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Antibiotic Resistance Genes in Minnesota Soil Bacteria from Areas of High and Low Ferric Iron

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Abstract

Naturally-occurring antibiotic resistance genes in soil bacteria represent a potentially important reservoir of genes that could contribute to antibiotic resistance of human pathogens. It has been reported that over 40 genes in bacterial genomes are controlled by concentrations of ferric iron. We examined the effect of soil metal content on the level of resistance to two antibiotics, ampicillin (Amp) and tetracycline (Tet), and the presence of multiple genes that code for efflux pump-mediated resistance. These pumps act to export toxins (e.g. heavy metals and antibiotics, perhaps). Because of this, growth in heavy metal-contaminated soils might select for antibiotic resistance. Ninety-six soil samples were collected over the course of two summers from areas of Minnesota with known high and low ferric iron, as reported by the US Dept. of Interior. Samples were plated on LB plates with either 10 mg/500mL Tet or 50 mg/500mL Amp. Tet resistance was the same in high and low iron soils (p = 0.63, sd = 0.02). Amp resistance was higher in samples from high iron soils only in 2015's data (**2015** p =0.002; **2016** p = 0.75, sd = 38.1). Distribution of resistance was, however, significant for Tet between iron concentrations ($p < 0.001$). Additionally, total DNA was extracted and PCRs with gel electrophoresis was used to determine the prevalence of 14 different efflux genes (acrB,D,F; emrB,E; mdfA; tehA; yhiV; mexF,Y; tetC,H,B,D) common to soil bacteria. In 2015, five of the eight genes studied were seen in high iron soil, while only one gene was detected in low iron soil. In 2016, four of six genes were found in samples from both soils. Ferric iron levels in the soils tested were not significantly correlated with Tet or Amp resistance levels in soil bacteria in 2016, but were correlated in 2015's data (Amp only). It is possible that other heavy metals play a more important role in selecting for antibiotic resistance than iron.

Justification and Objectives

An ever-growing concern in this modern day world is that of antibiotic resistance genes in the environment. These typically occur when antibiotics are used for medical treatments, and ultimately select for genes that code for antibiotic resistance. If these genes enter the food chain and incorporate into the human's body, they can spark issues for successful infection treatment at the medical level (Rolain et. al., 2012). The way these genes typically become integrated into the food chain is through phages, plasmids (Rower et. al., 2004), or agricultural application. The genes can end up creating complications through infections from the environment or virulence dissemination via increased antibiotic usage for infection treatments (Sengupta et. al., 2007).

An important physiological component of bacteria is efflux pumps (common, non-specific toxin exporters). These pumps are coded by some of the genes that are directly controlled by intracellular iron concentration levels (Sritharan, 2000). Iron is utilized in bacterial metabolism, unless it begins to reach toxic proportions. While the bacteria use their efflux pumps to export toxins, such as extra iron or other heavy metals, these pumps may at the same time be exporting antibiotics (Li et. al., 1995). This suggests that growth in heavy metal-contaminated soils may select for antibiotic resistance (Alonso et. al., 2001). Specific heavy metals of interest are copper, nickel, and iron. Thus, it is hypothesized that higher iron and increased heavy metal concentrations in the environment, soil in particular, can increase antibiotic resistance genes in soil bacteria as well as the presence of efflux pump genes in their DNA. The research will look into the correlations between heavy metal concentrations found in Minnesota soil in relation to the level of antibiotic resistance to two main antibiotics. This will allow for increased knowledge surrounding the relationship between potentially unsafe levels of antibiotic resistance and the amount of heavy metals in the soil.

Background

There is evidence that antibiotics have played a role in microbial metabolism for millions of years (Allen et. al., 2010). In natural environments, certain species of bacteria produce their own chemical compounds that are adept at killing other bacteria (Lupo et. al., 2012). This is done in order to outcompete other species for habitat, nutrients, and possible hosts. These compounds work in a variety of mechanisms. Some work by destroying the bacterial cell wall, disorganizing their peptidoglycan layer, or interrupting enzyme synthesis or signal cascades. It was not until relatively recently (Davies et. al., 2010) that humans have harnessed these natural capabilities of bacteria in order to purposefully and specifically target bacteria (i.e. infections, parasites) to kill them or to destroy their infectivity ability. Their medicinal purposes were not realized until the 1950s with penicillin (Berdy, 12). Antibiotics were seen to control and obliterate fungal and protozoal infections, to control pests on crops, to maintain health in livestock, and can help those with common physiological diseases (Ventola, 2015).

However, once these discoveries became known, use of antibiotics became common. One popular use for antibiotics is in animal husbandry and agricultural farm applications. Often, farmers will treat their livestock with antibiotics in order to protect their health and promote their growth. Once applied to the animals, antibiotics enter the manure, in which the opportunity for co-selection of resistance traits exists (Zhu et. al., 2013). One example of farm samples harboring antibiotic resistance genes is from a study on Chinese swine farms, which noticed they had significant levels of diverse and potentially mobile antibiotic resistance genes that correlated directly with antibiotic and metal concentrations in the farm soils (Zhu et. al., 2013).

The use of antibiotics in feed in agriculture has potential effects on humans as the environments harboring antibiotics often have enriched levels of transposons as well as antibiotic resistant genes. If humans interact with this environment, they are at higher risk of

picking up these genes and potentially experiencing health complications if they contract an infection (Van den Bogaard et. al., 2001). One retrospective analysis of pig and veal calf farmers identified that animal related methicillin resistant *S. aureus* ST398 from an area with a high density of pig farms led to an 82% increase in newly identified carriers of MRSA (Wulf et. al., 2010). Contracting MRSA can lead to difficulty in treatment of infection.

Furthermore, the overuse and misuse of antibiotics in treatment and preventative methods has been occurring consistently since the introduction of penicillin in the 1950s. Much overuse comes from pharmaceutical residues, animal husbandry, human wastes, and urban water systems (especially with poor flow-storage characteristics) (Chen et. al., 2012). This is an issue of concern, as microbial species are adept at evolving resistance to various antibiotics at exceptionally high rates. In the last 50 years, antibiotic resistance levels have continuously and rapidly increased (Davies and Davies, 2010). These increases in resistance are, in part, due to random mutations that spawn resistance, as well as gene transfers (Zhu et. al., 2013).

There are two main processes in which bacteria can acquire resistance to antibiotics. According to Drake (1991), bacteria can mutate spontaneously and obtain varied compositions or new mechanisms that aid them in surviving applied antibiotics. Another common way bacteria can gain resistance is through horizontal gene transfer (via plasmids, viruses and transformation). These processes permit existing resistance genes to spread to new and previously sensitive bacteria. Several different mechanisms of resistance have been identified, including antibiotic-altering enzymes, genetic variation affecting the target of the antibiotic action, antibiotic-degrading enzymes, and efflux pumps. These are conferred either through transduction, transformation, or vertical gene transfer. Commonly the genes travel through plasmids and transposons (Levy, 1998).

Plasmids allow for the exchange of genetic information via conjugation and transformation processes. Conjugation involves the physical interaction of two bacterial cells that exchange information while connected to one another via a conjugative pilus. Transformation involves the uptake of naked or “free” genetic information that becomes integrated into the new cell. These styles of gene exchange are relatively rapid and efficient. In fact, they are sometimes created in industry related to biofuels, agriculture, and environmental bioremediation.

