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Determining How Temperature Affects the Survival of Salmonella and E. coli Species in Sinking Creek: A Meta-Evaluation

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Determining How Temperature Affects the Survival of *Salmonella* and *E. coli* Species in Sinking Creek: A Meta-Evaluation

By

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Dr. Kurt J. Maier, Reader Date

Abstract

In recreational waters, pathogen pollution is a major concern for the USEPA. The USEPA is responsible for initiating the National 303(d) List of Impaired Surface Waters. Pathogen pollution from *E. coli* is a common reason why recreational waters are placed on the 303(d) list. *E. coli* O157 H:7 and other enteric pathogens can cause serious illness and even death. Sinking Creek is a part of the Watauga River watershed which runs through Carter and Washington county. Sinking Creek is currently listed on the 303(d) as impaired due to the presence of *E. coli*. Because of the known presence of *E. coli*, it is possible that other enteric pathogens may exist in the creek. The main objective of the study was to determine the presence of *E. coli* and *Salmonella* bacteria within Sinking Creek and was accomplished by using selective media to isolate the bacteria. The second goal of the study was to understand how various temperature treatments effect the growth of *Salmonella* and *E. coli* in Sinking Creek. Water samples from Sinking Creek were incubated at 4, 28, and 37°C. The third objective of the study was to determine how the survival of *Salmonella* and *E. coli* from Sinking Creek compared to samples taken from manure slurry via a meta-evaluation. It was predicted that *Salmonella* would not be present in water samples taken from Sinking Creek. It was further hypothesized that *E. coli* and any present *Salmonella* would have limited to no growth at 4°C and the most growth would occur at 37°C. A pilot study was conducted to determine the growth of microorganisms naturally present in Sinking Creek. The data from the pilot study was used to determine the creek's ability to support a healthy microbiota. Samples for the main experiment were taken from September 2019 to November 2019. The main experiment found that *Salmonella* was present in Sinking Creek at lower amounts than *E. coli*. Both *E. coli* and *Salmonella* grew significantly at 4°C. On average, the least amount of growth for *Salmonella* and *E. coli* was at 37°C. Most growth on R2A peaked at 5 days of incubation. Water incubated at 37°C showed the highest growth peaks at 5 days for all three selective plates. For all three selective plates, water incubated at 4°C peaked in growth between days 5 and 7. The results of the main experiment could have been affected by factors such as contamination. Another limitation of the study was that enumeration of the colony forming units became less accurate after larger colonies had formed. Replicating the main experiment over a longer period could indicate more representative growth curves. The meta-evaluation concluded there was no difference in decay rate between samples taken from water or manure. The results of the meta-evaluation disproved the hypothesis that manure would have lower decay rates than samples taken from water. A larger sample size is recommended to yield more representative results for the meta-evolution. Further replications of the main experiment are recommended along with studies sampling the presence of *Salmonella* and *E. coli* at various distances from the below wetlands site.

Table of Contents

Acknowledgements

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List of Tables

List of Figures

Introduction

Water and Human Health

The World Health Organization (WHO) estimates that 10% of the world's population does not have access to clean, potable water (WHO, 2019). Potable water refers to water that is free of pathogens or disease-causing agents (Levallois & Villanueva, 2019). Waterborne, diarrheal diseases are responsible for more than 485,000 deaths each year; this number is increasing as populations grow increasingly limited in potable water sources (WHO, 2019). Diseases which are most commonly associated with contaminated water are cholera, hepatitis A, dysentery, STEC producing *E. coli* infections, and typhoid fever as a result of *Salmonella* (WHO, 2019). The quality of water is a crucial element to public health. All sources of water have the potential to become contaminated; be it, public drinking water or water for recreational use. It is crucial that all bodies of water are monitored properly, and preventative measures be used to limit potential contamination.

Pathogenic organisms can infiltrate bodies of water in many ways. More commonly, contamination by bacteria occurs when fecal material enters a body of water. Many enteric pathogens, such as *E. coli*, infect their host via the fecal-to-oral route. *E. coli* contamination can occur due to agricultural runoff or cross connections of raw sewage; other pathogens may contaminate water by similar means (Levallois & Villanueva, 2019). Those in contact with contaminated water are at a significant risk of developing infection. Recreational water that is contaminated with bacteria can pose a significant threat to human health (Levallois & Villanueva, 2019). With increasing urbanization and development, contamination of both recreational and drinking water has increased (WHO, 2019).

To protect human health and the health of the environment, the United States Environmental Protection Agency (USEPA) has developed criteria and methods to predict the presence and limit exposure to pathogenic organisms. The USEPA's 303(d) list is part of the federal Clean Water Act. The Clean Water Act was promulgated in 1972 and established the basic requirements for regulating water sources (TDEC, 2017). Section 303(d) of the Clean Water Act requires states to develop a comprehensive list of all impaired lakes and streams (TDEC, 2017). The 303(d) list also incorporates streams which are expected to become impaired in two years (TDEC, 2017). As defined by the USEPA, an impaired stream is a body of water that exceeds the pollution criteria established by the Clean Water Act (TDEC, 2017). The total maximum daily load is the total amount a pollutant may be discharged into a stream. TMDL studies are required for streams on the 303(d) list until they can meet the requirements under the Clean Water Act (TDEC, 2017). Sinking Creek in Washington and Carter county in northeast Tennessee is an example of an impaired stream. Sinking Creek of the Watauga River Watershed in east Tennessee is listed on the Tennessee Department of Environment and Conservation's (TDEC) 303(d) list. The stream is considered impaired due to *E. coli* contamination which exceeds the TMDL. As determined by TDEC, Sinking Creek is impaired due to runoff from pasture grazing cattle (TDEC, 2017).

Survival of Pathogens in Water

There are many factors that influence the survival of pathogens in impaired streams. Bacteria such as *Salmonella* and *E. coli* require the appropriate pH, osmotic content, oxygen availability, and temperature to survive and reproduce. Furthermore, predation and competition for resources can limit the growth of pathogenic bacteria. Bacterial species can colonize and survive longer if nutrients are easily available (Farovitch, 2016). Competition for nutrients with other organisms

can limit the survival of certain species. Nutrient sources such as nitrate and phosphate can affect the presence of bacteria in streams (Farovitch, 2016). When streams experience significant nutrient contamination, from fertilizers and other anthropogenic sources, a buildup in organic material results in an increase in heterotrophic bacteria; with the correct pH and temperature, enteric pathogens can thrive in these conditions (Farovitch, 2016). Temperature is also an important determining factor for the survival of pathogens in aquatic environments. Enteric pathogens most often prefer temperatures close to that of warm-blooded mammals but can survive in colder temperatures outside of their host (Farovitch, 2016). Various organisms thrive best at different temperatures; for example, *E. coli* and *Salmonella* differ in optimum temperature for growth.

In laboratory experiments, selective media is used to isolate and enumerate the desired bacterial species; as the media contains factors that inhibit the growth of competing organisms. Selective media can inhibit competition by creating an environment that is hostile to other organisms yet is still capable of isolating the desired species; this is done by adjusting the pH, nutrient availability, or oxygen content of the media.

Salmonella spp.

Salmonella is a gram-negative bacterium belonging to the family *Enterobacteriaceae*. The rod-shaped, flagellated organism is the result of many food and waterborne disease outbreaks (CDC, 2011). *Salmonella* is transmitted via the fecal to oral route and is found in the gastrointestinal tract of organisms such as mammals, birds, and reptiles (CDC, 2011). *Salmonella* infections often result in gastroenteritis; which is characterized by severe vomiting and diarrhea within 12 to 72 hours of ingestion (CDC, 2011). The infection usually last four to seven days and does not

typically require medical intervention (CDC, 2011). In more serious cases, such as those involving children, the elderly, or the immune-compromised, *Salmonella* infections may result in hospitalization or death (CDC, 2011). The CDC (2011) and Scallan et al. (2011) have estimated that *Salmonella* causes 1.2 million illnesses, 23,000 hospitalizations, and 450 deaths in the United States annually. In the United States, *Salmonella* is mainly contracted through contaminated food products such as undercooked pork, chicken, or eggs. Fruits and vegetables may become contaminated from water compromised by fertilizer or other animal waste products (Winfield and Groisman, 2003).

Unlike similar enteric pathogens, *Salmonella* species can survive extended periods of time without a host (Winfield and Groisman, 2003). *Salmonella* typhi's cellular structure is comprised of a sturdy, bi-layered membrane. The organism uses a complex regulatory system which allows the cell to respond to environmental changes (Humphrey, 2004). In response to changes in the environment, *Salmonella* has demonstrated the ability to alter its genetic structure to enhance its chance of survival (Humphrey, 2004). Winfield and Groisman (2003) determined that *Salmonella* has a higher survival rate in aquatic environments than other pathogens. *Salmonella* can grow under aerobic and anerobic conditions; allowing it to proliferate in unique environments. A concern is *Salmonella's* ability to grow in impaired waters (Arrus et al., 2006).

Salmonella prefers warmer temperatures such as that of its host (37°C in humans) (Arrus et al., 2006). However, *Salmonella* can grow at temperatures as low as 4°C. Arrus et al. (2006) observed the growth of *Salmonella Typhimurium, Salmonella Agona, Salmonella Hadar, and Salmonella Oranienburg* in swine manure at temperatures of 4°C, 25°C, and 37°C. They found that *Salmonella* bacteria demonstrated significant growth at 4°C for the duration of the experiment. Arrus et al. (2006) determined the resilience of *Salmonella* species and their

resistance to environmental change. *Salmonella's* ability to survive and persist in the environment is a concern to human health regarding the potential to contract disease from recreational waters.

Pathogenic *E. coli*

E. coli is a gram-negative, rod-shaped bacterium naturally found in the intestinal tract of mammals such as cattle and humans. Like *Salmonella*, *E. coli* belongs to the family *Enterobacteriaceae*. Most species of *E. coli* are not harmful; however, some species can cause serious infection (CDC, 2014; Croxen et al., 2013). Pathogenic *E. coli* are largely responsible for human diarrheal diseases. Of the six pathogenic *E. coli* capable of causing diarrheal-like symptoms, Shiga-toxin producing (STEC) or *E. coli* O157 H:7 is most commonly associated with food and waterborne outbreaks (CDC, 2014; Croxen et al., 2013). A severe complication associated with a STEC infection is hemolytic uremic syndrome. Hemolytic uremic syndrome can be life threating in some cases (CDC, 2014). As an enteric pathogen, *E. coli* prefers the warm temperatures of its host; therefore, *E. coli* bacteria grow best at temperatures around 37° (Croxen et al., 2013). Assuming optimal environmental conditions, *E. coli* bacteria can survive in water between 4 to 12 weeks (Croxen et al., 2013).

