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A Preliminary Investigation of Fecal Contamination in the Silver Creek Watershed

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A Preliminary Investigation of Fecal Contamination in the Silver Creek Watershed

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By

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Mentors

Drs. Sheila Pressley and Jason Marion

Environmental Health Science

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ABSTRACT

This preliminary research strives to determine if a non-point source of contamination is contaminating a stream in Berea, Kentucky. The cattle feedlot is discharging runoff into the Silver Creek Watershed. I analyzed samples from Bogie Creek, John Ballard stream, and Silver Creek in order to determine the amount of phosphorus, nitrates, ammonia, and *E. coli* colony forming units present at each sample site. Precipitation and its effect on the samples was also considered. Bogie Creek and the other sample sites all exceeded EPA's recreational Maximum Contaminant Levels in Phosphorus, Total Nitrogen, and *E. coli* colonies. Nitrate, ammonia, and precipitation were all significantly associated with logCFUs with an R^2 value of 95.47%. Polymerase Chain Reaction (PCR) was also performed in order to determine the presence of Vancomycin Resistant Enterococci (VRE). According to PCR, universal bacteria were present but not VRE in our pure culture isolate from the VRE plate. PCR was not conducted on bovine specific mitochondrial DNA or enterococci. The research stresses the need for a more in depth study of the Silver Creek watershed and concrete remediation measures be taken.

Keywords: Thesis, Silver Creek, fecal, contamination

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INTRODUCTION

A property owner in Berea, Kentucky filed a complaint with local lawyer, Thomas Fitzgerald, concerning the discharge of a neighboring farm. The complainant's property borders Bogie farm and she presented concerns that the cattle farm was discharging contaminants into Bogie creek, a small creek adjacent to her property. After receiving the complaint, Fitzgerald contacted Dr. Jason Marion to seek a preliminary investigation of the creek. Upon reaching Bogie farm, Dr. Marion and I discovered that the stream connecting the two properties was an intermittent stream. The stream needed a rain event to occur in order for it to flow heavily enough to collect viable samples.

There was also the problem concerning the nature of the runoff from Bogie farm. While it flowed in a single stream from the farm, rocks covered the exit of the stream into Bogie creek, making it difficult to collect a significant amount of sample directly from Bogie farm's runoff. In consideration of this, samples would be collected from Bogie creek as well as the two ditches that flowed perpendicular to and in to Bogie creek. Bogie

creek's contaminants are the result of the combined contaminants of the two perpendicular ditches and the runoff from Bogie's farm.

In order to begin to understand the impact of Bogie farm on the Silver Creek watershed, samples were also collected at John Ballard stream which Bogie creek flowed into, and upstream and downstream of John Ballard's connection with Silver Creek. These sites were easily accessible by land and would give us a general idea of the level of pollution in one of Berea's and Central Kentucky's watersheds.

There is a great need for analyzing the watersheds throughout Kentucky. This need stems from the uses of the watershed. Although most of Silver Creek is not in use for drinking water, people still interact with the water through fishing or swimming. Furthermore, this is the same water that is being used by fish, domesticated animals, and to water our crops. The Environmental Protection Agency has standards for the amount of contaminants allowed in drinking water; it also has requirements for water that only has a recreational use designation, due to the potential for accidental ingestion, potential for infection of open wounds or cuts, or contamination of food or food products.

For bacteria that live in the guts of animals and can be expelled through feces, EPA has a Maximum Contaminant Level (MCL) for surface waters because even if water is not being used specifically for drinking, it can be ingested and cause harm. The EPA also regulates phosphorous and nitrogen, two nutrients that can also be found in abundance in fecal matter.

Phosphorus and nitrogen can damage the ecosystem of the water and be harmful to plants and animals that use it. The nutrients and bacteria affect the fish that residents will catch and eat or the water that they will use to water their farm and garden. It is

important to know how contaminated waterways are because this impacts the safety of food products. It also affects the way water should be treated if it is to be used for different purposes. While Bogie creek does not have any large wildlife using it, it is a tributary to John Ballard and Silver Creek which both support diverse aquatic and terrestrial ecosystems.

In 2011, a permit was issued to Bogie Farm which described their operation as an Animal Feeding Operation (AFO). This classification stipulates that the farm can have, at a maximum, 550 cattle on their premises (1). They were issued a Kentucky No Discharge Operational Permit (KNDOP), meaning that they were not permitted to discharge wastewater directly into a stream. It also mandated that no point source discharge of wastewater was allowed.

Point source pollution occurs when an operation releases waste in a concentrated, single stream or identifiable expulsion point. Non-point distribution is less easily identifiable as it could be dispersed though a wide area and is more difficult to identify the cause of the contamination. Non-point source pollution could be caused by agricultural runoff, urban runoff, domesticated animal waste, or wildlife waste.

In the scope of Bogie farm, this permit translated means that the farm was not allowed to expel waste directly into Kentucky's waterways. Furthermore, according to the permit, Bogie farm was to reduce the waste on its farm so that potential runoff would not be significant enough to contaminate Kentucky's water systems.

LITERARY REVIEW

Fecal Indicator Bacteria

While *Escherichia coli* and Enterococci are often used to detect the presence of fecal material, another fecal indicator bacteria (FIB) that has been used in studies is *Clostridium perfringens*. In one study utilizing this FIB, researchers analyzed the sediments in streams and rivers in order to determine if they had growth of different strains of *Clostridium*. They concluded that the ability of sediments to provide suitable habitats for bacteria depended on such seasonal attributes as temperature and precipitation as well as by the type of waste in the area. *C. perfringens* and *C. bifermentans* were both commonly present in wastes that derived from agricultural sewages, making them possible FIB to test for if further research was conducted (2).

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR), a test utilized in order to identify DNA strands, is frequently performed in order to identify the type of gut bacteria in the fecal material.

One important study was conducted in Australia concerning the presence of pathogens in bovine fecal matter by Marcus Klein, Leearna Broawn, and Robyn Tucker,

et al. This study specifically utilized qPCR to analyze bovine fecal material for 10 pathogens. When looking for the presence and frequency of DNA for these organisms, the researchers found a prevalence of pathogenic *E. coli*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Cryptosporidium* spp. and *Giardia* spp. in fresh feces, pen manure, harvested manure, and aged manure (3). The presence of so many pathogenic organisms in the manure of feedlot cattle illustrates the dangers of fecal contamination.

A report by Alexander Schriewer, Woutrina Miller, et al. examined the correlation between pathogen presence and fecal indicators; the authors investigated whether qPCR *Bacteroidales* assays or fecal indicator bacteria were more effective at predicting pathogen presence. They found that human fecal contamination was a more common fecal source than dogs or livestock. Again, the weather and seasons of California must be taken into consideration when evaluating this source. Traditional means of testing for fecal indicator bacteria—such as the growing of *E. coli* in cultures—have several problems not already considered. Aside from lack of host specificity, they also can multiply outside of the host body, and their absence does not prove an absence of pathogens as well. This report suggests that cattle samples using *Bacteroidales* as their genetic marker have a low probability of being present in positive testing samples, due to the similarities between them and horses. This is something which I will have to consider in relation to horses and deer, two potential grazers which might have similar *Bacteroidales* spp. living in their gastrointestinal tract. The authors postulate that qPCR was a more accurate way of predicting potential pathogens in a water source; this lends itself to the rational of performing these tests as well in determining the source of pollution (4).

