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Bacterial Diversity at an Abandoned Coal Mine in Southeast Kansas

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BACTERIAL DIVERSITY AT AN ABANDONED COAL MINE IN SOUTHEAST KANSAS

A Thesis Submitted to the Graduate School in Partial Fulfillment of the Requirements for the Degree of Master of Science

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An Abstract of the Thesis by
Rachel Bechtold

Acid mine drainage (AMD) is found in areas of abandoned coal mines in southeast Kansas as a result of mine waste rocks and tailings, and can create problems for the local environment. Soil bacterial populations may act as a reliable indicator of ecosystem health in these human-perturbed areas. The goals of the present study were to assess the bacterial diversity of an acid mine drainage site over a two-year period and to isolate acid-tolerant bacterial species for bioremediation purpose.

In fall (2015) and summer (2016), soil samples were aseptically collected from five locations representing diverse topography at an acid mine drainage site in southeast Kansas. Soil texture was evaluated and samples were chemically digested for physicochemical analysis using inductively-coupled plasma optical emission spectroscopy. Concentration of bacterial isolates was determined by counting CFUs after dilution plating on tryptic soy agar. Up to thirty morphologically different colonies from each annual sampling were characterized using physiological and biochemical tests and were further identified at the species level using 16S rRNA gene sequencing. In addition, acidophilic bacterial strains were screened using selective differential media.

Preliminary data showed that soil pH ranged from 2.5-6.8 and contained varied concentrations of arsenic, manganese, and iron. Total bacterial concentration was 10^2-10^8 CFU/g of soil over two samplings. Biochemical tests revealed a diverse metabolic potential of the bacterial population. Bacterial isolates for both fall and summer samples

were grown in citric acid buffer with varying pH of 3, 4, 5, and 6 and several were found to be acidophilic. PCR amplification of 16S rRNA gene and purification were completed before sending samples off for sequencing to Kansas State University; a phylogenetic analysis was completed for both sampling times and subsequently illustrated with neighbor-joining phylogenetic trees constructed using MEGA 7 software. Baseline measurements of bacterial diversity as well as soil chemistry in acid mine drainage sites in this region, are novel and the findings may have potential use in bioremediation of contaminated acid mine drainage sites.

Keywords: acid mine drainage, bacterial diversity, bioremediation

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CHAPTER I

INTRODUCTION

In southeast Kansas coal mining began in the early 1900s and was an active industry for more than half of a century (Johnson & Hallberg, 2003). The focus for Crawford County and some areas of Cherokee County in southeast Kansas had been the mining of coal, while further south, in Oklahoma, mining of lead, zinc, and iron was more prevalent (Johnson & Hallberg, 2003). Historically, few regulations existed for removal of coal resources and restoration of sites. It wasn't until 1979, when the state of Kansas filed Chapter 49-428, that the coal mining industry was legally required to restore land to its natural state at these mined sites.

Acid mine drainage (AMD) is a common waste product found in abandoned mine lands; this waste can pose a serious problem for the surrounding environment. Acid mine drainage is created when pyrite, a common coal by-product, is exposed to oxygen and to water. Pyrite –also referred to as iron disulfide- oxidizes to become sulfate and ferrous iron (Johnson & Halberg, 2005) through a series of redox reactions. Depending on the pH of the environment, ferrous iron may become the ferric iron, this form of iron leaches into the surrounding soil and water and is known as "yellowboy". Yellowboy gets its name from the color of iron that will precipitate and coat stream beds when the pH of AMD sites fluctuates near 3.5. The transformation of iron and sulfur can lead to

environmental damage toxicity of water, and becomes a hindrance to prokaryotic and eukaryotic life (Kolmert & Johnson, 2001). As this oxidation reaction occurs, the pH drops lower and escalating chemical reactions cause hydrogen ions and sulfuric acid to be created; this is what is known as the final product of AMD.

In order to restore these abandoned sites to their original state, various remediation techniques have been applied. Abiotic or biotic remediation may be used in mined sites where it is economically feasible; however, no perfect solution has been found. One possible remediation technique involves the use of sulfate- and iron-reducing acidophilic bacteria in AMD soil.

The pH levels of the AMD can vary from 0.77 -in extreme cases- to a more commonly found pH of 3 (Johnson & Halberg, 2003). Bacteria can survive in these extreme pH levels and acidic elemental conditions; they can also reverse the process of oxidation through reduction reactions. Because bacteria are naturally found in areas of AMD they are being applied in large numbers to clean up areas through bioremediation.

In successful cases of bacterial bioremediation, sulfate-reducing bacteria (SRB) have been shown to increase neutralization efforts when used with passive abiotic techniques like calcium carbonate limestone (Johnson & Halberg, 2005). Biotic factors can also be applied with bacterial bioremediation, for example, a wetland could be placed at a lower elevation than a mined land with current AMD. A biotic system would be drainage flowing down to the wetland, filtering through the reeds and systematically neutralizing the water with aid from acidophilic bacteria (Kalin & Wheeler, 2006; Sheoran & Sheoran, 2006) would be a completely biotic system.

Many acidophilic bacteria are present in AMD environments and are resilient to the low pH levels because of their protective cellular enzymes (Sharma et al, 2016). These bacteria may catalyze the iron that precipitates in low pH and reverse the oxidation process to form ferric iron (Branter & Senko, 2014), a more soluble form in these environments. As these chemical reactions reach equilibrium, the reduction reaction of the bacteria reverses the acidity and elemental precipitate and can bioremediate a mined site naturally.

Many sites in southeast Kansas could benefit by incorporating both bacterial remediation techniques in combination with abiotic methods. One such area is the Monahan Outdoor Education Center (MOEC, a part of Southeast Kansas Biological Station), a roughly 156 acre property located near Cherokee, KS and owned by Pittsburg State University. Monahan has been partially remediated but on the northern border of the property, a cap that was meant to contain the AMD has failed and a blowout has occurred, leaching acidic water into the surrounding soils and water. To remedy this problem, a wetland would be placed at a lower elevation than the land with current AMD damage, with any drainage flowing down to the wetland, filtering through the reeds and systematically neutralizing the water with aid from acidophilic bacteria (Kalin & Wheeler, 2006; Sheoran & Sheoran, 2006). As water flows through the reeds, large amounts of waste are assimilated in one of three ways: (a) nutrient uptake by plants, (b) bacterial degradation and decontamination, or (c) sedimentation (Sheoran & Sheoran, 2006).

Years of strip mining the coal seams in areas of Crawford and part of Cherokee counties in southeast Kansas is the single procedure that has most detrimentally affected

land by creating acid mine drainage (AMD); despite state legislation, many damaged sites still remain. MOEC has offered an opportunity to use established physicochemical analysis to further research remediated and un-remediated areas in an abandoned coal mine. This study is novel in trying to isolate acidophilic or sulfate-reducing bacterial species that could be used in bioremediation at MOEC.

CHAPTER II

MATERIALS AND METHODS

Sampling Procedures

Field Sampling:

Materials for this research were supplied by the PSU Department of Biology with collaboration from the Department of Geology, Kansas State University.

Sterilized equipment was taken to five pre-identified sites at the Monahan Outdoor Education Center (MOEC) to collect soil samples at a depth of five cm. These five geographically unique locations were chosen based on elevation, sunlight exposure, and proximity to water. The field sampling sites included the following areas of MOEC: grassland with mining fines (A), highest elevation/mound region (B), partial shade area of the artificially made wetland (C), failed remediated blowout (D), and the area of the wetland fully exposed to sunlight (E) (Figure 1).

Figure 1. Monahan, sampling site map

Soil samples were collected once in October of 2015 for initial base measurements of bacterial diversity and sampling was repeated in June of 2016 for follow-up measurement at previously visited sites. Air temperature readings were collected in the field for both dates; 16℃ in October of 2015 and 37℃ in June of 2016. Maximum temperatures were taken from records found at NOAA Data Tools, Daily Weather Records (National Oceanic and Atmospheric Administration: Data Records, 2016).

Two sets of soil samples were aseptically gathered using sterile equipment: a soil corer scoop, 50 ml Falcon tubes, 70% ethanol, sterile latex gloves, recording materials,

and a temperature probe. The five sites were given corresponding letter codes (A-E) and sampled twice per visit for a total of 10 site samples per season. Of the two sample sets per site, one was processed immediately for culturable bacterial analysis and later processed for elemental analysis. Additionally, soil pH and temperature were measured on site. Another sample set was preserved in -80°C for culture-independent metagenomics analysis in the future.

While at Monahan, the soil samples were collected at approximately five cm depth- it may be presumed that the soil was taken from the A horizon or A and O horizons in areas that were remediated (B, C, E) and from mining waste in unremediated sites (A and D). During sampling, a 50 ml sterile tube and its duplicate were filled 30-40 ml (~50 g) with each corresponding soil by using a sterilized soil scoop at a depth of five cm. Ethanol was used in between sites to clean the equipment.

Laboratory Techniques:

In the laboratory, characterization of soil bacteria was accomplished by analyzing morphology and physiology, observing growth on selective differential media, and reading biochemical test analysis. Media ingredients were procured from Difco media unless mentioned otherwise. Molecular characterization was also carried out using 16S rRNA gene amplification with universal primers and phylogenetic analysis.

Physicochemical Analysis: Bulk sediment extractions were performed in the Soil Chemistry Lab in the Department of Agronomy, Kansas State University. The method was an adaption of Premarathna et al. (2010), following a standard aqua regia digest with pre-treatment of 30% H_2O_2. Sediment samples were finely homogenized to <2 mm and weighed out to ≒0.5 g and placed in glass digestion tubes. Next, 2.5ml 30% $H_2O_2$was

added to each tube and set to equilibrate for ten minutes, followed by the further addition of 0.5 ml, and then equilibration for twelve hours. The next morning the samples were heated to 90℃ and digested until the volume was reduced to approximately 1ml. 2.5 ml aqua regia (1:3 HNO3:HCl; prepared just before adding) was then added to each tube and let to equilibrate for 12 hours. Samples were then heated in the following manner: 75℃ for thirty minutes, 90℃ for thirty minutes, 110℃ for thirty minutes, and then to 140℃ until the total volume was reduced to ≒1 ml. Tubes were vortex mixed at five speed for twenty seconds every fifteen minutes and the temperature of the block was monitored throughout the process. Once all samples had been reduced to <1 ml, they were diluted to 10ml with 0.1% HNO3, filtered through Whatman 42 filter paper and analyzed via a Varian 720-ES Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) (for Fe and Mn) and a Varian GTA 120 Graphite Tube Atomizer w/ AA 240Z Zeeman Atomic Absorption Spectrometer (GTA-AAS) (for As). Three NIST standards (Montana II) were digested and analyzed to ensure proper digestion, with yields of 101% (As), 84% (Fe) and 88% (Mn) obtained.

Dilution Plating: Samples were dilution plated on Difco (Sparks, MD) Bacto Tryptic Soy Agar (TSA) and incubated at 28°C in an Isotemp incubator for 48-72 hours. The concentration was calculated as colony forming units CFU/g of soil. Following dilution plating, morphologically different bacterial isolates were selected and streaked on TSA.

A total of 30 isolates were selected from the fall 2015 sampling set and 30 isolates were selected from the spring 2016 isolates. However, further analysis included 58

morphologically different viable isolates and were characterized following phenotypic and biochemical analyses.

Phenotypic Analyses: Colony morphology was noted and slides were prepared and viewed for motility, cell shape, and size. The slides were then prepared and viewed with Gram, acid fast, and Anthony's capsular stain. Spore staining was also performed for a subset of isolates using Malachite green stain. In Gram staining, glass slides were used for heat-fixing bacterial samples and flooded with Gram's crystal violet, iodine, 95% ethanol, and rinsed. Safranin was used as the counterstain. The slides were viewed under oil immersion to determine gram positive or gram negative results. All staining procedures were followed according to Microbiology Laboratory Theory & Application- Brief / Edition 2 (Leboffe & Pierce, 2016).

Biochemical Testing: Multiple biochemical tests were conducted: nitrate reduction, citrate utilization, cysteine desulfurization, phenylalanine deamination, indole production, gelatin hydrolysis, starch hydrolysis, catalase, and oxidase. Bergey's Manual (2000) may be referenced for the premade media used in lab research. Additionally, fermentation of carbohydrates including lactose, glucose, maltose, and sucrose were tested. All biochemical experimental procedures were followed according to Microbiology Laboratory Theory & Application- Brief / Edition 2 (Leboffe & Pierce, 2016).

Selective Differential Media: Eosin methylene blue (EMB), MacConkey (MAC), mannitol salt (MSA), and TSA plates were used for all isolates to determine growth and metabolic activity.

Molecular Characterization: Bacterial isolates were selected from TSA with a sterile toothpick and resuspended in 50 µl of sterile water. These samples were gently

mixed with a MidSci LabDoctor Vortex Mixer and placed in Bio-Rad C 1000 Touch Thermal Cycler to heat denature the genomic DNA at 95°C for ten minutes.

A total of 20 µl PCR reaction mixture included: one µl of bacterial DNA as template, 20 pmole of each primer (0.5 µl) 27F (AGAGTTTGATCCTGGCTCAG), (0.5 µl) 1492R (GGTTACCTTGTTACGACTT) (Lane et al, 1991, Biosynthsis Co.), 10 µl of Promega PCR Master Mix (Madison, WI), and the rest was sterile water.

PCR protocol followed was: denaturation at 95°C for three minutes prior to 29 cycles of the following: denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for one minute. Finally, the last extension step was at 72°C for 10 minutes. The infinite hold was set at 4°C.

