Environmental impacts of biocides used in hydraulic fracturing

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DEDICATION

To my parents, Irasema Ayala de Campa and Gerardo Campa, and my husband Özgür Özmen. Without their love and constant support, I would not be here. ¡Lo logramos familia!

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ABSTRACT

The goal of this dissertation was to better understand the environmental impacts of biocides used in unconventional oil and gas (UOG) practices. Specifically focusing on how industrial biocides may impact aquatic microbial communities, biocide degradation potential, and contribute to antimicrobial resistance propagation. Recently, the energy sector has seen a stark increase in biocide use, due to the dramatic growth in hydraulic fracturing (HF) operations. Biocides in HF are used to suppress microbial-induced corrosion, biofouling, and hydrogen sulfide production. The implications of biocide usage expansion, its impacts to antimicrobial resistance, and to environmental and public health risks are not fully understood.

To understand these knowledge gaps, microcosm-based studies were used to investigate the effect of biocide addition to both HF-impacted and unimpacted streams. The two most common HF biocides, glutaraldehyde and 2-2-dibromo-3-nitrilopropionamide (DBNPA), were used in two otherwise identical experiments. Degradation of the biocides and microbial community changes were measured over time. Results suggest that glutaraldehyde is more persistent in stream waters previously impacted by HF. However, the microbial community was able to tolerate it as shown by higher microbial diversity and biomass. The DBNPA microcosms experiment showed that previous HF impact, associated with higher total organic carbon, favors a less toxic and persistent DBNPA degradation pathway. Many unidentified brominated species were detected in both HF-impacted and unimpacted conditions. Whole genome sequencing of strains belonging to environmentally relevant genera enriched during the biocide microcosm and isolated in biocide plates were investigated to find relevant genes correlated with DBNPA resistance. Thirteen orthologous genes with predicted functions such as mobile elements (recombinase and terminase), efflux pumps, and possible enzymatic deactivation of the biocide were found. Finally, the knowledge gaps that need to be addressed to perform a risk assessment of antimicrobial resistance caused by biocide usage in UOG production were identified and discussed. This work should help oil and gas operators, environmental response teams, and regulators reach convergence about the risks and aquatic microbial community response to biocides in UOG production and help with preventive strategies and better formulations to minimize the effect this practice has in the environment.

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CHAPTER 1 INTRODUCTION

Background

Biocides are contaminants of concern in the unconventional oil and gas (UOG) industry. UOG is the extraction of hydrocarbon from low permeability strata, such as shale. UOG extraction is primarily done with hydraulic fracturing (HF) and horizontal drilling. HF is the method of extracting natural gas and oil from low permeability rocks using a mixture of more than 10 million liters (per horizontal well) of pressurized water, chemicals, and sand to create small fractures that release oil and gas from rock pockets[1]. In the U.S., this method has grown 702% since 2007 [2]. HF and horizontal drilling opened access to many previously unreachable hydrocarbon reserves, lowering gas prices in the U.S. and helping the country become the biggest hydrocarbon producer in the world [3]. The U.S. Energy Information Administration (U.S. EIA) forecasts that natural gas production in the U.S. will increase 29% between 2010 and 2035, and shale gas will account for 47% of total U.S. gas production [4]. Furthermore, the U.S. is now producing more natural gas than what it consumes, getting the country a step closer to energy independence[4].

Amid the proposed economic and energy security benefits of HF, many environmental questions and potential unforeseen environmental consequences remain. Reports of drinking water contamination with methane [5], elevated benzene content in groundwater [6], methane fugitive emissions and high greenhouse-gas footprint [7, 8], and limitations with waste water handling [9], among others have been increasing topics of concern. Understanding the impacts of surface spills, leaks, and disposal of ineffectually treated HF wastewater in stream-water and the environment is of extreme concern as there have been reported cases of the accumulation of toxic materials in groundwater, streams, soils, and sediments in some disposal sites[6, 10, 11].

HF fluid's chemical composition is proprietary and is company and basin dependent. However, gelling and foaming agents, friction reducers, crosslinkers, breakers, pH adjusters, corrosion inhibitors, iron control chemicals, clay stabilizers, surfactants, and biocides tend to be the main components [12]. Furthermore, biocides have been listed as one of the main chemicals of concern based on their toxicity and potential impact to the environment [1, 12]. Biocides are added to HF fluids to inhibit corrosive bacteria, bioclogging, and biofouling, which could cause equipment damage and/or failure and reduce the quality of the extracted hydrocarbons[13].These

biocides particularly target acid-producing bacteria (APB) and sulfate-reducing bacteria (SRB), which are both involved in microbial induced corrosion and souring of gas and oil [14]. In addition to potentially causing equipment failure and environmental contamination, the byproduct of this metabolism of SRB is hydrogen sulfide – a toxic gas – which may cause occupational safety and health concerns when fluids flow back to the surface[1].

Biocides are used in many other industries besides oil and gas. They are also used in animal husbandry, food production and packaging, hospitals, and industrial water systems, and are added to many consumer goods, including cosmetics, detergents, and paints [15-17]. There is growing concern over the use of biocides due to the link between biocide resistance and antibiotic resistance[18]. Previous studies have demonstrated a link between exposure to household biocides and development of antibiotic resistance[19-21]. However, there is sparse work on the impact of industrial biocides on environmental microbial communities.

Environmental microbes can be exposed to these industrial biocides through accidental releases of industrial wastewater or through other secondary routes. Due to the nature of biocide exposure to environmental microbial communities, the exposure is often at sublethal concentrations, which has been shown to select for resistance to these biocides and could potentially contribute to antimicrobial resistance in the environment[18, 22]. The available literature shows a wide discussion of the fate and possible environmental impacts of some of these practices. However, out of all these areas for biocides' industrial application, the energy sector has the fastest growing demand, because of the increase in HF operations[23].

The conditions within each gas harboring shale formation in the U.S. are different; thus, the use of biocides is dependent on the geological conditions, temperature, pressure, and biogeochemistry of the formation. Microbial dynamics are crucial in choosing the biocide, or combination of biocides, needed in the specific location. To determine which biocide to use, a 6-log or greater reduction should be observed for bacteria of concern, such as SRB and APB as per NACE TM0194 [24]. However, increasing reports have shown that biocide usage is not being optimized on a per well basis, and there is a reported active and diverse microbial community pre and post drilling[13, 25-28].

A number of different compounds are routinely used as biocides in HF operations, but glutaraldehyde (GA) and 2,2-dibromo-3-nitrilopropionamide (DBNPA) are used in over 50% of all HF operations that employ biocides[1]. GA and DBNPA are electrophilic biocides. The electron accepting aldehyde group in GA inhibits bacteria by reacting and damaging membrane proteins on the cell walls[29, 30]. DBNPA inhibits essential biological functions by reacting with sulfur-containing nucleophiles in the cell's components [1]. These biocides can reach the environment through a number of way: (1) surface spills into soil, surface water, and aquifers; (2) incomplete removal after water treatment; (3) groundwater contamination after equipment failure (leakage), and (4) unintended fractures or abandoned wells[1]. Furthermore, biocides reaching the environment could also occur at any point during the HF operation process such as: (1) the transportation of chemicals to the site; (2) mixing of HF fluids and chemicals on site; (4) injection of the HF fluids; (5) handling, collection, and storage of produced water; and (6) disposal of the produced water[31].

The fate and transport of the biocide depends on the environment conditions. Degradation of biocides can be classified as abiotic degradation or biotic biodegradation. Abiotic degradation of biocides can occur through hydrolysis, direct or indirect photolysis, and other chemical reactions with and without oxygen. Both temperature and pressure have been shown to play a role in dictating the rates of abiotic degradation. Biodegradation of biocides can also occur in water and sediments catalyzed by both aerobic and anaerobic microbes[1].

GA is water miscible and does not tend to bioaccumulate. Its hydrolysis half-life is pH dependent, having a shorter half-life at a more basic pH. GA can also be photo-degraded. Previous studies have shown that GA is biodegradable under aerobic and anaerobic conditions [32]. Despite its biodegradability, it is considered to be acutely toxic to both terrestrial and aquatic organisms – freshwater fish in particular. Under aerobic conditions GA can be biodegraded to carbon dioxide via glutaric acid oxidation to either alpha-hydroxyglutaric acid or beta-ketoglutaric acid, and under anaerobic conditions the biocide is metabolized to 1,5-pentanediol [32].

DBNPA is commonly used in water-cooling systems and paper manufacturing. It is somewhat water miscible with a solubility of 15,000 mg/L. Abiotic degradation of DBNPA can

occur through hydrolysis as well as photolysis. The hydrolysis half-life of this compound is pH dependent with faster degradation at a more basic pH. DBNPA is biodegradable under both aerobic and anaerobic conditions [33]. According to the EPA Registration Eligibility Decision (RED) for DBNPA, the compound can be degraded both aerobically and anaerobically into six compounds: oxalic acid, 2-cyanoactamide, bromoacetamide, dibromoacetic acid, bromoacetic acid, and dibromoacetonitrile. The daughter products of DBNPA biodegradation are the same under aerobic and anaerobic metabolism. However, the percent of individual daughter products and the half-lives of these daughter products varies depending on if it is aerobic or anaerobic degradation[33].

It is important to clarify the impact of industrial biocides associated with HF in the environment and the mechanism for biocide resistance. Bacteria can resist antimicrobials by secreting enzymes that deactivate the compound, by preventing the antimicrobial to get to the cell through efflux pumps, and by modifying the active target of the antimicrobial. These mechanisms of resistance to antimicrobials can be intrinsic or acquired. Intrinsic is an innate bacterial property associated with cellular structure, as is the case of the outer membrane in Gram-negative bacteria, or random genetic mutations not caused by an external stressor. Acquired resistance is the result of mutation or of the gain of mobile genetic elements such as plasmids or transposons caused by external stressors [18 34]. Relevant intrinsic properties that can be acquired through mobile genetic elements include: the capability of metabolizing or expelling recalcitrant and/or xenobiotic compounds from the inner cell, as is the case with microbes having monooxygenase enzymes [35] and efflux pumps [36]. Moreover, subsurface microorganisms capable of using recalcitrant aromatic carbon compounds for energy and carbon source were proven to have a higher incidence of plasmids carrying antimicrobial resistant genes (ARG) [37]. Acquired resistance is of higher concern, as antimicrobial resistant bacteria (ARB), and the mobile genetic elements they are carrying, are starting to be identified as contaminants of concern [38].

Many studies have investigated the mechanism of acquired resistance to household biocides, namely triclosan [21]. Much less is known about acquired resistance to industrial biocides such as GA and DBNPA. The mechanisms for GA resistance is beginning to be investigated under conditions commonly found in HF wastewater. Some studies have indicated that microbes

in produced water demonstrate a higher resistance to biocides than expected [14, 39 25 28]. These findings may indicate that biocides are not adequately inhibiting these corrosive bacteria in flowback water, the bacteria have developed resistance to the biocides, and/or subsurface bacteria are exhibiting intrinsic resistance due to reliance on recalcitrant carbon as a carbon and energy source (as presumably flowback water microbes may come from the subsurface). The increased resistance could be due to the interactions between the biocides and the chemicals in produced water and high salt concentration that activate efflux pumps[36 28, 40]. A recent study demonstrated that for a model organism, there is a biologically driven mechanism for biocide resistance to GA[40]. In this study, transcriptomics was used to identify the response of this model organism to biocide addition. They demonstrated that genes needed for osmotic stress, energy production and conversion, membrane integrity, and protein transport are up-regulated when the bacteria are exposed to HF produced water, which increased bacterial tolerance to biocide exposure. The same authors also discovered that GA resistance can also be mediated through an increase in efflux pumps, which will increase the rate of export of the biocide[40].

In the case for DBNPA, no studies have investigated the mechanism of microbial biocide resistance in the context of HF. The diversity of organisms resistant to DBNPA as well as the mechanism for biodegradation of DBNPA is not fully understood. This is of extreme concern as this is the second most commonly used biocide in HF operations, and yet very little is known about its effect on environmental microbial communities. It is therefore, of great importance to better understand the effect and unintended consequences of its use in the environment.

The goal of this thesis is to better understand the environmental impacts of HF an in particular how the use of industrial biocides in HF operations may contribute to development of acquired antimicrobial resistance in aquatic microbial communities. The approach to accomplish this goal involves microcosm-based studies to investigate the effect of biocide addition to both HF-impacted and pristine streams. Several streams in Western Pennsylvania were selected based on whether they have received inputs of HF wastewater or are in close proximity to active HF wells. Another set of three streams with no active HF operations was chosen as control streams. Microcosms amended with biocides were constructed using both sets of streams. The fate of the biocide, GA or DBNPA, was investigated through measuring rates of biocide degradation in

biotic and abiotic conditions comparing HF-impacted and pristine streams. The effect of biocide addition on the microbial community was investigated using next-generation sequencing of the 16S rRNA gene amplicon, and quantification of the 16S rRNA gene/mL. The biological mechanism of biocide resistance for DBNPA was investigated through comparative genomics of whole genome sequencing of bacteria from GA and DBNPA enrichments.

Finally, this dissertation ends with the identification of knowledge gaps that need to be addressed in order to identify the environmental and public health risks stemming from the propagation of ARG and ARB associated with biocides used in energy systems. Antimicrobial resistance (AMR) is a global health concern as many microbes are developing resistance to commonly used antimicrobials[41]. In the U.S., more than 2 million people acquire an antibiotic-resistant infection every year [42]. In 2016 the United Nations General Assembly had a meeting to discuss AMR—the fourth time it ever discussed public health issues—warning that if left unchecked AMR would disturb sustainable development, and public and environmental health. While the conversation of AMR has been centered on overuse of antibiotics in clinical and agricultural settings, biocides are also a contributor of AMR and more work is needed to establish their contribution to the environmental resistome.

Dissertation Overview

Streams and other surface waters commonly receive runoff of hydraulic fracturing accidental spills. However, to my knowledge, the environmental impacts to aquatic microbial communities caused by biocides used in hydraulic fracturing have not been studied before. Biocides have been previously identified as contaminants of concern in hydraulic fracturing. Therefore, the microbial community changes, degradation potential effects, and mechanistic understanding of biocide degradation and resistance are current knowledge gaps. With the importance of hydraulic fracturing for energy security, energy independence, and economic energy costs, these knowledge gaps are important for proper environmental remediation planning and environmental and public health protection.

Chapter 1 is the introduction to the dissertation. An overview of biocide usage in hydraulic fracturing, a description of glutaraldehyde and 2,2- dibromo-3-nitrilopropionamide, and a discussion with known/potential pathways of resistance.

Chapter 2 describes the impacts of the biocide glutaraldehyde on microbial community structure and degradation potential in streams impacted by hydraulic fracturing. Microbial communities of streams impacted and not-impacted by hydraulic fracturing were compared using 16S rRNA gene amplicon sequencing, and 16S rRNA gene copies/mL. Furthermore, biotic and abiotic degradation differences between hydraulic fracturing impacted and non-impacted streams were tracked my Ultra Performance Liquid Chromatography—Mass Spectrometry.

Chapter 3 discusses the impacts of the biocide 2,2-dibromo-3-nitrilopropionamide on microbial community structure and degradation potential in streams impacted by hydraulic fracturing. The same experimental design as chapter 2 is employed. This chapter also discusses if the microbial community differences observed between chapter 2 and 3 are biocide dependent.

Chapter 4 builds up on the findings from the previous chapters, and the genomic profiles of bacteria isolated after biocide enrichment are compared. The genera selected for comparison, *Paenibacillus* and *Bacillus,* were enriched in the microcosms, indicating their potential environmental relevance. Comparative genomics tools are used to identify genes correlated with 2,2-dibromo-3-nitrilopropionamide resistance.

Chapter 5 explores UOG production as a potential source of antimicrobial resistance caused by the use of biocides in HF practices. A coarse risk assessment framework was proposed, and key research gaps are identified.

Chapter 6 is a conclusion of the overall findings described in the dissertation. Key knowledge gaps that have now been address and what future work should be done in this arena are discussed.

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CHAPTER 2 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 stream water 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 that 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, crosslinkers, 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 photo-degraded[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 non-exposed 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 8,000-gallon spill of water and HF fluids[21]. Each of the HF-not impacted (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 S2.1 for a map of watersheds' location and refer to the Appendix for description of sample collection.

There was documented use of GA in wells associated with the three HF+ streams selected according to FracFocus.org[22]. Detailed selection of 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 observation 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 stream water, and prior to GA amendment 25 mL were collected for downstream DNA analyses. The amount of biocides used in HF fluids varies widely between 10 to 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 stream water were 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, negative biological controls were setup with stream water 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 day 7, 28, and 56. However, the day 0 sample depleted during total organic carbon analyses and were not analyzed by UPLC—HRMS. One mL of water from each microcosm was collected, filtered thought 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 x 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 ramp 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 $3x10^6$, resolution of 140,000, and a scan range of 90 to 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 Figure S2.2 and S2.3. Average HF+ and HF- concentrations with their respective standard error were reported. Refer to the Appendix 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. The acidification 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 Supplemental Information for 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 x 150 reads), according to 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 Appendix 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. De-multiplexing and quality filtering was 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 up to minimum 97% sequence identity was used. Open reference OTU picking was performed using the command pick_open_reference_otus.py using the UCLUST method [38] using 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 1,220 sequences. Alpha diversity, beta diversity and DESeq2[45] analyses were performed using un-rarefied OTU table as described in the Appendix.

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 based on 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 comparison the biocide concentration was $\log_{10}$ transformed and a baseline of 100mg/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 were then divided between biotic and abiotic. A CRD with repeated measures was applied to each. The same test 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 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 have 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 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 achieve

this objective, 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

Temperature, pH, conductivity, total dissolved solids, and salinity were measured at the time of sample collection and results are shown in Table S2.1. 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 UOG wastewater disposal facility, which suggests that the high salinity could have been caused either by 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 mg/L and 106.79 ± 5.1 mg/L in abiotic HF- control (Figure 2.1A). 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 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.

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 2.1B). 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 statistical 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 (100mg/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 Abiotic and Biotic Microcosms

It was observed that TOC decreased in the first 7 days (day 0 to 7) for both abiotic and biotic microcosms (Figure S2.5). 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, either by 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 S2.2). 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.

Quantification of 16S rRNA gene by qPCR

The abundance of 16S rRNA genes was determined from initial samples before addition of GA (Figure 2.2). All the pre-GA treatment 16S rRNA gene concentrations were on the order of 10^4 gene copies/ mL, averaging 4.03 x 10^4 gene copies/mL in the HF+ streams and 4.38 x 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 $\log_2$ FC. 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.

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 2.2). 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 2.3) 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.

The overall alpha diversity found in this study's HF- microcosms was higher than the HF+ microcosms pre-amendment 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 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 2.3). 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 the Appendix. Clustering by PC 1 explains 65.4% of the variation in 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 2.4). 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.

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 2.3) and by an increase in differentially abundant OTUs between day 0 and the next 4 sampling events (day 7, 21, 35, 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, seven days after addition of GA 239 OTUs were differentially enriched. 27 OTUs experienced a positive log_2FC while 213 OTUs experienced a negative log_2FC, and hence were inhibited by exposure to GA. The highest log_2FC corresponded to an OTU identified as the genus *Myroides* (19.09 log_2FC), followed by an OTU identified as *Robinsoniella* (18.64 log_2FC). 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 low abundant (< 2%) except for *Alcanivorax* (2.77 log_2FC). 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_2FC). 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_2FC), *Methylobacterium* (2.78 log_2FC), and *Bacillus* (2.06 log_2FC). There were not significant differences in enrichment between HF+ and HF- that passed the stringent 2 log_2FC cut-off that was imposed.

By day 35 there were 407 OTUs differentially enriched as compared to original, day 0 microbial population. These OTUs were classified as *Amphritea* (5.29 log_2FC), *Methylobacterium (*5.19 log_2FC), and *Beijerinckia* (3.23 log_2FC). Three OTUs were

differentially enriched in HF+ vs HF- at day 35. The genus *Acinetobacter* had a 3.60 log_2FC in HF-, while *Beijerinckia* and *Janthinobacterium* had an 8.17 and 3.94 log_2FC 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_2FC), *Robinsoniella* (10.61 log_2FC), *Methylobacterium* (6.02 log_2FC), and *Beijerinckia* (2.93 log_2FC). One OTU was differentially enriched in HF+ vs HF- at day 49. The genus *Beijerinckia* had an 8.97 log_2FC 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_2FC) and *Beijerinckia* (10.20 log_2FC), *Mycobacterium* (7.81 log_2FC), *Alcanivorax* (5.74 log_2FC), *Stenotrophomonas* (5.24 log_2FC), *Bacillus* (3.48 log_2FC), *Idiomarina* (3.28 log_2FC), and Burkholderia (3.04 log_2FC). Only one OTU identified as the genus *Beijerinckia* (9.36 log_2FC) 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_2FC), *Alcanivorax* (5.81 log_2FC), *Mycobacterium* (5.67 log_2FC), *Beijerinckia* (5.21 log_2FC), *Idiomarina* (4.42 log_2FC), *Bacillus* (3.13 log_2FC); 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_2FC) and *Idiomarina* (-5.10 log_2FC) were suppressed at day 56 no-GA as compared to day 0, and that *Myroides* (5.09 log_2FC) experienced an enrichment.

These enrichments over time suggest which OTUs were driving the response to GA. *Alcanivorax* was a dominant first responder, and after and 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 S2.6 B) indicating that it was able to adapt to GA presence and dominate. It 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 genus enriched 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], 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 S2.6 B); 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]* (Figure S2.6 B). 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 bacterial response mechanism 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 survival of GA.

These microcosms did not explore the impacts of high salinity in the 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 same 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 case of HF fluid spill containing GA and high salinity 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 S2.6 B) 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[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 the 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 in oxygen availability as study utilizing anaerobic microcosm inoculated with UOG impacted and not impacted sediment described a significant decrease in abundance after the addition of the biocide DBNPA[69]. Another interestingly enriched *Alphaproteobacteria* was the genus was *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, non-symbiotic, 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 (Table S2.5-S2.16) 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 biomass and richness loss 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 lack of degradation advantage suggests the difference in degradation rates might not be biotic alone but rather driven by abiotic-biotic interactions. HF- microcosms 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 S2.3), 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 S2.4). 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 is 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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Appendix A: Figures

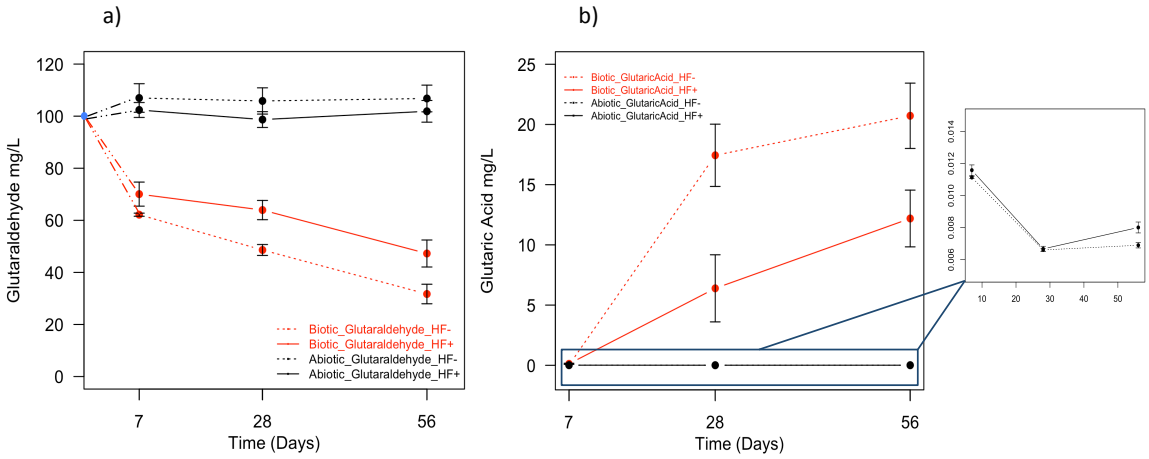


Figure 2.1 Biotic and abiotic degradation of glutaraldehyde and glutaric acid production over time.

(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).

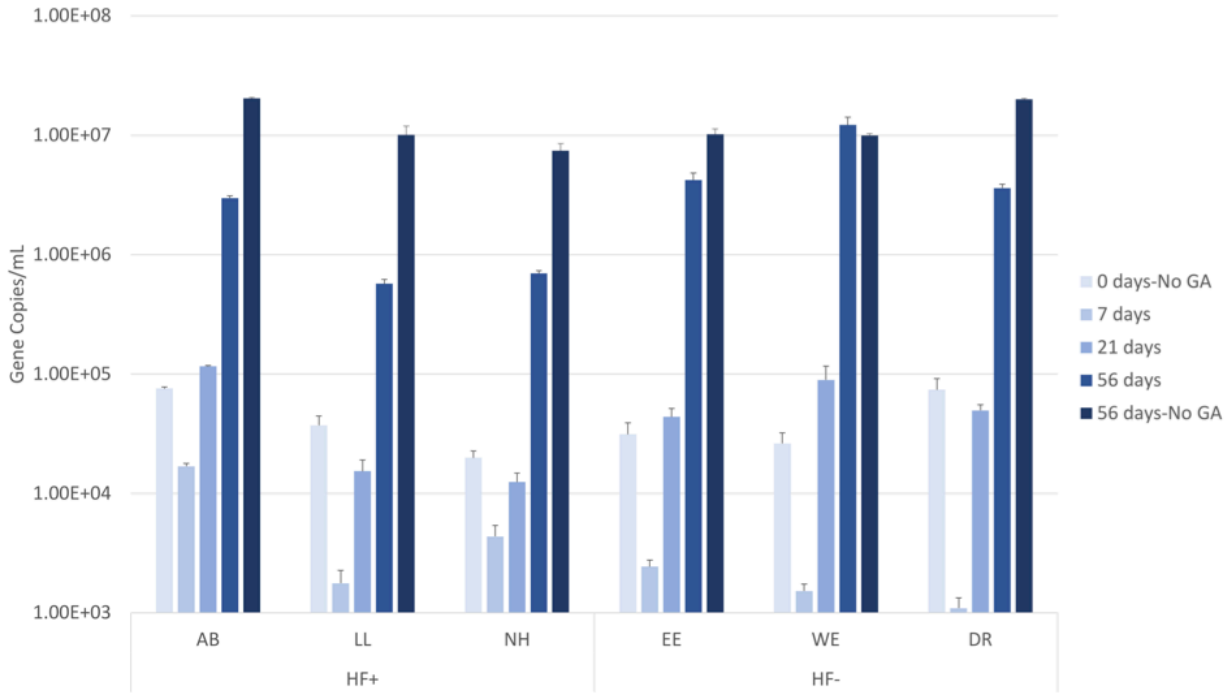


Figure 2.2 Impacts of glutaraldehyde in abundance of 16S rRNA gene over time.