QPCR, or quantitative PCR, does not require incubation and colonization of enterococci colonies in order to run a PCR assay. Unlike standard PCR, it does not require colonies to be grown before being processed as a PCR assay. It is a faster process that occurs in real time as, instead of the 18 or more hours it takes to cultivate enterococci colonies, it can take anywhere from 30 minutes to 4 hours to process. It is also more sensitive than enumerating *E. coli* cells because it can identify both cultivable and non-cultivable bacteria as long as it contains viable DNA. As *E. coli* counting is currently, viable, non-culturable cells cannot be grown on plates despite their viability at contaminating food or harming organisms.

Phylogenetic Microarray Analysis is used to determine the multiple bacteria present in a given sample and then match that up to a specific source. This method utilizes PCR to amplify the amount of DNA available, and then detects specific identifier bacteria which are only present in specific species and indicates which species are the source of the DNA. According to a study by Dubinsky, E. A., et al., *Clostridia*, *Bacilli*, and *Bacteroidetes spp*. were found in most of the grazing animals' samples. Human samples held other bacteria (5). Identification of bacteria and the differences between those found in humans and grazing mammals could help determine which bacteria should be focused on in future PCR analyses.

Other PCR methods use mitochondrial DNA (mtDNA) which is found in fecal material because animals shed the cells that form the lining of their digestive organs, such as the stomach lining, when they excrete other nonessentials. This differs primarily from other research in that most PCR assays are performed in order to find the DNA of gut microorganisms and bacteria as opposed to the DNA of the animal in question. This

relates to the research as it is a potential different manner of testing in order to find the same results. Studies have discovered that this testing has a high sensitivity and performs decently in identification of source even when there are more than one source in a given sample (6).

A 2008 study by William Schill and Melvin Mathes, agreed that testing for the presence and type of mtDNA a similarly viable option. They conducted multiple studies which led to an increasingly large data base from which to analyze and understand their research. They found that there was again a high specificity and selectivity to this PCR method and that cross-overs were detected.

This is particularly interesting in that it means that testing for mitochondrial DNA using PCR could result in information concerning the possibility of multiple sources. Waste from cattle feed lots, migrating birds, or humans could be detected and differentiated from each other, thus pointing to the source of the majority of the fecal contamination. A potential problem with this is that if DNA was present in too high amounts, it could inhibit their ability to discern other sources of DNA, this would still reveal what the main animal source is. The authors also consider the effect of other pathways from the animal to the water. This may explain why there likely would be more mtDNA than could be explained by fecal contamination alone (7).

Variables to Consider

Factors such as the rate of decay of the bacteria should be taken into consideration in order to determine the distance the fecal matter has traveled. It was discovered that

although sunlight does not have an impact on the rate of decay, temperature does. It is imperative to record such data as temperature so as to assess the amount of decay that has occurred. The rate of decay helps determine the amount of time spent traveling.

Weather and temperature are two components that scientists take into account when designing research studies in this area. Researchers Satoshi Okabe and Yoko Shimazu theorized that temperature and salinity of water would have a significant effect on the host-specific *Bacteroides-Prevotella* 16S rRNA genetic marker. They utilized four different strains of the genetic marker found in human, cow, pig, and one found in all three, and measured the amount and type using real time qPCR. The report explains that real-time qPCR simply means accessing the information from both PCR and qPCR methods at the same time; being able to identify the type and the amount of a strand of DNA. The authors determined that lower temperatures brought down the rate of decay in all four strains of bacteria. Salinity had no effect on the rate of decay. The researchers speculate that other factors such as predation and degradation were delayed due to colder temperatures (8). The possibility of competing microorganisms is something to consider, as well as the need to keep samples cold when not running tests on them. A delayed decay of genes might also mean that during the winter months, fecal matter is likely to stay active and dangerous longer.

A study performed in Kentucky over the last five years illustrates the impact of rainfall on water systems. During storms, the additional precipitation collects runoff and increases the nutrient and bacteria loading of a stream. Additionally, when a sample was collected during a storm, significant changes were observed in the amount of nutrients and *E. coli* levels in the water samples. The number of *E. coli* colonies present drastically

decreased as the storms progressed, leading to the conclusion that *E. coli* had the tendency to be washed into streams first and less so as the storm progressed.

Other variables to consider in this type of research include other potential sources of fecal contamination. We were unable to determine waste management practices for the area. Septic tank systems, which are utilized in approximately 20% of homes in the United States, and onsite septic wastewater treatment systems, which account for 37% of new homes, can both be major factors in contaminating a water system (9).

Properly working septic systems are odor free and allow plenty of space for fecal material to be filtered out of water. Due to age and neglect many such systems can easily deteriorate. The Silver Creek Watershed, however, is only about 7% residential. More than 75% of its land use is agricultural, which leads to the prediction that potential fecal contamination is a result of farms in the area rather than homeowners (10).

Microbial Source Tracking

Once travel time based on temperature and other factors is assessed, it becomes easier to work backwards to determine the source. On a given day, with a set temperature, which affects the rate of decay; rainfall; and the different transportation methods; a scientist could determine how far from the sample site bacteria have traveled. Using computer based modeling it is then possible to illustrate the pathway of fecal contamination and the most likely origin.

Samples collected are often the end results of the multiple variables that impact surface water systems. When testing water for pollution, researchers must look at data

with an eye towards what they hope to accomplish. Determining that a waterway is contaminated is no use if we cannot decipher the source of the contamination. Many studies attempt to identify the variables responsible for a particular data set so that they can then establish where a source or sources are located. By analyzing the various factors influencing surface water sources, it becomes easier to create targeted management techniques that are efficient and economical.

The point of such investigative studies is to determine the source of pollution. This is best accomplished by a compilation of data that includes an evaluation of the type(s) of contamination, weather patterns, analysis of potential point sources, and possible transportation pathways. A 2011 study utilized hydrodynamic and microbiological modeling in order to determine the starting location of fecal contamination (11). The modeling used information on the rate of decay of bacteria and other fecal indicators and the transportation methods of wind and water in order to locate the source of the contamination. Microbial fecal source tracking (MST) has been performed to illustrate the primary cause of particular pollution events and has also helped improved understanding of how pollutants travel to reach various water sources.

The SWAT model is another way of tracking the flow of streams. In order to portray accurate results, it requires such parameters as soil type, precipitation data, temperature, land cover area, and the location of the streams in the area. This model also takes into account such variables as the frequency of fertilizer application, crop rotation, and erosion rate so as to predict the amount and type of bacteria traveling from farms to waterways. In order to do this, the model simulates the travel of particles to which pathogenic bacteria might adhere (12).

