Fall 11-30-2020

Search for Antibiotic-Producing Microorganisms in Sites Affected by Acid Mine Drainage

Hannah A. Bussell
Eastern Kentucky University, Hannah_bussell3@mymail.eku.edu

EASTERN KENTUCKY UNIVERSITY

Search for Antibiotic-Producing Microorganisms in Sites Affected by Acid Mine Drainage

Honors Thesis

Submitted

In Partial Fulfillment

Of The

Requirements of HON 420

Fall 2020

By

Hannah Bussell

Faculty Mentor

Dr. Bill Staddon

Department of Biology

Search for Antibiotic-Producing Microorganisms in Sites Affected by Acid Mine Drainage

Hannah Bussell

Dr. Bill Staddon, Department of Biology

Antibiotic resistance in bacteria are an escalating problem. Little is known about the potential of microorganisms in sites affected by acid mine drainage (AMD) to be sources of new antibiotics. Sediments from AMD sites in Letcher County, Kentucky, were collected and microorganisms isolated. These samples were screened for their capacity to produce antimicrobial compounds active against known bacteria *E. coli* and *S. aureus*. The results showed no antimicrobial activity. This could be due to sample death, incorrect pH of media, or antibiotic resistance already present in the AMD site.

Keywords and phrases: Acid mine drainage, antibiotic, antibiotic resistance, microorganism, isolates, antimicrobial activity

Table of Contents

List of Figures

List of Tables

Acknowledgements

Dr. Bill Staddon

Mom & Dad

Introduction

1.1 Acid Mine Drainage

Acid mine drainage is the discharge of acidic water from coal or metal mines. It is often produced when sulfide-bearing material, such as iron sulfide-aggregated rocks, are exposed to oxygen and water (Akcil and Koldas 2004). Acid mine drainage is characterized by having a low pH and high concentrations of heavy metals and other toxic elements. AMD has been found to pollute ground and surface water along with the surrounding soils. These effects are categorized as chemical, physical, biological, and ecological, but the overall impact on communities affected by AMD include elimination of species, simplifying the food chain, and significantly reducing ecological stability (Gray 1997). When the sulfide-bearing material reacts with water and air, sulfuric acid is created. Acid mine drainage has an orange-yellow appearance (**Figure 1)**. This is due to the highly acidic AMD combining with more neutral conditions, causing the AMD pH to raise above 3. When the pH raises above 3, the dissolved iron precipitates out, causing the yellow-orange color (Jennings et al. 2008). When a mine drains this acid, it can upset the balance in rivers, streams, and aquatic life for hundreds, even thousands, of years. The fish, animals and plants in these affected sites are severely impacted due to the pH of the water dropping to a pH of 4 or lower (Jennings et al. 2008). The pH of the water could be similar to battery acid, which results in major fish kills. With the devastating effects of acid mine drainage, it is important for scientists to research the adverse environmental effects. By studying the effects, it is possible to predict the impacts of AMD in certain areas and come up with treatments to reverse these effects. Acid mine drainage is a world-wide problem and with advancing technologies, it has become possible to precent, mitigate and control AMD release.

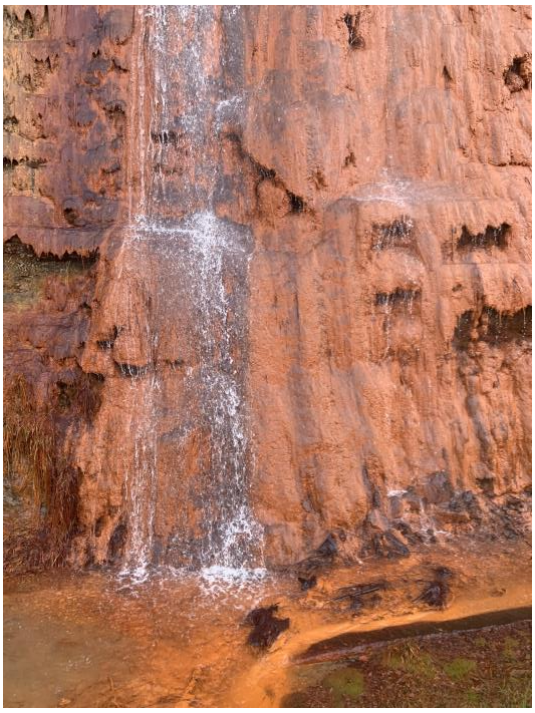

Figure 1. A site in Kentucky affected by acid mine drainage taken by Hannah Bussell on March 1st, 2020