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).

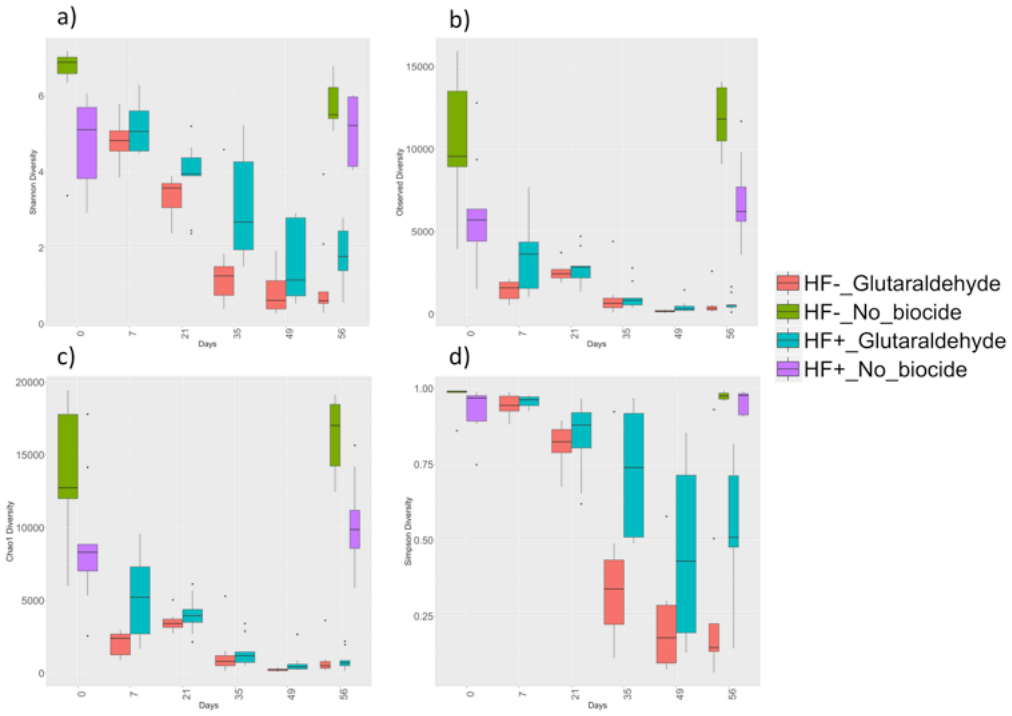


Figure 2.3 Alpha diversity measurements over time.

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, D) Simpson Diversity. Red and green box plots represent HF- glutaraldehyde (days 7 to 56) and no glutaraldehyde added. (day 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 represent 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.

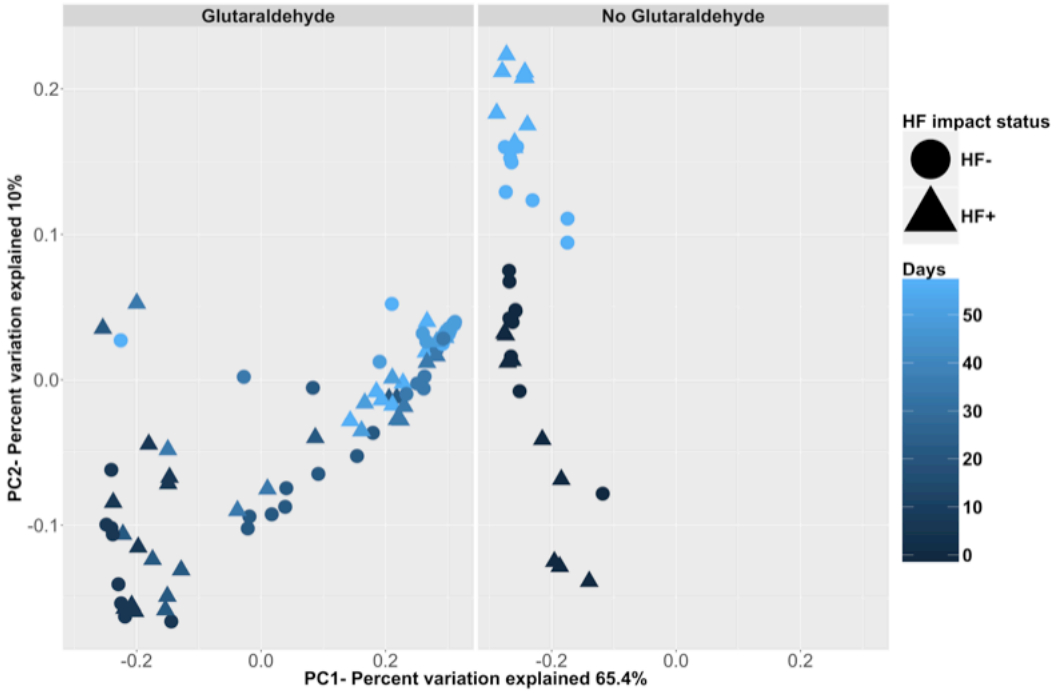
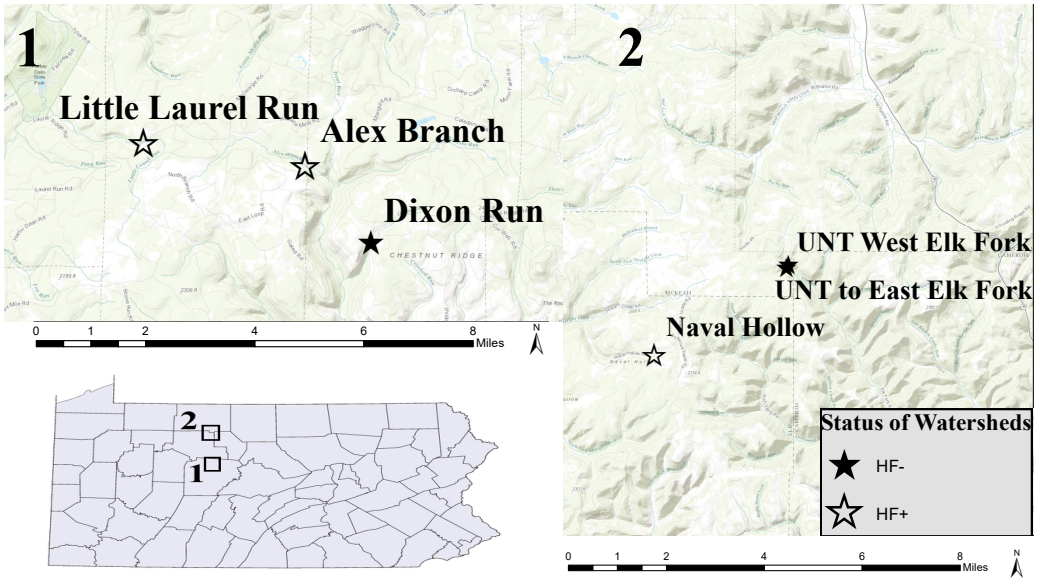


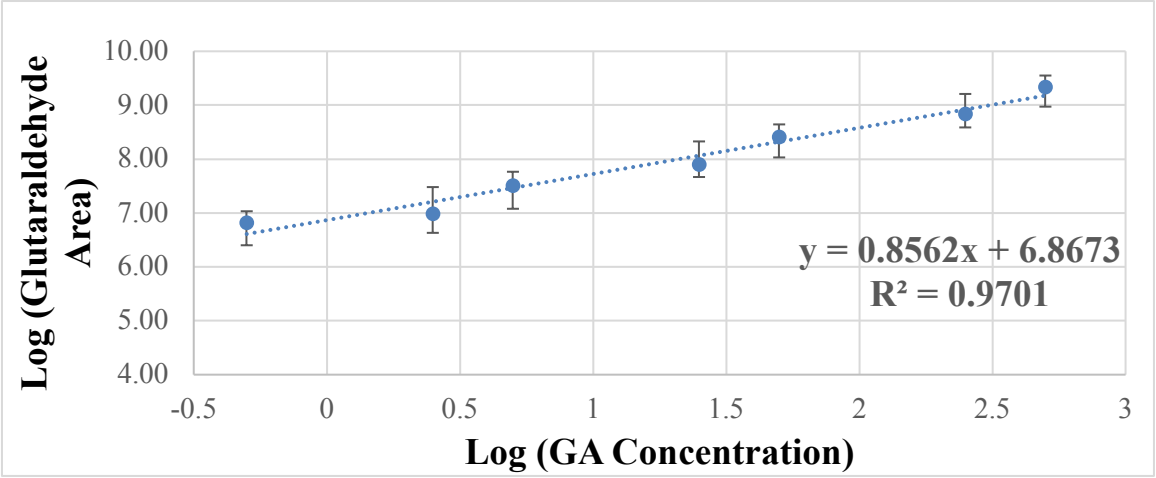
Figure 2.4 Beta Diversity

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%.

Supplemental Figures

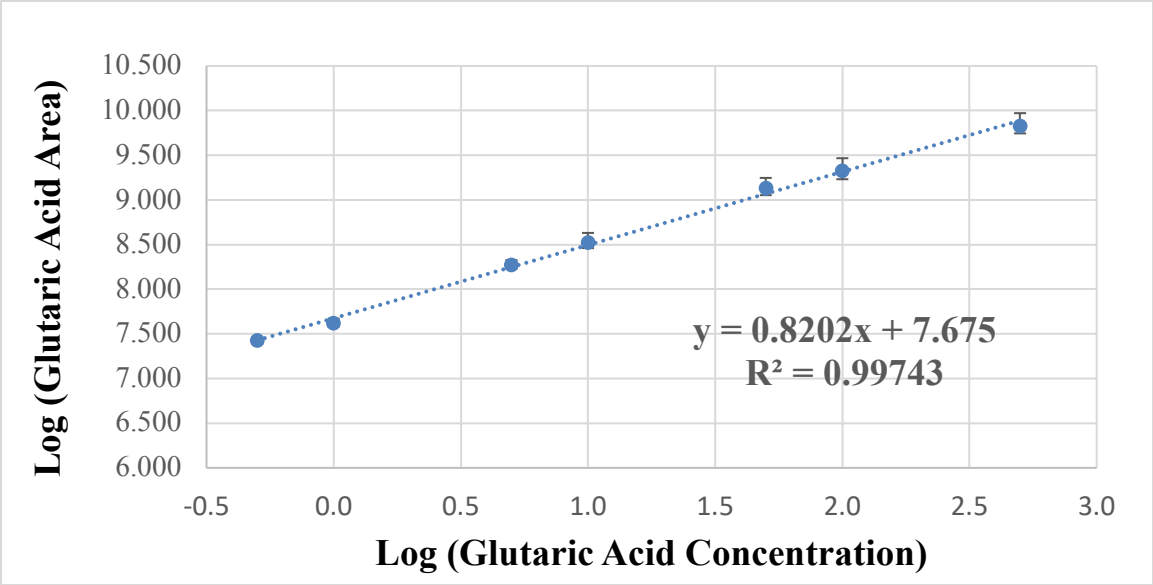


Supplemental Figure S2.1 Location of the Pennsylvania watersheds used as microcosms source water.



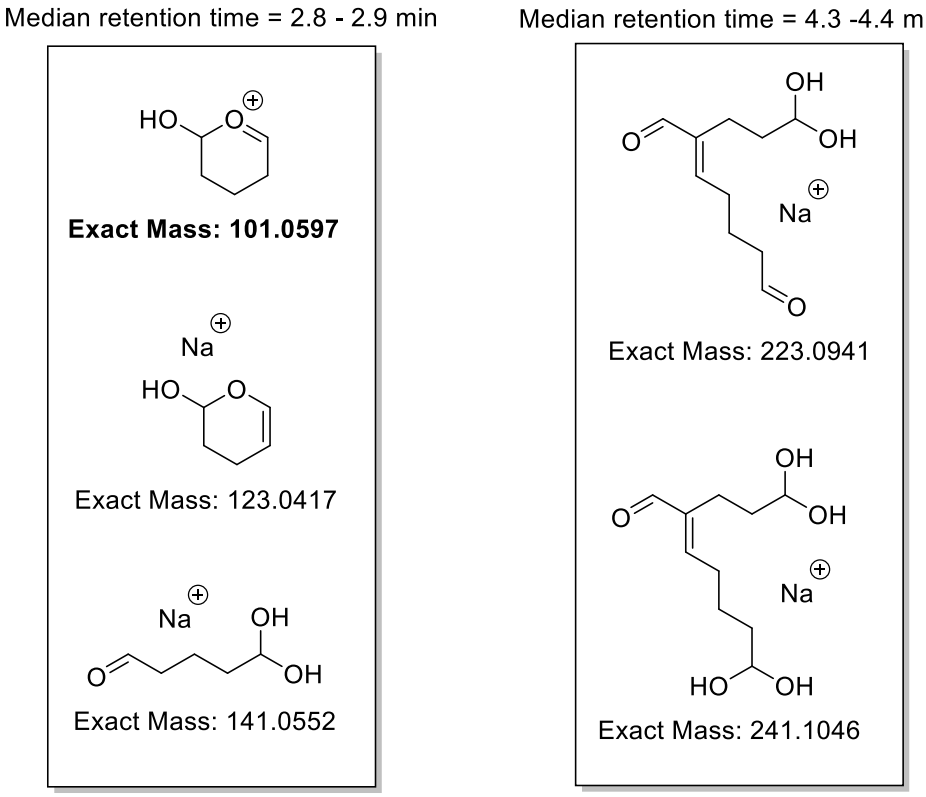
Supplemental Figure S2.2 Seven-point calibration curve for glutaraldehyde (GA).

The calibration curve is plotted as the log transformed total peak area versus log transformed concentration. Concentrations measured started at a concentration of 500 µM and was serially diluted to 0.5 µM.



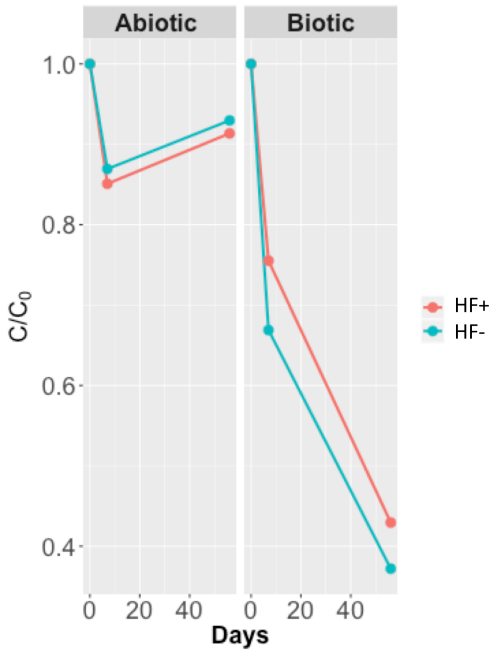
Supplemental Figure S2.3 Seven-point calibration curve for glutaric acid.

The calibration curve is plotted as the log transformed total peak area versus log transformed concentration. Concentrations measured started at 500 µM and was serially diluted to 0.5 µM.



Supplemental Figure S2.4 Putative detected forms of glutaraldehyde.

The molecular ion used for relative quantitation is the most abundant of the detected forms and is shown in bold.

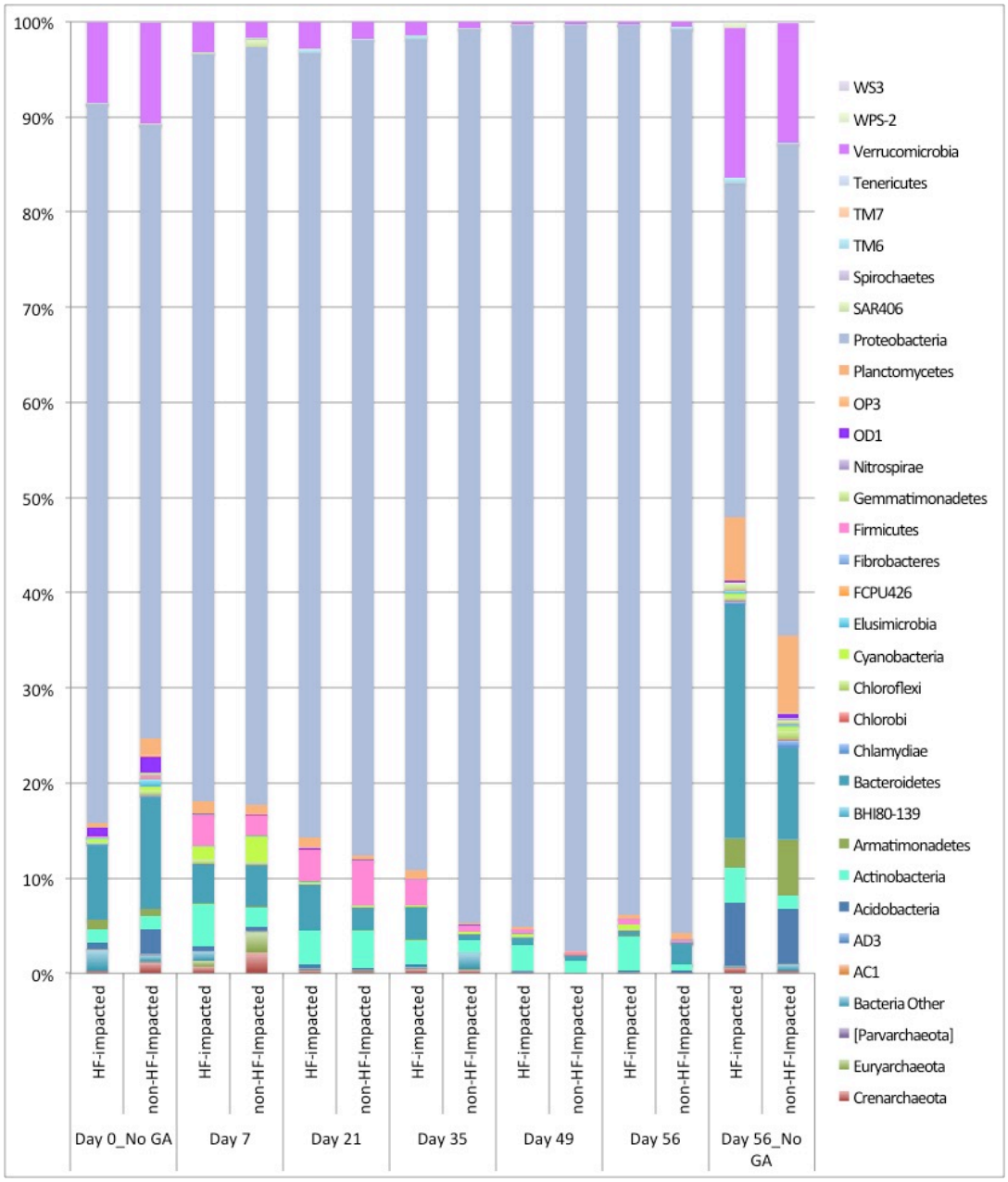


Supplemental Figure S2.5 Total Organic Carbon as a proxy of glutaraldehyde loss over time.

Supplemental Figure S2.6 Microbial Community Shifts Over Time.

A) Phylum level shifts over 56 days with and without glutaraldehyde addition, samples were average into HF-impacted and non-HF-impacted groups. B) Genus level shifts over 56 days, with and without glutaraldehyde addition, samples were average into HF-impacted and non-HF-impacted groups.

A)



Supplemental Figure S2.6 continued

B)

Supplemental Figure S2.6 continued

Appendix B: Tables

Supplemental Tables

Supplemental Table S2.1 Geochemical parameters.

Measurements taken on the field at time of sample collection. AB, LL, NH are streams with reported HF activity. EE, WE, DR have no reports of HF impacts.

	Stream	Temperature (°C)	pH	Conductivity (µS/cm)	Total Dissolved Solids (ppm)
HF+	Alex Branch (AB)	15.7	4.98	30.5	21.8
	Little Laurel (LL)	20.6	4.68	34.8	25
	UNT Naval Hollow (NH)	14.1	5.13	22.3	15.5
HF-	UNT East Elk Fork (EE)	13.2	7.3	43.4	30.8
	UNT West Elk Fork (WE)	13.6	6.48	34	24.1
	Dixon Run (DR)	11.7	5.71	23.8	16.9

Supplemental Table S2.2 Raw area data for the two-additional hydrate/adduct peaks and the two probable dimer peaks of GA

m/z	123.0411	141.0526	223.0951	241.1038
median Rt	2.9	2.9	4.3	4.4
Samples	Areas			
T1_-_AB1	1.57E+06	8.39E+06	4.63E+04	1.16E+06
T1_-_AB2	1.35E+06	7.87E+06	2.95E+04	9.85E+05
T1_-_AB3	1.44E+06	7.83E+06	2.11E+04	1.08E+06

Supplemental Table S2.2 continued

m/z	123.0411	141.0526	223.0951	241.1038
Samples	**Areas**			
T1_-_DR1	1.70E+06	9.62E+06	4.64E+05	2.19E+06
T1_-_DR2	1.52E+06	9.16E+06	3.23E+05	1.92E+06
T1_-_DR3	1.56E+06	8.24E+06	5.67E+05	2.28E+06
T1_-_EE1	1.38E+06	8.03E+06	7.93E+05	2.67E+06
T1_-_EE2	1.67E+06	8.67E+06	6.05E+05	2.48E+06
T1_-_EE3	1.74E+06	9.30E+06	5.38E+05	2.45E+06
T1_-_LL1	1.72E+06	9.00E+06	1.85E+05	1.73E+06
T1_-_LL2	1.43E+06	7.57E+06	1.04E+04	8.64E+05
T1_-_LL3	1.50E+06	8.43E+06	3.87E+04	1.05E+06
T1_-_NH1	1.54E+06	8.25E+06	1.00E+05	1.28E+06
T1_-_NH2	1.57E+06	8.44E+06	2.00E+05	1.77E+06
T1_-_NH3	1.35E+06	8.20E+06	4.24E+04	1.26E+06
T1_-_WE1	1.64E+06	8.36E+06	1.50E+05	1.47E+06
T1_-_WE2	1.15E+06	7.58E+06	3.21E+05	1.90E+06
T1_-_WE3	2.03E+06	1.04E+07	1.02E+05	1.41E+06
T1_AB1	9.46E+05	6.15E+06	1.12E+04	7.27E+05
T1_AB2	9.84E+05	6.26E+06	0.00E+00	4.55E+05
T1_AB3	1.52E+06	8.10E+06	0.00E+00	7.41E+05
T1_DR1	9.57E+05	6.61E+06	0.00E+00	5.83E+05
T1_DR2	8.68E+05	5.69E+06	0.00E+00	6.33E+05
T1_DR3	9.79E+05	6.84E+06	8.73E+03	7.93E+05
T1_EE1	9.13E+05	5.91E+06	7.48E+05	2.66E+06
T1_EE2	8.98E+05	5.77E+06	7.20E+05	2.61E+06
T1_EE3	8.90E+05	5.80E+06	6.28E+05	2.52E+06
T1_LL1	1.03E+06	6.26E+06	0.00E+00	4.18E+05

Supplemental Table S2.2 continued

m/z	123.0411	141.0526	223.0951	241.1038
Samples	**Areas**			
T1_LL2	8.56E+05	5.50E+06	0.00E+00	3.38E+05
T1_LL3	9.77E+05	6.07E+06	1.77E+04	3.57E+05
T1_NH1	9.07E+05	6.20E+06	4.03E+04	1.09E+06
T1_NH2	9.50E+05	6.39E+06	1.43E+05	1.47E+06
T1_NH3	9.27E+05	5.56E+06	8.56E+04	1.37E+06
T1_WE1	8.99E+05	6.06E+06	1.42E+05	1.34E+06
T1_WE2	9.57E+05	5.93E+06	1.23E+05	1.44E+06
T1_WE3	8.60E+05	5.83E+06	2.36E+04	9.14E+05
T4_-_AB1	1.61E+06	8.10E+06	1.73E+04	9.06E+05
T4_-_AB2	1.36E+06	7.82E+06	0.00E+00	7.19E+05
T4_-_AB3	1.34E+06	7.85E+06	6.05E+03	6.18E+05
T4_-_DR1	2.03E+06	8.77E+06	4.58E+05	2.11E+06
T4_-_DR2	1.41E+06	6.60E+06	2.39E+05	1.59E+06
T4_-_DR3	1.57E+06	8.10E+06	1.57E+05	1.37E+06
T4_-_EE1	1.50E+06	7.88E+06	7.33E+05	2.44E+06
T4_-_EE2	1.58E+06	8.27E+06	1.69E+06	3.83E+06
T4_-_EE3	1.37E+06	6.07E+06	2.27E+06	4.78E+06
T4_-_LL1	1.58E+06	7.88E+06	0.00E+00	4.95E+05
T4_-_LL2	1.54E+06	7.86E+06	1.23E+05	1.27E+06
T4_-_LL3	1.33E+06	7.42E+06	1.68E+03	4.64E+05
T4_-_NH1	1.63E+06	8.88E+06	7.93E+05	2.62E+06
T4_-_NH2	1.68E+06	8.81E+06	9.34E+05	2.64E+06
T4_-_NH3	1.20E+06	6.74E+06	7.00E+05	2.38E+06
T4_-_WE1	1.46E+06	7.75E+06	6.49E+05	2.34E+06

