+$ (sodium adduct) with a calculated exact mass of *m/z* 403.2302, which was an exact match to the measured mass. The compound was putatively identified as a mono-carboxylated PPG (PPG-COOH). Analogous to the PEG carboxylates, a double sodium adduct indicated that the sodium salt of the carboxyl group was present, albeit at trace levels (*m/z* 425.2129; Fig. S12). MS-MS was conducted using PPG-7-COOH as an example homologue (details in S2.2; Fig. S13). The chromatograph of PPG-4 is shown in Figure 5 because numerous isomers are discernable for relatively short-chained PPGs (see discussion below). PPG-COOH with three to ten propylene oxide units were identified (Table S9).

PPG-COOH was not detected in the day 0 microcosms (Fig. 5). A similar aerobic metabolic pathway as PEG has been reported for PPG: oxidation of a terminal alcohol (Fig. 5d) followed by cleavage of the terminal ether bond to produce PPG with one fewer propylene oxide unit.[32-34] The emergence of PPG-COOH corresponding with the removal of PPG demonstrates that PPG-COOH was formed in the microcosms (Fig. 5). Significant abiotic removal of PPG has

not been reported;[33, 36] thus, the transformation and removal observed in the microcosms was likely a result of biodegradation.

Multiple isomers were apparent for the PPG-COOH product. The number of isomers in the parent PPG increases exponentially for each additional propylene oxide unit due to non-symmetric hydroxyl groups on the monomer.[19, 33] For short-chained PPGs, individual isomers are apparent in the chromatography as distinct peaks with varying intensities. For instance, at least nine PPG-4 isomers can be discerned within the four readily-apparent peaks, and at least five isomers were observed for the corresponding PPG-4-COOH (Fig. 5b). The PPG-4-COOH isomer peaks had slightly different relative intensities compared to PPG-4, which indicates that different isomers were transformed to varying extents. Variable transformation for PPG isomers has been previously suggested.[33, 34]

In contrast to PEG, only a singly-carboxylated PPG product was identified. This is likely caused by the presence of both primary and secondary terminal alcohols in PPG isomers resulting from the non-symmetric monomer. PEGs are symmetric homopolymers with two terminal primary alcohol groups, both of which can be oxidized to form PEG-diCOOH. For PPG, oxidation of the terminal secondary alcohol to a carboxyl is not expected.[50] Thus, only PPG isomers with a terminal primary hydroxyl can be degraded to PPG-COOH. The formation of a ketone product from the terminal secondary hydroxyl has been reported;[32] however, we did not observe a PPG-ketone product in our microcosm experiments.

Degradation kinetics

Semi-quantitative analysis of PEG and PPG degradation was conducted using the HPLC/MS method. PEG-9 and PPG-6 were selected as an example PEG and PPG homologues,

respectively, based on relative abundance in the microcosms (Figs. 2 and 4). Figure 6 shows the relative response of PEG-9 and PPG-6 during the microcosm experiments, including both the oxic (filled symbols) and anoxic (open symbols) periods.

Under mixed oxygen- and nitrate-reducing conditions, PEG-9 was rapidly removed in all microcosms (Fig. 6a). First-order half-lives were similar between the four microcosms (Table 1). In the A-22 and B-14 microcosms, the half-lives were 0.4 and 1.1 d, respectively. In the A-611 and B-161 microcosms, PEG-9 was not detected by the day 1.5 sample; thus, the half-life was reported as <0.4 d. Under oxic conditions, PPG-6 degradation was faster for water collected at late production times ($t_{1/2}$ = 2.7 and 2.5 d in A-611 and B-161 microcosms, respectively) compared to early production times ($t_{1/2}$ = 7.5 and 14 d in A-22 and B-14 microcosms; Table 1, Fig. 6b). Our measured rates were consistent with PEG and PPG biodegradation rates reported under oxic conditions for similar initial concentrations and average molecular weights, by inoculum sources ranging from river water[29] to activated sludge.[30-32] Notably, the produced water mixture did not significantly inhibit or enhance PEG and PPG biodegradation compared to studies where these compounds were the only substrate. Biodegradation inhibition in mixtures of fracturing fluid organic constituents at high concentrations representative of the injected fluids has been previously reported.[26, 28]

Under oxic conditions, PPG degradation was both slower and more variable compared to the degradation of PEG (Fig. 6). While the occurrence of PEG-diCOOH demonstrates that PEGs can be degraded from both sides of the molecule, the mono-carboxylated PPG was the only PPG product identified in the microcosms. Thus, PPG was only degraded where a terminal primary hydroxyl was present and degradation was effectively blocked for isomers with terminal secondary hydroxyls. This likely contributed to slower degradation kinetics of PPG compared to

that of PEG. Differences in initial PPG concentrations may have contributed to the variable PPG degradation kinetics observed between microcosms because B-14 had both the highest estimated initial PPG concentration and the slowest degradation. A recent study under anoxic conditions suggested a concentration inhibitory effect on PPG biodegradation kinetics.[36] In our study, PPG-6 degradation was overall slower than PEG-9, but the relative succession of half-lives for the different microcosms was the same for both compounds (B-14 >A-22 >A-611≈B-161), which suggests that produced water composition also may have influenced degradation kinetics. Similar ATP concentrations in all microcosms during the period corresponding to PEG and PPG oxic degradation (10^{10}-10^{11} pg total ATP, Fig. S9) suggest comparable levels of overall biomass activity, and that a non-specific inhibitor such as a biocide likely did not affect the degradation kinetics. Competitive inhibition from a more labile or preferred carbon source present in the early-time produced water could have slowed the rate of PEG and PPG degradation relative to the waters from later production times. This is consistent with the rapid depletion of dissolved oxygen observed in the A-22 and B-14 microcosms compared to the A-611 and B-161 microcosms (Fig. 1), which could indicate more competition for available electron acceptors. TDS concentrations greater than 10,000 mg L^{-1} can inhibit biological activity;[7] however, TDS concentrations in the microcosms were below this threshold (1,920-3,260 mg L^{-1}, Table S7) and likely did not inhibit degradation.

