5-2016

Isolation and Characterization of Microbial Communities from Hydraulic Fracturing Fluids

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ISOLATION AND CHARACTERIZATION OF ANAEROBIC MICROBIAL COMMUNITIES FROM HYDRAULIC FRACTURING FLUIDS

An Honors Thesis Submitted to
the Department of Microbiology
in partial fulfillment of the Honors requirements

UNIVERSITY OF TENNESSEE

by
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MAY 4TH 2016

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by

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Isolation and Characterization of Anaerobic Microbial Communities from Hydraulic Fracturing Fluids

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ABSTRACT

Hydrocarbon production from hydraulic fracturing of gas shale in the US has skyrocketed and is projected to keep growing. This water intensive drilling process creates toxic wastewater without an efficient disposal method. Because this method involves injecting fluid 1-3 km deep into the Earth, it is likely that microbial communities adapted to extreme conditions of the subsurface have accumulated in the produced water. The goal of this study is to identify microorganisms that might have bioremediation capabilities for flowback water and characterize microbes isolated from fracking water samples under anaerobic conditions.

Water samples were obtained from hydraulic fracturing locations in the Marcellus shale of Pennsylvania. These water samples include six different collections of flowback water, a flowback mix tank, and three different treatment tanks. Inoculations from the water samples were grown under anaerobic conditions in high salinity marine media and halotolerant hydrocarbon degradation dependent media. Samples were also grown at ambient temperature and at 37°C. DNA was extracted, and 16S rRNA gene Sanger sequencing was used to identify individually isolated microbes. Illumina sequencing was used to yield genetic information about the overall microbial communities. The Biolog Omnilog, a high-throughput phenotype microarray, was used to determine the genotype-phenotype characteristics of some of the most significant isolates. Results show the presence of numerous anaerobic microbes with metabolic variability and bioremediation potential, including sulfate reducers and hydrocarbon degraders. There also were a considerable number of potential human pathogens identified with antibiotic resistance from biocide exposure.

INTRODUCTION

Emergence and Significance of Hydraulic Fracturing in the United States

Hydraulic fracturing has emerged as an unconventional means of tapping into oil and natural gas shales that were previously too deep to utilize. With the spike of oil and gas prices, advancements in drilling technology, and approval from some state governments and the Environmental Protection Agency (EPA) (1), the amount of fracking in the US has skyrocketed since 2007. Fracking poses an opportunity for major economic growth in the US and energy independence from foreign providers. By 2010, natural gas production from hydraulic fracturing had already risen to compromise 23% of total natural gas production in the US (2), and some reports indicate that fracking could constitute 38% of the US hydrocarbon energy portfolio by 2040 (3). In 2014, the US became the world's largest petroleum and natural gas producer by surpassing Russia and Saudi Arabia and continues to maintain that title (4).

What is "Fracking"?

Hydraulic fracturing (also known as "fracking") involves drilling 1-3 kilometers deep into the Earth to reach deep shales and then drilling horizontally through a sandstone layer along the shale (5) as displayed in Figure 1a and 1b. Layers of steel casing and cement coat the well bore to keep hydrocarbons from leaking out (6) (Figure 1a). Recent technological advancements in the horizontal drilling process and pipe casing structure have made the process more efficient (6, 7). Highly pressurized water-based fluid is then injected into the well to fracture the sandstone layer and release oil and/or natural gas from the shale (5). Once the pressure is released from the fracturing fluid, some fluid flows back out of the well and then oil and/or natural gas flow to the surface.

Concerns with Fracking

With the increase in alternative drilling methods, concerns have been raised about environmental safety and dangers to human health. One prominent concern with hydraulic fracturing is its connection to localized tremors. Most reports cite the relation of this drilling method to seismic activity is the injection of production fluid wastewater back into the subsurface (8, 9). Some studies propose links between wastewater injection and tremors with a magnitude as high as 5.6 on the Richter Scale and causing significant damage (10). Recent

increase in seismic activity in the midcontinent US has also been attributed to hydraulic fracturing (11). However, other researchers have reported that although wastewater injection process can cause seismic activity, the majority of wells have only caused micro-tremors and are rarely noticed by the surrounding community (12, 13). Another impending worry with hydraulic fracturing is the potential for methane, salt, and chemicals to leak into the groundwater supply (14). However other studies have shown that the leakage of saline water and methane into the groundwater is naturally caused by gas shales (15-17), and other publications have found no link of contamination from hydraulic fracturing to groundwater contamination (18). Although there is contradictory data on whether there is firm link between fracking activity and groundwater contamination, there are clear methane emission concerns (19, 20) and waste water handling issues (17, 21, 22). Many researchers advocate for tighter environmental regulations on hydraulic fracturing (23).

Flowback Water and Associated Chemicals

One impending environmental concern is the build up toxic fracking flowback water (1, 2). Hydraulic fracturing uses 15-20 million liters of pressurized water in the drilling process (3). The fracturing fluid used is composed mostly of water (90.6%), sand as a proppant (8.95%), and other chemicals (0.5-0.1%) which includes biocides, acids, corrosion inhibitors, clay stabilizers, friction reducers, gelling agents, and others (24). Approximately 10-14 days after fracturing the shale (2), between 10-70% of this injected fluid flows back out to the surface (3, 25). The fluid that flows back out of the well ("flowback water") consists of the injected water-sand mixture and the deep underground brine water created by breaking open the shale (2). This flowback water contains elevated levels of radium and barium (26) as well as arsenic and selenium (27). As can be expected, flowback water can be a dangerous pollutant if not contained and disposed of properly (1). Because of the dangers imposed by this flowback water, tighter regulations have been imposed only recently to block fracking companies from disposing of the flowback water in local water treatment facilities (17). A German study on hydraulic fracturing found that the only suitable method for treating high salinity flowback waste water was evaporation or crystallization (22). Currently, there are a variety of methods drilling and gas companies are using to manage this flowback wastewater (28). Many are using a variety of treatment methods on site to clean the wastewater to be released back into surface waters. It is also common to

inject the wastewater into deep geological formations presumably below the water table. Many companies also hold the wastewater in containment tanks and then reuse the fluid to fracture other shales. In some cases, it is being sprayed onto roads to suppress dust (28).

