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BIOCIDE RESISTANCE AS A RESULT OF EXPOSURE TO BIOCIDES USED IN HYDRAULIC FRACTURING

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BIOCIDE RESISTANCE AS A RESULT OF EXPOSURE TO BIOCIDES USED IN HYDRAULIC FRACTURING

By

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Preface

The focus of this report is biocide resistance as a result of hydraulic fracturing. The samples were collected by collaborators from Junita College (Regina Lamendella and Jeremy See Chen) and University of Tennessee (Terry Hazen and Maria Fernanda Campa). I performed all of the isolation studies, molecular characterization of the isolates and the biocide exposure studies.

I have been interested in resistance mechanisms for a while because of the growing concern with antibiotic resistance bacteria such as MRSA. Working on this project has been very interesting and I have learned a lot about lab techniques, biocides and the role the environment has in science. Although I worked on this project for only a short time, I feel like what I did matters and will help move the study of resistance forward.

The comparative genomics portion of the report focused on comparative genomics of Bacillus strains involved genomes that were sequenced as part of Waad Aljohani's thesis project. Waad isolated these strains and sequenced these genomes. I started with the raw data generated by Waad, but performed all of the downstream data analysis steps reported in this report

Acknowledgements

I would like to acknowledge the National Science Foundation for funding this project. Also, my advisor Stephen Techtmann and my committee members Paul Goetsch and Rupali Datta. This work was in part funded by NSF CBET 1804685.

Definitions

Biocides- Compounds that kill living cells.

Hydraulic Fracturing- The process of going into the earth to extract natural resources.

Biocide Resistance- Microbes that are not killed by biocides because they have developed ways to combat the biocides.

Amphiphilic Surfactants- Compounds that contain hydrophobic and hydrophilic components.

List of abbreviations

HF- Hydraulic fracturing

DBNPA- 2,2-dibromo-3-nitrilopropionamide

PBS- Phosphate-buffered saline

PCR- Polymerase chain reaction

rRNA- Ribosomal ribonucleic acid

Abstract

Microbial resistance to antimicrobials is an important topic to investigate not only for our health but for the environment. There is a growing concern about expanding microbial resistance to both antibiotics and other antimicrobials such as biocides. We wanted to know how bacteria previously exposed to hydraulic fracturing fluids, including biocides, would react to biocide exposure and what the mechanism of resistance looked like. In order to test this hypothesis bacterial isolates were obtained from water and sediment from a stream that had been previously exposed to a spill of hydraulic fracturing water. Thirty bacterial isolates were obtained from these samples that could withstand a moderate dose of two biocides (Glutaraldehyde and 2,2-dibromo-3-nitrilopropionamide (DBNPA)). These isolates then had their DNA extracted and taxonomy identified using 16S rRNA sequencing. We also tested minimum inhibitory concentrations (MIC) for each isolate against each biocide. This data however was not conclusive. Attempts were made to perform whole genome sequencing on these isolates to better understand resistance mechanisms. Sequencing was also attempted but due to complications was not completed. To increase our understanding of the mechanism of resistance a comparative genomic study was undertaken to compare between hydraulic fracturing associate *Bacillus* spp. and non-hydraulic fracturing associated relatives. Overall, isolates resistant to glutaraldehyde, DBNPA or both were obtained and identified. More isolates were found resistant to either glutaraldehyde or DBNPA not both. Meaning that the mechanisms of resistance may be different for the two biocides.

1 Introduction

Currently we are facing a problem with antibiotic resistance. Bacteria are becoming resistant to antibiotics and because of this are becoming harder to kill. In 2017 there were 120,000 new cases of MRSA (methicillin resistant *Staphylococcus aureus*) and 20,000 deaths (1). Another less known problem, however, is biocide resistance. Biocide resistance, like antibiotic resistance, leads to bacteria that are harder to kill (2).

Biocides are compounds that inactivate or kill living organisms through a variety of methods including cell lysis and oxidation. Antibiotics only target bacteria but biocides have the potential to kill any living cell and as such are not used to treat bacterial infections in humans. Biocides are used as disinfectants in a variety of different industries. However, increased use of biocides and disinfectants has led to the potential for an increase in bacterial resistance to biocides, which is a growing concern.

The increase in biocide and antibiotic resistance amongst microorganisms is seen in the emergence of biocide resistance in hospital acquired samples of *E. coli.* This resistance appears to indicate that our current disinfecting measures are being compromised (3). While biocides are often used in both household and medical settings to control microbial growth, industrial use of biocides is an expanding industry. One prominent industry where biocides are used frequently is hydraulic fracturing (HF) also known as fracking (4). The goal of this chapter is to understand the issues surrounding the potential for biocide use in hydraulic fracturing lead to increased biocide resistance in microbes from adjacent streams.

Hydraulic fracturing is the process of extracting oil and gas from under the earth. This process involves injection of water and chemicals are injected into gas harboring shale formations creating fractures through which fuels can then be extracted through these cracks in the earth. Since 2011 water useage has grown by over 770% meaning, produced water production has increased substainally. (5)

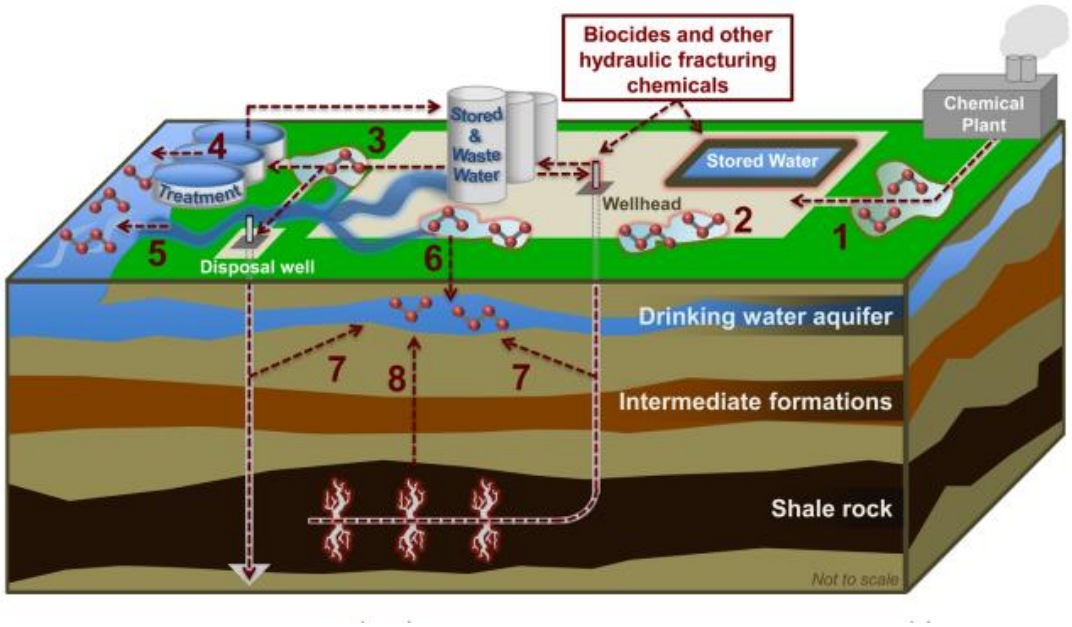
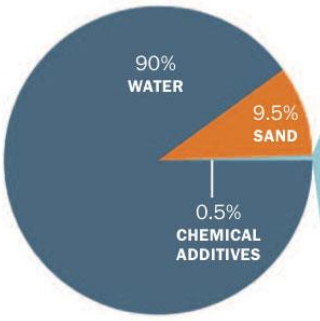