There was no evidence of further PEG or PPG degradation under anoxic conditions. In the A-22 and B-14 microcosms, low levels of PEG-9 remained at the oxic/anoxic transition (Fig. 6a), but no additional removal was observed. In the A-611 and B-161 microcosms, PEG-9 was not detected at the oxic/anoxic transition, likely because the longer oxic period allowed for more complete removal. Anaerobic biodegradation of PEGs has been reported at temperatures

≥35°C,[51, 52] so it is possible that either the anaerobic microbial community in the microcosms was unable to degrade PEG, or the kinetics were slow enough under ambient temperatures that no degradation was observed over the 84-86 d anoxic periods. Due to both different degradation rates and lengths of the oxic period for the four microcosms, varying levels of PPG-6 remained at the oxic/anoxic transition (Fig. 6b); however, no further removal was observed under anoxic conditions in any microcosm. Our observations are in agreement with studies that have reported PPG as recalcitrant under anaerobic conditions, particularly in the presence of microbial communities which have not been previously exposed to glycols.[36-38]

Microbial community dynamics and abundance of primary alcohol dehydrogenase genes

Analysis of microbial community dynamics and function based on metagenomes predicted from the 16S rRNA gene provide further insight into the PEG and PPG biodegradation pathway. After quality filtering and processing, 14,640-77,563 sequences per sample were obtained (Table S10). One triplicate sample of B-14 day 4 yielded very low sequence counts (3,010) and was not used in any analysis; therefore, the error bars associated with this sample represent a range of duplicate values. Exposure to produced water greatly decreased the diversity of sediment microbial communities: Shannon's diversity index was 10 in the aquifer sediments prior to exposure to the produced water and dropped to 5-7 by day 3-4 of the microcosm experiments. Similarly, microbial community richness decreased from over 3,500 OTUs in the aquifer sediments to 1,300-2,700 OTUs in microcosm sediment samples in 1-4 days.

Using a bioinformatics approach (PICRUSt), we searched for genes known to catalyze polyglycol chain shortening in metagenomes predicted from 16S rRNA gene sequences. While specific PEG- and PPG-degrading enzymes have been proposed,[35, 53] they remain poorly

characterized. Thus, primary alcohol dehydrogenase genes (PA-DH) were used as a proxy for polyglycol biotransformation under oxic conditions via terminal alcohol oxidation. PA-DH is found in several taxonomic groups, including *Pseudomonas,* when grown on a wide variety of alcohols and acts by oxidation of terminal primary hydroxyl groups.[54]

A sharp increase in the abundance of the PA-DH gene relative to the housekeeping gene *recA* (PA-DH/*recA*) was observed during the first 1-3 days of the A-22, A-611, and B-14 microcosms (Fig. 7), which coincided with the period of rapid PEG and PPG removal (Fig. 6). In the A-22 and B-14 microcosms, the relative abundance of PA-DH/*recA* initially spiked to approximately 0.40 normalized gene copies, followed by a decrease to <0.25. The relative abundance of PA-DH/*recA* in the A-611 microcosms initially increased to 0.52 and remained elevated for the duration of the microcosm experiment, which is consistent with faster removal observed in the late-time compared to the early-time produced waters. The prolonged presence of dissolved oxygen in A-611 microcosms compared to A-22 and B-14 (Fig. 1) also may have enabled the persistence of microbial taxa capable of encoding PA-DH genes, which are repressed under reducing conditions. Trace levels of the diol dehydratase gene *pduC*, associated with polyglycol chain shortening under anaerobic conditions,[36] were predicted in the microcosm samples; however, *pduC* abundance was at least three orders of magnitude less than that of PA-DH, which is consistent with the limited PEG and PPG removal observed after the microcosms changed from oxic to anoxic conditions.

Pseudomonas became highly enriched in all of the microcosms through time, increasing from 0.01% of the aquifer sediment community to 50-65% by day 3 and remaining between 20 and 50% of the terminal community in microcosm sediments (Fig. S14). *Pseudomonas* has been identified in produced fluids as an important member of hydraulically-fractured shale

communities[55-57] and was enriched during biodegradation of synthetic hydraulic fracturing fluid in soil-groundwater microcosms.[24] Many species of *Pseudomonas* have the enzymatic capacity to degrade PEGs; therefore, this taxa may contribute to PEG degradation in the microcosms.[58-61] The genus *Syntrophobotulus* was also detected, a taxa notable for fermenting glyoxylate,[62] a proposed product of aerobic PEG chain shortening.[34] Genes participating in oxic and anoxic transformations of glyoxylate were also predicted in the samples (K01638/EC2.3.3.9, K00015/EC1.1.1.26), suggesting that mineralization of these products may have occurred in the microcosms.