Williams-Nguyen et. al., 2015 presented a model of the effects antibiotics can have in various types of environments and on human health **(Figure 1)**. It is understood that an intentional addition of antibiotics results in a noticeable enrichment of antibiotic resistant bacteria. These bacteria, along with antibiotic resistant genes are linked, and the effects on human health, ecosystem function, and agricultural system productivity are displayed in **Figure 1** (Williams-Nguyen et. al., 2015).

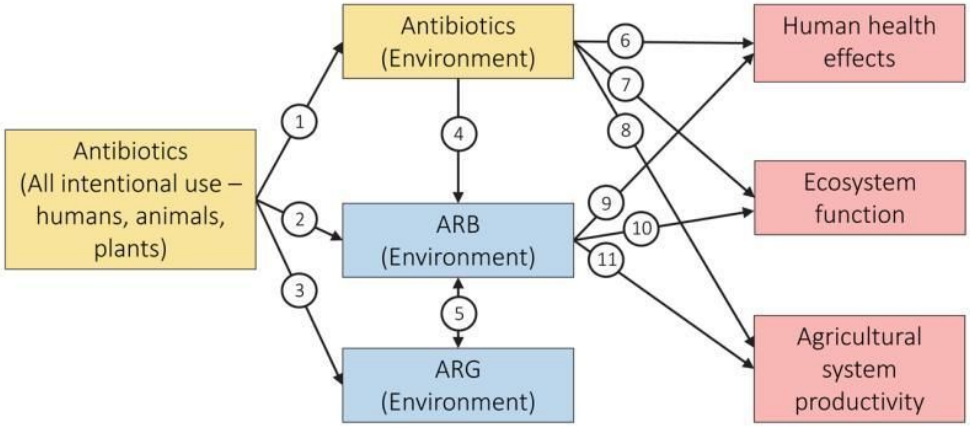

Figure 1. **Conceptual/causal model depicting hypothesized effects of antibiotics, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in agroecosystems and the environment.** *Yellow:* Active antibiotic compounds; *Blue:* Resistance elements, *Pink:* Outcomes of interest.

One specific and growing concern is that drug resistance could be transferred from genetically modified crops into water systems, produce, or livestock and finally into human

populations. This would require that the genetically modified crops contain vector components that encode resistance genes, which many do. (Chen et. al., 2012).

Now, another way that bacteria can evade antibiotic destruction is through the formation of biofilms. Biofilms are congregations of bacterial cells into a hydrated matrix of polysaccharide and protein (Stewart and Costerton, 2001). Biofilms are created through bacteria communicating with populations of one another via quorum sensing. Quorum sensing is the controlled expression of specific genes in response to extracellular signals produced by bacteria themselves (Soto et. al, 2013). Once a biofilm has been created, antibiotics cannot reach the individual cells to inhibit their growth and proliferation. Thus, biofilm formation can ultimately support the chronicity of infections. If biofilm formation occurs on medical devices or in damaged tissue in an organism, serious and life threatening effects can occur.

A number of genes have been shown to play roles in biofilm growth and overall resistance to antibiotics. Pode et. al. (2001) list a set of genes labeled ‘Mex_-Opr_’. MexAB-OprM is thought to cause resistance to fluoroquinolones, beta-lactams, tetracycline, macrolides, chloramphenicol, and other antibiotics. They are also known to prevent biofilm growth. MexCD-OprJ is known to cause resistance to tetracycline and some beta-lactams. MexEF-OprN is known to produce resistance to fluoroquinolones specifically. MexXY-OprM, a constituent of the outer-membrane, leads to a decreased susceptibility to fluoroquinolones and aminoglycosides. These genes were selected for investigation in this research project.

To expand on the types of drugs listed above, fluoroquinolones are a broad spectrum, systemic antibiotic commonly used to treat respiratory and urinary tract infections. This class is known to be active against both aerobic gram positive and gram negative bacteria. Tetracyclines are broad-spectrum, bacteriostatic antibiotics that are active against both gram positive and gram negative bacteria. Tetracyclines are often the antibiotic chosen to treat

livestock in agricultural applications. Tetracyclines work via binding to ribosomes at the 30S subunit and inhibiting protein synthesis (*NIH.gov*, 2016). Due to their popularity in agriculture and environmental applications, tetracycline resistance became of interest for this research.

Efflux pumps, listed earlier as a method of bacteria's resistance to antibiotics, are the focus of this research. Efflux pumps are relatively non-specific transporters that can remove molecules for the cytoplasm of a bacterial cell by transporting them across bacterial cytoplasmic membranes (Arabestani et. al., 2015). One purpose of these mechanisms is to export toxins via active transport. Additionally, it is known that bacteria use certain metallic compounds for metabolism. However, if heavy metal concentrations reach unsafe levels intracellularly, efflux pumps will work to export these metals in order to keep the cell healthy. Since these pumps are not specific in what they pump out, often other compounds will get pumped out of the cell.

It is known that drug efflux is common in Gram- bacteria. Gram- bacteria have both an inner membrane (IM) and an outer membrane (OM) with a peptidoglycan layer in between. Drugs enter through the OM by porin channels, specific protein channels, or the LPS-containing asymmetric lipid bilayer region (Li et. al., 2015). If the drugs continue, they will penetrate the IM via simple diffusion. Now, efflux is a transport system that is comprised of either a single-component pump or a multicomponent pump. Multicomponent pumps contain the pump itself, an OM channel protein, and an accessory membrane fusion protein. The single-component pumps transport the drug(s) from the cytosol of the cell to the periplasm (with the assistance of porins). The multicomponent pumps take the substrate (drug) from the periplasmic space and the IM and pump it directly out of the cell into the medium surrounding the cell. Efflux mechanisms are capable of allowing bacteria to be resistant to multiple types of drugs (Lee et. al., 2000). The method that systems such as efflux pumps arise is through genes acquired by the bacteria which code for the proteins required to synthesize these

compounds/mechanisms. For example, efflux genes encode multidrug efflux pumps/components of the pumps. These pumps are typically composed of an exit duct that remains anchored in the outer-membrane, an inner-membrane transporter, and a periplasmic adaptor protein connecting the two - allowing for easy transport of antibacterial drugs passing through the pump (Symmons et. al., 2009; **Figure 2**).

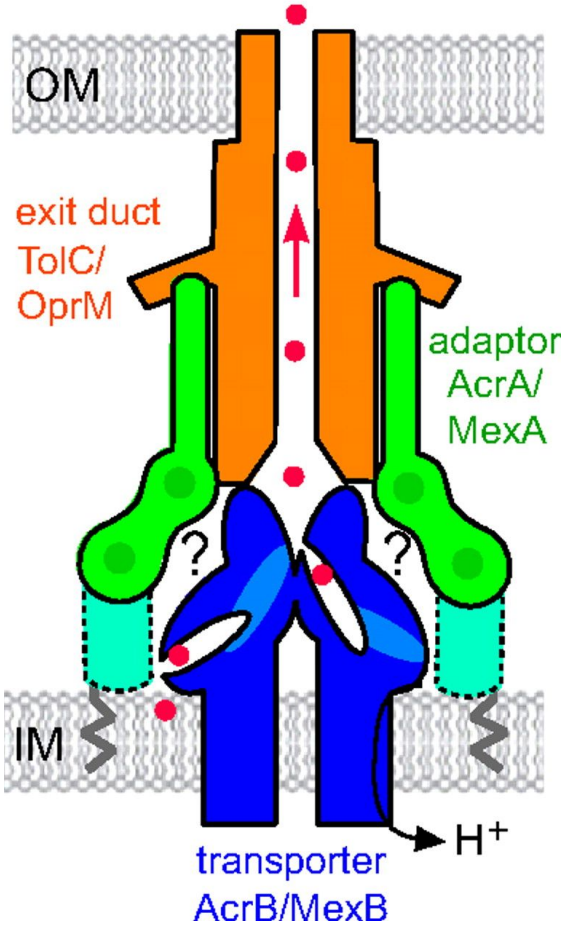

***Figure 2*. Schematic of a tripartite multidrug efflux pump (Symmons et. al., 2009).** Exit duct in outer-membrane (OM), integral inner-membrane (IM) transporter, and periplasmic adaptor. Dots show antibacterial drugs bound to pockets in transporter and passing through to the the outside of the cell.