Microbial Source Tracking has typically been performed using only one bacteria, usually the *Bacteroidales* 16S rRNA gene marker. Yong-Jin Lee, Mariosa Molina, et al, argue that utilizing multiple bacteria assays would create a more complete picture of the source of contamination. Specifically, they suggest using 16S rRNA and cattle-specific non-16S rRNA gene markers. Aside from providing a more in depth diagram of contamination in the area, the study determined that only Bac 5 markers would be present in water samples as well as other fecal samples. This shows that, while it is important to consider other means of source tracking, some microbial markers are not viable if held in a stream for too long(13).

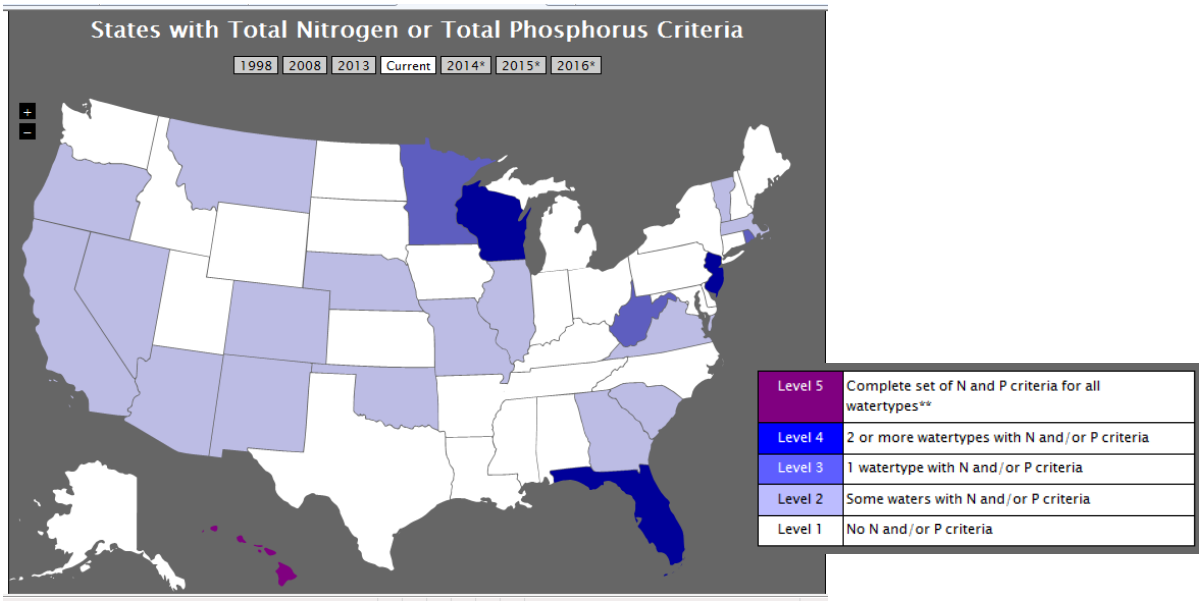

Figure 1: States with Total Nitrogen or Phosphorus Criteria

EPA's map of 'States with Total Nitrogen or Total Phosphorus Criteria' illustrates that only one state has a complete criteria for total nitrogen and phosphorus levels for all

watertypes, and that Kentucky is not alone in having none. As Kentucky has no standards for the nutrients we measured, we utilized EPA's recommended or Reference standards. This gave us the Reference standard for Phosphorus of 0.36 mg/L.

Table 1: Water Quality Assessment Status for Reporting Year 2010

Designated Use	Designated Use Group	Status
Fish Consumption	Aquatic Life Harvesting	Not Assessed
Primary Contact Recreation Water	Recreation	Not Assessed
Secondary Contact Recreation Water	Recreation	Not Assessed
Warm Water Aquatic Habitat	Fish, Shellfish, And Wildlife Protection And Propagation	Impaired

This table illustrates the designated uses and status from the 2010 Water body Report for Silver Creek 11.1 to 29.8, compiled by the Kentucky Environmental Protection Agency. This chart primarily illustrates that this section of the Silver Creek watershed has not been assessed for 75% of the designated uses for this stream, including recreational water contact. It also clarifies that the only designated use that this section of Silver Creek has been assessed for, Warm Water Aquatic Habitat, has an impaired status.

METHODOLOGY

Site Location

Our first objective was to identify several areas where we would like to collect samples. We hoped to collect from the Bogie stream itself, a larger stream of which it was a tributary, and both upstream and downstream of Silver Creek, in order to determine

the impact it held on the creek. Upon surveying the Complainant's land and the farm next to it, we were able to determine the nature of Bogie creek and devise a way to test it and the impact it may pose on waters downstream. This method included testing the two ditches lying perpendicular and the confluence location where Bogie stream was feeding into the roadside ditch. We determined that based on ease of access, our sample sites would include the ditches to the left and right of the farm; Bogie Creek; John Ballard Creek, which Bogie creek flows into; and upstream and downstream of where John Ballard enters Silver Creek.

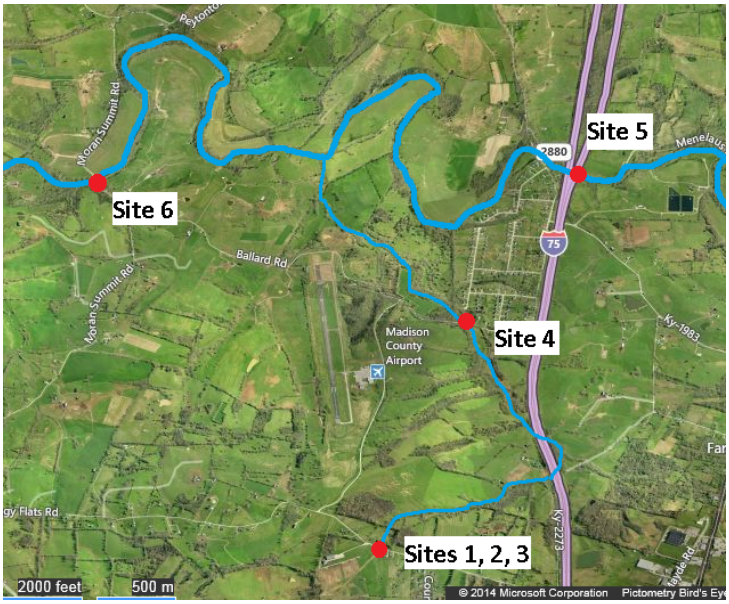

Figure 2: Map of Sample Sites

This map identifies the location of each of the six sample sites. Sites 1, 2, and 3 are located in the vicinity of Bogie Farm and includes Bogie Creek and two of its tributaries, the Ditch and Yard stream that flow into it. Bogie Creek is a tributary of John Ballard stream (also known as John Ballard Branch), which in turn is a tributary of Silver

Creek. Site 4 is located downstream from the connection point of Bogie Creek and John Ballard. Site 5 is upstream Silver Creek, located where Silver Creek crosses below the Interstate 75. Site 6 is downstream Silver Creek and is located where Silver Creek crosses under Moran Summit Road. All sample sites are in Madison County, Kentucky.