Supplemental Table S2.2 continued

m/z	123.0411	141.0526	223.0951	241.1038
Samples	**Areas**			
T4_-_WE2	1.21E+06	7.25E+06	3.15E+05	1.75E+06
T4_-_WE3	1.93E+06	8.74E+06	1.02E+06	2.85E+06
T4_AB1	7.24E+05	5.65E+06	4.45E+03	3.92E+05
T4_AB2	6.37E+05	5.10E+06	7.86E+03	5.11E+05
T4_AB3	8.86E+05	5.99E+06	0.00E+00	3.12E+05
T4_DR1	6.70E+05	4.75E+06	5.23E+03	2.55E+05
T4_DR2	7.48E+05	5.53E+06	6.22E+02	2.51E+05
T4_DR3	7.49E+05	5.58E+06	2.70E+03	3.26E+05
T4_EE1	7.37E+05	5.57E+06	2.14E+04	7.10E+05
T4_EE2	6.74E+05	5.06E+06	1.87E+04	9.51E+05
T4_EE3	7.43E+05	5.47E+06	1.19E+04	6.70E+05
T4_LL1	1.30E+06	7.67E+06	0.00E+00	6.31E+05
T4_LL2	9.73E+05	5.75E+06	4.41E+03	6.32E+05
T4_LL3	1.05E+06	7.09E+06	0.00E+00	4.75E+05
T4_NH1	8.90E+05	6.40E+06	5.89E+04	1.10E+06
T4_NH2	8.81E+05	5.68E+06	6.72E+05	2.49E+06
T4_NH3	9.38E+05	6.06E+06	5.08E+04	1.18E+06
T4_WE1	5.81E+05	4.77E+06	0.00E+00	3.78E+05
T4_WE2	4.67E+05	4.09E+06	0.00E+00	3.18E+05
T4_WE3	5.90E+05	4.59E+06	5.19E+03	2.75E+05
T8_-_AB1	1.62E+06	9.01E+06	1.66E+03	7.40E+05
T8_-_AB2	1.62E+06	8.24E+06	1.47E+04	7.86E+05
T8_-_AB3	1.39E+06	7.42E+06	0.00E+00	5.40E+05
T8_-_DR1	1.65E+06	8.94E+06	1.36E+06	3.42E+06
T8_-_DR2	1.38E+06	7.82E+06	4.18E+06	7.02E+06

Supplemental Table S2.2 continued

m/z	123.0411	141.0526	223.0951	241.1038
Samples	**Areas**			
T8_-_DR3	1.61E+06	7.61E+06	2.27E+06	4.39E+06
T8_-_EE1	1.82E+06	8.14E+06	5.22E+06	8.19E+06
T8_-_EE2	1.65E+06	8.33E+06	5.54E+06	8.66E+06
T8_-_EE3	2.03E+06	8.37E+06	5.45E+06	8.47E+06
T8_-_LL1	1.77E+06	8.52E+06	0.00E+00	4.42E+05
T8_-_LL2	1.23E+06	6.90E+06	1.88E+05	1.47E+06
T8_-_LL3	1.34E+06	7.15E+06	0.00E+00	4.46E+05
T8_-_NH1	1.33E+06	7.54E+06	2.27E+06	4.29E+06
T8_-_NH2	1.94E+06	8.24E+06	6.97E+06	1.07E+07
T8_-_NH3	1.50E+06	6.99E+06	2.04E+06	4.12E+06
T8_-_WE1	1.73E+06	7.88E+06	1.76E+06	3.83E+06
T8_-_WE2	1.19E+06	6.90E+06	1.69E+06	3.52E+06
T8_-_WE3	1.87E+06	8.29E+06	1.11E+06	3.02E+06
T8_AB1	3.38E+05	3.49E+06	9.31E+02	1.91E+05
T8_AB2	3.89E+05	3.60E+06	8.71E+03	2.01E+05
T8_AB3	5.79E+05	4.79E+06	8.10E+02	1.96E+05
T8_DR1	5.90E+05	4.12E+06	0.00E+00	2.43E+05
T8_DR2	6.04E+05	4.93E+06	1.17E+04	2.27E+05
T8_DR3	5.81E+05	4.33E+06	0.00E+00	2.12E+05
T8_EE1	4.88E+05	4.41E+06	8.33E+03	2.58E+05
T8_EE2	2.28E+05	2.60E+06	0.00E+00	1.77E+05
T8_EE3	9.57E+04	1.57E+06	1.04E+03	1.50E+05
T8_LL1	1.06E+06	6.42E+06	0.00E+00	2.90E+05
T8_LL2	9.05E+05	5.84E+06	1.13E+03	2.06E+05
T8_LL3	3.60E+05	3.42E+06	0.00E+00	1.24E+05

Supplemental Table S2.2 continued

m/z	123.0411	141.0526	223.0951	241.1038
Samples	**Areas**			
T8_NH1	8.61E+05	5.38E+06	2.55E+03	2.14E+05
T8_NH2	7.42E+05	5.23E+06	7.55E+03	2.21E+05
T8_NH3	6.15E+05	4.65E+06	0.00E+00	2.02E+05
T8_WE1	3.67E+05	3.57E+06	0.00E+00	2.55E+05
T8_WE2	3.16E+05	2.95E+06	4.36E+03	1.80E+05
T8_WE3	3.41E+05	3.17E+06	0.00E+00	2.09E+05
Blank1	1.31E+04	3.49E+05	3.44E+04	9.70E+04
Blank2	2.07E+03	6.05E+04	1.96E+04	4.13E+04
Blank3	0.00E+00	2.65E+04	1.93E+04	3.64E+04
Blank4	4.30E+02	3.74E+04	8.48E+03	3.72E+04
Blank5	0.00E+00	2.74E+04	0.00E+00	5.37E+04
Blank6	1.11E+03	2.65E+04	1.22E+04	3.18E+04
Blank7	1.85E+03	2.74E+04	1.23E+03	2.80E+04
Blank8	0.00E+00	4.91E+04	1.02E+03	2.56E+04

Supplemental Table S2.3 GA concentration over time shown by stream source.

Condition	Days	Impacted	Stream	101.0600 m/z (Area)	Concentration (mg/L)	Standard Error
Biotic	7	HF+	AB	3.85E+08	79.48160912	13.11212854
Biotic	7	HF+	LL	3.96E+08	66.7273629	3.801995694
Biotic	7	HF+	NH	3.81E+08	63.97547606	1.110752354
Biotic	7	HF-	EE	4.13E+08	60.70884643	3.801995694
Biotic	7	HF-	WE	3.95E+08	61.95103937	1.110752354
Biotic	7	HF-	DR	3.97E+08	63.8216446	1.255625153
Biotic	28	HF+	AB	3.64E+08	52.58146621	1.110752354

Supplemental Table S2.3 continued

Condition	Days	Impacted	Stream	101.0600 m/z (Area)	Concentration (mg/L)	Standard Error
Biotic	28	HF+	LL	3.75E+08	74.15880096	1.255625153
Biotic	28	HF+	NH	3.86E+08	65.08034858	0.335364966
Biotic	28	HF-	EE	3.92E+08	52.97379609	1.255625153
Biotic	28	HF-	WE	3.90E+08	41.42101544	0.335364966
Biotic	28	HF-	DR	4.12E+08	51.4239386	0.353042028
Biotic	56	HF+	AB	3.87E+08	34.18111899	0.335364966
Biotic	56	HF+	LL	3.73E+08	54.47144686	0.353042028
Biotic	56	HF+	NH	3.96E+08	53.11696095	2.563422329
Biotic	56	HF-	EE	4.34E+08	22.94410647	0.353042028
Biotic	56	HF-	WE	3.86E+08	28.34192364	2.563422329
Biotic	56	HF-	DR	3.83E+08	43.82851494	6.352693439
Abiotic	7	HF+	AB	3.11E+08	101.5543597	2.563422329
Abiotic	7	HF+	LL	2.68E+08	105.2199569	6.352693439
Abiotic	7	HF+	NH	2.59E+08	100.342735	0.490986499
Abiotic	7	HF-	EE	2.48E+08	110.2945362	6.352693439
Abiotic	7	HF-	WE	2.52E+08	105.2615509	0.490986499
Abiotic	7	HF-	DR	2.58E+08	105.3721828	1.458119011
Abiotic	28	HF+	AB	2.19E+08	95.35410966	0.490986499
Abiotic	28	HF+	LL	2.94E+08	98.65858198	1.458119011
Abiotic	28	HF+	NH	2.63E+08	102.0578332	1.95400352
Abiotic	28	HF-	EE	2.20E+08	103.7405871	1.458119011
Abiotic	28	HF-	WE	1.78E+08	103.4594802	1.95400352
Abiotic	28	HF-	DR	2.15E+08	110.309118	2.75098417
Abiotic	56	HF+	AB	1.51E+08	102.4418522	1.95400352
Abiotic	56	HF+	LL	2.24E+08	97.98890524	2.75098417

Supplemental Table S2.3 continued

Condition	Days	Impacted	Stream	101.0600 m/z (Area)	Concentration (mg/L)	Standard Error
Abiotic	56	HF+	NH	2.21E+08	105.1594224	3.186512269
Abiotic	56	HF-	EE	1.07E+08	117.0423702	2.75098417
Abiotic	56	HF-	WE	1.29E+08	102.0744244	3.186512269
Abiotic	56	HF-	DR	1.87E+08	101.2384625	12.72747454

Supplemental Table S2.4 pH changes over time in the microcosms.

The pH each group n=9 was averaged and standard deviation (SD) calculated. Day 0-No GA was measured prior to the start of the experiment. The other samples were measured after freeze-thaw. Days not shown were not measured because samples were depleted.

	Day 0- No GA		Day 14		Day 21		Day 28		Day 35		Day 49	
Microcosm group	pH	SD	pH	SD	pH	SD	pH	SD	pH	SD	pH	SD
HF+	5.3	0.6	7.8	0.2	6.9	0.9	8.4	0.0	6.2	1.0	4.2	0.1
HF-	6.5	0.3	7.7	0.3	7.4	0.2	8.1	0.2	5.2	0.5	4.1	0.1
HF- biotic	5.3	0.6	7.5	0.2	8.4	0.0	7.9	0.4	8.2	0.7	5.7	0.5
HF- abiotic	6.5	0.3	7.7	0.2	7.6	0.1	7.6	0.3	8.4	0.1	6.7	0.3

The following tables are available in attachments:

Supplemental Table S2.5 DESeq2 Results HF- vs HF+ Enrichment at Day 0 Prior to GA Addition

Supplemental Table S2.6 DESeq2 Results Day 7 Enrichment vs Day 0

Supplemental Table S2.7 DESeq2 Results Day 21 Enrichment vs Day 0

Supplemental Table S2.8 DESeq2 Results Day 35 Enrichment vs Day 0

Supplemental Table S2.9 DESeq2 Results Day 49 Enrichment vs Day 0

Supplemental Table S2.10 DESeq2 Results Day 56 Enrichment vs Day 0

Supplemental Table S2.11 DESeq2 Results HF- vs HF+ Enrichment at Day 7

Supplemental Table S2.12 DESeq2 Results HF- vs HF+ Enrichment at Day 35

Supplemental Table S2.13 DESeq2 Results HF- vs HF+ Enrichment at Day 49

Supplemental Table S2.14 DESeq2 Results HF- vs HF+ Enrichment at Day 56

Supplemental Table S2.15 DESeq2 Results Day 56 no-GA vs Day 0 no-GA

Supplemental Table S2.16 DESeq2 Results Day 56 vs Day 56 no-GA

Appendix C

Supplemental Methods

Sample Collection

Stream water was collected from HF+ and HF- streams in northwestern PA in June 2015 using sterile Nalgene bottles. All streams were sampled within a two-week period, and depending on the stream, were stored for 3 or 4 weeks at 4°C until use. Geochemical parameters, including temperature, pH, conductivity, total dissolved solids, and salinity were measured at the time of sample collection with a Eutech PCSTestr, 35 Multi-parameter test probe that was calibrated weekly using a three point-calibration27. Refer to Figure S2.1 for a map of watersheds' location.

GA Speciation

Given the probability that GA exists in equilibrium with many different hydrated forms, searches were conducted for any other GA related chromatographic peaks. In both the standard and experimental samples, a peak corresponding to the sodium adduct of the GA hydrate ([M+H_2O+Na+] = 141.0526 m/z) was observed (Table S2.2) at an identical retention time to the molecular ion (2.8 min), indicating that hydration/dehydration was occurring in-source. Likewise, the sodium adduct of molecular ion was observed ([M+H_2O+Na+] = 123.0417 m/z, r.t 2.8 min) (Table S2.2). However, these additional peaks were minor components compared to the observed molecular ion, with areas and intensities at least 1 order of magnitude lower to the molecular ion. The detection of multimeric forms of GA has been addressed in previous reports[52]. Ferrer and Thurman[52] analyzed GA (among other HF additives) and detected peaks for GA oligomers. The oligomers are formed in solution by aldol condensation instead of in-source, as evidenced by their separate retention times. In the current study, GA standards and samples produced a peak corresponding to the sodium adduct of the singly hydrated aldol dimer ([M+H_2O+Na+] = 223.0941 m/z, r.t. 4-5 min) (Table S2.2). Additionally, a peak was observed that corresponded to the mass of the sodium adduct of a doubly hydrated aldol dimer ([M+2H_2O+Na+] = 241.1038 m/z, r.t. 4-5 min) formed in-source (Table S2.2). In all cases, the

area and intensity of the dimer peak was at least 2 orders of magnitude lower than that of the parent molecular ion of GA. The mass range of the experiment excluded the sodium adduct of the doubly hydrated aldol trimer observed in past reports[52], and no other forms of the trimer were detected. Despite the presence of other detected forms of GA, the chromatographic peak for the chosen molecular ion of 101.0600 m/z is the best means of relative quantitation (Figure S2.4). If any environmental variable between streams influenced the detected amount of GA, then the changes can be reflected in the abiotic controls, which displayed constant GA among streams and over time as discussed below.

Detailed DNA Extraction Procedure and Library Preparation

The water collected was filtered through a 0.2 µm Sterivex nylon filter (Millipore Sigma, St. Louis, MO). Filters were frozen at -20°C until use. The plastic casing of the filter was cracked opened with sterile pliers in a biohazard hood. The filter was removed with sterile tweezers and cut with a sterile knife. The filter pieces were extracted for genomic DNA using PowerSoil DNA isolation kit (Qiagen) according to the manufacturer's manual. The v4 region of the 16S rRNA gene was amplified using the protocol described by Caporaso et al.[32] Primer dimers were removed using the Select-a-Size DNA Clean and Concentrator Kit (Zymo Research, Irvine, CA) following the manufacturer's instructions to remove fragments smaller than 300 bp. The size of the amplicons was visualized and quantified using a 2100 Bioanalyzer and DNA 1000 chip (Agilent Technologies, Santa Clara, CA). Samples were quantified using a Qubit Fluorometer (ThermoFisher Scientific) and pooled based on equimolar concentrations to a final pool of 10 nM. The concentration was verified using qPCR utilizing KAPA SYBR FAST qPCR kit (KAPA Biosystems, Wilmington, MA).

Quantification of Bacterial 16S rRNA Gene

Each 10 µl reaction was loaded using a QIAgility (Qiagen, Hilden, Germany) automated PCR loading robot. Each reaction contained 2 µl of template DNA, 3.94 µl of water, 4.00 µl of Applied Biosystems Power SYBR GreenPCR Master Mix (ThermoFisher Scientific, Waltham, MA), 0.03 µl of 300 nM forward primer and 300 nM reverse primer respectively.

Microbial diversity and differential abundance analyses

Alpha diversity, beta diversity and DESeq2[47] analyses were performed using un-rarefied OTU table. Difference in community evenness and richness between HF+ and HF- streams was measured using Simpson, Chao1, Observed diversity, and Shannon alpha diversity metrics using the Phyloseq [35] R[36] package. Alpha diversity results were used as a proxy for microbial resistance and resilience against GA. Microbial community alpha diversity values were rank transformed and compared using the same model as for 16S rRNA gene copies/mL. Beta diversity measures were calculated using weighted UniFrac distance matrix[48] and visualized using a principal coordinate analysis (PCoA). Finally, microbial community beta diversity was compared using a nested PERMANOVA using the adonis command in the VEGAN[49] R package

The DESeq2 [46] R package was used to find microbial taxa enriched through time by comparing each time point (day 7, 21, 35, 29, and 56) to the day 0 No-GA control. Day 56 was also compared to the day 56 No-GA control, and both day 0 and day 56 No-GA controls were also compared. At each time point comparison between HF+ and HF- was performed to identified differentially enriched taxa between HF impact status. The Wald test[46] was performed using the parametric fit-type and a Benjamini & Hochberg adjusted P-value with an alpha < 0.01 and reported OTUs had 2 $\log_2$ fold change or higher.

Supplemental Results

Microbial Community Description

All streams, independent of HF activity, had a dominant *Proteobacteria* population before addition of GA—more than 75% relative abundance in HF+ group and more than 65% in the HF- (Figure S2.6A). This was expected as *Proteobacteria* was the dominant phylum *in situ*[25] and was also observed in other streams impacted and not impacted by UOG[54]. The most dominant classes were *Beta, Alpha* and *Gammaproteobacteria* for the HF+ microcosms, and *Beta, Gamma,* and *Alphaproteobacteria* for the HF- microcosms, in that order. The HF- microcosms also had a slightly higher *Bacteroidetes, Verrucomicrobia,* and Acidobacteria populations than the HF+ microcosms. At day 7 after GA amendment, *Gammaproteobacteria* were the first responders, becoming 50.3% of the population in HF+ group compared to 59.7% in the HF- group. By day 21, *Gammaproteobacteria* populations decreased to 36.8% in HF+ and

40.1% in HF-, and *Alphaproteobacteria* was enriched reaching 28.2% in HF+ and 24.6% in HF-. By day 35, *Alphaproteobacteria* reached 57.6% abundance in HF+ streams and 84.9% in HF- streams. The trend of increasing *Alphaproteobacteria* population continued in both groups. By day 49, *Alphaproteobacteria* population reached 87.7% in HF+ and 92.5% in HF-. However, by day 56 *Gammaproteobacteria* reappeared at more than 12% of the population in the HF+ group, while *Betaproteobacteria* increased to 10% in HF- group. Conversely, at day 56, the no-GA control microcosms had more diverse microbial populations, reflecting a decrease of *Proteobacteria* over time. Only around 35% of the population consisted of *Proteobacteria* in HF+ streams, followed by approximately 25% *Bacteroidetes*, 15% *Verrucomicrobia*, and 5% *Planctomycetes*. Whereas in the HF- streams, the *Proteobacteria* population was close to 50%, followed by approximately 12% *Verrucomicrobia*, and 10% *Planctomycete*s and 10% *Bacteroidetes*.

At day 7 after addition of GA, *Gammaproteobacteria* was the only class that experienced a significant enrichment at this critical response phase to GA. This response was carried in both HF+ and HF- microcosms by the genera *Pseudoalteromonas* (19% HF+, 22% HF-)*, Unclassified Enterobacteriaceae (13.5% HF+, 16.8% HF-),* and *Alcanivorax (3.4% HF+, 4.6% HF-).* At the same time, the *genus Methylobacterium* member of the *Alphaproteobacteria* class was enriched from 0.1% in HF+ and 0.2% in HF- microcosms prior to GA amendment to 6.5% in HF+ and 1.6% in HF- microcosms (Figure S2.6B). Enrichment of this genus continued until day 49. At day 49, *Methylobacterium* accounted for 78% in HF+ and 92% in HF- microcosms and at day 56, 71% in HF+ and 84% in HF- microcosms. The no-GA control had less than 0.1% of *Methylobacterium* in either HF+ and HF-, showing that enrichment was due to the GA addition and not bottle effect.

The predominant microbial community shifts were overall similar in both sets of microcosms. The initial abundance of *Gammaproteobacteria* population at day 7, as compared to the microbial community prior to GA addition, could be associated with the bacterial stress response caused by GA.

CHAPTER 3 THE EFFECTS OF THE BIOCIDE 2-2-DIBROMO-3-NITRILOPROPIONAMIDE ON MICROBIAL COMMUNITY STRUCTURE AND DEGRADATION POTENTIAL IN STREAMS IMPACTED BY HYDRAULIC FRACTURING ACTIVITY

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Organized sampling expedition- NU, RL, and CJG

Collected samples-MFC, and CJG

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Provided equipment, data analyses training, and methods optimization- JY, and KC

Analyzed the data- MFC, and MPL. MPL was supervised by RLH

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Abstract

Unconventional natural gas production continues to rise, but the effects of hydraulic fracturing (HF) surface spills in aquatic systems are not fully understood. Here, a commonly used HF biocide 2,2-dibromo-3-nitrilopropionamide (DBNPA) was studied in HF-impacted vs HF-unimpacted aquatic systems to (1) compare the microbial community changes, (2) investigate differences in degradation potential of DBNPA, and (3) compare the results to a different HF biocide, glutaraldehyde. In this study, DBNPA and its by products were found to be more persistent than previously reported. High total organic carbon (TOC) associated with HF-impacted streams favors a less persistent degradation pathway as compared to low TOC in HF-unimpacted streams which tend towards a degradation pathway with intermediates that are more persistent and more toxic than DBNPA. Multiple unidentified brominated species may form as degradation or daughter products regardless of past HF impacts. Furthermore, the microbial community structure was different in HF-impacted vs unimpacted microcosms, which can affect degradation dynamics, pathways, and overall ecosystem functions. These findings are significant and may help to coordinate proper responses after HF spills and to formulate HF chemical mixtures that have minimal impact in the environment.

Introduction

Hydraulic fracturing (HF) has revolutionized the energy industry in the U.S. It has made previously unreachable oil and gas reserves available for extraction, and pushed the U.S. towards energy independence[1]. Multiple environmental concerns have also accompanied this energy and economic growth. Biocides are HF fluid components of concern based on their toxicity and potential impact to the environment [2, 3]. Biocides are used in HF operations to control microbial induced corrosion of casings and pipes, and gas souring caused by sulfate-reducing bacteria [2,3]. Biocides efficacy has been met with varying degrees of success due to potential resistance or inactivation of the biocides in HF conditions [2, 4-6]. The fate of these biocides in the environment, and their impact on microbial communities are poorly understood.

The biocide 2,2-dibromo-3-nitrilopropionamide (DBNPA) is the second most commonly used biocide after glutaraldehyde. DBNPA is a fast-acting electrophilic biocide. DBNPA inhibits

essential biological functions by reacting with sulfur-containing nucleophiles in the cell's organelles [7]. DBNPA has a solubility of 15,000 mg/L and in addition to HF, it is commonly used in water-cooling systems and paper manufacturing. DBNPA was demonstrated to be moderately toxic by oral and inhalation routes, corrosive to eyes, and caused developmental issues in animal studies[8, 9].

Abiotic degradation of DBNPA can occur through hydrolysis and photolysis [9]. The hydrolysis half-life of this compound is pH-dependent with faster degradation at a more alkaline pH. DBNPA is biodegradable under both aerobic and anaerobic conditions, with reported half-life or less than 4 hours for both at neutral pH [9]. There are two known biodegradation pathways of DBNPA. The first pathway involves the hydrolysis of DBNPA into dibromoacetonitrile (DBAN) → dibromoacetamide (DBAM) → dibromoacetic acid. DBAN is three times more toxic and more recalcitrant than DBNPA[7]. Dibromoacetic acid, a problematic disinfection-by-product[10], breaks down after what has been estimated to be over 300 days into glyoxylic acid, oxalic acid, and carbon dioxide[11]. However, a higher presence of total organic carbon (TOC) and/or nucleophilic reactions under ultraviolet light favors a second degradation pathway, where DBNPA is degraded to monobromonitrilopropionamide (MBNPA), a compound two times less toxic than DBNPA [12], and then to cyanoacetamide (CAM)[7, 11]. The products of DBNPA biodegradation are the same under aerobic and anaerobic metabolism. Still, the relative abundance of these daughter products and the half-lives of these daughter products varies if it is aerobic or anaerobic biodegradation [7, 9].

DBNPA can reach the environment in many ways; (1) surface spills into the soil, surface water, and aquifers; (2) incomplete removal after water treatment; (3) groundwater contamination after equipment failure (leakage), and (4) unintended fractures or abandoned wells[2]. It could also occur in HF operations e.g., (1) the transportation of chemicals to the site; (2) mixing of HF fluids and chemicals on site; (3) subsurface injection of the HF fluids; (4) handling, collection, and storage of produce water; and (5) disposal of the produced water [13]. Understanding the impacts of surface and shallow groundwater spills, leaks, and disposal of poorly treated HF wastewater in the environment is of extreme concern as there have been reported cases of the accumulation of toxic materials in groundwater, streams, soils, and

sediments at HF operating sites [14-18]. In addition to biocides reports of methane contamination in drinking water [19], high benzene content in groundwater [14], high methane fugitive emissions and greenhouse-gas footprint [20, 21], and limitations with wastewater handling[22], are also of concern.

This study aimed to (1) understand the local stream microbial community responses, and (2) degradation potential for DBNPA in streams impacted and not impacted by HF operations following the experimental design described in Campa et al. for glutaraldehyde[23]. DBNPA and glutaraldehyde microbial community response similarities and difference are also discussed. This study is one of the first times that DBNPA degradation and microbial community changes have been tracked simultaneously over time in aerobic stream waters impacted by HF.

Materials and Methods

Stream Selection and Sample Collection

For comparison purposes, sample collection was identical and done at the same time as Campa et al[23]. Briefly, sample selection employed GIS surveys, and Pennsylvania Department of Environmental Protection (PADEP) records to minimize watershed variation caused by industrial activities other than unconventional oil and gas (UOG) extraction. Streams selected were in forested areas, with no indication of past mining activity or other anthropogenic impacts in the PADEP records. HF-impacted (HF+) streams had high HF activity, with a history of surface spills in two streams (Alex Branch (AB) and Little Laurel (LL)) or more than 20 well-heads (unnamed tributary (UNT) and Naval Hollow (NH)) within the watershed. The spills occurred in 2009 when a pipe carrying flowback water burst, leaking into LL, and to a lesser extent to AB. In the same year, HF chemicals were accidentally spilled into AB. The three HF-not-impacted (HF-) streams had construction development involving well pads, but no HF activity had started. These streams were UNT East Elk (EE), UNT West Elk (WE), and Dixon Run (DR). Refer to Figure S3.1 for a map of watershed locations[23]. A detailed description of the sites, screening process and selection is described elsewhere [24-26].