On a grand scheme, environmental reservoirs of antibiotic resistance are not well understood. Resistance has been reported to correlate to areas affected by radiation and pollution, as well as metal contamination (Zhu et. al., 2013) and is harbored in livestock treated

with antibiotics. Resistance transfers between bacterial strains in intestinal microbiomes (Allen et. al., 2010), and it is known that it can be selected for when antibiotics are utilized when an actual infection does not exist, for example when low level antibiotics are used to stimulate growth in livestock.

There exists growing evidence that bacteria in soil environments are multi-drug resistant. Also, functional screening of the accumulating metagenomic databases are showing a previously unexpected density of resistance genes in soil environments. This has been termed the antibiotic resistome (D'Costa, et. al., 2007).

This project focuses on whether iron levels in the soil are associated with antibiotic resistance or the presence of efflux genes in the soil metagenome. Iron is sequestered and aids in biofilm formation, enzymatic processes, oxygen metabolism, electron transfer, DNA/RNA synthesis, and the production and release of siderophores (Goetz et. al., 2005). Siderophores assist bacteria by acting as ferric iron [Fe(III)] chelating agents (Ahmed and Holmstrom, 2014), allowing the iron to be available for the cells to complete the processes listed. Additionally, with the help of sulfur, iron aids in maintaining DNA integrity, gene regulation, RNA modification, and respiration. Lastly, iron is cofactor to many proteins (Ezraty and Barras, 2016).

However, upon reaching high levels of intracellular concentrations, iron can become toxic to bacteria. To prevent this issue, bacteria's efflux pumps can reduce the level of intracellular iron (Grass et. al., 2005). However, these pumps are non-specific to what exact compounds they are exporting. While removing the iron, they may indirectly be exporting antibiotics as well (Li et. al., 1995). Thus, it is assumed that in areas of high dissolved (ferric) iron, there may be measurably more antibiotic resistance since the efflux pumps may need to be removing the excess iron.

The questions posed for this project are: is there a greater proportion of antibiotic resistant bacteria in soils with high ferric iron concentrations? And, are efflux genes more prevalent in areas of high ferric iron?

Methods

Sample Collection

Soil was collected from four high and four low ferric iron concentration areas. Areas were determined as having high or low iron concentrations via a geological study completed by the U.S. Department of the Interior in 1984 on metal concentrations in soils (Shacklette and Boerngen, 1984; **Figure 3**). The study, while over 30 years old, detailed areas of Minnesota with relatively high and low amounts of ferric iron.

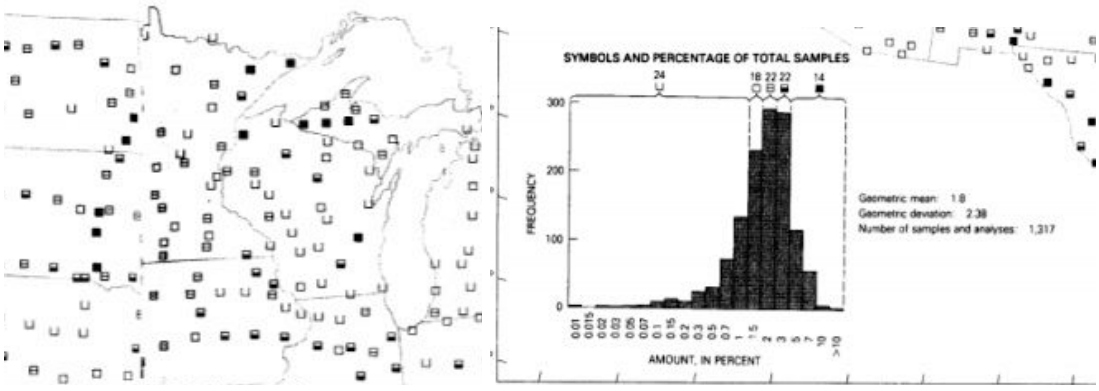

***Figure 3*. Map of Minnesota denoting locations of tested ferric iron concentration frequencies (Left: Minnesota and surrounding states, Right: Key with frequency and amount of iron, in percent).** Used to choose relative areas of Minnesota to travel to and collect soil samples for both summers of research. Latitudes and longitudes included in complete article. *Images adapted from Shacklette and Boerngen (1984).*

Figure 4 shows the exact locations of the collection sites. These sites were chosen due to their legality of access, public space availability, and population size (**Figure 4**). 48 samples

were retrieved from high and from low iron soil locations, half during the summer months of 2015 and half during the summer months of 2016.

Measuring Iron Concentrations

The iron levels were tested with the LaMotte Soil Kit. The average concentration from high iron soil ranged from 5.8 lbs/acre (2016) to 11 (2015) lbs/acre and the average from low iron soil ranged from 0.2 lbs/acre (2016) to 1 (2015) lb/acre. The samples were then submitted for complete metal analysis by the University of Minnesota's Research Analytical Laboratory (**Table 1, 2**). This was completed to validate the difference in iron concentrations as measured by the LaMotte Soil Kit. Additionally, the analyses completed by the University of Minnesota provided metal concentrations for over a dozen other metals, some of which human interaction is capable of altering (e.g. aluminum, copper).

Plating and Culture

The 2015 samples were plated on LB ampicillin nutrient plates at a 10^{-1} dilution and on LB nutrient plates at 10^{-3} and 10^{-4} dilutions. The samples from 2016 were plated on LB plus tetracycline media plates at a 10^{-1} dilution and on LB plus ampicillin media plates at 10^{-3} and 10^{-4} dilutions, and on LB nutrient plates at 10^{-3} and 10^{-4} dilutions. The plates incubated at 32ºC for 24 hours and the number of colony forming units (#CFU) were counted. If colony count reached over 300 CFUs, the samples were re-plated at the same dilution, and potentially diluted further to permit a countable #CFU. A two-tailed T-test was used to determine statistical significance between the high and low iron soil's antibiotic resistance. Kolmogorov-Smirnov's test was used to determine if the distributions of resistance were statistically significant.

DNA Extraction and PCR

To extract DNA from each soil sample, the MoBio PowerSoil DNA Isolation Kit was used and the protocol was followed with no changes made. The DNA extracted from 24 high iron and

24 low iron soil samples was then used in polymerase chain reactions (PCR) along with primers for 14 different efflux pump genes. Primers were utilized from previous research on the genes chosen for each year's project. 2015's primers were taken from Viveiros et. al., 2005. The genes tested for were: acrB, acrD, acrF, emrB, emrE, mdfA, tehA, and yhiV. The PCR ran with the following cycle:

Thermal cycler conditions: PCR activation at 95°C for 15 min, followed by 35 cycles of denaturation (94°C for 60 s each), then annealing (51°C to 53°C for 60 s depending on primers used), and lastly extension (72°C for 60 s). The primers were designed based on the E. coli K-12 complete genome (accession number NC_000913) (Viveiros et. al., 2005).