Hydraulic fracturing companies have been reluctant to fully release in entirety what chemicals are additives to the fracturing fluid, which has created some public concern (29). One study identified and characterized 81 different chemical additives in hydraulic fracturing fluids (30). Biocides are included in the fracturing fluid to reduce the growth of microbes such as sulfate reducers and acid producers that can corrode the pipes and sour the gas (31, 32). The gas industry cites quaternary ammonium compounds (QACs), 2,2-dibromo-3-nitrilopropionamide (DBNPA), and glutaraldehyde as the most commonly used biocides (30, 33). Again, the gas industry is reluctant to release exact details, research data shows that the biocide concentration in hydraulic fracturing fluids ranges from 10-800 ppm (30) depending on the geological characteristics of the shale. Glutaraldehyde is not only a biocide used in the gas industry; it also has commonly been used for disinfection and sterilization in hospitals at a concentration of 2% since the 1960s (34). Glutaraldehyde's primary mode of action involves causing intramolecular cross bridges of the tectonic acid chains in the cell wall (35) and has shown biocidal activity with Gram positive bacteria, Gram negative bacteria, bacterial spores, fungi, and viruses (34). Studies also note that biodegradation of the compound differs under anaerobic conditions compared to aerobic environments (36).

Potential Research with Flowback Water

Identifying bioremediation microbes that have the ability to break down some of the toxic components of flowback water would be incredibly advantageous to this growing industry. If an efficient method of bioremediation for flowback water can be optimized and implemented, this would alleviate many of the concerns with this toxic fluid contaminating the environment since the industry currently lacks efficient disposal methods. Fracking also involves a large consumption of water; direct water consumption for drilling and fracturing a well in the Marcellus shale region of Pennsylvania is estimated to be around 12,000 m^3 (or 3,170,000 gallons) (2). Effective bioremediation of flowback water could yield water that is clean enough to be released into local wastewater treatment plants; thereby, providing an opportunity for this water to be reused.

Because flowback water is coming into contact with the deep underground shale deposits, it is likely collecting novel microbial communities from the deep sub-terrestrial surface, and this build-up of different microbes would certainly increase if the water is being recycled to fracture other shales. The environment of the deep layers of rock are typically at pressures are 500 times greater than those found at the surface, temperatures exceed 70°C (1) and is virtually anaerobic (37). It has been suggested that many of these deep microbes would be spore-forming as well (37). These deep, halotolerant and anaerobic microbes could present extremophiles with novel capabilities. Oil companies are interested in the microbial communities in the flowback and produced water as well. Large populations of certain microbes, such as sulfate-reducers and iron-reducers, can clog and corrode pipes, sour the gas, and even be a health hazard to humans when H_2S gas is produced (25).

The Microbial Community

Previous studies have reported sulfate-reduction as a primary metabolic route for many subsurface microbes (37). Some anaerobic hydrocarbon degraders have been previous identified as well (38). A similar study on microbial community characterization of fracking fluids identified *Gammaproteobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Epsilonproteobacteria*, *Clostridia*, *Bacilli*, *Fusobacteria*, and *Flavobacteria* as the dominant bacterial classes in produced water samples and identified *Marinilabilia salmonicolor* as common anaerobic halotolerant microbial isolate (25). Another study identified *Halomonas*, *Marinobacter*, *Vibrio*, *Idiomaria*, and *Pseudomonas* as dominant taxa in hydraulic fracturing produced water samples (3). Another study demonstrated that *Marinobacter hydrocarbonclasticus* isolated from fracking fluids had an increased resistance to the biocide glutaraldehyde (39).

Goal of the Study

For our study, we have collected samples of flowback water from the Marcellus shale region of southwestern Pennsylvania. This portion of the study is focused only on anaerobic microbes from fracking flowback water. The goal of this study is to identify novel organisms by culturing, isolating, and sequencing individual microbes and to compare microbial communities isolated from fracking water samples by metagenomic sequencing of the entire community. We plan to culture and identify anaerobic microbial species from the obtained fracking fluids. We

can then perform physiological testing to determine their optimal conditions for resource acquisition, bioremediation capabilities, and resistance potential. 1) Our first hypothesis is that, given the unique conditions of the subsurface, hydraulic fracturing flowback water contains novel and unique microorganisms. 2) Secondly, we hypothesize that some microbial species isolated from hydraulic fracturing flowback water will display bioremediation capabilities. 3) Lastly, we hypothesize that given the use of biocides and chemicals in the hydraulic fracturing fluids, some microbial species will display biocide and antibiotic resistance.

MATERIALS AND METHODS

Sampling

Water samples were obtained from hydraulic fracturing locations in the Marcellus shale region of Pennsylvania in November 2014. These water samples include: six different flowback water collections, a sample of flowback mix tank fluid, and a sample from three different, individual flowback treatment tanks. All of the flowback samples contain raw flowback fluid, and all samples were collected from the same tank that contained a mixture of various wells flowback fluids. Three of the flowback samples also contained drill mud. Treatment tanks contain flowback water that is being treated with various acids and polymers. Each treatment sample comes from a different treatment tank in the remediation streamline. The mix tank is a collection of different treatment fluids for holding. After collection, all water samples were shipped and stored at 4°C until inoculation and filtration.

Microbial Isolations and Growth Conditions

Microbes were isolated under anaerobic conditions on plates using various media and differing temperatures. All experimental work was performed in an anaerobic chamber with a gas mixture of 5% CO^2, 5% H^2, and 90% N^2 gases (Airgas USA, St Louis MO). Two different types of media were used: a marine based media and a hydrocarbon-dependent media. BD Difco™ Marine Broth 2216 (Becton, Dickinson and Company, Franklin Lakes NJ) was used to isolate halotolerant microbes that may utilize various marine carbon sources. ONR7a + Angola oil + peptones were used as the other media as a means to isolate halotolerant microbes who can utilize crude oil as a carbon source. ONR7a is a synthetic medium that mimics seawater conditions but, on its own, lacks a principal carbon source (40). Oil sampled from the Angola region was used in a similar bioremediation project (41) and added to ONR7a medium as the sole carbon source. The two versions of media inoculated with hydraulic fracturing fluid samples were grown at two different temperatures: 21°C and 37°C. Ambient temperature, 21°C, was used because it is a baseline microbial growing condition and 37°C was used because of its clinical significance and because microbes in the subsurface are often accustomed to higher temperatures. In summary, we had four sets of anaerobic growth conditions: (1) 21°C marine, (2) 21°C ONR7a+oil, (3) 37°C marine, and (4) 37°C ONR7a+oil.