E. coli and other coliform bacteria can be used as indicators of stream impairment (USEPA, 2012). Because *E. coli* is found naturally in the intestinal tract of mammals, the presence of *E. coli* in streams could indicate fecal contamination (USEPA, 2012). The USEPA uses coliform bacteria as a basis for water quality assessment (Edberg et al., 2000). In fact, the USEPA refers to *E. coli* specifically as the best indicator of fecal contamination in surface waters (USEPA, 2012). Many peer-reviewed research articles argue fecal coliforms do not provide an accurate

prediction of water contamination (Edberg et al., 2000). Fecal coliforms can be indicative of pathogenic organisms but can also indicate contamination of bacteria naturally found in soil and surrounding vegetation. For this reason, some scholars find coliform bacteria to be an inaccurate indicator of stream impairment (USEPA, 2012). The USEPA still utilizes fecal coliforms as the standard test of water quality for drinking water; as contamination of drinking water by fecal coliforms indicates infiltration from an outside source (USEPA, 2012). In terms of *E. coli*, its presence in streams and other recreational waters is more likely to indicate the presence of fecal contamination than other coliforms. Many states still use *E. coli* as the main indicator of stream impairment (USEPA, 2012).

Goals and Objectives

There were three goals to this study. The primary goal of the study was to determine the presence of *Salmonella* and *E. coli* in samples taken from Sinking Creek below a wetland. The second goal of the study was to study how various temperature treatments effect the growth of *Salmonella* and *E. coli* in Sinking Creek. The growth patterns of *Salmonella* and *E. coli* were determined at temperatures of 4°C, 28°C, and 37°C. The growth trends of the organisms were determined over a period of 14 days per sample. The data collected for this part of the experiment can be used to provide information useful to assessing the overall health of the creek. The presence of the two pathogens could also indicate potential health hazard implications for those in contact with Sinking Creek. It was predicted that *E. coli* would have a significant presence in the creek samples (TDEC, 2017). This prediction was made provided Sinking Creek is listed on the TDEC 303(d) list as impaired due to *E. coli* contamination (TDEC, 2017). It was further predicted that the *E. coli* would exhibit the strongest growth trends at temperatures of 28 and 37°C (Croxen et al., 2013; Farovitch, 2016). It was also hypothesized that *E. coli* would no

longer exhibit growth even at 14 days in the flasks; and growth at 4°C would decrease over time far more quickly than the other temperature treatments (Croxen et al., 2013; Farovitch, 2016). It was hypothesized that *Salmonella* would not be present within the creek samples; as the TDEC 303(d) does not acknowledge the presence of the organism (TDEC, 2015). If *Salmonella* was present in the flasks, it was predicted that the organisms would grow best at temperatures of 37°C (Croxen et al., 2013; Farovitch, 2016). *Salmonella* was predicted to no longer exhibit growth past 5 to 7 days of incubation at the appropriate temperature.

The third goal of the study was to compare the result from the main experiment to that of published survival studies using *Salmonella* and *E. coli*. This was accomplished by completing a meta-evaluation of comparison data collected from various other published studies. A meta-evaluation was used over a meta-analysis because of the limited sources used in the statistical analysis. The sources used for the meta-evaluation evaluated the percent survival of *Salmonella* and *E. coli* in water and various other ecosystems. The meta-evaluation is important because it allows one to draw conclusions about the survival and reproducibility of the study. The data from the meta-evaluation can allow comparisons to be made between studies. Furthermore, the meta-evaluation can demonstrate the reproducibility between the main experiment and other published sources. Factors which may influence the reproducibility of the main experiment are geographic location, seasonal variability, and the sources the samples were taken from. Many of these factors affecting reproducibility were unavoidable for the main experiment. The meta-evaluation compares samples taken from fresh water, sea water, and manure. It was predicted that the results of the meta-evaluation would be in favor of the main experiment, and the main experiment would yield similar results to the comparison literature. The published literature for

manure was predicted to yield different results from the main experiment; and therefore, result in a difference somewhere in the ANOVA with 95% confidence.

Literature Analysis

Critical Review of Methods

Rhodes and Kator (1988) determined the survival of *Salmonella* and *E. coli* bacteria at varying temperatures in aquatic ecosystems. Rhodes and Kator did not apply temperature as a direct treatment; rather, they tested the presence of the bacteria at various seasons. Furthermore, Rhodes and Kator (1988) focused on calculating the survival of *Salmonella* and *E. coli* strictly in estuaries. The main experiment in this study tested fresh water over a similar stretch of seasonal variability. In terms of reproducibility, Rhodes and Kator's results are predicted to differ from the main experiment in that both *E. coli* and *Salmonella* were affected by the osmotic concertation of the estuary. Rhodes and Kator sampled for *Salmonella* and *E. coli* at temperatures ranging from 5.9 to 28.2°C; and this could prove a useful comparison to the main experiment. Rhodes and Kator (1988) found *Salmonella* growth at temperatures lower than 10°C; whereas, *E. coli* bacteria were found to die off at similar temperatures. Rhodes and Kator's conclusion could be used as a comparison to the main experiment and serve as an indicator of *Salmonella* and *E. coli's* survival at lower temperatures. A contradiction between Rhodes and Kator and the main experiment are the effects of salinity on bacterial growth. In a similar study, Sampson et al. (2006) determined the survival of *E. coli* in fresh water at 4, 10, 14, and 25°C. While Sampson et al. did not test *E. coli* at higher temperatures, the results of their study can be used as a comparison to the main experiment. Unlike Rhodes and Kator, Sampson et al. retrieved samples from fresh recreational waters; and sampling took place during the summer.

In addition to fresh water, other survival experiments have been conducted using animal manure. Though these samples were not taken from aquatic environments, they can serve as a comparison to the main experiment. Arrus et al. (2006) calculated the survival of *Salmonella* at 4, 25, and 37°C. As previously mentioned, Arrus et al. concluded that *Salmonella* is capable of growth at 4°C. In a similar study, Himathongkham et al. (1999) calculated the growth of *Salmonella* in cattle manure at 4, 20, and 37°C. Interestingly, the experiment found that *Salmonella* growth declined at 37°C (Himathongkham et al, 1999). Due to the similarities in the two study's' methods and temperature treatments, Arrus et al. and Himathongkham et al. were used for the meta-evaluation of the main experiment. The presence of higher nutrient content in the manure as compared to the water samples could contradict the results of the main experiment. It was predicted that Arrus et al. and Himathongkham et al. would have more growth when compared to the main experiment.

All published sources shared similar incubation temperatures (between 35 to 45°C). Furthermore, all published experiments afore mentioned used some form of selective media for *Salmonella* and *E. coli.* The main experiment used similar incubation temperatures (28°C for R2A, 37°C for MFC, and 35°C for XLD agar) and selective media to promote reproducibility between experiments. Though there are many sources which aim to determine the influence of temperature on the survival of *Salmonella* and *E. coli*, few experiments specifically test *Salmonella* and *E. coli at* 4, 28, and 37°C. The main experiment also determines the survival of *Salmonella* and *E. coli* that are already present within the creek, rather than inoculated into the sample.

Methods and Materials

The purpose of the main experiment was to determine the presence of pathogenic *E. coli* and *Salmonella* spp. in Sinking Creek. Temperature treatments were applied to each sample to evaluate the influence of temperature on the survival of *Salmonella* and *E. coli* in Sinking Creek. No other growth factors, such as pH or oxygen, were modified.

A pilot study was conducted prior to the main experiment to understand the concentration of total bacteria in water from Sinking Creek. The data from the pilot study was used to determine the creek's ability to support a healthy microbiota and the potential for competition between microorganisms within the creek (Farovitch, 2016). The existence of other microorganisms in the creek water could inhibit the growth of pathogenic bacteria such as *E. coli* and *Salmonella*. The pilot study was also conducted to estimate the feasibility of the main experiment. From the pilot study, it was found that the micro drop plating method and more frequent sampling times were best to measure organism growth.

Micro Drop Plating Method

The micro drop plating method was determined to be the most practical method of bacterial enumeration for the main experiment. Micro drop plating is often used to determine the number of organisms capable of growing in a given volume (Herigstad, Hamilton and Heersink, 2001). The method is cost effective and allows for multiple dilutions to be recorded on one plate. A drop plate is completed by micro-pipetting one or multiple 10 µL – 30 µL drops onto dry agar (Herigstad, Hamilton and Heersink, 2001). Chen, Nace, and Irwin (2003) found that the micro drop plating method was more cost effective because it reduced the number of plates used and amount of incubator space used vs. spread and pour plating methods. The pour plate method, a

method in which the liquid agar is poured over the sample, has the potential to kill organisms at high temperatures (Chen, Nace and Irwin, 2003). Likewise, the traditional spread plate method does not allow multiple dilutions to fit onto to a single plate (Chen, Nace and Irwin, 2003). Similar comparison studies have demonstrated the micro drop plating method to be the most beneficial with enumerating organisms (Herigstad, Hamilton and Heersink, 2001). However, the micro drop plating method is not standardized (Herigstad, Hamilton and Heersink, 2001). To deter variation in the main experiment, the same brand of pipette tips (Fisher disposable micro-pipette tips 10 µL -100 µL) were used. To minimize the risk of contamination, micro-pipette tips were autoclaved for 45 minutes on the setting liquid 15. In the main experiment, plates were divided into four sections and labeled per dilution. The micro drops were pipetted into each section according to the following figure:

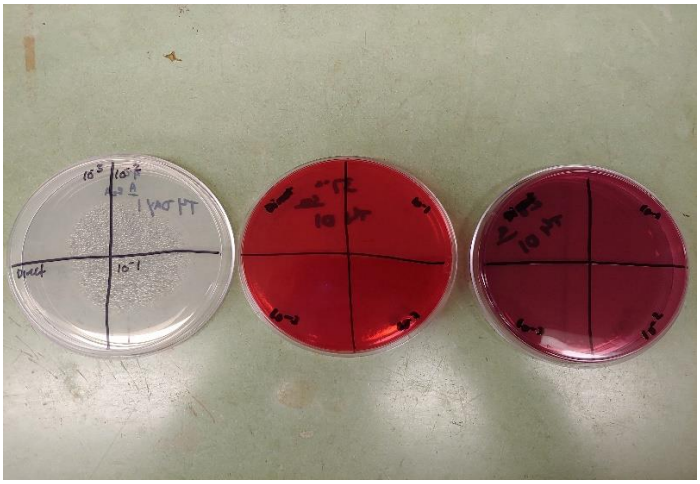

Figure 1: Photo Demonstrating How Plates Were Labeled in Accordance to the Drop-Plate Method. This figure exhibits the four sections allotted on each plate. Plates (staring far left) include R2A, XLD, and MFC agar.

