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Impacts of Glutaraldehyde on Microbial Community Structure and Degradation Potential in Streams Impacted by Hydraulic Fracturing

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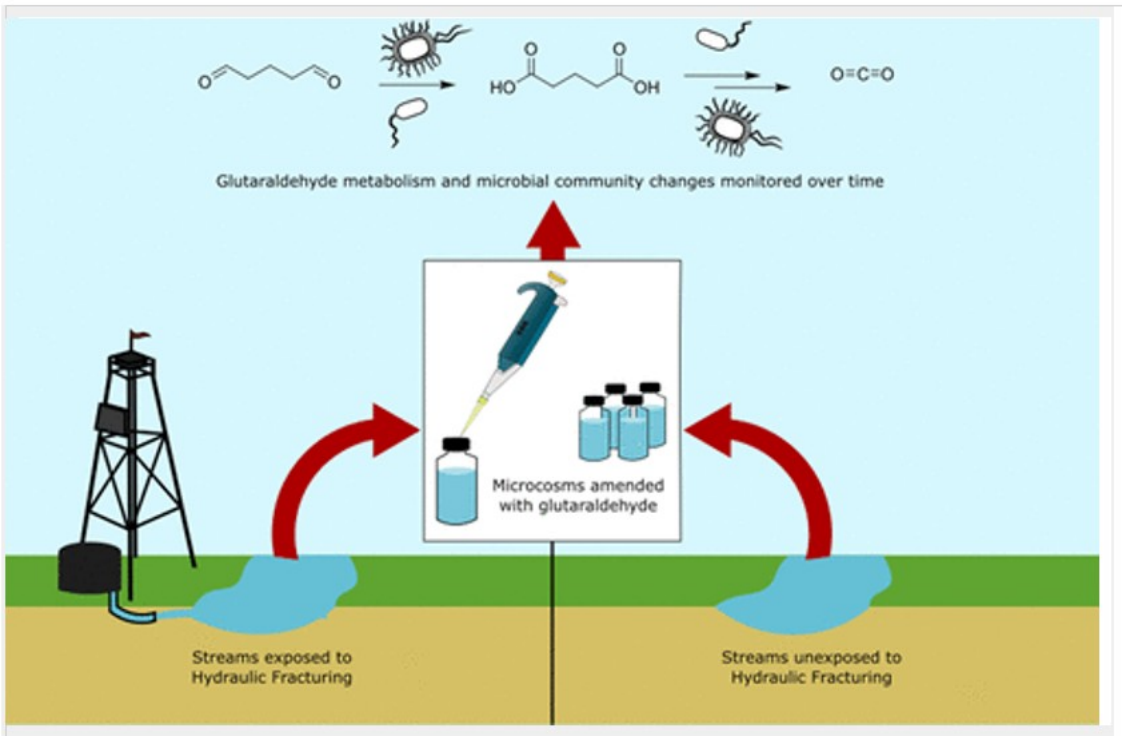
Abstract



The environmental impacts of hydraulic fracturing, particularly those of surface spills in aquatic ecosystems, are not fully understood. The goals of this study were to (1) understand the effect of previous exposure to hydraulic fracturing fluids on aquatic microbial community structure and (2) examine the impacts exposure has on biodegradation potential of the biocide glutaraldehyde. Microcosms were constructed from hydraulic fracturing-impacted and nonhydraulic fracturing-impacted streamwater within the Marcellus shale region in Pennsylvania. Microcosms were amended with glutaraldehyde and incubated aerobically for 56 days. Microbial community adaptation to glutaraldehyde was monitored using 16S rRNA gene amplicon sequencing and quantification by qPCR. Abiotic and biotic glutaraldehyde degradation was measured using ultra-performance liquid chromatography--high resolution mass spectrometry and total organic carbon. It was found that nonhydraulic fracturing-impacted microcosms biodegraded glutaraldehyde faster than the hydraulic fracturing-impacted microcosms, showing a decrease in degradation potential after exposure to hydraulic fracturing activity. Hydraulic fracturing-impacted microcosms showed higher richness after glutaraldehyde exposure compared to unimpacted streams, indicating an increased tolerance to glutaraldehyde in hydraulic fracturing impacted streams. Beta diversity and differential abundance analysis of sequence count data showed different bacterial enrichment for hydraulic fracturing-impacted and nonhydraulic fracturing-impacted microcosms after glutaraldehyde addition. These findings demonstrated a lasting effect on microbial community structure and

glutaraldehyde degradation potential in streams impacted by hydraulic fracturing operations.

Introduction

The use of hydraulic fracturing (HF) has grown 702% since 2007.(1) Since 2011, seven shale plays have been responsible for more than 90% of the oil and gas production growth in the U.S. The most productive of these plays is the Marcellus Shale in the northeastern U.S., producing more than 18 000 mcf of natural gas per day.(2) Despite the proposed economic and energy security benefits of HF, many environmental questions and potential unforeseen consequences remain. The exact mixture of chemicals and water (i.e., HF fluids) used in a HF job is proprietary and dependent on company and/or shale play geochemistry. However, HF fluids components often include gelling and foaming agents, friction reducers, cross-linkers, breakers, pH adjusters, corrosion inhibitors, iron-control chemicals, clay stabilizers, surfactants, and biocides.(3) Biocides are added to HF fluids to prevent the corrosion, bioclogging of pipes and equipment, and gas souring that are caused by sulfate-reducing bacteria and acid-producing bacteria. High volumes of HF fluids are injected under great pressure to crack open the shales deep beneath the surface. A portion of this fluid then resurfaces as wastewater, called “flowback” water. This flowback fluid requires special handling and disposal as improper disposal can alter geochemistry and have toxic effects in public and environmental health.(4) Biocides have been identified as some of the most toxic chemical additives in HF fluids.(3,5)

The efficacy of biocides in HF operations is unclear. Previous studies report active and diverse microbial communities in flowback waters despite biocide use.(6−12) Glutaraldehyde (GA) is the most commonly used biocide in HF.(5) There are a number of ways GA can degrade abiotically in the environment. The compound is water miscible and does not tend to bioaccumulate. It hydrolyzes as pH increases, and it can also be photodegraded.(13,14) Previous studies have shown that GA is biodegradable under aerobic and anaerobic conditions, but degradation rates can be affected by concentration, pH, salt, temperature, chemical interactions, and bacterial resistance.(13−17) Under aerobic conditions, GA can be biodegraded to carbon dioxide via glutaric acid, and under anaerobic conditions, the biocide is metabolized to 1,5-pentanediol.(14)Despite its biodegradability, GA is considered acutely toxic to both terrestrial and aquatic organisms, freshwater fish in particular, at concentrations as low as 2.5 mg/L for embryos and 4.7 mg/L in adult fish populations.(18)

To date, our review of the literature suggests that few if any studies have examined the fate of GA in an aquatic environment previously exposed to HF fluids. To address this gap, this study employs a combination of next generation sequencing and detailed chemical analysis. The goal of the study is to understand how GA affects aquatic microbial communities previously

exposed to HF fluids and to measure the degradation of GA in an exposed aquatic system as compared to a nonexposed aquatic system.