The primers used in the research completed in the summer of 2016 were taken from Aminov et. al., 2002 and from Poonsuk and Chuanchuen, 2014. The genes tested this year were tetC, tetH, mexB, mexD, mexF, and mexY. Thermal cycler conditions for the tet gene primers and the PCR were as follows: consisted of an initial denaturation period (94°C for 5 min), followed by 25 cycles at 94°C for 5 s and then 30 s of annealing and extension at 61°C, with a final extension at 61°C for 7 min upon completion of the 25 cycles (Aminov et. al., 2002). Thermal cycler conditions for the mex gene primers and their PCR were as follows: one pre-denaturation for 5 min at 95˚C and 30 cycles of denaturation for 45 s at 95˚C, annealing for 45 s at 54˚C and extension for 30 s at 72˚C, followed by final extension for 10 min at 72˚C (Poonsuk and Chuanchuen, 2014).

Gel electrophoresis was completed after the PCRs were ran. The gels were comprised of a 1.5% agarose gel with 1 to 2 drops of ethidium bromide (for DNA fragment visualization) to determine if the genes were present in the samples. The gels were placed in a BioRad UV light imager (BioRad GelDoc XR+) to view the presence of bands for the genes of interest. If the

bands appeared once, whether faint or clear, the samples were analyzed again to confirm their size and presence.

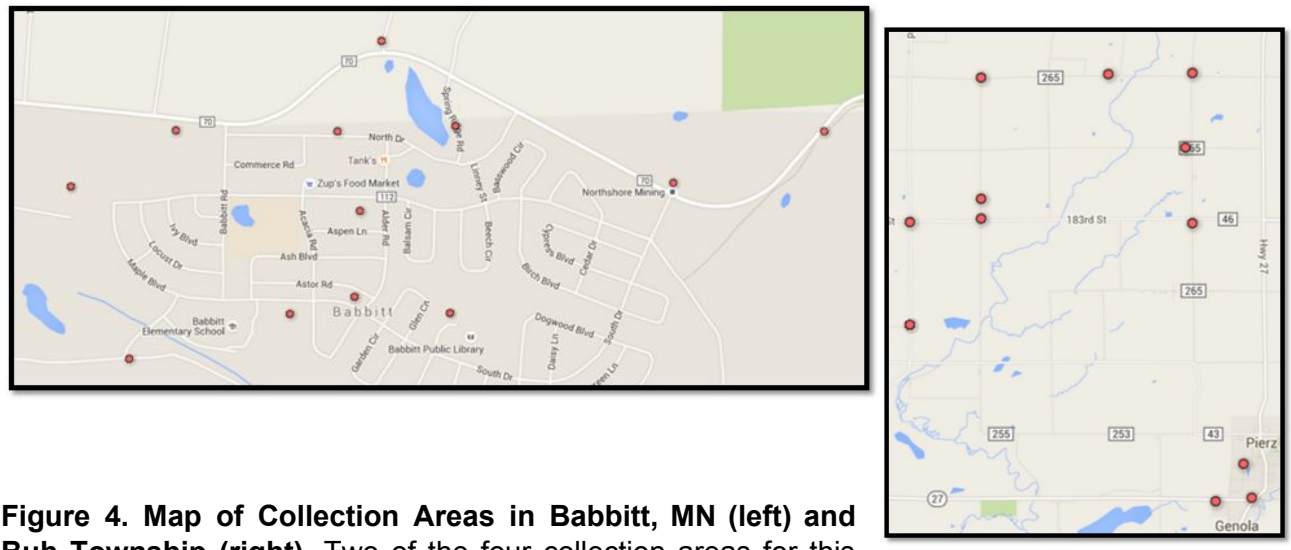

Figure 4. Map of Collection Areas in Babbitt, MN (left) and Buh Township (right). Two of the four collection areas for this project. The exact sites (red dots) were chosen due to ease and legality of access. The two other collection sites have similar maps for their respective cities (Morris, Ely, Babbitt, Buh County, Grand Marais, and Long Prairie, MN). All cities had relatively equivalent populations when compared from high to low iron soil and year to year.

Results and Discussion

Soil samples were collected from the various locations within the areas assigned as high and low iron concentrations, and samples were plated to measure resistance levels to ampicillin (2015) or to tetracycline (2016). As shown in **Figure 5a**, there was a greater proportion of ampicillin (Amp) resistant bacteria in soils with high ferric iron concentrations (p = 0.002).

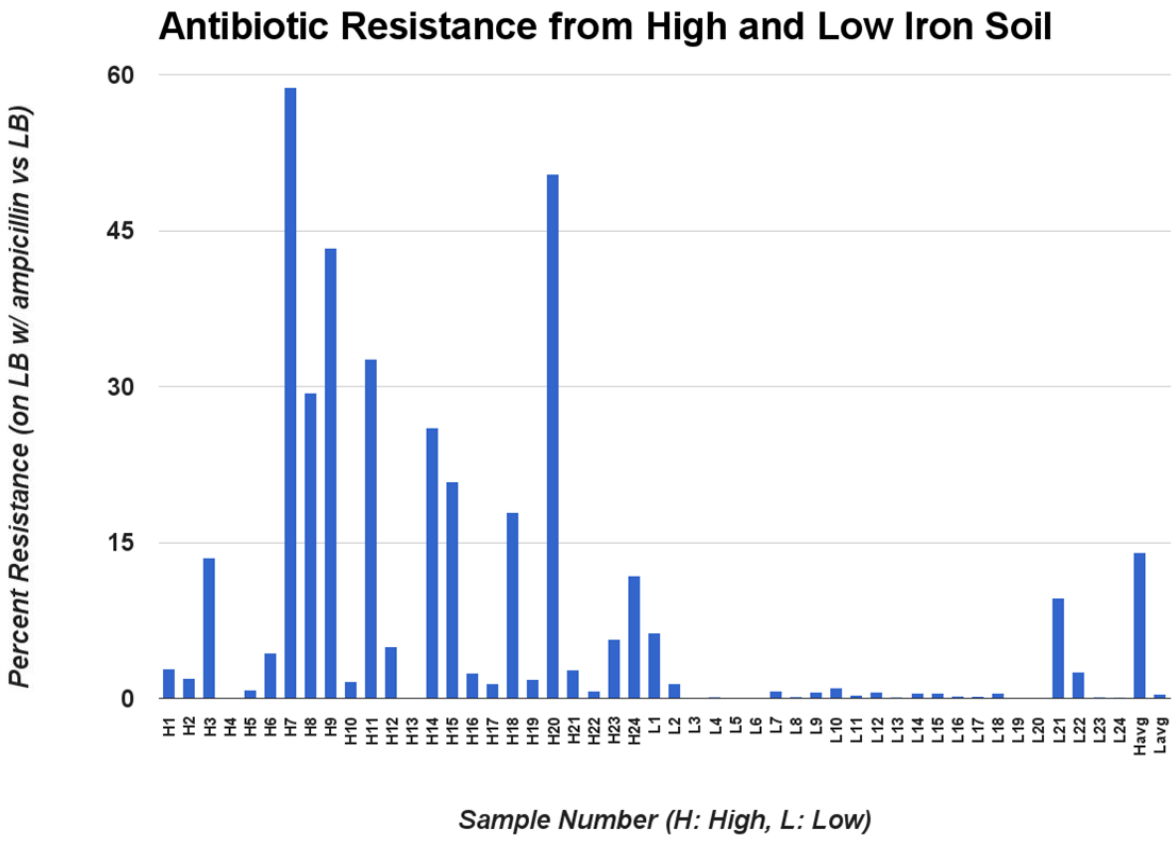

***Figure 5a*. Proportions of Antibiotic Resistance in High and Low Iron Soil, 2015.** Samples were plated on LB nutrient plates and LB nutrient plates with ampicillin (0.05g/500mL). The proportions of resistant colony forming units were calculated. The average percent of resistance was significantly higher (p = 0.002) for high iron (14%) compared to low iron soil (1.8%). The low iron soil's average lowers to ~0.92% when the outliers (L1, L21, L22) are removed.