Implications of PEG and PPG degradation in groundwater

Collectively, the predicted presence of genes associated with enzymatic pathways for the aerobic degradation of PEGs, PPGs, and their metabolites combined with the identification of mono- and di-carboxylated intermediate polyglycol products support the inference that PEG and PPG removal during the oxic phase was largely due to biodegradation. These results demonstrate the role that aerobic sediment microorganisms are likely to play in degrading PEG and PPG polymers, with implications for disruption of the natural microbial community (i.e., lower diversity and/or enrichment of key taxa) in the event of a release of produced water to shallow groundwater.

Given the persistence observed in our microcosms under anoxic conditions, PEGs and PPGs are more likely to be detected at sites where anoxic conditions prevail. The degradation products identified in our microcosms could help characterize releases of produced water to shallow groundwater even when the parent compounds have been largely transformed. The PPG-COOH product was persistent under both oxic and anoxic conditions (Figs. 5c and S15). Both carboxylated PEG products appeared to be persistent under anoxic conditions; however,

PEG-COOH and PEG-diCOOH emerged and were both subsequently degraded during the longer oxic periods in the A-611 and B-161 microcosms (Figs. 3c and S16). Thus, the PEG products may not be detected at sites with stable or prolonged oxic conditions.

Produced water composition is heterogeneous between different wells, formations, and production ages,[8-11] which could result in variable detection or degradation kinetics of PEGs and PPGs. While the detection of PEGs and PPGs in the A-611 and B-161 samples demonstrates that these compounds may be present long after well stimulation, the lower concentrations require sensitive analytical methods to detect. We found that PPGs were more persistent in produced waters with higher initial concentrations. We observed rapid PEG removal at the low concentrations present in the produced water microcosms; however, half-lives reported for high PEG concentrations, representative of initial hydraulic fracturing fluid composition, were an order of magnitude longer.[28] Rosenblum et al.[11] showed that concentrations of PEGs and PPGs in Denver-Julesburg Basin produced water decreased approximately 50% within the first two weeks of production. Thus, PEGs and PPGs are more likely to be detected at sites with a release shortly after production begins due to both higher initial concentrations and potentially slower degradation rates.

Given the frequency of accidental surface spills associated with unconventional oil and gas activities, there is a need to understand the natural attenuation rates and pathways of organic constituents in the produced fluids. Our results demonstrate the differences between PEG and PPG degradation rates and pathways. These insights may be utilized to better characterize shallow groundwater contamination following a release of produced water.

ASSOCIATED CONTENT

Supporting Information: Additional detail on methods; MS-MS for PEG-diCOOH and PPG-COOH products; 10 tables and 16 figures detailing compound identification, redox and ATP results, and microbial community analysis.

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CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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Table 1. Pseudo-first-order half-lives ($t_{1/2}$) and standard error fit during the oxic period of the produced water microcosm experiments.

microcosm	PEG-9 $t_{1/2}$ (d)	PPG-6 $t_{1/2}$ (d)
A-22	0.4 ± 0.1	7.5 ± 5.2
A-611	< 0.4	2.7 ± 0.5
B-14	1.1 ± 0.2	14 ± 3.7
B-161	< 0.4	2.5 ± 0.2

Figure 1. Dissolved oxygen (DO) *vs.* time in the A-22 (diamond symbols), A-611 (triangles), B-14 (squares), and B-161 (circles) microcosm experiments.