Materials and Methods

Stream Selection

Streams were selected using Pennsylvania (PA) Department of Environmental Protection records and GIS surveys. The sampling area was forested, and there were no physical indications of past mining activity prior to HF development in that region. The selected streams had minimum variation in watershed characteristics caused by anthropogenic impacts other than HF. There was no indication of conventional drilling, acid mine drainage, or other industrial activities. Each of the HF-impacted (HF+) streams selected had either a history of surface spills (stream names: Alex Branch (AB) and Little Laurel (LL))(19) or more than 20 well-heads (unnamed tributary (UNT) Naval Hollow (NH)) in the vicinity.(20) In 2009, LL received flowback from a broken pipe for over two months, to a lesser extent AB also received flowback from the same pipe. Furthermore, AB received input from an 8000-gallon spill of water and HF fluids.(21) Each of the HF-unimpacted (HF-) streams, UNT East Elk Fork (EE), UNT West Elk Fork (WE), and Dixon Run (DR), selected as baseline, had HF well construction in its vicinity, but no HF activity had commenced. Refer to Figure S1 for a map of the watersheds' location and refer to the Supporting Information for a description of sample collection.

There was documented use of GA in wells associated with the three HF+ streams selected according to FracFocus.org.(22) Details of the selection of the streams, screening process, collection, and description of the sites have been discussed elsewhere.(23−25) Past studies surveying these and other streams in central and northwestern PA showed that the microbial community composition and indicator taxa can be used to predict HF past exposure, even years after a documented spill.(23,26) Indicator taxa enriched in streams exposed to HF wastewater were also present in streams with adjacent HF operations but no history of spills. This suggests that direct spills are not the only source of HF impacts in the aquatic ecosystem.(23,26) In addition to persistence of microbial indicator taxa, streams in North Dakota impacted by flowback water spills maintained the geochemical and isotopic signatures of the spill for 4 years after documented spills.(27)

Microcosm Setup

Microcosms were established with 260 mL of streamwater, and prior to GA amendment, 25 mL was collected for downstream DNA analyses. The amount of biocides used in HF fluids varies widely between 10 and 800 mg/L.(3) Dow Chemicals has shown a 6-log reduction of acid-producing bacteria and sulfate-reducing bacteria, the standard in the oil and gas industry, at a concentration of 100 mg/L of GA.(28) Thus, the remaining 235 mL of streamwater was amended with 100 mg/L of GA. A 50% solution of GA (CAS

number 111-30-8, catalog number 340855) was bought from Sigma-Aldrich (St. Louis, MO, USA). Abiotic controls were autoclaved prior to GA amendment to measure abiotic biocide degradation. Additionally, biological controls were setup with streamwater and no GA addition to examine bottle effect on the microbial community. Both control sets had a volume of 20 mL. All microcosms were setup in triplicate and incubated for 56 days under minimal light exposure and at ambient temperature. Microcosms were uncovered only for sampling events and were shaken immediately prior to sampling.

Ultra-Performance Liquid Chromatography--High Resolution Mass Spectrometry (UPLC-HRMS)

Abiotic and biotic microcosms were sampled at day 0 before and after amendment of GA and at days 7, 28, and 56. However, the day 0 samples depleted during total organic carbon analysis and were not analyzed by UPLC--HRMS. One mL of water from each microcosm was collected, filtered through a 0.2 μm Sterivex nylon filter, and frozen at −20 °C until analysis at the University of Tennessee's Biological and Small Molecule Mass Spectrometry Core. The samples were diluted 1:10 with HPLC grade water. A 10 μL injection volume of each sample was subjected to UPLC separation (LC Dionex Ultimate 3000) on a Synergi 2.5 μm Hydro-RP 100 Å, 100 × 2 mm column (Phenomenex, Torrance, CA). Solvent A consisted of 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile. The separation gradient featured an initial ramp from 0% to 50% B over 6.5 min, and the conditions were held constant for 1 min. This was followed by a return to initial conditions over 0.25 min and a 3.5 min equilibration at 0% B for a total runtime of 11.25 min. The flow rate was held constant at 300 μL/min. Mass spectra were recorded in positive mode with an Orbitrap Exactive Plus mass spectrometer (Thermo Scientific, Waltham, MA) under the following parameters: Positive-mode heated electrospray ionization, sheath gas flow of 25 units, aux gas flow of 8 units, capillary temperature of 300 °C, aux gas heater temperature of 150 °C, spray voltage of 4.2 kV, ACG target of 3×10^6, resolution of 140 000, and a scan range of 90–300 m/z. GA was detected in positive mode as the [M + H] (m/z = 101.0600) with a retention time of 2.8 min. The GA metabolite glutaric acid (purchased from Sigma-Aldrich, CAS number 110-94-1, catalog number G3407) was measured with an identical instrument and column using an established negative-mode ion-pairing UPLC--HRMS method.(29,30) Concentrations were calculated using the standard curves available in Figures S2 and S3. Average HF+ and HF- concentrations with their respective standard error were reported. Refer to the Supporting Information for a description of GA speciation.

Total Organic Carbon (TOC)

TOC associated with GA was quantified at days 0, 7, and 56 using a Shimadzu TOC-L equipped with an ASI-L autosampler (Shimadzu, Kyoto, Japan). One mL of sample was filtered through a 0.2 μm Sterivex nylon filter

and then diluted 1:25 or 1:10 with DI water acidified to pH 3 with HCl. This released the inorganic carbon present in the samples. Samples were collected prior to GA addition to subtract the background TOC. GA standards were run to calculate TOC associated with GA. Time point 0 sample (after addition of biocide) for the biotic microcosms was depleted during preparation. However, as the same concentration of GA was added to both biotic and abiotic microcosms, the time point 0 TOC measurement for the abiotic microcosms was used to calculate percent loss for both.