1.2 Antibiotic Producing Microorganisms

A microorganism can only be seen when viewed under a microscope. Microorganisms include bacteria, algae, fungi, and protozoa. Antibiotics are a group of secondary metabolites, which are synthesized by microorganisms. Bacteria and fungi are the main microorganisms used in secondary metabolite accumulation. It was discovered in the 19th century that anti-infective compounds could be made by different microorganisms. These anti-infective compounds became known as antibiotics. An antibiotic can be defined as a chemical substance derived from microorganisms which have the capacity of inhibiting growth and even destroying other microorganisms (Rokem et al. 2007). An example of this would be the antibiotic Penicillin which is used to treat bacterial meningitis, strep throat, and other serious infections and is produced by the microorganism *Penicillium chrysogenum*. Microorganisms

are still widely used in modern medicine to produce antibiotics. These antibiotics are produced industrially by a process of fermentation (LibreTexts 2020). Fermentation is a metabolic process where organic molecules are converted into another substance. In the case of industrial produced antibiotics, the antibiotic-producing microorganisms are grown in large containers of liquid growth medium. The microorganisms used in fermentation differ from wild microorganisms. Lab grown microorganisms are genetically modified to yield the maximum about of antibiotics (LibreTexts 2020). During fermentation, the population size, temperature, oxygen concentration, pH, and nutrient levels are closely monitored to help ensure that the maximum yield is reached. To test microorganisms for antibiotics, a screening process must be completed, where isolates of the microorganisms are cultured and then tested on test organisms. Inhibition of these test organisms indicate an antibiotic is present.

1.3 Antibiotic Resistance

Antibiotic resistance is an escalating problem in modern medicine and is seen as a major challenge worldwide. Antibiotic resistance can be linked to bacteria that have experienced mutation or horizontal gene transfer driven by the pressure of antibiotics used in therapeutic settings (Martínez 2008). These pressures are created by over-prescription of antibiotics, overuse of antibiotics in livestock and fish farming, patients failing to finish the entire antibiotic course, poor infection control, poor hygiene, poor sanitation, and the absence of new antibiotics being discovered. Prior studies have found that animal husbandry, aquaculture, and plant production are significant factors in antibiotic resistance. This is due to cross contamination of antibiotics and bacteria between animals and humans. These cross contaminations can be seen in the environment and in the food humans eat (**Figure 2**). The most substantial reservoir of multidrug-resistant Gram-negative bacteria, such as

Enterobacteriaceae and *Pseudomonas aeruginosa*, is the gut of humans and animals, particularly those who are receiving antibiotics (Wellington et al. 2013). When the waste of these humans and animals harboring multidrug-resistant bacteria contaminate water, food, and the environment, the multidrug-resistant bacteria are able to spread.