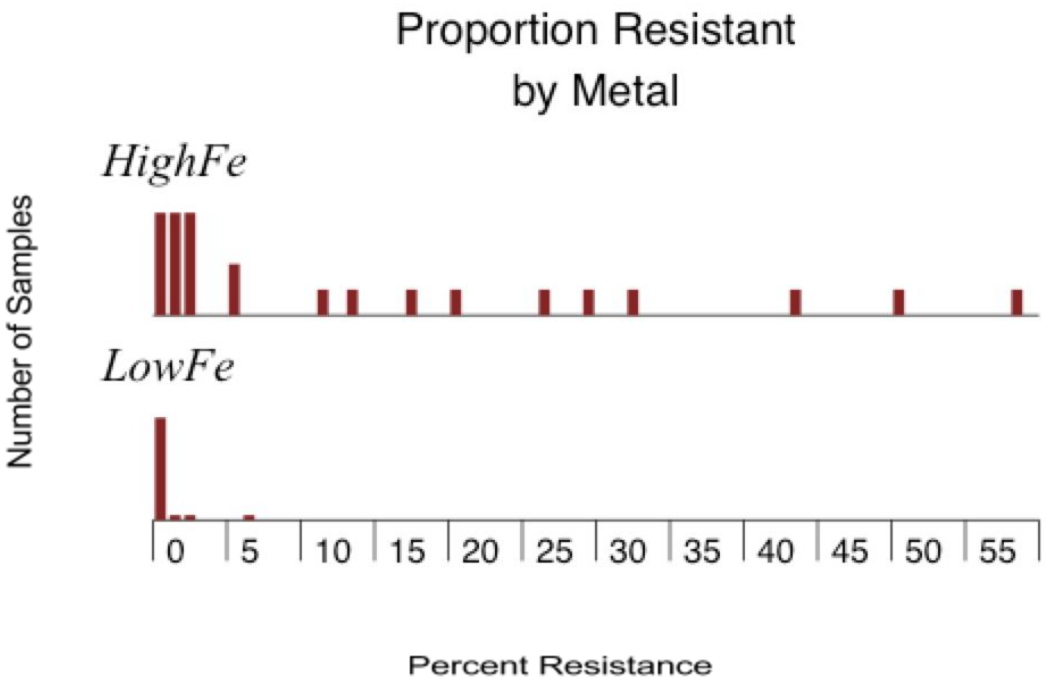

***Figure 5b*. Pattern of proportions of antibiotic resistance in correlation to iron concentrations, 2015.** Mean resistance is significantly different between the two iron concentration levels ($p < 0.001$; Kolmogorov-Smirnov test)

Figure 5b shows the significant difference in the distribution of proportion of resistance from high to low iron. Additionally, there were no significant differences based on land usage (Equal Means ANOVA; $p = 0.435$).

From the 2016 collection data, there was not a significantly greater proportion of antibiotic resistant bacteria in soils with high ferric iron concentrations (**Figure 6a**). Specifically, average antibiotic resistance to ampicillin was not significantly lower ($p = 0.75$) in low iron soil than it was in high iron soil. Also, the average antibiotic resistance to tetracycline (Tet) was not significantly lower ($p = 0.964$) when tested with the t-test in low iron soil than in high iron soil. However, as **Figure 6b** shows, the distribution of percent Tet resistance was skewed.

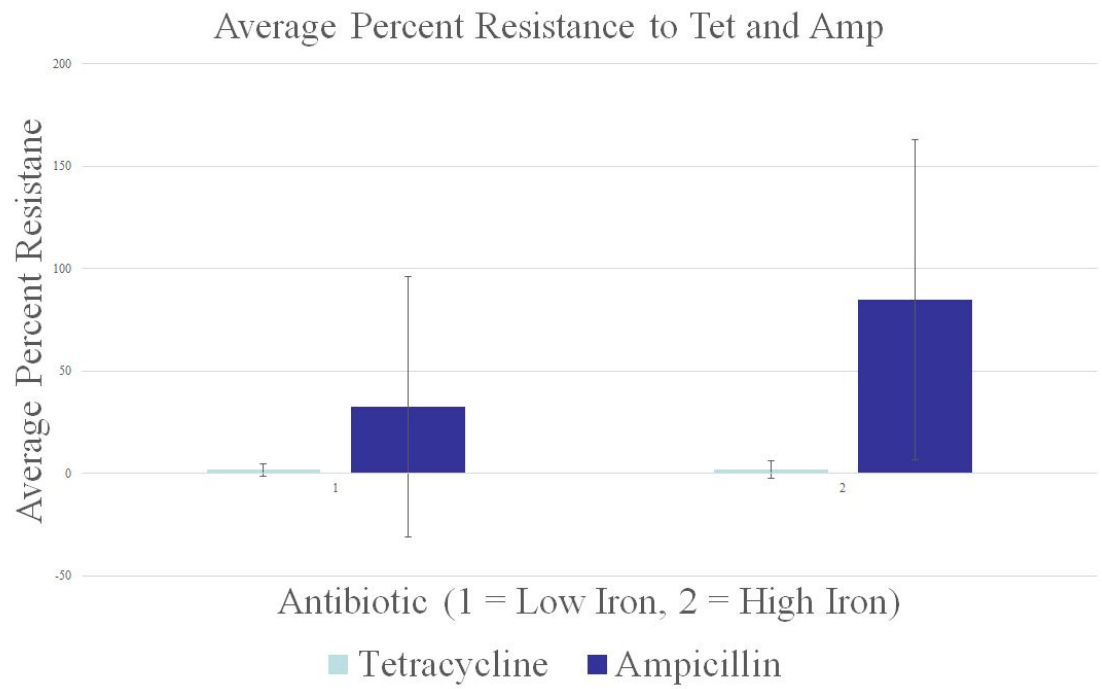

***Figure 6a*. Proportions of Antibiotic Resistance in High and Low Iron Soil.** Samples were plated on LB nutrient plates and LB nutrient plates with tetracycline (0.1g/500mL) and with ampicillin (0.05g/500mL). The proportions of resistant colony forming units were calculated. The average percent of resistance to tetracycline was not significantly different (p=.75) for high iron (1.79%) compared to low iron soil (1.76%). The average percent resistance to ampicillin was not significantly difference (p= .63) for low iron (32.59%) compared to high iron (85.11%). Outliers were removed.