Figure 1.1 The Hydraulic Fracturing Process (6)

There are multiple concerns with hydraulic fracturing currently, one of the biggest being the potential for environmental contamination. Although most concerns surrounding contamination are for chemical and radioactive contamination there is also the concern for biocide contamination (7). According to ExxonMobil their hydraulic fracturing fluid is 98 to 99.5 percent water and sand with the last bit containing chemicals. These chemicals are used to make the process easier, reduce friction and prevent micribial buildup etc. (6). While many of these chemicals are used in household

products, there is concern about the potential for large releases of these chemicals into the environment.



Compound	Purpose	Common application
Acids	Helps dissolve minerals and initiate fissure in rock (pre-fracture)	Swimming pool cleaner
Sodium Chloride	Allows a delayed breakdown of the gel polymer chains	Table salt
Polyacrylamide	Minimizes the friction between fluid and pipe	Water treatment, soil conditioner
Ethylene Glycol	Prevents scale deposits in the pipe	Automotive anti-freeze, deicing agent, household cleaners
Borate Salts	Maintains fluid viscosity as temperature increases	Laundry detergent, hand soap, cosmetics
Sodium/Potassium Carbonate	Maintains effectiveness of other components, such as crosslinkers	Washing soda, detergent, soap, water softener, glass, ceramics
Glutaraldehyde	Eliminates bacteria in the water	Disinfectant, sterilization of medical and dental equipment
Guar Gum	Thickens the water to suspend the sand	Thickener in cosmetics, baked goods, ice cream, toothpaste, sauces
Citric Acid	Prevents precipitation of metal oxides	Food additive; food and beverages; lemon juice
Isopropanol	Used to increase the viscosity of the fracture fluid	Glass cleaner, antiperspirant, hair coloring

Source: DOE, GWPC: Modern Gas Shale Development in the United States: A Primer (2009).

Figure 1.2 Components of Hydraulic Fracturing Fluids (8)

1.1 Biocides

Diverse biocides are used in HF operations and are used to control microbial growth in the shale formation in an effort to prevent biofouling of equipment and souring of the extracted oil and gas. Like previously stated, biocides are agents that kill or inhibit growth of prokaryotic and eukaryotic cells. Biocides are classified by their mechanism of action.

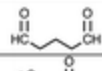
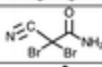
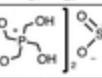
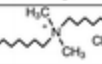
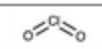
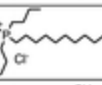
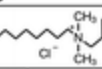
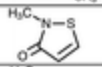
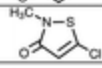
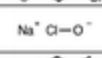
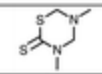
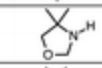
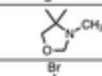
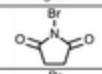
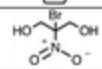
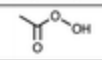
Trade Name & CAS No.	Chemical Structure	Chemical Formula	MOA	Freq. of Use
Glutaraldehyde 111-30-8		$C_5H_8O_2$	E	27%
Dibromo-nitrilopropionamide 10222-01-2		$C_3H_2Br_2N_2O$	E	24%
Tetrakis hydroxymethyl phosphonium sulfate 55566-30-8		$[(HOCH_2)_4P]_2SO_4$	E	9%
Didecyl dimethyl ammonium chloride 7173-51-5		$C_{22}H_{48}NCl$	L	8%
Chlorine dioxide 10049-04-4		ClO_2	O	8%
Tributyl tetradecyl phosphonium chloride 81741-28-8		$C_{26}H_{56}PCl$	L	4%
Alkyl dimethyl benzyl ammonium chloride 68424-85-1		$C_{19}H_{34}NCl$	L	3%
Methylisothiazolinone 2682-20-4		C_4H_5NOS	E	3%
Chloro-methylisothiazolinone 26172-55-4		C_4H_4NOSCl	E	3%
Sodium Hypochlorite 7681-52-9		NaClO	O	3%
Dazomet 533-74-4		$C_5H_{10}N_2S_2$	E	2%
Dimethyloxazolidine 51200-87-4		$C_5H_{11}NO$	E	2%
Trimethyloxazolidine 75673-43-7		$C_6H_{13}NO$	E	2%
N-Bromosuccinimide 128-08-5		$C_4H_4BrNO_2$	E	1%
Bronopol 52-51-7		$C_3H_6BrNO_4$	E	<1%
Peracetic acid 79-21-0		$C_2H_4O_3$	O	<1%

Figure 1.3 Commonly Used Biocides (6)

Different sources classify biocides differently but the majority of these sources separate biocides into two categories. Those categories are lytic and electrophilic biocides (5). Lytic biocides are amphiphilic surfactants. They typically work by disrupting the membrane and cell wall causing the cells to lyse. Electrophilic biocides usually have functional groups that accept electrons. This causes the biocides to react with chemical groups that have more electrons. Glutaraldehyde, a commonly used biocide is an example of an electrophilic biocide (5). Glutaraldehyde is used in 27% of hydraulic fracturing

operations whereas DBNPA (2,2-dibromo-3-nitrilopropionamide), another commonly used electrophilic biocide, is used in 24% of HF operations (5).