Collection of stream water from three HF+ and three HF- streams in northwestern Pennsylvania occurred in June 2015. Samples were collected in sterile Nalgene bottles and stored

at 4°C until use. Conductivity, pH, temperature, and total dissolved solids were measured at collection time using a weekly calibrated Eutech PCSTestr 35 Multi-parameter test probe.

Microcosm Setup

Dow Chemicals' literature showed effective kill (> 6 log reduction) of acid producing bacteria (APB) and sulfate-reducing bacteria (SRB) using 25 mg/L of DBNPA[27]; nevertheless, biocide usage in HF is highly variable with reports between 10 to 800 mg/L[3]. Thus, microcosms were constructed using 125 mg/L DBNPA in 235 mL of stream water. DBNPA was purchased from Sigma-Aldrich (CAS 10222-01-2). Abiotic controls were autoclaved to kill all microbes present and were used to measure abiotic degradation of DBNPA. Negative biological controls (No-DBNPA controls) were used to examine the bottle effect in microbial communities with no biocide added. Controls were set at a volume of 20 mL. All microcosms were set in triplicates at room temperature under aerobic conditions for 56 days. Samples were collected every seven days for chemical analysis and day 0, 7, 21, 35, 49, and 56 for microbial analyses. Total organic carbon was measured before the beginning of the experiment using a Shimadzu TOC-L Series analyzer with ASI-L autosampler (Shimadzu, Kyoto, Japan) following the protocol described in Campa et al[23].

Quantification of DBNPA using HPLC-DAD

Every week, one mL of microcosm water was collected to compare the difference between rates of abiotic and biotic DBNPA degradation in HF+ and HF- microcosms. After collection, samples were filter-sterilized using 0.2 µm nylon filter, acidified to pH 2.5 with phosphoric acid to minimize hydrolysis of DBNPA as described by Blanchard *et al.*, 1987[7], and were then frozen at -20°C until analysis.

DBNPA quantification was performed with high-performance liquid chromatography (HPLC) using a modified version of the method described by Blanchard *et al.*, 1987. An Agilent Eclipse XDB-C18 column (5 µm, 4.6 x 150 mm) and diode array detector (DAD) was used at a detection wavelength 210 nm. The mobile phases and elution gradient were as follows: from 1-6 mins, 75% deionized water (adjusted to pH 2.5 with phosphoric acid) and 25% acetonitrile, at min 6, 40% phosphoric acid, 60% acetonitrile, at min 7-10 15% phosphoric acid and 85%

acetonitrile, at min 11 75% phosphoric acid and 25% acetonitrile, and at min 13 75% phosphoric acid and 25% acetonitrile. Calculation of half-life was done using the $N_{(t)}=N_0(0.5)^{t/t_{(.5)}}$, where N_0 was the original amount added, 125 mg/L.

Detection of DBNPA Degradation Products Using Nano-High Performance Liquid Chromatography-High-Resolution Mass Spectrometry

Filtered stream water samples were kept frozen in amber bottles in the dark at -20°C until analysis by nano-liquid chromatography-high-resolution mass spectrometry (nano-HPLC-HRMS). Measurements were collected using a Dionex UltiMate 3000 HPLC pump (ThermoFisher Scientific) coupled to an LTQ-Orbitrap Velos Pro mass spectrometer (ThermoFisher Scientific) equipped with a nano-electrospray ionization (ESI) source (Proxeon, Denmark) operated in positive mode under direct control of the XCalibur software, v2.2 SP1.48 (ThermoFisher Scientific). The nano-electrospray column/emitter was prepared manually in-house using 100 µm i.d. fused-silica (Polymicro Technologies) which was laser-pulled and pressure-packed to 20 cm with Kinetex C18-RP material (5 µm, 100 Å, Phenomenex). The column was aligned in front of the MS capillary inlet, and 300 nL of the sample was manually injected directly onto the column. LC/MS-grade acetonitrile (ACN) and water (both degassed) were purchased from EMD Millipore, and formic acid (FA) from Sigma-Aldrich. Nano-flow rates were achieved with a split-flow setup prior to the injection loop (~250 nL min^{-1} at the nano-spray tip) and separations were conducted by initially holding at 100% A (95% ACN/5% H_2O/0.1% FA) for 5 min, increasing linearly over 60 min to 100% B (70% ACN/30% H_2O/0.1% FA), and then holding at 100% B for 5 min before re-equilibrating the column at 100% A for 20 min prior the next injection.

The mass spectrometer was externally calibrated for mass accuracy on the day of analysis using the positive calibration solution (Pierce, ThermoFisher Scientific). The ESI source capillary voltage was set to 3.0 kV and the capillary temperature to 275°C. High-resolution full scans were acquired in centroid mode at a resolving power of 30,000 over a mass range of 50 – 1000 *m/z*. Fragmentation data (MS^2) were also collected using collision-induced dissociation (CID, $He_{(g)}$) and a data-dependent acquisition approach on the top 5 most abundant ions in each

MS^1 full scan. High-resolution (15,000 resolving power) MS^2 spectra were collected using a 2 *m/z* precursor isolation width, and an optimized 30% normalized CID energy for fragmentation. Raw LC/MS data were analyzed using the Thermo XCalibur Qual Software. Integrated LC peak areas were obtained from the extracted ion chromatograms (10 ppm tolerance).

Quantification of Bacterial 16S rRNA Gene

25 mL of water was filtered through a 0.2 µm nylon filter (Sterivex), and frozen at -20°C until use. The frozen filter was cut with sterile pliers. The filter membrane was cut with a sterile razor and DNA was extracted from the membrane using Mo Bio PowerSoil DNA isolation kit following manufacturers specifications. Universal bacterial primers Bac1055YF and Bac1392R were used to quantify the 16S rRNA gene in a QuantStudio 12K Flex Real-Time PCR system (ThermoFisher Scientific). For reaction mixture, and qPCR parameters refer to Campa et al[23].

16S rRNA Gene Amplicon Library Preparation and Sequencing

After DNA extraction the v4 region of the 16S rRNA gene was amplified using the primers and protocol described by Caporaso et al.[28] Refer to Campa et al.[23], for a description of library preparation. The final libraries were run in the Illumina MiSeq (San Diego, CA, USA) using a v2 (2 x 150 reads) kit following manufacturer's specifications.

16S rRNA Gene Amplicon Sequencing Data Analyses

Data analyses were done in QIIME (version 1.9.1) and the Phyloseq[29] and Vegan[30] packages in R following the protocol described in Campa et al[23]. DESeq2 [31] R package was used to identify differentially enriched taxa through time and between HF+ and HF- microcosms at each time point following the protocol in Campa et al[23]. using a cutoff of 2 $\log_2$ fold change or higher, and a Bonferroni adjusted p-value of 0.01.

Statistics

For comparison purposes, the statistics were performed using the same tests and software as in Campa et al[23]. To understand the effect of DBNPA on microbial community, 16S rRNA gene abundance was compared using a complete randomized design (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 in the R nlme package[32]. The least squares means were computed and separated with Bonferroni method using the R emmeans package[33]. 16S rRNA gene copies/mL were log10 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 used. To determine the differences between HF+ and HF- at day 0, an independent sample t-test was performed with data for only that time point. Microbial community alpha diversity values were rank transformed and compared using the same model as for 16S rRNA gene copies/mL. Finally, microbial community beta diversity was compared using a nested PERMANOVA using the adonis command in the R VEGAN[30] package. All statistical tests were performed using R, and p-value significance were set at p= 0.05.

Accession Numbers and Data Availability

Mass spectrometry data was uploaded to the Center for Computation Mass Spectrometry (UCSD) online database MassIVE. The MassIVE ID number is MSV000082488. Microbial 16S rRNA gene amplicon sequences for both DBNPA treated microcosm and the glutaraldehyde treated microcosms were deposited in NCBI Sequence Read Archive (SRA) in SRA accession SRP151211 under BioProject PRJNA476929 as Biosamples SAMN09459387 to SAMN09459570, and SAMN09475542 to SAMN09475579.

Results and Discussion

Abiotic and Biotic Degradation of DBNPA Over Time

We evaluated the degradation of DBNPA over 56 days using both biotic and abiotic microcosms constructed from HF+ and HF- streams. There are only a few peer-reviewed papers reporting degradation half-life of DBNPA[7, 11], and none of them are under conditions found after HF impacts. DBNPA previously reported half-lives are hours to days depending on pH (longer half-lives at more acidic pH, exponential trend). While it was expected that DBNPA would degrade consistently over time, quantification by HPLC-DAD revealed unusual degradation curves at two of the HF+ sites with documented spills—AB and LL—which could not be

attributed to human error, or equipment malfunction (Figure 3.1 and Figure S3.2). The half-life for HF- abiotic was 8.49 d and 5.79 d for biotic conditions. The half-live for HF+ was 30.11 d for abiotic and 70.25 d for biotic. These half-lives are slower than what would be expected from previously reported, based on pH (HF- average pH at the beginning of the experiment was 6.5)[11]. Based on pH alone, it would be expected that HF+ (average pH of 4.9) would have a slower half-life than HF-, which seemed to be the case here. It is possible that there may have been a coeluting compound that also absorbed in the same region or interfered with the HPLC-DAD measurement as seen by the spike at day 14 (Figure 3.1) due to chromophores and/or similarities in the degradation products that might not have been able to be separated with the method used[34].

In both the biotic and abiotic samples, a sharp increase in the DBNPA signal at day 14 of the incubation was observed (Figure S3.2). To evaluate whether something may be contributing to the signal besides DBNPA (i.e. a contaminant or degradation products that may absorb at the same wavelength and have the same retention time as DBNPA) we analyzed the biotic and abiotic samples from days 0, 7, 14, 21, and 28, from the HF+ sites AB and LL, and also two HF- sites for comparison, WE and EE, using nano-HPLC-HRMS. Using high mass accuracy measurements (+/- 5 ppm) and fragmentation data, we qualitatively evaluated the resulting LC-MS data by searching for DBNPA and known degradation products and by comparing the number of brominated compounds detected. Using relative abundance values and integrated peak areas, we also quantitatively evaluated the trends of these compounds across the 5 time points within each sample set.

The DBNPA molecular ion ($[M+H]^+ = 240.8606$ *m/z*) was not detected in most of the samples analyzed, which may be due to prolonged storage or multiple freeze-thaw cycles, as the samples were frozen after collection, thawed for HPLC-DAD analysis, and then frozen and thawed again for HRMS analysis. Though, because bromine (Br) has a unique isotopic signature (Figure S3), we did observe multiple other brominated species—some known DBNPA degradation products, but also many previously-unreported species and potentially novel degradation products. Across the four sites (WE, EE, AB, LL), five time points (day 0 to 28), and two microcosm conditions (biotic or abiotic) analyzed (n = 40), we observed 18 brominated species including DBNPA and four known degradation products—CAM, MBNPA, DBAN, and

DBAM. The detected mass to charge ratio, predicted elemental formula, and putative structure of some of these brominated products are described in Table S3.1. With the exception of the WE sample set, more brominated species were detected in the abiotic samples compared to the biotic samples, and in the HF- samples than the HF+ samples (Figure S3.4), which may indicate enhanced microbial processing of brominated compounds in HF+ microcosms. Similar to the trend observed by HPLC-DAD, the number of brominated species detected by LC-MS in the HF+ AB and LL abiotic samples increased sharply from day 0 to day 14 (Figure S3.4). The total "brominated signal"—summed integrated peak areas at each time point—also showed a sharp increase in signal at day 14 for the abiotic HF+ samples (Figure S3.5). While not as strong, the two abiotic HF- sample sets also showed an increase in signal at day 14. For the biotic samples, a steady increase in brominated signal over time was observed regardless of microcosm, with the highest signal occurring at day 21. It is important to note that only one replicate of each sample set was analyzed by LC-MS here. Despite this limitation, the quantitative trends correlate well with the initial HPLC-DAD measurement suggesting these brominated degradation products may indeed have impacted the signal response in the initial measurement.

After evaluating the total number of brominated compounds observed, we identified which compounds were known degradation products, adducts, or complexes. Degradation products from both known pathways were detected in the abiotic samples from one HF- site (EE) and both HF+ sites (AB and LL), as well as the biotic samples from EE and LL (Figure S3.6 and Table S3.1). Yet, only DBAM, the end product of the toxic pathway, was found in the biotic and abiotic samples from the WE site. Comparatively, only CAM and MBNPA, intermediates from the nontoxic pathway, were observed in the biotic samples from one of the HF+ sites (AB), suggesting a possible microbial preference for the nontoxic pathway at the HF+ sites. Alternatively, Blanchard et al.[7] showed that preference over degradation pathways is TOC-dependent, with higher TOC selecting for the second, less toxic pathway, with MBNPA as an intermediate. Here, we measured TOC at day 0 of the experiment (Table S3.2). The HF+ samples had significantly more TOC ($P = 0.02$, t-test) than the HF- samples with averages of 7.81 mg/L and 4.09 mg/L, respectively. This could explain why we saw more of a preference for the nontoxic pathway at the HF+ sites. Other factors to consider include different enzymatic

capabilities of the microbial communities present in the samples or that differing water chemistries may favor one pathway over another. The measured water chemistry *in situ* were discussed in Campa et al[23]. Temperature (HF+: 16.8°C, HF-: 12.8°C), pH (HF+: 4.9, HF-: 6.5), conductivity (HF+: 29.2 μS/cm, HF-: 33.7 μS/cm), and total dissolved solids (HF+: 20.8 ppm, HF-: 23.9 ppm) were measured. Even though the differences were not statistically significant the differences such as pH may affect the stability of DBNPA. This observation is also supported by cluster analysis as the detected brominated species clustered by HF impact history (Figure S3.6). Overall, these results suggest that DBAM, and other brominated species, may be persistent degradation products of DBNPA that, depending on the history of the watershed, may be favored over the less toxic pathway.

Quantification of 16S rRNA gene by qPCR

16S rRNA gene abundance was quantified before biocide addition, at 7, 21 and 56 days after biocide addition to observe the effect DBNPA had on bacterial biomass. The starting 16S rRNA gene concentrations for all microcosms were in the range of 10^4 gene copies/ mL, averaging 4.03 x 10^4 gene copies/mL in the HF+ streams and 4.38 x 10^4 gene copies/mL in HF- streams. Seven days after addition of DBNPA a decrease of -0.16 $\log_2$ fold change (FC) in 16S rRNA gene copies/mL was observed in HF+ microcosms, and a small increase of 0.22 $\log_2$ FC was observed in HF- microcosms. By day 21 16S rRNA gene copy/mL increased in both microcosms, 0.62 $\log_2$ FC in HF+ and 3.6 $\log_2$ FC in HF-. By day 56 the HF+ experienced a 4.9 $\log_2$ FC and HF- experienced a 3.9 $\log_2$ FC. In comparison at day 56 both no-biocide control, HF+ and HF-, experienced an 8.3 $\log_2$ FC from their initial gene copies/mL at day 0. The difference between 16S rRNA gene copies in HF+ and HF- microcosms before DBNPA addition was not statistically significant. After addition of DBNPA, the difference through time (day 7 to 56) between HF+ and HF- microcosms was statistically significant ($p < 0.05$). HF+ microcosms had a higher 16S rRNA gene copies/mL on average by day 56 as compared to HF- (Figure 3.2) even though 16S rRNA gene copies/mL first decrease in HF+ after DBNPA addition while experiencing a small increase in HF-. The no-biocide control microcosms did not show this trend, as they experienced the same $\log_2$FC over time, indicating that the difference seen in the

DBNPA treated microcosms is due to initial DBNPA impact to the microbial community, followed by response and adaptation to DBNPA.

Measurement of the 16S rRNA gene throughout the experiment shows that the HF- microbial communities were initially more resistant and tolerant to the DBNPA perturbation, as shown by their overall positive log-fold change in the gene copy number at day 7. However, through time HF+ showed strong adaptation to DBNPA, as their log-fold changes surpass HF- and overall gene copies/mL was an order of magnitude higher than HF-. Thus, overall HF+ could quickly adapt to resist and tolerate DBNPA better than HF-. It has been shown that previously perturbed microbial communities can better resist new perturbations[35]. These results are also consistent with the results from Campa et al.[23], of the same experiment ran with the biocide glutaraldehyde, showing that the adaptation response is not biocide specific and that in these microcosms HF+ microbial community can quickly adapt to the biocide presence as shown by the increase in 16S rRNA gene copies/mL over time.

Microbial Community Diversity Changes

Before DBNPA addition HF- microcosms had an overall higher evenness and richness than HF+, in spite of, after addition of DBNPA evenness and richness were affected through time. Using Shannon diversity, HF+ microcosms experienced a smaller decrease in evenness and richness--even though HF- had an overall higher diversity (Figure 3.3a) ($P < 0.01$). Meanwhile, while not statistically significant, Simpson diversity (Figure 3.3d) which also account for the abundance of species present, detected minimal changes in diversity over time except for HF- at day 21, still, diversity bounced back by day 35. In contrast, Chao1 ($P < 0.05$) and Observed Diversity ($P < 0.05$) measurements (Figure 3.3b and 3.3c) give higher importance to unique and rare OTUs and thus experienced a more prominent decrease in diversity as fewer OTUs dominated overtime. These alpha diversity measurements suggest that the microcosms contain a higher quantity of OTUs able to tolerate and adapt to DBNPA as compared to having just a few OTUs becoming enriched as it the case of glutaraldehyde[23]. The difference in alpha diversity between glutaraldehyde and DBNPA is another indication of DBNPA's low microbial control efficacy over time. In comparison, when comparing day 0 with day 56 control to test bottle

effect, the changes detected by day were not significant, and HF- maintained higher diversity than HF.

Weighted Unifrac beta diversity revealed that there was a different phylogenetic response between HF+ and HF- populations. The weighted Unifrac response was plotted on a PCoA, PC 1, explained 23.5 % of the sample variance, while PC2, explained 16.2% of the sample variance (Figure 3.4). The DBNPA treated microcosms cluster by past HF exposure (P = 0.001, ADONIS) and by day (P = 0.001, ADONIS). The response through time between the samples treated with DBNPA and not treated was phylogenetically different through time (P=0.001, ADONIS). The phylogenetic response shows the addition of DBNPA causes a microbial shift that is different between HF+ and HF- groups of streams, and it is different from the shift caused by bottle effect.

Weighted Unifrac beta diversity (Figure 3.4) showed a distinct phylogenetic response between HF+ and HF- microcosms. This was similar to what was observed in Campa et al.[23], yet glutaraldehyde showed more significant phylogenetic distances with a 65% axis, while DBNPA had an axis of 16% explaining the distance between the groups, showing that the response and phylogenetic changes due to DBNPA addition were not as pronounced as for glutaraldehyde. Even though both are electrophilic biocides, DBNPA is a fast kill biocide while glutaraldehyde biocidal properties are longer lasting.[2] Glutaraldehyde is also more persistent over time[23], potentially explaining the more pronounced differences in phylogenetic distribution of glutaraldehyde treated microcosms over time.

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

Microorganisms in headwater ecosystems are environmental regulators of natural geochemical cycles and organic matter cycling[36, 37]. Microorganisms are also very sensitive to perturbation making them good sensors of environmental change and can be used for contaminant tracking[38]. The initial bacterial population in all microcosms, regardless of previous HF activities, was predominantly *Proteobacteria* prior to DBNPA amendment—more than 75% relative abundance in HF+ and more than 65% in the HF- group. However, the dominant classes differ with *Beta*, *Alpha*, and *Gammaproteobacteria* in the HF+ and *Beta, Gamma,* and

Alphaproteobacteria for the HF- group. These dominant classes were expected as previous studies on these streams show a dominant *Proteobacteria* population and a strong correlation between *Gammaproteobacteria* and HF+ streams[24, 39]. Microbial community taxa plots (Figure S3.7a and S3.7b) show differential enrichment of multiple taxa through time. *Gammaproteobacteria* were the first responders with *Idiomarinaceae* being the most dominant family. By day 35, *Alphaproteobacteria,* specifically *Methylobacterium* was the most dominant taxa. By day 56 there is no clear dominant Genus. The microbial community showed more resilience than when exposed to glutaraldehyde, as more members of the community were able to tolerate DBNPA[23]. This difference in resilience can be attributed to microbial and chemical dynamics as the resilience can also be driven by the depletion of biocidal properties, and glutaraldehyde is more persistent over time than DBNPA[23, 40, 41].

Specifically, seven days after addition of DBNPA there were 29 differentially enriched OTUs. Of those, 24 were enriched, and five were suppressed (Table S3.4). The two OTUs with the highest enrichment corresponded to AEGEAN 185 (7.43 $\log_2$FC) from the SAR404 phylum, and SAR 324 (7.26 $\log_2$FC) a member of the class *Deltaproteobacteria.* Both these OTUs were found in the glutaraldehyde enrichment done with the same samples[23]. The metabolic profile of AEGEAN 185 is unknown, but it matches to sequences of a clone library from the North Aegean Sea[42]. Sequenced members of SAR 324 have demonstrated the presence of methane monooxygenase and dehalogenases that could aid in the co-metabolization of halogenated compounds such as DBNPA[43-45]. The only enriched OTU that had a relative abundance of more than 2% at some point throughout the incubation was *Alcanivorax* (3.27 $\log_2$FC). *Alcanivorax* is a known oil degrader and was also enriched in the glutaraldehyde microcosms, showing the wide range of xenobiotic compounds it is capable of tolerating, and even possibly degrading[23]. In addition to the three previously mentioned OTUs 9 other OTUs were both enriched with glutaraldehyde and with DBNPA. Those were *Achromobacter*, *Synechococcus,* SarSea-WGS and Artic95A-2 from the SAR 406 clade*, Acidimicrobiales, Nitrospina, Sphingopyxis* and *Euryarchaeota* Marine group II and III. Of the five suppressed OTUs, three were from the order *Burkholderiales. Note: Achromobacter* has been occasionly reported to be an opportunistic pathogen[46]. Differential enrichment analysis between HF+ and HF- at day 7 showed 51 taxa

were enriched, 30 in HF+ and 21 in HF- (Table S3.9). The most substantial log FC was *Micrococcus* (6.14 $\log_2$FC), and the OTUs with more than 2% abundance enriched in HF+ were *Verrucomicrobiaceae, Caulobacteraceae, Janthinobacterium, Novosphingobium, Oxalobacteraceae,* and *Limnohabitans.*

Day 21 (Table S3.5), 35 (Table S3.6), 49 (Table S3.7), and 56 (Table S3.8) followed a similar trend with 105, 100, 97, and 103 differentially enriched taxa compared to day 0. Through time, many OTUs related to marine environments such as *Idiomarina,* SAR 324, Aegean-185, *Alteromondaceae,* ZD017, *Halomonas*, and *Alcanivorax* were enriched. This enrichment is interesting as osmotic regulation and efflux pumps have been liked to tolerance to other biocides[47-50] but had not been reported for DBNPA. Marine organisms are found in low abundance in freshwater streams and they can bloom when conditions are favorable,[51] which indicates a potential competitive advantage of halotolerant bacteria to DBNPA. Halotolerant *Halomonadaceae* was shown to be enriched in HF exposed anaerobic sediments treated with DBNPA[52]. *Dietzia, Bacillus, Methylobacterium, Verrucomicrobiaceae, Novosphingobium, Caulobacteracea,* among others were differentially enriched between HF+ and HF- microcosms (refer to Table S3.10 – Table S3.13). HF+ enrichment as compared to HF- included *Verrucomicrobiaceae* and *Caulobacteraceae* which were shown to be susceptible to low dosage of DBNPA in sediments not exposed to HF[52], confirming that *Verrucomicrobiaceae* and *Caulobacteraceae* may build a competitive advantage to DBNPA in HF-impacted streams.

At day 56, the no-DBNPA control had 209 differentially enriched OTUs compared to day 0, which can be attributed to bottle effect (Table S3.14). *Zhouia, Caulobacter, Prosthecobacter, Halomonas, Planctomyces, Gemmata, Cytophagacea, Rhodobacter* may be attributed to bottle effect. Meanwhile, at day 56 the experimental and no-DBNPA control had 181 differentially enriched taxa of those 111 were enriched in the experimental microcosms which can be attributed to the DBNPA addition (Table S15). Thus, *Bacillus, Idiomarina, Glaciecola, Alcanivorax, Acinetobacter, Vibrio, Dietzia, Methylobacterium, Pseudoalteromonas, Marinobacter, Novosphingobium, Stenotrophomonas, Burkholderia, Oxalobacteraceae,* show tolerance and adaptation to DBNPA.

Another study used .0025% v/v DBNPA with and without the addition of FeOOH as an electron donor in microcosms with sediment inoculum downstream from a UOG wastewater treatment facility comparing it to inoculum from upstream as a control to better understand microbial community changes in anaerobic microbial communities[52]. That study found three enriched families in the impacted microcosms with no FeOOH of those *Halomonadaceae*, and *Staphylococcaceae* were also found in this study. Conversely, the impacted microcosm with FeOOH as an electron donor had six enriched families, of those we detected *Rhodospirillaceae* enriched over time in HF+ as compared to HF- (Tables S3.9 to S3.13), *Ignavibacteriaceae* (enriched day 21and 35 Table S3.5 and S3.6). However, the study by Mumford et al.[52] only sample at day 42 after incubation, and the low DBNPA concentration, sediment, and anaerobic conditions used are expected to results in wide differences between that study and the one described here.

Similar taxonomic groups, such as *Methylobacterium, Idiomarina, Bacillus, Alcanivorax,* among others, were enriched when exposing the same stream water to DBNPA or glutaraldehyde[23]. The resemblance in enriched taxa may indicate that the mechanism of tolerance and resistance is similar for these two electrophilic biocides. Previous studies have shown that glutaraldehyde resistance may be caused by the expression of efflux pumps[49, 53]. Nevertheless, the mechanisms for DBNPA resistance is not known and functional genomics and transcriptomics analyses are needed to better understand this mechanism.

Even though a microbial genetic pathway for DBNPA biodegradation has not been previously determined, as a halogenated compound, it is likely that the aerobic degradation pathways would involve cometabolism, aerobic assimilation, or reductive dehalogenation (though, reductive dehalogenation is generally more common in anaerobic conditions)[54, 55]. Many of the OTUs enriched overtime have been previously identified as being capable of degrading or co-metabolizing xenobiotic compounds. In the DBNPA non-toxic degradation pathway 2, the bromines are substituted by hydrogen, which could be achieved by microbial reductive dehalogenation[56], and by abiotic mechanisms. For example, AB and LL, both HF+ streams, favored pathway 2, for both biotic and abiotic conditions, but MBNPA was an order of magnitude higher in biotic conditions, showing that microbial degradation had an active role, but

there were other abiotic interactions that aid in the degradation of DBNPA. Further research is needed to understand which microbes can use DBNPA as a carbon source, electron donor, or electron acceptor in metabolism.