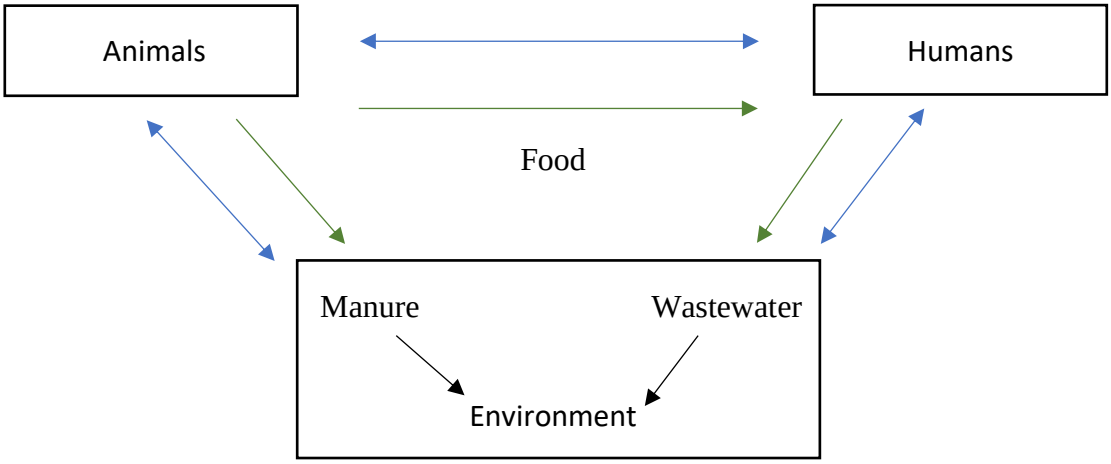

Figure 2. Exchange of antibiotics and bacteria between animals and humans. The blue lines represent bacteria while the green lines represent antibiotics. Bacteria is exchanged between humans or animals and the environment from direct spread or runoff. Antibiotics are spread from animals or humans to the environment through urine and feces. The urine and feces lead to manure for animals and wastewater for humans, both of which return to the lakes, rivers, and/or soils of the environment. While bacteria can be transferred both ways between humans and animals, antibiotics from animals are transferred to humans when animals are consumed (Martínez 2008).

1.4 Microorganisms Affected by AMD

Changes in natural ecosystems alter the population dynamics of microorganisms, including antibiotic production and resistance (Martínez 2008). The microorganism found in AMD are classified as acidophiles. Acidophiles are organism that survive and thrive in acidic

environments where the pH ranges from 1 to 5. These organisms are able to survive in extreme acidic conditions due to their ability to maintain pH homeostasis. A variety of mechanisms, such as passive and active regulation, and characteristics, such as a reversed membrane potential, highly impermeable cell membrane, and a predominance of secondary transporters, help acidophiles maintain an internal cellular pH at a constant level (Austin and Dopson 2007). Acid mine drainage conditions can vary from site to site. This implies that there are a variety of acidophilic microorganism in AMD. It has been found that the acidophilic microbial communities found in AMD are dominated by iron/sulfur-oxidizing microbes (Teng et al. 2017). Antibiotic resistance has been discovered in sites affected by AMD. AMD environment contaminated with several toxic metals are thought to serves as reservoirs for antibiotic resistance genes. There is little to no research conducted on antibiotic-producing microorganism affected by acid mine drainage. Due to the harsh environment acid mine drainage creates, there is potential that a new antibiotic can be discovered in these acid mine drainage communities due to antibiotic-producing organisms having to adapt.

The goal of this research project was to study sites affected by acid mine drainage to see if the microorganisms affected by the acidic conditions were producing new and/or already discovered antibiotics. It is reasonable to assume that there could be antibiotic production in these communities because most antibiotics are produced by environmental organisms. With the extreme acidic environment of AMD, the antibiotic-producing microorganisms could be adapting and changing, which could lead to a production of a new antibiotic.

1.5 Letcher County

The locational focus of this study was in Letcher County, Kentucky. Letcher County is located in the South-East corner of Kentucky (**Figure 3**). Letcher County has a rich mining history dating back to the early 19th century. Research has found that the mining of certain minerals like gold, copper, and nickel are associated with acid mine drainage problems. While AMD is a naturally occurring process, mining increases the AMD amount because it increases the quantity of sulfides exposed (Akcil and Koldas 2004). There are multiple areas in Letcher County affected by acid mine drainage. The largest pollution of acid mine drainage in Letcher County occurred on March 18th, 2016 when AMD invaded Pine Creek. On this day, a mining company was mining at the head of Pine creek when they accidentally drilled into an old underground mine. The water that had been stored in the mine was very acidic and had a high iron content. The water rushed out of the mine and overflowed into Pine Creek. The AMD from the old mine flooded the creek with its signature orange color (**Figure 4**) and acidified the environmental community, killing the aquatic life present (**Figure 5**). This spill affected the health of Letcher County residents because Pine Creek connects to the Kentucky River, which serves as the municipal drinking water intake that serves Whitesburg, the city in Letcher County. While the communities near coal mines suffer ecologically, economically, and in human health, it is hoped that AMD could be beneficial in fighting antibiotic resistance by producing a new antibiotic.