Agarose Gel Electrophoresis: Gel media was prepared from one-percent agarose and 1X Tris base, acetic acid and EDTA buffer (TAE)) and ~3.5 µl (recommended: 1 µl per 20 ml gel) iNtRON RedSafe Nucleic Acid Staining Solution (20,000x). The gel was poured into a Fisher Biotech Electrophoresis Mini Horizontal Unit then left to solidify before being placed in 1X TAE buffer with ¼ inch to ½ inch TAE buffer covering the agarose gel.

Loading samples were prepared as follows: In 10 µl, three µl of sterile water, two µl loading buffer (MIDSCI), and five µl of PCR reaction. A Bullseye 100 base pair (bp) DNA ladder (MIDSCI) was pipetted in the first well in the amount of five µl. The samples were run at 80 volts for 75 minutes and the gel was removed from the buffer solution to visualize under ultraviolet light Electrophoresis Systems 312 Transilluminator (Fisher Scientific).

Purification of PCR Product: Rest of the PCR reaction was purified using ZYMO RESEARCH Clean and Concentrator DNA (PCR) Clean following instructions from the manufacturer, (http://www.zymoresearch.com/downloads/dl/file/id/112/d7010i.pdf). As a minor modification 14 µl Elution Buffer was substituted with sterile water.

Quantitation of DNA: Nanodrop Lite (Thermo Scientific) was used to take readings for 58 isolates in order to record 260/280 ratios and ng/µl (Appendix A).

DNA Sequencing: The isolates were identified at the species level after PCR amplification and 16S rRNA gene sequencing using universal eubacterial primers at Kansas State University Sequencing Facility.

Phylogenetic Analysis: Obtained sequences were run on CodonCode Aligner to create fasta files for upload to MOLE MEGABlast on the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/moleblast/moleblast.cgi) in order to create traditional, radial, circular, or cladogram phylogenetic trees (Appendix B). MEGAblast software (version 7) was used for traditional phylogenetic tree and bacterial identification display. A Neighbor-Joining phylogenetic tree was created using aligned sequences in MEGA7 software.

Isolation of Acidophilic Bacteria: Tryptic Soy Broth was mixed with citric acid monohydrate $C_6H_8O_7.H_2O$ 0.1 M (Sigma Chemical Company, St. Louis, MO) and 0.1 M trisodium citrate dihydrate $Na_3C_6H_5O_7.2H2O$ (Fisher Scientific, Fair Lawn, NJ) to prepare media with varying pH levels. Before beginning acid media tests, all isolates were restreaked on TSA to check the viability. Agar media was used with acid buffer but did

not solidify at a pH lower than five; therefore, further the media was prepared in broth with lower pH values.

The TSB as a liquid medium for acid buffer base is in a ratio for 30 g/L of water. The TSB media was divided into equal halves and labeled either Solution A or Solution B. Solution A contained 0.1 M citric acid monohydrate and Solution B contained 0.1 M trisodium citrate dihydrate. Per 250 ml of TSB, 5.25 grams of solution component A and 8.08g of solution component B were weighed and mixed (Appendix C).

Four acid buffer solutions of various pH were made from 1 L (500 ml A, 500 ml B) of acid media in TSB. Each pH media was prepared in separate 500 ml Erlenmeyer flask with a combination of Solution A and B as mentioned below:

A/B Ratio in mL	PH 3	PH 4	PH 5	PH 6
Solution A (mL)	205	147.5	87.5	28.75
Solution B (mL)	45	102.5	162.5	221.25

When pH media was thoroughly mixed, flasks were autoclaved at 121°C, 15 psi for 15 minutes and then placed in a warm water bath at 28°C for 45 minutes. After cooling, the media was pipetted to sterile polystyrene 24-well plate (2 ml capacity per well, Becton Dickinson). Fresh bacterial colonies were suspended in 500 µl 0.9% saline solution for each isolate for an OD at 600nm of ~0.5. Ten µl of suspension inoculated into one ml of acid media in each well and growth was determined after 24-48 hours of incubation using Eppendorf Uvette for absorbency analysis in an Eppendorf biospectrophotometer (Appendix D).

Data Analysis: Linear regression was used in a correlation test to examine relationship between iron, arsenic, magnesium and pH; an alpha level of 0.05 was used.

A one-way ANOVA reviewed the colony-forming unit count between seasonal sampling to further understand the number of total bacteria with an alpha level of 0.05.

CHAPTER III

RESULTS

Physicochemical Analysis of Soil Samples

Soil samples collected at sites designated A-E at the Monahan Outdoor Education Center were chosen for their topography and proximity to light or water (Figure 1). Site D was chosen because damage had occurred to the remediation technique applied and a possible low pH value in the area was suspected due to this "blowout". A soil study completed by the United States Department of Agriculture (USDA) revealed the area sampled as having Coalvale silty clay (Fig. 2, Table 1) or Parson's silt loam.

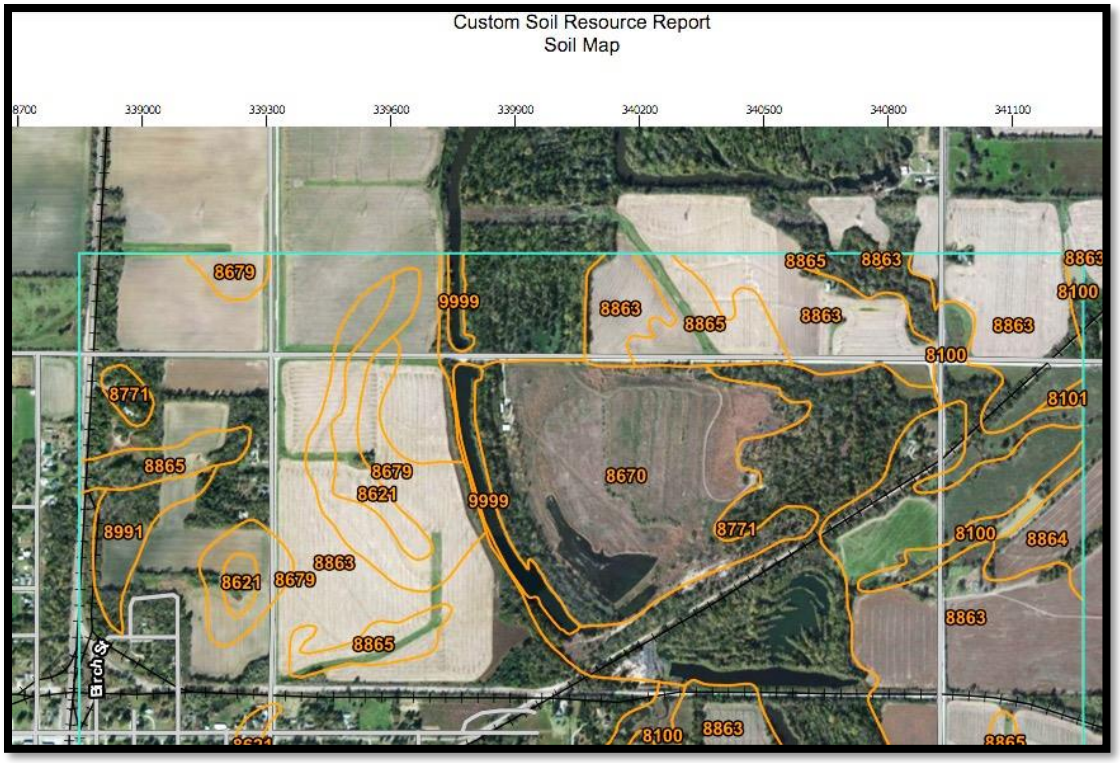


Figure 2. Monahan, USDA map (web soil survey, 2016). The USDA map shows 8670 as Coalvale clay and 8865 as Parson's silt loam in this aerial image.

Physicochemical characteristics of soil taken from the five sites were analyzed with facilities and collaboration through the Department of Geology at Kansas State University. In general, the five sampling sites of Monahan showed little variation in pH readings. Four sites had a pH of 6.6 with the exception of pH at location D which was found to be a pH of 2.6. The arsenic (As), Manganese (Mn), and Iron (Fe)- all soil elements- were measured in either mg/kg or g/kg and recorded.

Table 1. Physicochemical characteristics of soil, Kansas State Geology Department

Location/ Sample ID	Soil Type*	pH	As (mg/kg)	Mn (mg/kg)	Fe (g/kg)
Mine Soil (A)	Coalvale silty clay	6.6±0.0	10.1	614.7	28.7
Top Mound (B)	Coalvale silty clay	6.6±0.2	13.8	877.6	35.5
Wetland S. (C)	Coalvale silty clay	6.6±0.0	15.5	932.7	39
Ditch (D)	Parson's Silt Loam	2.6±0.2	10.4	433.4	169.6
Wetland N. (E)	Coalvale silty clay	6.6±0.0	8.5	978	34.1

*Data taken from United States Department of Agriculture, 2016

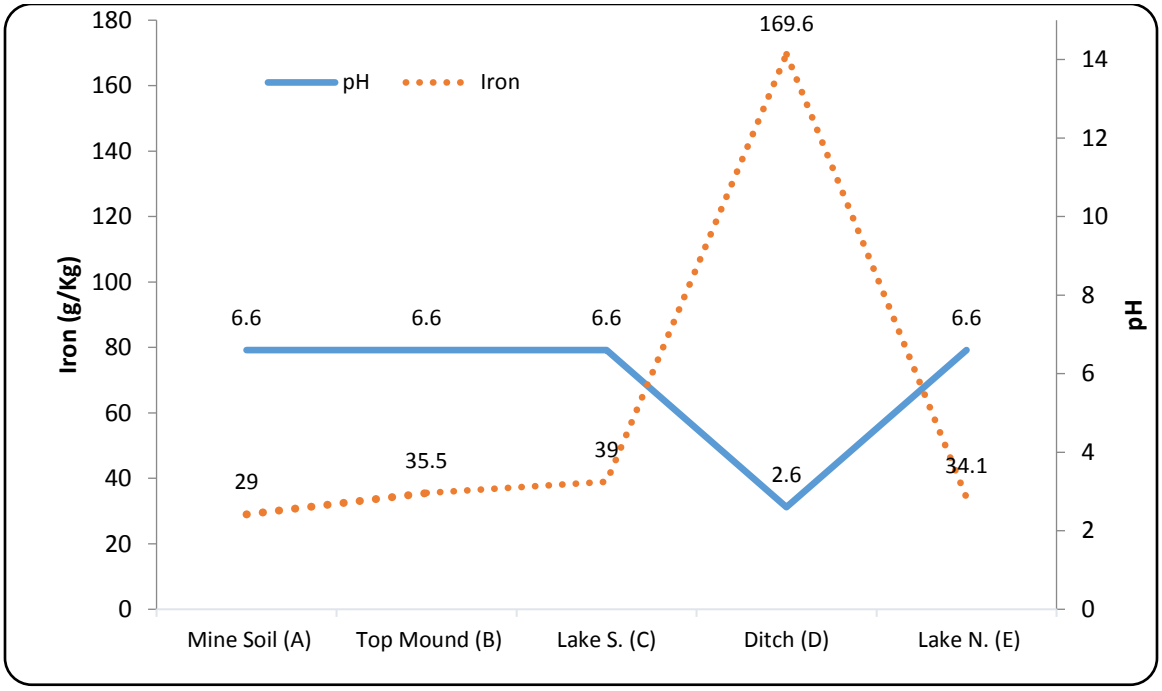


Figure 3. Relationship between acidity and iron, natural log. Site D had both high levels of acidity and high levels of iron (g/kg) indicating the presence of iron precipitate in a flux state.

Soil Bacterial Growth

Bacterial colony counts were completed on TSA media in the lab after sample collection in order to ascertain the level of dilution needed to pick up individual colonies from the

plates. Colony forming units (CFUs) were counted (Fig. 4, Fig. 5) and compared to site and to season. The average number of colonies on dilution plates for fall (45) were slightly higher than for summer (50) but no statistical significance was found between the total CFU.

Soil samples in suspension were spread at dilution rates of 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}. After growth at 28°C for 24-48 hours, single colonies were counted (Figs. 4 and 5). Sites A and E had the highest concentration of bacterial colonies while site D had few bacterial colonies.

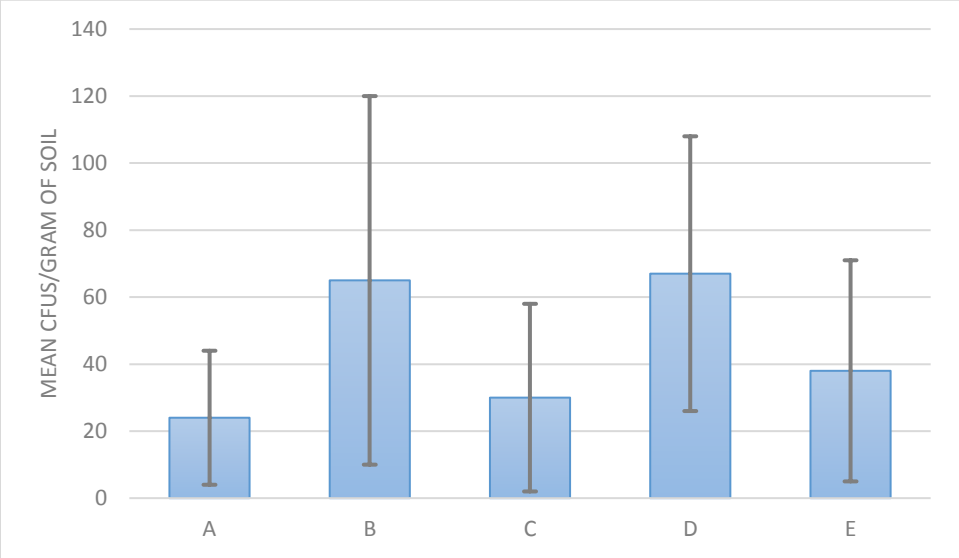


Figure 4. Bacterial concentration (fall 2015 sampling) after dilution plating on TSA. Bar graph with SEM values.