Environmental Implications

This experiment aimed to test if the bacterial response to DBNPA is biocide specific or if it triggered similar responses to other biocides. In a similar experiment using glutaraldehyde, we detected a distinct community being enriched after glutaraldehyde perturbation, and while the HF+ microbial community show higher tolerance to glutaraldehyde based on higher diversity and less log fold decrease of the 16S rRNA gene after glutaraldehyde perturbation, it was not able to degrade glutaraldehyde faster than HF-. We hypothesize the difference in degradation rates could be attributed to biotic-abiotic interactions as HF+ had acidic pH compared to HF- [23].

DBNPA caused a different microbial response than the biocide glutaraldehyde. Even though similar microbial groups were enriched, a more diverse microbial population was able to resist DBNPA more than glutaraldehyde. The different microbial response may be caused by the DBNPA fast-kill approach, where its biocidal activity is more potent at the moment of initial contact, while glutaraldehyde works over a period of days to weeks. However, similar to what was observed with glutaraldehyde *Gammaproteobacteria* were the first responders, with *Alphaproteobacteria* becoming more prevalent at the later time points, yet at more specific taxonomic levels such as genus, a more diverse taxa were detected in the DBNPA microcosm. To our knowledge, this study was the first focusing on the microbial response to DBNPA in surface aquatic environments impacted by HF while simultaneously tracking DBNPA degradation.

This study revealed that DBNPA could be persistent in water previously impacted by HF, but the high TOC present in streams that have been affected by HF favors the non-toxic degradation pathway of DBNPA. Previously unaffected streams with lower TOC may favor the DBAN degradation product which is more toxic and persistent than DBNPA itself. Difference in degradation pathways is of extreme importance, as this environmental persistence may further retard microbial attenuation in the environment, potentially requiring intervention to stimulate

the affected area to enhance the preference for the MBNPA, non-toxic, degradation pathway. Environmental persistence of the brominated disinfectant by products can cause harm to the public and environmental health. For example, persistence of these side products may affect ecosystem function, affecting microbial primary production having a cascading effect to higher tropic levels. Broad HF impacts have already shown to affect micro and macroinvertebrates, fish and other aquatic organisms in the streams used as source water for the microcosms [25, 57].

More research is needed to better understand when one pathway is preferred over the other to inform UOG operators, so they can adequately handle an HF chemical spill containing DBNPA. DBNPA may not persist in the environment, but its brominated degradation products, such as DBAN, have a longer half-life and could be more harmful to the public and environmental health.

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Appendix D: Figures

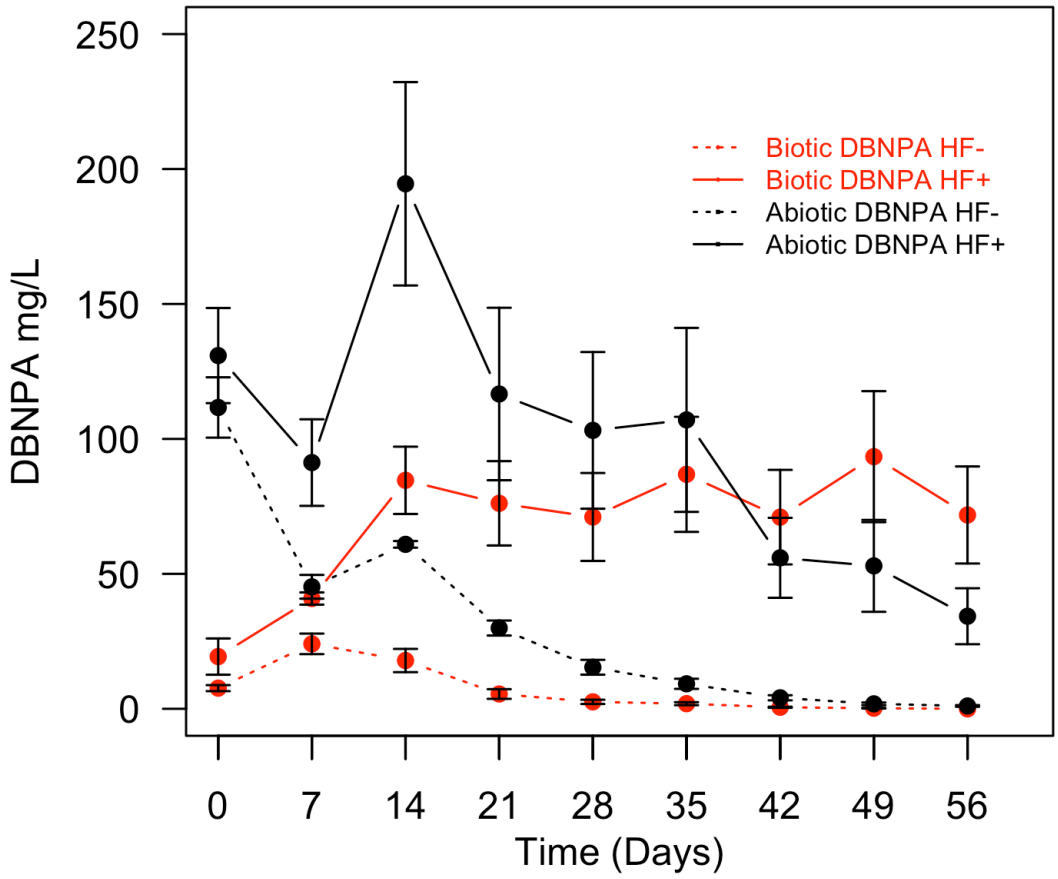


Figure 3.1 Biotic and abiotic degradation of DBNPA over time.

The red lines represent the biotic microcosms while the black lines represent the abiotic. Each dashed line represents the HF-unimpacted microcosm (n=9, three source streams and three replications) while the solid lines are HF-impacted microcosms (n= 9). Error bars represent one standard error.

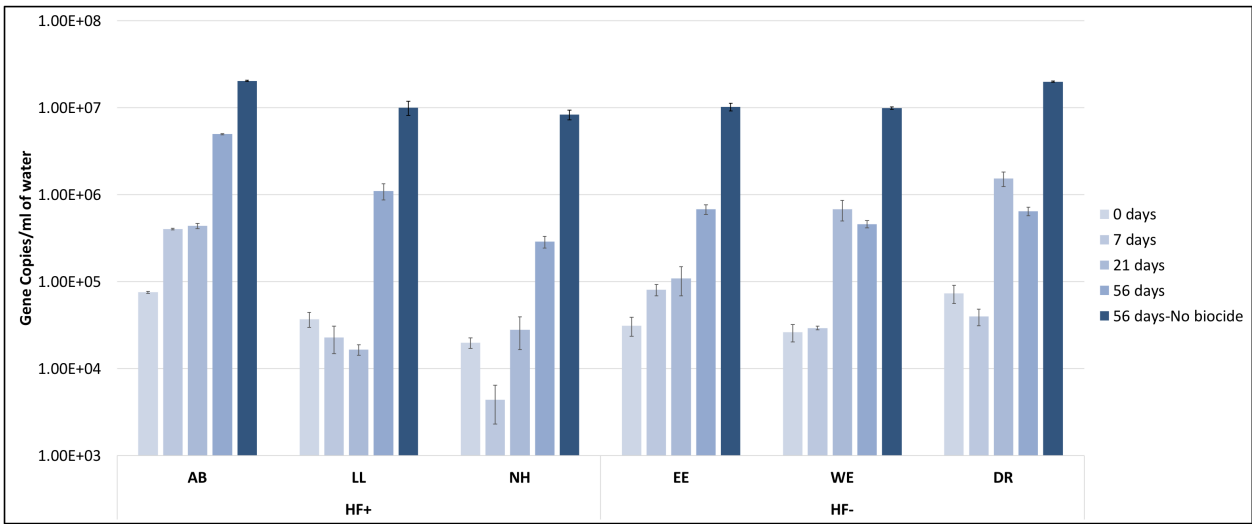


Figure 3.2 Impacts of DBNPA in abundance of 16S rRNA gene copies/mL over time.

Data shown is divided by HF-impacted (first three clusters, AB, LL, NH) and HF-unimpacted (EE, WE, DR) microcosms at day 0 before DBNPA addition, day 7, 21, and 56 after DBNPA addition, and day 56 no-DBNPA added control. The bars are color on a gradient over time, with the last bar representing the no-DBNPA control at day 56. Each bar represents n=3, and the error bars represent one standard error.

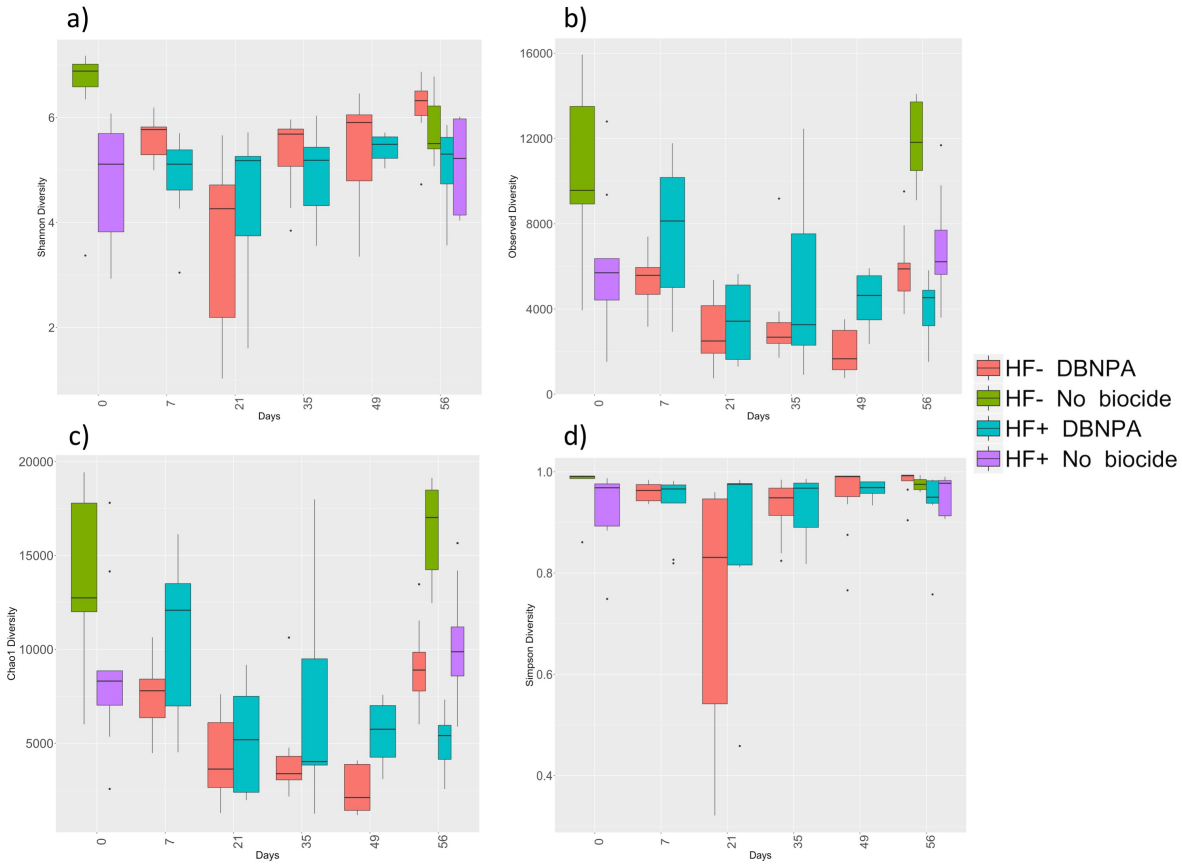


Figure 3.3 Four different richness and evenness alpha diversity estimators comparing HF impacted and HF-unimpacted microcosms over time.

The estimators used were (a) Shannon Diversity, (b) Observed Diversity, (c) Chao1, and (d) Simpson Diversity. Red and green represent HF-unimpacted microcosms. Red boxes represent the changes after DBNPA addition in HF-unimpacted (days 7 to 56), while the green boxes represent the alpha diversity without DBNPA addition in HF- (day 0 and 56). Blue and purple boxes represent HF-impacted microcosms. Blue boxes represent the changes after DBNPA addition in HF-impacted (days 7 to 56), while the purple boxes represent the alpha diversity without DBNPA addition in HF- (day 0 and 56). The box and whisker plot described the distribution of the data points. The beginning of the whiskers to the beginning of the box are the upper and lower quartiles. The box represents the interquartile range, which represents 50% of the data points (n=9). The vertical line inside the box represents the median.

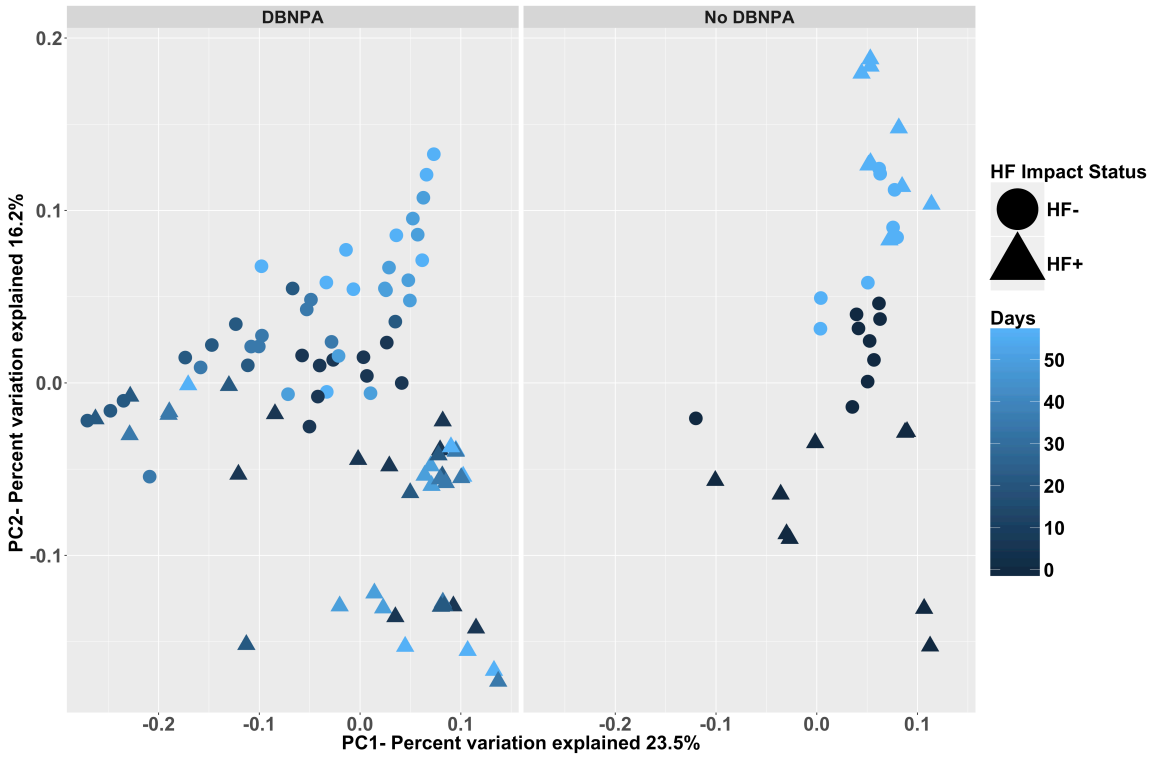
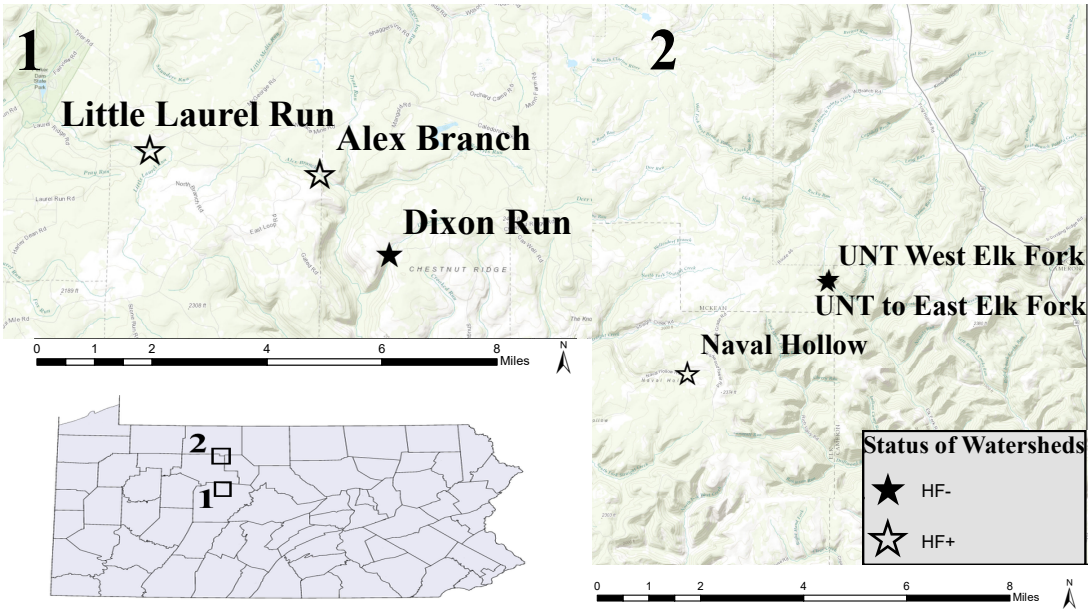


Figure 3.4 Principal coordinate analysis plot of phylogenetic microbial community changes over time calculated using weighted Unifrac beta diversity estimator.

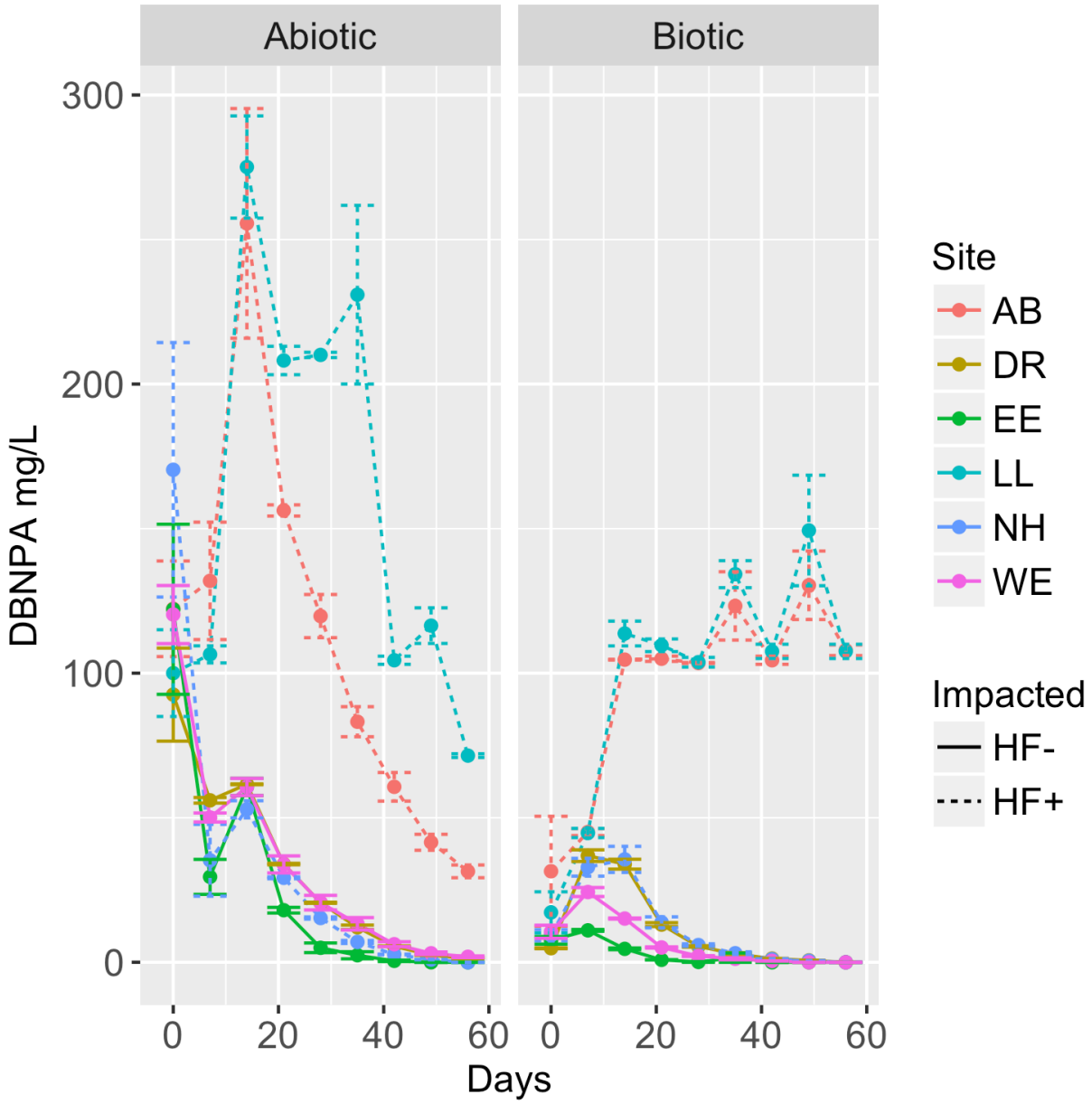
The left panel displays the HF- impacted, and HF-unimpacted microcosms perturbed with DBNPA as compared to the no-DBNPA control displayed in the right panel. Circles represent HF-unimpacted microcosms, and triangles represent HF-impacted microcosms. Temporal changes are displayed in a color gradient from dark blue (day 0) to light blue (day 56).

Supplemental Figures



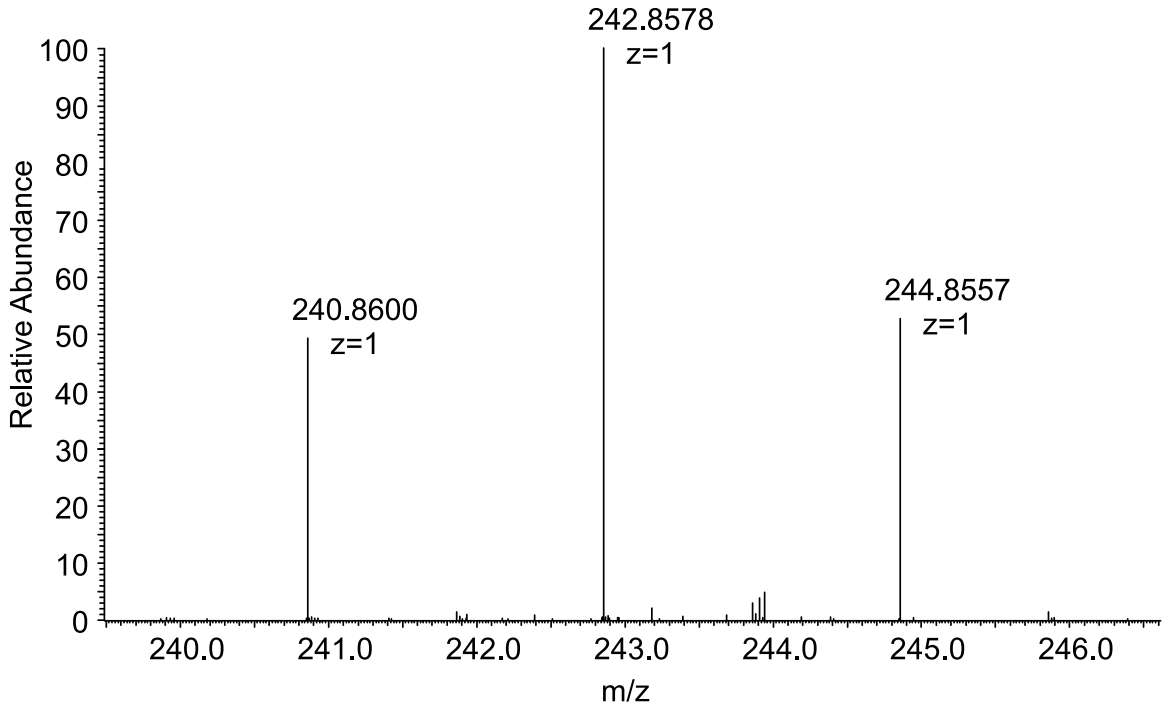
Supplemental Figure S3.1 Map of Pennsylvania watersheds used for microcosm study.

After Campa et al. 2018.