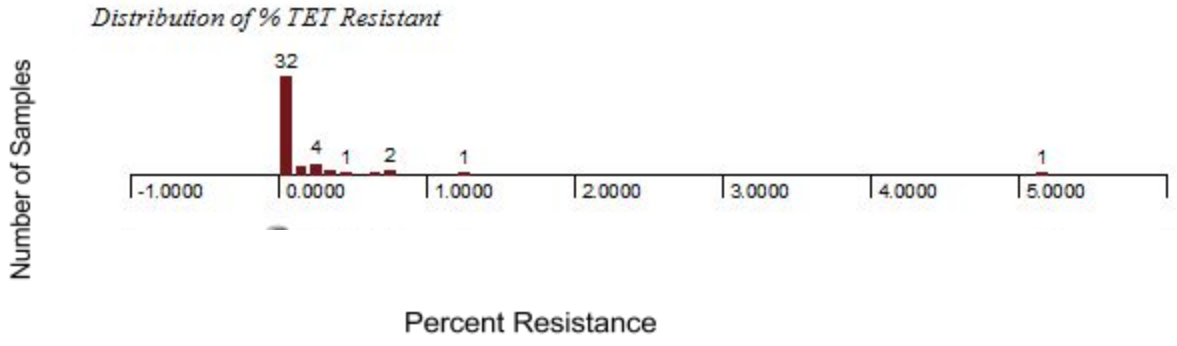

***Figure 6b*. Distribution of Overall Percent Tet Resistance (2016).** All samples were under 1% resistant except for noticeable two outliers. One outlier was slightly above 1% and one was above 5% resistant.

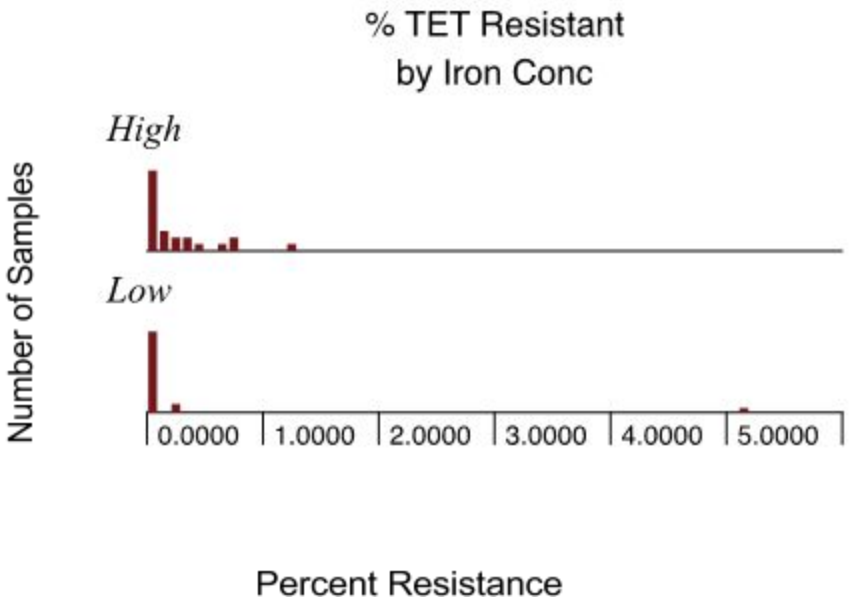

***Figure 6c*. Distribution of percent Tet resistance by iron concentration.** There is a significant difference between the distribution of tetracycline resistant colonies collected from high iron and low iron areas ($p < 0.001$).

Even though the means between concentrations are not significantly different, the distributions of Tet resistance between high and low iron, analyzed using the Kolmogorov-Smirnov test, were significantly different ($p < 0.001$). A distribution of the Tet resistance by iron concentration aids in visualizing this difference (**Figure 6c**).

Soil analysis conducted by the Research Analytics Lab at the University of Minnesota in St. Paul was completed on four randomly chosen samples both summers. Two samples were from the high iron soils and two were from the low iron soils, as indicated by the crude measurements achieved via the LaMotte Soil Kit. The analysis provided the concentrations of 14 other heavy metals in addition to iron. Certain metals showed similar high and low trends following the iron concentrations (**Tables 1 and 2**).

Heavy Metal Concentrations from Four Samples from 2015

Sample	High 1 (Sample 12)	High 2 (Sample 23)	Low 1 (Sample 9)	Low 2 (Sample 19)
Average Fe (lbs/acre)	15		0	
Fe (ppm)	17700.5	12757	4530.2	8011.4
Al	9949.8	8.94	48.4	3754.4
B	8.29	5494	48.4	5.77
Cd	5677.2	0.01	3724.5	20215
Ca	0.036	21.289	0.01	0.107
Cr	14.662	21.289	10.609	7.054
Cu	14.7405	31.206	9.969	2.734
K	386.175	957.69	471.45	393.33
Mg	8079.25	7491.4	2911.4173	8275.9
Mn	225.09	484.53	173.62	187.6
Na	1041.55	416.53	266.99	124.99
Ni	43.289	27.299	8.958	6.215
P	247.07	802.35	229.2	223.57
Pb	4.0055	19.259	4.062	3.907
Zn	28.7405	76.213	28.495	20.037

***Table 1*. Metal Concentrations in Soil, 2015.** The crude iron test (LaMotte) gave results in lbs/acre (shown in blue). The soil analysis completed by the University of Minnesota shows results in mg/kg (ppm) (shown in orange and grey). Samples tested were chosen at random from samples that specifically had iron conc. of 15 lbs/acre or 0 lbs/acre.

Heavy Metal Concentrations (ppm) of Four Samples from 2016

Sample	High 1 (Sample 11)	High 2 (Sample 18)	Low 1 (Sample 7)	Low 2 (Sample 21)
Average Fe (lbs/acre)	25439.5		10547.78	
Fe (ppm)	16587	34292	80760.55	13019
Al	8250.9	14172	3983.75	8415.4
B	5.866	5.891	13.179	10.684
Ca	4064.8	7982.2	54234.5	22792
Cd	2.634	5.531	1.25	2.255
Cr	33.149	20.210	7.6045	17.345
Cu	23.461	59.496	9.544	12.426
K	857.06	663.54	1253.65	1439.3
Mg	4204.7	8693.3	16995.5	7449.4
Mn	414.26	579.53	626.8	556.37
Na	85.307	1776.8	95.775	143.29
Ni	24.187	35.236	8.52	11.215
P	1051.10	813.74	677.88	803.12
Pb	13.054	5.701	10.017	8.960
Zn	50.722	60.570	37.319	80.868

***Table 2*. Metal Concentrations in Soil from 2016.** The crude iron test (LaMotte) gave results in lbs/acre (shown in blue). The soil analysis completed by the University of Minnesota shows results in mg/kg (ppm) (shown in orange and grey). Samples tested were chosen at random from samples that specifically had iron conc. of 15 lbs/acre or 0 lbs/acre. Some metals had high concentrations where iron was high, and low concentrations where iron was low. These include: Al, Cr, Cu, and Ni. Some metals showed opposite trends. These include: B, Ca, K, and Mg.

After completion of PCRs for half of the high iron and half of the low iron samples, gel electrophoresis results were visualized for bands on the gels correlating to various efflux genes known to be tied to efflux of Amp (2015 and 2016) or Tet (2016). An example of a positive gel band is shown in Appendix 2. Overall, efflux genes were more prevalent in areas of high ferric iron (**Table 3 and 4**). From 2015's research, only one of the eight genes appeared in low iron soil (**Table 3** and **Figure 7**). Three genes were never discovered in high iron samples form 2015 (**Table 3**).

Efflux Genes present in samples from 2015

Sample #	acrB	acrD	acrF	emrB	emrE	mdfA	tehA	yhiV
H1								
H2								
H3								
H10		x	x				x	
H12								x
H14								
H16								
H18								
H19								
H21								
H22						x		
H23								
L1								
L2								
L5								
L8								
L11								
L12								
L14								
L15			x					
L18								
L19								
L21								

***Table 3*. Efflux Genes Present in High (H) and Low (L) Iron Soil, 2015.** PCR and gels were ran for half of all samples collected (chosen randomly). If bands appeared once, those samples were ran again to raise confidence. 5 efflux pump genes were seen in high iron soil, but only one efflux gene was present for low iron soil.