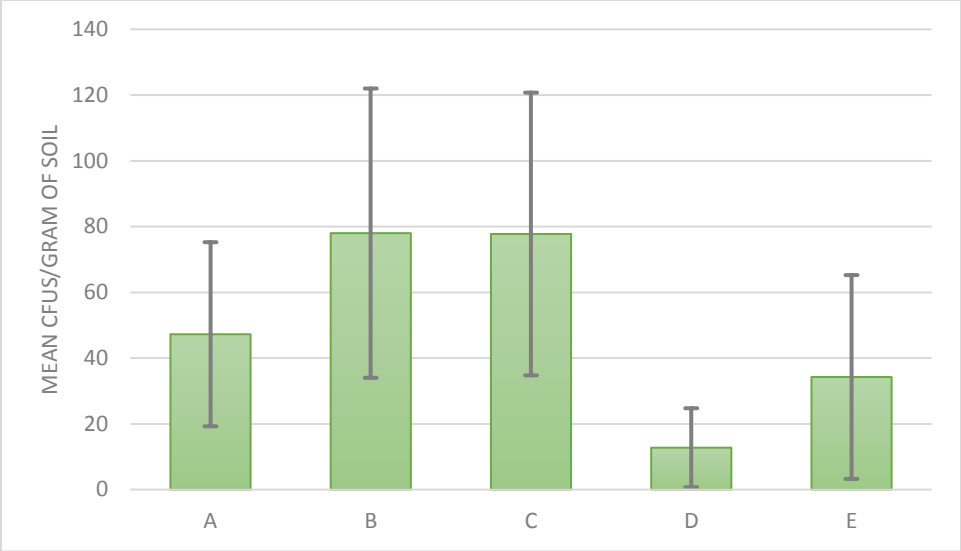


Figure 5. Bacterial concentration (summer 2016 sampling) after dilution plating on TSA. Bar graph with SEM values.

Morphology Confirmation

Following a count of colony forming units, 30 pure and visually unique bacterial colonies were transferred to Tryptic Soy Agar (TSA) resulting in one single bacterial isolate per plate. These bacterial isolates were viewed with a wet mount slide, evaluated for mobility, and categorized by their colony shape, odor, color, and size (Appendix E, F).

Staining and Carbohydrate Fermentation

After staining with Malachite green spore stain three isolates were found to be positive for endospores. Gram staining evaluated shape (rod or coccus), size (thick, thin or short, long), and any unique qualifiers of all the 58 isolates (Tables 2 and 3).

Carbohydrate fermentation tests for lactose, sucrose, glucose and maltose revealed two isolates that could ferment all four sugars and eight that could ferment up to three sugars. The main carbohydrate which was utilized was glucose (41%), closely followed by sucrose (38%) (Fig. 6).

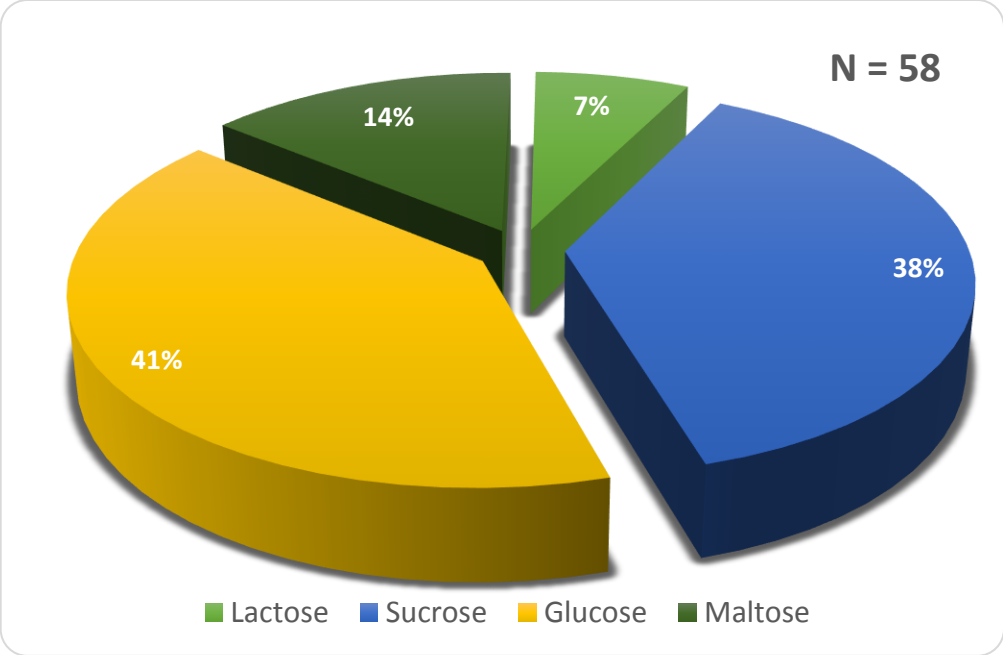


Figure 6. Carbohydrate fermentation by soil isolates.

Biochemical Test Results

Gram staining results were further confirmed by growing the isolates on selective differential agar media: 29 positive on eosin methylene blue (EMB), nine positive on MacConkey (MAC), and 28 positive on mannitol salt agar (MSA). Isolates were subjected to various biochemical tests (Tables 2, 3, 4, 5) to determine their metabolic diversity among sampling sites and among isolates. No clear duplicates were found during biochemical testing and all 58 isolates were preserved. Additionally, SIM stab results showed A1 (*Paenibacillus dendritiformis*) and E7 (*Bacillus subterraneus*) produced the enzyme cysteine desulfurase and C2 (*Pantoea agglomerans*) as indole positive from fall samples. The biochemical tests yielded diverse results for all isolates and thus DNA 16s rRNA sequencing was completed on all surviving samples. Bacteria were sequenced and identified as aerobic Gram positive bacilli although three isolates were cocci: *Micrococcus luteus*, *Rhodococcus jialingiae*, and *Macrococcus caseolyticus*.

Table 2. Gram stain, bacterial cell shape*, and selective differential media results of bacterial isolates from fall 2015.

Isolate	G +/- & Shape	EMB G-	MSA G+	MAC G-
A1	+			
A2	+			
A3	+		X	
A4	+			
A5	-	X		
A6	+			
A7	+	X		
B1	+		X	
B2	+, coccus			
B3	-			
B4	+			
B5	+			
C1	-	X		X
C2	-	X	X	X
C3	-	X		X
C4	+	X	X	
C5	-	X		
C6	-	X		X
C7	-, coccus			
C8	+		X	
D1	-			X
D2	-			
D3	-	X		
E1	-		X	
E2	+			
E3	-		X	
E4	+	X	X	
E5	+			
E6	+		X	
E7	-			

***Shape is typically rod unless noted otherwise**

Table 3. Gram stain, bacterial cell shape*, and selective differential media results of

bacterial isolates from summer 2016.

Isolate	G +/- & Shape	EMB G-	MSA G+	MAC G-
A1	-			
A2	-			
A3	+, coccus		X	
A4	+, coccus		X	
A5	+		X	X
B1	+			
B2	+			X
B3	+			
B4	-			
B5	+		X	
B6	+		X	
B7	-	X		
C1	+		X	
C2	+	X	X	
C3	+	X	X	
C4	+		X	
D1	+	X	X	
D2	+	X	X	
D3	+		X	
D4	+	X	X	
D5	+			
E1	+	X		
E2	+			
E3	+		X	
E4	-			
E5	+		X	X
E6	+			
E7	+			X

***Shape is typically rod unless noted otherwise**

Table 4. Biochemical test results of bacterial isolates from fall 2015

Isolate	Peroxidase	Catalase	Nitrate Reduction	Citrate utilization	Starch hydrolysis	Gelatin hydrolysis	MR	VP	Phenyl Alanine	Litmus	Urea
A1		X			X	X					
A2		X									
A3		X			X	X					
A4						X					
A5											
A6	X	X				X					
A7	X	X				X					
B1		X			X	X					
B2											
B3		X	X								
B4		X		X	X	X					
B5		X	X	X		X					
C1	X	X	X	X							
C2	X	X		X							
C3		X	X	X							
C4	X	X	X	X		X					
C5		X	X			X					
C6		X		X		X					
C7		X				X					
C8	X	X				X					
D1	X	X				X					
D2	X	X	X	X							
D3	X	X				X					X
E1	X	X				X					
E2		X									
E3	X	X	X		X	X					
E4		X			X	X					X
E5	X	X	X	X		X					
E6		X				X					
E7		X	X								

Table 5. Biochemical test results of bacterial isolates from summer 2016

Isolate	*Oxidase*	*Catalase*	*Nitrate Reduction*	*Citrate utilization*	*Starch hydrolysis*	*Gelatin hydrolysis*	*MR*	*VP*	*Phenyl Alanine*	*Litmus*	*Urea*
A1		X	X								X
A2		X			X						
A3		X			X						
A4		X	X								
A5		X							X		
B1									X		
B2		X	X								
B3		X			X						
B4		X	X		X						
B5		X	X	X	X					X alk	
B6	X	X	X	X						X alk	
B7	X	X		X	X					X alk	
C1		X					X				
C2		X			X						
C3		X			X					X alk	
C4		X		X	X		X			X alk	
D1		X					X				
D2		X		X	X			X		X alk	
D3		X		X				X		X alk	
D4		X	X				X	X			
D5		X	X	X	X		X	X		X alk	
E1		X	X	X	X					X alk	
E2		X	X								
E3		X	X								
E4		X					X	X			
E5		X	X				X	X			
E6		X	X								
E7		X	X	X	X					X alk	

Growth on Acid Media

Certain bacterial isolates grew well in the TSB solution with citric acid buffer. Bacterial isolates that grew at pH levels of 3, 4, 5, & 6 are included in Appendix G. A total of 21 unique isolates and 18 acidophilic strains were isolated on selective acid media (Fig. 7) and were identified using DNA sequencing.

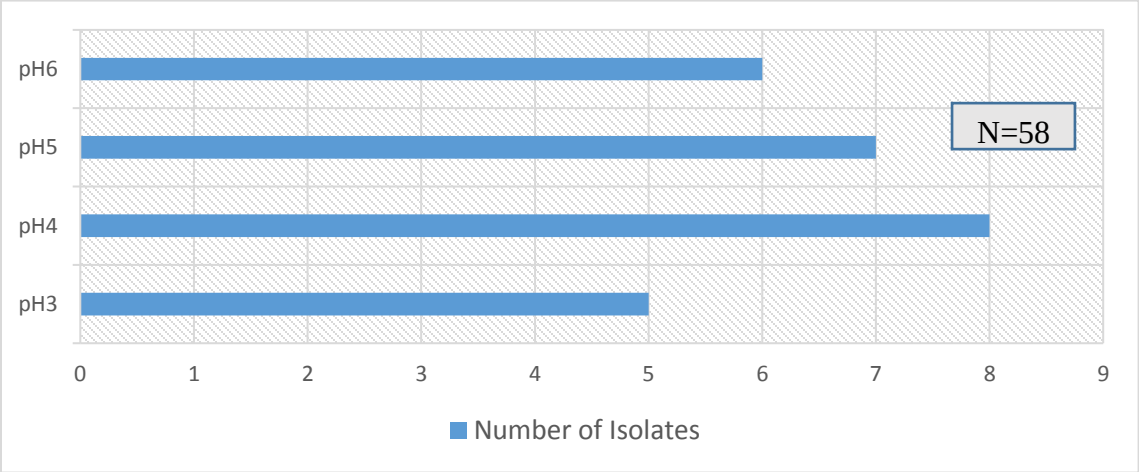


Figure 7. Growth of bacterial isolates in varying pH levels of citric acid buffer media.

Identification of Isolates at the Species Level

The majority of bacterial species belonged to phyla Firmicutes, followed by Actinobacteria and Proteobacteria (Fig. 8). All three phyla were found in the fall 2015 sampling but summer 2016 lacked Proteobacteria isolates.