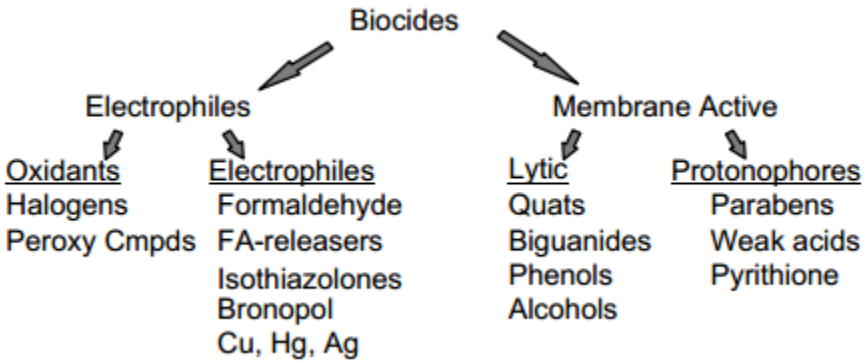


Figure 1.4 Biocide Classes (9)

1.1.1 Glutaraldehyde and DBNPA (2,2-dibromo-3-nitrilopropionamide)

Glutaraldehyde is an electrophilic biocide commonly used in multiple industries including HF and as a disinfectant in hospitals. In HF, glutaraldehyde is the most commonly used biocide (9). Electrophilic biocides such as glutaraldehyde work by reacting with electron rich chemical groups. These chemical groups are found in components of the cell membranes and cell walls (9). DBNPA is another commonly used biocide in HF. It is, however, more potent than glutaraldehyde and therefore not commonly used as a disinfectant in hospitals (6). DBNPA reacts with sulfur containing nucleophiles releasing bromine. Thus, disrupting key components within the cell (9).

1.2 Environmental Factors

There are multiple environmental factors that should be taken into account when considering biocide resistance and HF. Hydraulic fracturing has a great potential for contaminating the environment due to the large volume of chemicals used and highly hazardous nature of the wastewater. There are many places in the process where spills can happen.

When thinking of HF environmental contamination, the first thing that comes to mind is produced water spills and is considered one of the primary sources of HF wastewater. Produced water is the water that has already been used in HF. Produced water contains all the chemicals, including biocides, that have been used in the HF process. Releases of produced water are often one of the primary routes through which HF operations have led to environmental contamination..

1.3 Impact on Aquatic Communities

The potential for contamination of areas near HF sites is high. Any time the hydraulic fracturing fluids or the water produced by hydraulic fracturing is being transported there is a potential for an accidental spill. Biocides can contaminate soil and water through a variety of means and originate from not only HF but also households using them as disinfectants (10). Exposure to biocides is known to have multiple effects on microbial communities in the affected areas. One such outcome of HF fluid contamination is differential enrichment of microbial groups. Differential enrichment is the phenomena where different bacteria are selected for due to changes in the

environmental conditions. Differential enrichment often is exhibited by an overabundance of certain microbial groups able to tolerate the exposure compared to groups. These bacteria are different than those that are normally in the non-impacted environment. Once exposed to HF fluids including biocides, microbial communities have been shown to demonstrate different community compositions (10). In one study in streams in the Marcellus shale region, they found that pH drove changes in the stream microbial communities after exposure to biocides. They also found that methanotrophic and methanogenic bacteria were enriched in HF-associated streams(14). Another study in stream in the Marcellus shale region found that there were different operational taxonomic units (OTUs) in streams impacted by HF operations relative to non impacted streams and these communites were enriched in bacteria that could live in saline environments and some that could do hydrocarbon degradation (15). These impacted streams have also been shown to generate resistance hot spots which are a biological risk (11) One study demonstrated that triclosan exposure resulted in triclosan resistance as well as a unique community composition (12,13). In a study on hydraulic fracturing fluids and how they affect microbial communities by Lozano *et al.* They tested 6 different HF fluid combinations to look for different community compositions, the findings show that the combinations that contained the biocides were similar to each other and different from those combinations without biocides in their fluids (10).

Further studies have investigated the impact of HF-associated biocides to stream microbial communities. These studies have shown that biocide tolerance increases after exposure to the biocides. One such study sought to better understand how biocides affect

the environment through testing how glutaraldehyde impacted streams adjacent to HF operations in comparison to control streams. They tested for community adaption and the degradation of the biocides. This study found that glutaraldehyde was degraded faster by communities not exposed previously to HF biocides. Additionally, this study found that the microbial communities that were exposed to glutaraldehyde maintained species richness at higher levels compared to the non-HF impacted streams. This suggests that streams near HF activities had developed a tolerance to glutaraldehyde. These impacts may have larger ecosystem-wide implications (11).

In another study focusing on DBNPA samples from streams both impacted and not impacted by hydraulic fracturing were compared. The bacterial communities had different responses to the presence of DBNPA. DBNPA remained longer in the communities that had been exposed to the biocides previously, but these microbial communites were more tolerant to the biocide (16). A similar study was done with glutaraldehyde was done and they found similar results. Glutaraldehyde remained in the samples that had previously been exposed to the biocide longer but were more tolerant (11).

Biocides can have a variety of effects on not only the microbial composition but also wildlife. One such example would be invertebrates such as oysters exposed to biocides. In one study there was evidence suggesting that the by-products of oxidative biocides have detrimental effects on oyster larvae (17).

1.4 Resistance Mechanisms

Previous work has indicated that in streams adjacent to HF operations have altered microbial communities. Furthermore, recent work has suggested that the organisms living in these streams have higher resistance to biocides. This resistance could be a result of multiple mechanisms bacteria use to combat and resist biocides. Many of these mechanisms show similarities to resistance mechanisms used against antibiotics (18). These mechanisms of resistance can be broken down into two categories, intrinsic and acquired. Intrinsic resistance would be qualities that the bacterial cells already have such as spore formation. Acquired resistance would be mechanisms the cells ascertained through horizontal gene transfer or mutation (19).

Some examples of intrinsic resistance mechanisms would be changes in cell permeability, enzymes that degrade the biocides, spore formation, and biofilm formation (20). Permeability deals with the cell membrane and cell wall. If the cell membrane is less permeable and cells have thicker cell wall, it would be more difficult for the antimicrobials to enter the cell and thus these cells would be more resistant. These differences in cell envelope structure result in some bacterial types having more resistance than others. When it comes to enzymes that degrade the biocides, gram negative bacteria have an advantage. Gram negative bacterial cell walls have many more enzymes in them when compared to gram positive bacteria. Gram negative bacteria can have these enzymes because they have a periplasmic space whereas gram positive bacteria have only layers of peptidoglycan (21).