Selective Media

XLD or xylose lysine deoxycholate agar is one of the more common mediums used to isolate *Salmonella* and *Shigella* Species (Nye et al., 2002). XLD plates utilize a phenol red indicator and appear red in color once dried. *Salmonella* species will appear pink or red, with a darker center. Other coliform bacteria will appear bright yellow on the plate (Nye et al., 2002). XLD agar was chosen for the main experiment because of its ease of use and ability to isolate *Salmonella* species with more accuracy than that of some other mediums (Nye et al., 2002).

MFC agar is commonly used to isolate *E. coli* and other fecal coliform bacteria (USGS, 2007). The method is frequently used in laboratories given its inexpensive and easy to use (USGS, 2007). The medium itself contains several agents which allow it to differentiate between other bacterial species. For example, the rosolic acid within MFC inhibits the growth of bacteria other than fecal coliforms (USGS, 2007). The bile salts present in the agar inhibit the growth of non-enteric bacteria (USGS, 2007). Aniline blue within the agar exclusively allows fecal coliforms to ferment lactose; which results in a distinct color change of the medium (USGS, 2007). On MFC agar, *E. coli* will appear green/blue with a dark center (USGS, 2007). Other coliform bacteria will appear red or yellow in color on the MFC agar (USGS, 2007).

Site Description

Sinking Creek is located on the Watauga River Watershed in Johnson City, Tennessee. The creek has a long-standing history of impairment. Sinking Creek was listed on the TDEC 2016 303(d) list as impaired due to the presence of *E. coli* (TDEC, 2017). TDEC estimates the likely source of *E. coli* was runoff from pasture grazing cattle on the creek (TDEC, 2017). TDEC did not

elaborate on the potential for the presence of *Salmonella* in the creek; however, given *E. coli* and *Salmonella* are closely related organisms, favoring similar conditions, this experiment assumed that *Salmonella* was likely present in the creek.

The importance in finding these organisms in Sinking creek is that some sections of the creek serve as recreational parks for nearby residents. Jacob's Park is located above the wetlands of Sinking creek and was established in 2016. The park was founded upon the death of a young boy, Jacob Francisco, who passed in 2004 as a result of an *E. coli* O157 H:7 infection (City of Johnson City, 2019). The cause of the boy's infection is unknown but is was previously assumed he contracted the bacterium from swimming in Sinking Creek (City of Johnson City, 2019). The presence of *E. coli* and the potential existence of *Salmonella* in the creek is incredibly important regarding maintaining a healthy environment for those living around and visiting the creek.

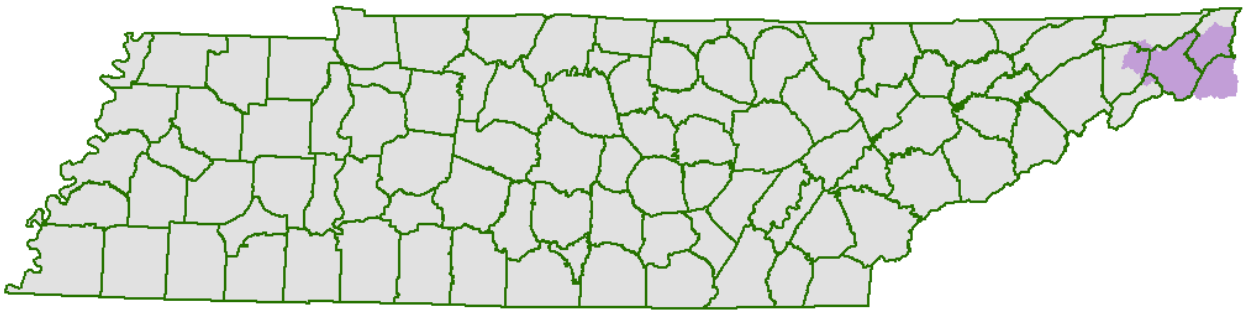

Figure 2. **Drainage area of the Watauga River Watershed (TDEC, 2002).** The figure above indicates the general area of the Watauga River watershed in which part is drained into Sinking creek.

Figure 3. Photo Taken of Sinking Creek Below Wetlands at a High-Water Level. This photo, taken at the beginning of sampling for the pilot study, indicates the general area in which samples were taken from the creek.

The sampling site for the experiment was the site Below Wetlands (BW). The Below Wetlands was chosen because of its potential to "filter" microorganisms that are in creek. Samples were taken from this site specifically because it was believed that the wetlands prevented pathogenic organisms such as *E. coli* and *Salmonella* from continuing downstream. Collecting data from this site would further indicate the effectiveness of the wetlands and the true capability of the two organisms to proliferate in the creek.

Physical Assessment of Sinking Creek

With each sample collected, a visual assessment of the creek was conducted. Bank erosion, presence of vegetation, and the presence of macroinvertebrate species was noted with each visit to the site. Based on these observations, it was evident the creek was impaired. The riparian zone

exhibited little vegetative growth and there were no visible macroinvertebrate populations recorded (EPA, 2004). Furthermore, heavy sedimentation was evident during some sampling site visits (EPA, 2004). Physical assessment of the creek was conducted over a wide period of seasonal variability; allowing variation in the creek to be observed with each sampling period. Seasonal variability also played a significant role in understanding the growth patterns of microorganisms within the creek.

Figure 4: Photos Taken to Demonstrate the Sedimentation and Impairment of Sinking Creek. These photos were taken at the time of sampling for the main experiment. Here, sedimentation and the presence of high nutrient content were noted. Also depicted in the photo, is a used flip-flop; which was among other anthropogenic waste found at the site.

Environmental Parameters

Environmental parameters were taken for the main experiment prior to sampling. Dissolved oxygen, pH, conductivity, and temperature were taken at the center flowline of the creek using the Hanna HI 9828 pH/ORP/EC/DO meter. Environmental parameters were not taken for the pilot experiment.

Sample Collection

Two water samples for the pilot study were collected; one sample collected in February 2019 and on sample from March 2019. Water samples were collected using 18 oz. Whirl-Pak bags. One Whirl-Pak was filled per sample. Water samples were collected using a grab sampling method from the center flowline of the creek. Samples were kept in a walk-in cooler in between each plating. The walk-in cooler was kept at a constant 4°C to prevent further bacterial growth.

Samples for the main experiment were collected from September 30th, 2019 to November 18th, 2019. Water for the experiment was collected using a Nalgene one-liter sampling bottle. Three sampling bottles were used for each collection. Water for each sample was taken using a grab sampling method from the center flow-line of the creek. The samples were kept in a 4°C walk-in cooler for no more than 24 hours prior to plating.

The Pilot Study

Three flasks were filled with water from Sinking Creek. As a control, three flasks were filled with deionized water. The flasks were kept at room temperature (approximately 28°C) in between each round of plating. Each flask was sealed with parafilm as an effort to prevent contamination. One plate was used for each flask; for a total of six plates. Plates were then

divided into four sections corresponding to each dilution. Dilutions were initially prepared by filling 18 test tubes with 4.5 ml of deionized water. The tubes were then autoclaved for 45 minutes. After sterilization, the tubes were sealed and allowed to cool in a walk-in cooler for an additional 45 minutes. Test tubes were given time to cool to prevent inadvertently killing the organisms within the sample. The serial dilutions were prepared by pipetting .5 ml of water from each flask and placing it into its corresponding test tube. This process was continued with each tube until a dilution of 10^{-3} was created for every flask.

The media used for the pilot study was R2A. R2A was a non-selective growth media to enumerate total countable bacteria. The R2A was made by mixing 18.2 grams of R2A powder and 1000 ml of water into a large, 2-liter flask. The mixture was brought to a boil on a hot plate and then sterilized using an autoclave. The agar was then poured evenly into all six plates and allowed to dry for approximately 30 minutes. After the serial dilutions were made, 10 µL were taken from each tube and inoculated onto to the plate within its corresponding section (Naghili et al., 2013). Each section of the plate had a total of three drops or 30 µL total (Naghili et al., 2013). The direct drops were taken from the flask and inoculated onto the plate. The method used for this experiment was the micro drop method (Naghili et al., 2013). The micro drops were given time to dry before inverting and storing the inoculated plates at room temperature for 24 hours. After incubating at room temperature, the plates were counted, and bacterial concentration expressed as colony forming units. Colony forming units (CFU) per milliliter were determined using the following formula:

1.) Number of colonies / volume plated x dilution

After calculating CFU/ml, the log was taken of each value to find log (CFU/ml). The average log (CFU/ml) were calculated between the A, B, and C replications of each temperature. The process was continued each week to extrapolate potential growth patterns in the Sinking Creek samples.

The Main Experiment

Three flasks were used per each temperature treatment and labeled 4°C, 37°C, and 28°C. All nine flasks were filled with 100 ml of Sinking creek sample water. One flask, per each temperature, treatment was filled with 100 ml of sterile deionized water. The deionized water was treated by autoclaving for 45 min. All 12 flasks were then placed at the appropriate temperature and marked as hour 0. The flasks were left at the appropriate temperature for a total of 14 days. R2A agar was used for enumerating general colony-forming units. The R2A agar was prepared as before, using 18.2 grams of R2A powder in 1000 ml of deionized water. XLD agar was used for isolating *Salmonella* spp. and was prepared by mixing 55 g of XLD powder into 1000 ml of deionized water (as per the manufacture's instruction). The XLD agar was then boiled until it became a translucent red. The XLD was not autoclaved. Lastly, MFC broth was used for isolating *E. coli* and other coliform bacteria. MFC agar was prepared by adding 37 g of MFC broth and 17 g of agar to 1,000 ml of deionized water. 0.1 g of rosolic acid was mixed with 10 ml of 0.2 N of NaOH and added to the MFC agar. The MFC agar was then brought to a boil on a hot plate until it became slightly translucent. Plates were prepared by adding approximately 25 ml of the appropriate media to 100 mm x 15 mm Fisher, disposable cell culture dishes. The plates filled with agar were stored at room temperature and allowed to dry for one week prior to plating. The plates were kept in a sealed incubator to prevent cross-contamination of the agar.