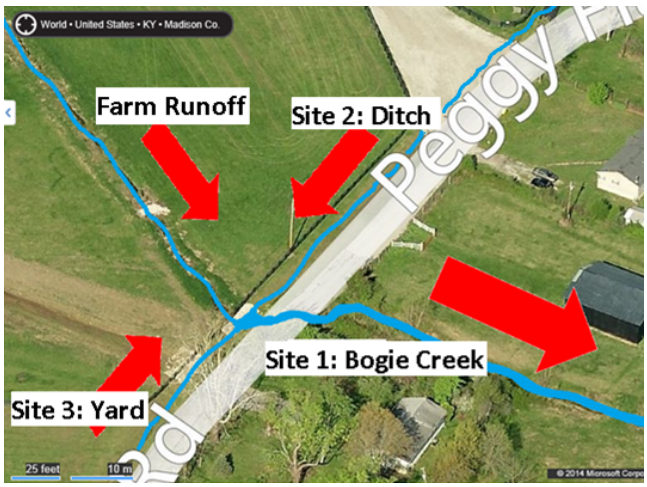

Figure 3: Map of sites at Bogie Farm

This map provides a close-up of the flow of water at the location of Bogie's Farm. The farm runoff drains directly from the farm and merges with the Yard ditch, which flows from Bogie's residence, and the Ditch, which flows from the Complainant's residence through the Bogie Farm driveway. These three ditches converge to form the mouth of Bogie Creek.

Precipitation Measurements

Table 2: Precipitation Measurements

Date	Precipitation
9/29/2013	0.00 inches
10/7/2013	3.0 inches
10/16/2013	0.25 inches
11/16/2013	0.50 inches
12/6/2013	2.5 inches
2/2/2014	0.50 inches

Due to Bogie Creek being an intermittent stream, we gathered our samples after a significant amount of precipitation had occurred (Table 2). Following periods of high precipitation, we collected two samples in WhirlPacks® from each of our six sites. Precipitation levels were important due to the intermittent nature of flow in Bogie Creek. The low levels of precipitation prevented data collection on two occasions, all other days we were able to collect from all sample sites. The intermittent nature of Bogie Creek and its tributaries prevented true random sampling as effort was focused on days with significant rainfall.

Factors analyzed

We performed two tests from each sample site to determine Total Phosphorous, Nitrate, Ammonia, and the amount of colony forming units (CFU), specifically, *Escherichia coli* colony forming units. We also tested for pH, conductivity, total

dissolved solids or TDS using the Hach conductivity meter. Further testing was conducted for the presence of Vancomycin and Ciprofloxacin resistant enterococci.

Samples were collected on September 29th, 2013, October 7th and 16th, November 16th, December 6th, and February 2nd, 2014. Information regarding each collection was recorded into Table 4. Samples were transported back to a water laboratory at Eastern Kentucky University. Tests for Nitrates, Ammonia, *E. coli* CFUs, and Phosphorous tests were all started within six hours of sample collection. All samples were held in refrigeration at 5º Celsius until they were assessed.

Phosphorus: Phosphorus was tested because an increase in Phosphorus increases the growth of algae and other plants, thus reducing oxygen levels which leads to the death of fish and other aquatic animals. In testing for the amount of phosphorous present, the USEPA PhosVer(R) 3 with Acid Persulfate Digestion Method was utilized. It involved adding the sample and Potassium Persulfate Powder to a vial then heating each vial for thirty minutes to 150º C. After cooling to room temperature, Sodium Hydroxide standard was added. PhosVer 3 Powder was then added and the vial was read on the Hach DR 2700™ Spectrophotometer for absorbance and estimated concentration.

Total Nitrogen Total Nitrogen levels are a combination of Nitrate, Ammonia, and Nitrite levels. It is necessary to test for because Total Nitrogen as it is a measure of the impact of fecal-associated sources such as fertilizer, sewage, and agricultural runoff. Specifically, Nitrate in water can cause blue baby syndrome or death in infants and Ammonia in elevated levels can cause mutations in fish organs and alter the growth rate, or cause death in aquatic life. In order to test for Nitrate levels, the Hach DR 2800 spectrophotometer was utilized using the Test-n-Tube Method for determining Nitrate by

the Dimethylphenol Method, using TNTplus vials in accordance with Hach Method 10206; and the Nessler Method was utilized to test for Ammonia levels.

***E. coli*:** We tested for the amount of *E. coli* fecal coliforms present by incubating diluted samples on modified mTEC agar plates. Samples were diluted with Dulbecco's Phosphate Buffered Saline 1x with Calcium and Magnesium. Diluted samples were filtered and incubated for two hours at 35.5º C and then for 18-22 hours at 44.5º C using the Membrane Filtration Method (EPA 1603). Magenta colored colonies were deemed to be *E. coli* colonies.

Polymerase Chain Reaction

In an effort to learn more about what type of problems may be present in fecal contaminated water sources, the samples collected on February 2nd were also tested using Polymerase Chain Reaction. To determine the abundance of drug resistant bacteria in the Silver Creek watershed the samples were tested for Vancomycin and Ciprofloxacin resistant enterococci.

Vancomycin resistant bacteria were tested for by looking at the DNA primers that would code for such an adaptation. The Universal primer pair that detects bacteria presence was also utilized. Vancomycin is an antibiotic that many farmers give their cattle so they may be more resistant to bacterial infection and for weight gain. If Vancomycin resistant genes were found using PCR, then a much larger problem would be occurring, and dire steps would be encouraged to remedy or further investigate the problem. When performing PCR, we used Primer Pairs 43 and 44 to test for VanA, 45

and 46 for Van B, 66 and 67 for VanC, 68 and 69 for VanC-2, and the Universal Bacteria Primer Pair 39 and 40.

Polymerase Chain Reaction occurred by growth of the bacteria in a media, isolation of the genes utilizing the Promega kit, and cycling the vials of DNA through varying temperatures so they replicate many times in four hours. Polymerase Chain Reaction works by increasing the temperature until it is approximately boiling; this denatures the double strands of a DNA helicase so they separate into two strands. The temperature then drops so that the Primers bind to complementary DNA brackets. The temperature then increases again so that the Enzyme Taq Polymerase adds nucleotides to the strands to create whole DNA strands (14).

An agarose gel was then created to use electrophoresis to determine the length of the strands of DNA present. Electrophoresis involves filling each of many wells in the agarose gel in with a sample, an electrical current is then run through the gel and the pieces of DNA in each sample travel across the gel from the negative to the positively charged side. Smaller pieces move further and you can therefore determine the size of a piece of DNA based on the distance it has traveled. By also including a ladder or standard in one of the wells, you can compare the samples to the standard to determine if a particular gene is present or not.

For the purpose of our tests, the collected samples were plated on HardyCHROM™ VRE Agars, (Vancomycin-Resistant Enterococci) and MacConkey Agars with Ciprofloxacin which tests for ciprofloxacin-resistant *E. coli*. The VRE agars would grow *E. faecalis* as red colonies and *E. faecium* as blue colonies. If they grew on the plates, viable colonies were selected from the VRE agars and inoculated in tubes of

traditional broth with 20 microliters of Vancomycin added. Colonies could only grow on both the plates and in the broth if they had some type of resistance gene.