16S rRNA Gene Amplicon Library Preparation and Sequencing

To profile the taxonomic diversity and microbial community composition, a marker gene, 16S rRNA, was used. Bacterial community changes can be used as biosensors for contamination even after the contaminants are fully degraded.(31) In the case of these streams, prior exposure to GA may lead to microbial adaptation, which may affect the degradation of GA. To test this hypothesis, 25 mL of water from the GA amended microcosms was filtered for DNA collection to track microbial community changes and perform qPCR for the 16S rRNA gene. Samples were collected prior to GA amendment at day 0 and at days 7, 21, 35, 49, and 56. The no-GA control microcosms were sacrificially sampled at day 56 to perform the same DNA analyses. Refer to the Supporting Information for the extraction protocol and sequencing library preparation. Final pools of 10 nM each were run in an Illumina MiSeq (San Diego, CA) using a v2 kit (2 × 150 reads), according to the manufacturer's manual.

Quantification of Bacterial 16S rRNA Gene

qPCR amplification was performed for days 0, 7, 21, 56, and 56 no-GA using the universal bacterial primers Bac1055YF(32,33) and Bac1392R.(33) The qPCR reactions were performed in a QuantStudio 12K Flex Real-Time PCR system (ThermoFisher Scientific) using the qPCR cycle parameters described in Ritalahti et al.(32) Refer to the Supporting Information for reaction volumes and concentrations.

16S rRNA Gene Amplicon Sequencing Data Analyses

Data analyses were performed using the QIIME pipeline (version 1.9.1) (34) and the Phyloseq(35)package in R.(36) Briefly, the forward and reverse raw reads were joined using the assembler fastqjoin(37) embedded in QIIME. Demultiplexing and quality filtering were performed at an average Q-score of more than 19. The sequences were then chimera filtered using the UCHIME method and applying the USEARCH program.(38,39) Both de novo and reference-based chimera detection were used. For the reference-based detection, the Greengenes database (version May 2013)(40) filtered to a minimum of 97% sequence identity was used. Open reference OTU picking was performed with the command pick_open_reference_otus.py using the UCLUST method(38)and the Greengenes database as described above. Representative sequences for each OTU were aligned using the PyNAST

method,(41) and taxonomy was assigned to each representative sequence using the RDP classifier(42) trained against the Greengenes database. (40,43,44) OTUs were then filtered to remove sequences with counts below 0.005%. The samples were then rarefied to 1220 sequences. Alpha diversity, beta diversity, and DESeq2(45) analyses were performed using an unrarefied OTU table as described in the Supporting Information.

Statistics

Geochemical parameters were compared between HF+ and HF- microcosms using a *t* test. GA degradation over time was compared between HF+ and HF- microcosms to test if degradation rates changed on the basis of impact status. Degradation between biotic and abiotic samples was also compared to test if the main driver of degradation was biotic or abiotic. To do this, the biocide concentration was $\log_{10}$ transformed and a baseline of 100 mg/L was used for day 0. A complete randomized design (CRD) with a split–split plot was used. Impact statuses (HF+ and HF-) were assigned to the whole plot and applied to two levels of conditions (biotic and abiotic) for the sub-plot. Microcosms' samples were taken for measurement at days 7, 28, and 56 (sub-sub-plot). Data was then divided between biotic and abiotic. A CRD with repeated measures was applied to each. The same was performed with glutaric acid concentrations. The mixed effect ANOVA method was employed to analyze the data using SAS 9.4 and least-squares means separated with a Bonferroni method. The alpha level was set at $P = 0.05$. A Pearson correlation of initial pH and GA concentrations was performed for day 56.

The 16S rRNA gene abundance was compared to understand the effect previous exposure to HF fluids has on aquatic microbial community structure after GA addition. This was done using a CRD with split plot using impact status (HF+ vs HF-) as the whole plot factor and time (days) as the split plot factors using a mixed effect ANOVA model (R nlme package(46)). The least-squares means were computed and separated with the Bonferroni method (R emmeans package(47)). 16S rRNA gene copies/mL were $\log_{10}$ transformed to meet normality and variance assumptions for ANOVA. To compare the no-biocide control at day 0 and at the end of the experiment (day 56), the same model was run. To determine differences between HF+ and HF- at day 0, an independent sample *t*test was run with data for only that time point.

Results and Discussion

The objective of this study was to understand the lasting effect of HF impacts on the biocide resistance and degradation potential of surface water microbial communities. To do this, GA, the most common biocide used in the HF industry, was added to microcosms of water from streams impacted and not impacted by HF as determined by previously published studies. (23−25,48)

Physiochemical Parameters of Stream Water in Situ

Temperature, pH, conductivity, and total dissolved solids were measured at the time of sample collection, and results are shown in Table S1. HF+ streams had an average temperature of 16.8 °C, and HF- streams had an average temperature of 12.8 °C. HF+ streams had an acidic pH averaging 4.9, while HF- had a neutral pH of 6.5. The average conductivity for HF+ streams was 29.2 µS/cm and for HF- streams, 33.7 µS/cm. Finally, the average total dissolved solids for HF+ was 20.8 ppm and for HF-, 23.9. There were no statistically significant differences in the physiochemical parameters between HF+ and HF-.

HF is the most common method used in unconventional oil and gas (UOG) extraction. It is worth noting that others have documented higher conductivity in surface waters impacted by UOG activity,(49) as UOG wastewaters are high in salinity.(6,12) The streams described by Akob et al. (49) were impacted by their proximity to the UOG wastewater disposal facility, which suggests that the high salinity could have been caused by either a recent spill or constant inflow of wastewater to the streams. In that study, the pathway of contaminants to disposal facility could not be assessed. However, a 5 year-long study of these 6 streams and others in northwestern PA consistently showed that pH was the only statistically different measured parameter between the impacted and not impacted streams,(26) indicating that a one-time spill is not enough to alter conductivity for a long time as input waste is diluted over time.

GA Abiotic and Biotic Degradation Over Time Measured with UPLC--HRMS

It was observed that abiotic degradation of GA was negligible and independent of HF impact status and the difference in GA concentrations between HF+ and HF- abiotic microcosms through time was not statistically significant. The final concentration of GA in the abiotic HF+ control was 101.9 ± 4.2 and 106.79 ± 5.1 mg/L in abiotic HF- control (Figure 1a). Additionally, biotic degradation of GA was detected in both HF+ and HF- microcosms. The final concentration of GA in the HF+ biotic microcosms was 47.3 ± 5.2 mg/L, and in the HF- biotic microcosms, it was 31.7 ± 3.8 mg/L. The difference in degradation over time was found to be statistically significant ($P < 0.05$). The HF- communities degraded GA faster by day 56, a 68.3% removal of GA with half-life of 33.8 d, while HF+ experienced a 52.7% removal with half-life of 51.9 d.