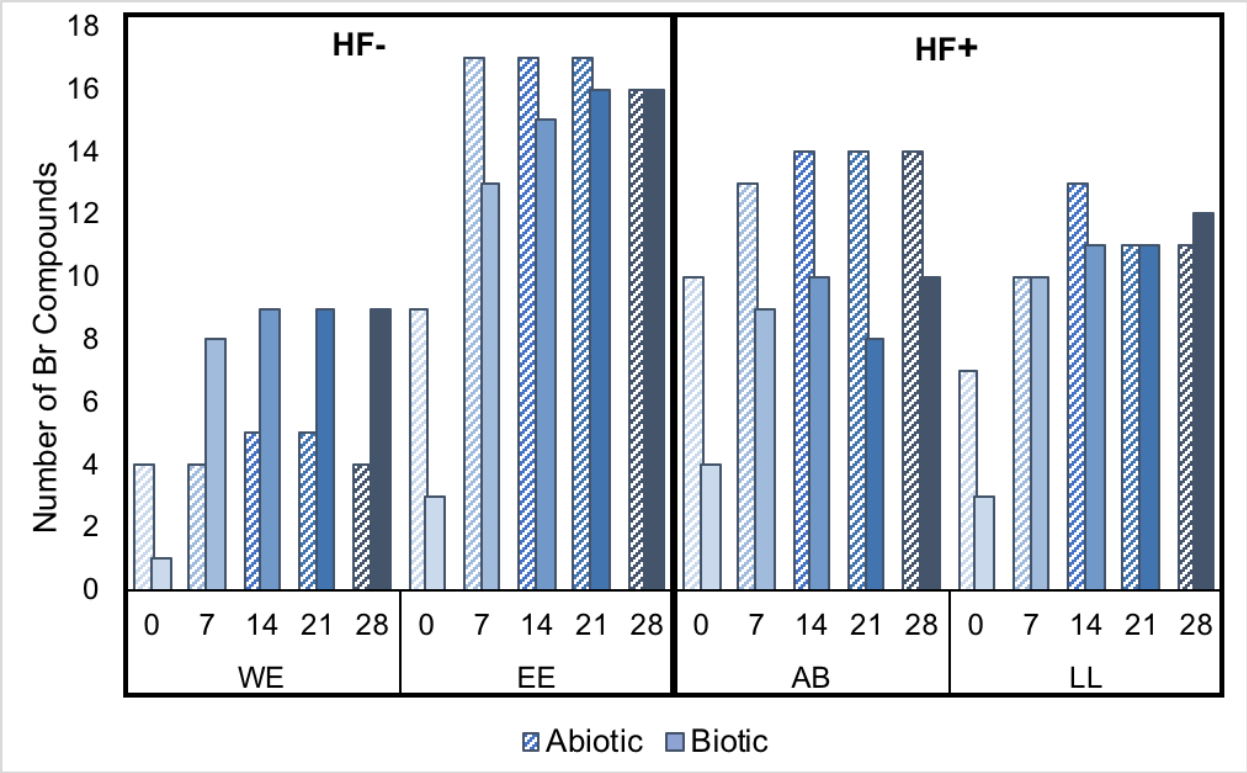
Supplemental Figure S3.2 Abiotic and biotic DBNPA degradation over time.

Data is presented by water source visualized by different colors. HF-impacted line trends are displayed with a solid line, while HF-unimpacted are displayed with a dashed line. Each data point is n=3, and the error bars represent one standard error.



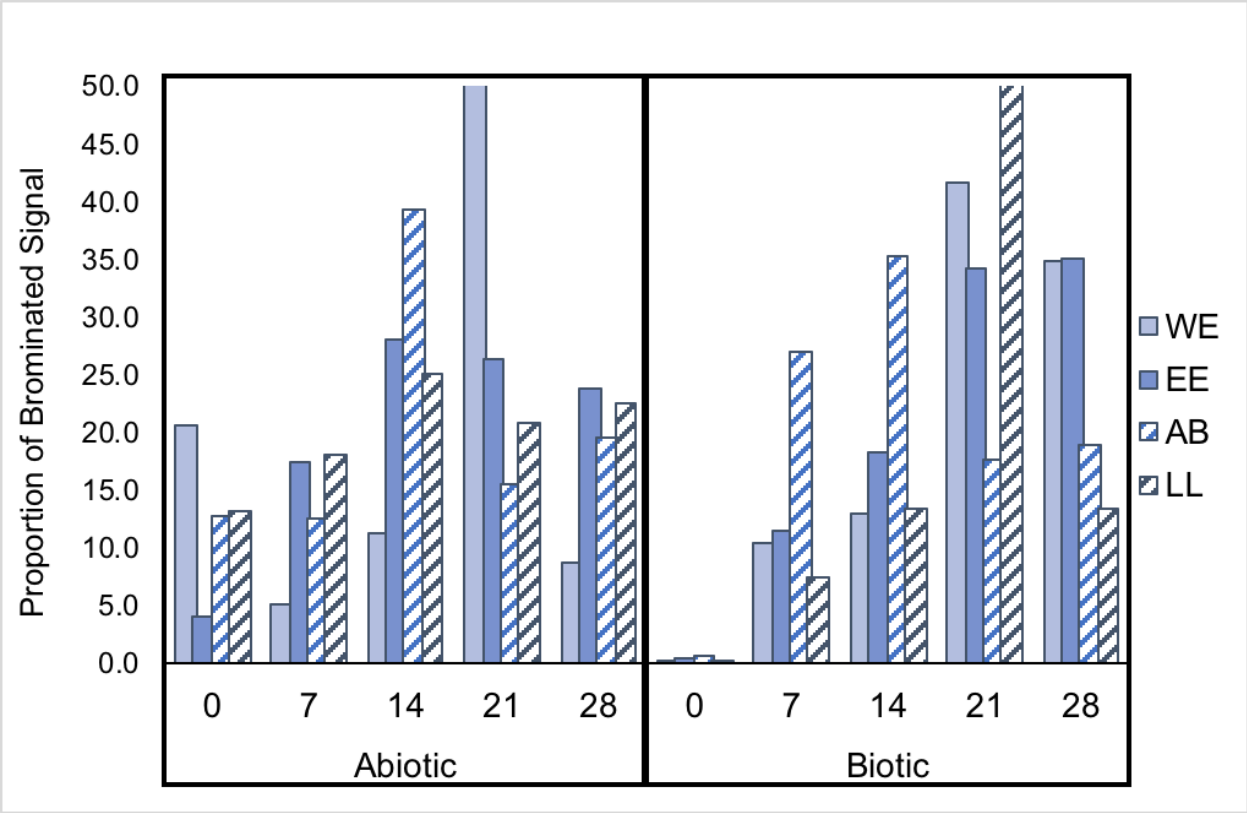
Supplemental Figure S3.3 High-resolution mass spectrum of DBNPA standard.

The DBNPA standard (monoisotopic mass: 239.8534 Da) was collected by direct infusion in positive-ion mode showing characteristic isotopic signature for dibrominated compounds. The monoisotopic ion ($[M+H]^+$) can be seen at 240.8600 *m/z* (Δ2.5 ppm mass error), the M+2 at 242.8578 *m/z*, and the M+4 at 244.8557 *m/z*.



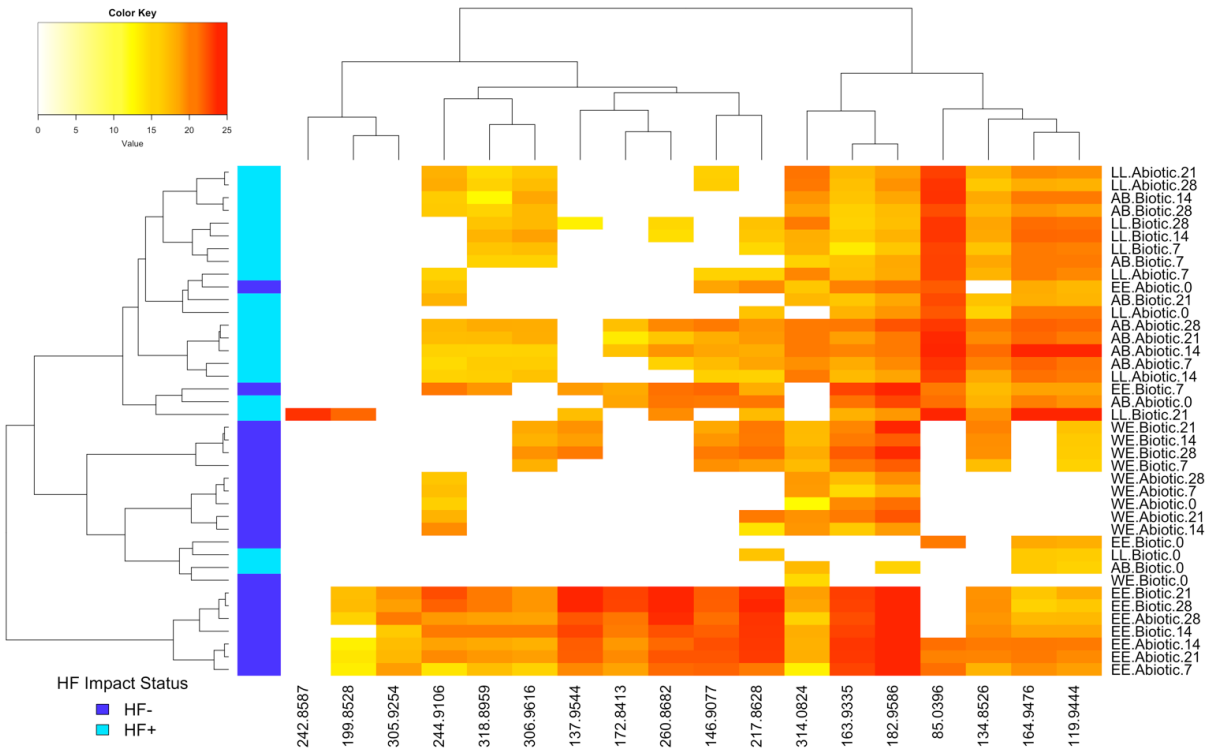
Supplemental Figure S3.4 Number of brominated species detected by nano-HPLC-HRMS.

Brominated species detected in two HF- (left) and two HF+ (right) sets of microcosm samples, biotic and abiotic, from days 0, 7, 14, 21, and 28. Abiotic samples are indicated by textured bars and biotic samples are shown with solid color.



Supplemental Figure S3.5 Summed peak areas for all brominated compounds

Summed peak of all brominated compounds at each time point (0, 7, 14, and 28 days), normalized to each sample set (stream), analyzed by nano-HPLC-HRMS. The abiotic samples are shown on the left and the biotic on the right with the HF+ samples indicated by textured bars and the HF- samples shown with solid color.



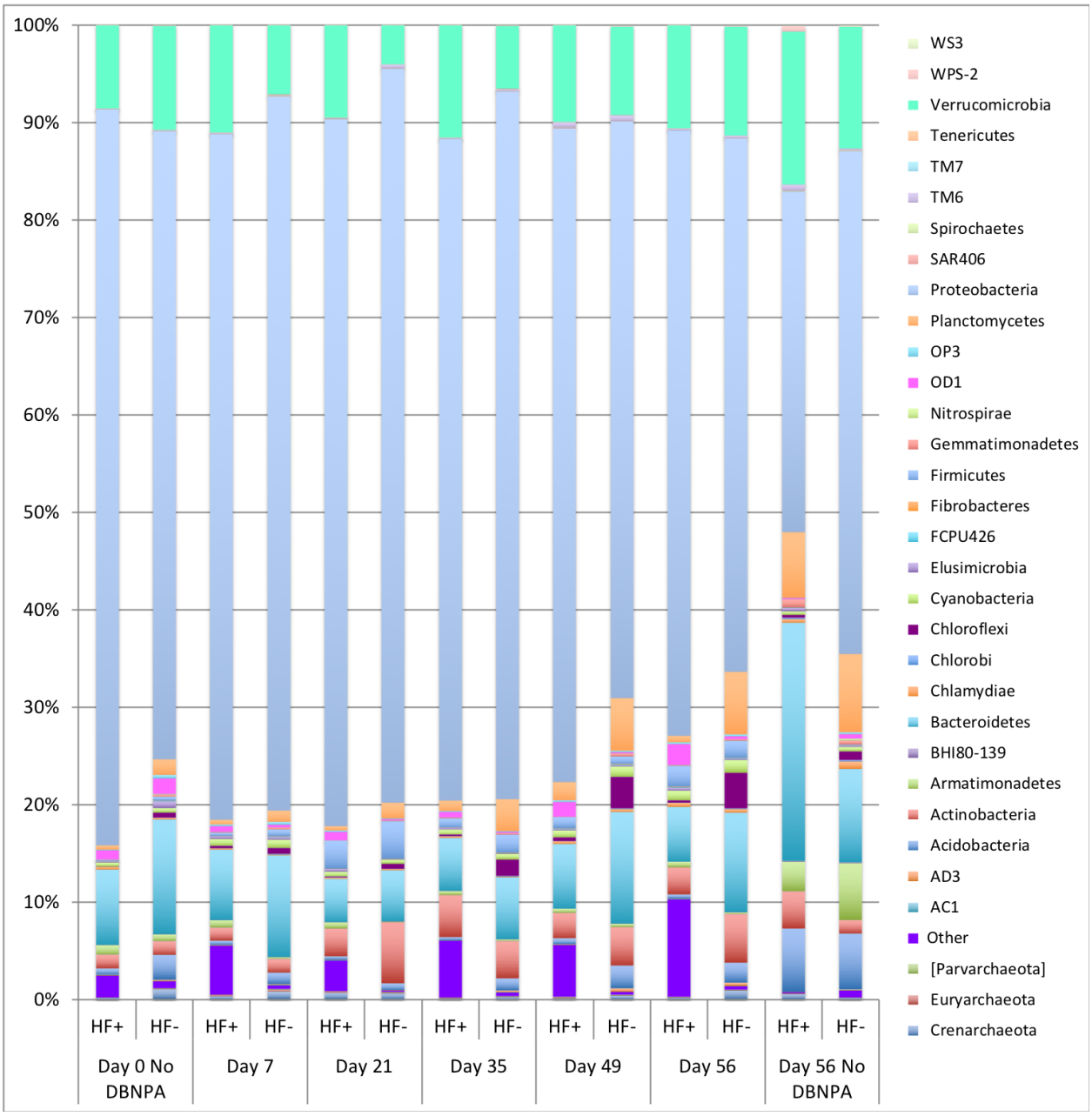
Supplemental Figure S3.6. Heat map of the normalized log_2 peak areas for brominated species detected by nano-HPLC-HRMS.

The dendrograms cluster samples using the Ward method of agglomeration. Rows represent samples (described by stream location, condition, and day of collection) and columns represent m/z ratios of the brominates species detected. The left dendrogram clusters first by HF+ or HF- streams, and then by abiotic and biotic microcosms. The top dendrogram is clustered by brominated species that varied similarly across the data set.

Supplemental Figure S3.7 Microbial community taxonomy changes over time.

The first set is the microbial community prior to DBNPA addition, then day 7 to 56 after DBNPA addition, and the last set is the no-DBNPA control at day 56. A) Phylum microbial community changes over time. B) Genus level microbial community changes over time.

A)



Supplemental Figure S3.7 continued

B)

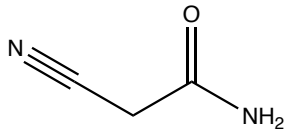
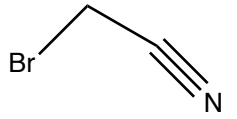
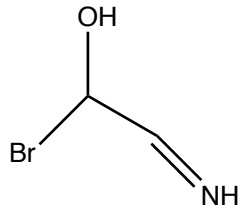
Supplemental Figure S3.7 continued

Appendix E: Tables

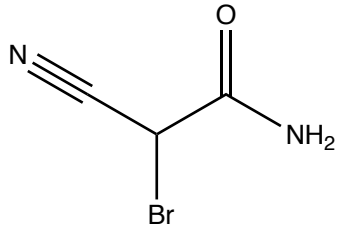
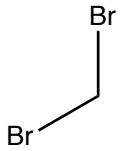
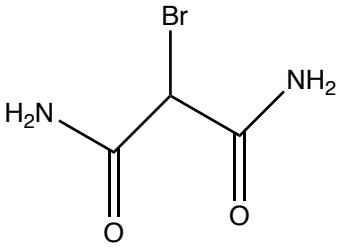
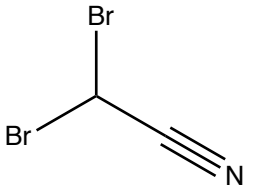
Supplemental Tables

Supplemental Table S3.1 Putative DBNPA brominated degradation products detected by nano-HPLC-HRMS.

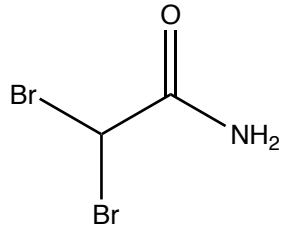
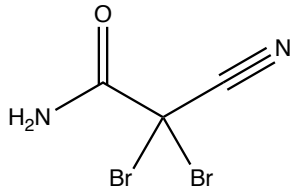
The elemental formula was predicted using the formula predictor in Xcalibur Software (Thermo Fisher Scientific) and the putative structure was confirmed using ChemDraw (PerkinElmer).

m/z	**Predicted Elemental Formula**	**Putative Structure**
85.0396	$C_3H_5O_2N_2$	 cyanoacetamide CAM
119.9444	C_2H_3NBr	 *2-bromoacetonitrile*
134.8526	C_2O_2Br	
137.9544	C_2H_5ONBr	 *1-bromo-2-iminoethan-1-ol (119 + H_2O)*
146.9077	C_3O_2Br	
163.9335	$C_3H_3O_2NBr$	*119 + CO_2*

Supplemental Table S3.1 continued

m/z	Predicted Elemental Formula	Putative Structure
164.9476	$C_3H_3BrN_2O$	 monobromnitrilopropionamide MBNPA
172.8413	$CHBr_2$	 *Dibromomethane* *DBAN - CN*
182.9586	$C_3H_6O_2N_2Br$	 *2-bromomalonamide* *MBNPA + H_2O*
199.8528	C_2HBr_2N	 dibromoacetonitrile DBAN

Supplemental Table S3.1 continued

m/z	Predicted Elemental Formula	Putative Structure
217.8628	$C_2H_3Br_2NO$	 dibromoacetamide DBAM
242.8587	$C_3H_2Br_2N_2O$	 DBNPA
244.9106	$C_4H_9N_2Br_2$	
260.8682	$C_4H_4O_2NBr_2$	*DBNPA + H_2O*
305.9254	$C_3H_{10}ON_6Br_2$	
306.9616	$C_8H_4O5N_3Br$	
314.0824	$C_{13}H_{19}N_4Br$	
318.8959	$C_9H_5N_3Br_2$	

Supplemental Table S3.2 Total Organic Carbon (TOC) concentration in source water prior to DBNPA addition.

Site	TOC (mg/L)	Standard Error
AB	8.57	2.14
LL	8.04	2.60
NH	6.84	1.62
EE	1.70	.62
WE	4.83	1.30
DR	5.73	2.06

The following supplemental table can be found as attachments:

Supplemental Table S3.3 DESeq2 Results HF- vs HF+ Enrichment at Day 0 Prior to DBNPA Addition

Supplemental Table S3.4 DESeq2 Results Enrichment Day 7 vs Day 0

Supplemental Table S3.5 DESeq2 Results Enrichment Day 21 vs Day 0

Supplemental Table S3.6 DESeq2 Results Enrichment Day 35 vs Day 0

Supplemental Table S3.7 DESeq2 Results Enrichment Day 49 vs Day 0

Supplemental Table S3.8 DESeq2 Results Enrichment Day 56 vs Day 0

Supplemental Table S3.9 DESeq2 Results Differentially Enriched HF- vs HF+ at Day 7

Supplemental Table S3.10 DESeq2 Results Differentially Enriched HF- vs HF+ at Day 21

Supplemental Table S3.11 DESeq2 Results Differentially Enriched HF- vs HF+ at Day 35

Supplemental Table S3.12 DESeq2 Results Differentially Enriched HF- vs HF+ at Day 49

Supplemental Table S3.13 DESeq2 Results Differentially Enriched HF- vs HF+ at Day 56

Supplemental Table S3.14 DESeq2 Results Enrichment Day 56 no-DBNPA vs Day 0

Supplemental Table S3.15 DESeq2 Results Enrichment Day 56 vs Day 56 no-DBNPA

Appendix F

Supplemental Results

By day 7 after DBNPA amendment, HF+ composition slightly changed, with *Gammaproteobacteria* getting slightly enriched, meanwhile in the HF- group *Gammaproteobacteria* population doubled. By day 21, *Gammaproteobacteria* enrichment was still slower in the HF+ group than in HF-. Gammaproteobacteria represented over 35% percent of HF+ and close to 60% of HF-. The dominant *Gammaproteobacteria* Family at this time point was *Idiomarinaaceae*. By day 35 the dominant Class was *Alphaproteobacteria* in both HF+ and HF-, dominated by Methylobacteriaceae, 15% in HF+ and 30% in HF-, followed by Sphingomonadaceae which was almost 10% in HF+ and almost 5% in HF-. By day 49 *Alphaproteobacteria* abundance decreased in both groups, but the decrease was more pronounced in the HF- group with *Bacteroidetes* and *Planctomycetes* getting enriched in the HF- group.

By day 56 unclassified bacteria were more than 10% of the relative abundance in HF+ while it was only ~1% in the HF-. *Acidobacteria* and *Chloroflexi* were present in higher percentage in HF- than in HF+, while *OD1* was more prominent in HF+. The phylum *Verrucomicrobia* stayed constant for most of the incubation in HF+ while in HF- group *Verrucomicrobia* observed a gradual decrease after addition of DBNPA, reaching its lowest point at day 21. The biocide control groups at day 56 observed a decline in *Proteobacteria* population (particularly *Gammaproteobacteria*), the decline was more prominent in HF+, which also observed a higher increase in *Verrucomicrobia* and *Bacteroidetes.* Refer to Figure S3.7 for a figure of taxonomic changes through time.

CHAPTER 4 COMPARATIVE GENOMICS ELUCIDATES THE BACTERIAL MECHANISM OF RESISTANCE TO THE BIOCIDE 2-2-DIBROMO-3-NITRILOPROPIONAMIDE

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Author Contributions:

Conceived or designed experiments- MFC with input from SMT, TCH

Isolated, purified and extracted DNA- SB, MFC

KBase discussions, training, and method development- BHA, MFC

Useful bioinformatics discussion- RM, BHA

PI of the JGI community sequencing project- RL

Wrote the paper- MFC with input from BHA, RM, SB and TCH

Abstract

The biocide 2-2-dibromo-3-nitrilopropionamide (DBNPA) is commonly used in an array of industries including unconventional oil and gas (UOG) production. This study employs comparative genomics of environmental strains from streams impacted by UOG activities. The strains selected based on enrichment with the biocide DBNPA or glutaraldehyde were from the *Paenibacillus* and *Bacillus* genera. The genome of a publicly available *Paenibacillus* strain was used as reference. These strains were compared using the "Build Pangenome with OrthoMCL" app in KBase to elucidate the mechanism of resistance to DBNPA. Thirteen orthologous proteins were found. Their putative functions include mobile elements (recombinase and terminase), efflux pumps, and possible enzymatic deactivation of the biocide. These findings provide a first look into the potential mechanism of DBNPA resistance.

Introduction

Biocides are used in oil and gas extraction as a microbial control method to minimize microbial induced corrosion, bioclogging, and gas souring[1]. Biocide use in hydraulic fracturing (HF) has become an increased topic of concern due to high toxicity and less than ideal efficacy[1, 2]. HF is an unconventional method of oil and gas extraction where hydrocarbons are extracted from low permeable strata such as shale using highly pressurized fluids containing chemicals and sands. The average well uses between 1,000 m^3 to 30,000 m^3 of HF fluid per year[3]. HF fluid is mostly composed of water, sand, and around 0.5 to 2% chemicals such as proppants, frictions reducers, surfactants, pH adjusters, iron control, corrosion inhibitors, and biocides[3-5]. Of these additives, biocides have been repeatedly listed as chemicals of concern due to their human and environmental toxicity[1, 4], and as a potential source of environmental antimicrobial resistance. While the use of biocide is common, the resistance mechanisms of many of the most common biocides are still not known. One of those biocides is 2-2-dibromo-3-nitrilopropionamide (DBNPA). Although DBNPA is the second most commonly used biocide in HF[1], little is known about its microbial resistance mechanism.

DBNPA is an electrophilic halogenated biocide. It was first synthesized for use in industrial waters, but its use has expanded to paper manufacturing, cooling towers, and oil and

gas production. Its "fast-kill" approach makes it appropriate for preprocessing sterilization of HF fluids, and its fast decomposition kinetics decreases its persistence in the environment. However, its degradation kinetics pathways, and thus degradation products, depend on abiotic conditions such as total organic carbon (TOC) present in the sample, exposure to UV light, and pH. At higher TOC concentration, DBNPA degrades to → dibromoacetonitrile (DBAN) → dibromoacetamide (DBAM), while at low TOC concentration DBNPA degrades to → monobromonitrilopropionamide (MBNPA) → cyanoacetamide (CAM). DBAN is more toxic and recalcitrant than DBNPA itself, and we previously showed that many other brominated compounds are formed through these pathways as well[6]. We are just recently starting to understand the environmental implication of DBNPA, specifically in the HF lifecycle process.

This paper aims to better understand the microbial genetic responses associated with DBNPA resistance. To do so, we collected isolates after a 56-day incubation in stream water with DBNPA. The genera *Paenibacillus* and *Bacillus* were differentially enriched in the microbial community of the microcosm after exposure to either biocide, DBNPA[6] or glutaraldehyde[7] as compared to pre-amendment, indicating a competitive advantage in these genera as compared to the rest of the environmental community. Microbial isolates of these taxa were obtained from the microcosms and grown in media with biocide amendment. The draft genomes of these isolates were used to elucidate the microbial genetic response to DBNPA and similarities and differences between glutaraldehyde resistance in bacteria.

Methods

Bacterial isolation, DNA extraction, and 16S rRNA Gene Sequencing

Stream water was collected from impacted and unimpacted HF streams in Northwestern Pennsylvania (PA). Samples collected were used to set microcosms to test the difference in biocide microbial response between streams impacted and unimpacted by HF[6, 7]. The microcosms were set with a volume of 230 mL of stream water. The glutaraldehyde microcosms were amended with 100 mg/L and the DBNPA microcosms were amended with 125 mg/L. At the end of the experiment (56 days), water from the microcosms was spread plated onto a non-selective, general purpose medium to stimulate initial growth from the starved microbial

community and induced colony formation. In this case, Nutrient medium (BD Difco™, ThermoFisher Scientific) agar plates. After inoculation, plates were stored in aerobic conditions at ambient room temperature (~21°C) for 5-14 days to allow time for any viable environmental colony-forming microbes.