Figure 3. Location of Letcher County highlighted in red on a map of Kentucky.

Figure 4. The orange coloration of acid mine drainage seen in Pine Creek, Kentucky, taken by Tarence Ray on March 18th, 2016.

Figure 5. A dead turtle on the banks of Pine Creek after acid mine drainage leaked from the old mine taken by Tarence Ray on March 18th, 2016.

Methods and Procedure

2.1 Creating Buffers

Before the experiment began, full strength, 1/10 strength, and regular TSA was made. Prior research indicated that antibiotic-producing microorganisms grew best at a pH around 3.5-4. The initial goal was to create buffers that had a pH of 3.5. Different combinations of 0.1M potassium hydrogen phthalate, 0.5 M HCl, and distilled water were combined with 0.4 g of TSA agar in 50 mL beakers. After each buffer was heated to boiling while being stirred, the media was allowed to solidify at room temperature. The pH meter was calibrated to a pH 4, and the media's pH was read (**Table 1).** It should also be noted that the 0.5 HCl was prepared in December of 2019 from old concentrated HCl, so it is not likely to be exactly 0.5 M.

Table 1. Amounts of each component and pH of medias made by Hannah Bussell on January 1st, 2020.

0.1 potassium hydrogen phthalate (mL)	0.5 M HCl (mL)	DI H_2O (mL)	pH of media after cooling
5.0	1.0	4.0	3.32
5.0	0.9	4.1	3.43
5.0	0.8	4.2	3.67
5.0	0.7	4.3	3.83
5.0	0.6	4.4	3.90
5.0	0.5	4.5	4.11
5.0	0.4	4.6	4.27
5.0	0.3	4.7	4.35
5.0	0.2	4.8	4.57
5.0	0.1	4.9	4.68
5.0	0.0	5.0	4.78

The next step of preparation included creating a pH 4 Buffer that is 1/10 strength TSA. To do this, 11 different combinations of 0.1M potassium hydrogen phthalate, 0.5 M HCl, and distilled water were also used. The difference with this buffer is that there was 0.4 g of TSA powder/Bacto Agar Mixture. To make the TSA/Bacto Agar mixture, 0.52 g of TSA powder was mixed with 1.76 g of Bacto Agar. After each buffer was heating to boiling while being stirred, the media was allowed to solidify at room temperature. The pH meter was calibrated to a pH 4, and the media's pH was read (**Table 2**).

Table 2. Amounts of each component and pH of medias made by Hannah Bussell on February 5th, 2020.

0.1 potassium hydrogen phthalate (mL)	0.5 M HCl (mL)	DI H_2O (mL)	pH of media after cooling
5.0	1.0	4.0	2.70
5.0	0.9	4.1	2.77
5.0	0.8	4.2	2.87
5.0	0.7	4.3	3.02
5.0	0.6	4.4	3.18
5.0	0.5	4.5	3.37
5.0	0.4	4.6	3.53
5.0	0.3	4.7	3.70
5.0	0.2	4.8	3.95
5.0	0.1	4.9	4.34
5.0	0.0	5.0	4.66

The main focus was to make buffers at a pH 4. It was also decided that some media should be made at a pH 3.5, also. Three different TSA mixtures were made. The first was full strength TSA at pH 3.5. This was made in a 1000 mL media flask. 250 mL of 0.1 potassium hydrogen phthalate, 45 mL of 0.5 M HCl, 205 mL of water, and 20 g of TSA powder was added

to the media flask. The contents were brought to a boil while being stirred until the media was clear. The media was then refrigerated and left to be poured later.

The second mixture was a 1/10 strength TSA at pH 3.5. This was made in a 1000 mL media flask. 250 mL of 0.1 potassium hydrogen phthalate, 24 mL of 0.5 M HCl, 226 mL of water, 2 g of TSA powder, and 6.75 g of Bacto Agar was added to the media flask. The contents were brought to a boil while being stirred until the media was clear. The media was then refrigerated and left to be poured later.