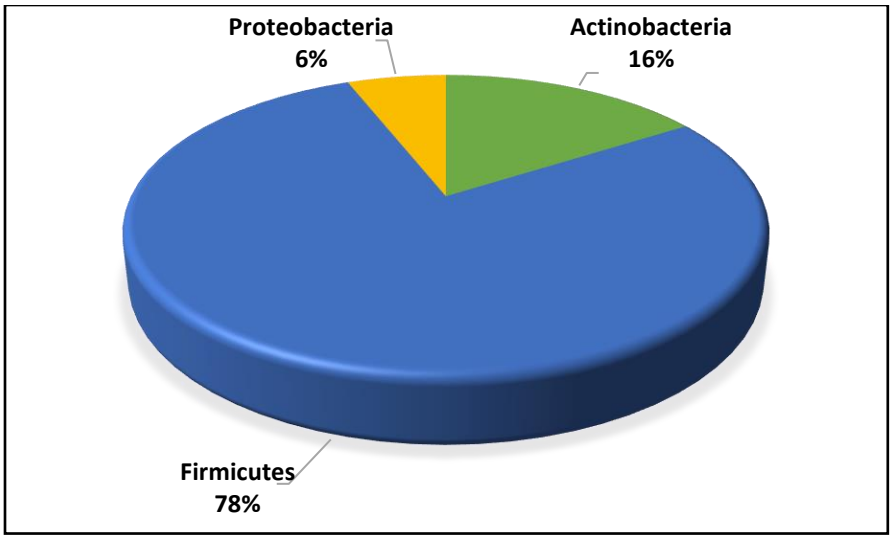


Figure 8. Phyla associated with bacterial species

Table 6. Identification of bacterial isolates at genus and/or species level

Fall	*Genus species*	Summer	*Genus species*
A1	*Paenibacillus dendritiformis*	A1	*Arthrobacter phenanthrenivorans*
A2	*Bacillus indicus*	A2	*X*
A3	*Fictibacillus nanhaiensis*	A3	*Macrococcus caseolyticus*
A4	*Fictibacillus phosphorivorans*	A4	*X*
A5	*Paenibacillus alvei*	A5	*X*
A6	*X*	B1	*Bacillus pumilus*
A7	*Curtobacterium flaccumfaciens*	B2	*X*
B1	*Bacillus megaterium*	B3	*Brevibacterium frigoritolerans*
B2	*Microbacterium oleivorans*	B4	*Paenibacillus amylolyticus*
B3	*Bacillus simplex*	B5	*X*
B4	*Arthrobacter nitroguajacolicus*	B6	*Bacillus thuringiensis*
B5	*Bacillus simplex*	B7	*Bacillus thuringiensis*
C1	*Pseudomonas lini*	C1	*Exiguobacterium profundum*
C2	*Pantoea agglomerans*	C2	*Bacillus idriensis*
C3	*Pseudomonas syringae*	C3	*Fictibacillus phosphorivorans*
C4	*Bacillus simplex*	C4	*Bacillus pumilus*
C5	*Fontibacillus panacisegetis*	D1	*Jeotgalibacillus campisalis*
C6	*X*	D2	*Bacillus pumilus*
C7	*Micrococcus luteus*	D3	*X*
C8	*Psychrobacillus psychrodurans*	D4	*Bacillus toyonensis*
D1	*Bacillus simplex*	D5	*Brevibacterium frigoritolerans*
D2	*Brevibacterium frigoritolerans*	E1	*Bacillus simplex*
D3	*Frigoribacterium endophyticum*	E2	*Psychrobacillus psychrodurans*
E1	*Bacillus indicus*	E3	*Brevibacterium frigoritolerans*
E2	*Bacillus horikoshii*	E4	*Arthrobacter oxydans*
E3	*Fictibacillus phosphorivorans*	E5	*Rhodococcus jialingiae*
E4	*Bacillus megaterium*	E6	*Bacillus simplex*
E5	*Brevibacterium frigoritolerans*	E7	*Terribacillus saccharophilus*
E6	*Bacillus marisflavi*		
E7	*Bacillus subterraneus*		

DNA sequence analysis of 16S rRNA gene revealed a subset of the diverse soil bacterial species from the sampling sites (Table 6). A fragment of up to 800 base pairs of the gene sequence was analyzed and closest matches were identified using MegaBLAST 7

program. Codon Code Aligner was used to check the quality of chromatogram and alignment (Fig. 9). Figures 10 and 11 depict the neighbor joining phylogenetic trees generated using MEGA7 software.

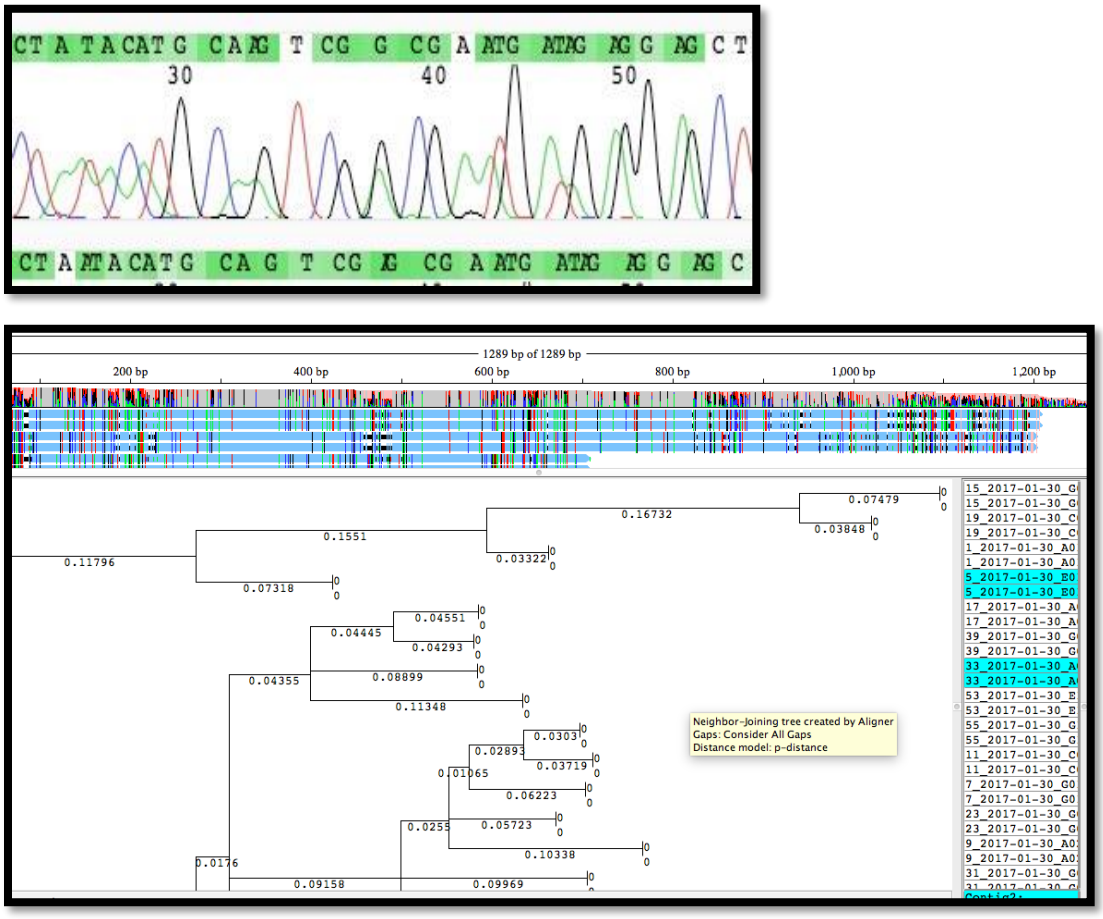


Figure 9. Representative screenshot of chromatogram and alignment using Codon Code Aligner.

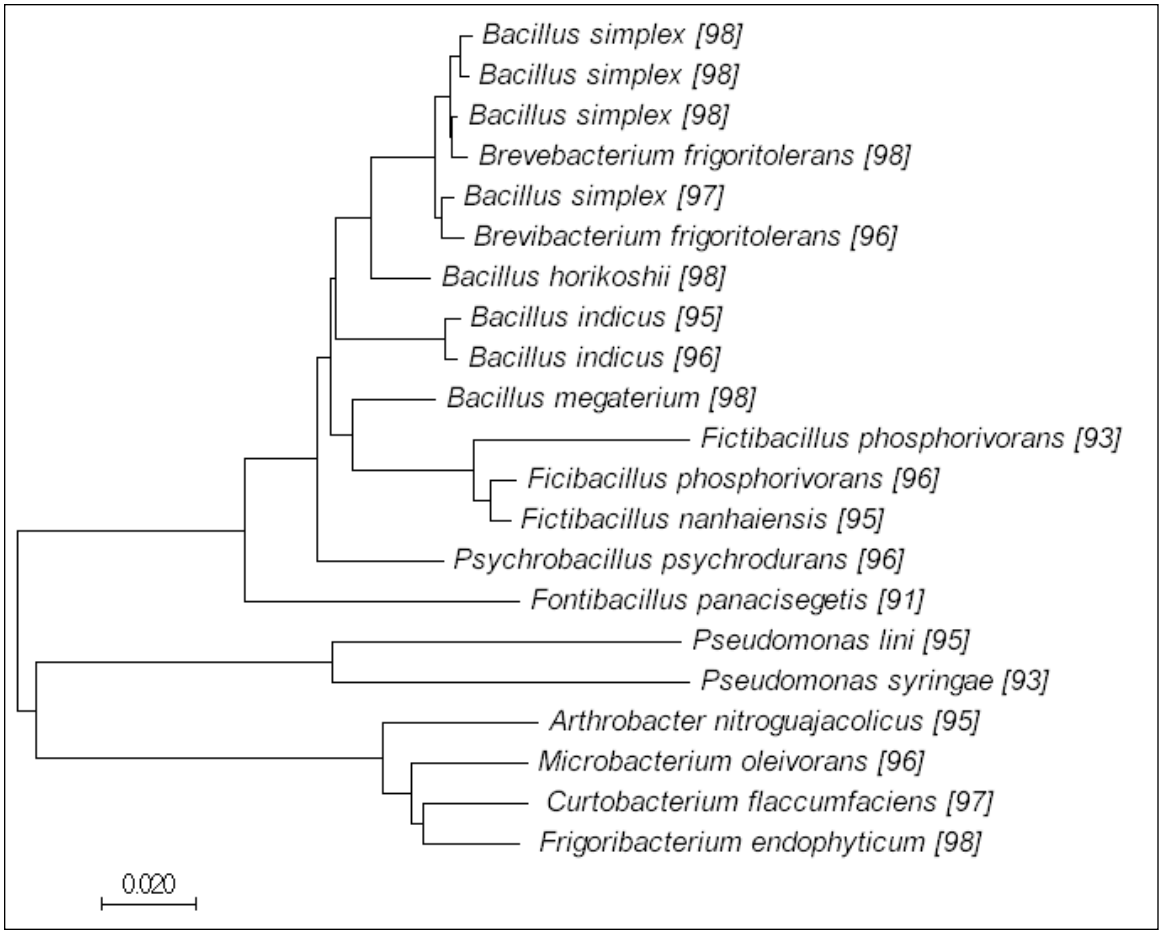


Figure 10. Neighbor-joining phylogenetic tree illustrating the evolutionary relationship among bacterial isolates from fall sampling (n=30). Number in bracket indicates % similarity index. The bar represents two nucleotide base substitution per 100 bases. Nine isolates were not included in analysis due to poor sequencing data.

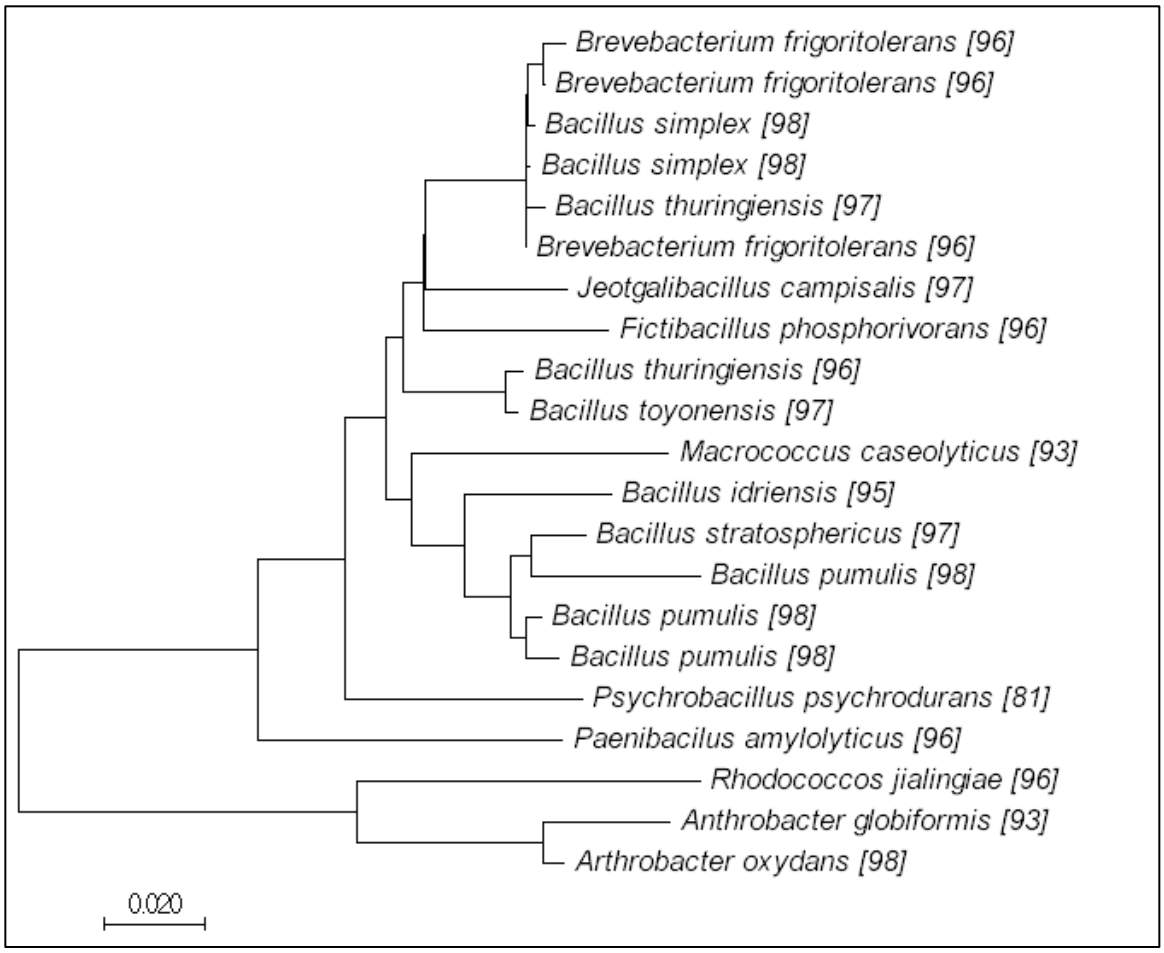


Figure 11. Neighbor-joining phylogenetic tree illustrating the evolutionary relationship among bacterial isolates from summer sampling (n=21). Number in bracket indicates % similarity index. The bar represents 2 nucleotide base substitution per 100 bases. Seven isolates were not included in analysis due to poor sequencing data.

