5-2020

Downstream Survival of Total Bacteria, Escherichia coli and Salmonella spp. in Sinking Creek and Evaluation of the Effectiveness of the Implemented Constructed Wetland

Blaire Brooks

Downstream Survival of Total Bacteria, *Escherichia coli* and *Salmonella spp.* in Sinking Creek and Evaluation of the Effectiveness of the Implemented Constructed Wetland

---

By

Blaire Brooks

Spring 2020

An Undergraduate Thesis Submitted in Partial Fulfillment of the Requirements for the Environmental Health Honors-in-Discipline Program,

College of Public Health,

East Tennessee State University

Key Words: *Escherichia coli*, *Salmonella*, Bacteria, Survival, Sinking Creek

Blaire E. Brooks Date

Dr. Phillip R. Scheuerman, Thesis Mentor Date

Dr. Kurt J. Maier, Reader Date

Abstract

Access to good quality, pathogen-free water is a necessity for human life. Pathogen-contaminated water poses a threat to human health, and steps must be taken to minimize that risk using remediation techniques, such as constructed wetlands. Sinking Creek is a tributary of the Watauga River that was placed on the 2016 303(d) list published by the Tennessee Department of Environment and Conservation due to the presence of *Escherichia coli*. Because of this impairment, a constructed wetland was placed in Sinking Creek to decrease the downstream transport of pathogens. Knowing this, three primary goals were made for this experiment. The first goal was to establish the seasonal presence of *E. coli*, *Salmonella spp.*, and other culturable bacteria in Sinking Creek. The second goal was to determine the concentration patterns of *E. coli*, *Salmonella*, and other culturable bacteria as water in Sinking Creek flows downstream. The third goal was to use the data to analyze the effectiveness of the constructed wetland in Sinking Creek and its ability to decrease bacterial concentrations downstream. To achieve these goals, water samples were collected every Wednesday from January 29th to March 11th from four sites on Sinking Creek: two upstream from the constructed wetland and two downstream from the constructed wetland. The samples were plated on mFC, XLD, and R2A agar using the micro drop technique to establish the presence of *E. coli*, *Salmonella*, and other culturable bacteria, respectively. It was hypothesized that, because of the placement of the wetland, concentrations of *E. coli*, *Salmonella*, and other culturable bacteria would be lower at Sites 3 and 4 than at Sites 1 and 2, but this hypothesis was disproved. Data analysis and statistical tests displayed that all bacterial concentrations were higher at Sites 3 and 4 than at Sites 1 and 2. From this, it was concluded that the constructed wetland is not functioning as it was intended, and the increase in bacterial concentrations at Sites 3 and 4 suggest that there is most likely a source of fecal contamination below the wetland.

Table of Contents

Abstract 2

Dedication 4

Acknowledgements 5

List of Tables 6

List of Figures 7

Introduction and Literature Review 8

Materials and Methods 15

Results 21

Discussion 31

Recommendations 35

Conclusions 36

References 37

Dedication

I would like to dedicate this thesis to my mother, Jacqui Schollenberger, my stepfather, Jason Schollenberger, and my sister, Jadyn Brooks, who been my biggest fans throughout this process, as well as my entire academic career. I am so thankful for each of their encouraging words and endless support. I hope that my perseverance through this project has shown my little sister that, with determination, she is capable to achieve her biggest goals and fulfill her wildest dreams.

Acknowledgements

I am forever grateful to the Department of Environmental Health for cultivating my passions and interests, helping me to succeed in many academic efforts, and for ensuring that I am prepared to enter the workforce. I would first like to thank Dr. Phillip R. Scheuerman for his help as my mentor throughout this process, as well as for much of my collegiate experience. I would also like to thank my reader, Dr. Kurt Maier, for his assistance in the completion of this project, and for his role as an academic, professional, and personal advisor. Additionally, I would like to thank Mr. Charles Patton and Mr. Brian Evanshen for always ensuring my access to the materials in the laboratory that were necessary to complete this project. And of course, I cannot forget about my fellow ENVH cubby-inhabitants for their never-ending support and friendship. Lastly, I would like to thank the Honors-in-Discipline Scholars Program for their support and aid in my ability to continue higher education.

List of Tables

1. **Table 1**: Average Concentration (CFU/mL) of Total Culturable Bacteria Each Week for Each Site

2. **Table 2**: ANOVA: Single Factor to Determine Significance of Total Culturable Bacteria Concentration Between Weeks

3. **Table 3**: ANOVA: Single Factor to Determine Significance of Total Culturable Bacteria Concentration Between Sites

4. **Table 4**: Average Concentration (CFU/mL) of *E. coli* Each Week for Each Site

5. **Table 5**: ANOVA: Single Factor to Determine Significance of *E. coli* Concentration Between Weeks

6. **Table 6**: ANOVA: Single Factor to Determine Significance of *E. coli* Concentration Between Sites

7. **Table 7**: Average Concentration (CFU/mL) of *Salmonella* Each Week for Each Site

8. **Table 8**: ANOVA: Single Factor to Determine Significance of *Salmonella* Concentration Between Weeks

9. **Table 9**: ANOVA: Single Factor to Determine Significance of *Salmonella* Concentration Between Sites

10. **Table 10**: ANOVA: Single Factor to Determine Significance of All Organism Concentration Between Weeks

11. **Table 11**: ANOVA: Single Factor to Determine Significance of All Organism Concentration Between Sites

12. **Table 12**: ANOVA: Two-Factor with Replication to Determine Significance Between All Organism Concentration and Week

13. **Table 13**: ANOVA: Two-Factor with Replication to Determine Significance Between All Organism Concentration and Site

List of Figures

1. **Figure 1**: Map of Sampling Sites Along Sinking Creek.
2. **Figure 2**: Average Total Culturable Bacteria Concentration at Each Site Over 7 Weeks
3. **Figure 3**: Average Total Culturable Bacteria Concentration at Sites 1 – 4 Over Time
4. **Figure 4**: Average *E. coli* Concentration at Each Site Over 7 Weeks
5. **Figure 5**: Average *E. coli* Concentration at Sites 1 – 4 Over Time
6. **Figure 6**: Average *Salmonella* Concentration at Each Site Over 7 Weeks
7. **Figure 7**: Average *Salmonella* Concentration at Sites 1 – 4 Over Time
8. **Figure 8**: Average Concentration of Total Culturable Bacteria, *E. coli*, and *Salmonella* Over 7 Weeks

Introduction and Literature Review

Pathogens and Water

Water is a necessity to support human life, and Levantesi, et al. (2012) suggest that one-third of the world experiences water scarcity or water stress. Not only is availability of water expected to become more limited, but the impact of pathogens on public health is expected to increase due to the increase of the human population and certain human activities (Levantesi et al., 2012). Simply having access to water is not enough; good quality, pathogen-free water is necessary to promote good health in humans. It is estimated that 50% of deaths associated with waterborne disease are due to microbial agents that cause gastrointestinal illness (Cabral, 2010). In addition to exposure through drinking water, exposure to waterborne pathogens can occur through recreational and agricultural usage (Bosch, Guix, Sano, & Pinto, 2008).

A primary issue that is associated with the presence of pathogens in water is that their concentration may be high enough to pose a threat to human health but may not be high enough for adequate detection (Bosch, Guix, Sano, & Pinto, 2008). Even with mechanisms of detection that are widely used, such as fecal coliforms as indicators of contamination, there are scholars who suggest that detection is not accurate (Edberg, 2000). Through research, it has been suggested that fecal indicator bacteria could be found naturally in aquatic environments, which would skew data regarding sources of fecal contamination (Haack et al., 2009).

Because of the global presence of pathogens in water bodies, it is important to understand what conditions promote and discourage the growth of pathogenic organisms in open environments so that measures can be taken to minimize the human health risk (Pandey et al., 2014). Survivability of pathogens, in any environment outside of where they have the highest degree of fitness, depends on the environmental conditions present.

Escherichia coli

Escherichia coli is a gram-negative, rod-shaped bacteria belonging to the *Enterobacteriaceae* family (Lampel, 2014; Winfield & Groisman, 2003). It is an enteric organism that naturally colonizes human and ruminant animal gastrointestinal (GI) tracts. These strains are harmless, but there are sub-groups of *E. coli* that are pathogenic in nature, some causing respiratory illnesses, and others causing gastrointestinal illnesses (Lampel, 2014). The pathogenic *E. coli* can be divided into six pathotypes: enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), diffusely adherent *E. coli* (DAEC), and enterohemorrhagic *E. coli* (EHEC). Some strains of *E. coli* are categorized as "Shiga toxin producing" *E. coli* (STEC), the enterohemorrhagic strain being one of them (CDC, 2019; Wells et al., 1991).