Spore formation is another formidable mechanism of resistance to biocides. A spore is a form that certain bacterial cells can take that increases its resistance to outside stressors such as temperature, acidity and of course, biocides. When in the spore state the cell does not grow. Spores can germinate into an active state once the environment is in more favorable conditions. Lastly, biofilm formation is a mechanism of biocide and antimicrobial resistance. A biofilm forms when many cells get together and collectively decide to enter a biofilm state. Biofilms are diverse but they are known to have more resistance than individual cells (20). In a biofilm the bacteria are held together with exopolysaccharide (EPS). This EPS makes it more difficult for biocides to enter the cells (20).

Intrinsic changes in gene expression have also been observed to confer resistance to biocides. Some bacteria already have the ability to resist biocides but do not normally express this resistance. One example would be bacteria that can express increased amounts of efflux pumps but do not normally have that level of expression (20). In one study by Vikram *et al.* RNA sequencing was used to observe any gene expression changes in *Pseudomonas spp.* after exposure to glutaraldehyde. They found that there was an increase in efflux pump expression in the cells that were exposed to glutaraldehyde. After that efflux pump inhibitors were used to test if the efflux pumps were what was causing the resistance to the biocide (22).

Acquired resistance is another common way that cells can obtain resistance to biocides. Acquired resistance can happen through the transfer of plasmids, which are obtained during horizontal gene transfer. These plasmids may contain genes that allow

for changes in phenotype that result in increased resistance to the antimicrobial. These types of genes could code for enzymes that breakdown the antimicrobial or contain other proteins that confer enhanced resistance. Acquired resistance is also known to happen due to mutation, which happens at a faster rate in bacteria than it does in multicellular organisms (23). Mutations may alter the target site for biocides or allow for increased expression of certain enzymes that may alter the permeability of the cell and thus lead to increased resistance. Not all bacterial cells however can use horizontal gene transfer due to the CRISPR-Cas system (24).

One more well-known story of horizontal gene transfer is the case of MSRA. *S. aures* obtained a methicillin resistant plasmid through horizontal gene transfer and as such as caused huge problems in the medical field (25). There are three ways horizontal gene transfer occurs, transformation, transduction and conjugation. Transformation is when one cell releases DNA, this can be for a variety of reasons such as lysis of the cell. This DNA is then taken up by a living cell and incorporated into the living cell's DNA. This is usually mediated by a shock of some kind such as heat shock or an electric shock in the lab but in the environment, cells have a natural capacity towards uptake (25). Transduction is horizontal gene transfer mediated by a viral bacteriophage. The bacteriophage when reproducing in the donor cell takes up some of the bacterial cell's DNA. It then infects a new cell with both its viral DNA along with the donor cells DNA (26). Lastly, there is conjugation involving plasmids. This process is when two cells form a bridge to transfer their DNA through. The donor cell connects to the recipient cell and

sends a plasmid to it. All three of these methods can be stopped by CRISPR-cas9 system (27).

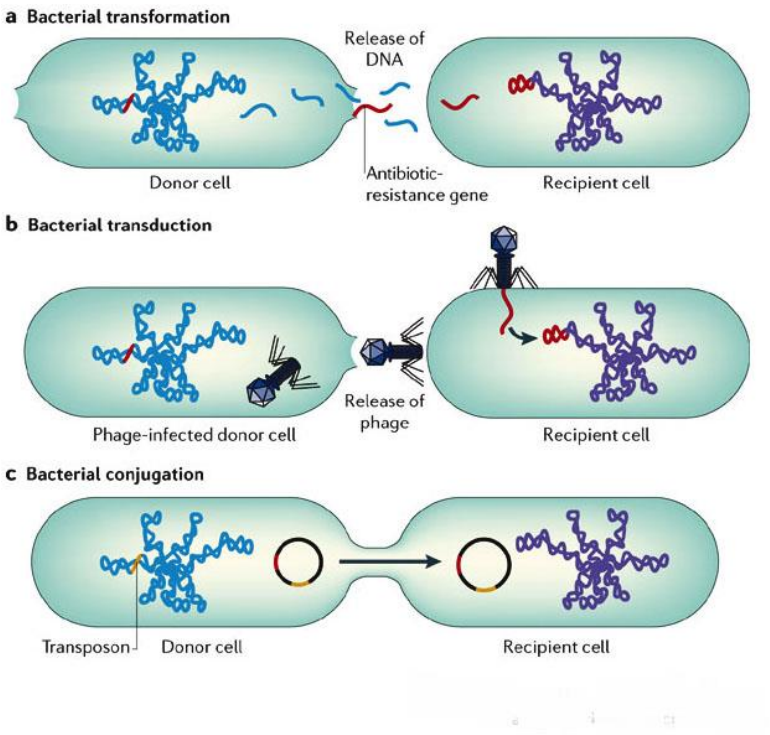
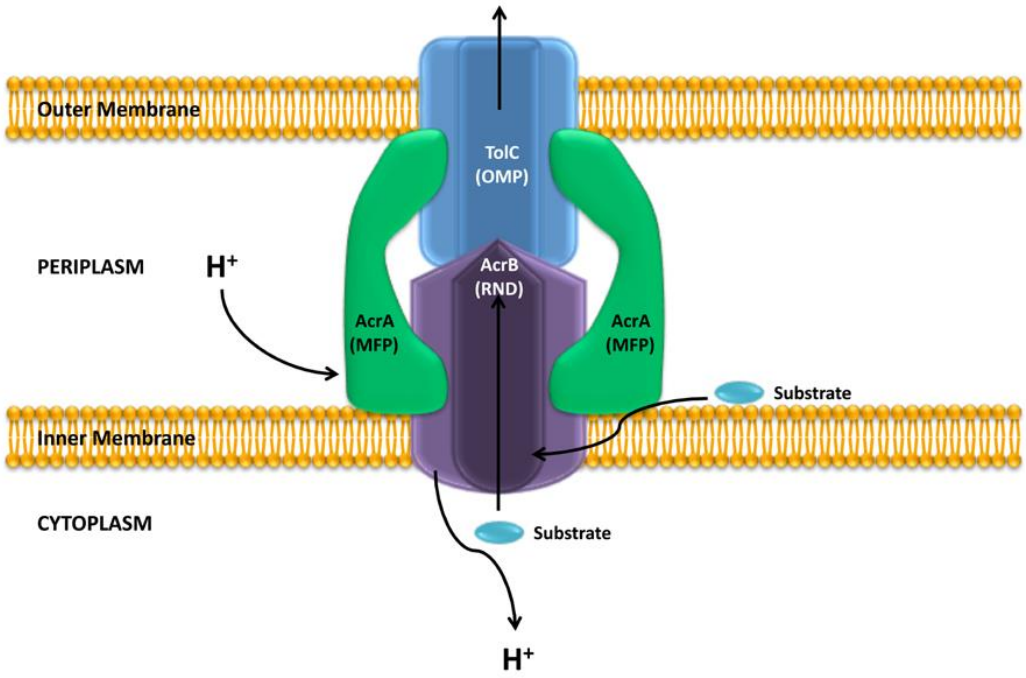