After incubating for 24 hours, serial dilutions were made from each flask. Using the micro-drop plating method, three drops from each serial dilution tube (totaling 30 µL) were dropped onto the appropriate section of the plate. Originally, the plates were placed at room temperature and incubated for 24 hours. It was later determined that the MFC plates were best incubated at 37°C, XLD at 35°C, and R2A at room temperature. It was also determined that the plates exhibited the best growth at 48 hours of incubation. For the remainder of the experiment, the selective media were incubated at the appropriate temperature and counted at 48 hours. This process was continued using the same flasks for incubation at days 5, 7, and 14. Plates were counted and the average colony forming units were determined using the afore mentioned formula. For the control experiments, the DI water was initially not sterilized via the autoclave. Beginning Temperature 3, the control DI water was autoclaved and allowed to cool before plating.

For both experiments, those plates which exhibited uncontrolled fungal contamination were labeled "contaminated" and counted as zero. Non-*E. coli* colony forming units were counted and recorded separately from *E. coli* on the MFC plates. Likewise, *Salmonella* and general coliform bacteria were counted and recorded separately on the XLD plates. *Salmonella* species were counted strictly as those that were pink and dark in the center; as plain pink colonies could indicate other organisms (Nye et al., 2002). Yellow colonies were counted as general coliform bacteria; as this could further indicate the presence of *E. coli.* The bacteria were enumerated under magnified lens using a New Brunswick Scientific model C-110 colony counter.

Meta-evaluation

The purpose of the meta-evaluation was to compare the conclusions of the main experiment to similar published sources. All experiments used in the meta-evaluation shared the commonality

of temperature as a treatment for the growth *Salmonella* and *E. coli*. The meta-evaluation was completed by compiling data from the comparison studies. The decay rate was calculated from the average direct and/or unfiltered log CFU/ml of each study and then compared to the decay rate from the main experiment. The decay rate was calculated using the following formula:

2.) $k = [\ln(C_0/C)]/(T)$

Whereas, C_0 = initial concentration

C = final concentration

T = incubation time

The value k was written as k [hour $^{-1}$]. Decay coefficients which were expressed as k [day $^{-1}$], were converted to hours. A meta-evaluation was not completed for the pilot study or controls of the experiment. An ANOVA was used to determine the strength of comparison between the main experiment and the published sources.

Results

Between August and November, the environmental parameters for Sinking Creek fluctuated significantly. The lowest pH recorded was 6.52 on October 9th, 2019. The highest pH recorded was 9.11 on October 16th, 2019. The creek experienced the most fluctuation in dissolved oxygen. The lowest dissolved oxygen recorded was 0.00 mg/L on October 29th, 2019; however, pH and conductivity were relatively normal. Dissolved oxygen levels increased as sampling continued into the later months of the year. The highest dissolved oxygen concentration was 9.01 mg/L on November 5, 2019. Conductivity remained relatively stable; the highest conductivity being 426 µS/cm on September 12th, 2019. The lowest recorded conductivity was 278 µS/cm on October 16th, 2019. Conductivity was not recorded on August 30th or September 9th.

Table 1: Environmental Parameters Taken for the Duration of the Primary Experiment.
(Those listed as N/A failed to receive an instrument reading)

Date	pH	Dissolved O_2 (mg/L)	Conductivity (µS/cm)
8/30	8.50	2.01	N/A
9/9	8.10	2.91	N/A
9/12	7.95	4.67	426
9/16	7.03	2.61	423
9/30	6.91	1.01	410
10/9	6.52	0.59	421
10/16	9.11	5.21	278
10/29	8.30	0.00	391
11/5	9.08	9.01	398
11/18	7.7	8.76	356

The Pilot Study

Tables 2-4 indicate the direct counts observed on each plate of the pilot study; these counts were later converted to colony forming units/ml. In general, the largest amount of growth occurred on the direct counts of all three pilot studies. All three studies mostly followed the trend of decreasing growth the longer the samples were incubated. All three pilot studies decreased as dilutions increased. A control was used for all three pilot studies. The control of pilot study #1 indicated significant amounts of contamination; as seen in table 2. Control pilot studies #1 and #3 did not have contamination.

Table 2: Direct Plate Counts Taken from Pilot Study #1 (Conducted 02/08 2019)

Day 1					
Water	A	D: 90	10^{-1}: 90	10^{-2}: 14	10^{-3}: 9
	B	D: 90	10^{-1}: 36	10^{-2}: 6	10^{-3}: 1
	C	D: 90	10^{-1}: 46	10^{-2}: 3	10^{-3}: 16
Control (Sterile Water)	A	D: 47	10^{-1}: 1	10^{-2}: 1	10^{-3}: 1
	B	D: 25	10^{-1}: 1	10^{-2}: 1	10^{-3}: 1
	C	D: 32	10^{-1}: 7	10^{-2}:1	10^{-3}: 1
Day 7					
Water	A	D: 24	10^{-1}: 17	10^{-2}: 2	10^{-3}: 1
	B	D: 23	10^{-1}: 7	10^{-2}: 1	10^{-3}: 1
	C	D: 30	10^{-1}: 24	10^{-2}: 9	10^{-3}: 1
Control (Sterile Water)	A	D: 90	10^{-1}: 53	10^{-2}: 19	10^{-3}: 1
	B	D: 45	10^{-1}: 45	10^{-2}: 23	10^{-3}: 8
	C	D: 90	10^{-1}: 81	10^{-2}: 24	10^{-3}: 31

Table 3: Direct Plate Counts Taken from Pilot Study #2 (Conducted 3/22/2019)

Day 1					
Water	A	D: 21	10^{-1}: 6	10^{-2}: 1	10^{-3}: 0
	B	D: 53	10^{-1}: 7	10^{-2}: 2	10^{-3}: 0
	C	D: 48	10^{-1}: 17	10^{-2}: 2	10^{-3}: 2
Control (Sterile Water)	A	D: 0	10^{-1}: 0	10^{-2}: 0	10^{-3}: 0
	B	D: 0	10^{-1}: 0	10^{-2}: 0	10^{-3}: 0
	C	D: 0	10^{-1}: 0	10^{-2}: 0	10^{-3}: 0
Day 7					
Water	A	D: 40	10^{-1}: 25	10^{-2}: 8	10^{-3}: 1
	B	D: 8	10^{-1}: 4	10^{-2}:1	10^{-3}: 0
	C	D: 20	10^{-1}: 2	10^{-2}: 0	10^{-3}: 0
Control (Sterile Water)	A	D: 3	10^{-1}: 0	10^{-2}: 0	10^{-3}: 0
	B	D: 0	10^{-1}: 0	10^{-2}: 0	10^{-3}: 0
	C	D: 0	10^{-1}: 0	10^{-2}: 0	10^{-3}: 0

Table 4: Direct Plate Counts Taken from Pilot Study #3 (Conducted 4/15/2019)

		Day 1			
Water	A	D: 90	10^{-1}: 80	10^{-2}: 3	10^{-3}: 1
	B	D: 40	10^{-1}: 22	10^{-2}: 2	10^{-3}: 1
	C	D: 36	10^{-1}: 23	10^{-2}: 1	10^{-3}: 1
Control (Sterile Water)	A	D: 0	10^{-1}: 0	10^{-2}: 0	10^{-3}: 0
	B	D: 0	10^{-1}: 0	10^{-2}: 0	10^{-3}: 0
	C	D: 0	10^{-1}: 0	10^{-2}: 0	10^{-3}: 0

Pilot study #1 demonstrated the growth of general colony forming units on R2A; which were incubated at 28°C for a maximum of 7 days. All dilutions indicate a decrease in growth between one and seven days of incubation. The 10^{-1} dilution remains mostly unchanged and only slightly decreases as it approaches day seven.

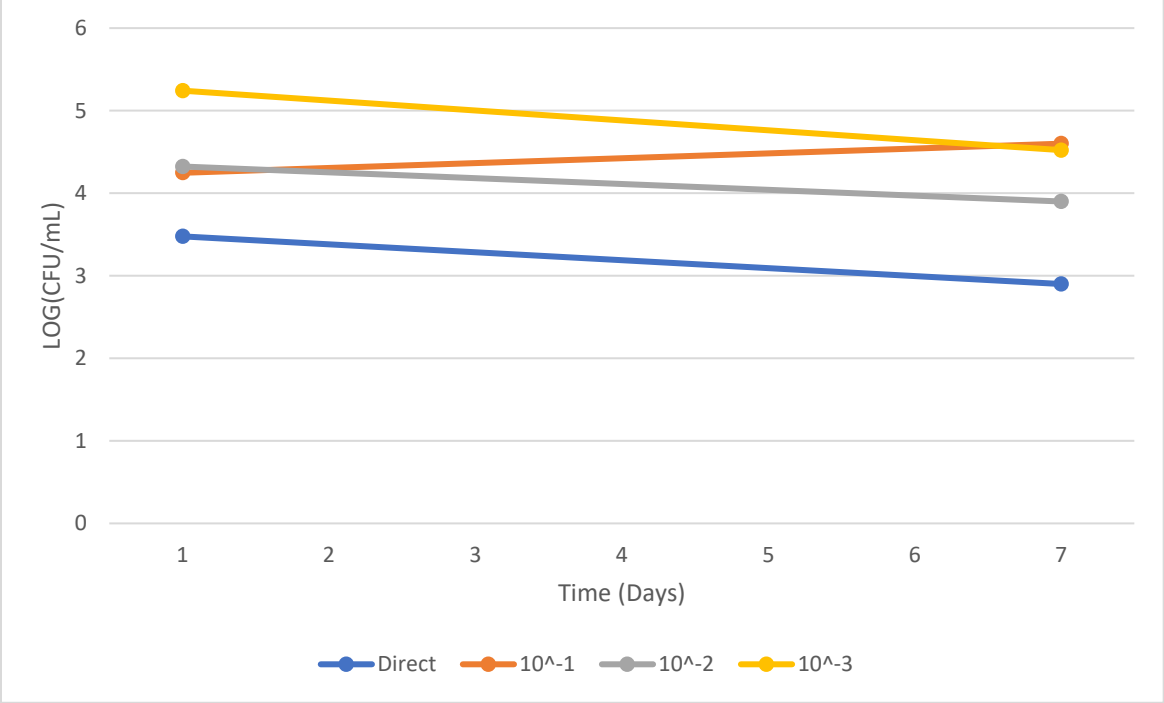

Figure 5: Graph Depicting the Growth Curve of Pilot Study #1.