E. coli O157:H7 is classified as an EHEC strain, making it a Shiga-toxin producing strain of *E. coli*. It was first recognized as a pathogenic risk to humans in 1982 and has since been associated with severe symptoms of gastroenteritis. *E. coli* O157:H7 is predominantly a foodborne organism but can be waterborne due to the grazing of animals and their release of contaminated waste into a water body (Chalmers, Arid, & Bolton, 2000). Globally, *E. coli* O157:H7 is responsible for close to 75% of EHEC infections (Lampel, 2014). In the United States, it is estimated that 265,000 STEC infections occur annually, and of those, nearly 36% are cause by *E. coli* O157:H7 (CDC, 2014). Shiga-like toxin has been linked to a cause of hemolytic uremic syndrome (HUS) and other serious health problems (Wells et al., 1991). Because the *E. coli* group thrives in the GI tract of humans and animals, it has a lower survival rate in open environments (Winfield & Groisman, 2003).

The GI tracts of human and animal hosts are the ideal environment for *E. coli* to grow and reproduce in because they are of warm temperatures and have an excess of nutrients to support growth. After studies on the survival of *E. coli* as it transfers from the primary to

secondary environment, it is often concluded that *E. coli* can survive in open environments but may struggle to grow and reproduce. This is due to a decrease in available nutrients, due to competition, and a wide range of temperatures that *E. coli* must adapt to for productive fitness (van Elsas et al., 2011; Winfield & Groisman, 2003).

Salmonella spp.

Salmonella also belong to the *Enterobacteriaceae* family. Like *E. coli*, *Salmonella* is a rod-shaped, Gram-negative bacteria, of which some species can naturally be found in the gastrointestinal tracts of both humans and animals (Lampel, 2014; Liu, Whitehouse, & Li, 2018). The disease caused by *Salmonella* is called salmonellosis (Lampel, 2014). *Salmonella* is listed in the top five pathogens associated with foodborne illness, but research indicates that waterborne outbreaks can occur due to the drinking of water contaminated with raw-sewage and the irrigation of crops with contaminated water, which contributes to a significant portion of *Salmonella* outbreaks (Liu, Whitehouse, & Li, 2018). Cases of *Salmonella* outbreaks tend to follow a seasonal pattern with higher rates in the summer than in the winter (Haley, Cole, & Lipp, 2009). *Salmonella* species can be divided into two main groups, *Salmonella enterica* being the largest public health concern of the two (Lampel, 2014).

The serotype of *S. enterica* that is primarily associated with water contaminated by raw sewage is *Salmonella* Typhimurium, which causes typhoid fever (Lampel, 2014). Typhoid fever elicits similar symptoms of other gastrointestinal illnesses but will often also present with a high fever of 103°F-104°F and a rash (Lampel, 2014). A significant difference between *Salmonella* Typhimurium and the nontyphoidal species is that *Salmonella* Typhimurium is only found in the human gastrointestinal tract, hence why outbreaks are most often associated with raw sewage contamination (Ashurst, Truong, & Woodbury, 2019). It is estimated that ~2,000 cases of typhoid fever occur each year in the United States, and, when left untreated, the mortality rate can reach to 10% (Lampel, 2014).

Waterborne Associated Outbreaks of *E. coli* and *Salmonella*

The transmission of *E. coli* is often foodborne or person-to-person with cattle a primary source of infection (Chalmers, Aird, & Bolton, 2000). Waterborne transmission becomes possible when farm animals graze in nearby water sources and act as a source of fecal contamination (Chalmers, Aird, & Bolton, 2000). The first reported outbreak in the United States of *E. coli* O157:H7 infection associated with drinking water was in Missouri in 1989, and since then, several others have occurred (Olsen et al., 2002). In 1998, an outbreak occurred near Alpine, Wyoming, where more than 150 cases of severe gastrointestinal issues among residents and visitors were observed and 71 cases of *E. coli* O157:H7 were confirmed (Olsen et al., 2002). Upon investigation, it was determined that the Alpine municipal water system, which was fed by an underground spring, had been contaminated when surface water that contained deer and elk feces leached into the town's aquifer. Because the investigation began several weeks after the outbreak, the Environmental Protection Agency was unable to detect the presence of *E. coli* O157:H7 in the water storage tank, but ruled that the reason for this is because *E. coli* O157:H7 survives long periods of time in cold water by entering into a viable but non-culturable state (Olsen et al., 2002).

Over the years, the reporting of waterborne outbreaks of *Salmonella* has increased and its presence has been detected in both freshwaters and marine waters, which indicates the high probability that its transmission through water will occur (Haley, Cole, & Lipp, 2009). An area of the United States that consistently reports high case rates of waterborne salmonellosis is the coastal plain of southern Georgia, specifically the Little River watershed. In 2007, the Little River district had a case rate of 58.4 per 100,000, while the United States had a case rate of 14.9 per 100,000 (Haley, Cole, & Lipp, 2009). In this area, surface waters are used for recreation and irrigation, while groundwater is used for domestic water supply, but these two systems are significantly interconnected. Because of this interconnectedness, septic systems, irrigation

systems, and wildlife throughout the area act as potenital sources of contamination to the municipal water system in this district (Haley, Cole, & Lipp, 2009).

Transport and Fate of Bacteria in Surface Waters

It is estimated that ~13% of all streams in the United States are impaired by pathogens (Hellweger, 2009). Through research, it has been seen that the transport of pathogens by surface runoff undoubtedly leads to increases in the concentrations of these pathogens found in watersheds (Ferguson et al., 2003). When pathogens enter surface waters, they are immediately dispersed through the water, and their presence is dependent on the aquatic environment and various parameters (Wilkes et al., 2011). Their density is usually lessened through natural mechanisms such as dilution, decay, and settling, but accumulation and growth in soil or sediment can increase their density (Hellweger, 2009). One of the main issues with detecting pathogens, specifically fecal bacteria, in surface waters is that, to survive in stressful environments, they can enter a state in which they are alive but not culturable, known as “viable but non-culturable” (Hellweger, 2009). This means that these pathogens can be present and viable in an environment, but not detectable, causing them to remain unknown as a risk to the public. Knowing this makes understanding how bacteria act in surface waters and attempting to use different models to estimate their environmental fate even more important from a public health perspective (Ferguson et al., 2003).

The survival, fate, and transport of bacterial indicators and pathogens can be affected by the implementation of a constructed wetland (Smith, Gordon, Madani, & Stratton, 2005). Constructed wetlands act as a biofilter by using aquatic components such as vegetation, soil, and sediment to trap bacteria. Within the wetland, a wide variety of mechanisms can be used to help with the filtration of pathogens from the water including but not limited to sedimentation, predation, oxidation, and natural die-off (Weber & Legge, 2008). When done properly, constructed wetlands have proven to be effective mechanisms of bacteria removal (Smith,

Gordon, Madani, & Stratton, 2005). Many studies have shown pathogen removal efficiency reach upwards of 99.99% with the implementation of constructed wetlands in surface water systems (Weber & Legge, 2008). Not only have constructed wetlands proven to be effective at the removal and inactivation of pathogens, but they are usually a low-cost, low-maintenance option for working to improve water quality (Weber & Legge, 2008).

Effect of Land Use on Pathogens in Surface Waters

Because pathogens can enter surface water through different means, such as irrigation, livestock, and cross-contamination from septic systems, it is necessary to know what category of land usage has the greatest impact on the presence of pathogens in surface water. A study done in Ontario, Canada from 2004-2008 evaluated the associations between land use and pathogen presence in a nearby river basin (Wilkes et al., 2011). Like the areas surrounding Sinking Creek, the river basin in eastern Canada is found in a rural, agriculturally dominated area. Because of this, *E. coli* O157:H7 and *Salmonella enterica*, were selected because they are pathogens often associated with agricultural processes (Wilkes et al., 2011).

For *E. coli* O157:H7 it was determined that the increased presence of livestock upstream was directly correlated with the increased occurance of *E. coli* found in surface water (Wilkes et al., 2011). It was also found that 20% of *E. coli* detections were in locations where cattle had direct access to the river, as opposed to just the occurance of agricultural runoff (Wilkes et al., 2011). For *Salmonella spp.* it was determined that high surface water discharge events such as runoff and agricultural drainage promoted the presence of *Salmonella spp.* in surface water, partially due to the resuspension of trapped bacteria during any forceful expulsion of water (Wilkes et al., 2011).