Figure 1.5 Horizontal gene transfer (28)

One commonly observed mode of resistance to biocides and other antimicrobials are efflux pumps. Efflux pumps can be found across all living organisms. Efflux pumps are pumps in the cell membranes that pump various molecules in and out of the cell. When it comes to resistance the commonly found efflux pumps pump the toxic compounds out of the cell. These efflux pumps can be specific to particular molecules, or they can be more universal (29).



1.6 RND Efflux Pump (22)

There are five groups of efflux pumps that deal with substances such as biocides. They are as follows, resistance-nodulation-division (RND) family, the major facilitator super family (MFS), the ATP-binding cassette (ABC) superfamily, the small multidrug

resistance (SMR) family and lastly the (DMT) drug/metabolite transporter superfamily. The efflux pumps associated the most with antibiotic resistance and biocide resistance are multidrug resistance efflux pumps (MDR) (30).

2 Experimental

2.1 Methods

We set out to better understand the potential for biocide exposure in streams impact by HF to result in biocide resistance of bacterial isolates from those streams and the mechanism for biocide resistance by testing bacterial isolates for biocide resistance. We obtained the samples from a stream in Pennsylvania called Lower Gray Run. The samples were both sediment and water. The main focus was the water samples as the sediment samples contained many fungi. The stream had previously been impacted by a spill of HF wastewater. Therefore, we believe that the stream was previously exposed to biocides.

Samples were collected in the fall of 2018. Attempts were made to isolate biocide resistant strains from these impacted samples. The next step was to isolate the bacteria from the samples. The samples were treated with biocides and then plated. Ten isolates were treated with glutaraldehyde, ten were treated with DBNPA and ten were treated with both glutaraldehyde and DBNPA. Samples were treated with both glutaraldehyde and DBNPA at a concentration of 100 ppm. This concentration was chosen as it was used in previous microcosm studies of the impact of biocide on stream microbial communities in Western Pennsylvania (31). A stock solution of 10,000 ppm was made for each biocide. For glutaraldehyde, a stock at 25% of glutaraldehyde was diluted in PBS, 10 µl of glutaraldehyde and 2.5 ml of phosphate-buffered saline (PBS) A 100 ppm solution of. DBNPA solution was made from 0.025 g of powdered DBNPA and 2.5 ml of ethanol.

Each biocide was added to the sample water at a concentration of 100 ppm and left to sit at room temperature for an hour. This solution (200 µl) was then spread around the R2 agar petri dishes until dry. The plates were then incubated at 30 °C for two days. Isolates were then chosen from the colonies hat grew on the plate. These isolates were then streaked again on the same type of agar. The plates were streaked quadrant style with flaming between each streak. This process was done three times to obtain isolates. In total, 30 isolates were obtained. Ten for each treatment, being glutaraldehyde, DBNPA and both glutaraldehyde and DBNPA.

Freezer stocks were then made for the 30 isolates. This was done by growing a colony in 5ml of nutrient broth for 2 days at 30 °C shaking between 180-200 rpm. Per each freezer stock 1ml of the bacteria and 500 µl of 30% glycerol and nutrient broth solution were added together into a cryovial. The 30% glycerol and nutrient broth solution was 15 ml glycerol and 35 ml nutrient broth that was then sterile filtered through a 0.2 µm membrane. The freezer stocks were then stored in a -80°C freezer.

Next the bacterial isolates needed to be prepared for DNA extraction. The first step was to pellet the cells grown in liquid medium as described above. Two ml of the cultured cells were added into 2 ml Eppendorf tubes. The cells were pelleted using the centrifuge at 10,000 rpm for 10 minutes. The supernatant was then removed. The pellet was then resuspended in 200 µl of water and bashing beads were added to the lysis tube. Also, in the tube was 750 µl of lysis solution. DNA was extracted from the cells using the Zymo Fungal/Bacterial DNA Miniprep kit (Zymo Research).

The tubes were then placed on the bead beater for 100s at 5.5 m/s. Next, the lysis tubes were placed on the centrifuge for 1 min at 10,000 x g. Then, 400 µl were transferred from the lysis tube to a collection tube and spun at 7,000 x g for 1 min. 1,200 µl of genomic lysis buffer was added to the filtrate from the collection tube. Next, 800 µl was added twice to the column and collection tube complex and spun at 10,000 x g for 1 min. 200 µl of prewash buffer was then added and the complex was spun at 10,000 x g for 1 min. Lastly, 500 µl of DNA was buffer was added and the complex was spun at 10k for 1 min. The product was transferred to a new tube. 100 µl of elute was added and then the product was spun at 10,000 x g for 30s.

The next step was to identify the bacterial isolates. To do this PCR was done to obtain the 16S rRNA sequences of the isolates. Two and a half microliters of the microbial DNA were taken and amplicon PCR forward and reverse primers were added. Here we used 27f and 1492R to amplify the nearly full length 16S rRNA gene from each isolate Both 1 micromolar measuring 5 µl. 2x Phusion master mix measuring 12.5 µl was also added. The PCR cycle was 95 °C for 3 min to start followed by a cycle of 95 °C for 30s, 55 °C for 30 s then 75 °C for 30s. Once the cycling is complete it was followed by 72 °C for 5 mins and lastly hold at 4 °C.

To make sure the amplification was a success we ran a gel. The gel consisted of 0.84 g of agarose and 70 ml of 1.TAE. This was then microwaved for 1 min. Two µl of SYBR safe DNA gel stain was added then. The gel was then poured and let to sit for 20 mins to allow it to solidify. The TAE was then added to fill the dock. Loading buffer was used to color each sample as well. The gel ran at 70 v for 45 mins.