Colonies were selected for isolation based on visual inspection and morphology. Selected bacterial colonies were subject to growth challenge testing in which they were exposed to increasing concentrations of the biocide in the culture medium to select for bacterial colonies that displayed some level of resistance to the respective biocide. Colonies sourced from the glutaraldehyde microcosms were transferred to Nutrient medium + 100 mg/L glutaraldehyde agar plates during isolation. The glutaraldehyde resistant colonies were then transferred and cultured by increasing step concentrations of glutaraldehyde until 800 mg/L was reached and growth maintained. Bacterial colonies retained viability at 800 mg/L glutaraldehyde but could not be transferred to higher concentrations since higher concentration of glutaraldehyde affected the integrity of the agar. Similarly, colonies sourced from the DBNPA microcosms were then transferred to Nutrient medium + 100 mg/L of DBNPA agar during isolation. The DBNPA resistant colonies were then transferred and cultured by increasing step concentrations of DBNPA until 500 mg/L was reached. However, culture vitality was not maintained at this concentration of 500ppm DBNPA so colonies from a 200ppm DBNPA isolation were used. After colonies had been isolated and selected for resistance via microcosms enrichment and subsequently confirmed by biocide addition culturing technique, cells were transferred to un-amended (no biocide addition) Nutrient broth for downstream DNA extraction.

After liquid cultures of isolates reached turbidity, samples were centrifuged and supernatant decanted, leaving the cell pellet. The remaining cell pellet was and extracted using MoBio UltraClean Microbial DNA extraction kit (MoBio Laboratories, Carlsbad, CA) following manufacturer's instructions. The 16S rRNA gene was amplified using bacterial primers 27F (AGA GTT TGA TCC TGG CTC AG) and 1492R (ACG GCT ACC TTG TTA CGA CTT) (IDT Laboratories, Skokie, IL)[8]. Purity was checked by sequencing the amplicons using Sanger sequencing at the University of Tennessee Genomics Core Facility. The taxonomy of the isolates

was determined by querying the nearly full-length 16S rRNA gene sequence against the curated taxonomy database SILVA[9].

For comparison purposes, only isolates from microcosms constructed with water from Alex Branch (Pennsylvania, USA), a stream impacted by a HF spill, were selected for downstream analyses. Furthermore, only genera that were enriched in the microcosms after biocide addition were selected for sequencing. The selected strains from the DBNPA enrichment were designated *Paenibacillus pabuli* strain DB3, *Paenibacillus taichungensis* strain DB4, *Bacillus mycoides* strain DB1, and *Bacillus sp.* strain DB2. Only one strain was successfully sequenced from the glutaraldehyde enrichment, *Paenibacillus pabuli* strain NG13.

Sequencing and comparative genomics

DNA from the five selected isolates was sent to the Department of Energy (DOE) Joint Genome Institute (JGI), where the genomes were sequenced on an Illumina HiSeq-2500 1TB, assembled, and annotated using their pipeline[10, 11]. The assembled reads were uploaded to KBase and genomes were reannotated using the RAST pipeline (Annotate Microbial Assembly app) within the KBase platform[12, 13]. The "FastANI" app in KBase was used to compute the whole genome similarity using Average Nucleotide Identity using alignment-free approximate sequence mapping[14, 15]. Using the five isolates and *Paenibacillus pabuli* NBRC as reference (this is a publicly available reference strain), a genome set was created using the "Build GenomeSet". This genome set was then used to build a pangenome. The pangenome was built using the "Build Pangenome with OrthoMCL" app using the default parameters. The pangenome is generated by clustering gene features in each genome into homologous gene families. The pangenome data object generated by this app allows for viewing each cluster as a table of functions across each constituent genome, which can be analyzed for presence or absence of features across the set of genomes. Homology and function were also compared using the "Compare Genomes from Pangenome" app, which generated a similar table with representative functions and presence and absence among the six strains. The table was then queried using R to find orthologs and functions only present in the biocide treated strains and the difference between the DBNPA and glutaraldehyde treated strains.

Accession numbers and data availability

The genomes are publicly available at JGI Genome Portal and can be accessed through NCBI using the following BioSample IDs: SAMN09062494 (*P. pabuli* strain DB-3), SAMN09062974 (*P. taichungensis* strain DB-4), SAMN09062736 (*P. pabuli* strain NG-13), SAMN09062741 (*B. sp* strain DB-2), and SAMN09062822 (*B. mycoides* DB-1). For reference and comparison purposes the publicly available genome of Paenibacillus pabuli NBRC 13638 (BioSample: SAMD00034166) was used.

Results and Discussion

Comparative genomics enables the investigation and discovery of genes that relate observed phenotypic differences between closely related organisms[16]. In this study, comparative genomics tools were used to identify genes correlated with DBNPA resistance. Both *Paenibacillus* and *Bacillus* are members of the order Bacillales. Members of this order have repeatedly been found in HF produced and flowback water independent of formation location[17-19]. Not only has this order been reported in HF flowback and produces water, but the specific genera have been previously enriched in microcosms constructed with stream water impacted by HF amended with biocides[6, 7]. Thus, demonstrating their ecological significance and importance as model organisms to better understand the genetic mechanism of DBNPA resistance.

General genome statistics

The *P. pabuli* strain DB-3 was assembled into 34 contigs and its GC content was 46.25%. The *P. taichungensis* strain DB-4 was assembled into 29 contigs and had the same GC content as strain DB-3. The *P. pabuli* strain NG-13 was assembled into 38 contigs and had a GC content of 46.27%. The *Bacillus* strains had a smaller genome with lower overall GC content of 35.22%. *B. sp* strain DB-2 was assembled into 44 contigs and *B. mycoides* strain DB-1 was assembled into 72 contigs. The control strain *P. pabuli* NBRC was previously assembled into 58 contigs. The number of predicted protein-coding genes was 6,504 (*P. pabuli* strain DB-3), 6,661 (*P. taichungensis* strain DB-4), 6,696 (*P. pabuli* strain NG-13), 6,008 (*B. sp* strain DB-2), 6,014 (*B. mycoides* DB-1) and 6,894 (*P. pabuli* NBRC). This is described in Table 4.1.

FastANI was applied to characterize the similarity between the isolates in this study (Table 4.2). Results show that the isolated *Paenibacillus* species have an ANI estimated index of 98.75% to 99.99% to one another. However, similarity of the isolates with *Paenibacillus pabuli* NBRC are only between 88.89 to 89.06%. This is unexpected as intraspecies strain similarity is expected to be higher than 96% [20-22]. Different species within a genus, or interspecies similarity is more variable, ranging from 62 to 100%[21]. It was previously documented that ecological speciation and mobilome could cause lower ANI measurements[20], and thus it is possible that the biocide enrichment, specifically mobile genetic elements additions into the genome, caused the dispersity between the isolates described here and the *P. pabuli* NBRC reference. The *Bacillus spp*. are 99.99% similar to each other, but below the 80% threshold with the *Paenibacillus* strains and thus ANI is not appropriate for comparison[20].

Pangenome and genome comparisons

A pangenome was computed to determine the core genome of the six isolates and which genes might be gained or are different providing a phenotypic advantage to resist the biocide DBNPA. Results show there are 1,560 core functions between the 6 strains. There was a total of 38,890 protein coding gene families, of which 35,368 were in homolog families shared between at least two strains and 3,522 were in singleton families (Table 4.3).

At a finer resolution (Table 4.4) *P. pabuli* DB-3 had 2,757 homologous proteins with predicted functions and shared all but 7 functions with *P. taichungensis* DB-4. Meanwhile *P. pabuli DB-3* and *P. pabuli NG-13,* same species, but strains isolated with different biocides, have 2,691 predicted protein functions in common and 66 different. These 66 different protein functions are the ones of most interest to elucidate the different mechanisms of glutaraldehyde and DBNPA resistance. For comparison purposes, *P. pabuli* NBRC, selected from GenBank records, was added to this pangenome. *P. pabuli* NBRC has 2,823 proteins with predicted functions, 312 different from DB-3, 315 from NG-13 and DB-4. These are all protein functions that may have a role in biocide resistance and response.

The *Bacillus* isolates are from the same order as the *Paenibacillus*, and thus potentially could serve as comparisons of resistance mechanisms that are shared among more

phylogenetically distant bacteria *B. mycoides* DB-1 has 3,893 protein functions and shared all but one with *B. DB2*, which has 3,899 predicted protein functions. Overall the *Paenibacillus* isolates shared 1,630-1,645 protein functions with the *Bacillus* isolates but 2,263-2,248 were different.

The functional significance of pangenome orthologs was explored. A total of 13,014 ortholog clusters were detected, 2,110 of which were shared among the six genomes. The pangenome was queried to only focus on orthologs present in all isolates except NBRC and NG-13, removing what is expected to be core functional genes and resistance genes not specific to DBNPA such as those coding for ABC efflux pumps which are shared among glutaraldehyde and DBNPA resistant strains. This effort yielded 13 ortholog clusters that were present in all isolates but not in NBRC or NG-13. The predicted functions of these 13 ortholog clusters according to the SEED ortholog database [23](Table 4.5) were; cassette chromosome recombinase B (CcrB), chloramphenicol acetyltransferase (EC 2.3.1.28), L-lysine permease, L-O-lysylphosphatidylglycerol synthase (EC 2.3.2.3) , Major Facilitator Superfamily (MFS) general substrate transporter, O-acetylhomoserine sulfhydrylase (EC 2.5.1.49) / O-succinylhomoserine sulfhydrylase (EC 2.5.1.48), phage terminase large subunit, phosphoenolpyruvate phosphomutase (EC 5.4.2.9), prophage Clp protease-like protein, transcriptional regulator in the ArsS family, and two conserved hypothetical proteins.

These 13 ortholog clusters encode functions relevant to conferring resistance to a xenobiotic stressor. For example, CrrB, there are two ortholog clusters that have this function. A CcrB is used in genetic recombination to receive foreign DNA through transformation, transduction, and/or conjugation and has been described as mediating the integration antimicrobial resistance genes in bacteria such as *S. aurerus*[22]. Chloramphenicol acetyltransferase provides resistance to chloramphenicol, a halogenated antibiotic. These gene tend to be present in mobile elements and confer resistance to chloramphenicol through enzymatic inactivation[24, 25]. Intrinsic resistance has not been observed previously, indicating chloramphenicol acetyltransferase is most likely gained after environmental pressures[25]. The fact that all of the DBNPA resistant strains have it may indicate it has a role in the resistance. This gene also confers resistant to florfenicol[26], the fluorinated version of chloramphenicol, and while

there are no previous publications indicating resistance to brominated compounds, this could be the case. Further investigation is needed to confirm its cross resistance to other halogenated compounds and if chloramphenicol acetyltransferase is able to inactivate DBNPA by acetylation.

The L-lysine permease is notable as it has been hypothesized that as part of DBNPA mechanism of action would focus on the electrophilic double brominated C-2 of DBNPA would be the location of a nucleophilic attack[27]. It was expected that sulfur containing methionine and cysteine would lead the attack, but that lysine and arginine may play a role. The presence of this L-lysine permease involved in amino-acid efflux, may indicate that for these strains lysine may be part of the response to achieve DBNPA degradation[27].

MFS general substrate transporters are efflux pumps known to confer antimicrobial resistance[28, 29], they tend to be ubiquitous and not specific thus further research is needed to understand their role in DBNPA resistance. Bacteriophages are also ubiquitous in bacterial genomes, and contribute a substantial amount of genetic material to the mobilome, including antimicrobial resistance genes[30]. Phage terminase large subunit is needed to package viral DNA[31] indicating that there are mobile genetic element insertions in these genomes, which are not present in the reference control NBRC nor the NG-13.

Finally, ArsS family transcriptional regulators act as metal sensors that up-regulate gene expression in the presence of metal ions and suppress when there is no stressor[32]. Heavy metal presence can co-select for mechanisms of antimicrobial resistance as they tend to be together in gene clusters in mobile genetic elements. Further investigation of the synteny of the gene within the genomes will be needed to confirm if it was co-selected with an antimicrobial resistance mechanism[33].

Preliminary conclusions and future work

This ongoing investigation has led to identifying 13 orthologous groups that may have a role in DBNPA resistance. These genes will need to be explored further to better understand their role in DBNPA resistance. For example, the major facilitator superfamily (MFS) had not been detected in DBNPA or HF related biocide use. This is of interest as efflux pumps are ubiquitous and the type of efflux pumps tend to be conserved among different members of the order

Bacillales is common among HF fluids and is of interest as it has been identified as having a role in microbial induced corrosion and increased resistance to biocides[18]. This information may serve to formulate optimized antimicrobial that target these efflux pumps. Furthermore, non-homologous functions should al explored to identify their role in DBNPA resistance as well as non-shared function among the isolate that may provide unique resistance mechanisms

In order to confirm whether these genes have a direct role in DBNPA resistance, these experiments will need to be repeated with RNAseq to ensure that they are transcribed in the presence of DBNPA. Gene knock-out experiments could also be done to determine the role these 13 ortholog clusters have on DBNPA resistance. More mechanistic information about biocide resistance is needed to better understand and prevent the co-selection for biocide and antibiotic resistance.

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Appendix G: Tables

Table 4.1 General genome information

Strain Name	Genome Size	Gene Count (IMG)	Gene Count (KBase)	GC content	DNA Contigs	CRISPR Count
P. pabuli DB-3	7,090,257	6,504	6,617	46.25%	34	5
P. taichungensis DB-4	7,083,592	6,475	6,661	46.25%	29	5
P. pabuli NG-13	7,114,510	6,491	6,696	46.27%	38	1
B. sp DB-2	5,798,937	5,905	6,008	35.22%	44	1
B. mycoides DB-1	5,794,236	5,940	6,014	35.22%	72	1
P. Pabuli NBRC	7,326,056	6,565	6,894	46.52%	58	N/A

Table 4.2 Pairwise FastANI Results

Query	Reference	ANI estimate (%)	Matches	Total
P. pabuli NBRC	*P. pabuli* NG13	88.9812	1825	2415
P. taichungensis DB4	*P. pabuli* NBRC	89.014	1794	2348
P. pabuli NG13	*P. pabuli* NBRC	89.0166	1815	2353
P. pabuli NBRC	*P. pabuli* DB3	89.0224	1825	2415
P. pabuli NBRC	*P. taichungensis* DB4	89.044	1809	2415
P. pabuli DB3	*P. pabuli* NBRC	89.0574	1808	2347
P. taichungensis DB4	*P. pabuli* NG13	98.7542	2222	2348
P. pabuli NG13	*P. taichungensis* DB4	98.77	2216	2353
P. pabuli DB3	*P. pabuli* NG13	98.7744	2221	2347
P. pabuli NG13	*P. pabuli* DB3	98.7934	2214	2353
Bacillus sp. DB2	*B. mycoides* DB1	99.9923	1884	1910
P. taichungensis DB4	*P. pabuli* DB3	99.9936	2347	2348
B. mycoides DB1	*Bacillus* sp. DB2	99.9956	1877	1893
P. pabuli DB3	*P. taichungensis* DB4	99.9983	2336	2347

Table 4.3 Output from "Build Pangenome with OrthoMCL"

Genome	# Genes	Homologs	Homolog Families	Singletons
P. taichungensis DB-4	6661	6297	6175	364
P. pabuli NBRC	6894	5461	5226	1433
P. pabuli NG-13	6696	6022	5895	674
P. pabuli DB-3	6617	6293	6171	324
B. sp. DB-2	6008	5650	5466	358
B. mycoides DB-1	6014	5645	5467	369

Table 4.4 Overall genomes comparison

Genome	Legend	G0	G1	G2	G3	G4	G5
G0-*P. taichungensis* DB-4	# of families:	6540	5811	4993	6165	2254	2254
	# of functions:	2753	2689	2508	2750	1631	1632
G1-*P. pabuli* NG-13	# of families:	5811	6570	5003	5808	2264	2264
	# of functions:	2689	2757	2508	2691	1630	1631
G2-*P. pabuli* NBRC	# of families:	4993	5003	6660	4992	2254	2254
	# of functions:	2508	2508	2823	2511	1644	1645
G3-*P. pabuli* DB-3	# of families:	6165	5808	4992	6496	2255	2255
	# of functions:	2750	2691	2511	2757	1632	1633
G4-*B. mycoides* DB-1	# of families:	2254	2264	2254	2255	5837	5466
	# of functions:	1631	1630	1644	1632	3893	3892
G5-*B. sp.* DB-2	# of families:	2254	2264	2254	2255	5466	5825
	# of functions:	1632	1631	1645	1633	3892	3899

Table 4.5 Ortholog cluster identified with a putative role in DBNPA resistance. Columns 3-6 identify the coding sequence were the cluster is found.

Cluster	**Representative function**	**B. DB.2**	**B. *mycoides* DB-1**	**P. *pabuli* DB-3**	**P. *taichungensis* DB-4**
cluster5160	Cassette chromosome recombinase B	B.DB2.CDS.318	*B. mycoides*DB1.CDS.594	*P. pabuli* DB3.CDS.3684	*P. taichungensis* DB4.CDS.2423
cluster5162	Cassette chromosome recombinase B	B.DB2.CDS.323	*B. mycoide*s DB1.CDS.589	*P. pabuli* DB3.CDS.4226	*P. taichungensis* DB4.CDS.4358
cluster5166	Chloramphenicol acetyltransferase (EC 2.3.1.28)	B.DB2.CDS.1845	*B. mycoides* DB1.CDS.3785	*P. pabuli* DB3.CDS.4684	*P. taichungensis* DB4.CDS.4817
cluster5157	FIG01226333: hypothetical protein	B.DB2.CDS.4146	*B. mycoides* DB1.CDS.2199	*P. pabuli* DB3.CDS.756	*P. taichungensis* DB4.CDS.1231

Table 4.5 continued

Cluster	**Representative function**	**B. DB.2**	**B. *mycoides* DB-1**	**P. *pabuli* DB-3**	**P. *taichungensis* DB-4**
cluster5163	FIG01234501: hypothetical protein	B.DB2.CDS.3620	*B. mycoides* DB1.CDS.3206	*P. pabuli* DB3.CDS.4245	*P. taichungensis* DB4.CDS.4377
cluster5161	Llysine permease	B.DB2.CDS.4410	*B. mycoides* DB1.CDS.2462	*P. pabuli* DB3.CDS.3531	*P. taichungensis* DB4.CDS.4037
cluster5167	LOlysylphosphatidylglycerol synthase (EC 2.3.2.3)	B.DB2.CDS.2834	*B. mycoides* DB1.CDS.2917	*P. pabuli* DB3.CDS.6501	*P. taichungensis* DB4.CDS.6530
cluster5159	MFS general substrate transporter	B.DB2.CDS.722	*B. mycoides* DB1.CDS.1226	*P. pabuli* DB3.CDS.3660	*P. taichungensis* DB4.CDS.2399

Table 4.5 continued

Cluster	**Representative function**	**B. DB.2**	**B. *mycoides* DB-1**	**P. *pabuli* DB-3**	**P. *taichungensis* DB-4**
cluster5168	Oacetylhomoserine sulfhydrylase (EC 2.5.1.49) / Osuccinylhomoserine sulfhydrylase (EC 2.5.1.48)	B.DB2.CDS.497	*B. mycoides* DB1.CDS.3841	*P. pabuli* DB3.CDS.6494	*P. taichungensis* DB4.CDS.6538
cluster5164	Phage terminase large subunit	B.DB2.CDS.2424	*B. mycoides* DB1.CDS.1510	*P. pabuli* DB3.CDS.4279	*P. taichungensis* DB4.CDS.4411
cluster3814	Phosphoenolpyruvate phosphomutase (EC 5.4.2.9)	B.DB2.CDS.4406	*B. mycoides* DB1.CDS.2458	*P. pabuli* DB3.CDS.684	*P. taichungensis* DB4.CDS.693
cluster5165	Prophage Clp proteaselike protein	B.DB2.CDS.2426	*B. mycoides* DB1.CDS.1508	*P. pabuli* DB3.CDS.4282	*P. taichungensis* DB4.CDS.4414

Table 4.5 continued

Cluster	**Representative function**	**B. DB.2**	**B. *mycoides* DB-1**	**P. *pabuli* DB-3**	**P. *taichungensis* DB-4**
cluster5158	Transcriptional regulator, ArsR family	B.DB2.CDS.5521	*B. mycoides* DB1.CDS.4841	*P. pabuli* DB3.CDS.3659	*P. taichungensis* DB4.CDS.2398

CHAPTER 5 UNCONVENTIONAL OIL AND GAS ENERGY SYSTEMS: AN UNIDENTIFIED HOTSPOT OF ANTIMICROBIAL RESISTANCE GENES AND ANTIMICROBIAL RESISTANT BACTERIA

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Abstract

Biocides are used in unconventional oil and gas (UOG) practices, such as hydraulic fracturing, to control microbial growth. Unwanted microbial growth can cause gas souring, pipeline clogging, and microbial induced corrosion of equipment and transportation pipes; UOG operators are using many techniques only because conventional oil and gas operations have used them in the past, and biocide use optimization has not been a priority. Indeed, biocide efficacy has been put into questioned as microbial surveys show an active microbial community in hydraulic fracturing produced and flowback water. Flowback water presents an increased risk to surface aquifers and rivers/lakes near the UOG operations that the conventional oil and gas operations don't have. Some biocides, and their degradation products, have been highlighted as chemicals of concern for their toxicity towards human and environmental health especially considering UOG operations. The selective antimicrobial pressure they cause has not been seriously considered. This article discusses the potential pathways of environmental biocide exposure, identifies potential risks, and highlights important knowledge gaps that need to be addressed to properly incorporate antimicrobial resistance risk into UOG environmental and health risk assessments.

Introduction

Oil and gas extraction may create underappreciated hotspots for antimicrobial resistance. Antimicrobial agents, in this case, biocides, are used in oil and gas extraction to mitigate microbially induced corrosion of equipment and costly gas souring caused by microbes[1]. Traditional or conventional hydrocarbon extraction involves extraction from high-permeability, highly pressurized strata such as limestone, while unconventional oil and gas (UOG) refers to hydrocarbon extraction from low permeability strata such as shale, using hydraulic fracturing (HF) coupled with horizontal drilling. Biocides are used in conventional oil and gas practices to prevent oil and gas souring after extraction and during pipeline transportation. Biocides play a markedly different role in unconventional reservoirs, which rely on biodegradable thickening agents, such as guar gum, and other chemicals to ensure fluid stability which can be compromised by microbial activity. HF injection fluid is needed to stimulate the reservoir and

extract the hydrocarbons. The final volume of HF injection fluid may exceed 10 million liters per horizontal well employing more than 500 mg/L of biocide[1]. Between 30 to 90% of the injection fluid does not resurface. Injection fluid that does resurface is referred to as "flowback" waste[1]. Both injection and flowback provide environmental exposure to biocides, and importantly, to microbes causing potential resistance to the biocide[2].

Different biocides, with different antimicrobial mechanisms, are currently used in UOG. Biocides may be used by themselves or in combination with other biocides to increase maximum biocidal activity. Common biocides used are glutaraldehyde (27% of HF jobs), 2-2-dibromo-3-nitrilopropionamide (DBNPA) (24%), tetrakis hydroxymethyl phosphonium sulfate (THPS) (9%), Didecyl dimethyl ammonium chloride (DDAC) (8%), chlorine dioxide (8%), among others that are used at frequencies of 4% or lower[1]. These compounds can be grouped as oxidizing and non-oxidizing. The oxidizing group employs free radicals to attack cellular components, an example of this group is chlorine dioxide. The non-oxidizing biocides have two primary mechanisms, electrophilic and lytic. Electrophilic biocides efficacy comes from their negatively charged functional groups that react with positively charges functional groups in the cell wall, crosslinking amino and nucleic acids leading to cell wall damage and eventual cytoplasm coagulation[1]. Examples of biocides in this category are glutaraldehyde and DBNPA. Conversely, lytic biocides work by binding to anionic functional groups on the cell membrane, perturbing the cell wall bilayer and disrupting the osmotic regulation capacities of the microbe and finally lysing the cell[1]. Quaternary ammonium compounds are members of this group; DDAC is an example. The difference in biocide mode of action may trigger different resistance mechanisms in bacteria. Thus, not all biocide may cause the same selective pressure in the environment.

HF biocides microbial dynamics, mobility, degradation, physiochemical characteristics, and toxicity have been discussed by others before[1, 3, 4]. However, the environmental and public health implications of the selective pressure they may cause have mostly been ignored. In this piece, we identify potential environmental exposure to HF biocides, current knowledge gaps, and potential alternatives to biocides. Addressing this knowledge gaps may aid UOG operators and environmental regulators mitigate risk.

Human health and environmental health

Stringfellow et al. (2014), reviewed the physical, chemical, and biological characteristics of chemicals used in HF fluids and concluded that biocides are of much concern due to high human and environmental toxicity[3]. Using the United Nations standards in the Globally Harmonized System of Classification and Labelling of Chemicals (GHS), they assigned toxicity levels to commonly used chemicals in UOG, such as gelling and foaming agents, friction reducers, crosslinkers, breakers, pH adjusters, biocides, corrosion inhibitors, among others. These authors concluded that biocides were of utmost concern as the chemical category contains the most toxic compounds. GHS classification goes from 1 to 5, with category 1 being the most toxic. For example, glutaraldehyde is a GHS 1 chemical, DBNPA meets criteria for GHS 1, THPS is a GHS category 3 chemical, and DDAC, a quaternary ammonium compound, is also a GHS category 3.

In addition to their direct toxicity to human and environmental health as micropollutants, biocides can also indirectly affect human and environmental health through the selective pressure of the remaining sub-lethal concentration of biocide and the resistant bacteria that survived the biocide dosage. The selective pressure of the sublethal/remaining biocide may serve as breeding environments for antimicrobial resistant bacteria (ARB) and subsequently the spread of antimicrobial resistant genes (ARG)[5-9] For example, exposure to quaternary ammonium biocides at a subinhibitory concentration can cause resistance to the specific biocide and a variety of clinically relevant antibiotic by enriching for ARB and by gaining ARG[10].

ARG may provide resistance to specific antimicrobial agents or confer nonspecific resistance via multidrug efflux pumps. ARG can be part of the microbial core genome or can be embedded in mobile elements such as plasmids, transposons, and integrons that may carry resistance to the specific antimicrobial agents and be transferred to other microbes through horizontal gene transfer. The non-specificity of the multidrug efflux pumps makes them particularly relevant for antibiotic-biocide cross-resistance[11]. Environmental exposure to subinhibitory concentrations of the antibiotic tetracycline of 10 μg/L, 150 times below the minimal inhibitory concentration, was enough to drive horizontal transfer of antibiotic

resistance[12]. Unfortunately, data on the minimal concentrations to drive horizontal transfer of resistance in the environment for most of the typical biocides are not available.