Goals and Objectives

After analyzing data which determined a baseline for the total bacteria present in Site 3 of Sinking Creek, as well as how the alteration of temperature affected the concentration of both *E. coli* and *Salmonella* over time, it was decided that evaluating the changes in concentration of these pathogens as Sinking Creek flows downstream was necessary (Liner, 2019).

There were three primary goals of this experiment. The first goal was to further establish the seasonal presence of *E. coli*, *Salmonella,* and other culturable bacteria in Sinking Creek. This experiment was performed from the end of January through mid-March, which allowed for sampling during a wide range of weather condition, each one with the potential to alter pathogen concentration and presence. The second goal was to determine the concentration patterns of *E. coli*, *Salmonella,* and other culturable bacteria as water in Sinking Creek flows downstream. The third goal of this experiment was to use this data to analyze the effectiveness of the constructed wetland in Sinking Creek and its ability to decrease bacterial concentrations downstream. The four sites that were analyzed in this experiment surrounded the constructed wetland in roughly equal distances on each side. By choosing these sampling sites, pathogen presence as water flows through the wetland could be monitored for any changes in concentration. It was hypothesized that, because of the intended function of the wetland, the concentrations of bacteria downstream from the wetland (Sites 3 and 4) would be significantly lower than the concentrations upstream of the wetland (Sites 1 and 2).

Materials and Methods

Sampling Sites

Sinking Creek is a tributary of the Watauga River, running through Johnson City, TN, in Washington County and Carter County. As of the 2016 303(d) list published by the Tennessee Department of Environment and Conservation, Sinking Creek is an impaired stream due to the presence of *E. coli* from agricultural discharge (TDEC, 2017). Because of this impairment, a wetland was constructed in the stream to act as a filter to decrease the downstream travel of pathogens and other contaminants. The sampling sites for this experiment were chosen strategically around the location of the wetland to evaluate its effectiveness. Four sampling sites (1-4) were chosen, two above the wetland and two below (Figure 1). In addition to the four sampling sites on Sinking Creek, a control sample of sterilized water was used to verify that the aseptic procedures used were adequate and to inform the researcher of any potential contamination.

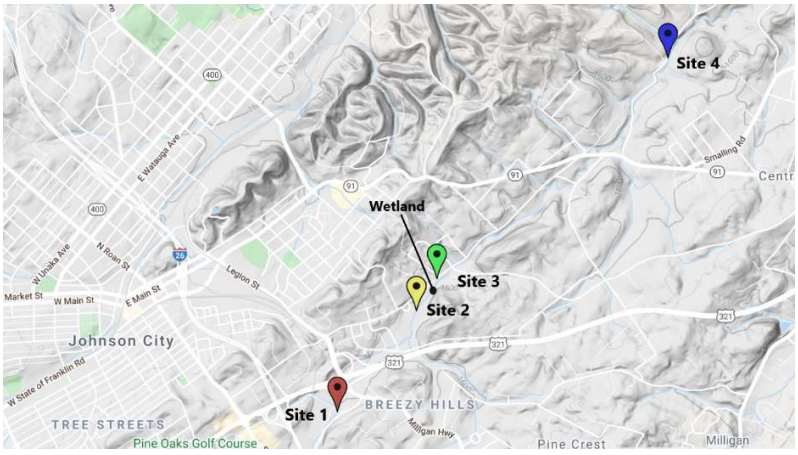

Figure 1. Map of Sampling Sites Along Sinking Creek. This map shows the four sampling sites along Sinking Creek, with the red marker indicating Site 1, yellow marker indicating Site 2, green marker indicating Site 3, and blue marker indicating Site 4. The black dot placed in between Site 2 and Site 3 indicates the location of the wetland.

Sampling Technique

Water samples were collected every Wednesday between January 29th, 2020 and March 11th, 2020. One water sample was collected from each of the four sites each time samples were collected. Samples were collected using large Whirl-Pak bags. At each site, the bag was opened, placed in the main flow line of the stream at mid-depth, and filled one-third of the way. All four water samples were stored in a walk-in refrigerator at 4°C for no more than 2 hours once returned to the lab.

Since the weather was expected to fluctuate significantly throughout the timeframe of the experiment, certain environmental parameters were recorded at each site on each sampling date. The parameters recorded were pH, conductivity, dissolved oxygen, flow rate, average depth, water temperature, and ambient temperature. In addition, any significant weather events, such as a heavy rain or snow were recorded.

Plating Media

To enumerate organisms from Sinking Creek, both non-selective and selective media were used. R2A is a non-selective media that was used to enumerate the total countable bacteria in the samples. The R2A was prepared, as listed in the instructions, by adding 18.2 g of the powdered R2A agar to 1,000 mL of deionized water in a 2,000 mL Erlenmeyer flask. A stir bar was added to the flask and the opening of the flask was covered with aluminum foil. The R2A agar was heated to a boil using a hot plate, while stirred at a constant rate. Once boiled, the R2A agar was sterilized in the autoclave for 45 minutes at 121°C. Immediately after sterilization, the R2A agar was poured evenly into 30-45 100 mm x 15 mm plastic petri dishes. The petri dishes were cooled, inverted, and left to dry until needed.

The two types of selective media used for this experiment were mFC and XLD agar. mFC is a type of media that is typically used to enumerate fecal coliform bacteria, specifically *E.*

coli (USGS, 2007). The presence of rosolic acid in the media prevents the growth of non-fecal coliform bacteria, while certain bile salts prevent the growth of non-enteric bacteria (USGS, 2007). The growth of fecal bacteria on the medium can be seen by a distinct blue-colored colony, which indicate that lactose fermentation has occurred (USGS, 2007). For this experiment, the mFC agar was prepared by combining 37 g of mFC broth and 17 g of agar. This was added to 1,000 mL of deionized water in a 2,000 mL Erlenmeyer flask. An important component of mFC agar is rosolic acid. To add this, 0.1 g of rosolic acid was added to 10 mL of 0.2 N NaOH, and this solution was mixed into the agar in the flask. A stir bar was added to the flask and the opening of the flask was covered with aluminum foil. The agar was heated to a boil using a hot plate, while stirred at a constant rate. Once boiled, the agar was poured evenly into 50-55 100 mm x 15 mm plastic petri dishes. The petri dishes were cooled, inverted, and left to dry until needed.

XLD (xylose lysine deoxycholate) is a type of agar used to enumerate enteric bacteria, specifically *Salmonella* and *Shigella* (Taylor & Schelhart, 1971). XLD agar has been found to work equally as well, if not better than other media created to isolate these organisms, such as Salmonella-Shigella (SS) agar, because of its ability to limit false positives (Taylor & Schelhart, 1971). When grown on XLD agar, *Salmonella* colonies appear as red or pink with small, black centers (Park, Ryu, & Kang, 2012). For this experiment, the XLD agar was prepared, as listed in the instructions, by adding 55 g of the powdered agar to 1,000 mL of deionized water in a 2,000 mL Erlenmeyer flask. A stir bar was added to the flask and the opening of the flask was covered with aluminum foil. The agar was heated to a boil using a hot plate, while stirred at a constant rate. Once boiled, the agar was poured evenly into 40-45 100 mm x 15 mm plastic petri dishes. The petri dishes were cooled, inverted, and left to dry until needed.

Each type of media was made and poured into plates at least 3 days before samples were plated to allow for adequate drying of the plates and to minimize the spreading of the micro drops across the media (see below).

Plating Method

Water samples taken were plated on the appropriate media using the drop plate method (Naghili, 2013). The drop plate method uses a single petri plate to enumerate organisms in four dilutions. This is accomplished by dividing the petri plate into four sections, one for each serial dilution concentration, and distributing small sample amounts (10 – 30 µl) onto each section in three drops per dilution. The drop plate method is often chosen as the preferred plating mechanism for experiments because it saves time and resources (Naghili, 2013). Although this method is widely used, it is not standardized; drop size often varies between 10 – 30 µL (Naghili, 2013). For the purpose of these experiments, 30 µL of the sample was plated on the corresponding section of the petri plate in 10 µL drops.

Experimental Procedure

Serial dilutions of each sample were made by placing 4.5 mL of deionized water in each of 15 small test tubes. Fifteen test tubes were used because 3 serial dilutions were made from each of the 5 water samples (Note: the fourth dilution used came directly from the collected sample). The dilution tubes were capped and placed in the autoclave for 15 minutes at 121°C to be sterilized. The dilution tubes were left to cool for 45 minutes before creating the serial dilutions to avoid killing any bacteria present in the sample that would be added into the tubes. For each of the 5 samples, the serial dilutions were made by adding 0.5 mL of water directly from the sample into one of the test tubes to make a 10^{-1} dilution. From there, 0.5 mL was taken from the 10^{-1} dilution tube and added to another test tube to make a 10^{-2} dilution, and this process was repeated until dilutions of 10^{-1} to 10^{-3} were made for each sample.