The x-axis indicates the incubation time in days. The y-axis of the graph indicates the log of the average colony forming units/µL. Samples were incubated between 1 and 7 days. The samples were incubated at 28°C (room temperature). This process was repeated for pilot studies #2 and #3.

In pilot study #2, the 10^{-2} dilution increased in growth as time increased. The other dilutions died-off as time increased.

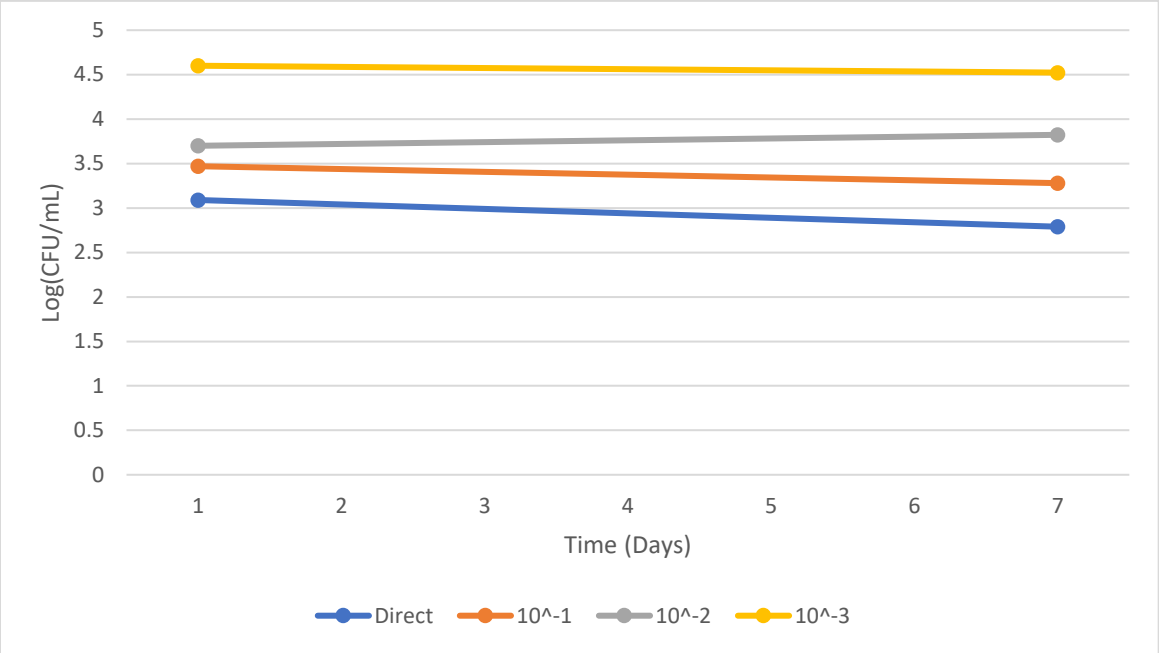

Figure 6: Graph Depicting the Growth Curve of Pilot Study #2

In pilot study #3, dilutions 10^{-2} and 10^{-3} increased significantly in growth as time increased. 10^{-1} increased slightly as time increased. The direct plating followed the typical trend and died off as time increased.

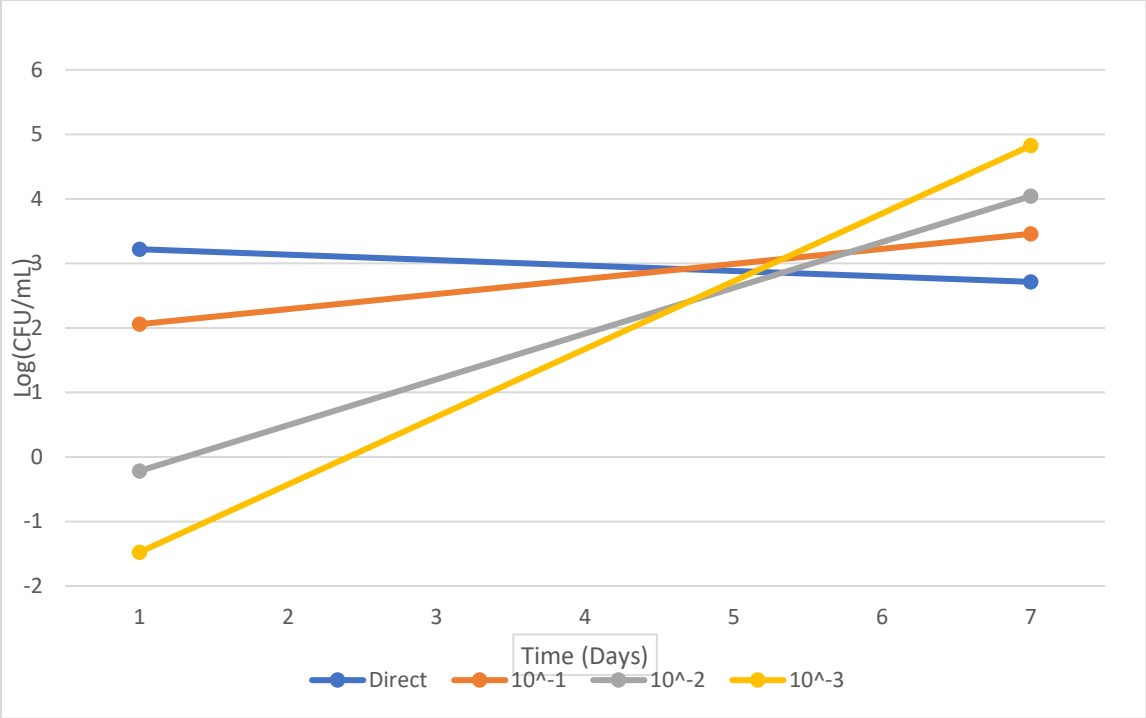

Figure 7: Graph Depicting the Growth Curve of Pilot Study #3

The Main Experiment

Growth curves were generated for all of Temperature Treatments 1 and 4. For each figure, the x-axis represents incubation time in days. The y-axis indicates the log (CFU/ml) calculated from the direct plate counts. Each line of the graph corresponds to each dilution.

Temperature Treatment 1 at 4°C increased in bacterial growth for all three plates as time increase. The direct and 10^{-1} dilutions of general bacteria on R2A resulted in the greatest increase as time increased. Dilution 10^{-2} on R2A resulted in the least amount of change as time increased. At 4°C, there was little to no growth of *Salmonella*. The greatest amount of *Salmonella* growth occurred between day 7 and 14 on the direct sample plating. *E. coli* at 4°C resulted in a decrease in growth between days 5 and 7; followed by an increase in log (CFU/ml) between day 7 and 14.

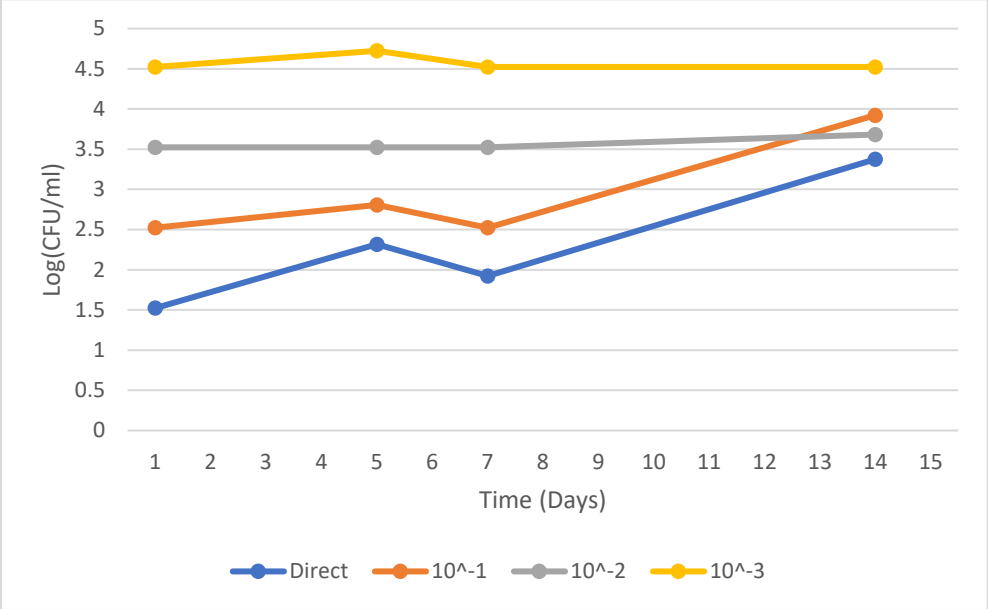

Figure 8: Growth Curve of General Bacteria on R2A at 4°C (Temperature Treatment 1)

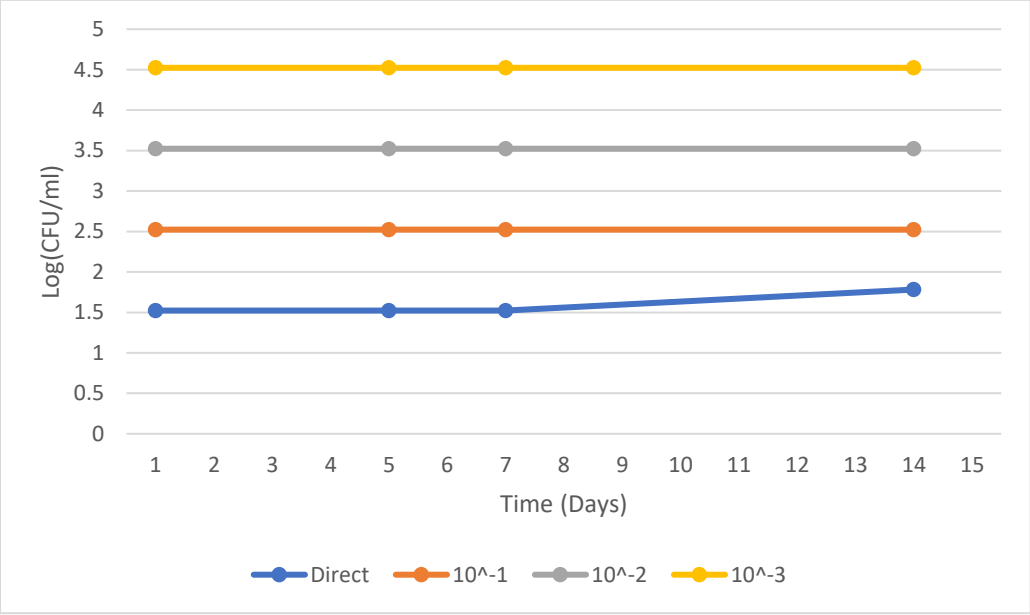

Figure 9: Growth Curve of *Salmonella* on XLD at 4°C (Temperature Treatment 1)