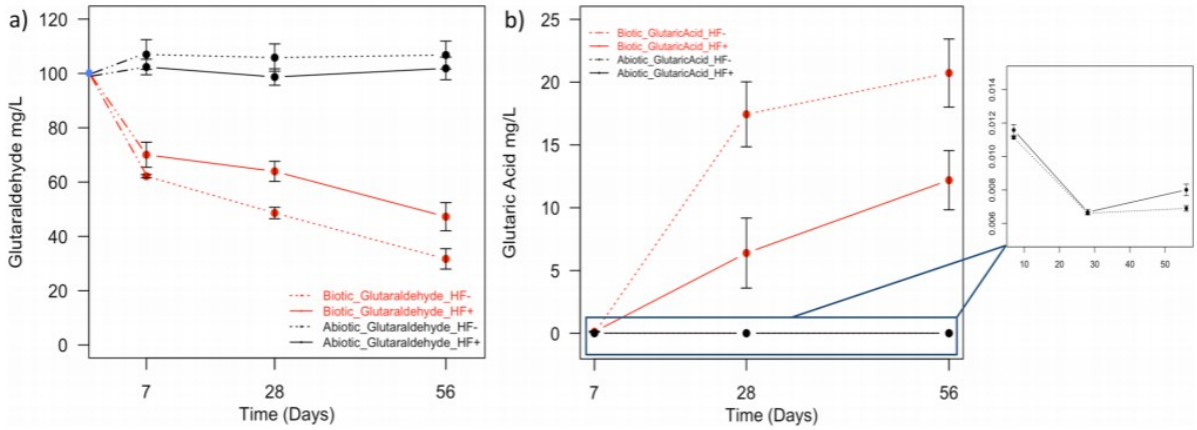


Figure 1. Biotic and abiotic degradation of glutaraldehyde and glutaric acid production over time. (a) Biotic and abiotic degradation of glutaraldehyde in HF+ and HF- microcosms. The blue dot represents the added amount of GA, 100 mg/L. (b) Biotic and abiotic production of glutaric acid in HF+ and HF- microcosms; the zoom in graph shows abiotic concentration over time. Error bars represent one standard error (n = 9).

Glutaric acid is a known degradation product of the oxidation of GA.(13) Glutaric acid was produced in the microcosms, validating the GA degradation measurements. Minimal production of glutaric acid was observed in the abiotic microcosms, with pronounced production in the biotic microcosms (Figure 1b). By day 56, abiotic HF+ microcosms produced 8.0 ± 1.0 µg/L of glutaric acid and abiotic HF- microcosms produced 6.9 ± 0.5 µg/L. This difference was statistically significant ($P < 0.05$). Meanwhile, by day 56, 12.2 ± 2.4 mg/L of glutaric acid was produced in the HF+ biotic microcosms and the HF- biotic microcosm produced 20.7 ± 2.7 mg/L. The difference between the abiotic and biotic glutaric acid production and the difference between biotic HF+ and biotic HF- glutaric acid production over time were also statistically significant ($P < 0.05$). The steady increase of glutaric acid in the biotic microcosms as compared to the abiotic microcosms shows that the main pathway of GA depletion after day 7 is microbially mediated.

Other studies have shown abiotic degradation of GA in oxic and anoxic conditions, but their experimental conditions included soil, where GA can be lost to sorption.(14,16) However, in this study, the rate of biotic degradation in both HF+ and HF- microcosms was slower than the rates reported in the review by Leung.(14) Leung describes the degradation of lower concentrations (0.9 to 50 mg/L) than the study described here (100 mg/L), and GA degradation was indirectly quantified in the review using oxygen, carbon dioxide, or dissolved organic carbon measurements as proxies for GA degradation.(14) Leung reported a variable GA half-life of 0.4–24 d, due to enhanced microbial inhibition at higher GA concentrations, which increases the half-life of GA. Another study measuring the biodegradation of GA in combination with 5 other HF chemicals also showed an increase in GA half-life at increasing concentrations.(16) In that study, microcosms containing 100 mg/L of GA did not experience more biodegradation than the abiotic controls, indicating complete microbial inhibition, with an extrapolated half-

life of more than 93 d. The addition of 5 other HF chemicals could have exacerbated microbial toxicity, particularly as the inoculum in those microcosms came from pristine soil with no previous exposure to HF chemicals.(16) In this study, the HF+ source water had prior exposure to HF, and there was no competing chemical interactions or toxicity to inhibit microbes other than GA.

GA Associated TOC in Biotic and Abiotic Microcosms

It was observed that TOC decreased in the first 7 days (day 0 to 7) for both abiotic and biotic microcosms (Figure S5). After the initial TOC reduction, abiotic microcosms stayed constant, and by day 56, there was 8.64% removal in HF+ and 7.04% removal in HF-. In contrast, the biotic microcosms observed a higher TOC removal by day 56, 57.06% removal in HF+ and 62.81% removal in HF-. These findings agree with the trends observed with direct GA and glutaric acid measurements by UPLC--HRMS, showing a pronounced difference between biotic and abiotic degradation and HF- microcosms degrading GA faster than HF+.

The decrease in TOC after GA addition may suggest a decrease of GA in the first 7 days in both biotic and abiotic microcosms. After 7 days there was no decrease in the abiotic samples. This correlates to what McLaughlin et al. (15) observed in their microcosms with agricultural topsoil and synthetic surface water. However, they attributed this effect to GA absorption into the soil, by either physiosorption or chemisorption. Because the microcosms described here did not have sediment as a confounding variable, it is likely that the observed initial depletion was from less prominent reversible GA hydrates forming in solution (Table S5). The results indicate that GA persists longer in a sediment free aquatic environment than in a sediment–water matrix such as the one described by McLaughlin et al.(15) as their reported half-life for GA was 10 d. Previous HF impacts may increase GA persistence in the environment.

qPCR

The abundance of 16S rRNA genes was determined from initial samples before addition of GA (Figure 2). All the pre-GA treatment 16S rRNA gene concentrations were on the order of 10^4 gene copies/mL, averaging 4.03×10^4 gene copies/mL in the HF+ streams and 4.38×10^4 gene copies/mL in HF- streams. The difference between HF+ and HF- was not statistically significant. Seven days after addition of GA, 16S rRNA gene copy number observed a $\log_2$-fold change (FC) in all microcosms independent of HF impacted status. HF+ microcosms showed a smaller change with an average of -2.92 $\log_2$ FC compared to -4.62 $\log_2$ FC in HF- microcosms. However, by day 21, the bacterial population recovered, returning to the original order of magnitude and with all streams surpassing the original gene copies except for NH (HF+) and DR (HF-), which were slightly lower. HF+ streams had an average of -0.45 $\log_2$ FC from the original gene copies/mL on day 21, while HF- streams had surpassed the original concentration with an average of

0.56 log_2FC. Finally, by day 56, all of the microcosms underwent 16S rRNA gene enrichment, exhibiting a higher enrichment on HF- microcosms. Additionally, HF+ microcosms underwent a 4.79 log_2 FC from day 0, while HF- was 7.18 log_2 FC. The difference through time (day 7 to 56) between HF+ and HF- was statistically significant ($P < 0.05$). In contrast, at day 56, the no-GA controls had a similar log_2 FC, independent of previous HF status. No-GA HF+ microcosms had an average of 8.23 log_2 FC while no-GA HF- has an 8.34 log_2 FC, which was not statistically significant. When the same time points were compared, microcosms with no GA had higher 16S rRNA gene copies/mL at day 56 than the GA-amended microcosm. This can be attributed to the GA-free microcosms not experiencing inhibited growth and having sufficient nutrients from the source water to promote growth. Thus, without GA addition, the biomass of the microbial communities increased to the same final gene copies/mL, showing that the difference in gene copies/mL between the GA-amended HF+ and HF- microcosms can be attributed to the microbial community response to GA.