Table 3: Primers Used

Primer	Gene	Name	Sequence
39	U Bac	39-27F	AGAGTTTGATCCTGGCTCAG
40	U Bac	40-1492R	GGTTACCTTACGACTT
43	Van(a)	43van(a)F	ATGAATAGAATAAAAGTTGCAATAC
44	Van(a)	44van(a)R	CCCCTTTAACGCTAATACGAT
45	Van(b)	45van(b)F	CCCGAATTTCAAATGATTGAAAA
46	Van(b)	46van(b)R	CGCCATCCTCCTGCAAAA
66	vanC-1F	66vanC123-FOR	GATGGCWGTATCCAAGGA
67	vanC-1R	67vanC1-REV	GTGATCGTGGCGCTG
68	vanC-2/3F	68vanC123-FOR	GATGGCWGTATCCAAGGA
69	vanC-2/3R	69vanC23-REV	ATCGAAAAAGCCGTCTAC

RESULTS

Table 4: Results

Date	Prec (inch)	Phos Avg	Nitrate Avg	Ammonia Avg	TN (Nit + Amm)	Spec. Cond.	TDS	pH	CFU Avg
/29/2013	0	0.955	0.621	0.11	0.731	572	274	8	68.6666666
0/7/2013	3	0.75	1.1	0.265	1.365	407	196	8	241
/16/2013	0.25	0.77	0.601	0.17	0.771	587	282	7.6	65.3333333
/16/2013	0.5	1.93	1.655	1.46	3.115	358	170	6.8	2051
2/6/2013	2.5	1.125	1.26	0.745	2.005	272	129	7.4	1734
2/2/2014	0.5	0.775	1.285	0.66	1.945	488	234	7.5	1405
/29/2013	0	0.635	0.238	0.155	0.393	493	236	8.2	39.6666666
0/7/2013	3	1.085	0.5	0.23	0.73	421	201	7.9	1940
/16/2013	0.25	0.695	0.5815	0.14	0.7215	542	260	7.9	130.333333
/16/2013	0.5	1.035	1.235	0.385	1.62	514	247	7.7	586
2/6/2013	2.5	1.605	1.565	0.785	2.35	284	135	7.3	18973.3333
2/2/2014	0.5	0.63	1.315	0.535	1.85	545	260	7.9	3198.333333
/29/2013	0	0.55	0.222	0.185	0.407	721	348	7.8	434
0/7/2013	3	0.715	0.456	0.335	0.791	456	218	7.8	2010
/16/2013	0.25	0.64	0.1315	0.175	0.3065	745	357	7.6	279.666666
/16/2013	0.5	0.935	0.977	0.845	1.822	447	24	7.9	21693.3333
2/6/2013	2.5	1.51	1.21	1.345	2.555	238	112	7.3	22916.6666
2/2/2014	0.5	0.505	1.29	0.56	1.85	641	308	7.8	1475
0/7/2013	3		0.6695	0.785	1.4545	488	239	8	123.333333
/16/2013	0.5	0.47	0.971	0.64	1.611	316	151	7.8	648
2/6/2013	2.5	0.471	1.105	1.25	2.355	206	98	7.2	9259.33333
2/2/2014	0.5	0.26	0.877	0.295	1.172	1530	747	7.6	345
0/7/2013	3		1.345	0.77	2.115	466	223	8.2	1283.33333
/16/2013	0.5	0.7	1.2	2.085	3.285	367	175	7.4	775
2/6/2013	2.5	4.95	2.28	0.885	3.165	285	135	7.5	8913.33333
2/2/2014	0.5	0.575	0.757	2.26	3.017	391	187	7.3	1625
0/7/2013	3	0.595	1.82	0.795	2.615	495	223	7.9	8000
/16/2013	0.5	1.64	4.99	1.405	6.395	535	257	6.9	141266.666
2/6/2013	2.5	2.17	1.595	1.665	3.26	210	99	7.4	7005
2/2/2014	0.5	0.585	2.205	0.63	2.835	510	244	7	7700

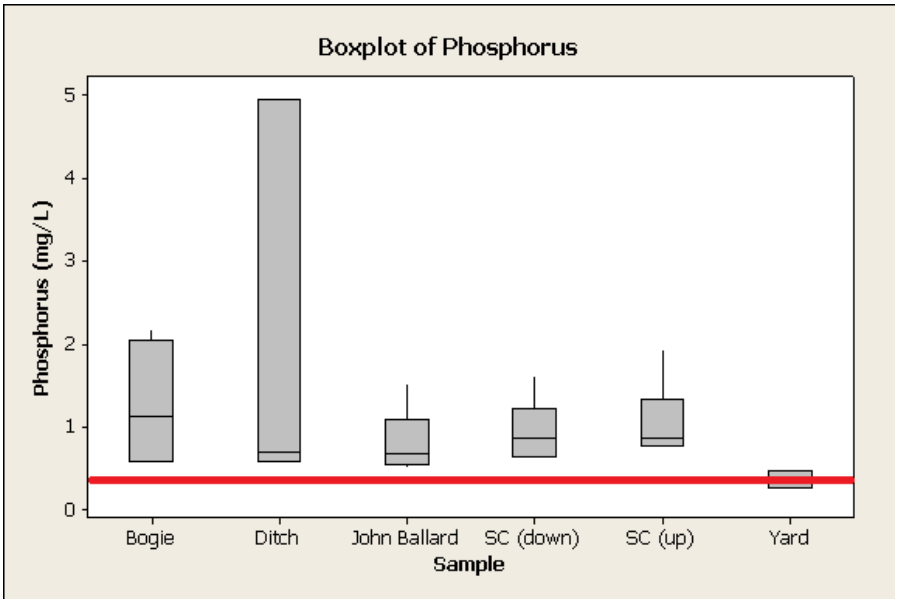

Figure 4: Phosphorus Boxplot

EPA suggests Phosphorus levels no higher than 0.36 mg/L, illustrated by the line on the Boxplot. Bogie Creek consistently had Phosphorus levels above 1 mg/L, this may have been caused by the high levels of Phosphorus in the Ditch feeding into Bogie Creek.