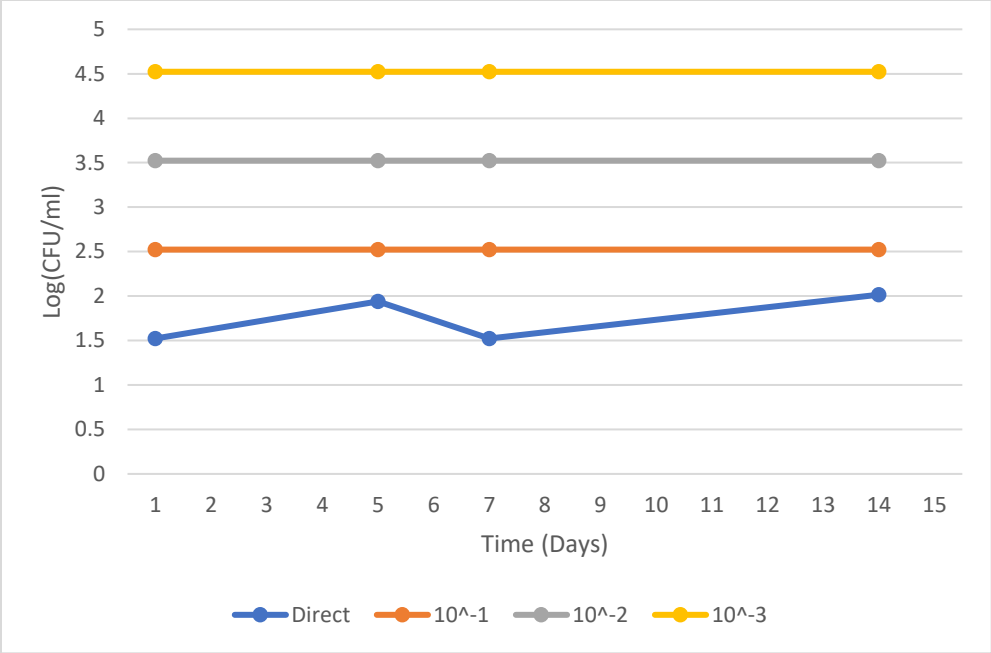

Figure 10: Growth Curve of *E. coli* on MFC at 4°C (Temperature Treatment 1)

Bacterial growth on Temperature Treatment 1 at 28°C generally decreased as incubation time increased for all three plates. General bacterial growth at 28°C did not result in growth at dilutions 10^{-2} and 10^{-1}. The direct sample and 10^{-1} dilution resulted in a decrease in log (CFU/ml) between days 5 and 7. There was no *Salmonella* growth at 28°C for Temperature Treatment 1. There was little *E. coli* growth at 28°C. Most *E. coli* bacteria peaked at 5 days. *E. coli* bacteria did not grow beyond 5 days.

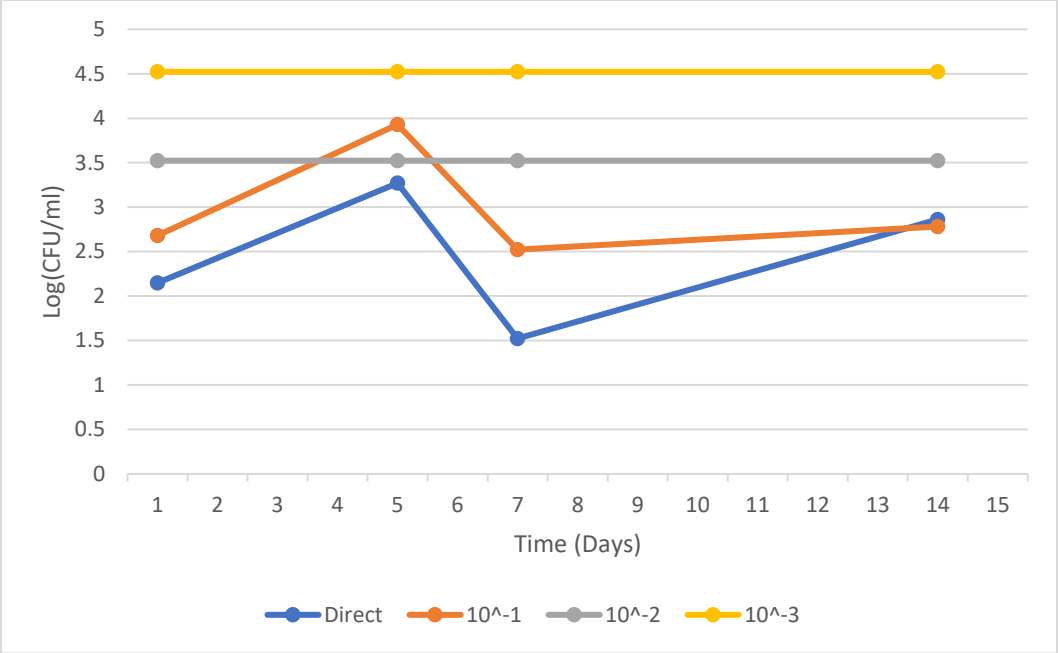

Figure 11: Growth Curve of General Bacteria on R2A at 28°C (Temperature Treatment 1)

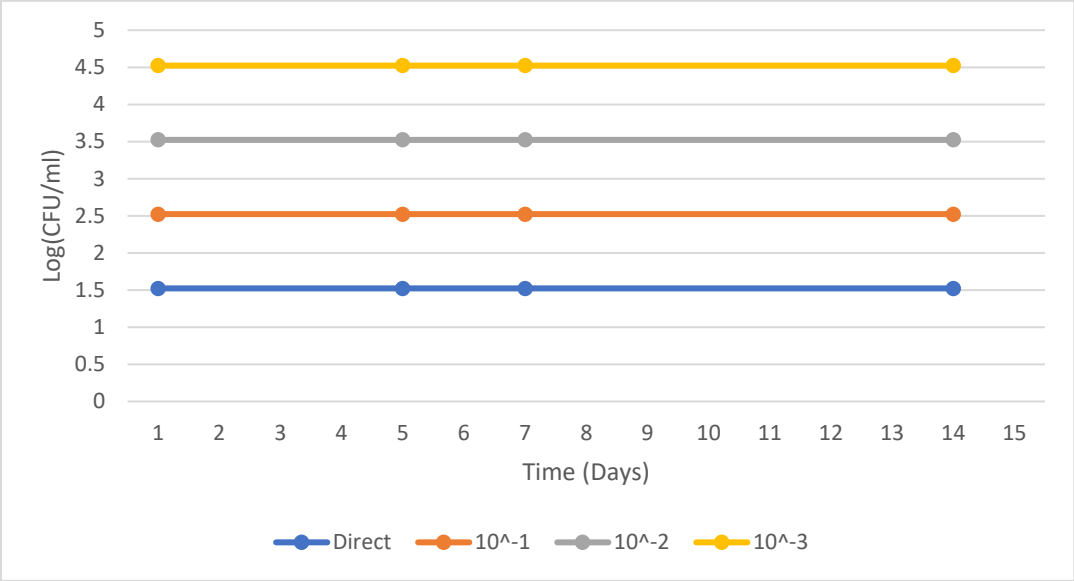

Figure 12: Growth of *Salmonella* on XLD at 28°C (Temperature Treatment 1)

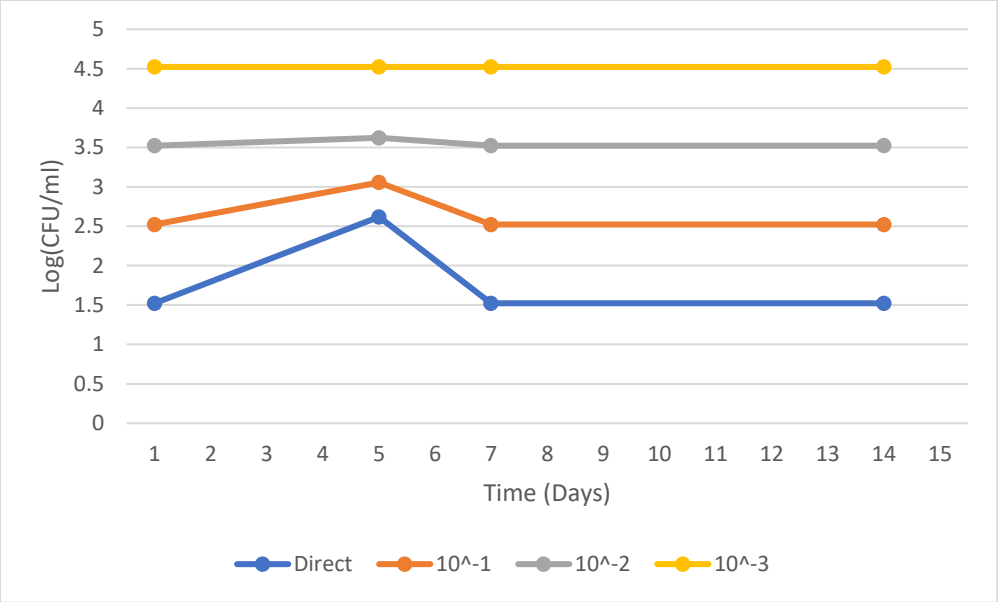

Figure 13: Growth of *E. coli* on MFC at 28°C (Temperature Treatment 1)

Bacterial growth on Temperature Treatment 1 at 37°C peaked at day 5 and decreased at day 7 and 14 for all three plates. General bacteria at 37°C did not exhibit any growth at 10^{-3}. There was no growth for *Salmonella* at 37°C. *E. coli* did not exhibit growth at dilutions 10^{-2} and 10^{-3}.