DNA Extraction, Amplification and Sequencing

Liquid cultures of each isolate were spun down to a cell pellet, and DNA was extracted from the pellet. DNA extraction was performed using a MoBio PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad CA). Concentrations and quality of extracted DNA were determined by measuring concentrations and 260/280 and 260/230 ratios on a NanoDrop spectrophotometer (Thermo Scientific, Waltham MA). DNA samples were then subject to PCR, and the 16S rRNA region of DNA was amplified using 27F and 1492R primers. All amplified DNA concentrations were further confirmed by Qubit® Fluorometric Quantitation (Life Technologies, Carlsbad CA). All PCR-amplified DNA samples were purified using Zymo DNA Clean & Concentrator Kit^{TM}-5 (Zymo Research Corporation, Irvine CA). The amplified, purified DNA samples were then submitted to the University of Tennessee Microbial Biology Resource Facility for 16S rRNA gene Sanger sequencing.

Analysis of Sequencing Data

The quality of extracted DNA was assessed using the nucleotide chromatogram provided by Geospiza's FinchTV 1.4 DNA Sequence Analysis Software. The sequence was then submitted to the NCBI BLAST® database. Only samples with a match of 98% or greater were considered in the study.

Microbial Isolates

To date, nearly 100 microbial species have been isolated under anaerobic conditions. Many of those were prepared in anaerobic glycerol stocks (concentration of 15% glycerol in marine media) and stored at -80°C for later use. Some of those microbial isolates are still in the pipeline for extraction and identification. However, for now, our efforts have become concentrated on performing further physiological testing on a selected group of identified isolates. Twelve bacteria isolates (Table 2) were selected for further physiological characterization.

Glutaraldehyde Resistance Testing

Those microbial isolates selected for further physiological testing were re-grown from glycerol stocks at their corresponding temperatures. Isolates were inoculated onto media plates at

a concentration of 50 ppm glutaraldehyde. Three media conditions were used: ONR7a + 50ppm glutaraldehyde, ONR7a + 50ppm glutaraldehyde + peptones, and Difco™ Marine Media 2216 + 50ppm glutaraldehyde. ONR7a + 50ppm glutaraldehyde was used as a halotolerant biocide degradation dependent media in which glutaraldehyde served as the sole carbon source for the microbial isolate in testing. ONR7a+ 50 ppm glutaraldehyde + peptones was used to analyze organism's resistance to the biocide with an added generic carbon source. Difco™ marine media contains many various carbon sources in a halotolerant environment. Marine media + 50 ppm glutaraldehyde was used to demonstrate if the microbial isolate is able to grow in the presence of 50 ppm glutaraldehyde with an abundance of carbon sources.

Antibiotic Resistance Testing

The Biolog GEN III Omnilog® ID system (Biolog, Hayward CA) which is a high-throughput phenotype microarray system was used for antibiotic resistance testing of selected microbial isolates. Isolates were grown in 96 well PM plates with a marine media base, and Biolog Redox Dye Mix A (a microbial respiration indicator dye). Before inoculation, all PM plates were placed in the anaerobic chamber for 24 hours at 21°C to allow additional oxygen to diffuse out of the plate. After inoculation, PM plates were a placed in Biolog PM GAS anaerobic bags with two MGC Ageless® oxygen absorber packets (Mitsubishi Gas Chemical America, New York NY) and then vacuum sealed inside the anaerobic chamber. All isolates were grown on Biolog MicroArray PM Plate 9 MicroPlate™ to measure various osmotic/ionic responses and environmental conditions and PM Plate 11C MicroPlate™ to measure for various antibiotic sensitivities.

PM Plate 9 MicroPlate™ contains the various conditions: NaCl concentrations 1-10%, NaCl 6% + betaine, NaCl 6% + N,N-dimethyl glycine, NaCl 6% + sarcosine, NaCl 6% + dimethyl suphonyl propionate, NaCl 6% + MOPS, NaCl 6% + ectoine, NaCl 6% + choline, NaCl 6% + phosphoryl choline, NaCl 6% + creatine, NaCl 6% + creatinine, NaCl 6% + L-carnitine, NaCl 6% + KCl, NaCl 6% + proline, NaCl 6% + N-Acetyl L-Glutamine, NaCl 6% + β-glutamic acid, NaCl 6% + γ-amino-N-butyric Acid, NaCl 6% + glutathione, NaCl 6% + glycerol, NaCl 6% + trehalose, NaCl 6% + trimethylamine-N-oxide, NaCl 6% + trimethylamine, NaCl 6% + octopine, NaCl 6% + trigonelline, potassium chloride concentrations 3-6%, sodium sulfate concentrations 2-5%, ethylene glycol concentrations 5-20%, sodium formate

concentrations 1-6%, urea concentrations 2-7%, sodium lactate 1-12%, sodium phosphate pH 7 20-200mM, sodium benzonate pH 5.2 50-200 mM, aluminum sulfate pH 8 10-100mM, sodium nitrate 10-100mM, and sodium nitrite 10-100mM.

PM Plate 11C contains 24 different antibiotic compounds: Amikacin. Chlortetracycline, Lincomycin, Amoxicillin, Cloxacillin, Lomefloxacin, Bleomycin, Colistin, Minocycline, Capreomycin, Demeclocycline, Nafcillin, Cefazolin, Enoxacin, Nalidixic Acid, Chloramphenicol, Erythromycin, Neomycin, Ceftriaxone (Rocephin), Gentamicin, Potassium sulfate, Cephalothin, Kanamycin, and Ofloxacin. Each antibiotic was inoculated onto the plate with a four step increase in the concentration gradient.

(It is important to note here that a mechanical issue with the Omnilog occurred and delayed data collection of all microbial isolates for several weeks, and only endpoint growth data was successfully obtained for one selected isolate- *Marinobacter hydrocarbonclasticus*.)

RESULTS*

*****It is important to note that these results are still considered preliminary. The project is very much still ongoing, and results mentioned here do not signify the completion of the project.

To date, 37 different taxa have been isolated and identified in the hydraulic fracturing fluids at various conditions (Table 1). Although, several more microbial isolates are still in the pipeline for extraction and identification. Most isolated organisms have been derived from flowback fluids or treatment tanks; also it is noteworthy that most identified taxa are from either of the two major sources but not both. The source conditions for these microbes do not overlap for the most part. More taxa prefer the higher temperature of 37°C compared to ambient temperature. More taxa were able to survive on the marine base media than ONR7a+oil media. *Vibrio* was by far the most abundant genus. Although duplicates are not reported in Table 1, *M. hydrocarbonclasticus* was increasingly common. Many isolates (including *Sunxiuqinia* and *Marinilabiliaceae*) hail from the *Bacteroidetes* family.