The last mixture was regular TSA. This was also made in a 1000 mL media flask. 500 mL of distilled water and 20 g of TSA powder was added to the media flask. The contents were brought to a boil while being stirred until the media was clear. The media was then refrigerated and left to be poured later.

2.2 Collection of Samples

The samples of acid mine drainage affected sites were all collected within Letcher County. Most of the samples were collected within the city limits of Whitesburg. Two separate collections were done. The first collection took place on March 1st, 2020. On this day, Letcher County was surveyed, and sites of orange colored water were collected. Six samples from different collection sites were gathered. When a sample was collected, the pH was taken first, then the coordinates were written down (**Table 3**).

Table 3. Sample numbers, coordinates, and pH of samples collected from Letcher County by Hannah Bussell on March 1st, 2020.

Sample Number	Coordinates	pH
1	N: 37.14666 W: -82.82200	3.7
2	N: 37.14697 W: -82.82192	3.6
3	N: 37.12669 W: -82.74273	6.0
4	N: 37.11963 W: -82.86952	5.6
5	N: 37.22959 W: -83.00430	6.0
6	N: 37.36299 W: -83.25518	5.4

The second collection of samples occurred on May 30th, 2020. Only three samples were taken, and they were all collected from N: 37.14697 W: -82.82192. The reason for this collection was to conduct more experiments on the sample with the correct pH. During this collection, the pH of each sample was checked to ensure the correct pH (**Figure 6**).

Figure 6. Image of sample #2 collection site at N: 37.14697 W: -82.82192 collected by Hannah Bussell on March 1st and May 20th, 2020.

2.3 Preliminary Experiment

Using the samples that were collected, the previously made TSA plates were streaked and spread with different amounts of sample #2. The streak plate technique is where the loop and wire tool is flamed so that the tool is sterilized. Once sterilized, the loop is cooled and dipped into the sample and streaked onto one side of the agar plate (1 in **Figure 7**). Next, the loop is sterilized, cooled in the agar, and streaked onto another side of the plate (2 in **Figure 7**). The loop is sterilized one final time and streaked on another side of the plate (3 in **Figure 7**). A streak plate was made for the full strength, 1/10 strength, and the regular TSA plates. The spread plate technique is where an amount of the sample is poured onto the plate and a tool that is shaped like a miniature hockey stick is used to spread the sample evenly over the surface (**Figure 8**). A 10 μL spread plate and a 100 μL spread plate was made for the full strength, 1/10 strength, and regular TSA.

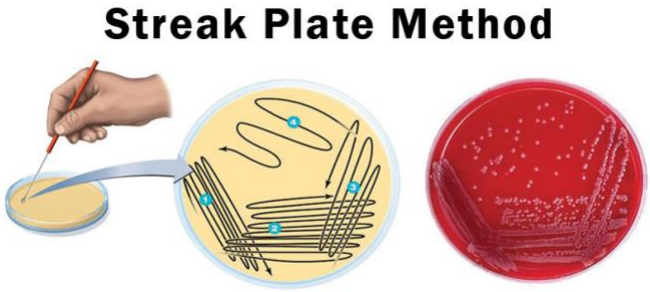

Figure 7. Streak plate technique

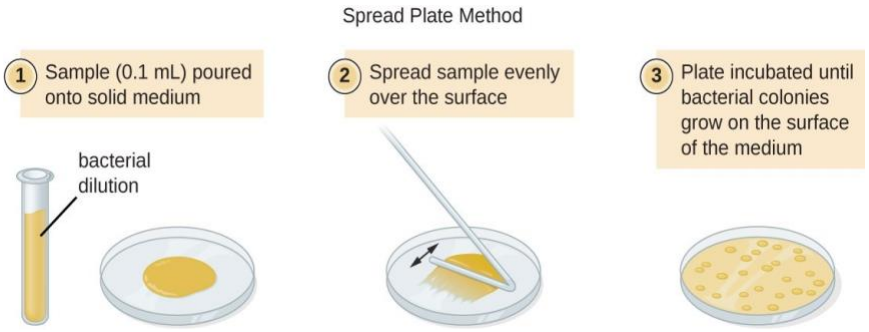

Figure 8. Spread plate technique

Due to there being no growth seen on the full-strength plates, it was decided that the plates no longer needed to be acidified. Regular TSA plates with a neutral pH 7 were made. The plates were then streaked and spread with sample #2 and then placed into an incubator to grow. The colonies that grew on these regular TSA plates were scraped off the plates with a sterilized loop tool and placed onto square grid plates for storage.