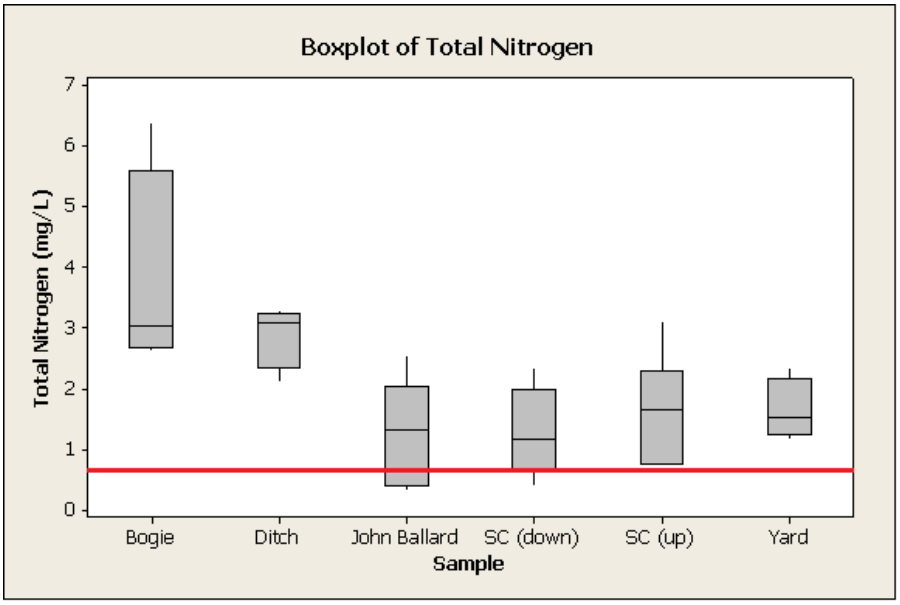

Figure 5: Total Nitrogen Boxplot

Total Nitrogen is a combination of the measurements of Nitrite, Nitrate, and Ammonia. Our data used measurements from Nitrate and Ammonia. Even with the exclusion of Nitrite, we were still able to determine that median levels of Total Nitrogen exceeded EPA's regulated Maximum Contaminant Levels of 0.69 mg/L. With the exception of a few sample days from John Ballard Creek and downstream Silver Creek sites, all of the data is above the EPA's MCL's for total nitrogen. These high levels would suggest that the farm is a source of contamination for Bogie Creek.

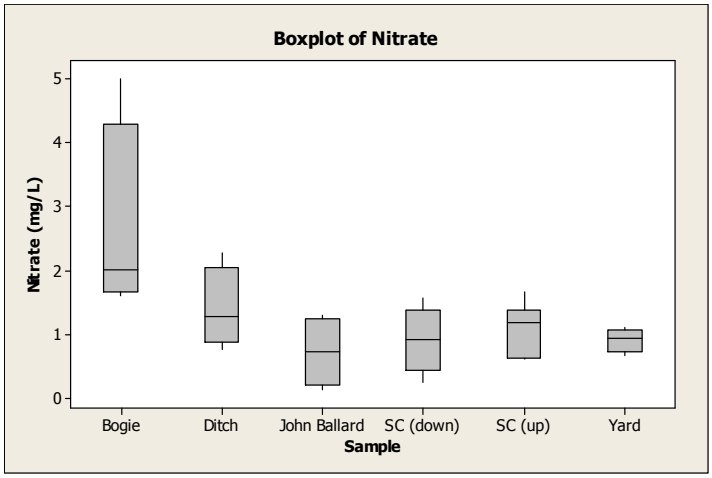

Figure 6: Nitrate Boxplot

Figure 6 illustrates how much higher the Bogie Creek data points are in comparison to the other sample sites.

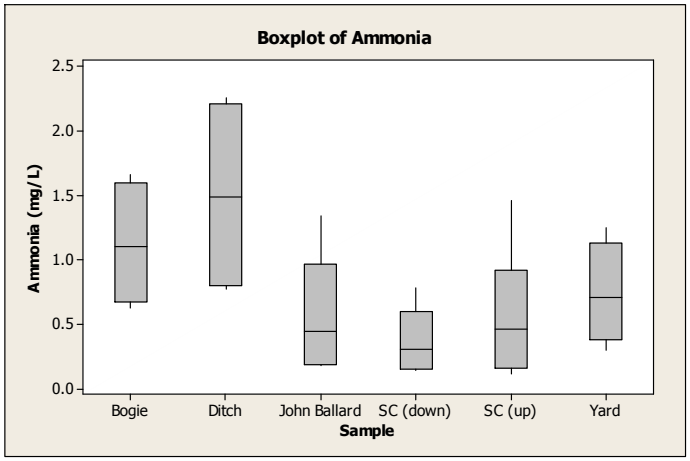

Figure 7: Ammonia Boxplot

Figure 7 illustrates that the Ditch sample site has higher levels of ammonia than others and probably had a high impact level on the Bogie Creek.

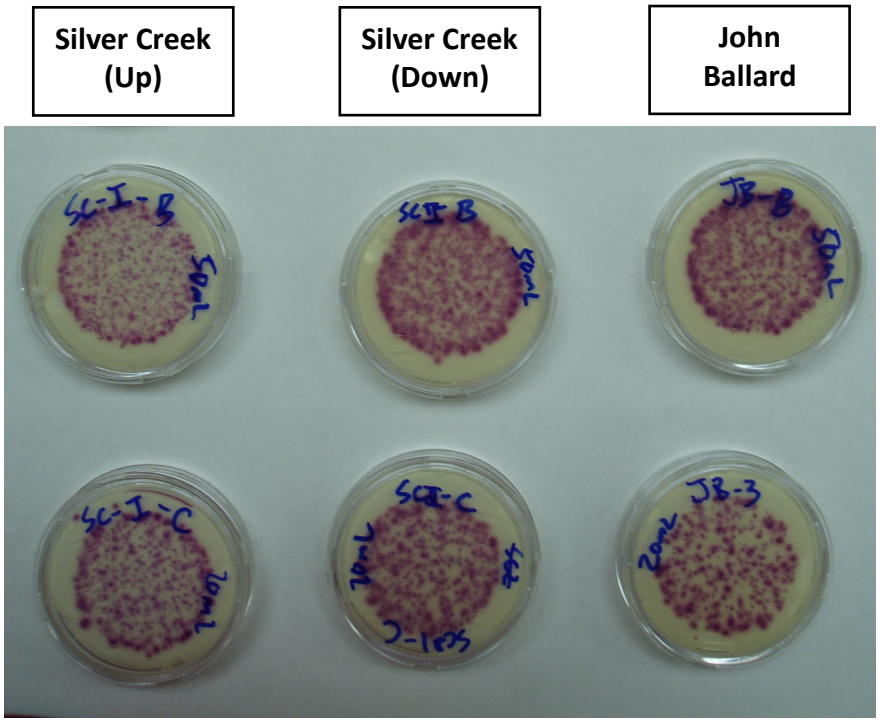

Figure 8: E. coli: Silver Creek and John Ballard

Figure 8 represents samples taken from the Silver Creek and John Ballard sampling sites on October 7th. Here we were testing for *E. coli* colonies. Due to the high level of bacteria present in these creeks, we were forced to dilute them by 50% and 80%. As you can see, on the 50% diluted plates, it is extremely difficult to identify the individual colonies. By diluting it further, we were able to identify the colonies to a greater extent. As you can see, there is a high level of *E. coli* present in each of the samples collected.