The next step was PCR cleanup. 20 µl of Axyprep Magneti beads were added to each well. After mixing each well the magnetic stand was used to attract the magnetic beads. The supernatant was then discarded. The beads were then washed twice with 80% ethanol, approximately 200 µl. The beads were then left to dry for 10 mins. The tray was then removed from the magnet and 52.5 µl of 10mM Tris at pH 8 was added to the plate. It was incubated for two minutes then put back on the magnet for 2 minutes. Lastly 50 µl of the supernatant were transferred to a new plate.

To then determine the concentration of the PCR products we did Qubit broad range DNA quantification. The working solution of the QuantIT dye needs to be diluted to 1:200 in the dsDNA high sensitivity buffer. Two standards were used for the standard curve. For the standards 190 µl of the working solution was added to 10 microliters of the standards. They were then vortexed for 2-3s. For the sample tubes working solution was added to each tube so that the final volume in each tube was 200 microliters. We used 2 microliters of DNA for each sample. The tubes were then left to incubate at room temperature for 2 mins. The Qbit machine was then used to read each value in ng/microliter. These 16S rRNA genes were sequenced from both direction at the University of Tennessee DNA sequencing core. To analyze the 16S rRNA sequences, the forward and reverse reads were assembled into the nearly full-length 16S rRNA. The taxonomy of the isolates was identified by using BLASTn against the non-redundant database. The best hit was considered to be the closest relative for these strains. Additionally, the taxonomy was confirmed using the RDP classifier.

To better understand the potential mechanisms for biocide resistance, we attempted whole genome sequencing. Whole genome library prep was performed using the Illumina NexteraXT library prep kit. The protocol is as follows. 10 µl of TD was added to 5 µl of gDNA. 5 µl of ATM were added to each well and then mixed via pipetting. In a minicentrifuge the tubes were spun for a few seconds. The tubes were then placec in the thermal cycler for tagmentation. The cycle is 72 °C for 3 min followed by 12 cycles of 95 °C for 10s, 55 °C for 30s and 75 °C for 30s. Then 72 °C for 5 mins and lastly hold at 10 °C. Once the cycle was finished 5 µl of NT was added to each well and mixed. The tubes were centrifuged in the minicentrifuge again. The tubes were then left to incubate at room temperature for 5 mins. After that the tubes were placed on ice for 5 mins. The primers added to the tubes for library prep depends on the number of samples and the kit used. Once library prep is done bead cleanup is done once again in the same matter as stated previously. The libraries were sequenced using the Illumina MiSeq. Unfortunately, the sequencing run failed and the libraries were not able to sequence.

In order to see the extent of the resistance for the 30 isolates we did minimum inhibitory concentration testing, also known as obtaining MICs. Fresh cultures were grown by inoculating 5mL of nutrient broth with 200 µl of freezer stock. They were left to incubate on a shaker for 2 days. MICs were prepared on 96 well plates. Each isolate was tested for the MIC of glutaraldehyde, DBNPA and for both biocides. For example, with DBNPA 1% DBNPA was added to PEG 300. Solutions were prepared for 50 ppm, 75 ppm, 100 ppm, 250 ppm, 500 ppm, 1250 ppm and 2500 ppm. 180 µl of the biocide solution were added to 20 µl of culture for each well on the 96 well MIC plates. The

plates were then incubated on a shaker at 30 ° C at 180 rpm for 2 days. Growth was measured by recording the OD 600 for each well using a plate reader.

Because we were unable to obtain genome sequencing data from these stream isolates in time, we analyzed data from a previous study. This data includes isolates obtained from flowback water. In this previous study they analyzed the antibiotic resistance genes from these isolates (31). However, in the previous study the flowback water isolates were not compared with their non-flowback relatives. Here we sought to perform some comparative genomics between *Bacillus* sp from flowback water with non-flowback Bacillus strains to identify differences between these strains that may be.

The strains used for this experiment were *Bacillus* strains D23, DG33 and G16 and they were compared to *Bacillus cereus* and *Bacillus subtilis* respectively. The first step was to download the data. After the data was downloaded, we did a quality check using fastqc and cutadapt (32-33). Cutadapt works by removing adaptors and polyA tails. fastqc then was used to visualize the new data.

Both reference-based assembly and *de novo* assembly was done on the data. For reference-based assembly the genomes of *Bacillus cereus* and *Bacillus subtilis* were downloaded from the NCBI data base (34). Bowtie2 was used to map the raw reads to the reference genomes (35).

de novo assembly was done using SPAdes on the three target strains. The quality of the assemblies were assessed with QUAST. SPAdes works by generating de Bruijn graphs, this removes bubbles and chimeras from the reads. The distance between k-mers

is estimated next (36). Finally, another graph is generated this time being a paired assembly graph. Contigs are then generated last (29). QUAST is then used to check the quality of the generated data (37).

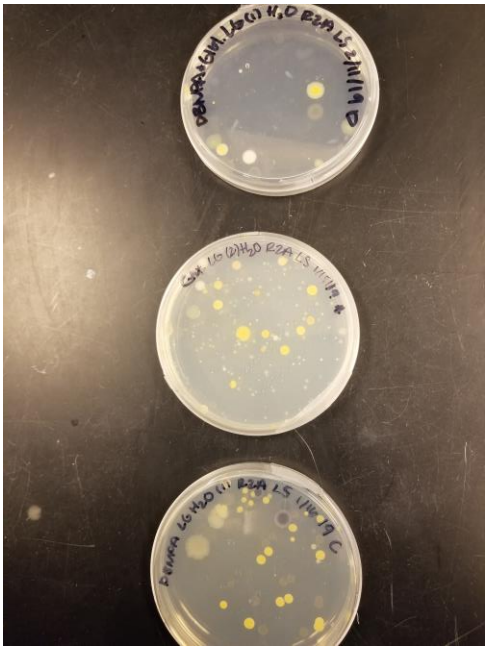
The *de novo* assembled genomes and the strains for comparison were annotated using PROKKA analysis. PROKKA was used to annotate the *de novo* assembled genomes. Prokka works using the generated contigs to identify the elements in the genomes and classify those genes (38). *Bacillus subtilis* (NC_000964.3) and *Bacillus cereus* (NC_004722.1) were used for comparison. Antibiotic resistance genes were annotated using Resistance Gene Indentifier at the Comprehensive Antibiotic Resistance Database (39). verage nucleotide identity was used to get a fine scale analysis of relatedness using the ANI calculator tool (40).