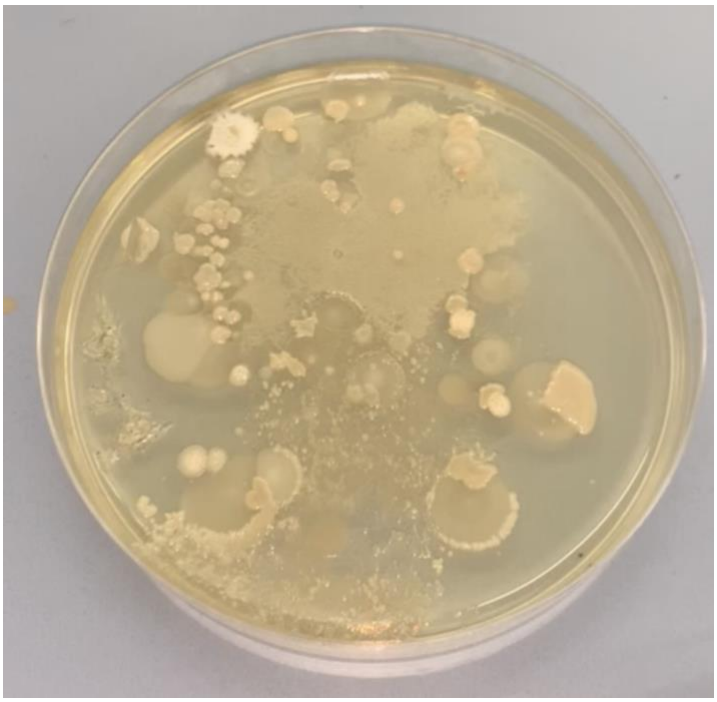

Figure 9. Regular TSA plate with the colonies that grew on it after incubation.

2.4 Antimicrobial Activity Assay Preparation

Tryptic soy broth (TSB) was made to begin the antimicrobial activity assay preparation. The isolates from the grid plates were sterilely transferred to 5 mL of TSB in stumpy tubes. The stumpy tubes were incubated on an angle at 37^{o}C and were shaken at 50 rpm overnight to gather nutrients. 1 mL of each culture was then transferred to sterile microcentrifuge tubes. The tubes were then frozen at -20^{o}C until the assay.

2.5 Antimicrobial Activity Assay

To begin the antimicrobial activity assay, the isolates were thawed and centrifuged at 14,000 x g for 3 minutes. Grid plates were gathered, and wells were poked into each square. A swab was used to spread *E. coli* and *S. aureus* thinly on different grid plates with wells. The first well of each plate was filled with 15 μL of TSB, which was used as the negative control. The second well of each plate was filled with 15 μL of Doxycycline, which was used as a positive control. 15 μL of supernatant from each isolate was added to the wells. The plates were then incubated right side up overnight at 37^{o}C. The plates were then examined for evidence of growth inhibition.

Results

3.1 *E. coli* and *S. aureus* Plates

The results of the antimicrobial activity assay for the *E. coli* plate can be seen below in **Figure 10**. The first well of the plate in the top left-hand corner was the negative control. There was growth seen here, which was expected. The second well was filled with Doxycycline, which was used as the positive control. There was no growth here, which was also expected. None of the supernatants inhibited the growth of *E. coli.*

The results of the antimicrobial activity assay for the *S. aureus* plate can be seen below in **Figure 11**. The first well of the plate in the top left-hand corner was the negative control. There was growth seen here, which was expected. The second well was filled with Doxycycline, which was used as the positive control. There was no growth here, which was also expected. None of the supernatants inhibited the growth of *S. aureus.*

Figure 10. Results of the *E. coli* antimicrobial activity assay taken by Hannah Bussell on September 18th, 2020.