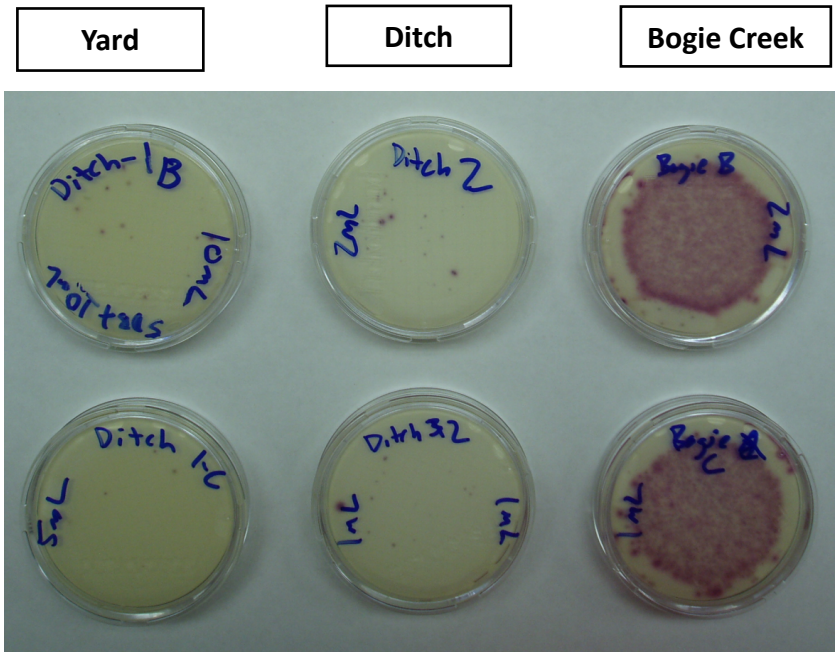

Figure 9: E. coli: Bogie Farm sites

Figure 9 represents samples taken from the Bogie farm. Once again, due to the high levels of *E. coli* contamination, it was necessary to dilute the samples. We were forced to dilute these samples from between 90 and 99%. The Yard samples were diluted to 90% and 95%. Both the Ditch and Bogie Creek samples were diluted to 98% and 99%. As we increase the dilution, it becomes easier to distinguish individual colonies;

however, Bogie Creek is still so contaminated that even at 99% dilution, colony differentiation was nigh impossible.

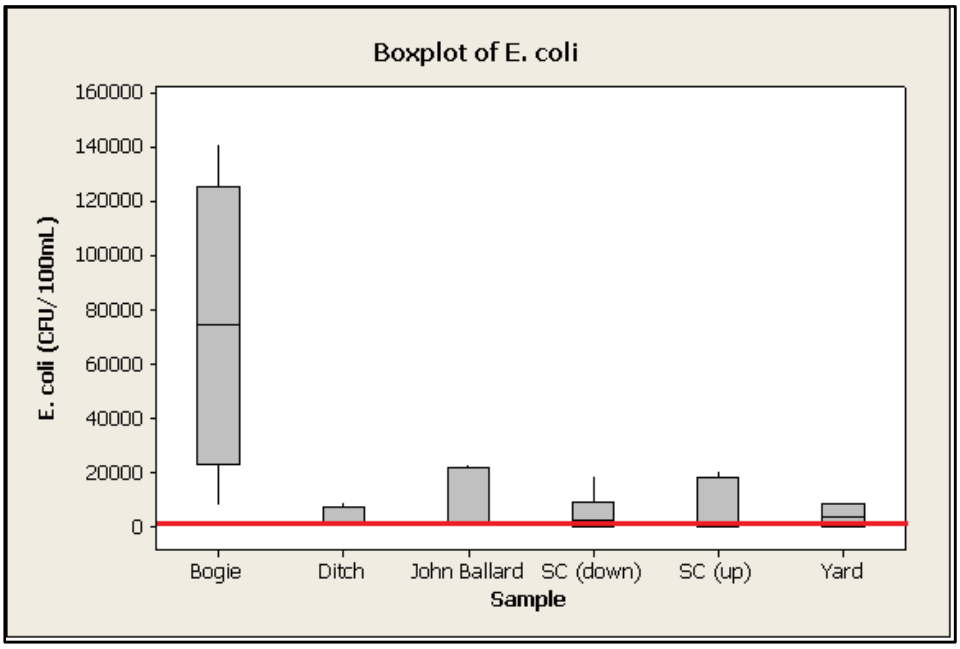

Figure 10: E. coli Boxplot

Figure 10 represents the distribution of the daily average *E. coli* values determined throughout the project period. EPA's Maximum Contaminant Levels are depicted by the line at 200 Colony Forming Units per 100 mL. All of Bogie Creek's samples measured extremely high in comparison to EPA's recreational water *E. coli* MCL. The median for Bogie Creek is near 80,000 CFU/100 mL versus a much lower amount that is orders of magnitude lower for all other sites. All other sample sites had at least two days' worth of data higher than the MCL.

Table 5: Linear Regression Model

Variable	**Coefficient**	***P* value**
Ammonia (mg/L)	0.7811	0.005
Nitrate (mg/L)	0.5563	0.003
24 hour Rainfall (in)	0.27830	0.005
Constant	1.7180	

A linear Regression Model was constructed and the model adjusted for sample sites, ammonia, nitrate and rainfall, and resulted in significant association with all of the variables ($p < 0.05$). The adjusted R^2 for the model was 65.47%, explaining a substantial amount of the variability in *E. coli* levels. The other, unexplained, 34% of variability could be due to other factors such as time, temperature, the effects of previous rainfall, or researcher error.

The Linear Regression Model presents an application so other scientists can predict log CFUs of *E. coli* for this watershed during wet weather conditions. Measurements of nutrients and precipitation, inserted into the model, would result in rapid analysis of water quality so that quick notification or warning of hazardous conditions could occur. The model also demonstrates that ammonia, nitrate, and rainfall are all predictors of log CFUs of *E. coli* after adjusting for sample location. Furthermore, it lends credence that the pattern of increased *E.* coli occur due to animal-associated or runoff-associated sewage. Nitrogen and ammonia are typically found in fresh fecal sewage. If phosphorus had been statistically significant in predicting log CFU, there would be more cause to consider that the contamination was caused by fertilizer runoff.

Rainfall is a large predictor of the amount of colony forming units present in the water system. After a heavy rain event it is dangerous to use these streams for recreational uses such as playing and fishing.

Table 6: Vancomycin and Ciprofloxacin Resistant Enterococci

Sample	Date	Prec (inch)	CFU Avg	LogCFU	*E. faecalis* Avg	*E. faecium* Avg	Cipro Avg
SC (up)	12/6/2013	2.5	17340	4.239049	966.6667	2530	
SC (up)	2/2/2014	0.5	1405	3.147676	1610	2130	20
SC (down)	12/6/2013	2.5	18973.33	4.278144	1500	4033.333	
SC (down)	2/2/2014	0.5	3198.333	3.504924	1670	2560	79
John Ballard	12/6/2013	2.5	22916.67	4.360151	693.3333	4386.667	
John Ballard	2/2/2014	0.5	1475	3.168792	1480	225	217
Yard	12/6/2013	2.5	9259.333	3.96658	430	260	
Yard	2/2/2014	0.5	345	2.537819	240	60	19
Ditch	12/6/2013	2.5	8913.333	3.95004	265	281.6667	
Ditch	2/2/2014	0.5	1625	3.210853	200	360	0
Bogie	12/6/2013	2.5	70050	4.845408	506.6667	2466.667	
Bogie	2/2/2014	0.5	7700	3.886491	2100	600	850

We tested for the presence of Vancomycin Resistant Enterococci (VRE) on two separate occasions, December 6th, 2013 and February 2nd, 2014. We tested for the presence of Ciprofloxacin resistant enterococci on February 2nd, 2014. Colonies of Ciprofloxacin resistant enterococci were present for most of the sample sites (Table 6) . VRE colonies on the plates grew with *E. faecalis* showing as red colonies and *E. faecium*

as blue colonies. The presence of both types of enterococci suggested that PCR should be performed in order to confirm the results that VRE were present.