The step by step code for this comparative analysis can be found on the GitHub page at the end of this paper.

2.2 Results

After treatment of impacted stream water and sediment with the biocides, we have been able to recover approximately 10^{2} CFU/ml from Glutaraldehyde treated water and sediment, approximately 10^{2} CFU/ml from DBNPA treated, and 10^{1} CFU/ml after the combination treatment. This suggests that the cocktail of the two biocides is more effective at microbial control in these settings. However, the high number of colonies obtained indicates a robust population of biocide-resistant microbes in these streams. Thirty isolates were obtained from the water samples collected from Lower

Gray Run. These strains had diverse morphologies. Many of the colonies were pale or yellow colored. A number of these strains produced a dark purple color during isolations. These 30 isolates were then used for further study.



2.1 Isolate plates from top to bottom both glutaraldehyde and DBNPA, DBNPA and glutaraldehyde.

After isolation of the strains, DNA was extracted from each of the strains. The concentration of these strains varied greatly. Several of the strains didn't not have detectable DNA. This could be due to either limited cell growth in the liquid medium or issues associated with DNA purification. The samples that had detectable DNA were used for 16S rRNA sequencing to identify the taxonomy of the isolates.

	1	2	3	4	5	6	7	8	9	10
DBNPA	7.09	1.48	22.1		3.57	5.08	3.10	9.54	3.12	4.00
Glut.	14	9.47	10.1			11.7	1.64			
Both	3.21	1.37		5.24						8.78

2.1 Table of Qbit scores after PCR. All values are in ng/microliter.

The 16S rRNA gene was sequenced for many of the isolates. Some of the sequencing reactions failed either due to the contamination of the strain or due to insufficient DNA in the starting reaction. Of the strains that produced at good 16S rRNA sequence, the taxonomy was classified for them.

Strain Name	Genus of closest relative
DBNPA 1	*Janthinobacterium*
DBNPA 2	*Janthinobacterium*
DBNPA 3	*Pseudomonas*
DBNPA 6	*Pedobacter*
DBNPA 8	*Novosphingobium*
DBNPA 9	*Janthinobacterium*
Glutaraldehyde 1	*Caulobacter*
Glutaraldehyde 2	*Caulobacter*
Glutaraldehyde 3	*Pseudomonas*
Glutaraldehyde 6	*Caulobacter*
Glutaraldehyde 7	*Caulobacter*
Both 2	*Caulobacter*
Both 6	*Bacillus*

Names of bacterial isolates after 2 rounds of naming

Janthinobacterium s a bacterium that shows up a few times in the data but only after exposure to DBNPA alone. It can be found in both water and soil and it has a distinctive dark purple color (39). This color comes from violacein, an insoluble compound the cells create. Violacein is known to be toxic for bacteria, fungi, viruses and protozoa. These bacteria are also known to create its own antibiotics (39).

Pseudomonas was another bacterium isolated from the samples. *Pseudomonas* is a gamma proteobacteria commonly found in soil and water. *Pseudomonas* is both a pathogen to plants and humans. It is known to be resistant to many commonly used antibiotics and it also has minimal needs for growth (40).

Pedobacter is known to be resistant to multiple antibiotics including colistin. *Pedobacter* is more commonly found in soil but can also be found in water. All strains are multidrug resistant strains. *Pedobacter* is thought to be an environmental superbug (41).

Novosphingobium are members of alpha proteobacteria. They are gram negative bacteria but unlike most gran negative bacteria they have a glycosphingolipid layer instead of a lipopolysaccharide layer. The glycosphingolipid layer is characteristic of eukaryotic cells. They can live in a wide variety of places but most isolates that have been researched have come from human samples (42).

Caulobacter is another bacterium that is commonly found in water samples both glutaraldehyde and in the isolates treated with both biocides. Like all the other found

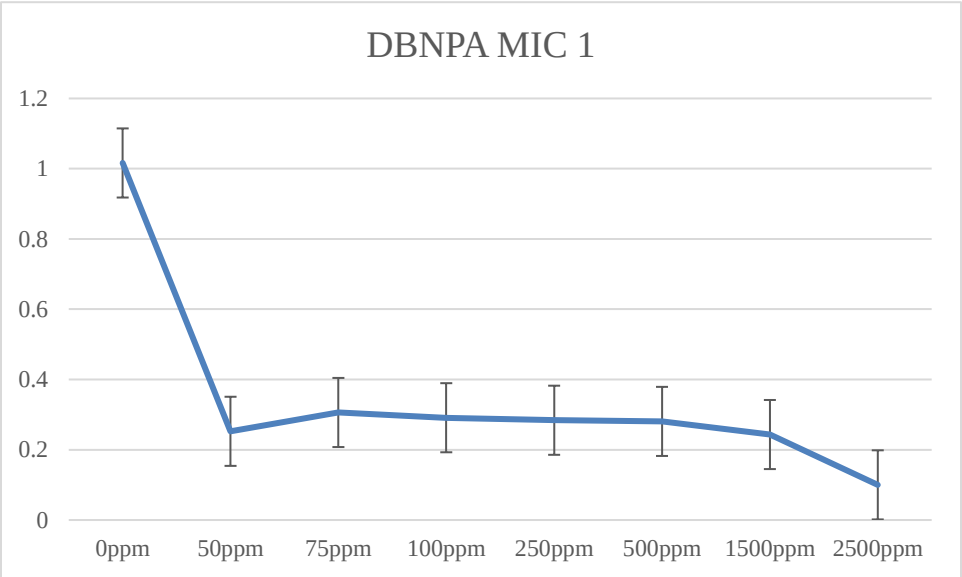
bacteria it is also a gram-negative bacterium. This bacterium has been known to do well in nutrient poor areas and is said to have a complex life cycle (43).

The last kind of bacteria that we recovered was *Bacillus. Bacillus* are commonly known for being spore formers. The spore form is known to have greater resistance to a variety of environmental stressors. They are also found in soil and water environments (44).

The common trend in the bacteria isolated after biocide treatment was that they are all gram negative except Bacillus and they were all commonly found in water and soil environments. Gram negative bacteria are known to be more resistant than gram positive bacteria. They are also known to be more pathogenic. It also makes sense that these bacteria were all commonly found in soil and aquatic environments because that was the focus of the study. It was interesting that spore forming strains were only isolated in the double biocide treatment. This is in contrast to the findings in Waad Aljohani's thesis in which the spore forming Bacillus strains were the most resistant to the biocides.