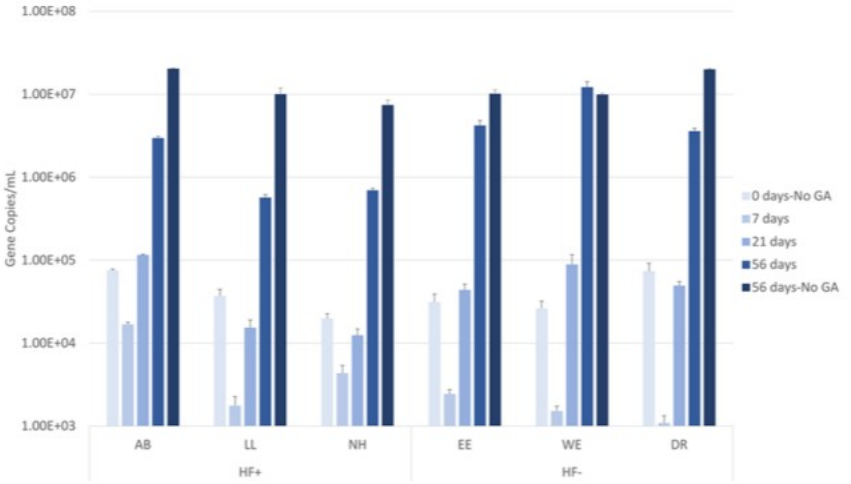


Figure 2. Impacts of glutaraldehyde in abundance of 16S rRNA gene over time. The first three clusters are the HF-impacted streams, and the last three clusters represent the non-HF-impacted streams. Data point “56 days-No GA” represents bottle effect on the microcosms as no GA was added. Error bars represent one standard error ($n = 3$).

Quantification of the 16S rRNA gene also showed that HF+ microcosms were able to tolerate and resist the biocide better than HF- microcosms at day 7, the critical response phase to GA biocidal action (Figure 2). However, both HF+ and HF- microbial communities recovered rapidly after 21 days suggesting adaptation by certain microbial populations and enrichment of those microbes able to tolerate and resist GA in both the HF+ and HF- water, especially as GA’s concentration decreases over time. Furthermore, the differences in 16S rRNA gene copies over time showed that HF+ and HF- microcosms had a distinct adaptation and tolerance to GA.

Microbial Community Changes Between HF+ and HF- Over Time

Richness, as measured by Shannon, Observed Species, and Chao1, showed that before GA amendment HF- streams were more diverse than HF+ streams ($P < 0.05$) (Figure 3) while the difference was not significant for Simpson alpha diversity measurements. Seven days after addition of GA, HF+ maintained higher richness and evenness than HF-, a significant trend observed with Chao1, and Observed diversity measurements ($P < 0.01$ through the duration of the experiment) but not with Simpson and Shannon. The interaction between impact status (HF+ and HF-) and days was not significant. A comparison of no-GA control microcosms at day 0 and day 56 showed that there were no significant changes in alpha diversity (Observed, Chao1, and Simpson) over time except with Shannon diversity ($P < 0.05$). Thus, the control (no GA added) at day 56 maintained high diversity, comparable to the diversity before GA addition, independent of HF-impact status. This shows that the diversity differences observed after GA addition are not confounded by the bottle effect.