Before plating the serial dilutions, each of the petri dishes was labeled. For each of the five sampling sites, three replicas were plated to ensure more accurate results, and they were labeled A, B, and C. This resulted in a total of 15 R2A, 15 mFC, and 15 XLD plates that were used each week. The four sections of all the plate were labeled as direct, 10^{-1}, 10^{-2}, and 10^{-3}, and each plate was labeled with the site number and the assigned replica letter.

The plates were placed under a biological safety hood (BSL 2) to reduce contamination of the plates before the samples were plated. The micro drop technique was performed using a 10-100 µL micropipette and plastic micropipette tips, dispensing 10-30 µL of each sample (1-5) onto the corresponding plate and section. A total of 45 plates were used each week. Once the samples were plated, the plates were left for 5-10 minutes to allow the micro drops to dry.

The plates were inverted during incubation to avoid contamination of the samples from condensation in the lid of the plate. The R2A plates were incubated at room temperature, but the mFC and XLD plates were incubated at 37°C and 35°C, respectively, to encourage rapid growth of the organisms. For the first five weeks of the experiment, the plates were incubated for 48 hours and then counted. During the fifth week, overgrowth of colonies was seen, so for the last two weeks of the experiment, the plates were only incubated for 24 hours. Bacterial growth for each micro drop (0.01 mL) on each plate was counted and in colony forming units (CFU).

Calculations and Data Analysis

Each recorded colony count was converted to CFU/mL using the following formula: (CFU x serial dilution) / plating volume. The values were converted to CFU/mL for each micro drop and the three micro drops for each plate section were averaged. This data was used to created graphs to compare colony concentration between organisms, sites, and times. Using Excel, the averages, the geometric means, and the geometric standard deviations of the three

replicas for each site and dilution were calculated. This data was used to determine which serial dilution worked best for enumeration of each organism.

Two *Single Factor ANOVAs* were performed for each organism: one to determine the variation of concentration between weeks and one to determine the variation of concentration between sites. To determine the variation of concentration between weeks, the CFU/mL values for each site each week were averaged, and vice versa for variation of concentration between sites. Two additional Single Factor ANOVAs were performed the same way to determine the variation of concentration between weeks and sites of all three organisms combined.

Two *Two-Factor: With Replication ANOVAs* were run. The first was run to determine the variation between concentration of all three organisms, concentration at all four sites, and the interaction between the two variables. The second was run to determine the variation between concentration of all three organisms, concentration during all seven weeks, and the interaction between the two variables. Like the Single Factor ANOVAs, the CFU/mL values were averaged appropriately to run the analysis.

Results

Total Bacteria Concentration Data

The data collected at all four sites for concentration of total culturable bacteria in Sinking Creek over the seven-week experiment was compiled and analyzed by creating a summary table (Table 1) and figures (Figures 2-3), and statistical analyses to determine statistical significance of the results (Tables 2-3).

Table 1. Average Concentration (CFU/mL) of Total Culturable Bacteria Each Week for Each Site. Each week, three sample replicas from each site were plated on R2A agar and the total culturable bacteria were counted and recorded as CFUs. The average site concentrations (CFU/mL) were calculated and reported for each week of the experiment. The 'X' for Week 5 - Site 2 representing that no sample was collected because the site was inaccessible.

	SITE			
WEEK	**1**	**2**	**3**	**4**
1	222.22	222.22	222.22	166.66
2	6222.22	9777.77	7222.22	11000
3	1055.55	2444.44	2777.77	2222.22
4	833.33	1777.77	1055.55	1777.77
5	1444.44	X	3333.33	2555.55
6	833.33	888.88	1333.33	1000
7	500	555.55	555.55	666.66

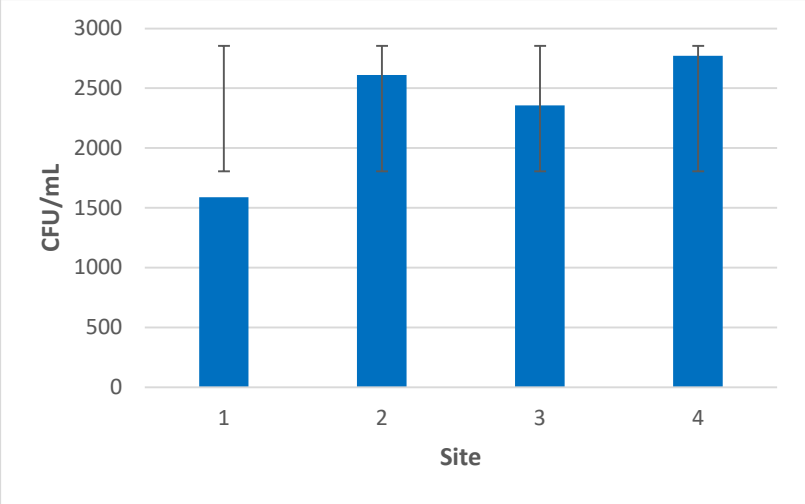

Figure 2. Average Total Culturable Bacteria Concentration at Each Site Over 7 Weeks. For each site, the recorded average concentrations of total culturable bacteria (CFU/mL) each week

were averaged to determine average site concentration for the entire experiment. Each bar on the graph represents a different sampling site experiment average. Error bars represent standard deviation.

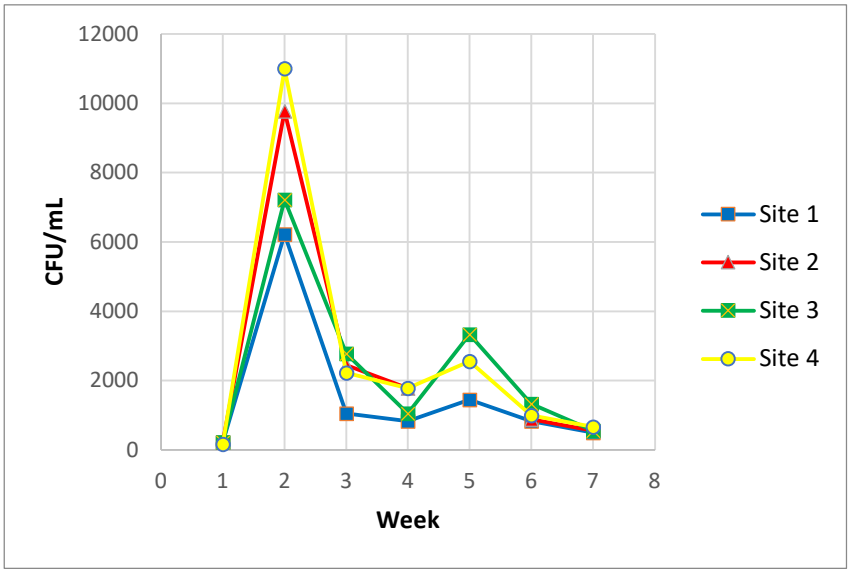

Figure 3. Average Total Culturable Bacteria Concentration at Sites 1 – 4 Over Time. The recorded average concentration of total bacteria (CFU/mL) for each site were plotted for each week to show the change in bacterial concentrations at each site from week-to-week. The blue line represents site 1, the red line represents site 2, the green line represents site 3, and the yellow line represents site 4. The line gap for Site 2 between Week 4 and Week 6 represents that Site 2 was inaccessible for sample collection during Week 5.

Table 2. ANOVA: Single Factor to Determine Significance of Total Bacteria Concentration Between Weeks. A single factor ANOVA was run to determine the statistical significance of total bacteria concentration between each week of the experiment. Statistical significance was determined based on comparison of the F value to the F critical value, and the size of the P-value (≤ 0.05).

ANOVA						
Source of Variation	*SS*	*df*	*MS*	***F***	***P-value***	***F crit***
Between Groups	196323045.3	6	32720507.54	34.3311	1.64509E-09	2.598978
Within Groups	19061728.4	20	953086.4198			
Total	215384773.7	26				

Table 3. ANOVA: Single Factor to Determine Significance of Total Culturable Bacteria Concentration Between Sites. A single factor ANOVA was run to determine the statistical significance of total bacteria concentration between each sampling site. Statistical significance was determined based on comparison of the F value to the F critical value, and the size of the P-value (≤ 0.05).