Of those 37 identified taxa, 12 have been selected for further physiological study based on their distinctive characteristics (Table 2).

The first physiological test involved monitoring of selected isolates response to glutaraldehyde. None of the selected isolates were able to successfully grow on ONR7a media with 50 ppm glutaraldehyde as the sole carbon source. Furthermore, the selected isolates were still not able to grow on the ONR7a medium with 50 ppm glutaraldehyde even with the addition of peptone as a generic carbon source. All of the selected isolates were able to grow on the marine based media with various carbon sources but with 50 ppm of glutaraldehyde added as a potential growth deterrent (Table 3); thereby, suggesting some level of resistance to the biocide.

As previously noted, endpoint assessment of growth in physiological conditions was only available for *Marinobacter hydrocarbonclasticus*. Survival outcomes of the isolate involving osmotic and ionic stressors (PM Plate 9) and various antibiotic concentrations (PM Plate 11C) are displayed in Figure 2 and Figure 3. This isolate of *M. hydrocarbonclasticus* showed growth in all available concentrations of the following: NaCl, KCl, sodium sulfate, ethylene glycol, sodium phosphate, ammonium sulfate, sodium nitrate, and 6% NaCl + various carbon sources (Figure 2). *M. hydrocarbonclasticus* showed growth in at least the first 3 of 4 concentration

gradient increases of the following antibiotics: Amikacin. Chlortetracycline, Amoxicillin, Bleomycin, Colistin, Capreomycin, Demeclocycline, Cefazolin, Enoxacin, Ceftriaxone (Rocephin), Gentamicin, Cephalothin, Kanamycin, and Ofloxacin (Figure 3).

DISCUSSION/CONCLUSIONS

Our results show a fairly diverse array of microbial species from the hydraulic fracturing fluids (Table 1). Although many are halotolerant, that is expected because of the high salt content in the brine that is mixed with flowback and the nature of the meteoric porewater in the formation. There are also several isolates such as *Bacillus cereus*, *Acinetobacter*, and *Klebsiella* that have a potential to be human pathogens. Given the diverse array of taxa and the unique qualities of some selected isolates (Table 2) it is very likely that some microbial isolates show unique bioremediation qualities or resistance to biocides and antibiotics.

The first experimental testing of the selected isolates and glutaraldehyde resistance (Table 3) demonstrates that the microbes were not able to utilize glutaraldehyde as their sole carbon source, but they did survive in a marine media with various carbon sources and glutaraldehyde added as biocide. This suggests that the isolated species require some other carbon source other than glutaraldehyde to live under anaerobic conditions. However, the isolates still failed to grow in an ONR7a medium + glutaraldehyde when peptones were added. Peptones generally act as a generic carbon source for all bacterial species. The only significant difference between ONR7a+peptones and Difco^{TM} Marine media 2216 is the addition of yeast extract. It is very likely that these organisms require some of the amino acids, vitamins, and carbohydrates that are provided by yeast extract for survival.

Marinobacter hydrocarbonclasticus survived at all NaCl concentrations (Figure 2), even those as high as 10% NaCl, which is nearly three times the concentration of NaCl found typical seawater (42). *M. hydrocarbonclasticus* is already well regarded as a hydrocarbon degrader, but this data demonstrates its ability to grow anaerobically in extremely high salt conditions as well. This is unique because extremophile bioremediation bacteria are the ideal candidates for the clean-up of polluted habitats like fracking fluid (43). *M. hydrocarbonclasticus* also survived at every 6% NaCl condition regardless what type of osmolyte or carbon source was added. This could be indicative of the idea proposed by previous researchers that the high salinity environment induces a stress response in the cell population that changes gene expression (39). *M. hydrocarbonclasticus* could also grow in various concentrations of: urea, sodium nitrate, and ammonium sulfate. These are all common components of fertilizer. This could indicate the ability of this strain of *M. hydrocarbonclasticus* to bioremediate areas of fertilizer runoff and agricultural waste. Notably, *M. hydrocarbonclasticus* could also survive in all concentrations of

ethylene glycol. Ethylene glycol is an important precursor in the development of polymers and plastics, which could mean this organism potential for the bioremediation of plastic waste products.

Without detailed analysis of the contents of PM plate 11C (Figure 3), it is very apparent that this strain of *M. hydrocarbonoclasticus* has strong antibiotic resistance. Some of the most notable antibiotic compounds are Amikacin, Colistin, and Kanamycin, which are often used to treat multi-drug resistant pathogen infections. Also notable is the isolate's resistance to Ceftriaxone (Roecephin) which is a commonly used broad spectrum antibiotic. This data suggests that this strain of *M. hydrocarbonclasticus* is resistant to some of the more powerful antibiotics available for prescription. It is important to keep in mind, however, that *M. hydrocarbonclasticus* is not a pathogenic organism. Furthermore, antibiotic resistant organisms found in the environment, particularly in more extreme conditions such as the subsurface, have been noted to be antibiotic resistant for over a decade (44, 45). We do not yet know if the antibiotic resistance found in *M. hydrocarbonoclasticus* is constitutive or in plasmids. Plasmid resistance could allow this resistance to be transferred to other bacteria via horizontal gene transfer mechanisms, making it much more significant to human health.

The first hypothesis of this study states that given the unique conditions of the subsurface, hydraulic fracturing flowback water contains novel and unique microorganisms. The data presented in Table 1 displays a wide range of taxa isolated under various conditions (Table 1) from hydraulic fracturing fluids. The information in Table 2 presents some unique qualities about each selected microbial isolate (Table 2). This data presents a diverse microbial population found in flowback water with noteworthy qualities and therefore fails to reject our initial hypothesis.

Our second hypothesis states that some microbial species isolated from hydraulic fracturing flowback water will display bioremediation capabilities. Four microbial species isolated from flowback water (*Marinobacter hydrocarbonclasticus*, *Marinobacterium georgiense*, *Marinilabilia salmonicolor*, and *Stappia indica*) were selected for further physiological analysis because of previous publications that noted their bioremediation capabilities (Table 2). Although we have yet to perform thorough physiological testing of all the

microbial isolates to confirm their bioremediation potential, the information presented from previous studies thus far does not reject our second hypothesis.