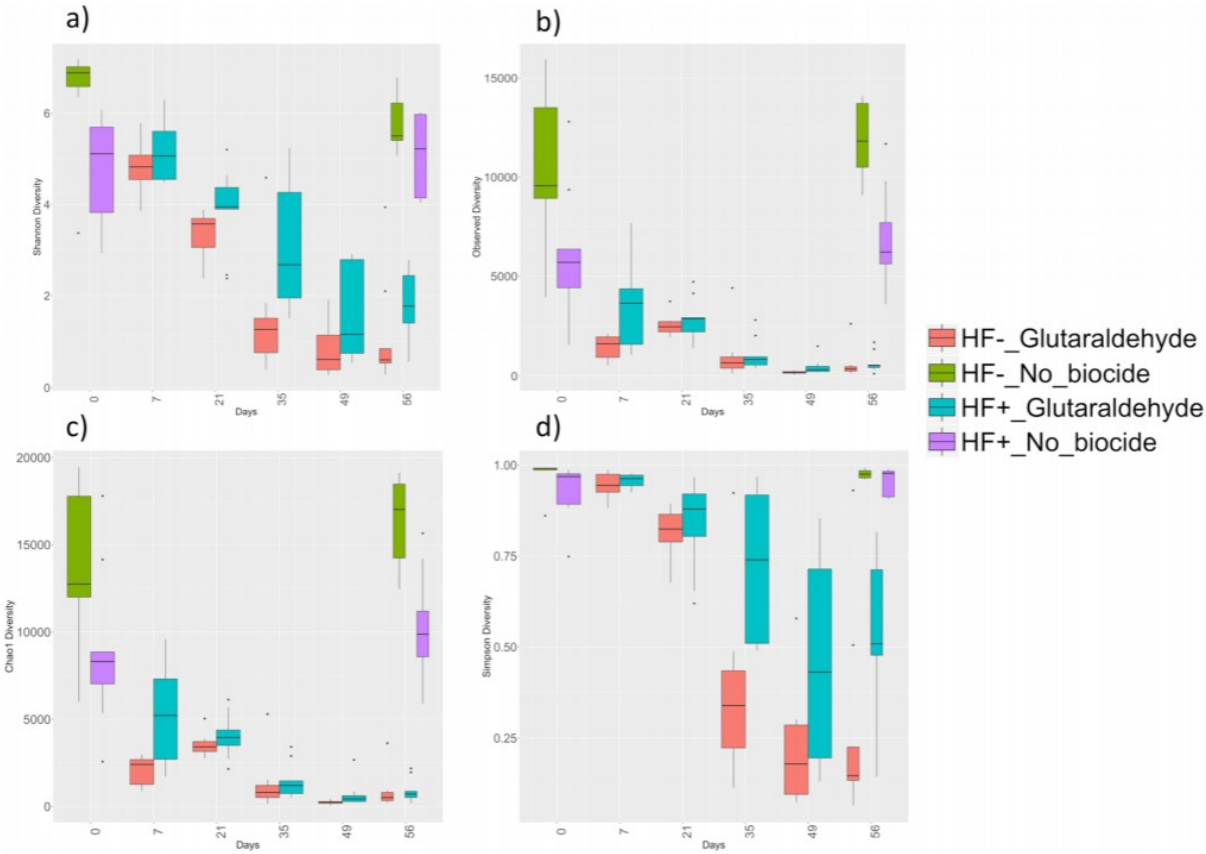


Figure 3. Alpha diversity measurements over time. Different richness and evenness alpha diversity estimators comparing HF+ and HF- microcosms over time; the estimators used were (a) Shannon Diversity, (b) Observed Diversity, (c) Chao1, and (d) Simpson Diversity. Red and green box plots represent HF+ glutaraldehyde (days 7 to 56) and no glutaraldehyde added (days 0 and 56 only). Blue and purple box plots represent HF- glutaraldehyde and no glutaraldehyde added. The box plots show the distribution of the data points: upper whisker to the beginning of the box is the first quartile, beginning of box to median represents the second quartile of the data, median to end of box is third quartile, and end of box to lower whisker is the fourth quartile.

The overall alpha diversity found in this study's HF- microcosms was higher than the HF+ microcosms preamendment of GA. This is in agreement with the in situ study that examined these streams and other streams in the region.(26) After amendment of GA, HF+ microcosms maintained higher richness than HF- streams when calculating diversity with metrics that focus on unique OTUs (Observed) and the importance of rare OTUs (Chao1), whereas evenness seems to be decreasing through time as a couple of taxa dominated over time in both HF+ and HF- microcosms as seen by similar Simpson and Shannon diversity trends between the groups (Figure 3). High diversity and richness in a community after a perturbation is a sign of adaptation to chronic exposure to perturbations.(50) This shows that more unique members of the HF+ microbial community were able to tolerate and resist the biocide than HF- microbial communities.

Beta diversity was calculated using weighted UniFrac distance matrix. Data was ordinated using a Principal Coordinate Analysis (PCoA) as described in SI methods. Clustering by PC 1 explains 65.4% of the variation in the microbial community, while clustering by PC 2 explains 10%. Results showed a visible clustering by days and impact status (HF+ and HF-) in the GA added microcosms by both PC1 and PC2, while the no-GA microcosm mostly clustered by PC 2 (Figure 4). Statistically significant differences were observed between HF+ and HF- microbial communities ($P < 0.01$), treatments (GA vs No Biocide with $P < 0.001$), treatments through time ($P < 0.001$), the interaction between impact status (HF+ and HF-) and treatments ($P < 0.02$), and the interaction between impact status, treatments, and days ($P < 0.03$). Results showed that the microbial community response to the biocide in these microcosms included phylogenetically distinct organisms based on previous exposure to HF activity.

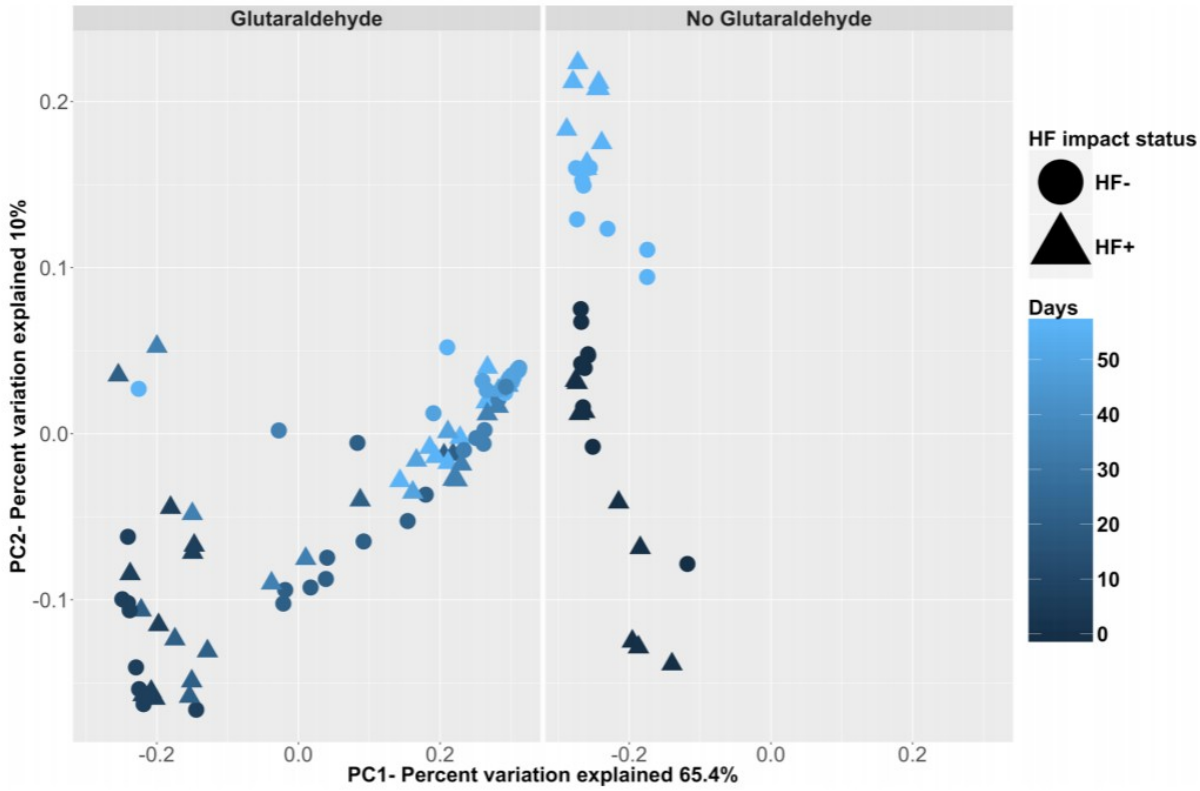


Figure 4. Principal coordinate analysis (PCoA) plot of phylogenetic microbial community changes over time in HF+ and HF- impacted microcosms amended or unamended with glutaraldehyde as described by weighted Unifrac beta diversity measurements. PC1 explains 65.4% of the variation while PC2 explains 10%.