ANOVA						
Source of Variation	*SS*	*df*	*MS*	***F***	***P-value***	***F crit***
Between Groups	5692533.804	3	1897511.268	0.208128	0.889720155	3.027998
Within Groups	209692239.9	23	9117053.907			
Total	215384773.7	26				

E. coli Concentration Data

The data collected at all four sites for *E. coli* concentration in Sinking Creek over the seven-week experiment was compiled and analyzed by creating a summary table (Table 4) and figures (Figures 4-5), and statistical analyses to determine statistical significance of the results (Tables 5-6).

Table 4. Average Concentration (CFU/mL) of *E. coli* Each Week for Each Site. Each week, three sample replicas from each site were plated on mFC agar and *E. coli* colonies were counted and recorded as CFUs. The average site concentrations (CFU/mL) were calculated and reported for each week of the experiment. The 'X' for Week 5 -Site 2 representing that no sample was collected because the site was inaccessible.

	SITE			
WEEK	**1**	**2**	**3**	**4**
1	233.33	911.11	16.67	350
2	1311.11	1022.22	1966.67	1744.44
3	855.56	633.33	655.56	955.56
4	316.67	433.33	361.11	744.44
5	200	X	238.89	222.22
6	144.44	177.78	77.78	183.33
7	188.89	238.89	188.89	222.22

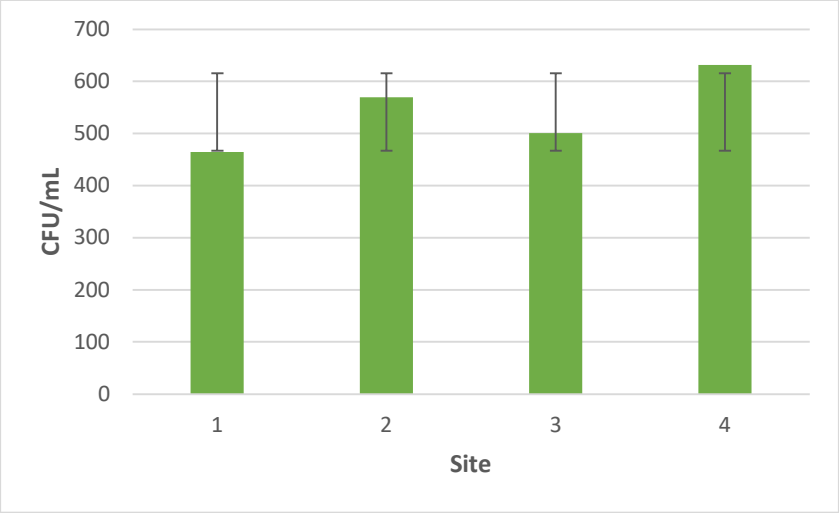

Figure 4. Average *E. coli* Concentration at Each Site Over 7 Weeks. For each site, the recorded average concentrations of *E. coli* (CFU/mL) each week were averaged to determine average site concentration for the entire experiment. Each bar on the graph represents a different sampling site experiment average. Error bars represent standard deviation.

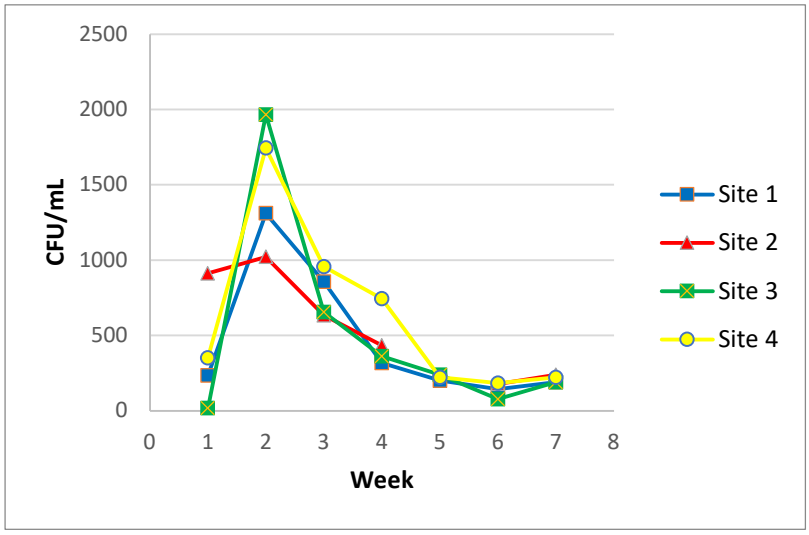

Figure 5. Average *E. coli* Concentration at Sites 1 – 4 Over Time. The recorded average concentration of *E. coli* (CFU/mL) for each site were plotted for each week to show the change in bacterial concentration at each site from week-to-week. The blue line represents site 1, the red line represents site 2, the green line represents site 3, and the yellow line represents site 4. The line gap for Site 2 between Week 4 and Week 6 represents that Site 2 was inaccessible for sample collection during Week 5.

Table 5. ANOVA: Single Factor to Determine Significance of *E. coli* Concentration Between Weeks. A single factor ANOVA was run to determine the statistical significance of *E. coli* concentrations between each week of the experiment. Statistical significance was determined based on comparison of the F value to the F critical value, and the size of the P-value (≤ 0.05).

ANOVA						
Source of Variation	*SS*	*df*	*MS*	***F***	***P-value***	***F crit***
Between Groups	5485848	6	914307.9	15.59621	1.33516E-06	2.598978
Within Groups	1172474	20	58623.71			
Total	6658322	26				

Table 6. ANOVA: Single Factor to Determine Significance of *E. coli* Concentration Between Sites. A single factor ANOVA was run to determine the statistical significance of *E. coli* concentrations between each sampling site. Statistical significance was determined based on comparison of the F value to the F critical value, and the size of the P-value (≤ 0.05).

ANOVA						
Source of Variation	*SS*	*df*	*MS*	***F***	***P-value***	***F crit***
Between Groups	115004	3	38334.67	0.134748	0.938319617	3.027998
Within Groups	6543318	23	284492.1			
Total	6658322	26				

<u>*Salmonella* Concentration Data</u>

The data collected at all four sites for *Salmonella* concentration in Sinking Creek over the seven-week experiment was compiled and analyzed by creating a summary table (Table 7) and figures (Figures 6-7), and statistical analyses to determine statistical significance of the results (Tables 8-9).

Table 7. Average Concentration (CFU/mL) of *Salmonella* Each Week for Each Site. Each week, three sample replicas from each site were plated on XLD agar and *Salmonella* colonies were counted and recorded as CFUs. The average site concentrations (CFU/mL) were calculated and reported for each week of the experiment. The 'X' for Week 5 -Site 2 representing that no sample was collected because the site was inaccessible.

	SITE			
WEEK	**1**	**2**	**3**	**4**
1	16.67	16.67	16.67	16.67
2	288.89	422.22	722.22	522.22
3	122.22	183.33	216.67	122.22
4	100	183.33	133.33	200
5	55.56	X	55.56	77.78
6	50	55.56	61.11	72.22
7	72.22	66.67	94.44	77.78

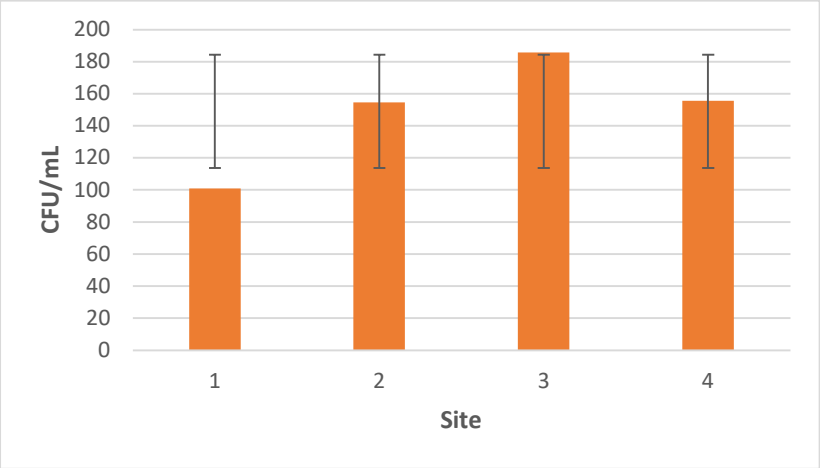

Figure 6. Average *Salmonella* Concentration at Each Site Over 7 Weeks. For each site, the recorded average concentrations of *Salmonella* (CFU/mL) each week were averaged to determine average site concentration for the entire experiment. Each bar on the graph represents a different sampling site experiment average. Error bars represent standard deviation.