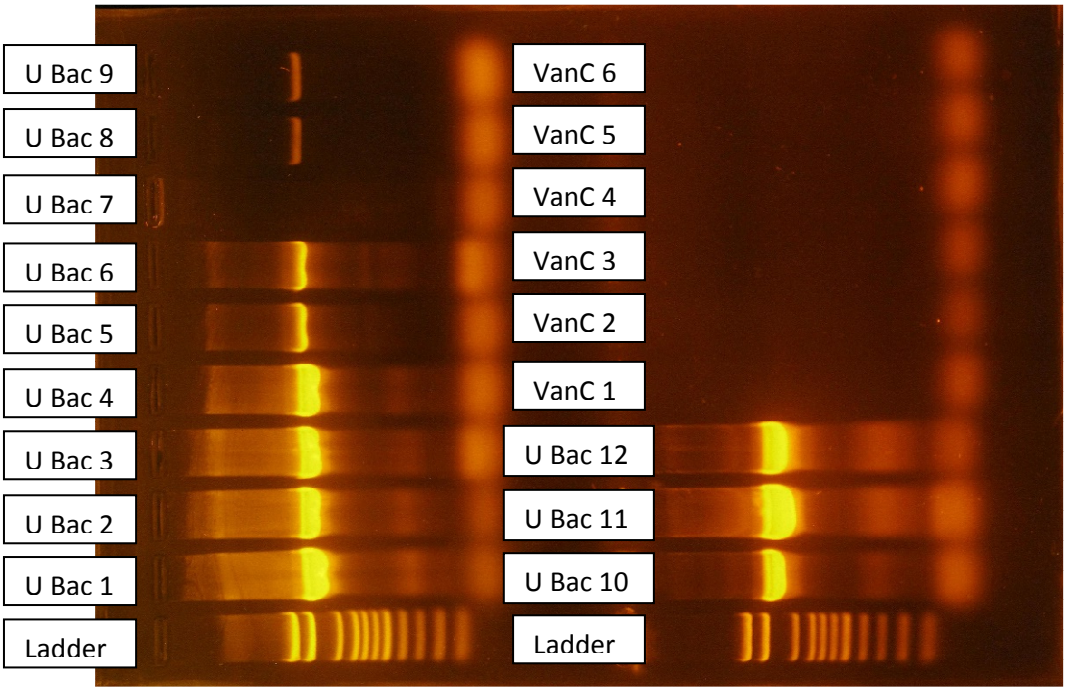

Figure 11: PCR results: Universal Bacteria and Van(c)

Twelve samples were tested using PCR for the Vancomycin Resistant genes. This figure illustrates the results of the PCR gels of the samples when using the Universal Bacteria primers and the first six samples tested using the Vancomycin C primers. Genes were visible for the Universal Primer, illustrating the presence of bacteria in the samples. Despite the growth of colonies on Vancomycin resistant media, the six samples shown on this agarose gel were indicative for the other results using VRE primers. There were no visible positives for any of the Vancomycin Resistant primers that were tested in the sample batch.

LIMITATIONS

The nature of Bogie Creek created problems for sample collection. When we first arrived for testing, and surveillance of the land, we noticed that it was an intermittent stream and the runoff from the farm especially would not occur in adequate enough amounts unless it was raining. This made it particularly problematic to collect samples and created a dilemma when trying to find suitable depths for sampling water. Furthermore, the owner of the cattle farm changed his farming habits as we were testing. Bogie creek is a collection of the runoff of the farm's waste and two ditches that ran perpendicular to Bogie Creek and the farm. We adapted our sampling procedure by collecting samples from both ditches and Bogie Creek, by doing this, we were able to determine the extent of the impact of the farm runoff.

Limitations of this study included difficulty measuring the movement of the cattle. At one point we were informed that they were no longer on the area of land in question. Furthermore, lack of accurate and precise recording was not remedied. Due to limitations of time and recourses, we were unable to evaluate the impact of multiple farms on this land area or how this could affect the community near Berea's health and the health of all those who live downstream from this area of Berea's farming community.

Fecal indicator bacteria, such as the cultured *E. coli* plates we used, do not indicate whether the environmental source was directly from an animal or if it came from

soil, sediments, or other non-fecal sources. We had not the time nor the funding to perform many PCRs that would determine if cattle were the only source of the waste product found in the area. As other animals and humans could have contaminated the area, it is imperative to know the source of the problem. Additionally, accurate tracking of where the fecal contamination was created could not be determined. While the contamination likely came from the farm in question, it cannot be stated as fact beyond a reasonable doubt. Confounders such as the concentration of fecal matter in storm water, preexisting fecal matter in the area, waste products from other agricultural and wild animals all play a role in the accuracy of the data and conclusions.

In a more complete study, not only would temperature and rate of decay be analyzed, but information on the potential methods of transportation for the bovine fecal bacteria would also be collected. Researchers should collect data on wind, water, or other modes of transport concerning their speed, direction, and force, so as to create a better idea of the direction and length fecal matter may have traveled. This can then be modeled using a Linear Regression Model.

The lack of random sampling could also have an impact on the data collected as samples could only be collected during significant precipitation events or immediately following such events.

We were able to test the bacteria recovered from our samples for Vancomycin Resistant genes. Critiques of this method suggest that they are generating false-negatives (unsuccessfully augmenting a DNA strand) through inhibitory factors and false-positives (amplifying a DNA strand that is not common or not present) due to the number of temperature changes in PCR methodology (15).

CONCLUSION

This preliminary research serves as proof that there is a problem concerning fecal material in the Silver Creek Watershed. It is clear we have bacteria present in worrying amounts. Every time we sampled the stream of interest, the density of colony forming units of *E. coli* exceeded the Maximum Contaminant Levels and the PCR using Universal Primers also prove this is true. In this case, however, the phrase "dilution is the solution to" fecal contamination proves at least partially true as the *E. coli* colonies formed were much fewer in number in John Ballard Branch and Silver Creek than in Bogie Creek.

As an exploratory study, further research should be conducted to determine the effect of each farm in the area on the potability and overall safety of the water as well as any potential impacts on treatment methods required in any downstream water supplies. Time limitations mean that a comprehensive map could not be created. Future research on this subject should include one that identifies the location of and type of agricultural farms, residential homes, sewage systems, watershed topography, natural or manmade barriers, and other potentially influential factors that will affect how fast fecal matter enters and travels through the surface water system as well as the pathways used.

It would also be important to note what potential management practices could be or are being put into place to reduce the amount of fecal contamination entering this water system. Barriers such as strips of land to soak up or slow down polluted water, use of animal feces as manure fertilizer placed on other crops, and treatment of water before it reaches streams are all topics which should be addressed in a more in-depth study.

Specifically a study that includes this should discuss the potential successes of each barrier or remediation technique.

ENDNOTES

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