Selective pressure that drive ARB enrichment and ARG horizontal transfer can create a "hotspot" for resistance. Hotspots are areas in the environment that serve as a bioreactor, or breeding ground, for the enrichment of ARB and, overtime and through selective pressure, enhance the transfer and acquisition of ARG. It is essential to identify, monitor, and, if possible, remediate or even prevent these hotspots to stop the inadvertent spread of antimicrobial resistance and the spread of so-called superbugs. Antimicrobial resistance is currently one of the biggest threats for public and environmental health. Naturally occurring antimicrobial resistance has been previously observed and discussed[13-17]. However, anthropogenic stresses are the most significant selective pressures in antimicrobial hotspots[14]. Multiple anthropogenic hotspots have been identified through the years. Hotspots caused by pharmaceutical, agricultural, and municipal wastewater effluent which continue to be a focus of prevention and monitoring efforts, have received the most attention[18-20]. We argue that oil and gas extraction process could be an underappreciated hotspot in the literature, practice, and regulation arena, one that should not be ignored.

Potential routes of environmental biocide exposure

Data on UOG spills are highly variable because of the diversity of state laws, and because disclosure responsibility falls on well operators or owners[21]. A comprehensive study on four UOG high activity states—Colorado, New Mexico, North Dakota, and Pennsylvania, found annual spill rates of up to 15%[21]. Spills occurred through blowouts, drilling processes, storage, flowlines, transportation, equipment failure, and wellhead malfunction[21]. Furthermore, Kahrilas et al. identified additional pathways of biocide exposure to the environment[1], and we have expanded those sources to include soil, surface water, groundwater, animal and human exposure. Figure 5.1 summarized these pathways of exposure that can lead to ARG and ARB to disseminate and propagate.

One of the limiting factors to monitor and/or remediate these potential sources of exposure is reporting[21]. Environmental regulation varies by state, and there may be a culture that

limits the reporting of accidents or spills (e.g., if jobs are jeopardized), causing many spills to be unaccounted for[21]. The variation in reporting and local regulations limits the capability of companies or states to intervene and promptly clean the spill. Furthermore, incomplete removal of biocides/antimicrobials and in turn ARB and ARG in waste water treatment facilities has been a hot topic of discussion[18], and is beyond the scope of this article.

In addition to the direct pathways and exposure to biocides associated with the well lifecycle, some states allow for flowback water to be used for road salting[22] and agricultural purposes[23]. Furthermore, to decrease the stress in local water resources, some operators are reusing the flowback water to fracture new wells[24]. Such activities, while well intended, may further expand the dissemination reach of biocides, ARB, and ARG.

It is still unclear what the environmental fate and transport of most HF biocides would be. Biocides in surface spills would have a different fate than biocides present in HF fluids down borehole, due to the chemical interactions, temperature, pressure, and oxygen fluctuations down borehole. To our knowledge, only one study has explored the fate and transport of a biocide down the borehole. The study showed that glutaraldehyde would have limited antimicrobial activity in an alkaline and/or hot formation, but it would be more persistent in lower temperatures, higher acidity and/or salinity[25]. Glutaraldehyde would also polymerize to dimers and trimers at different high temperatures and salinities found in shales, but not at different pressured or shale contents. The polymerization of glutaraldehyde affects its biocidal properties, as monomeric glutaraldehyde has two possible bacteria crosslinking sites, while polymerized forms only have one[26, 27]. A limitation of this study is that microbial interactions were not explored. Thus, it is unclear if the limited biocidal properties are enough to cause selective pressure for ARB. It was previously shown that subsurface microorganism harbor higher concentration of mobile genetic elements, such as plasmids, that harbor mechanisms to degrade recalcitrant compounds such as polycyclic aromatic hydrocarbons, and they also confer high incidence of antimicrobial resistance[28, 29]. The difference between surface and subsurface microbial communities is of extreme interest as biocide selection is based on 6 log reduction of commercial planktonic bacterial cells, conditions far from what is encounter down borehole.

Surface spills of biocides would encounter a different fate. For example, glutaraldehyde would undergo chemical or physical absorption with soil, decreasing its reactive availability and biocidal efficiency[30]. Glutaraldehyde can also be more persistent in areas previously impacted by UOG activity[31]. It is unclear if limited biocidal efficiency can still cause a selective pressure for ARB, but even small concentrations (~5 mg/L) caused a microbial inhibition effect, presumably capable of causing selective pressure[32].

Fate and transport studies of other HF biocides have not been conducted in the context of HF, but there are studies about their uses in other industries. For example, QAC biocides, such as DDAC, are ubiquitous in domestic and industrial products and tend to accumulate in wastewater treatment plants. Subsequently, they are introduced in the environment as wastewater treatment plant effluent or sludge[33]. Once in the environment, they can either degrade or undergo absorption into the soil or different substrates with the potential for leaching. Sorption is faster than degradation. The longer the alkyl chains, the more recalcitrant the compound, and long microbial exposure to QACs was shown to select for resistance to clinically relevant antibiotic[10, 33, 34]. Even though borehole transformation studies have not been performed on QAC biocides, they have been detected in flowback samples, meaning it is not all depleted or biotransformed downborehole[35].

Evidence for risk to humans and the environment

While there are human and animal studies of the direct toxicity of some biocides (and other chemicals) used in HF,[1, 3, 36-38] a systematic measure of ARB enrichment potential is not considered and lacking in the literature. The literature discussing potential risks of UOG focus on water availability[39, 40], groundwater contamination[41], surface spills and their repercussions in the environment[42, 43], primarily from the standpoint of chemical detection[21, 22]. Little, if any, attention has been paid to the implications of UOG biocides for the environmental microbial community. Environmental surveys associated with UOG have mostly focused on the microbial phylogeny of pre-and post- production water[44] or the phylogeny of impacted sites[43, 45]. Only a couple of studies focus on the functional potential of microbial communities in flowback water[46, 47] and even fewer on the functional potential of microbial communities in UOG impacted sites[48].

Regardless of biocide usage and the harsh/extreme conditions (i.e. anoxic, high pressure, salt and temperature) present down borehole, microbes were active and present in the produced and flowback water [47, 49, 50], evidence that conditions and dosage of biocide use are not enough to completely inhibit bacteria[51]. Biocides are not completely inhibiting their targets; however, they do trigger the microbial defense mechanism which include, 1) sporulation, 2) biocide inactivation by enzymatic action, 3) efflux pumps, 4) wall composition adaptation, 5) biofilm formation and exopolysaccharides secretion, and 6) acquisition of resistant genes[1, 4].

Impacts from a UOG wastewater disposal facility to nearby aquifers enriched for the following anaerobic microbial orders: *Desulfuromonodales, Anaerolineales,* and *Syntrophobacterales*, and *Clostridiales*. In contrast, Rhizobiales, Myxococcales, and Sphingobacteriales were enriched aerobic orders[42]. Functional potential studies show that known pathways of antimicrobial resistance such as stress response, sporulation, dormancy, and efflux pumps are present or upregulated in HF wastewater[46, 50]. Surface water impacted by HF activities also report genetic markers for antimicrobial resistance, efflux pumps, and dormancy, all associated with stress caused by biocides[48].

Microbial functional potential analyses of those same UOG impacted aquifers revealed detection of 43 ARGs. Using qPCR, those 43 ARGs were quantified. Of those, 8 were above the limit of detection in all sites (background and impacted) and 11 unique ARGs in one of the impacted aquifers[48]. The functionality of the enriched genes is mostly multidrug efflux pumps (arc*B* and mex*B*), which are not antibiotic specific but can aid in antibiotic resistance[52]. Multidrug efflux pumps have been identified as enriched in the presence of HF biocides[53]. Fahrenfeld et al., compared the profiles and quantities of ARG found in the HF impacted streams, as compared to municipal wastewater that was previously labeled as "hot spot" of antibiotic resistance and a salt river that has experienced anthropogenic impact[48]. More ARG types were detected in the HF impacted stream compared to the anthropogenically impacted salt river, while the actual concentrations of ARG ppm in the sites were comparable. However, the municipal wastewater contained more ARG types and generally higher ppm levels of ARG. Currently there is no consensus or regulation that defines what are acceptable levels of ARG in wastewater treatment plants nor anthropogenically impacted aquifers[18].

There has not been sufficient research to determine/ quantify the risks to humans and the environment from the biocides used in HF. However, multidrug efflux pumps are frequently reported in human infectious diseases, and the AcrAB-MexAB family is the most common[54]. Those efflux pumps have been detected at high levels in HF impacted areas and reported as mechanisms for resistance for glutaraldehyde, the most commonly used HF biocide. However, a method to distinguish between core resistant genes within the microbial community and mobile genetic elements acquired due to selective pressure caused by HF biocides is unclear.

Martinez et al., proposed a conservative redefinition of a resistance gene, focusing on the risk associated with the particular gene selected[55]. They claimed that not all ARG pose the same risk, for example, intrinsic ARG part of the general genome of specific microbial taxa does not impose the same risk as an ARG located within a mobile genetic element which has a higher probability of being horizontally transferred to other bacteria. Furthermore, Martinez et al., also considered the evidence of risk, which they categorized in different levels. They outlined seven resistance readiness conditions (RESCon), with RESCon 1 being the highest risk, and RESCon 7 being the lowest risk based on identity, functional evaluation (demonstrated not only predicted), and mobility[55]. Others argued that Martinez et al., downplayed the risk caused by mobile genetic elements that have not been yet detected in human pathogens, but their detection would significantly affect public health[56]. Applying a combination of those concepts, such as utilizing a framework that quantifies risk based on functional evaluation and mobility without ignoring the threat caused by unknown ARG and mobile genetic elements, to the study of UOG impacted ecosystems would better assess the risk associated from ARG derived from HF usage.

AcrAB-MexAB efflux pumps (found in high concentration in UOG impacted sites[48]) would be categorized as low-level risk based on Martinez et al. risk rating scheme as their resistance is not antibiotic specific. However, to fully analyze their risk we would need to determine if they are in mobile genetic elements, or if they are in high abundance because of the taxa enriched due to other chemical parameters of UOG wastewater. By understanding if the ARG genes are in mobile genetic elements, we could better understand the range of risks for UOG workers, people living near the impacted area, and community-wide risk.

Using Kahrilas et al.[1] compiled list of frequently used biocides according to the self-disclosure HF chemical data base FracFocus.org, we compiled the reported microbial genetic responses to common HF biocides (Table 5.1). We found that 6 out of 16 biocides do not have a reported microbial genetic resistance mechanism. The 10 biocides that have reported mechanisms of resistance, seem to confer broad resistance to other antimicrobials and antibiotics. Resistance to QAC biocides, seems to be carried in mobile genetic elements carrying other resistance genes[57], it is unclear if the resistance response for other biocides are also in mobile genetic elements.

Conclusion

Current HF risk assessments do not include the potential risk of antimicrobial resistance due to the biocides used. To categorize risk, we need to determine the probability and the severity of an event occurring. More standardized risk assessment field tests are required to understand and quantify the potential risk of bacterial resistance. However, in the case of ARG from UOG, precise and robust results are not currently available because microbial ecology surveys of UOG impacted sites that include functional genomics are scarce. Access to UOG areas are limited, some spills may go unreported, and the regulation around UOG varies by state/country.

Even though the level of risk of UOG antimicrobial resistance cannot be determined precisely at this time, it does not mean the risk should be ignored. The risk is not limited only to occupational hazards. Spills can impact soil, surface water, and groundwater, making it easier for ARG to reach different environmental niches than the original environmental reservoir (such as well and/or holding pond). Furthermore, beneficial uses of HF wastewater, such as reuse could also contribute to the unintended propagation of antimicrobial resistance, and areas were HF wastewater is used for irrigation or road salting should be monitored for ARGs.

Functional genomic surveys detecting ARG should discuss if the ARG is present in mobile elements and if it is detected across different taxa. In case of a spill, not only should the chemicals be monitored in the environment, but functional genomics surveys should be included in the spill response and compared to upstream of background areas. Doing so will help us to

understand if the spill caused an increase in ARG and if those ARG are due to intrinsic resistance from the taxa enriched or caused by mobile genetic elements. This distinction matters as broad resistant ARG in mobile genetic elements can be more easily horizontally transferred into potential pathogenic bacteria. Further, we echo the call of others asking for ARG to be treated as micropollutants and the establishment of a maximum ARG concentration which is deemed acceptable based on its risk to public and human health.[66]

Efforts should also be placed into better understanding the microbial resistance mechanisms to common HF biocides to better understand their impacts in the environment. Lastly, optimization of biocide usage may decrease the concentrations needed to reach the desire effects lowering the repercussion of its use. There are other successful methods of bacterial control such as competitive exclusion. This method utilizes a more thermodynamically favorable electron donor to help microbes outcompete microbes with undesirable mechanisms[4]. For example, when trying to reduce sulfate reducing (SRB) bacteria, common culprits of microbial induced corrosion, addition of nitrate may help microbes with nitrate reducing metabolism outcompete SRB bacteria[67, 68].

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Appendix H: Figures

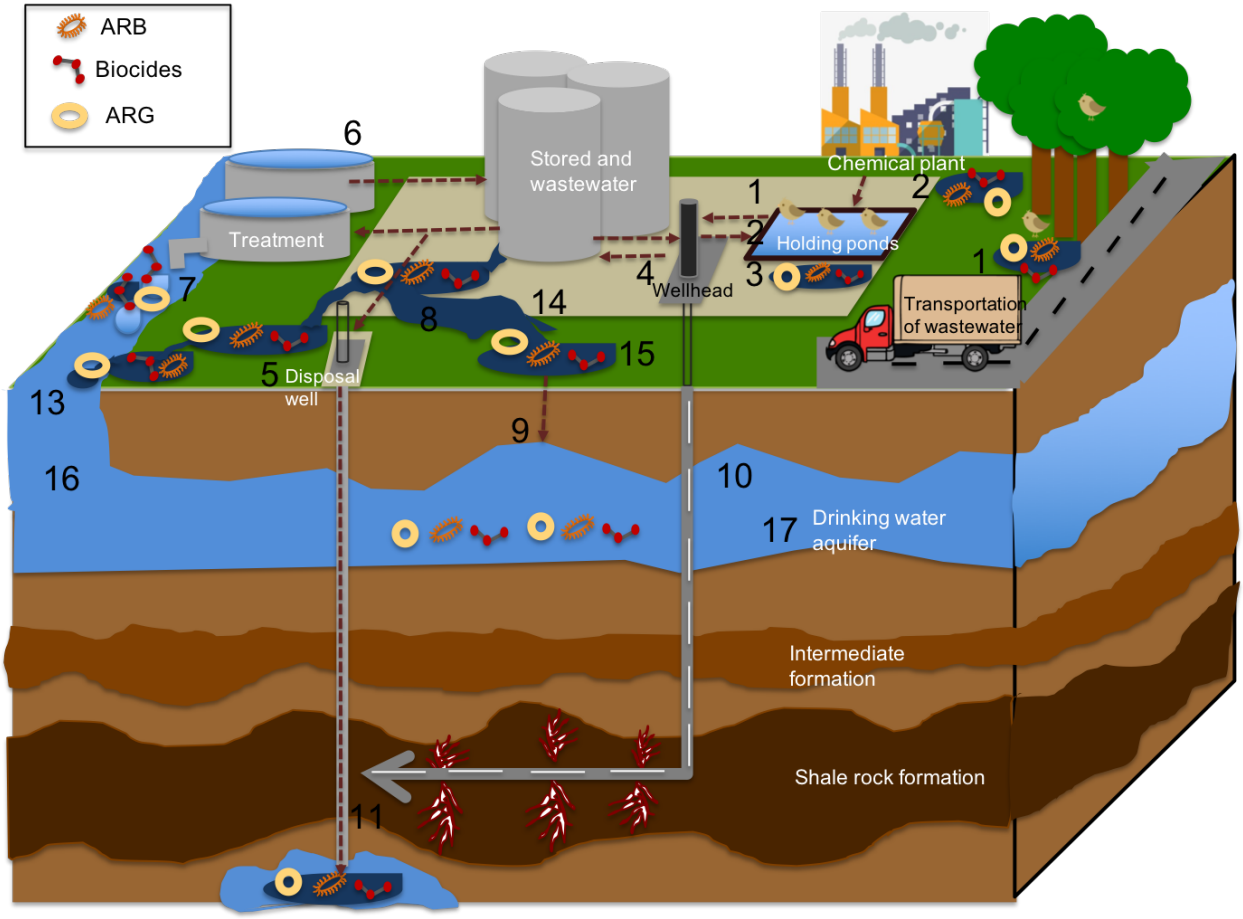

Figure 5.1 Potential sources of ARB and ARG release.

Soil exposure from, 1) transportation spills, 2) chemical plant spills, 3) holding pond spills, 4) wellhead spills, 5) disposal well spills, 6) incomplete removal in treatment plants, sludge applied to agricultural top-soil. *Surface water exposure from*, 7) surface spill runoff, 8) incomplete removal in a treatment plant, effluent disposed of in streams. *Shallow groundwater from*, 9) surface spills leaching into shallow aquifers, 10) borehole leakage, fault lines, and abandoned wells, 11) via induced fractures. *Animal exposure,* 12) contact with holding ponds, 13) contact with eluent from a treatment plant, 14) contact with spills. *Human exposure,* 15) exposure with spills, 16) consumption of exposed stream water, 17) consumption of exposed groundwater. Figure adapted from Kahrilas et al., 2014[1].

Appendix I: Tables

Table 5.1 Frequently used hydraulic fracturing biocides.

Their reported mode of action, microbial resistance response, and specificity of the response.

Biocide name and Cas No.	**Chemical Formula**	**Frequency of use**	**Biocide mode of action**	**Microbial Genetic Resistance Response**	**Is the known genetic response biocide specific?**
Glutaraldehyde 111-30-8	$C_5H_8O_2$	27%	Electrophilic	Efflux pumps[53, 58] Heat-shock like response[59]	No, efflux pumps confer broad resistance.
2-2-Dibromo-3-nitrilopropionamide 10222-01-2	$C_3H_2Br_2N_2O$	24%	Electrophilic	Not known	N/A
Tetrakis hydroxymethyl phosphonium sulfate 55566-30-88	$[(HOCH_2)_4P]_2SO_4$	9%	Electrophilic	Not clear[59]	N/A

Table 5.1 continued

Biocide name and Cas No.	**Chemical Formula**	**Frequency of use**	**Biocide mode of action**	**Microbial Genetic Resistance Response**	**Is the known genetic response biocide specific?**
Didecyl dimethyl ammonium chloride 7173-51-5	$C_{22}H_{48}NCl$	8%	Lytic	*qacA* and homologs *qacB-H/J/Z,* and other multidrug efflux pumps[57]	QAC resistance genes are commonly found on plasmids with other multi-drug-resistance genes[57].
Chlorine dioxide 10049-04-4	ClO_2	8%	Oxidizing	σ^B, CtsR, and HrcA[60]	Not known
Tributyl tetradecyl phosphonium chloride 81741-28-8	$C_{26}H_{56}PCl$	4%	Lytic	*qacA* and homologs *qacB-H/J/Z,* and other multidrug efflux pumps[57]	QAC resistance genes are commonly found on plasmids with other multi-drug-resistance genes[57].

Table 5.1 continued

Biocide name and Cas No.	**Chemical Formula**	**Frequency of use**	**Biocide mode of action**	**Microbial Genetic Resistance Response**	**Is the known genetic response biocide specific?**
Alkyl dimethyl benzyl ammonium chloride 68424-85-1	$C_{19}H_{34}NCl$	3%	Lytic	*qacA* and homologs *qacB-H/J/Z,* and other multidrug efflux pumps[57]	QAC resistance genes are commonly found on plasmids with other multi-drug-resistance genes[57].
Methylisothiazolinone 2682-20-4	C_4H_5NOS	3%	Electrophilic	RND efflux pumps[61]	Efflux pumps confer broad non-specific resistance

Table 5.1 continued

Biocide name and Cas No.	**Chemical Formula**	**Frequency of use**	**Biocide mode of action**	**Microbial Genetic Resistance Response**	**Is the known genetic response biocide specific?**
Chloro-methylisothiazolinone 26172-55-4	C_4H_4NOSCl	3%	Electrophilic	RND efflux pumps[61]	Efflux pumps confer broad non-specific resistance
Sodium Hypochlorite 7681-52-9	NaClO	3%	Oxidizing	*ohr, ahpC, and ahpF,* ROS response[62]	This are genes confer resistance to oxidative stress.[62]
Dazomet 533-74-4	$C_5H_{10}N_2S_2$	2%	Electrophilic	Not known	NA
Dimethyloxazolidine 51200-87-4	$C_5H_{11}NO$	2%	Electrophilic	Not known	NA
Trimethyloxazolidine 75673-43-7	$C_6H_{14}NO$	2%	Electrophilic	Not known	NA

Table 5.1 continued

Biocide name and Cas No.	**Chemical Formula**	**Frequency of use**	**Biocide mode of action**	**Microbial Genetic Resistance Response**	**Is the known genetic response biocide specific?**
N-Bromosuccinimide 128-08-5	$C_4H_4BrNO_2$	1%	Electrophilic	Not known	NA
Bronopol 52-51-7	$C_3H_6BrNO_4$	<1%	Electrophilic	*rpos*[63]	No, *rpos* also has a role in antibiotic resistance.[64]
Peracetic acid 79-21-0	$C_2H_4O_3$	<1%	Oxidizing	Tetracyclines ARG[65]	Not clear.

CHAPTER 6 CONCLUSION

The focus of this dissertation was to evaluate the environmental implications of the biocides used in hydraulic fracturing (HF). Within the second chapter, the microbial response to glutaraldehyde, the most commonly used HF biocide, was explored. This microcosm-based experiment revealed that glutaraldehyde is more persistent in aquatic environments previously exposed to HF operations, even though more members of the microbial community were able to resist and tolerate glutaraldehyde as shown by higher diversity and biomass measures. We hypothesize that the glutaraldehyde persistence is in part due to biotic-abiotic interactions, such as the lower pH in HF-impacted microcosms. This study was the first of its kind to simultaneously track microbial response and biodegradation in streams previously impacted by HF. These findings are very relevant to HF operators and environmental regulators, so they can consider better HF fluid formulations to favor conditions that would help accelerate biodegradation of glutaraldehyde in case of a spill.

The third chapter explored the microbial response to the biocide DBNPA using the same conditions and source water as in chapter two. DBNPA has two known degradation pathways. Higher total organic carbon associated with HF-impacted streams favors a less persistent degradation pathway while low total organic carbon favors intermediates that are more persistent and more toxic than DBNPA. We also discovered that multiple unidentified brominated species that may form as degradation or daughter products of DBNPA regardless of previous HF impact. Furthermore, as compared to glutaraldehyde, DBNPA is less persistent and can degrade abiotically, even at high concentrations, thus the microbial community was less affected by DBNPA than by glutaraldehyde. Similar enriched species were detected in both biocide experiments, such as *Methylobacterium* and *Bacillus*, and an enrichment of bacteria commonly found in marine environments such as *Idiomarina* and *Alcanivorax.* But overall, the microbial community enrichments were different between HF-impacted and unimpacted microcosms. These findings show that DBNPA may be more problematic when spilled in pristine environments with low total organic carbon. These findings may help to coordinate proper response efforts after a HF spill and to ameliorate the effects of brominated compounds in the environment.

The fourth chapter aimed to elucidate the microbial mechanisms of resistance to DBNPA. Bacteria were isolated from the last day of the microcosms (both DBNPA and glutaraldehyde). The isolated bacteria were placed on plates with increasing concentrations of the biocides and the surviving colonies were isolated, extracted, identified using Sanger sequencing, and sent for whole genome sequencing at the JGI. The selected five isolates for downstream analyses were from the genus *Paenibacillus* and *Bacillus*, which were enriched in both microcosm experiments, and have been also detected in flowback water. Using comparative analyses, we found 13 ortholog genes that were present in all the isolates treated with DBNPA and not in the isolates treated with glutaraldehyde nor in the *Paenibacillus* used as reference. These ortholog genes encode functions that may have a role in DBNPA resistance, such as cassette chromosome recombinase B, chloramphenicol acetyltransferase, L-lysine permease, and major facilitator superfamily. These results are promising and have helped narrow the hypothesis, but more research is needed, particularly functional comparisons of non-homologous genes and transcriptomic studies to understand if the genes are taking an active role in DBNPA resistance.

Finally, the fourth chapter explores the environmental and public health risk associated with the biocides used in HF in the context of co-selection for antibiotic resistance. This chapter reviews the current knowledge and highlights important knowledge gaps that need to be addressed to perform a risk assessment. Currently, we do not know the resistance mechanism for many of the commonly used HF biocides; this information is essential to assess if resistance (1) is biocide specific or (2) could co-select for a broad spectrum of antimicrobials and antibiotics. Understanding these knowledge gaps is critical as resistance to common antibiotics is becoming increasingly common, leaving many populations vulnerable to complications or death by common infections.

Overall, this dissertation addresses important knowledge gaps that will help oil and gas operators, environmental response teams, and regulators better understand the risks associated with biocides. The knowledge found can also aid with prevention strategies and better formulations to minimize the effect this practice has on the environment.

VITA

Maria Fernanda Campa was born and raised in Monterrey, Mexico. She graduated from the American School Foundation of Monterrey in 2008. She obtained her Bachelor of Science in Engineering Science- Nanomedicine from the University of Virginia in 2012. She joined Oak Ridge National Laboratory (ORNL) as a Higher Education Research Experience intern from 2012 to 2013 under the mentorship of Dr. Amy Wolfe where she studied the societal implications of emerging technologies. She then became a Bredesen Center Graduate Research Fellow at the University of Tennessee and ORNL where she completed her PhD in Energy Science and Engineering under Dr. Terry Hazen studying the environmental implications of biocides used in hydraulic fracturing. She has authored 11 publications, 1 book chapter, and presented her work in numerous national and international conferences. She has been awarded 8 awards for her research including, International Society of Microbial Ecology Travel Grant to attend the ISME 17 conference in Leipzig, Germany (2018), the Outstanding Abstract Award by the American Society of Microbiology Microbe Annual meeting (2017), and the third place in the poster competition at the Women in STEM Research Symposium (2015). Maria Fernanda has a passion for emerging technologies and their implication in environmental and public health and plans to continue pursuing this passion as a post-doctoral fellow.