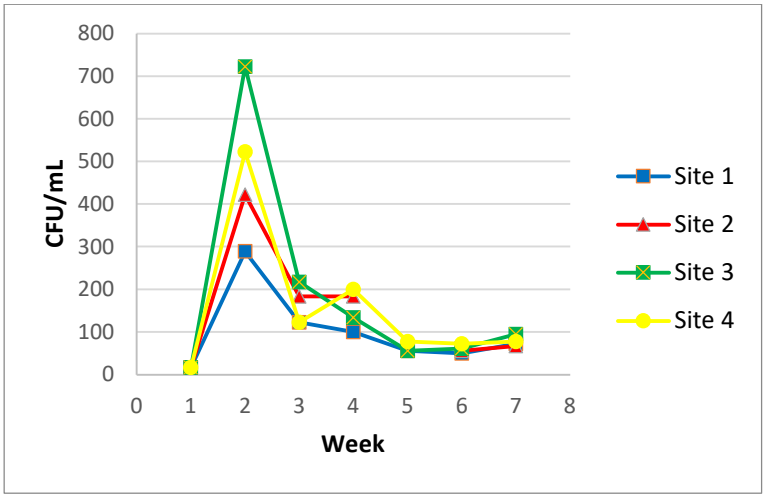

Figure 7. Average *Salmonella* Concentration at Sites 1 – 4 Over Time. The recorded average concentrations of *Salmonella* (CFU/mL) for each site were plotted for each week to show the change in bacterial concentration at each site from week-to-week. The blue line represents site 1, the red line represents site 2, the green line represents site 3, and the yellow line represents site 4. The line gap for Site 2 between Week 4 and Week 6 represents that Site 2 was inaccessible for sample collection during Week 5.

Table 8. ANOVA: Single Factor to Determine Significance of *Salmonella* Concentration Between Weeks. A single factor ANOVA was run to determine the statistical significance of *Salmonella* concentration between each week of the experiment. Statistical significance was determined based on comparison of the F value to the F critical value, and the size of the P-value (≤ 0.05).

ANOVA						
Source of Variation	*SS*	*df*	*MS*	***F***	***P-value***	***F crit***
Between Groups	607219.4	6	101203.2	17.76184	4.7E-07	2.598978
Within Groups	113955.8	20	5697.788			
Total	721175.1	26				

Table 9. ANOVA: Single Factor to Determine Significance of *Salmonella* Concentration Between Sites. A single factor ANOVA was run to determine the statistical significance of *Salmonella* concentrations between each sampling site. Statistical significance was determined based on comparison of the F value to the F critical value, and the size of the P-value (≤ 0.05).

ANOVA						
Source of Variation	*SS*	*df*	*MS*	***F***	***P-value***	***F crit***
Between Groups	26193.5	3	8731.166	0.288953	0.832893	3.027998
Within Groups	694981.6	23	30216.59			
Total	721175.1	26				

Comparison Between Concentration Data for All Organisms

The raw data for concentration of each organism over the seven-week experiment at each site was consulted and compared through the use of a bar graph (Figure 8), while several statistical tests were run to determine the statistical significance of the results (Tables 10-13).

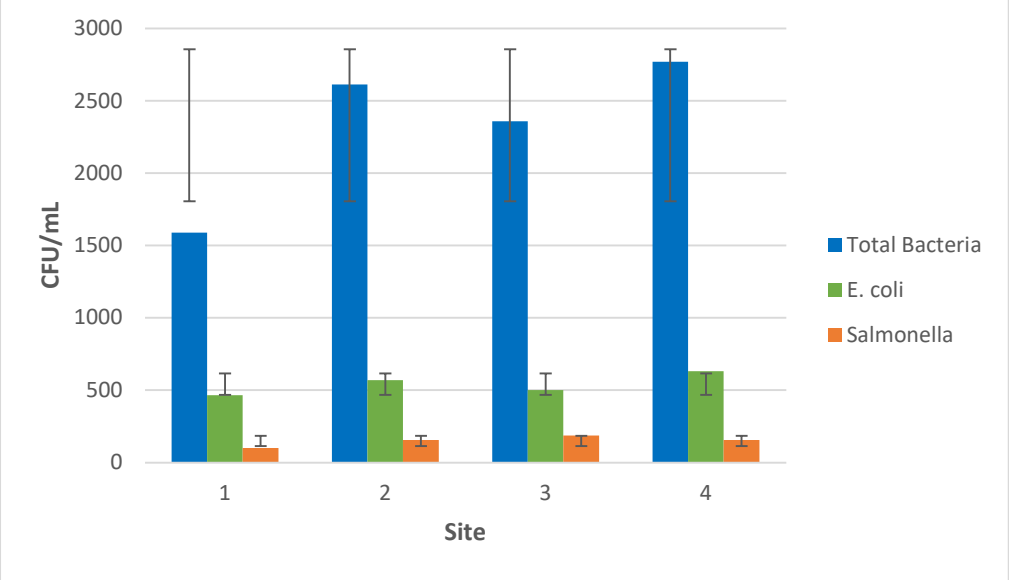

Figure 8. Average Concentration of Total Culturable Bacteria, *E. coli*, and *Salmonella* Over 7 Weeks. The recorded concentrations (CFU/mL) for total bacteria, *E. coli*, and *Salmonella* present in Sinking Creek each week were averaged based on site to determine average concentration of all organisms over the entire experiment. The blue bars represent total bacteria site averages, the green bars represent *E. coli* site averages, and the orange bars represent *Salmonella* site averages. Error bars represent standard deviation.

Table 10. ANOVA: Single Factor to Determine Significance of All Organism Concentration Between Weeks. A single factor ANOVA was run to determine the statistical significance of all organism concentration between each week of the experiment. Statistical significance was determined based on comparison of the F value to the F critical value, and the size of the P-value (≤ 0.05).

ANOVA						
Source of Variation	*SS*	*df*	*MS*	***F***	***P-value***	***F crit***
Between Groups	17446075	2	8723038	3.087908	0.070311	3.554557
Within Groups	50848242	18	2824902			
Total	68294318	20				

Table 11. ANOVA: Single Factor to Determine Significance of All Organism Concentration Between Sites. A single factor ANOVA was run to determine the statistical significance of all organism concentration between each sampling site. Statistical significance was determined based on comparison of the F value to the F critical value, and the size of the P-value (≤ 0.05).

ANOVA						
Source of Variation	*SS*	*df*	*MS*	***F***	***P-value***	***F crit***
Between Groups	9969185.88	2	4984593	60.51697	6.03721E-06	4.256495
Within Groups	741301.808	9	82366.87			
Total	10710487.7	11				

Table 12. ANOVA: Two-Factor with Replication to Determine Significance Between All Organism Concentration and Site. A two factor ANOVA was run to determine the statistical significance between all organism concentrations and sample site. The difference between sites ("sample"), difference between organisms ("columns"), and the interaction between the two variables were analyzed. Statistical significance was determined based on comparison of the F value to the F critical value, and the size of the P-value (≤ 0.05).

ANOVA						
Source of Variation	*SS*	*df*	*MS*	***F***	***P-value***	***F crit***
Sample	2363840.021	3	787946.6735	0.254321177	0.858012911	2.731807
Columns	69784301.15	2	34892150.57	11.26194588	5.55229E-05	3.123907
Interaction	2825272.634	6	470878.7723	0.151982929	0.988090163	2.227404
Within	223072892.4	72	3098234.617			
Total	298046306.2	83				

Table 13. ANOVA: Two-Factor with Replication to Determine Significance Between All Organism Concentration and Week. A two factor ANOVA was run to determine the statistical significance between all organism concentration and week of the experiment. The difference between weeks ("sample"), difference between organisms ("columns"), and the interaction between the two variables were analyzed. Statistical significance was determined based on comparison of the F value to the F critical value, and the size of the P-value (≤ 0.05).

ANOVA						
Source of Variation	*SS*	*df*	*MS*	***F***	***P-value***	***F crit***
Sample	96648711.05	6	16108118.5	40.80623	6.93413E-20	2.246408
Columns	69784301.15	2	34892150.6	88.39126	5.1877E-19	3.142809
Interaction	106744258.5	12	8895354.88	22.53434	2.3678E-18	1.909325
Within	24869035.49	63	394746.595			
Total	298046306.2	83				

Discussion

The first goal of this experiment was to establish the presence of *E. coli*, *Salmonella*, and other bacteria in Sinking Creek. This was done by simply culturing the organisms present in the water samples from each site using selective and non-selective media, using basic organism identification, and counting colonies. The three replica concentrations (CFU/mL) from each site were averaged for each week of the experiment to show the average site concentration for total culturable bacteria, *E. coli*, and *Salmonella* (Tables 1, 4, and 7). These data were compiled for all sites and weeks, except for Week 5 – Site 2. During Week 5, Site 2 was unreachable due to an inmate litter clean-up, so a water sample was not collected, and no concentrations were recorded (Tables 1, 4, and 7).