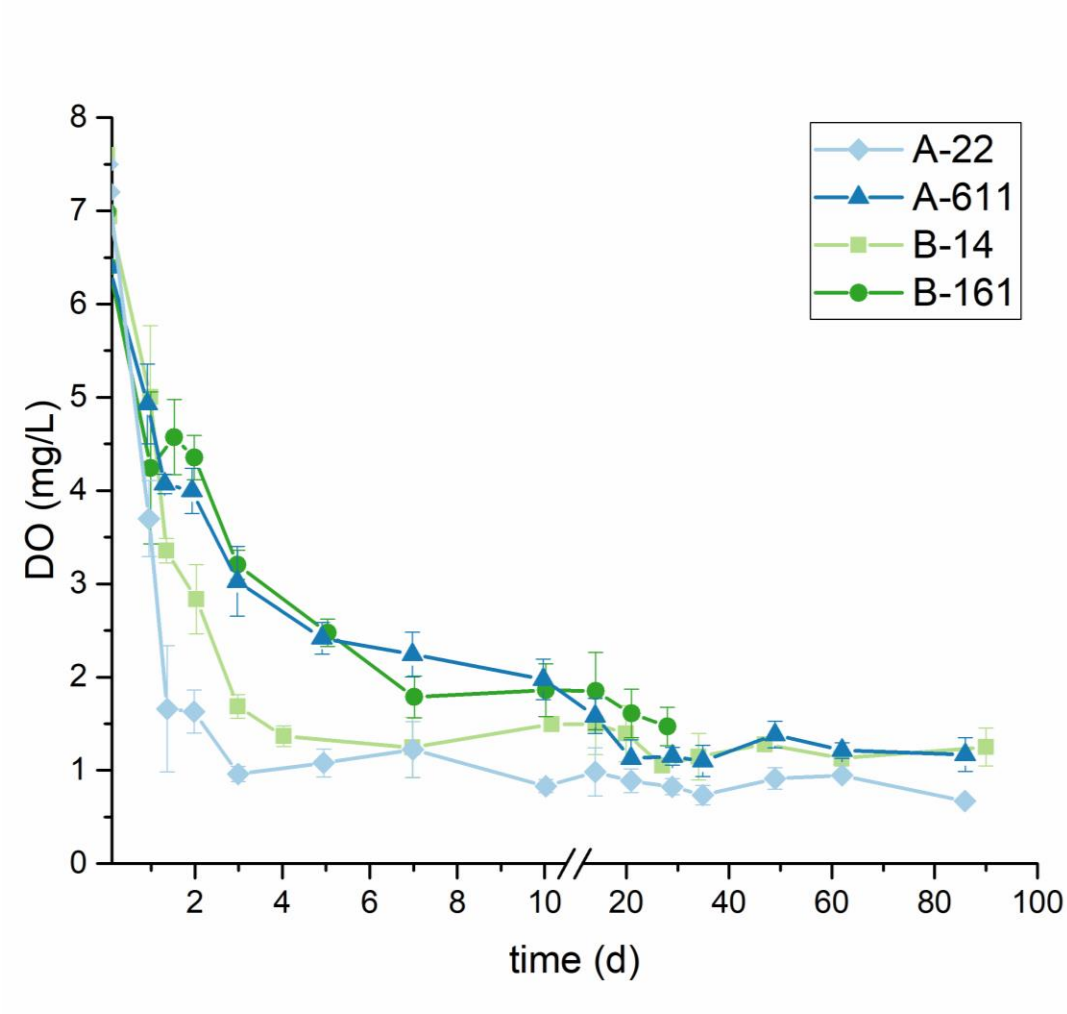


Figure 2. Initial (day 0) distribution of PEG homologues in (a) A-22, (b) A-611, (c) B-14, and (d) B-161 microcosms.

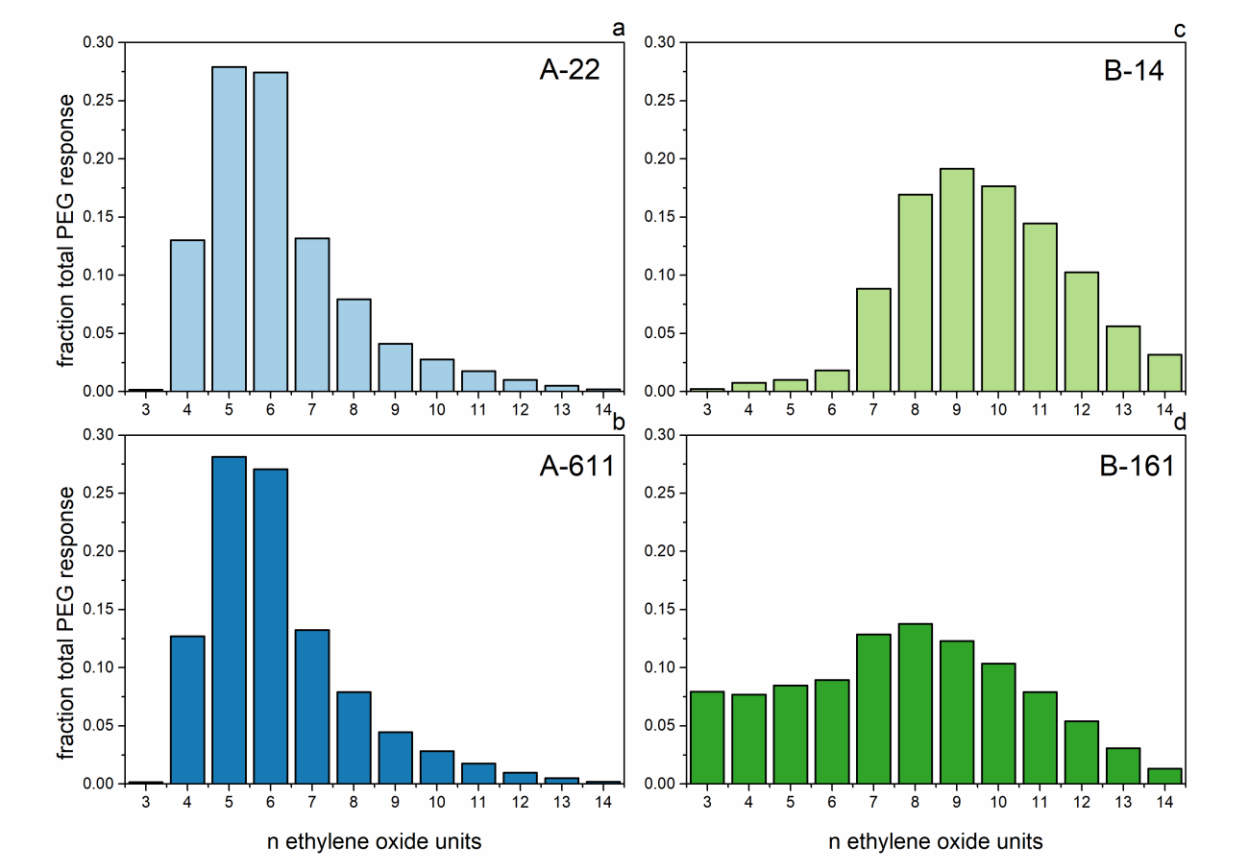


Figure 3. Chromatographic separation of PEG-9 (black trace) and corresponding products PEG-9-COOH (green trace) and PEG-9-diCOOH (orange trace) on (a) day 0 and (b) day 4 of the B-14 microcosm experiment, and (c) response (integrated peak area) of the three compounds relative to PEG-9 ($C/C_{0,parent}$) during the microcosm experiment. The aerobic metabolic pathway is shown (d).