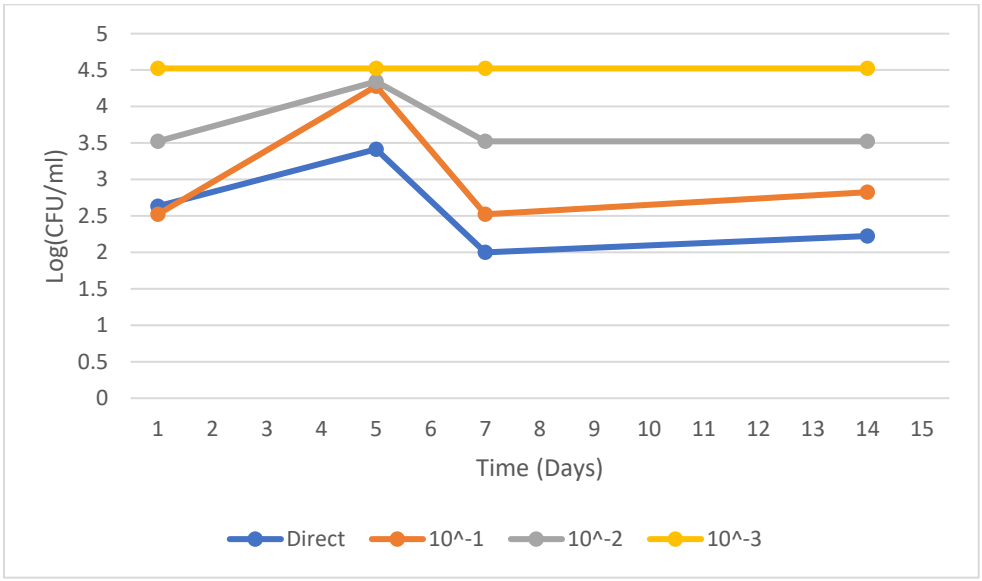

Figure 14: Growth Curve of General Bacteria on R2A at 37°C (Temperature Treatment 1)

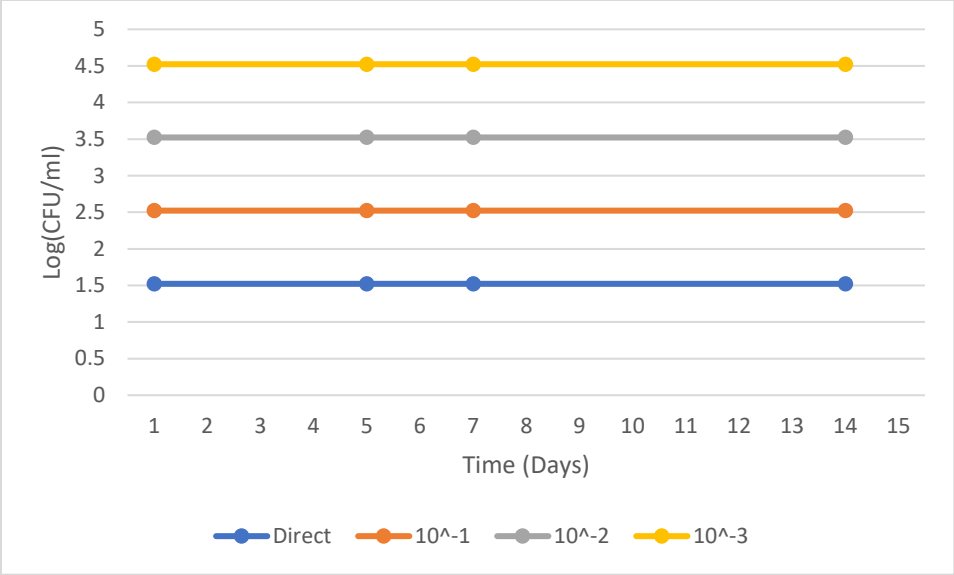

Figure 15: Growth Curve of *Salmonella* on XLD at 37°C (Temperature Treatment 1)

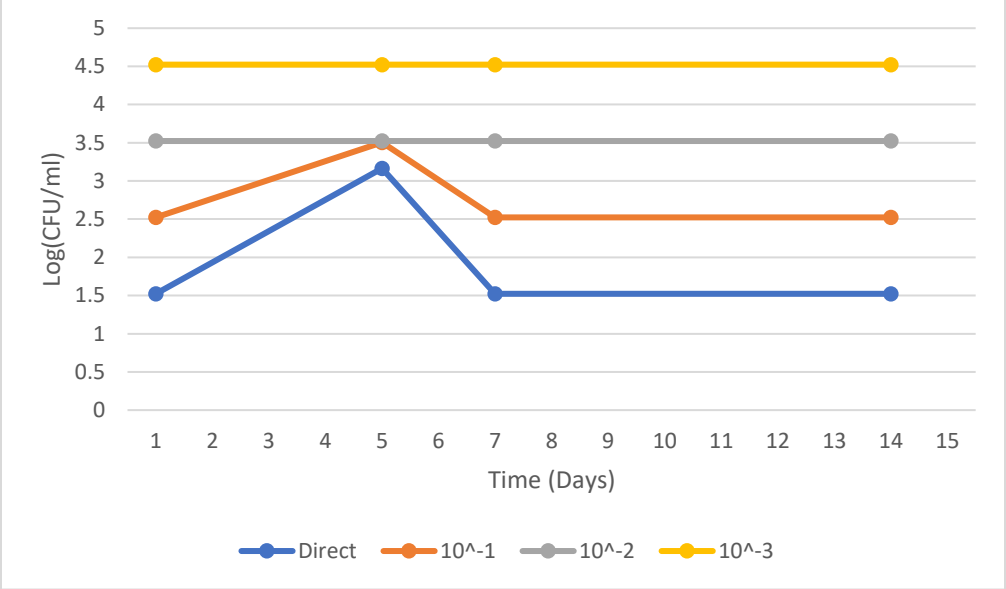

Figure 16: Growth Curve of *E. coli* on MFC at 37°C (Temperature Treatment 1)

Temperature Treatment 4 at 4°C resulted in a decrease in log (CFU/ml) across all three plates. General bacteria and *Salmonella* mostly peaked at day 5; while *E. coli* growth peaked at day 7.

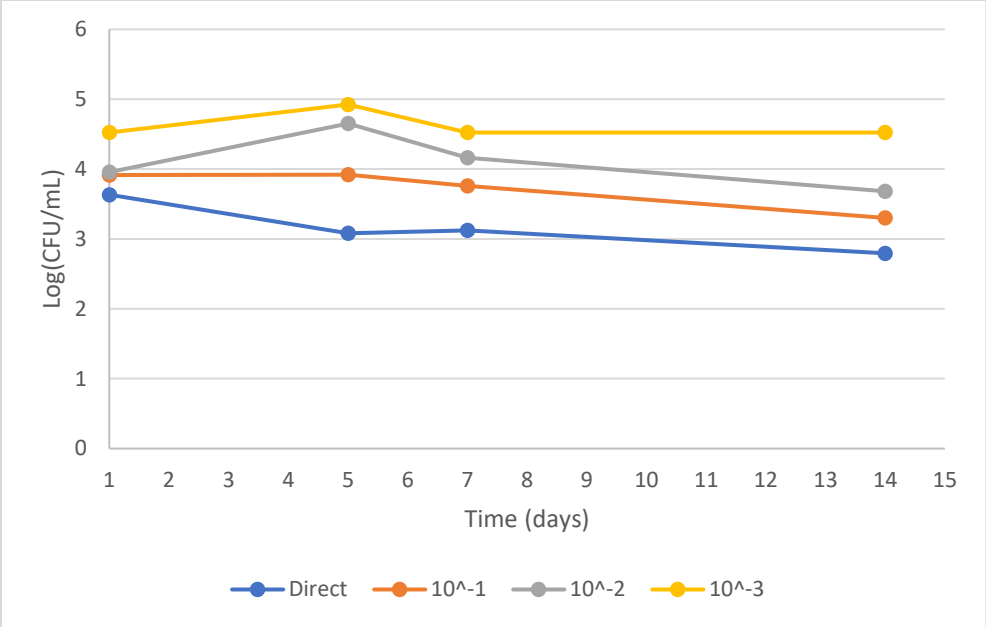

Figure 17: Growth Curve of General Bacteria on R2A at 4°C (Temperature Treatment 4)

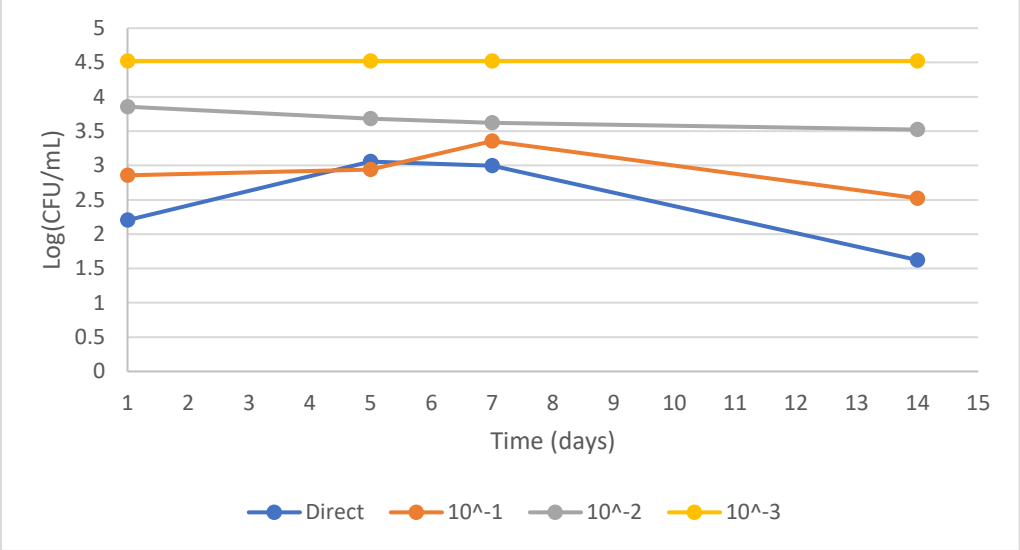

Figure 18: Growth Curve of *Salmonella* on XLD 4°C (Temperature Treatment 4)

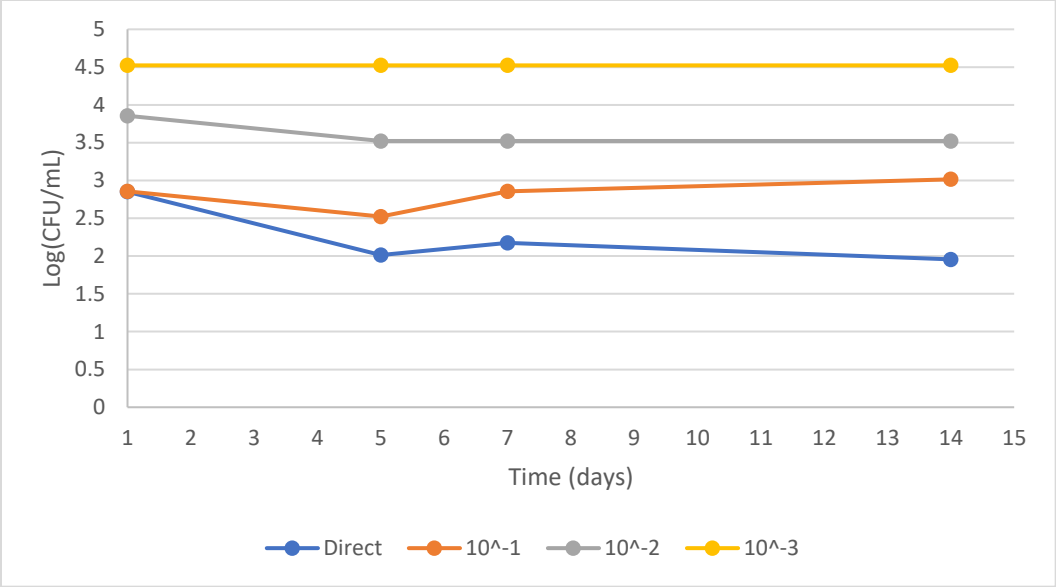

Figure 19: Growth Curve *E. coli* on MFC at 4°C (Temperature Treatment 4)

For all three plates, the number of log (CFU/ml) peaked at 5 days for 28°C. For all three plates, there was no growth beyond day 5. For all three plates, dilution 10^{-3} resulted in no growth.