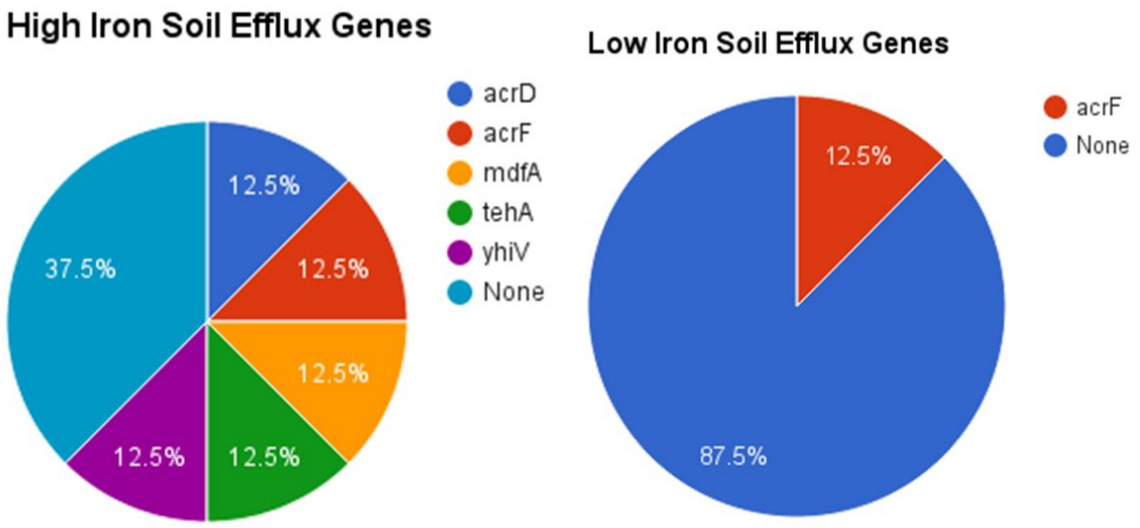

***Figure 7*. Efflux Genes Present in Soil Bacteria, 2015.** In high iron soil bacteria, five of the eight efflux genes were present from at least one sample in gels ran for the samples from Babbitt and Grand Marais, MN. For low iron soil bacteria, one efflux gene appeared. Overall, efflux genes acrB, emrB, and emrE never appeared in any tested samples.

Five of the six efflux genes from 2016's research were present in samples from high iron, while only one of the six efflux genes was present in samples from low iron areas. Four of six genes were present in samples from low iron soil. (**Table 4** and **Figure 8**). TetC was the most common efflux gene (detected in 16 of 24 samples) (**Table 4**). Five of six efflux genes that were investigated were present in at least one sample, as shown in **Table 4**.

Efflux Genes present in samples from 2016

Sample #	tetC	tetH	mexB	mexD	mexF	mexY
L1	x					
L4	x				x	
L5	x					
L6	x					
L7	x					
L10	x					
L12	x					
L15	x		x	x	x	
L16	x		x	x		
L20			x		x	
L21	x			x		
L24	x				x	
H1	x		x	x		
H4			x			
H9	x			x		
H12				x	x	
H14	x			x		
H16	x	x				
H18				x		
H20	x					

Table 4. Efflux Genes Present in High (H) and Low (L) Iron Soil, 2016. PCR and gels were run for half of all samples collected (chosen randomly). If bands appeared once, those samples were run again to raise confidence.

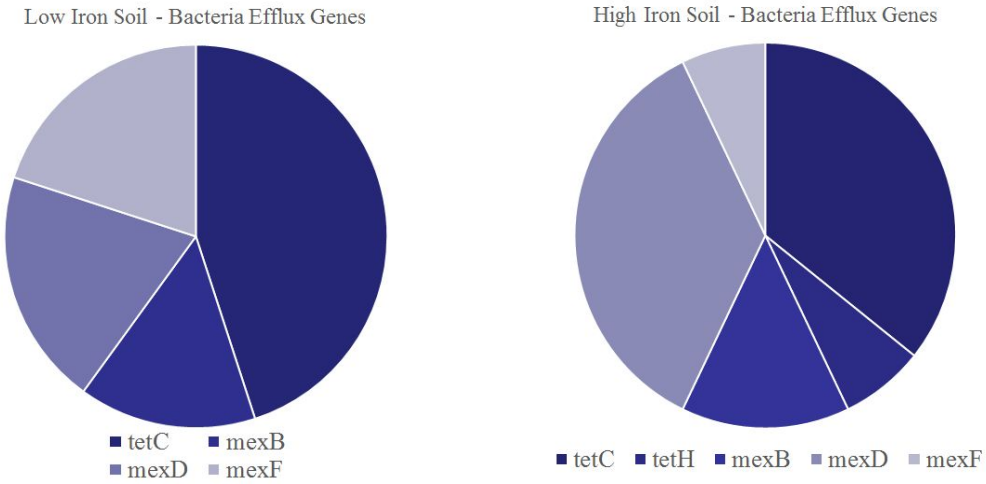

***Figure 8*. Proportion of Efflux Genes Present in Soil Bacteria, 2016.** In high iron soil bacteria, five of the six genes were present from at least one sample in gels ran for the samples from Ely and Two Harbors, MN. For low iron soil bacteria, four efflux genes appeared. Overall, efflux gene mexY never appeared in any tested samples.

Discussion

In previous research from Poonsuk and Chuanchuen (2014), the Mex efflux genes researched in this study were detected in samples from clinical isolates, and now this project expands on these findings by showing that these genes also exist in non-clinical, public environments. Tet-related efflux genes studied by Aminov et. al. (2002) percolated into the groundwater samples from animals fed with antibiotics and were detectable in the laboratory. Thus, the Tet genes studied in this research that were found in ground samples were not necessarily a surprising finding. Aminov et. al. (2002) hypothesized that the Tet genes are capable of mobility and are typically persistent in the general environment. The implications of this involve how the genes studied in this research could potentially be taken up from the environment by humans and potentially cause antibiotic resistant infection that would be difficult

to treat. The genes could be transferred from environment to human via soil particles, animal fecal matter, or general plasmid dispersal (Udikovic-Kolic, et. al., 2014).

Interestingly, there were noticeably high levels of aluminum, calcium, and magnesium when iron was also high in the soil samples analyzed by Research Analytical Laboratory at the University of Minnesota. It would be interesting to investigate the pathways in which these metals interact with bacteria and to see what levels are toxic to individual bacteria species. Perhaps these metals play a role in increasing antibiotic resistance, as iron has been seen to do as well (Knapp et. al., 2011). Furthermore, these findings sparked the question: What specific level(s) of iron are toxic to certain bacteria species? This was not a focus of this project but could reveal novel findings pertinent to the field of antibiotic resistance.

It is curious why not all of the efflux genes tested for were not seen to be present in every sample. There exists no overarching and well-defined consensus relating to the function of antibiotic resistant genes in the soil microbiome (Allen et. al., 2010). The pattern of resistant genes observed here and the variation between samples have no obvious, clear explanation. This may be due to the different bacterial ecosystems playing a causal factor, or the fact that the ability to efflux may be innate simply more frequently in some species compared to others. Additionally, Arabestani et. al. (2015) note that certain organisms have the ability to develop multi-drug resistance through efflux pump mechanisms at increased rates. This research did not sequence the samples using 16s RNAseq methods due to how the DNA extracted from the soil contains a mixture of all bacterial species (not just those that were culturable). Next generation sequencing is also not applicable, as it would not show which species the antibiotic resistance genes were from specifically.