Figure 11. Results of the *S. aureus* antimicrobial activity assay taken by Hannah Bussell on September 18th, 2020.

Discussion

4.1 Antimicrobial Activity

The results indicate that there was no antimicrobial activity. Antimicrobial activity can be classified as inhibiting the growth of bacteria, preventing the formation of microbial colonies, and destroying microorganisms. No supernatant in wells 3-36 showcased any inhibition of *E. coli* or *S. aureus* growth. It is known that *E. coli* and *S. aureus* can be inhibited because in this experiment, Doxycycline was used to inhibit both bacteria. Prior research of acid mine drainage and other toxic environments suggests that antibiotic resistance is present even in highly acidic conditions. Several studies show that there is a close relationship between antibiotic-resistant phenotypes and resistance to other toxic compounds, like heavy metals, that require co-resistance or cross-resistance mechanisms. There is also evidence of antibiotic resistant *E. coli* in AMD sites contaminated with heavy metals. Antibiotic resistance in toxic environments is thought to occur because there are higher relative metabolic potentials of genes in AMD due to elevated microbial competition (Kuang et al. 2016). A likely reason for no inhibition in the results for the *E. coli* plate would be that antibiotic resistant strains of *E. coli* were already present in the samples collected. This could also be possible for the *S. aureus* plate.

4.2 Media and Acidophiles

During this experiment, it was a challenge to find media that would grow the samples. When trying to acidify the media with 0.1 potassium hydrogen phthalate and 0.5 M HCl, it was found that the cultures did not grow on the pH 3.5 media. The cultures did grow, though, on regular TSA media at a pH of 7.3. With acidophiles being known to thrive in acidic conditions, this was an anomaly. In order for this experiment to work, solid and liquid media that can grow

acidophiles needs to be made. A solid and liquid media for isolating and cultivating acidophilic and acid-tolerant sulfate-reducing bacteria has been discovered and could be used for continuation of this experiment. The solid media consists of glycerol (as an electron donor), 2% agarose at pH 7, and 1 M $FeSO_4$ (as the sink for hydrogen sulphide) (Ñancucheo et al. 2016). The liquid media was made with glycerol, 0.01% yeast extract, and 1 M $FeSO_4$. Both the liquid and solid media had pH's of 3.4-4.0. The solid and liquid media were successful in growing and cultivating acidophiles.

Conclusion

Antibiotic resistance in bacteria is an escalating problem. Little is known about the potential of microorganisms in sites affected acid mine drainage (AMD) to be sources of new antibiotics. The samples from Letcher County, Kentucky, were ran through an antimicrobial activity assay, where they displayed no antimicrobial activity. The reason for this could be due to bacteria death, incorrect pH of the media, or due to antibiotic resistance already present in the acid mine drainage community. In order for to continue the search for antibiotic-producing microorganisms in sites affected by acid mine drainage, a media to support and grow acidophiles would need to be made.

References

Akcil A, Koldas S. 2006. Acid Mine Drainage (AMD): Causes, treatment and case studies. J Clean Product. 14(1): 1139-1145.

Andersson D, Balaban N, Baquero F, Courvalin P, Glaser P, Gophna U, Kishony R, Molin S, Tønjum T. 2020. Antibiotic resistance: Turning evolutionary principles into clinical reality. FEMS Microbio Rev. 44: 171-188.

Austin C, Dopson M. 2007. Life in acid: pH homeostasis in acidophiles. Trends Microbio. 15(4):165-171.

Bhuyan A, Yadav R, Samanta R. 2017. Isolation and characterization of antibiotic producing bacteria from soil samples of Dibrugarh District, Assam. Internat J Pharma Chem & Biolo Sci. 7(3):193-201.

Chen L, Hu M, Huang L, Hua Z, Kuang J, Li S, Shu W. 2015. Comparative metagenomic and metatranscriptomic analyses of microbial communities in acid mine drainage. ISME J. 9(7):1579-1592.