CHAPTER IV

DISCUSSION

Monahan Outdoor Education Center, a part of Southeast Kansas Biological Station, is roughly 156 acres of partially remediated land that was previously strip mined for coal. Acquired by Pittsburg State University, it was used in this study as a representative mined land in southeast Kansas. The site was mined until the 1950's and partially reclaimed in 1984, at this time, man-made soil, limestone, straw, and organic matter were placed on the soil surface (Pittsburg State University, 2016). The site was chosen because of it's accessibility and known low pH levels at the site of a blowout.

Bacteria (biotic) can be applied to contaminated coal mine sites ensuring that bioremediation occurs naturally without abiotic additions. Using inorganic nutrients, bacteria can reverse the oxidation process of AMD through the reduction of inorganic nutrients (redox reactions) and effectively neutralize the acidic components in the environment. Our hypothesis stated that total bacterial diversity would differ in seasonal comparison as well as when remediated versus non-remediated sites were compared. We also assumed that sulfate-reducing (SRB) or iron-reducing bacteria (IRB) would be found in Site D, where the pH level was more acidic due to a failed remediation and blowout.

Fewer colony forming units were found when samples were taken from AMD site D. It is normal for the number of viable bacteria to decrease in areas of high acidity;

rather than finding 30-300 average CFU per dilution plate lower numbers are seen after a wildfire, exposure to heavy metals or mine drainage waste (Schmidt et al., 2004). A count can include soil CFU with other bacterial and fungal colonies (Ogunmwonyi et al., 2008); however, numbers of colonies decreased in acidic environments. In mined land, sometimes numbers aren't consistent but this may be attributed to the media chosen on which to grow CFU, the standard Tryptone Soy Agar media has a count of 30-300 colonies (Zhu, 2008).

Physicochemical Analysis

No unexpected levels of iron, arsenic, or manganese were found at any of the five sites though iron levels were high at Site D; this was expected because of the low pH in the area. Forms of iron may precipitate at a pH around 3.5, unlike most other metals which precipitate at higher pH values (LEO Enviro Sci, 2011). Additionally, iron content in the soils may be inhibited by manganese content (Rahman et al., 2013) as these two elements are constantly in flux with one another. Site D had lower levels of managanese and high levels of iron. While the red tint of iron may be visible with the naked eye in areas of AMD, only certain forms of iron will precipitate in these regions when the pH is above 3.5. This type of iron may produce a substance known as "Yellowboy" or iron precipitate that will turn stream surfaces the representative red of iron (Sun et al., 2016). In this study, Site D had a low pH value and a high iron content, especially when compared to other sampling sites specifically at Monahan. Proteobacteria and ferrous iron oxidizing microbes were found further along the streambed, away from acidic areas, but ferric iron oxidizing bacteria were found closer to the low pH levels. Iron can have an

effect on both arsenic (As) and manganese (Mn) in soil since it inhibits both elements at higher concentration (Rahman et al., 2013) and can be detrimental if in precipitate form.

Five sites were sampled and 60 isolates were initially viable; however, two isolates from summer sampling became non-viable during culture transfers. In order to identify the bacterial isolates, various biochemical tests were performed and bacterial growth was evaluated on selective differential media or with staining procedures. Spore staining was not performed on summer isolates due to limited availability of resources.

The biochemical tests showed high metabolic diversity. The 16S rRNA gene sequencing data grouped the bacterial isolates into the following phyla: *Firmicutes*, *Actinobacteria*, and *Proteobacteria* (Fig. 8). Some of the isolates that were identified were found to be actively used in bioremediation techniques (discussed below). In the acidic site (D) SRB and IRB were not specifically found, this could be due to the initial isolation media (TSA) used in the study.

Acidophilic bacteria, however, were found throughout the sampling sites. Fall isolates had a greater number of acidophiles. There was no representative isolate belonging to phylum *Proteobacteria* in summer 2016 samples.

Bacterial Characteristics

Bacteria were mainly found to be aerobic Gram positive bacilli although three isolates were cocci: *Micrococcus luteus, Rhodococcus jialingiae,* and *Macrococcus caseolyticus*. While in juvenile form, *Arthrobacter oxydans* can be a coccus form as well, becoming a rod as it matures. Of note are several species that were found to be either waste-degrading or acidophilic bacteria. *Paenibacillus* was identified as an acidophile; in addition, a nitrogen-fixer, a psychrophile, and few antibiotic-producers were identified

among the acidophiles. Another isolate identified as *Brevebacterium* has a high tolerance to arsenic and sulfur and is commonly found in AMD.

As industry and science become more committed to the use of biotechnology in remediation, the most efficient bacteria are evaluated for their limitations and advantages. Some are able to reduce hydrocarbons, solvents, and metals and can be applied to contaminated sites but three aspects must be analyzed before full-scale application: biochemistry, bioavailability, and bioactivity of the organisms (Dua, Singh et al, 2002). Of the isolates sampled from MOEC, some were found to be reducing bacteria, some were found to be bioremediative, a few examples follow:

Exiguobacterium profundum

Often grown in lab on nutrient agar, the yellow or orange-pigmented *profundum* reduces nitrate to nitrite. It works somewhat like an antioxidant in that it can find free-radicals in the environment (Arulselvi) and destroy them. *E. profundum* is a producer of Astaxanthin, the yellow pigment associated with the species.

Rhodococcus jialingiae

Already used in bioremediation, this gram-positive bacteria is oil-degrading, a psychrophile, non-motile, aerobic, and non-sporulating (Wang, Zhichun et. al. 2010). Part of the Actinobacteria phyla, it is often found in areas of waste and sludge.

Pantoea agglomerans

P. agglomerans is a gammaproteobacterium, endophytic diazotrophic bacterium that can form the metabolite of tryptophan, indole; and is known for it's ability to create a biofilm, thus protecting plants by inhibiting pathogens and promoting growth.

Brevibacterium frigoritolerans

Known as a phorate degrader, this genus actively seeks out agricultural chemicals (herbicide, fungicide, pesticide) to metabolize and nullify so that the chemical structure is broken down to a less-harmful component. Mammals are less affected after this bioremediation and degradation process and do well in acidophilic environments (Arya and Sharma 2014).

Paenibacillus alvei &Paenibacillus dendritiformis

The genus *Paenibacillus* has been used as a biosurfactant in removing sludge, polycyclic aromatic hydrocarbons (PAH) and other oils from contaminated surfaces (Bezza and Chirwa 2015). This genus has the ability to swarm and to use quorum sensing in some environments (Strain, Hernández et al. 2013). The species *dendritiformis* is particularly able to produce a lipopeptidal biosurfactant. Biosurfactants and biofilms are useful because they contribute to the viability of the bacteria in an area to withstand destruction.

Pseudomonas lini

P. lini is of interest in this study because of its ability to solubilize certain minerals, primarily phosphorous, and because of its tolerance for low-pH systems. The bacteria that have been found to do well in a pH of 3, 4, or 5 also have future potential in application to damaged sites for either neutralization of the environment through chemical reduction reaction or by simply protecting and enhancing plant growth (Ehsan, Ahmed et al. 2016).

Future Application- Bench Scale Trials

Cryodessication, or lyophilization of bacteria is a process in which the cells can be freeze-dried and then topically applied to an unremediated site. Before

cryodessication, a large bacterial mass need to be produced in nutrient rich media. This is followed by dehydration and then is stored for application. This could easily be accomplished with bench-scale trials before attempting to apply the technique in on-site.

After assessing bacterial diversity in our study, a species that may be of interest was found. *P. agglomerans* is a gammaproteobacterium, endophytic diazotrophic bacterium; and is known for it's ability to create a biofilm, thus protecting plants by inhibiting pathogens and promoting growth (Dutkiewicz et al., 2014). This bacteria is already used in bioremediation and the degradation of harmful biocides (Walterson & Stavrinides, 2015) and as an acid-tolerant bacteria, it could be used in AMD cleanup. Current literature suggests that research is particularly interested in this phyla because of the ability to self-sustain (Corsiniet et al., 2016) and because it is a well-known and easy to propagate bacterium. Already there are publications reviewing the efficacy of lyophilized samples that are to be used in bioremediative techniques. The viability for temperature and storage (Torres et al., 2014) are shown to reflect maximum hardiness at -20 °C. Packaging and storage are of considerable interest for bench-scale cryodessication and environmental application for future studies at Monahan. Although no statistical significance was noted in bacterial diversity overall between seasonal samplings, future tests could be run on total metagenomic analysis to evaluate site and season-specific differences and to specifically look for this species of bacteria.

If log growth occurs in the bacteria introduced to the environment, the damaged site would be altered as the bacteria use inorganic materials as metabolites, reversing oxidation and acidification. When the environment reaches more neutral pH levels, the number of acidophilic bacteria are reduced. Nevertheless, this enrichment, dehydration,

and spray delivery method of bacterial mass may be able to degrade environmental pollutants and restore the environmental health.

REFERENCES

Alghamdi, A. G. (2016). *Rehabilitation of waste materials near lead and zinc mining sites in Galena, Kansas.* Kansas State University.

Ali, N., Dashti, N., Salamah, S., Al-Awadhi, H., Sorkhoh, N., & Radwan, S. (2016). Autochthonous bioaugmentation with environmental samples rich in hydrocarbonoclastic bacteria for bench-scale bioremediation of oily seawater and desert soil. *Environmental Science and Pollution Research, 23*(9), 8686-8698.

Arulselvi, I., Sasidharan, P., Raja, R., Karthik, C., & Gurumayum, R. S. (2013). Isolation and characterization of yellow pigment producing Exiguobacterium sps. *Journal of Biochemical Technology, 4*(4), 632-635.

Arya, R., & Sharma, A. K. (2014). Screening, isolation and characterization of Brevibacillus borstelensis for the bioremediation of carbendazim. *J Environ Sci Sustainability, 2*(1), 12-14.

Bergey, D. H., Buchanan, R. E., Gibbons, N. E., & American Society for Microbiology. (2000). *Bergey's manual of determinative bacteriology*. Baltimore: Williams & Wilkins.

Bezza, F. A., & Chirwa, E. M. N. (2015). Biosurfactant from Paenibacillus dendritiformis and its application in assisting polycyclic aromatic hydrocarbon (PAH) and motor oil sludge removal from contaminated soil and sand media. *Process Safety and Environmental Protection, 98*, 354-364.

Brantner, J. S., & Senko, J. M. (2014). Response of soil-associated microbial communities to intrusion of coal mine-derived acid mine drainage. *Environmental science & technology, 48*(15), 8556-8563.

Bundy, J. G., Campbell, C. D., & Paton, G. I. (2001). Comparison of response of six different luminescent bacterial bioassays to bioremediation of five contrasting oils. *Journal of Environmental Monitoring, 3*(4), 404-410.

Chockalingam, E., & Subramanian, S. (2006). Studies on removal of metal ions and sulphate reduction using rice husk and Desulfotomaculum nigrificans with reference to remediation of acid mine drainage. *Chemosphere, 62*(5), 699-708.

Corsini, G., Valdés, N., Pradel, P., Tello, M., Cottet, L., Muiño, L., . . . Gonzalez, A. R. (2016). Draft genome sequence of a copper-resistant marine bacterium, Pantoea agglomerans strain LMAE-2, a bacterial strain with potential use in bioremediation. *Genome Announcements, 4*(3), e00525-00516.

Costa, M., & Duarte, J. (2005). Bioremediation of acid mine drainage using acidic soil and organic wastes for promoting sulphate-reducing bacteria activity on a column reactor. *Water, Air, & Soil Pollution, 165*(1), 325-345.

Dutkiewicz, J., Mackiewicz, B., Lemieszek, M. K., Golec, M., & Milanowski, J. (2014). Pantoea agglomerans: a marvelous bacterium of evil and good. Part I. Deleterious effects: Dust-borne endotoxins and allergens-focus on cotton dust. *Annals of agricultural and environmental medicine: AAEM, 22*(4), 576-588.

Dutkiewicz, J., Mackiewicz, B., Lemieszek, M. K., Golec, M., & Milanowski, J. (2016). Pantoea agglomerans: a mysterious bacterium of evil and good. Part IV. Beneficial effects. *Annals of agricultural and environmental medicine: AAEM, 23*(2), 206-222.

Ehsan, M., Ahmed, I., Hayat, R., Iqbal, M., Bibi, N., & Khalid, N. (2016). Molecular Identification and Characterization of Phosphate Solubilizing Pseudomonas sp. Isolated from Rhizosphere of Mash Bean (Vigna Mungo L.) for Growth Promotion in Wheat. *Journal of Agricultural Science and Technology, 18*(3), 775-788.

Erbilgin, O., Bowen, B. P., Kosina, S. M., Jenkins, S., Lau, R. K., & Northen, T. R. (2017). Dynamic substrate preferences predict metabolic properties of a simple microbial consortium. *BMC bioinformatics, 18*(1), 57.

Glaeser, S. P., Dott, W., Busse, H.-J., & Kämpfer, P. (2013). Fictibacillus phosphorivorans gen. nov., sp. nov. and proposal to reclassify Bacillus arsenicus, Bacillus barbaricus, Bacillus macauensis, Bacillus nanhaiensis, Bacillus rigui, Bacillus solisalsi and Bacillus gelatini in the genus Fictibacillus. *International journal of systematic and evolutionary microbiology, 63*(8), 2934-2944.

Gudiña, E. J., Fernandes, E. C., Rodrigues, A. I., Teixeira, J. A., & Rodrigues, L. R. (2015). Biosurfactant production by Bacillus subtilis using corn steep liquor as culture medium.

Heckly, R. J. (1961). Preservation of bacteria by lyophilization. *Advances in applied microbiology, 3*, 1-76.

Hu, Z., Ma, B., Zhang, M., & Wang, P. (2009). Bioremediation of sulfuric contamination from coal waste by sulfate-reducing bacteria (SRB). *Journal of China Coal Society, 34*(3), 400-404.