To determine the extent of resistance to each of the biocides, Minimum Inhibitory Concentrations (MICs) were determined for each strain. The MIC data was not always easy to read as some strains did not have graphs that matched what was expected or there was a reverse trend, but we did get some results. From the data we could not decisively say how resistant the isolates were overall. For the ones where an MIC was able to be determined (Figure 2.2). The minimum inhibitory concentration was often the lowest concentration of biocide. This indicates that while many of the isoaltes were able to

survive the initial treatment with biocides during isolation, they were not able to grow in low concentrations of the biocides.



2.2 A sample graph of the MIC data. The x axis is in ppm and the y axis is absorption.

2.2.1 Comparative Genomics of Bacillus species

From the data analysis done on the *Bacillus* strains we found that the quality of the reads was good for the strains sequenced in our lab. Using fastqc there were no red flags presented to us after the quality control was performed.

The reference-based genome sam files showed that the raw reads and the reference genomes were very close with almost 100% matches. The *de novo* data showed similar results with the GC content and number of Ns being similar. These two methods

giving similar results is good because it was the same results just gained two different ways. The data in table 2.3 suggests that the strains were are quite different from one another in that they varied in every category. All of these assemblies were very good assemblies with very few contigs, relatively long longest contigs and high N50 values.

Qualities	D23	DG33	G16
Number of Contigs	42	50	68
Length assembly	4338878	4234615	4258983
Length of Longest Contig	576084	706250	411992
N50	239015	279343	259571
L50	6	5	7

Table 2.3 *de novo* assembly statistics

These genomes were then annotated and compared with the annotations from the non-HF related strains. These results indicated that the strains were in fact different from one another in regards to their genes. Overall, these genomes contain similar numbers of genes. *B. cereus* is the exception with almost one thousand more genes than the other strains substantially more tRNAs. Additionally, there were substantial differences in the numbers of antibiotic resistance genes. We hypothesized that HF-associated strains

would have increased numbers of resistance genes. However, the type strain of *B. subtilis* had the highest number of antibiotic resistance genes.

Qualities	D23	DG33	G16	*B. subtilis*	*B. cereus*
Number of CDS	4330	4152	4145	4214	5529
Number of tRNAs	88	85	84	87	108
Number of CRISPRs	0	0	0	0	0
Number of hypothetical proteins	1471	1309	1290	1352	2589
Number of antibiotic resistance genes	4	4	4	9	0

Table 2.4 PROKKA annotation data

To better understand the relatedness of these strains and their non-HF relatives, we used Average Nucleotide Identity to compare these strains. Using an ANI calculator and the fasta files of the contigs the following table was obtained. As you can see the three isolates were more related to each other than *B. subtilis* and *B. cereus.* Out of the two type strains, *B. subtilis* had more in common with the experimentally obtained strains.

	G16	DG33	D23	B. subtilis	B. cereus
G16		99.09	98.98	92.95	68.22
DG33	2,821,577		98.94	92.86	68.18
D23	2,934,691	2,912,639		93.00	68.36
B. subtilis	2,741,227	2,609,928	2,656,500		68.71
B. cereus	546,534	538,501	525,863	564,904	

Table 2.5 Percent identity upper triangle and genome alignment lower triangle

We also wanted to determine the number and identity of the resistance genes. To do that we used the resistance gene identifier on CARD. Perfect and strict hits were considered.

Organism	Number of Hits
D23	4
DG33	4
G16	4
B. subtilis	9 (2 strict)
B. cereus	0 (2 strict)

Table 2.6 Number of resistance genes

D23	1	2	3	4
AMR gene family	small multidrug resistance (SMR) antibiotic efflux pump	small multidrug resistance (SMR) antibiotic efflux pump	macrolide phosphotransferase (MPH)	daptomycin resistant pgsA
Resistance Mechanism	antibiotic efflux	antibiotic efflux	antibiotic inactivation	antibiotic target alteration
%ID	96.19	93.75	81.05	97.37
Length of ref seq	100.00	100.00	100.00	100.00

Table 2.7 D23 Resistance genes

DG33	1	2	3	4
AMR Gene Family	small multidrug resistance (SMR) antibiotic efflux pump	small multidrug resistance (SMR) antibiotic efflux pump	macrolide phosphotransferase (MPH)	daptomycin resistant pgsA
Resistance Mechanism	antibiotic efflux	antibiotic efflux	antibiotic inactivation	antibiotic target alteration
%ID	95.24	93.75	80.72	97.37
Length of ref seq	100.00	100.00	100.00	100.00

Table 2.8 DG33 Resistance genes

G16	1	2	3	4
AMR Gene Family	macrolide phosphotransferase (MPH)	small multidrug resistance (SMR) antibiotic efflux pump	small multidrug resistance (SMR) antibiotic efflux pump	protein variant model
Resistance Mechanism	antibiotic inactivation	antibiotic efflux	antibiotic efflux	peptide antibiotic
%ID	81.37	93.75	96.19	97.11
Length of ref seq	100.00	100.00	100.00	100.00

Table 2.9 G16 Resistance genes

As you can see from the above tables the most common mode of resistance is the efflux pumps. *B. subtilis* had many resistance genes, many of which were also efflux pumps. This demonstrates that there are similarities between the strains and also that efflux pumps are a common mode of biocide resistance.

2.3 Conclusions and future directions

Biocide research is important because it has a very real impact on life. Biocide resistance can lead to many complications when it comes to battling bacterial infections among other things. We set out to determine if the we could better understand the differences between bacteria exposed to biocides and their wildtypes along with determining if we could find bacteria resistant to DBNPA. In the end we could see some bacteria that were resistant to DBNPA, but the MIC data did now show how resistant to DBNPA they were both in general and in comparison to glutaraldehyde and those exposed to both.

Like previously stated, the bacteria isolated were all gram negative. This is in contrast to previous studies that have shown spore forming gram positives were commonly isolated from flowback water. It is possible that something about the bacteria being gram-negative helped them be resistant to the biocides. Also, many of the bacteria isolated were able to survive in low nutrient conditions which could mean something about their metabolism is helping them withstand biocides. Our results indicate that a diverse group of culturable strains were able to be isolated from impact streams and that they had varying resistance to biocides.

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