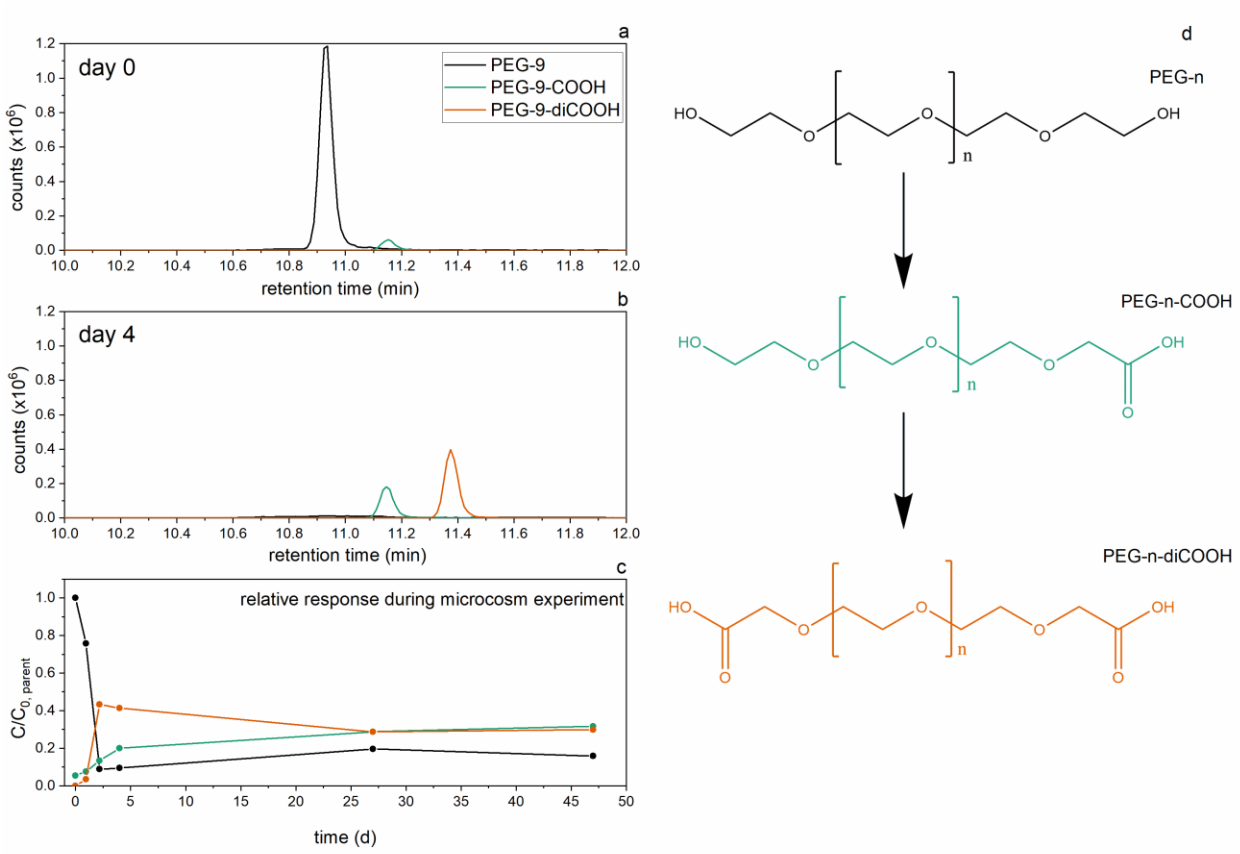


Figure 4. Initial (day 0) distribution of PPG homologues in (a) A-22, (b) A-611, (c) B-14, and (d) B-161 microcosms.