Just by observing the raw data for average concentration of each organism at each site for the duration of the experiment (Tables 1, 4, and 7), there are no significant trends, except for the spike in concentration at all four sites during Week 2. While sampling during Week 1 of the experiment, Johnson City, TN received 0 inches of rain, but on the day of sampling for Week 2, Johnson City received ~0.5 inches of rain with accumulation, which lead to significant flooding of the banks at each sampling site (U.S. Department of Commerce, 2020). Rain plays a significant role in the stirring up and dispersal of pathogens in surface water through the splashing of rain as it lands in the water, and by increasing pathogen concentrations through runoff (Fitt, McCartney, & Walklate, 1989). As the intensity of the rain that day increased, it most likely disturbed pathogens that had accumulated within the sediment and washed pathogens into the creek, causing the samples collected that day to have a higher concentration of bacteria present than the samples from the previous week, as well as future weeks.

The second goal of this experiment was to determine the concentration patterns of *E. coli*, *Salmonella*, and other bacteria as water in Sinking Creek flows downstream. This was done by determining the average concentration of total bacteria, *E. coli*, and *Salmonella* at each site

for the whole experiment to determine which site contained the highest organism concentrations.

The overall average concentration for total culturable bacteria and *E. coli* followed a similar site pattern; as water flowed downstream from Site 1 to Site 2, bacterial concentrations increased slightly, then concentrations decreased slightly at Site 3, and spiked back up at Site 4 (Figures 2 and 4). For total culturable bacteria and *E. coli*, Site 1 had the lowest average concentration and Site 4 had the highest average concentration (Figures 2 and 4). But, the overall average concentration for *Salmonella* did not follow this same pattern. For *Salmonella*, Site 1 had the lowest average concentration, but Site 3 proved to have the highest average concentration (Figure 6). In addition to concentration patterns by site, evident and expected concentration patterns for individual organisms were observed. At all four sites, the average concentration for total culturable bacteria significantly exceeded the average concentration for both *E. coli* and *Salmonella* (Figure 8). This confirms that Sinking Creek most likely has an abundant microbial population made up of organisms that are easily cultured using non-selective media and maybe acting as competition to foreign species like *E. coli* and *Salmonella. Salmonella*, on the other hand, showed the lowest average concentration for all four sites, but the difference compared to the concentration of *E. coli* was less significant (Figure 8). This makes sense because *E. coli* and *Salmonella* favor similar environments for growth (Arrus et al., 2006).

Looking at concentration patterns over time, total culturable bacteria, *E. coli*, and *Salmonella* all followed a similar pattern. For each one, average concentration for all four sites was the lowest during the first week and experienced a drastic spike during Week 2 because of the heavy rain (Figures 3, 5, and 7). Following the spike, organism concentrations experienced a steep drop in Week 3, and then slowly decreased between Week 3 and Week 5 (Figures 3, 5, and 7). The exception to this was total culturable bacteria concentrations for Week 5. Instead of

gradually decreasing like concentrations of *E. coli* and *Salmonella*, a small spike was observed for Sites 1, 3, and 4 (Figure 3). One reason for this could have been the water temperature at the time of sampling. A trend of decreasing aquatic temperature was seen from Week 3 (10.12°C) to Week 5 (9.92°C), which most likely contributed to the decline in concentrations of *E. coli* and *Salmonella* during that time because, despite having the potential to grow at temperatures as low as 4°C, these organisms survive best near 37°C (Arrus et al., 2006). As for the spike in total bacteria concentrations, some of the unidentified bacteria cultured on R2A media may be species whose optimal growth temperatures coincide with the low temperatures that were measured during Week 5.

Statistical Significance within the Experiment

Analysis of variance (ANOVA) was used to determine the statistical significance of the results. Each ANOVA was calculated with $\alpha = 0.05$, where statistical significance is proven by a P-value less than 0.05 and an F value greater than the F critical value. One exception was made to this rule; in one case (Table 10), the P-value was less than 0.1, but the F value (3.09) was slightly less than the F critical value (3.55). It was still concluded as statistically significant because of the low N and larger variability in environmental samples an alpha level of 0.1 is often used for environmental sampling, and the difference between the F and F critical values was insignificant.

For each organism, two one-way ANOVAs were calculated; one to determine the significance of organism concentration between weeks and one to determine the significance of organism concentration between sites. For all three organisms, statistical significance was found when evaluating variation between concentration and week (Tables 2, 5, and 8). This meaning that with 95+% certainty there is a difference in the weekly concentration of each organism. Using ANOVA to compare organism concentrations between weeks, a statistically significant trend was found, which supports the observation that there is a difference in the

weekly concentration of organisms (Table 10). In contrast, statistical significance was not found for any organism when evaluating the variation between concentration and site, meaning that it cannot be confirmed with 95+% certainty that there is a difference in organism concentration at each site (Tables 3, 6, and 9). This can be seen visually by looking at the standard deviation error bars on Figures 2, 4, 6, and 8. All of the error bars for the bar graphs overlap and are the same, which shows that the concentration differences for the sites were not statistically significant. Despite not finding significant variation between concentrations of individual organisms and site, significance was found between all organisms and site (Table 11). This makes sense because, individually, the average concentrations of the organisms look very close with little variation (Figures 2, 4, and 6), but, when combined, the difference is more radical (Figure 8).

Two-way ANOVAs were used to analyze the interaction between 1) the combined average concentrations of all organisms and weeks and 2) the combined average concentrations of all organisms and sites. The interaction between combined organism concentrations and the week was found to be statistically significant (Table 12), meaning that it is with 95+% certainty that all organism concentrations varied by week, thus supporting the results of the one-way ANOVAs. The interaction between combined organism concentration and the site was found to not be statistically significant (Table 13) meaning than it cannot be said with 95+% confidence that all organism concentration varies by site, which is inconsistent with what was determined by the one-way ANOVAs. But, the F value, once again, is only slightly lower than the F critical values and the P-value barely exceeds 0.1 (Table 13). So, even though this is inconsistent with other findings, it only slightly misses the mark to be qualified as statistically significant.

Wetland Effectiveness

The third goal of this experiment was to analyze the effectiveness of the constructed wetland in Sinking Creek, and its ability to reduce downstream bacterial concentrations. The wetland implementation within Sinking Creek was intended to act as a filter between Sites 2 and 3, specifically to reduce *E. coli* concentrations downstream since contamination with *E. coli* is an important issue for Sinking Creek. Research has been done on the removal of pathogens from water systems through the use of physical, chemical, and biological factors in a constructed wetland, so, based on this, it would be expected that a wetland would be an effective mechanism of remediation for Sinking Creek (Wu et al., 2016). The hypothesis was made that, because of the intended function of the wetland, the concentrations of bacteria at Sites 3 and 4 would be significantly lower than the concentrations at Sites 1 and 2, but this was disproved. The overall average concentrations for total culturable bacteria, *E. coli*, and *Salmonella* were all found to be higher at Sites 3 and 4 than at Sites 1 and 2 (Figures 2, 4, and 6). In most cases, even looking at the raw data, in which all concentrations for each site and week are displayed, the concentrations for Sites 3 and 4 were higher than those for Sites 1 and 2 (Tables 1, 4, and 7).

This data shows and supports that idea that the wetland is not effectively performing the job for which it was created. In addition, the increase in bacterial concentrations at Sites 3 and 4 suggests that there is most likely a source of fecal contamination below the wetland that is causing increased concentrations of pathogens as compared to Sites 1 and 2.

Recommendations

Because it is apparent that the wetland is not functioning as effectively as it should be, it is recommended that steps be taken to restore the wetland to a functional state, such as restoring ecological integrity, natural structure, native species, and using bioengineering techniques to fix issues (U.S. Environmental Protection Agency, 2018). It is also recommended

that research and investigation be continued to determine the source of fecal contamination below the wetland. Once the source is determined, consideration should be made as to whether the original wetland should be extended, a second wetland implemented, or a different approach entirely needs to be taken to continue in the remediation efforts started with the wetland implementation.