Differentially Enriched Taxa Over Time and in HF+ and HF- Microcosms

Overall, many members of the original microbial community in HF+ and HF- microcosms were not able to tolerate GA over time as seen by a decrease in diversity (Figure 3) and by an increase in differentially abundant OTUs between day 0 and the next 4 sampling events (days 7, 21, 35, and 49). By the last sampling event, day 56, the number of differentially abundant OTUs decreases, a sign of population resilience, and/or GA reaching concentrations below inhibition level.

Specifically, 7 days after addition of GA, 239 OTUs were differentially enriched. Twenty-seven OTUs experienced a positive $\log_2$ FC while 212 OTUs experienced a negative $\log_2$ FC and, hence, were inhibited by exposure to GA. The highest $\log_2$ FC corresponded to an OTU identified as the genus *Myroides* (19.09 $\log_2$ FC), followed by OTU identified as *Robinsoniella* (18.64 $\log_2$FC). Interestingly, 6 OTUs corresponding to the marine clade SAR406 were also enriched (all corresponding to Family A714017 but different or unclassified genus). However, all of these enriched OTUs were in low abundance (<2%) except for *Alcanivorax* (2.77 $\log_2$ FC). There were 71 differentially enriched OTUs between HF+ and HF- prior to the addition of GA. Seven days after addition, only one OTU was differentially

enriched between HF+ and HF- identified as *Psychroserpens* (7.80 $\log_2$ FC). However, it was at low abundance (below 2%). By day 21, there were 315 OTUs differentially enriched as compared to the original pre-GA population. Eight OTUs were enriched at this time point. The only OTUs with abundance of more than 2% of the population were *Idiomarina* (4.90 $\log_2$ FC), *Methylobacterium* (2.78 $\log_2$ FC), and *Bacillus* (2.06 $\log_2$ FC). There were not significant differences in enrichment between HF+ and HF- that passed the stringent 2 $\log_2$ FC cutoff that was imposed.

By day 35, there were 407 OTUs differentially enriched as compared to the original, day 0 microbial population. These OTUs were classified as *Amphritea* (5.29 $\log_2$ FC), *Methylobacterium*(5.19 $\log_2$ FC), and *Beijerinckia* (3.23 $\log_2$ FC). Three OTUs were differentially enriched in HF+ vs HF- at day 35. The genus *Acinetobacter* had a 3.60 $\log_2$ FC in HF-, while *Beijerinckia* and *Janthinobacterium* had an 8.17 and 3.94 $\log_2$ FC, respectively, in HF+. By day 49, there were 419 differentially enriched OTUs as compared to the pre-GA microbial population. Only four OTUs were positively enriched at day 49; those OTUs correspond to *Myroides* (14.00 $\log_2$ FC), *Robinsoniella* (10.61 $\log_2$ FC), *Methylobacterium* (6.02 $\log_2$ FC), and *Beijerinckia* (2.93 $\log_2$ FC). One OTU was differentially enriched in HF+ vs HF- at day 49. The genus *Beijerinckia* had an 8.97 $\log_2$ FC in HF+ as compared to HF-.

By day 56, there were 174 differentially enriched OTUs. Of those, 66 were enriched in day 56 as compared to day 0. The ones with more than 2% abundance were *Methylobacterium* (12.19 $\log_2$FC), *Beijerinckia* (10.20 $\log_2$ FC), *Mycobacterium* (7.81 $\log_2$ FC), *Alcanivorax* (5.74 $\log_2$ FC), *Stenotrophomonas* (5.24 $\log_2$ FC), *Bacillus* (3.48 $\log_2$ FC), *Idiomarina* (3.28 $\log_2$ FC), and *Burkholderia* (3.04 $\log_2$ FC). Only one OTU identified as the genus *Beijerinckia* (9.36 $\log_2$ FC) was enriched in HF+ microcosms as compared to the HF-. Day 56 GA-microcosms were also compared to no-GA microcosms at day 56. There were 263 enriched OTUs, of those 44 were enriched in the GA microcosms: *Methylobacterium* (10.31 $\log_2$ FC), *Alcanivorax* (5.81 $\log_2$ FC), *Mycobacterium* (5.67 $\log_2$ FC), *Beijerinckia* (5.21 $\log_2$ FC), *Idiomarina* (4.42 $\log_2$ FC), and *Bacillus*(3.13 $\log_2$ FC); day 0 and day 56 no-GA microcosms were also compared to see how the community changed over time due to bottle effect. There were 209 differentially enriched OTUs. It is worth noting that *Bacillus* (-2.77 $\log_2$ FC) and *Idiomarina* (-5.10 $\log_2$ FC) were suppressed at day 56 no-GA as compared to day 0 and that *Myroides* (5.09 $\log_2$ FC) experienced an enrichment.

These enrichments over time suggest which OTUs were driving the response to GA. *Alcanivorax*was a dominant first responder, and after an adaptation period, *Idiomarina*, *Methylobacterium*, and *Bacillus* responded as well. *Methylobacterium* differential enrichment continued until the end of the experiment, dominating in abundance (71% in HF+ and 84% in HF- microcosms at day 56, Figure S6B), indicating that it was able to adapt to GA

presence and dominate. It is worth noting that it was not enriched right after GA addition, possibly indicating that a lag period was needed for adaptation. By day 35, other than *Methylobacterium*, *Beijerinckia* is worth highlighting, as it was preferentially enriched in HF+ microcosms. The trend of *Methylobacterium* and *Beijerinckia*continued until the end of the experiment. In addition, by day 56, *Alcanivorax* and *Idiomarina* were enriched when comparing both day 56 with day 0 no-GA and with day 56-no-GA.

Studied members of the enriched genus can provide better understanding of the interactions at play. *Alcanivorax* are commonly found in hydrocarbon-impacted marine environments and have been observed to degrade alkanes and other hydrocarbons and use them as their sole carbon source,(51) and the alkane degradation pathway employs aldehyde dehydrogenases, (52,53)which may help this genus thrive and possibly help degrade GA. Furthermore, isolated strains of *Alcanivorax* spp. were shown to be resistant to antimicrobials by the use of efflux pumps,(54)which could also facilitate tolerance for GA. *Idiomarina* is frequently detected in hydrocarbon-rich environments such as oil spills;(51) HF produce water and flowback,(9) but their role and/or mechanisms in hydrocarbon degradation is unknown. It is possible that enrichment of *Idiomarina*is also associated with the aldehyde dehydrogenases. *Alcanivorax* and *Idiomarina* are members of the *Gammaproteobacteria* class, which observed enrichment after a week of exposure to GA (Figure S6B); enrichments of this class have been observed in aquatic environments after perturbations from hydrocarbon sources, sewage runoff, antimicrobials, and other anthropogenic sources.(55) Most of the enriched *Gammaproteobacteria* families are known to be halotolerant such as *Alteromonadaceae*,(56) *Pseudoalteromonadaceae*, (57) *Alcanivoracaceae*,(58)*Idiomarinaceae*,(59) and *Halmonadaceae*(60) (Fig ure S6B). Moreover, Vikram et al.(11) showed that genes needed for responding to osmotic stress, membrane integrity, and protein transport are up-regulated when the bacteria are exposed to HF produced and flowback water, and this up-regulation was correlated with increased bacterial tolerance to biocide exposure. Another recent study indicated that, in pathogens, GA resistance can be mediated through an increase in efflux pumps, which will increase the rate of export of the biocide.(61) It has also been reported that efflux pump encoding genes increase in downstream UOG impacted surface water, which may be a bacterial response mechanism to stress caused by HF chemicals and high salinity.(62) This could help explain why *Gammaproteobacteria* associated with saline aquatic environments are enriched after GA addition, since the mechanisms to control osmotic stress might be a key genetic trait for microbial survival against GA.