Lastly, our third hypothesis states that given the use of biocides and chemicals in the hydraulic fracturing fluids, some microbial species will display biocide and antibiotic resistance. All of the isolates selected for further physiological study showed a resistance to the biocide glutaraldehyde at least 50 ppm in a halotolerant environment (Table 3). The only isolate that has undergone physiological testing thus far in the study, *Marinobacter hydrocarbonclasticus*, confirms the potential for antibiotic resistance (Figure 3). Although this data is not comprehensive of the microbial community, it does suggest that some microbial species in flowback water contain some level of biocide and antibiotic resistance and therefore fails to reject the third hypothesis. We intend to perform further studies to analyze in detail the data behind our hypotheses; our preliminary data does not reject our initial hypotheses.

FUTURE WORK

Identifying Resistance Limitations in Selected Isolates

The thing remaining to be done on this project will be the twelve microbial isolates selected for further physiological characterization using the Biolog Omnilog® ID System. Isolates will be grown in triplicates in 96 well plates in concentrations of glutaraldehyde ranging from 0-2000 ppm to measure the limit of their resistance to the biocide. Then bacterial isolates will be grown in two different types of media: a marine base media BD Difco™ Marine Broth 2216 (Becton, Dickinson and Company, Franklin Lakes NJ) and nutrient base media Difco™ Nutrient Broth Isolates will be grown in nutrient base media to eliminate the confounding variable that an upregulation antibiotic resistance and biocide resistance genes could be caused by the stressful environment of high salinity as proposed by some researchers (39). Those selected twelve isolate will also be studied using the kinetic data collection on Biolog MicroArray PM Plate 11C MicroPlate™ to test for various antibiotic chemical sensitivities and PM Plate 9 MicroPlate™ PM plate 9 to test for various osomic/ionic responses.

The Future and Overall Goals of the Microbial Community in Fracking Fluids Project

Once all microbial isolates have been fully isolated and identified, we can further identify a species role in the microbial community by comparing it to the metagenomic data. Additionally, the anaerobic species isolates can be compared to the aerobic species isolates, and unique differences noted. The overall scope of this project is to identify bioremediation capabilities in microbial isolates as well as any unique physiological features including what impact they might have on human health. This question can be further investigated with the physiological data of selected isolates. We can also investigate if the antibiotic resistance genes found in our microbial isolates are constitutive or in plasmids by performing whole genome sequencing

New Samples from the Permian Basin Drilling Region in Texas

Recently, more samples have been obtained from Permian Basin in southwest Texas. Those samples include: water from a hydraulic fracturing runoff pit, produced water from a vertical well, and a well flow (production fluids) from a vertical well. Those water samples have been inoculated on four media conditions: marine, nutrient, ONR7a+Angola oil, and

ONR7a+50ppm of glutaraldehyde. More microbial species will be isolated and identified from these testing sites over time. Each of these water samples have also been filtered through an Omnipore® 0.2 Micron filter to collect the microbial community biomass. Those filters have been extracted and will be used in metagenomic Illumina® sequencing. This data will allow us to compare the microbial communities from the Marcellus Shale region of Pennsylvania to those in the Permian Basin of Texas as well as compare the differences in microbial species found in hydraulic fracturing fluids to those found in classic vertical drill well waters.

ROLE IN PROJECT

I, Sheridan Brewer, started working as an undergraduate research assistant in the Hazen Lab in the summer of 2013; however, I did not start assisting with this project until December of 2014. Since then, I have drafted and submitted a MICR 402 (Advanced Microbiology Research) manuscript on the early basis of this project. I have presented to members of our lab with this project on numerous occasions. I have also presented a poster on this project at the University of Tennessee Exhibition of Undergraduate Research and Creative Achievement (Eureca) in 2015 and 2016. I gave an oral presentation on this project at the Southeastern Biogeochemical Symposium in March 2016. Recently, I have also submitted an abstract for a poster presentation on this project the American Society of Microbiology 2016 Conference in Boston, MA, which got accepted! More specifically, I independently performed all the laboratory work this project under the guidance and assistance of Maria Fernanda Campa. I provided my recommendations for the advancement of the project on occasions and assisted with design of some experiments. At this point, data analysis and compilation for this project is not yet complex. However, I did independently perform all the BLAST identifications of microbial isolates and analysis of the integrity of their 16S sequences. Furthermore, I appropriately recorded all microbial isolates and recorded their identifications in a running database. I then did preliminary research on the identified isolates and identified those that I thought showed unique significance to the project and warranted further physiological study.

The entirety of this project was performed with the oversight of Bredesen Center Ph.D. student, Maria Fernanda Campa. Maria collected the hydraulic fracturing flowback and treatment water samples from the Pennsylvania region. Maria provided training with the use of the Biolog Omnilog and some data analysis features. Maria also revised all manuscripts, presentations, and other scientific writings associated with this project. She led the direction and served as the leading mentor for the project.

Dr. Stephen Techtmann was a previous post-doctoral research fellow in the Hazen Lab who is now at Michigan Technological University. During his time at University of Tennessee, he greatly assisted in the training and data analysis for myself and many other students in the lab.

He also provided some initial guidance with the start-up of this project in 2015. And currently, his students at MTU are working with isolates obtained from hydraulic fracturing fluids and providing information on some genomic qualities as well as physiological differences. He also continues to provide recommendations with the fracking project.

Katie Fitzgerald and Amanda Garcia de Matos Armal assisted with extraction and identification of our many microbial isolates. Amanda also provided some comparative data with microbial isolates from hydraulic fracturing fluids in aerobic conditions. Julian Fortney provided the training of some of the complex laboratory equipment and also provided recommendations with the operation of the anaerobic chamber and specifications the experiment. Dominique Joyner provided training with some equipment as well and oversaw the operations of the laboratory at the University of Tennessee and Oak Ridge National Lab.

Dr. Terry Hazen oversaw all research endeavors with this project, provided funding, and served as the overall research mentor for the laboratory. He also approved all scientific writings and presentations associated with the ongoing project as well as offered recommendations.