Jing, Q., Zhang, M., Liu, X., Li, Y., Wang, Z., & Wen, J. (2017). Bench-scale microbial remediation of the model acid mine drainage: Effects of nutrients and microbes on the source bioremediation. *International Biodeterioration & Biodegradation*.

Johnson, A. W., Gutiérrez, M., Gouzie, D., & McAliley, L. R. (2016). State of remediation and metal toxicity in the Tri-State Mining District, USA. *Chemosphere, 144*, 1132-1141.

Johnson, D. B., & Hallberg, K. B. (2003). The microbiology of acidic mine waters. *Research in microbiology, 154*(7), 466-473.

Johnson, D. B., & Hallberg, K. B. (2005). Acid mine drainage remediation options: a review. *Science of the Total Environment, 338*(1), 3-14.

Kalin, M., Fyson, A., & Wheeler, W. N. (2006). The chemistry of conventional and alternative treatment systems for the neutralization of acid mine drainage. *Science of the Total Environment, 366*(2), 395-408.

Kiran, M. G., Pakshirajan, K., & Das, G. (2017). Heavy metal removal from multicomponent system by sulfate reducing bacteria: Mechanism and cell surface characterization. *Journal of Hazardous Materials, 324*, 62-70.

Kishimoto, N., & Tano, T. (1987). Acidophilic heterotrophic bacteria isolated from acidic mine drainage, sewage, and soils. *The Journal of General and Applied Microbiology, 33*(1), 11-25.

Kolmert, Å., & Johnson, D. B. (2001). Remediation of acidic waste waters using immobilised, acidophilic sulfate-reducing bacteria. *Journal of Chemical Technology and Biotechnology, 76*(8), 836-843.

Le Pape, P., Battaglia-Brunet, F., Parmentier, M., Joulian, C., Gassaud, C., Fernandez-Rojo, L., . . . Olivi, L. (2017). Complete removal of arsenic and zinc from a

heavily contaminated acid mine drainage via an indigenous SRB consortium. *Journal of Hazardous Materials, 321*, 764-772.
Li, J.-t., Liang, Z.-w., Jia, P., Liu, J., Xu, Y.-j., Chen, Y.-j., . . . Shu, W.-s. (2017). Effects of a bacterial consortium from acid mine drainage on cadmium phytoextraction and indigenous soil microbial community. *Plant and Soil*, 1-12.
Li, S.-H., Yu, X.-Y., Park, D.-J., Hozzein, W. N., Kim, C.-J., Shu, W.-S., . . . Li, W.-J. (2015). Rhodococcus soli sp. nov., an actinobacterium isolated from soil using a resuscitative technique. *Antonie Van Leeuwenhoek, 107*(2), 357-366.
Li, X., Wu, Y., Zhang, C., Liu, Y., Zeng, G., Tang, X., . . . Lan, S. (2016). Immobilizing of heavy metals in sediments contaminated by nonferrous metals smelting plant sewage with sulfate reducing bacteria and micro zero valent iron. *Chemical Engineering Journal, 306*, 393-400.
Luptakova, A., & Kusnierova, M. (2005). Bioremediation of acid mine drainage contaminated by SRB. *Hydrometallurgy, 77*(1), 97-102.
Najafi, A., Roostaazad, R., Soleimani, M., Arabian, D., Moazed, M., Rahimpour, M., & Mazinani, S. (2015). Comparison and modification of models in production of biosurfactant for Paenibacillus alvei and Bacillus mycoides and its effect on MEOR efficiency. *Journal of Petroleum Science and Engineering, 128*, 177-183.
National Climate Data Center. Data records. 2017. Retrieved from: https://www.ncdc.noaa.gov/cdo-web/datatools/records
Park, Y., Kook, M., Ngo, H. T., Kim, K.-Y., Park, S.-Y., Mavlonov, G. T., & Yi, T.-H. (2014). Arthrobacter bambusae sp. nov., isolated from soil of a bamboo grove. *International journal of systematic and evolutionary microbiology, 64*(9), 3069-3074.
Peiravi, M., Mote, S. R., Mohanty, M. K., & Liu, J. (2017). Bioelectrochemical Treatment of Acid Mine Drainage (AMD) from an Abandoned Coal Mine Under Aerobic Condition. *Journal of Hazardous Materials*.
Pham, V. H., Kim, J., & Jeong, S.-W. (2014). Enhanced isolation and culture of highly efficient psychrophilic oil-degrading bacteria from oil-contaminated soils in South Korea. *Journal of environmental biology, 35*(6), 1145.
Sen, A., & Johnson, B. (1999). Acidophilic sulphate-reducing bacteria: candidates for bioremediation of acid mine drainage. *Process metallurgy, 9*, 709-718.
Sharma, A., Parashar, D., & Satyanarayana, T. (2016). Acidophilic Microbes: Biology and Applications *Biotechnology of Extremophiles:* (pp. 215-241): Springer.
Sheoran, A., & Sheoran, V. (2006). Heavy metal removal mechanism of acid mine drainage in wetlands: a critical review. *Minerals engineering, 19*(2), 105-116.
Strain, S. K., Hernández, R. A., Ben-jacob, E., & Florin, E.-l. (2013). Periodic reversals in Paenibacillus dendritiformis swarming.
Togo, C. A., Mutambanengwe, C. C. Z., & Whiteley, C. G. (2008). Decolourisation and degradation of textile dyes using a sulphate reducing bacteria (SRB)–biodigester microflora co-culture. *African journal of Biotechnology, 7*(2).
Torres, R., Solsona, C., Viñas, I., Usall, J., Plaza, P., & Teixidó, N. (2014). Optimization of packaging and storage conditions of a freeze-dried Pantoea agglomerans formulation for controlling postharvest diseases in fruit. *Journal of applied microbiology, 117*(1), 173-184.

United States Department of Agriculture. 2016. Retrieved from: https://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspx

United States National Library of Medicine. MOLE Blast. 2017. Retrieved from: https://blast.ncbi.nlm.nih.gov/moleblast/moleblast.cgi

Walterson, A. M., & Stavrinides, J. (2015). Pantoea: insights into a highly versatile and diverse genus within the Enterobacteriaceae. *FEMS microbiology reviews*, fuv027.

Yu, X., Jiang, J., Liang, C., Zhang, X., Wang, J., Shen, D., & Feng, Y. (2016). Indole affects the formation of multicellular aggregate structures in Pantoea agglomerans YS19. *The Journal of General and Applied Microbiology*, *62*(1), 31-37.

Zhou, Q., Chen, Y., Yang, M., Li, W., & Deng, L. (2013). Enhanced bioremediation of heavy metal from effluent by sulfate-reducing bacteria with copper–iron bimetallic particles support. *Bioresource technology*, *136*, 413-417.

APPENDIX

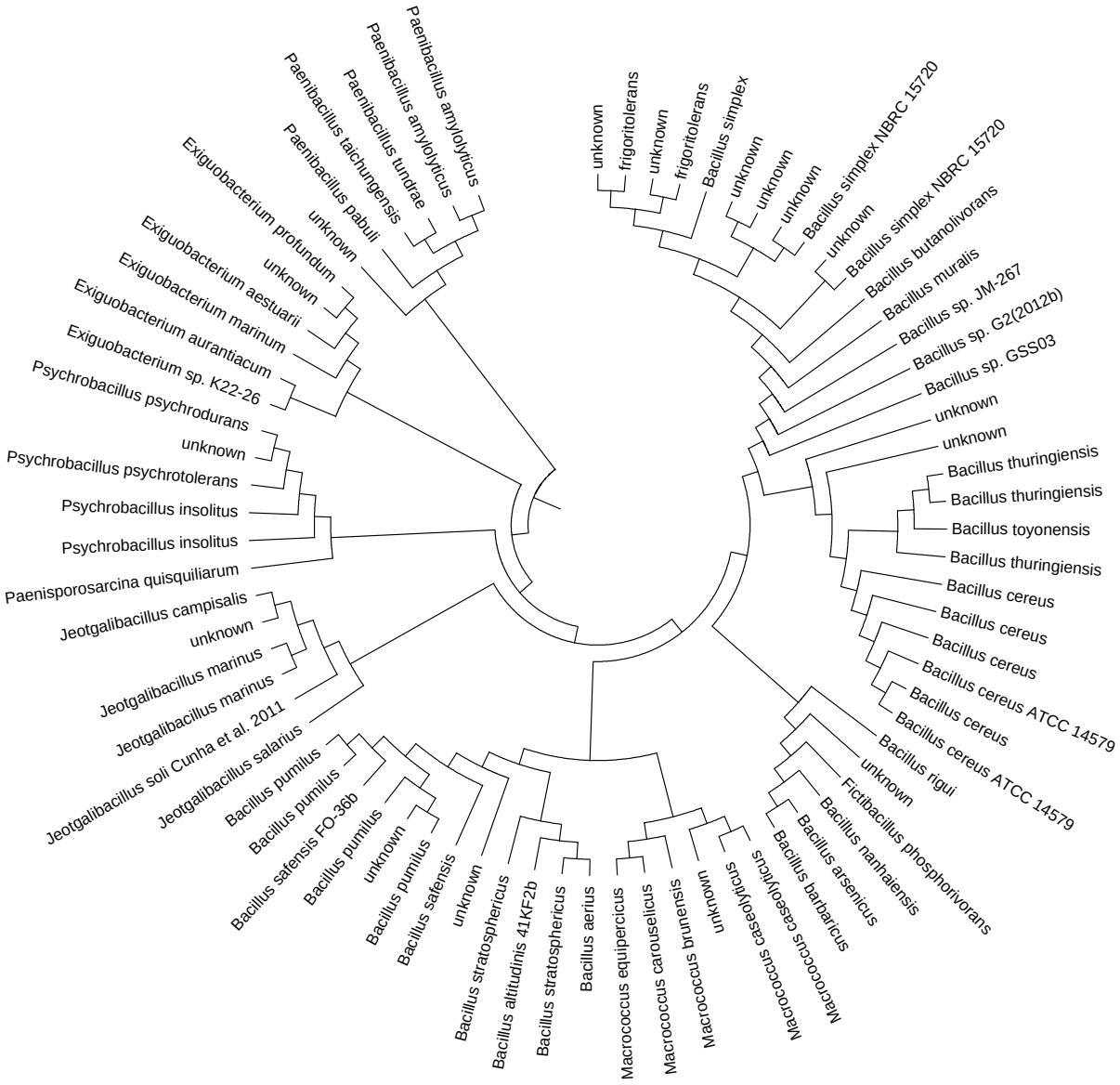
APPENDIX A

Quantitation

Nanodrop Lite	LL 2015 ISOLATES			Nanodrop Lite	Summer 2016 ISC
Sample	ng/ul	260/280	Factor 50	Sample	
A1	80.8 79.2	1.82 1.86		A1	19.8
A2	47.1	1.66		A2	62.6
A3	23.9	1.76		A3	37.5
A4	32.1	1.49		A4	11
A5	9.8	1.61		A5	54.6
A6	33.4	1.82			
A7	59.1	1.2			
B1	137.6 69.8	1.23 1.71		B1	43.8
B2	56	1.8		B2	6.5
B3	37.6	1.75		B3	151.6
B4	27.8	1.8		B4	50.6
B5	69.4	1.83		B5	53.1
				B6	86
				B7	46.7
C1	45.1	1.83		C1	60.3
C2	46.4	1.79		C2	60.1
C3	40.7	1.8		C3	21.8
C4	273.6 47.0	1.37 1.82		C4	26.7
C5	33.9	1.78			
C6	55.6	1.79			
C7	31.3	1.77			
C8	9.3	1.82			
D1	41.6	1.74		D1	46.3
D2	51.2	1.64		D2	33.5
D3	58.2	1.36		D3	31.9
				D4	41.1
				D5	39.8
E1	141.1 53	1.45 1.64		E1	48.6
E2	35.8	1.79		E2	92.1
E3	57.4	1.79		E3	50.5
E4	60.5	1.82 1.86		E4	60.5
E5	104/115	1.76 1.71		E5	148
E6	76.3	1.76		E6	41.3
	30.6	1.74		E7	54.7

APPENDIX B

Radial Phylogenetic Tree
MEGA Blast Software; Illustrating Fall 2015 and Summer 2016 Bacterial Isolates



APPENDIX C

Citric Acid Buffer

Follow mix found on Promega site for Citric Acid Buffer: https://www.promega.com/-/media/files/resources/paguide/a4/chap15a4.pdf?la=en

Use this site for M calculations if needed: http://www.cytographica.com/lab/molar.html

The procedure used for this lab was as follows:
1. Decide on pH, then on how much of Solution A and B are needed in mL
2. To make 500 mL of the buffer, make about 250mL of A and B separately
3. Make TSB broth as liquid medium for buffer powders

TSB is 30g/1L

Molecular weight is by 100mL .1M solution

4. Calculate molecular weight times amount of TSB being made (ex. 250mL= 2.5x)
5. Add Solution A powder to TSB in correct ratio in separate container
6. Do the same for Solution B powder (all based on PH)

7. Now to each container mix proportion of A to B in new flasks after measuring for PH
8. In each vial use 1.3% bacto agar (.013xmL=grams needed)
9. Autoclave
10. Warm water bath
11. Pour media
12. Streak plates with 8 each- may have to first find out if bacteria are viable by streaking them on TSA

APPENDIX D

Acid Buffer Growth Response (Absorbency Values)

PH	Summer 16	A5	B1	B5	B7	C3	C4	D1	D2	E5	E7
3		0.13	0.045	0.19	0.02	0.043	0.063	0.062	0.12	0.034	0.038
4		ND	0.055	0.103	0.032	0.025	0.062	0.029	0.001	0.021	0.026
5		0.082	2.632	1.156	0.445	0.022	0.056	0.024	0	0.007	0.039
6		2.843	2.283	2.897	2.83	0.026	0.123	0.022	2.153	0.296	0.524
TSB		1.394	1.529	2.504	1.336	1.833	1.05	2.844	1.648	0.838	2.975