Florence A, Vremont D, Farasin J, Freel K, Chiboub O, Fouteai S, Barbe V. 2018. Adaptation in toxic environments: Comparative genomics of loci carrying antibiotic resistance genes derived from acid mine drainage waters. Enviro Sci Poll Rsrch internat. 25(2):1470-1483.

Gray N. 1997. Enviromental impact and remediation of acid mine drainage: A management problem. Envrio Geo. 30(1): 62-71.

Guo J, Wang Q, Wang X, Wang F, Yao J, Zhu H. 2015. Horizontal gene transfer in an acid mine drainage microbial community. BCM Genom. 16(1):1-11.

Hallberg K. 2010. New perspectives in acid mine drainage microbiology. Hydromet. 10(3-4): 448-453.

Jennings S, Neuman D, Blicker P. 2008. Acid Mine Drainage and Effects on Fish Health and Ecology: A Review. U.S. Fish Wild Serv. 1-26.

Kuang J, Huang L, He Z, Chen L, Hua Z, Jia P, Li S, Liu J, Zhou J, Shu W. 2016. Predicting taxonomic and functional structure of microbial communities in acid mine drainage. ISME Jour. 10(1): 1527-1539.

LibreTexts. 2020. Industrial Production of Antibiotics. https://bio.libretexts.org/Bookshelves/Microbiology/Book%3A_Microbiology_(Boundless)/17%3A_Industrial_Microbiology/17.2%3A_Microbial_Products_in_the_Health_Industry/17.2A%3A_Industrial_Production_of_Antibiotics.

Martínez J. 2008. Antibiotics and Antibiotic Resistance Genes in Natural Environments. Sci Mag. 321: 365-367.

Ñancucheo I, Rowe O, Hedrich S, Johnson D. 2016. Solid and liquid media for isolating and cultivating acidophilic and acid-tolerant sulfate-reducing bacteria. FEMS Microbio Let. 363(10): 1-5.

Ogwara H. 2019. Comparison of Antibiotic resistance Mechanisms in Antibiotic-Producing and Pathogenic Bacteria. Molecules. 24(19):3430.

Ploetze F, Chiboub O, Lièvremont D, Farasin J, Freel K, Fouteau S, Barbe V. 2018. Adaption in toxic environments: Comparative genomics of loci carrying antibiotic resistance genes derived from acid mine drainage waters. Enviro Sci Pullu Resear. 25(1):1470-1483.

Ray T. 2016. What happened on Pine Creek?. App Voic. https://appvoices.org/2016/04/12/what-happened-on-pine-creek/.

Richlen M. Microbial Life in Acidic Environments. Microb Life. https://serc.carleton.edu/microb

elife/extreme/acidic/index.html.

Rodrigues V, Henao D, Oliveira V, Ottoboni L. 2018. Functional diversity and biotechnological potential of heterotrophic bacteria isolated from Brazilian copper mine envrionments. Oxid Comm. 41(3):440-461.

Rokem J, Lantz A, Nielsen J. 2007. Systems biology of antibiotic production by microorganism. Nat Prod Rep. 24(1): 1262-1287.

Sun W, Xiao E, Krumins V, Dong Y, Xiao T, Ning Z, Chen H, Xiao Q. 2016. Characterization of the microbial community composition and the distribution of Fe-metabolizing bacteria in a creek contaminated by acid mine drainage. Appl Microbiol & Biotech. 100(19):8523-8535.

Teng W, Kuang J, Luo Z, Shu W. 2017. Microbial Diversity and Community Assembly across Environmental Gradients in Acid Mine Drainage. Biogeochem AMD. 7(6): 1-10.

Wellington E, Boxall A, Cross P, Fell E, Gaze W, Hawkey P, Rollings A, Jones D, Lee N, Otten W, Thomas C, Williams A. 2013. The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. Lancet Infect Dis. 13: 155-165.

Woappi Y, Gabani P, Singh A, Singh O. 2016. Antibiotrophs: The complexity of antibiotic-subsisting and antibiotic-resistant microorganisms. Crit Rev Microbiol. 42(1):17-30.