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DATA APPENDICES

LIST OF TABLES

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Isolate Identified Taxon	Frack Water Source			Temperature		Media Type	
	Flowback	Mix Tank	Treatment	21°C	37°C	Marine	ORN7a+oil
Acinetobacter sp.	x				x	x	
Aeromonas salmonicida	x	x		x	x	x	x
Bacillus cereus	x	x		x	x	x	x
Bacillus firmus	x				x	x	
Bacillus thuringensis	x	x			x	x	
Bacteroidetes sp.			x	x		x	
Breoghania corrubedonensis			x		x		x
Citrobacter freundii			x	x		x	
Dietzia sp.		x			x	x	
Donghicola xiamenensis			x		x		x
Enterobacter aerogenes			x	x			x
Halomonas sp.	x	x	x	x	x	x	x
Idomarina/Psuedoidomarina	x	x			x	x	
Klebsiella oxytoca			x	x	x	x	x
Mangrovibacter plantisponsor			x		x		x
Marinilabilia salmonicolor			x		x	x	
Marinobacter hydrocarbonclasticus	x				x	x	x
Marinobacterium georgiense	x			x	x	x	x
Martelella mediterranea			x		x	x	
Proteus penneri			x		x	x	
Proteus vulgaris			x		x	x	
Raoultella ornithinolytica			x	x			x
Rhodobacter	x		x	x			x
Roseobacter/Oceanicola	x			x			x
Schewanella pultrefaciens		x		x		x	

Shewanella fodinae			x	x			x
Sphingobacteria	x				x	x	
Stappia Indica			x		x	x	
Suxiuqina	x				x	x	
Tadarida brasiliensis	x				x		x
Vibrio alginolyticus	x				x	x	
Vibrio anguillarum	x	x	x		x	x	x
Vibrio diabolicus	x				x	x	x
Vibrio harveyi	x			x			x
Vibrio parahaemolyticus	x			x			x
Vibrio planitsponsor			x		x	x	

Table 1. List of Microbial Taxa Identified and Their Respective Conditions. This table identifies every microbial taxa that has been purely isolated and identified from hydraulic fracturing fluids in anaerobic conditions so far in this study. An "x" indicates organism was isolated at those conditions during the culturing process. (It is worth noting, however, that some isolates have yet to be extracted and identified; so this list is not comprehensive of the study.) The table also demonstrates the source of the isolate in the frack fluid system as well as the preferred growing temperature and media used to isolate the organism. There are no duplicates listed in this table so any isolate that is in existence at multiple conditions of the same type indicates there was two or more types of that species found in the isolate data, and some isolates compromised a range of conditions.

Taxa	Unique Quality	Additional Info
Rauoltella orinthinolytica/ Klebsiella oxytoca[1]	Biocide/Antibiotic Resistance Potential	Taxa is in the Enterobacteriaceae family which has a reputation for producing antibiotic-resistant strains, particularly with carbapenems (47). These strains have also been reported to cause enteric fever (48) and histamine poisoning in fish (49).
Suxiuqinia	Biocide/Antibiotic Resistance Potential	Species identified in a publication noting its capability to form biofilms and adhere to metal surfaces as well as corrode pipes (50). Likely a target of bioicides.
Bacteriodietes sp.	Biocide/Antibiotic Resistance Potential	Isolate was noted as a sulfur reducer during isolation (produced a black precipitate). It is also commonly associated with the human gut and feces. Taxa is commonly noted as being antibiotic resistant, particularly with beta-lactams and aminoglycosides (51).
Acinetobacter sp.	Biocide/Antibiotic Resistance Potential	Some Acinetobacter species (particular Acinetobacter baumannii) are noted to be serious Gram-negative antibiotic resistant pathogens (52).
Marinobacter hydrocarbonclasticus	Biodegredation Potential	Species is a well-known seawater hydrocarbon degrader (53). Isolate was selected to analyze hydrocarbon degradation potential under anaerobic conditions and compare to isolates from other locations (such as oil seeps) (53). Also species was isolated in another fracking microbial study and showed glutaraldehyde resistance (39).
Marinobacterium georgiense	Biodegredation Potential	Species was originally identified in lignin degradation study and was also noted to degrade hydrocarbons and aromatic compounds (54).
Marinilabilia salmonicolor	Biodegredation Potential	*Marinilabilia* genus is chemo-organotrophic and is noted to be able to degrade a number biomacromolecules. Taxa was originally isolated form marine mud with decaying algae (55).
Stappia indica	Biodegredation Potential	Another study isolated the species from the seawater of the Indian Ocean and characterized the species as being able to degrade PAHs. Some strains have been noted to show antibiotic resistance as well (56).
Schwanella pultrefaciens	Diverse Metabolism	Species is very metabolically diverse and can reduce metals and radionuclides (57). A considerable amount of research has been directed toward the species use as a microbial fuel cell (58).
Rhodobacter sp.	Diverse Metabolism	Isolate was noted as sulfur reducer during isolation (produced black precipitate). Taxon is known to be remarkably metabolically diverse (59)
Idiomarina sp.	Novel Isolate	Although a common marine organism, numerous studies note the species as being “strictly aerobic”(60) (61). This makes the species potentially novel because we isolated the taxon in anaerobic conditions.

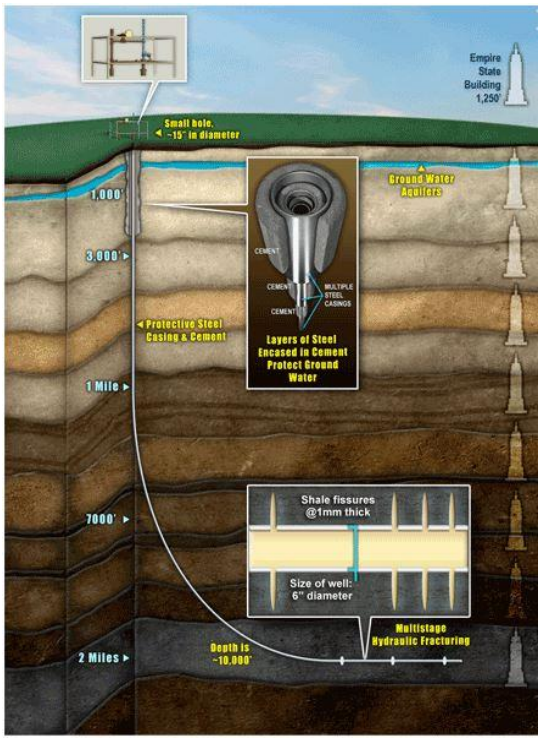
Table 2. Microbial Isolates Selected for Further Study and Their Unique Characteristics Reported from Previous Studies. The species or taxon name is reported in the far left column. Isolates are grouped according to their unique potentials and the general reasons for selected are noted in the middle column. The far right column contains additionally detailed information regarding the isolates as reported from previous publications.