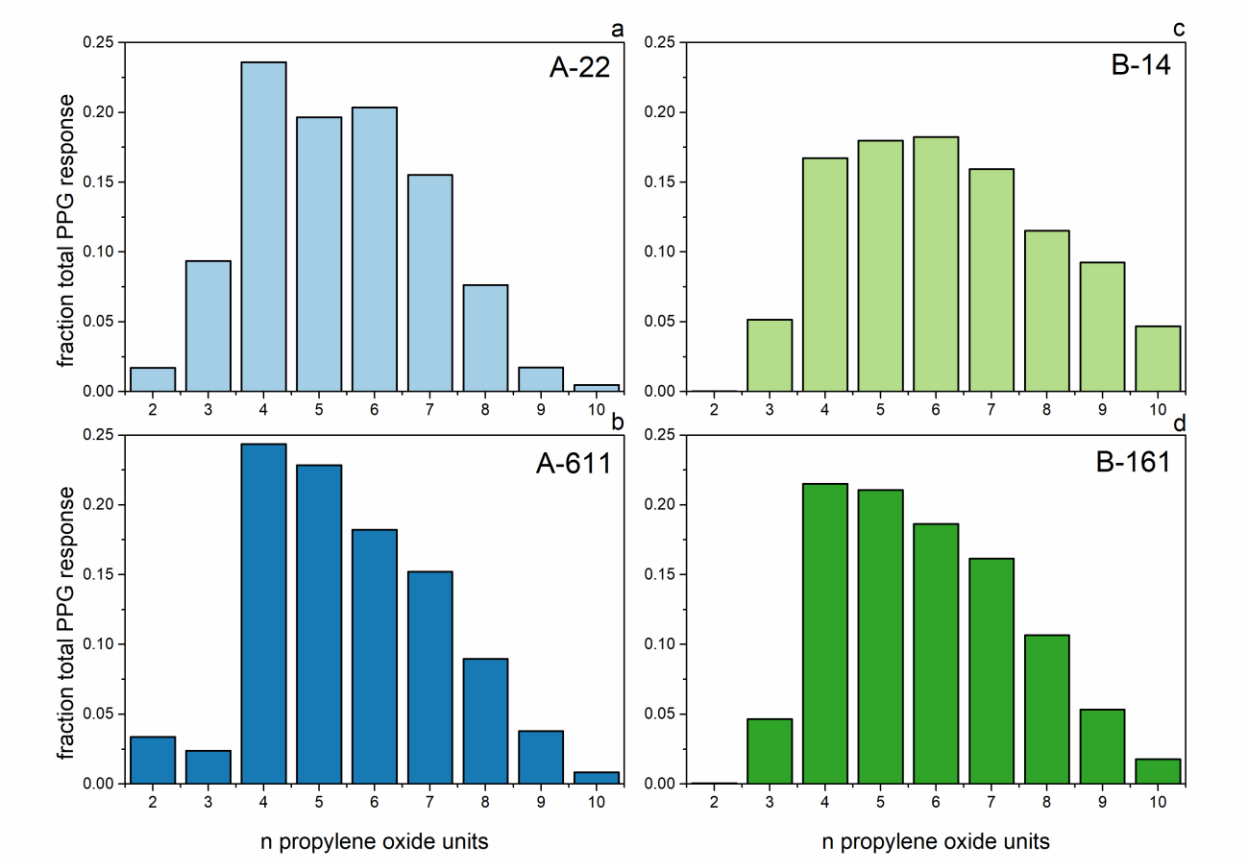


Figure 5. Chromatographic separation of PPG-4 (black trace) and corresponding product PPG-4-COOH (green trace) on (a) day 0 and (b) day 4 of the B-14 microcosm experiment, and (c) response (integrated peak area) of the three compounds relative to PPG-4 ($C/C_{0,parent}$) during the microcosm experiment. The aerobic metabolic pathway is shown (d).