Conclusions

Through this study, the presence of *E. coli*, *Salmonella*, and other bacteria in Sinking Creek was confirmed. It was determined that weather can have a significant impact on the concentration patterns of these organisms from week-to-week. By observing average concentrations of total culturable bacteria and comparing them to the average concentrations of *E. coli* and *Salmonella*, it was seen that Sinking Creek most likely contains an abundant population of easily cultured bacteria that serve as a source of competition for foreign species. Similar organism concentration patterns were found between sites and weeks, and that data could be used in future projects to estimate pathogen locations and the best steps for remediation. Using ANOVA, statistical significance was found between organism concentration and week, and, in most cases, statistical significance was not found between organism concentration and site. After analyzing all data, the initial hypothesis was disproved by displaying that all bacterial concentrations were higher at Sites 3 and 4 than at Sites 1 and 2, proving that the implemented wetland is not functioning properly, and will require further research and action to restore it to its intended state.

References

Arrus, K. M., Holley, R. A., Ominski, K. H., Tenuta, M., & Blank, G. (2006). Influence of temperature on *Salmonella* survival in hog manure slurry and seasonal temperature profiles in farm manure storage reservoirs. *Livestock Science*, *102*(3), 226-236.

Ashurst, J. V., Truong, J., & Woodbury, B. (2019). Salmonella Typhi. In *StatPearls [Internet]*. StatPearls Publishing.

Baker-Austin, C., & Dopson, M. (2007). Life in acid: pH homeostasis in acidophiles. *Trends in Microbiology*, *15*(4), 165–171. doi: 10.1016/j.tim.2007.02.005

Bosch, A., Guix, S., Sano, D., & Pinto, R. M. (2008). New tools for the study and direct surveillance of viral pathogens in water. *Current Opinion in Biotechnology*, *19*(3), 295-301.

Cabral, J. P. (2010). Water microbiology. Bacterial pathogens and water. *International journal of environmental research and public health*, *7*(10), 3657-3703.

Center for Disease Control and Prevention. (2019, July 15). E. coli (Escherichia coli). Retrieved from https://www.cdc.gov/ecoli/index.html

Chalmers, R. M., Aird, H., & Bolton, F. J. (2000). Waterborne Escherichia coli O157. *Journal of Applied Microbiology*, *88*(S1), 124S-132S.

Edberg, S. C. L., Rice, E. W., Karlin, R. J., & Allen, M. J. (2000). Escherichia coli: the best biological drinking water indicator for public health protection. *Journal of applied microbiology*, *88*(S1), 106S-116S.

Ferguson, C., Husman, A. M. D. R., Altavilla, N., Deere, D., & Ashbolt, N. (2003). Fate and transport of surface water pathogens in watersheds.

Fitt, B. D., McCartney, H. A., & Walklate, P. J. (1989). The role of rain in dispersal of pathogen inoculum. *Annual review of phytopathology*, *27*(1), 241-270.

Haack, S. K., Duris, J. W., Fogarty, L. R., Kolpin, D. W., Focazio, M. J., Furlong, E. T., & Meyer, M. T. (2009). Comparing wastewater chemicals, indicator bacteria concentrations, and bacterial pathogen genes as fecal pollution indicators. *Journal of environmental quality*, *38*(1), 248-258.

Haley, B. J., Cole, D. J., & Lipp, E. K. (2009). Distribution, diversity, and seasonality of waterborne salmonellae in a rural watershed. *Appl. Environ. Microbiol.*, *75*(5), 1248-1255.

Hellweger, F. L., Bucci, V., Litman, M. R., Gu, A. Z., & Onnis-Hayden, A. (2009). Biphasic decay kinetics of fecal bacteria in surface water not a density effect. *Journal of Environmental Engineering*, *135*(5), 372-376.

Lampel, K. A. (2014). *Bad Bug Book: foodborne pathogenic microorganisms and natural toxins handbook*. North Charleston, SC: CreateSpace.

Levantesi, C., Bonadonna, L., Briancesco, R., Grohmann, E., Toze, S., & Tandoi, V. (2012). Salmonella in surface and drinking water: occurrence and water-mediated transmission. *Food Research International*, *45*(2), 587-602.

Liner, M. (2019). Determining How Temperature Affects the Survival of *Salmonella* and *E. coli* Species in Sinking Creek: A Meta-Evaluation (Unpublished undergraduate thesis). East Tennessee State University, Johnson City, TN.

Liu, H., Whitehouse, C. A., & Li, B. (2018). Presence and Persistence of *Salmonella* in Water: The Impact on Microbial Quality of Water and Food Safety. *Frontiers in public health*, *6*, 159. https://doi.org/10.3389/fpubh.2018.00159

Naghili, H., Tajik, H., Mardani, K., Razavi Rouhani, S. M., Ehsani, A., & Zare, P. (2013). Validation of drop plate technique for bacterial enumeration by parametric and nonparametric tests. *Veterinary research forum: an international quarterly journal*, *4*(3), 179–183.

Olsen, S. J., Miller, G., Breuer, T., Kennedy, M., Higgins, C., Walford, J., McKee, G., Fox, K., Bibb, W., & Mead, P. (2002). A waterborne outbreak of Escherichia coli O157:H7 infections and hemolytic uremic syndrome: implications for rural water systems. *Emerging infectious diseases*, *8*(4), 370–375. https://doi.org/10.3201/eid0804.000218

Pandey, P. K., Kass, P. H., Soupir, M. L., Biswas, S., & Singh, V. P. (2014). Contamination of water resources by pathogenic bacteria. *AMB Express*, *4*, 51. doi:10.1186/s13568-014-0051-x

Park, S. H., Ryu, S., & Kang, D. H. (2012). Development of an improved selective and differential medium for isolation of Salmonella spp. *Journal of clinical microbiology*, *50*(10), 3222–3226. https://doi.org/10.1128/JCM.01228-12

Saarinen, K., Laakso, J., Lindström, L., & Ketola, T. (2018). Adaptation to fluctuations in temperature by nine species of bacteria. *Ecology and evolution*, *8*(5), 2901–2910. doi:10.1002/ece3.3823

Smith, E., Gordon, R., Madani, A., & Stratton, G. (2005). Pathogen removal by agricultural constructed wetlands in cold climates. *Journal of Environmental Informatics*, *6*(1), 46-50.

Taylor, W. I., & Schelhart, D. (1971). Isolation of Shigellae: VIII. Comparison of Xylose Lysine Deoxycholate Agar, Hektoen Enteric Agar, Salmonella-Shigella Agar, and Eosin Methylene Blue Agar with Stool Specimens. *Appl. Environ. Microbiol.*, *21*(1), 32-37.

Tennessee Department of Environment and Conservation. (2017). Proposed final year 2016 303(d) list. Planning and Standards Unit Division of Water Resources: Tennessee Department of Environment and Conservation.

U.S. Department of Commerce. (2020). Advanced Hydrologic Prediction Service. Retrieved from https://water.weather.gov/precip/

U.S. Environmental Protection Agency. (2018). Principles of Wetland Restoration. Retrieved from https://www.epa.gov/wetlands/principles-wetland-restoration

United States Geological Survey [USGS]. (2007). Analytical Methods. Retrieved from https://oh.water.usgs.gov/OWML/micro_methods_mFC_agar.htm

van Elsas, J. D., Semenov, A. V., Costa, R., & Trevors, J. T. (2011). Survival of Escherichia coli in the environment: fundamental and public health aspects. *The ISME journal*, *5*(2), 173–183. doi:10.1038/ismej.2010.80

Weber, K. P., & Legge, R. L. (2008). Pathogen removal in constructed wetlands. *Wetlands: Ecology, Conservation and Restoration*, 176-211.

Wells, J. G., Shipman, L. D., Greene, K. D., Sowers, E. G., Green, J. H., Cameron, D. N., ... & Ostroff, S. M. (1991). Isolation of Escherichia coli serotype O157: H7 and other Shiga-like-toxin-producing E. coli from dairy cattle. *Journal of Clinical Microbiology*, *29*(5), 985-989.

Wilkes, G., Edge, T. A., Gannon, V. P. J., Jokinen, C., Lyautey, E., Neumann, N. F., ... & Lapen, D. R. (2011). Associations among pathogenic bacteria, parasites, and environmental and land use factors in multiple mixed-use watersheds. *Water Research*, *45*(18), 5807-5825.

Winfield, M. D., & Groisman, E. A. (2003). Role of nonhost environments in the lifestyles of Salmonella and Escherichia coli. *Appl. Environ. Microbiol.*, *69*(7), 3687-3694.

Wu, S., Carvalho, P. N., Müller, J. A., Manoj, V. R., & Dong, R. (2016). Sanitation in constructed wetlands: a review on the removal of human pathogens and fecal indicators. *Science of the Total Environment*, *541*, 8-22.