[1] *K. oxytoca and R. orinthinolytica are both in the Enterobacteriacae family are still largely to be considered the same species although there have been recent recommendations to split the Klebsiella genus into the two subgenera based on phylogenetic analyses (46)

Taxa	ONR7a + 50ppm Glutaraldehyde	ONR7a+peptones+ 50ppm Glutaraldehyde	Marine + 50ppm Glutaraldehyde
Rauoltella orinthinolytica/ Klebsiella oxytoca	-	-	+
Suxiugina	-	-	+
Bacteriodites sp.	-	-	+
Acinteobacter sp.	-	-	+
Marinobacter hydrocarbonclasticus	-	-	+
Marinobacterium georgiense	-	-	+
Marinilabilia salmonicolor	-	-	+
Stappia indica	-	-	+
Schwanella pultrefaciens	-	-	+
Rhodobacter sp.	-	-	+
Idiomarina sp.	-	-	+

Table 3. Selected Isolates and Responses to Glutaraldehyde. The names of each taxon are displayed in the far left column. The second column reports the growth responses of each taxon when grown in the seawater mimic media where 50 ppm of glutaraldehyde is the only carbon source. The third column reports the growth responses of each taxon when grown in the seawater mimic media with 50 ppm glutaraldehyde as well as the addition of peptones as a generic carbon source. The fourth column reports the growth of each taxon in marine based media with various carbon sources but 50ppm was added to potentially inhibit growth. A minus (-) sign indicates no identifiable CFUs and a plus (+) sign indicates one or more identifiable CFUs.

1a) Cross-section of a Typical Horizontal Well (Anadarko Petroleum Corporation)



1b) The Hydraulic Fracturing Water Cycle (US Environmental Protection Agency)

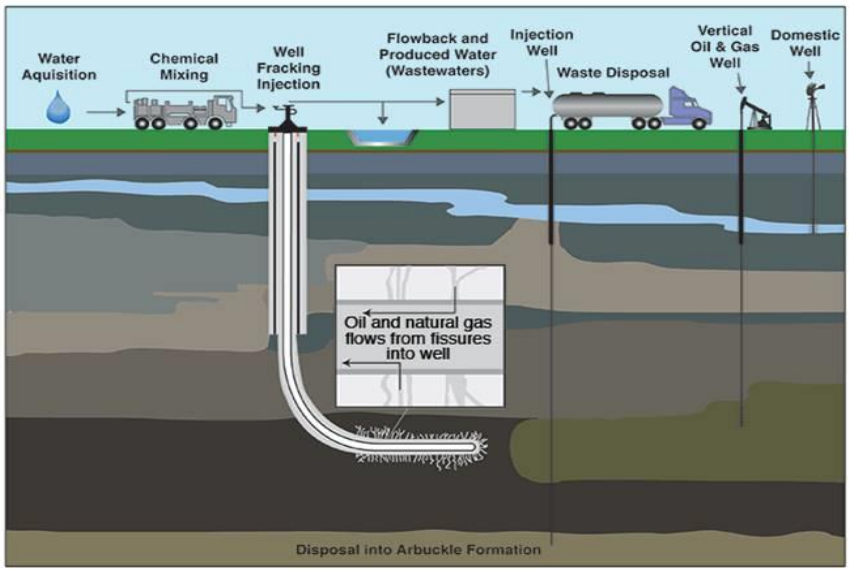


Figure 1a and 1b. The Hydraulic Fracturing Process and Details of Structure. Figure 1a "Cross Section of a Typical Horizontal Well" (62) shows detail of the depths and casing layers and components of the drilling process. Figure 1b "The Hydraulic Fracturing Water Cycle" (63) summaries the hydraulic fracturing drilling process including wastewater handling and disposal and also gives comparison to more conventional drilling methods.

PM9 MicroPlate™ Osmolytes

A1 NaCl 1%	A2 NaCl 2%	A3 NaCl 3%	A4 NaCl 4%	A5 NaCl 5%	A6 NaCl 5.5%	A7 NaCl 6%	A8 NaCl 6.5%	A9 NaCl 7%	A10 NaCl 8%	A11 NaCl 9%	A12 NaCl 10%
B1 NaCl 6%	B2 NaCl 6% + Betaine	B3 NaCl 6% + N-N Dimethyl Glycine	B4 NaCl 6% + Sarcosine	B5 NaCl 6% + Dimethyl sulphonyl propionate	B6 NaCl 6% + MOPS	B7 NaCl 6% + Ectoine	B8 NaCl 6% + Choline	B9 NaCl 6% + Phosphoryl Choline	B10 NaCl 6% + Creatine	B11 NaCl 6% + Creatinine	B12 NaCl 6% + L- Carnitine
C1 NaCl 6% + KCl	C2 NaCl 6% + L-Proline	C3 NaCl 6% + N-Acetyl L-Glutamine	C4 NaCl 6% + β-Glutamic Acid	C5 NaCl 6% + γ–Amino -N-Butyric Acid	C6 NaCl 6% + Glutathione	C7 NaCl 6% + Glycerol	C8 NaCl 6% + Trehalose	C9 NaCl 6% + Trimethylamine-N-oxide	C10 NaCl 6% + Trimethylamine	C11 NaCl 6% + Octopine	C12 NaCl 6% + Trigonelline
D1 Potassium chloride 3%	D2 Potassium chloride 4%	D3 Potassium chloride 5%	D4 Potassium chloride 6%	D5 Sodium sulfate 2%	D6 Sodium sulfate 3%	D7 Sodium sulfate 4%	D8 Sodium sulfate 5%	D9 Ethylene glycol 5%	D10 Ethylene glycol 10%	D11 Ethylene glycol 15%	D12 Ethylene glycol 20%
E1 Sodium formate 1%	E2 Sodium formate 2%	E3 Sodium formate 3%	E4 Sodium formate 4%	E5 Sodium formate 5%	E6 Sodium formate 6%	E7 Urea 2%	E8 Urea 3%	E9 Urea 4%	E10 Urea 5%	E11 Urea 6%	E12 Urea 7%
F1 Sodium Lactate 1%	F2 Sodium Lactate 2%	F3 Sodium Lactate 3%	F4 Sodium Lactate 4%	F5 Sodium Lactate 5%	F6 Sodium Lactate 6%	F7 Sodium Lactate 7%	F8 Sodium Lactate 8%	F9 Sodium Lactate 9%	F10 Sodium Lactate 10%	F11 Sodium Lactate 11%	F12 Sodium Lactate 12%
G1 Sodium Phosphate pH 7 20mM	G2 Sodium Phosphate pH 7 50mM	G3 Sodium Phosphate pH 7 100mM	G4 Sodium Phosphate pH 7 200mM	G5 Sodium Benzoate pH 5.2 20mM	G6 Sodium Benzoate pH 5.2 50mM	G7 Sodium Benzoate pH5.2 100mM	G8 Sodium Benzoate pH 5.2 200mM	G9 Ammonium sulfate pH8 10mM	G10 Ammonium sulfate pH 8 20mM	G11 Ammonium sulfate pH 8 50mM	G12 Ammonium sulfate pH8 100mM
H1 Sodium Nitrate 10mM	H2 Sodium Nitrate 20mM	H3 Sodium Nitrate 40mM	H4 Sodium Nitrate 60mM	H5 Sodium Nitrate 80mM	H6 Sodium Nitrate 100mM	H7 Sodium Nitrite 10mM	H8 Sodium Nitrite 20mM	H9 Sodium Nitrite 40mM	H10 Sodium Nitrite 60mM	H11 Sodium Nitrite 80mM	H12 Sodium Nitrite 100mM