These microcosms experiments did not explore the impacts of high salinity with respect to microbial response and degradation of GA. High salinity might affect the tolerance to GA as shown by Vikram et al.;(11) however, as

shown by this work, higher tolerance does not translate to higher degradation. Another study showed inhibited biotic degradation of GA in a mixture with 30 000 mg/L NaCl and two other HF chemicals on agricultural top soil as compared to GA alone, while the abiotic degradation of the GA, NaCl, and HF chemicals was faster than GA alone.(15)Degradation of low concentration (1.5–3.0 mg/L) GA has also been shown in seawater and its native organisms.(14) Halotolerant microbes seem to be able to degrade GA; however, it is unclear how salt would affect degradation rates in freshwater streams in the case of a HF fluid spill containing GA and the high salt content associated with HF flowback.

The increase in *Alphaproteobacteria* (accounting for more than 90% of the microbial community in the microcosms after day 49 of GA amendment, Figure S6B) as the microbial system adapted to the GA perturbation suggests that this bacterial class is better at tolerating the GA as a stressor in the long term compared to *Beta* and *Gammaproteobacteria*. *Alphaproteobacteria* are known to experience horizontal gene transfer more frequently than other *Proteobacteria*, and their extensive genomes are known to have a larger number of mobile elements.(63) This may contribute to the higher "memory effect" or adaptation detected in the HF+ aquatic microbial community with genetic material being shared between the sediment's sessile microbial community, the epilithic bacteria from rocks, and the free-floating microbes collected for the microcosm setups. (64)Moreover, *Alphaproteobacteria* are Gram-negative and, therefore, are known to be more resilient to antimicrobials because of their outer membrane, as compared to Gram-positive bacteria.(65)The genus *Methylobacterium* was the most abundant *Alphaproteobacteria* in both HF+ and HF- streams; however, it is more dominant in HF- streams, representing 84% of the population by day 56. The Family *Methylobacteriaceae* are commonly found in the environment growing on single carbon compounds, the microbe's sole energy source, in addition to more complex carbon compounds.(66) Enrichment of methylotrophs has also been observed in studies pertaining to triclosan and quaternary ammonium antimicrobials and other environmental pollutants like hydrocarbons and chlorinated compounds as these bacteria are able to cometabolize these pollutants through the production of methane monooxygenase.(67,68) However, *Methylobacteriaceae* response might be antimicrobial specific and dependent on oxygen availability as a study utilizing anaerobic microcosm inoculated with UOG impacted and unimpacted sediment described a significant decrease in abundance of after the addition of the biocide DBNPA.(69) Another interestingly enriched *Alphaproteobacteria* was the genus *Beijerinckia*, preferentially enriched in HF+ microcosms. These genera are members of the order *Rhizobiales,* which has similarly been detected in streams adjacent to UOG disposal facilities.(49)Isolated members of this genera have been shown

to be nitrogen-fixing, nonsymbiotic, chemo-heterotrophic bacteria capable of degrading recalcitrant aromatic compounds because of their methanotrophic capabilities.(70)

Overall, the microbial communities of HF+ and HF- microcosms had different phylogenetic responses to the addition of GA even though *Methylobacteriaceae* was the most dominant taxa in both. The phylogenetic differences are driven by lower abundance microbes (Tables S5–S16) that respond to GA based on past HF activity exposure. HF- had a more prominent negative response to GA, as seen by biomass and richness loss. This suggests HF fluids exposure causes different microbial responses and adaptation to the biocide GA.

A long list of studies have described the adaptation of microbes to chemical stressors, which they then use as energy sources or acquire the ability to cometabolize.(71) An increase in this effect has previously been observed in ecosystems that were exposed to contaminants;(72) however, adaptation did not provide a degradation advantage to GA in the HF+ microcosms. This suggests the difference in degradation rates might not be biotic alone but rather driven by abiotic–biotic interactions. HF- microcosms source water had a more neutral pH (average pH = 6.5) compared to the acidic pH of the three HF+ streams (average pH = 4.9), and the pH was negatively correlated (Pearson = −0.83) to the concentration of GA at day 56. Thus, higher pH experienced more biodegradation of GA. Higher pH difference has the potential to affect the availability of reactive sites in the microbial cell walls surface, causing a faster biocidal effect (and a faster depletion/deactivation of GA).(73) These factors may explain why GA decreases more rapidly over time in the HF- streams. The site with the most GA depletion by day 56 was EE (Table S3), the HF- stream with the highest pH (pH = 7.3).

However, the microcosms did not maintain constant pH over the incubation period; independent of HF impact status, source water location, or biotic or abiotic conditions, all of the microcosms pH increased over time (Table S4). While GA is more stable at lower pH, its bactericidal properties are impaired in acidic environments where there are fewer available active sites on the cell wall. This effect of pH will require more future studies, but it is still our hypothesis that it is affecting GA degradation in a number of ways.

As explained above, the microbial community from HF+ microcosms was shown to better tolerate the biocide; however, it did not degrade the biocide faster than HF- microcosms. Therefore, further studies of the microbial mechanisms driving biodegradation and adaptation to GA at varying pH and salinity are needed to better understand the nuances of the abiotic–biotic interactions and microbial genetics driving GA biodegradation.

Environmental Implications of This Study

This study shows that there are long lasting effects in streams impacted by HF, which need to be considered for environmental impact assessment and

bioremediation strategies. Abiotic factors such as acidified pH may affect the microbial community's ability to respond to a second or continuous exposure to HF waste, causing HF chemicals to be more persistent in the environment than expected. As HF practices keep expanding worldwide, this knowledge can help bioremediation efforts to optimize natural attenuation and aid HF companies to make better decisions about amendments to use in HF fluids.

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