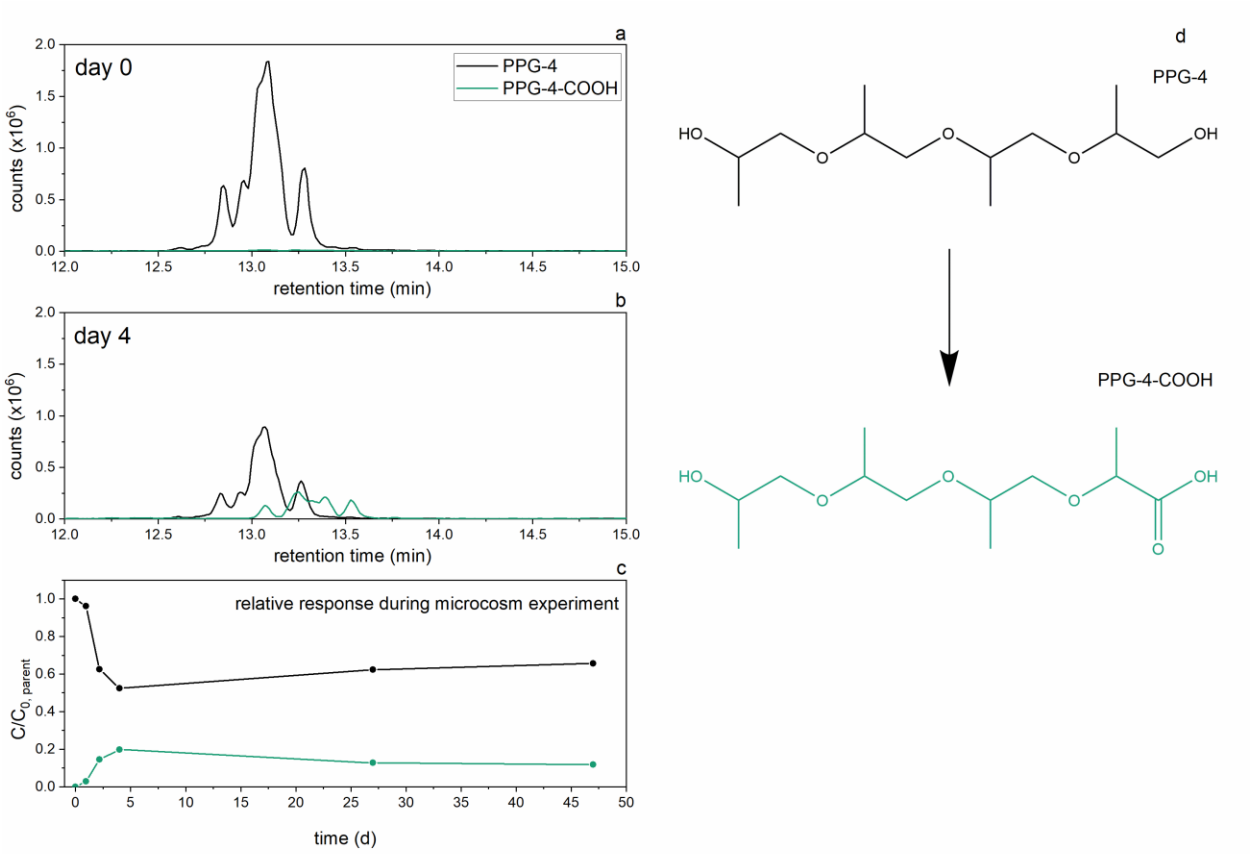


Figure 6. Degradation of (a) PEG-9 and (b) PPG-6 in A-22 (diamond symbols), A-611 (triangles), B-14 (squares), and B-161 (circles) microcosms. Oxic and anoxic periods are represented by solid and open symbols, respectively. An axis break at day 10 is used to show detail for rapid degradation.

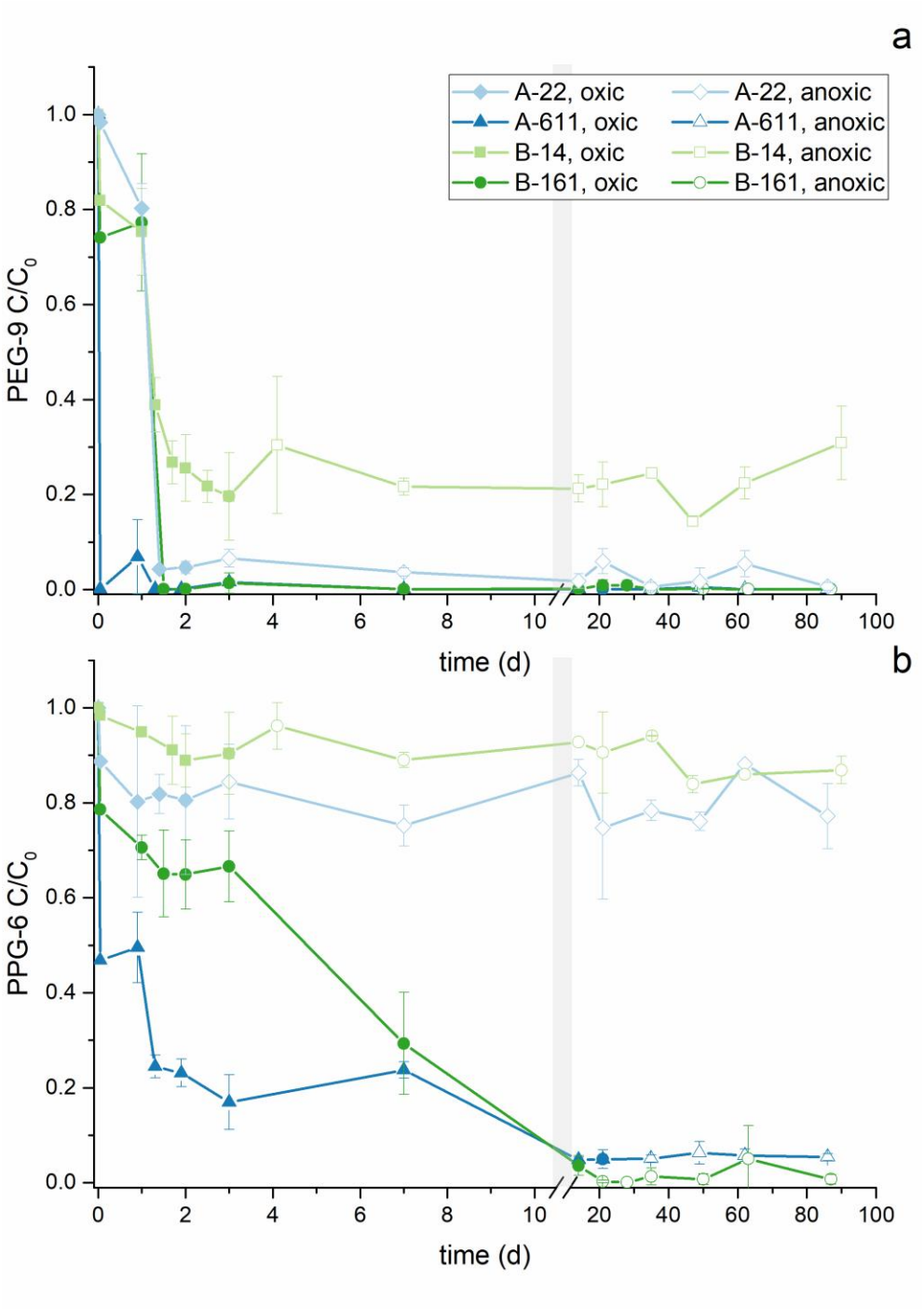
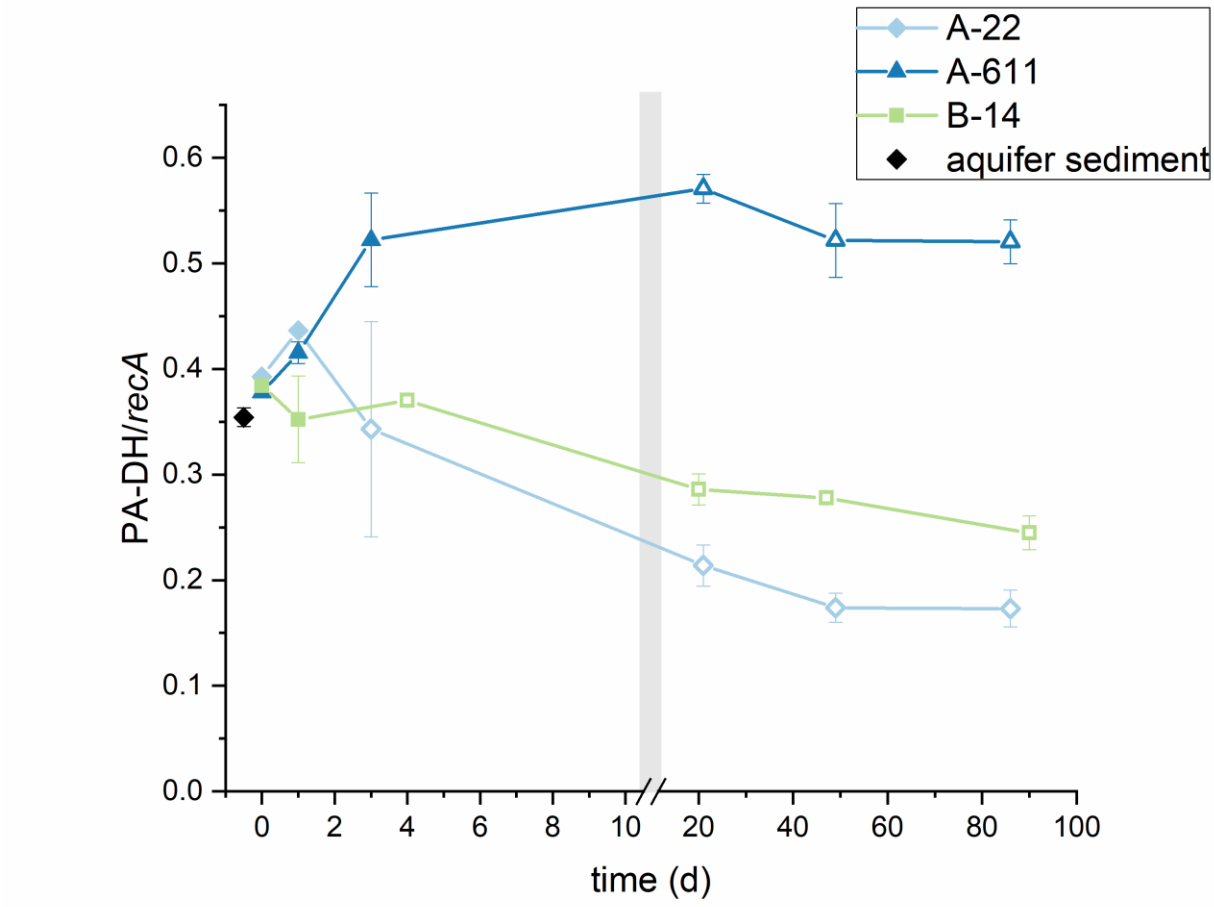