Figure 2. ***Marinobacter hydrocarbonclasticus*** **response to various osmotic and ionic stresses.** Those cells enclosed in an orange perimeter indicate survival of the organism (*Marinobacter hydrocarbonclasticus*) in the indicated environment and therefore some level of resistance to the conditions.

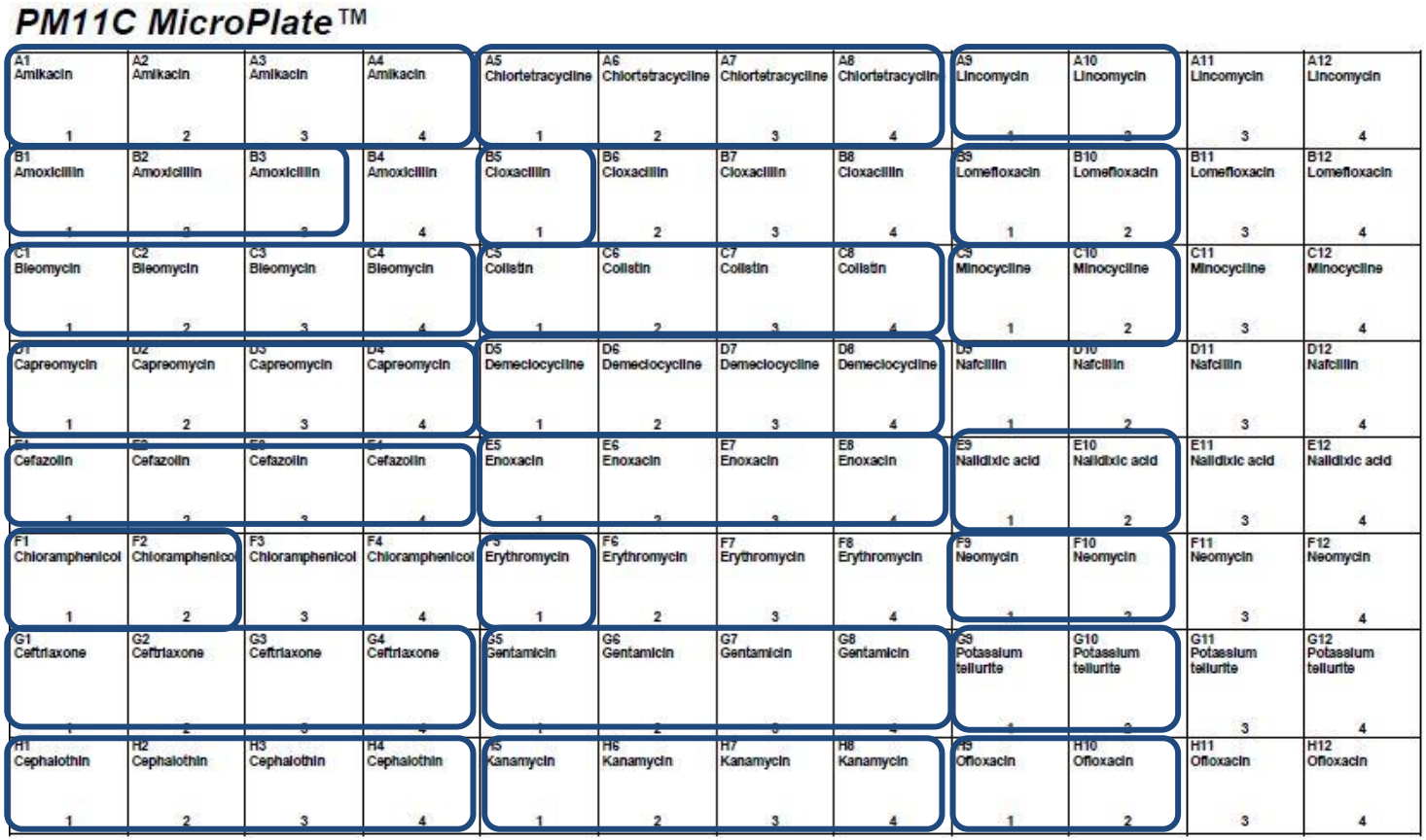


Figure 3. ***Marinobacter hydrocarbonclasticus*** **response to various antibiotics at increasing concentrations.** Those cells enclosed in a blue perimeter indicate survival of the organism (*Marinobacter hydrocarbonclasticus*) in the indicated environment and therefore some level of resistance to the specified antibiotic. There are 24 antibiotics included on this plate: Amikacin, Chlortectracycline, Lincomycin, Amoxicillin, Cloxacillin, Lomefloxacin, Bleomycin, Colistin, Minocycline, Capreomycin, Cemeclocycline, Nafcillin, Cefazolin, Enoxacin, Nalidixic acid, Chloramphenicol, Erythromycin, Neomycin, Ceftriaxone, Gentamicin, Potassium tellurite, Cephalothin, Kanamycin, and Ofloxacin (respectively). Each antibiotic has a four step increase in concentration from left to right in each row.

ACKNOWLEDGEMENTS

The author expresses his deep appreciation to Hazen Lab manager, Dominique C. Joyner, for her mentorship throughout the process as well as other colleagues from the Hazen lab for their continual input and support.