PH	Fall 15	A1	A5	C1	C2	C3	D1	D3	E5	E6
3		0.91	0.244	0.214	0.163	0.126	0.13	0.074	0.19	0.092
4		0.037	0.073	0.11	0.135	0.111	0.092	0.084	0.161	0.068
5		0.041	0.165	0.106	0.075	0.071	0.02	0.063	ND	0.089
6		2.285	2.15	0.092	0.106	0.132	0.057	0.07	0.098	0.06
TSB		2.399	0.156	0.92	2.317	0.851	2.654	1.708	1.619	2.385

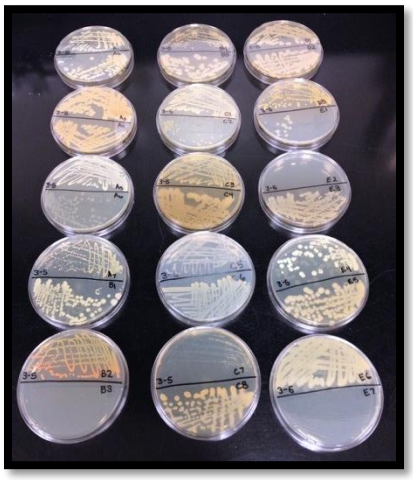
Trial Repeat

PH	Summer 16	D1	B1	B5	B7	C3	C4	D1	D2	E5	E7
3		0.60	0.17	1.02	0.35	0.33	0.42	0.37	0.63	0.07	0.40
4		0.38	0.57	0.30	0.39	0.27	0.21	0.66	0.39	0.46	0.34
5		0.24	1.97	0.53	0.84	0.22	0.52	0.89	0.68	0.78	0.20
6		0.47		1.85	1.96	0.42	0.32	1.05	1.37	1.20	0.90
TSB		1.11	1.43	0.24	0.69	0.71	1.94	0.84	0.91	0.52	0.61

PH	Fall 15	A1	A5	C1	C2	C3	D1	D3	E5	E6	A7	C6	E7
3		0.00	0.05	0.11	0.06	0.03	0.61	0.60	0.12	0.20	0.29	0.13	0.13
4		0.03	0.05	0.15	0.15	0.31	0.56	0.46	0.54	0.27	0.33	0.11	0.19
5		0.02	0.04	0.09	0.13	0.32	0.44	0.60	0.21	0.32	0.36	0.05	0.26
6		0.01	0.01	0.49	0.99	1.09	0.32	0.67	0.32	0.47	0.31	0.05	0.30
TSB		1.02	0.76	1.92	1.12	0.90	0.47	0.18	0.62	0.04	0.67	0.45	0.64

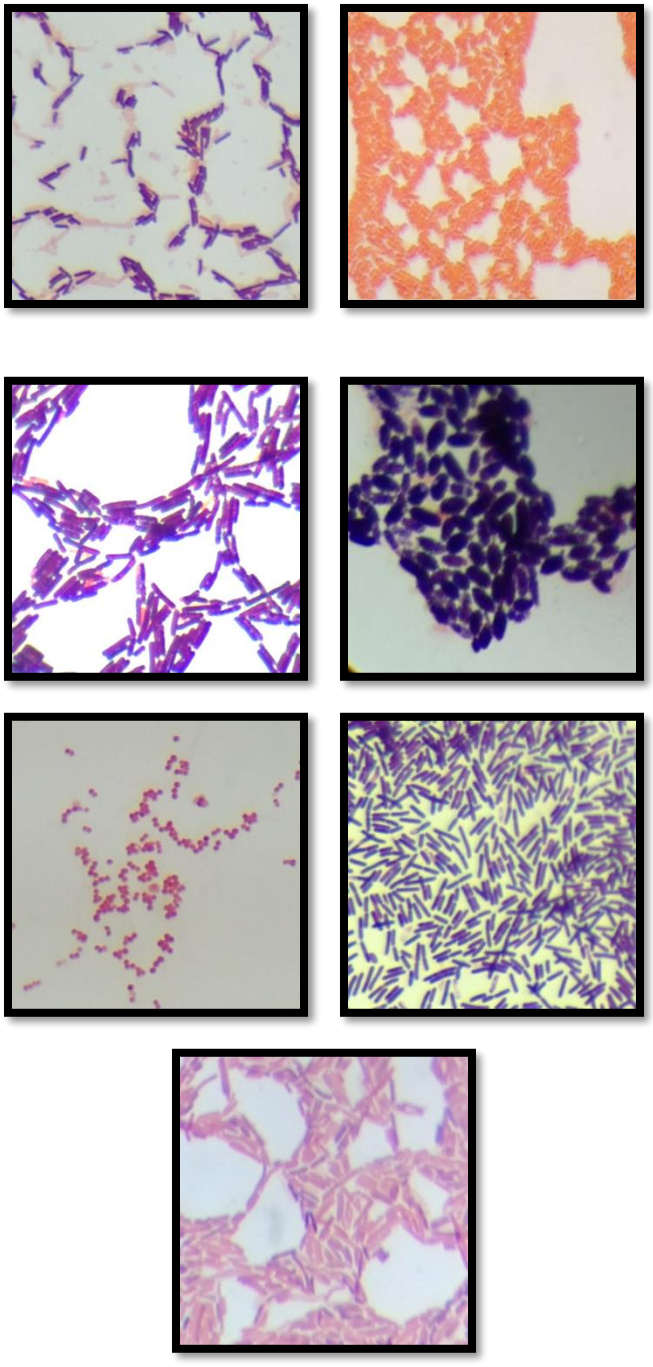
APPENDIX E

Colony Growth on TSA



APPENDIX F

Gram Stain Images



APPENDIX G

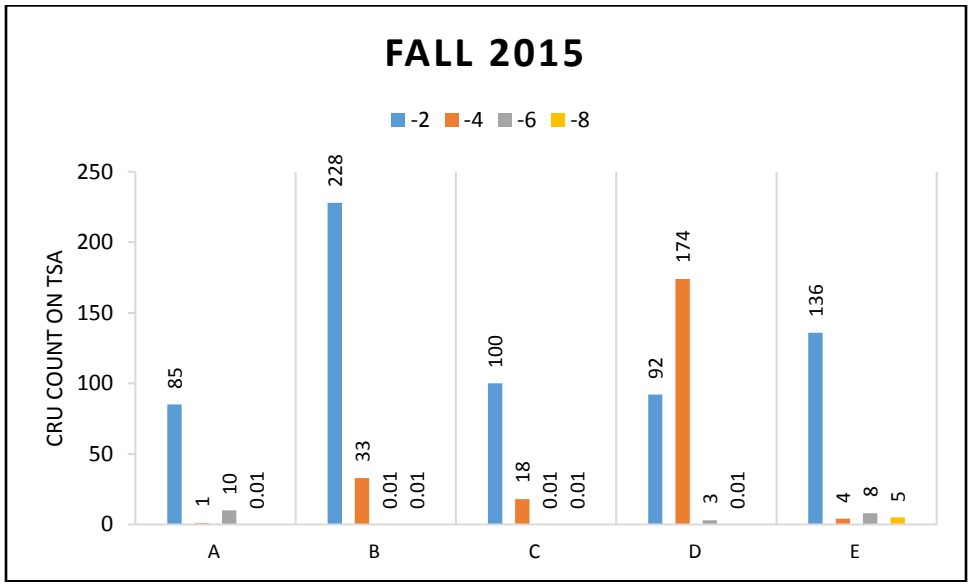
Acidophilic Bacteria (bold)

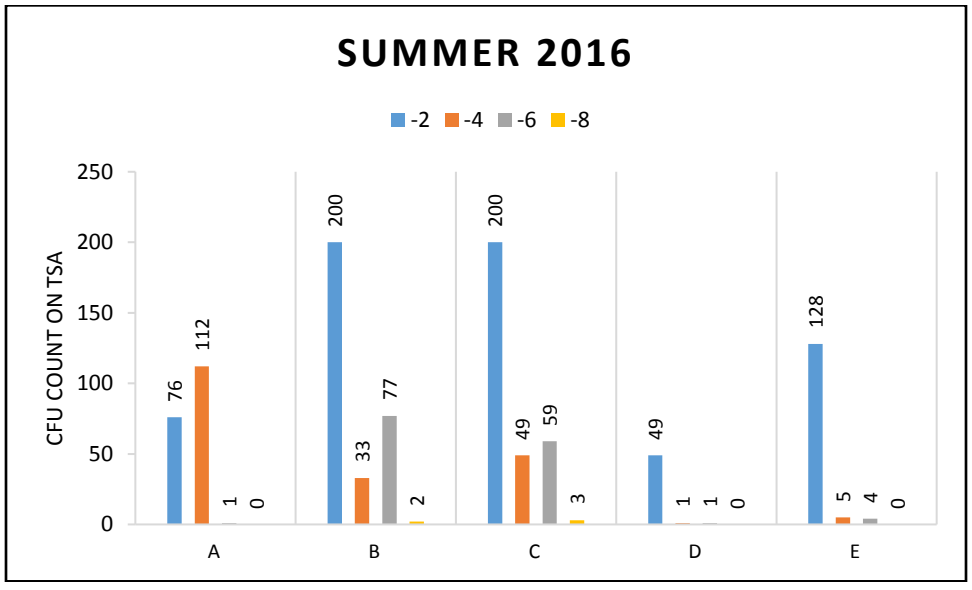
FALL 2015	260/280	*Genus and species*	% Identity	Alternative Identity	Acidophilic
A1	**1.82 1.86**	***Paenibacillus dendritiformis***	**97**		**X**
A2	1.66	*Bacillus indicus*	95	*Bacillus cibi*	
A3	1.76	*Fictibacillus nanhaiensis*			
A4	1.49	*Fictibacillus phosphorivorans*			
A5	**1.61**	***Paenibacillus alvei***	**95**		**X**
A6	1.82				
A7	**1.2**	***Curtobacterium flaccumfaciens***	**97**		**X**
B1	1.23 1.71	*Bacillus megaterium*	98		
B2	1.8	*Microbacterium oleivorans*	96	*Microbacterium paraoxydans*	
B3	1.75	*Bacillus simplex*	98		
B4	1.8	*Arthrobacter nitroguajacolicus*	97	*Arthrobacter aurescens*	
B5	1.83	*Bacillus simplex*	97	*Micrococcineae bacterium*	
C1	**1.83**	***Pseudomonas lini***	**95**		**X**
C2	**1.79**	***Pantoea agglomerans***	**96**	***Pantoea alli***	**X**
C3	**1.8**	***Pseudomonas syringae***	**93**		**X**
C4	1.37 1.82	*Bacillus simplex*	98	*Brevebacterium frigoritolerans*	
C5	1.78	*Fontibacillus panacisegetis*	91		
C6	**1.79**	***Stenotrophomonas rhizophilia***	**93**		**X**
C7	1.77	*Micrococcus luteus*	93		
C8	1.82	*Psychrobacillus psychrodurans*	96		
D1	**1.74**	***Bacillus simplex***	**98**		**X**
D2	1.64	*Brevibacterium] frigoritolerans*	98		
D3	**1.36**	***Frigoribacterium endophyticum***	**98**	***Frigoribacterium faeni***	**X**
E1	1.45 1.64	*Bacillus indicus*	96	*Bacillus cibi*	
E2	1.79	*Bacillus horikoshii*	98		
E3	1.79	*Fictibacillus phosphorivorans*	93	*Fictibacillus nanhaiensis*	
E4	**1.82 1.86**	***Bacillus megaterium***	**98**	***Bacillus aryabhattai***	**X**
E5	**1.76 1.71**	***Brevibacterium] frigoritolerans***	**96**		**X**
E6	**1.76**	***Bacillus marisflavi***	**92**		**X**
E7	1.74	*Bacillus subterraneus*	97		

SUMMER 2016					
A1	1.69	*Anthrobacter globiformis*	93		
A2	1.34				
A3	1.69	*Macrococcus caseolyticus*	93		
A4	1.34				
A5	**1.71**				**X**
B1	**1.66**	***Bacillus pumilus***	**98**	***Bacillus safensis***	**X**
B2	1.6				
B3	1.37	*Brevibacterium frigoritolerans*	96		
B4	1.71	*Paenibacillus amylolyticus*	96		
B5	**1.79**	***Bacillus stratosphericus***	**99**		**X**
B6	1.6	*Bacillus thuringiensis*	97	*Bacillus cerus*	
B7	**1.79**	***Bacillus simplex***	**97**		**X**
C1	1.61	*Exiguobacterium profundum*	98	*Exiguobacterium aestuarii*	
C2	1.82	*Bacillus idriensis*	95		
C3	**1.79**	***Fictibacillus phosphorivorans***	**96**		**X**
C4	**1.7**	***Bacillus pumilus***	**98**	***Bacillus safensis***	**X**
D1	**1.81**	***Jeotgalibacillus campisalis***	**97**		**X**
D2	**1.42**	***Bacillus pumilus***	**98**	***Bacillus safensis***	**X**
D3	1.84	*Bacillus stratosphericus*	97	*Bacillus altitudinis*	
D4	1.63	*Bacillus toyonensis*	97	*Bacillus thuringiensis*	
D5	1.84	*Brevibacterium frigoritolerans*	98	*Bacillus simplex*	
E1	1.38	*Bacillus simplex*	98	*Bacillus butanolivorans*	
E2	1.83	*Psychrobacillus psychrodurans*	81		
E3	1.86	*Brevibacterium frigoritolerans*	96		
E4	1.45	*Arthrobacter oxydans*	98	*Arthrobacter polychromogenes*	
E5	**1.38**	***Rhodococcus jialingiae***	**96**	***Rhodococcus gingshengii***	**X**
E6	1.74	*Bacillus simplex*	98		
E7	**1.82**	***Terribacillus saccharophilus***	**97**	***Terribacillus goriensis***	**X**