Figure 7. Abundance of the primary alcohol dehydrogenase gene (PA-DH) relative to a housekeeping gene (*recA*) generated from metagenomes predicted from the 16S rRNA gene of microbial communities in the A-22 (diamond symbols), A-611 (triangles), and B-14 (squares) microcosms, in addition to the aquifer sediment prior to exposure to produced water (black diamond symbol). Oxic and anoxic periods are represented by solid and open symbols, respectively.



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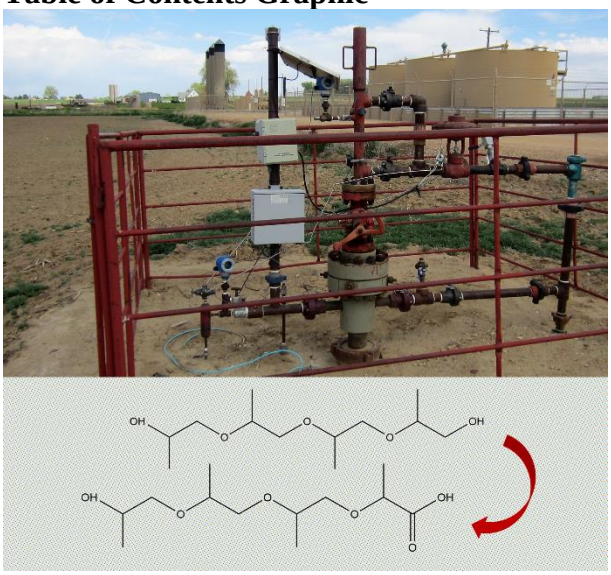

Polyethylene glycol, polypropylene glycol, and their degradation products could be utilized to better characterize shallow groundwater contamination following a spill of produced water.