In conclusion, this study has demonstrated that antibiotic resistance to ampicillin and tetracycline is distributed in the soil microbiome of Minnesota. Rates of resistance can vary

widely from location to location, however, the scale to which they vary is not well-defined. In the research completed in 2015, there was a significant difference in the rate of resistance between high and low iron soils. There was no specific correlation between the rate of resistance and the ferric iron concentrations of the soil from research conducted in 2016. However, there was a significant difference in the pattern of Tet resistance rates for samples collected from areas of high and areas of low iron soils. This research shows that iron content may play a role in influencing resistance gene content, yet further studies must be completed to confirm this notion. Lastly, Tet resistance efflux genes were found in samples from both high and low iron soil. At least one Tet resistance gene was present in 83% of samples analyzed, indicating that these genes are widely distributed and fairly common in soil bacteria.

Overall, this research supports that antibiotic resistance genes for tetracycline and ampicillin are widely distributed in the soil, and that iron levels may have an influential role in the levels of the genes in the natural environment.

Future Directions

Future research should include a collection of a broader range of iron level soils in attempts to define correlations more specifically. Also, it could be advantageous to conduct a detailed metal analysis and explore correlations with all heavy metals (see **Table 1, Table 2**). Perhaps other metals have a more influential role in promotion of resistance than iron. For example, Seiler and Berendonk (2012) found that contamination with mercury, cadmium, copper, and zinc play potential roles in co-selecting for antibiotic resistance in agriculture and aquaculture environments.

Testing resistance at various levels of the bacteria biome environment may benefit this field of research. For example, future research could involve taking a vertical core or horizontal

sample of the soil (e.g. 100 mm in length) and sampling every centimeter to examine if resistance levels, as well as efflux gene presence, change significantly in such short distances.

Lastly, testing on other antibiotic plate types (e.g. sulfonamides, streptomycin, oxymycin) could reveal further insight into resistant patterns. In conjunction with this, testing for the presence of other resistance genes would aid in mapping out what types of resistance exist in certain locations.

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Summer 2015

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Appendix 1.

Collection Site Geographic Location Data (15 - collected in 2015, 16 - collected in 2016, H - high iron soils, L - low iron soils)

Site	Latitude	Longitude
15H1	47.709	-91.945
15H2	47.714282	-91.945819
15H3	47.708489	-91.940447
15H4	47.708459	-91.948088
15H5	47.707018	-91.95576
15H6	47.712531	-91.958565
15H7	47.711742	-91.944753
15H8	47.714316	-91.953548
15H9	47.717178	-91.943721
15H10	47.71447	-91.940195
15H11	47.712657	-91.929831
15H12	47.714282	-91.922614
15H13	47.453805	-90.195641
15H14	47.45211	-90.1953364
15H15	47.452424	-90.194998
15H16	47.458489	-90.2021919

15H17	47.458564	-90.2023212
15H18	47.452039	-90.2143999
15H19	47.444575	-90.202949
15H20	47.445469	-90.2031445
15H21	47.44558	-90.1959473
15H22	47.452635	-90.1953897
15H23	47.458662	-9020242664
15H24	47.435222	-90.2621037

15L1	46.04191	-94.127325
15L2	46.05727	-94.125336
15L3	46.056979	-94.149939
15L4	46.05625	-94.187419
15L5	46.031472	-94.20637
15L6	46.005899	-94.208541
15L7	46.026759	-94.198967
15L8	46.027508	-94.187422
15L9	46.026514	-94.12535
15L10	45.970587	-94.108028
15L11	45.969838	-94.118617

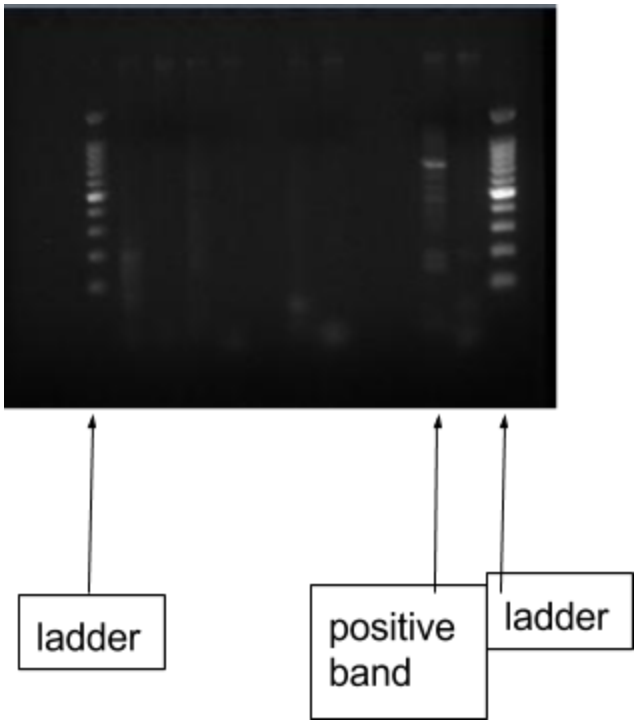
15L12	45.97744	-94.110226
15L13	45.896296	-94.751211
15L14	45.904458	-94.859856
15L15	45.905227	-94.870992
15L16	45.911594	-94.873919
15L17	45.919081	-94.865376
15L18	45.93297	-94.864845
15L19	45.96599	-94.861367
15L20	45.979944	-94.862215
15L21	45.988247	-94.864901
15L22	45.998353	-94.864764
15L23	45.030871	-94.866224
15L24	46.005122	-94.896935
16H1	47.901317	-91.871284
16H2	47.898639	-91.868447
16H3	47.891321	-91.8674
16H4	47.899885	-91.86087
16H5	47.878991	-91.849091
16H6	47.904266	-91.840196
16H7	47.906129	-91.854504

16H8	47.906691	-91.861111
16H9	47.912537	-91.860332
16H10	47.906385	-91.87711
16H11	47.903087	-91.855428
16H12	47.901376	-91.835243
16H13	47.024536	-91.661544
16H14	47.029348	-91.671026
16H15	47.032897	-91.671389
16H16	47.026613	-91.680591
16H17	47.040513	-91.680312
16H18	47.023286	-91.685481
16H19	47.024918	-91.698264
16H20	47.022441	-91.694769
16H21	47.01793	-91.698508
16H22	47.011153	-91.706286
16H23	47.020991	-91.672397
16H24	47.019005	-91.668716
16L1	45.558679	-95.539127
16L2	45.55949	-95.538919
16L3	45.587265	-95.526746

16L4	45.560186	-95.53953
16L5	45.615065	-95.717145
16L6	45.611015	-95.799677
16L7	45.591796	-95.887661
16L8	45.590579	-95.899559
16L9	45.580044	-95.903413
16L10	45.584818	-95.904579
16L11	45.633263	-95.96855
16L12	45.71627	-95.767045
16L13	45.935547	-95.488148
16L14	45.904471	-95.45048
16L15	45.894426	-95.403849
16L16	45.890789	-95.376415
16L17	45.898745	-95.370201
16L18	45.910159	-95.374897
16L19	45.895996	-95.352779
16L20	45.862519	-95.378363
16L21	45.845602	-95.386561
16L22	45.848541	-95.362556
16L23	45.875708	-95.355145

16L24	45.835207	-95.153897

Appendix 2.



Example gel result image. Shows one positive band and two ladders. For a band to be deemed positive it had to be clearly visible and located at approximately the correct base pair size listed in past literature. Not shown: the ladder's base pair size key.