APPENDIX H

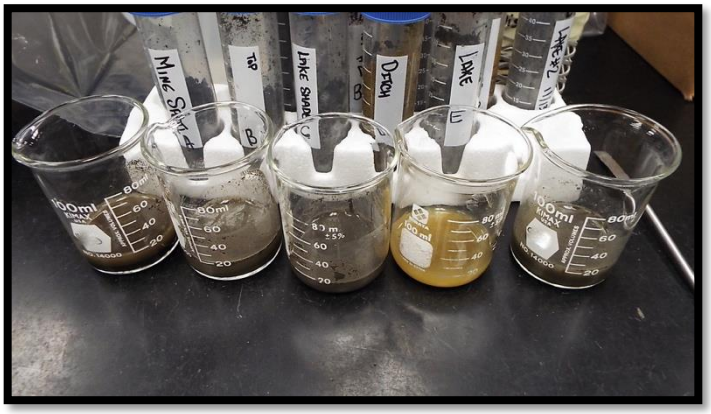
Site with Total CFU Count on TSA Media Dilutions 10^





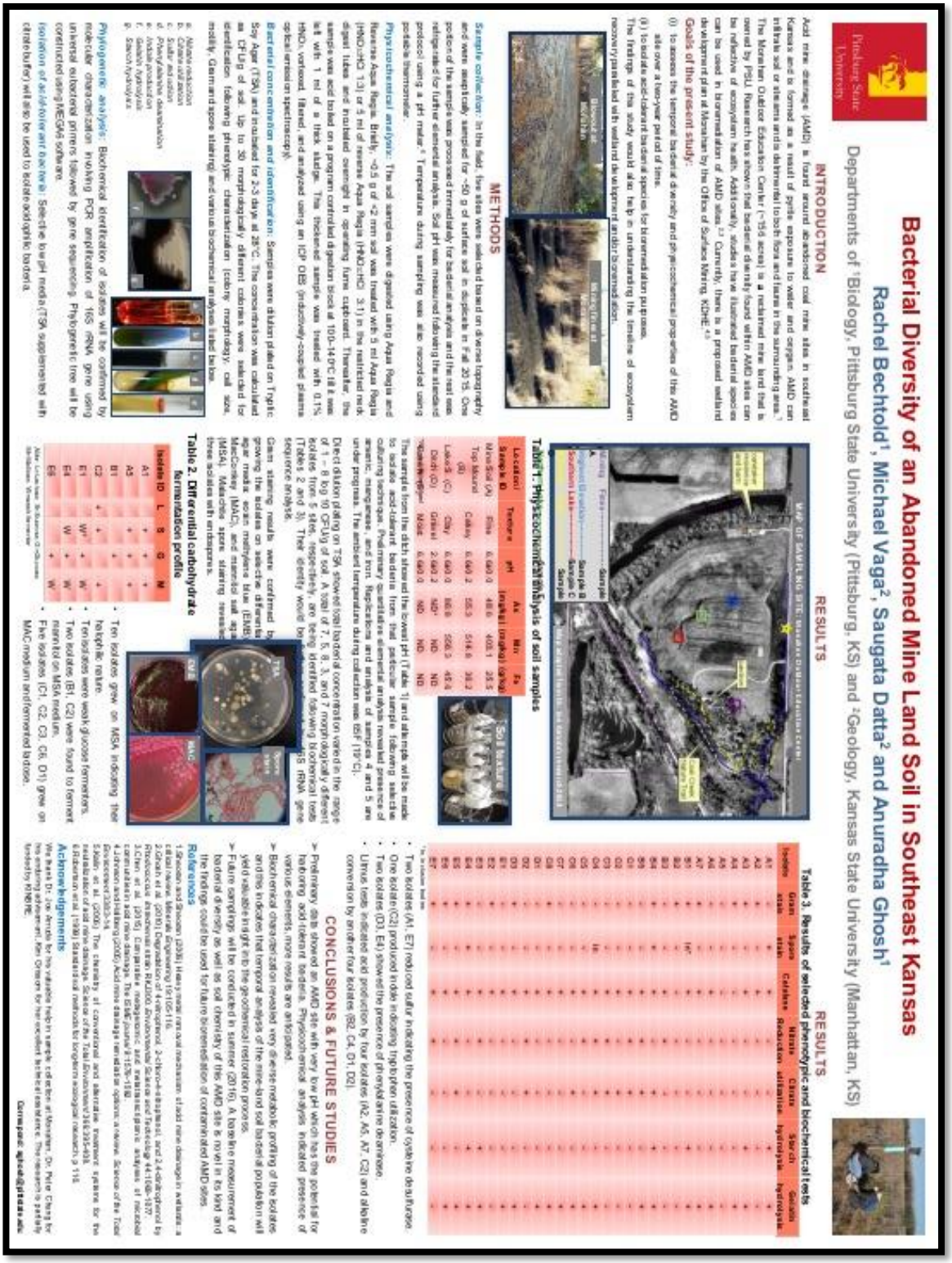
APPENDIX I

Site Images



APPENDIX J

PDF of Research Posters



Bacterial Diversity of an Abandoned Mine Land Soil in Southeast Kansas

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INTRODUCTION

Acid mine drainage (AMD) is found around abandoned coal mine sites in southeast Kansas and is formed as a result of pyrite exposure to water and oxygen. AMD can infiltrate soil or streams and is detrimental to both flora and fauna in the surrounding area.[1] Research has shown that bacterial diversity of AMD sites is reflective of ecosystem health; additionally, studies have illustrated bacterial species can be used in bioremediation of AMD sites.[2,3] The Monahan Outdoor Education Center (~156 acres) is a partially reclaimed mine land owned by PSU (Fig. 1). Given the potential for serious environmental damage and burdensome reclamation costs, it is practical to seek long-term, cost effective treatments for AMD. Isolation of acidophilic and/or sulfate-reducing bacteria could be used along with anoxic limestone drains or wetlands to expedite the process of bioremediation and restoration of natural habitat for plants and animals.[4,5]

Goals of the present study:

(i) To assess the temporal bacterial diversity and physicochemical properties of this AMD site over a two-year period of time.

(ii) To isolate acid-tolerant bacterial species for bioremediation purposes.

The findings of this study would also help in understanding the timeline of ecosystem recovery paralleled with wetland development and/or bioremediation.

Figure 1. Areas for remediation at Monahan

METHODS

Sample collection: In the field, five sites were selected based on diverse topography and were aseptically sampled for ~50 g of surface soil in duplicate in Fall 2015 and in Summer 2016. One portion of the sample was processed immediately for bacterial analysis and the rest was refrigerated for further elemental analysis. Soil pH and temperature were measured on collection site.

Physicochemical analysis: The soil samples were digested using Aqua Regia and Reverse Aqua Regia (Fig. 2). Briefly, ~0.5 g of <2 mm soil was treated with 5 ml Aqua Regia (HNO_3:HCl 1:3) or 5 ml of reverse Aqua Regia (HNO_3:HCl 3:1) in the restricted neck digest tubes and incubated overnight in operating fume cupboard. Thereafter, the sample was acid boiled on a program controlled digestion block at 100–140°C till it was left with 1 ml of a thick sludge. This thickened sample was treated with 0.1% HNO_3 vortexed, filtered, and analyzed using an ICP OES (inductively-coupled plasma optical emission spectroscopy).

Figure 2. Soil characteristics

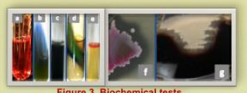


Figure 3. Biochemical tests

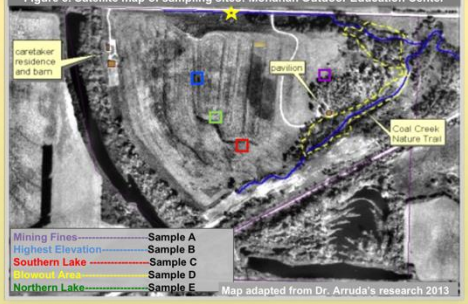
Bacterial concentration and identification: Samples were dilution plated on Tryptic Soy Agar (TSA) and incubated for 2-3 days at 28°C. The concentration was calculated as CFU/g of soil. Up to 58 morphologically different colonies were characterized following phenotypic and biochemical analyses such as (a) nitrate reduction, (b) citrate utilization, (c) cysteine desulfurization, (d) phenylalanine deamination, (e) indole production, (f) gelatin hydrolysis, (g) starch hydrolysis, catalase, oxidase, etc. (Fig. 3). Selective low pH media was used to isolate acidophilic bacterial isolates.

Phylogenetic analysis: Biochemical identification of isolates were confirmed by PCR amplification of 16S rRNA gene using eubacterial primers followed by gene sequencing (Fig. 4).

Figure 4. Representative gel electrophoresis of PCR amplicons from individual isolates

RESULTS

Figure 5. Satellite map of sampling sites: Monahan Outdoor Education Center



Location/ Sample ID	Soil Type	pH	As (g/kg)	Mn (g/kg)	Fe (g/kg)
Mine Soil (A)	Coalvale silty clay	6.6±0.0	0.010	0.614	28.7
Top Mound (B)	Coalvale silty clay	6.6±0.2	0.013	0.877	35.5
Lake S. (C)	Coalvale silty clay	6.6±0.0	0.015	0.932	39
Ditch (D)	Parson's Silt Loam	2.6±0.2	0.010	0.433	169.6
Lake N. (E)	Coalvale silty clay	6.6±0.0	0.008	0.978	34.1

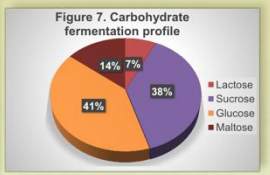
The sample from the ditch (D) showed the lowest pH (Table 1) and following selective culturing technique acidophilic bacteria were isolated from all sites. Preliminary quantitative elemental analysis revealed presence of arsenic, manganese, and iron. The ambient temperature during collection was 65F (19°C) in fall of 2015 and 100F (38°C) in summer 2016.

Direct dilution plating on TSA showed total bacterial concentration varied in the range of 1 – 8 log 10 CFU/g of soil (Fig. 6). A total of 58 morphologically different isolates from 5 sites were characterized and their identity was further confirmed by 16S rRNA gene sequence analysis.

Gram staining results were confirmed by growing the isolates on selective differential agar media: eosin methylene blue (EMB), MacConkey (MAC), and mannitol salt agar (MSA) (Fig. 6). Malachite spore staining revealed three isolates with endospores.

Figure 6. Growth on TSA and selective differential media

Figure 7. Carbohydrate fermentation profile



The majority of isolates could ferment glucose and sucrose followed by maltose and lactose (Fig. 7). Two isolates could ferment all four sugars and eight isolates could ferment three sugars.

RESULTS

Figure 8. Phenotypic and biochemical characterization of 58 isolates

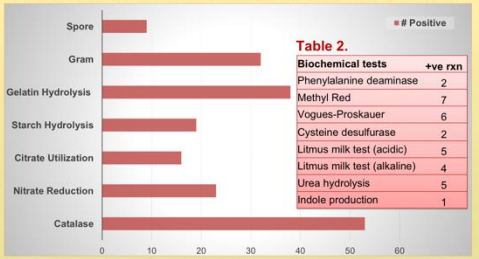


Table 2.

Biochemical tests	+ve rxn
Phenylalanine deaminase	2
Methyl Red	7
Vogues-Proskauer	6
Cysteine desulfurase	2
Litmus milk test (acidic)	5
Litmus milk test (alkaline)	4
Urea hydrolysis	5
Indole production	1

Biochemical tests revealed vast metabolic potential of bacterial isolates (Fig. 8 and Table 2). A total of 17 unique acidophilic strains were isolated on selective acid media and were identified using molecular approach (Table 3)

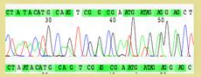


Table 3. Isolation and identification of acidophilic bacteria

pH3 (N=5)*	pH4 (N=8)	pH5 (N=7)	pH6 (N=6)
Pantoea agglomerans	*Bacillus simplex*	*Paenibacillus alvei*	*Bacillus megaterium*
Pseudomonas syringae	*Frigoribacterium endophyticum*	*Curtobacterium flaccumfaciens*	*Microbacterium oleivorans*
Bacillus subterraneus	*Jeotgalibacillus campisalis*	*Pantoea agglomerans*	*Bacillus thuringiensis*
Bacillus pumilus	*Bacillus pumilus*		*Bacillus pumilus*
Terribacillus saccharophilus	*Frigobacterium endophyticum*		

*unique isolates are presented in the table. A few isolates grew on a wide low pH range.

CONCLUSIONS & IMPACT OF THE STUDY

- Data showed an AMD site with very low pH has the potential for harboring acid-tolerant bacteria. Physicochemical analysis indicated presence of various soil elements; arsenic, manganese, and iron, and this is useful in selecting physical remediation strategies.
- Biochemical characterization revealed very diverse metabolic profiling of the isolates and majority of bacterial species belonged to common soil inhabitant phyla *Firmicutes* and *Proteobacteria*.

A baseline measurement of bacterial diversity and soil chemistry of AMD site is novel in its kind in southeast KS. Acidophilic bacterial strains from this study could be used along with anoxic limestone drains or wetlands to expedite the process of bioremediation and restoration of natural habitat for plants and animals.

References

1.Sheoran and Sheoran (2006). *Minerals Engineering* 19:105-116.
2.Ghosh et al. (2010). *Environmental Science and Technology* 44:1069-1077.
3.Chen et al. (2015). *The ISME journal* 9:1579-1592.
4.Johnson and Hallberg (2005). *Science of the Total Environment* 338:3-14.
5.Kalin et al. (2006). *Science of the Total Environment* 366:395-408.

Acknowledgements

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