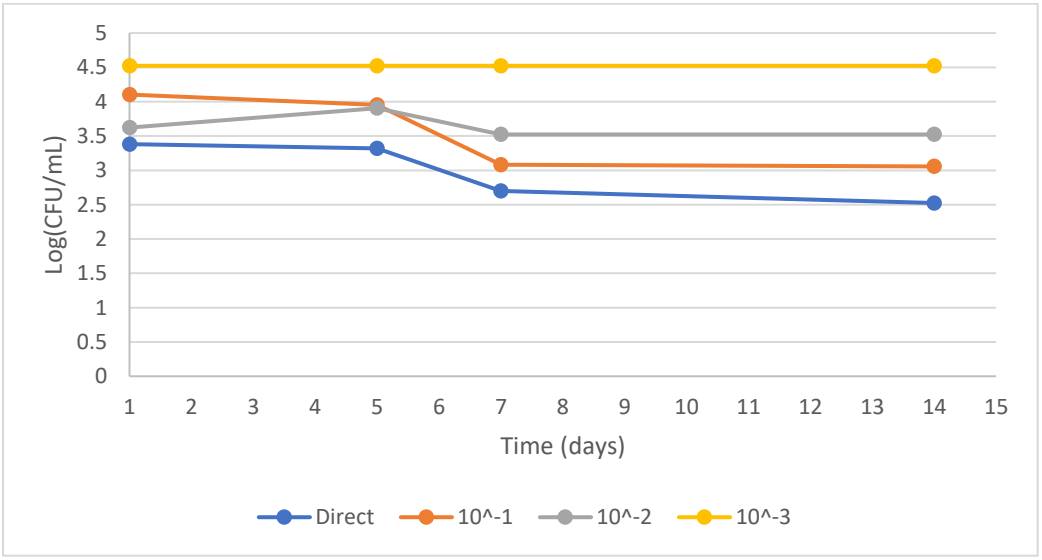

Figure 20: Growth Curve of General Bacteria on R2A at 28°C (Temperature Treatment 4)

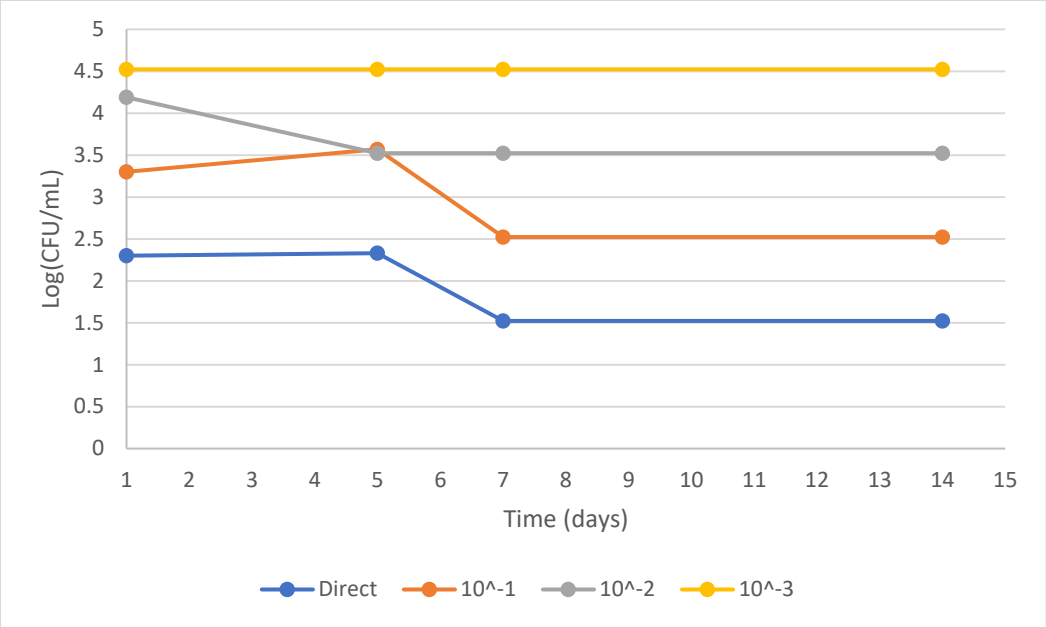

Figure 21: Growth Curve of *Salmonella* of XLD at 28°C (Temperature Treatment 4)

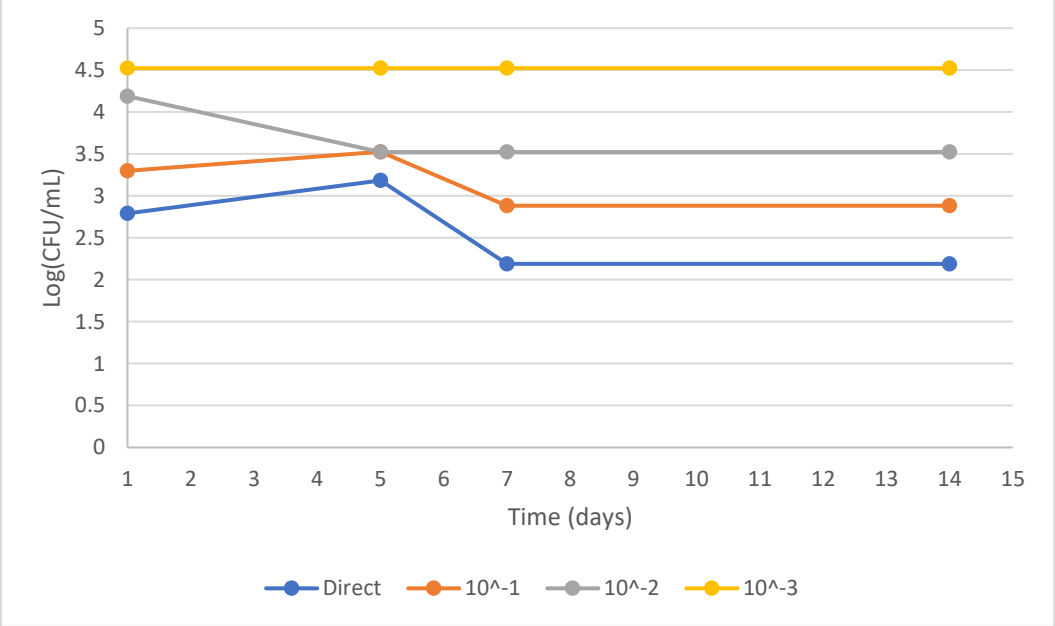

Figure 22: Growth Curve of *E. coli* on MFC at 28°C (Temperature Treatment 4)

For Temperature Treatment 4 at 37°C, general bacteria on R2A demonstrated the highest amount of growth on day5. The direct sample taken for *Salmonella* and *E. coli* peaked at day 1. There was no *Salmonella* and *E. coli* growth at dilutions 10^{-1}, 10^{-2}, and 10^{-3}.

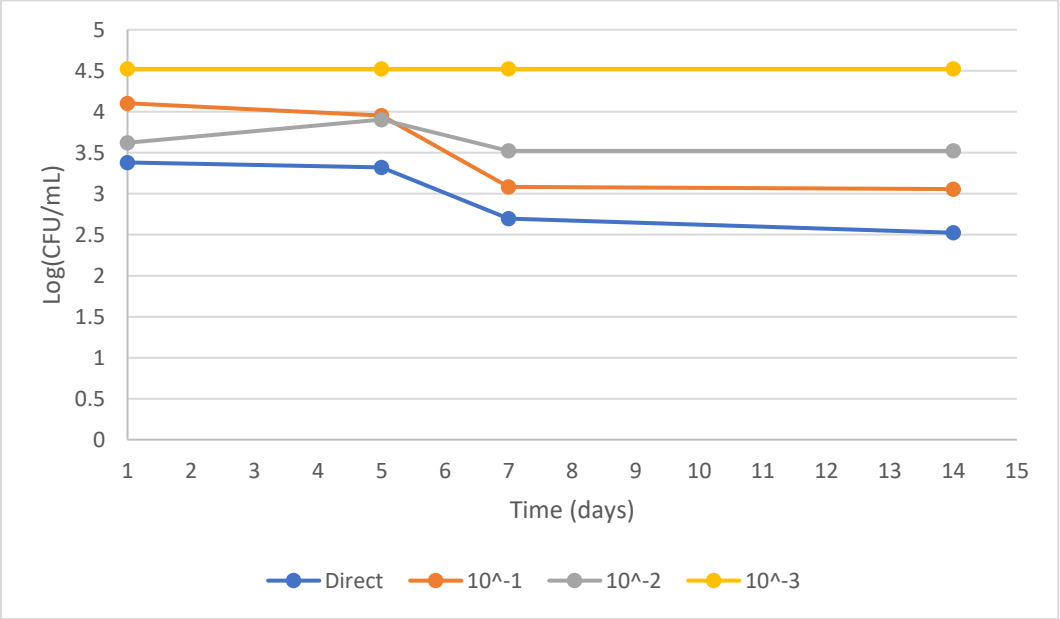

Figure 23: Growth Curve of General Bacteria on R2A at 37°C (Temperature Treatment 4)

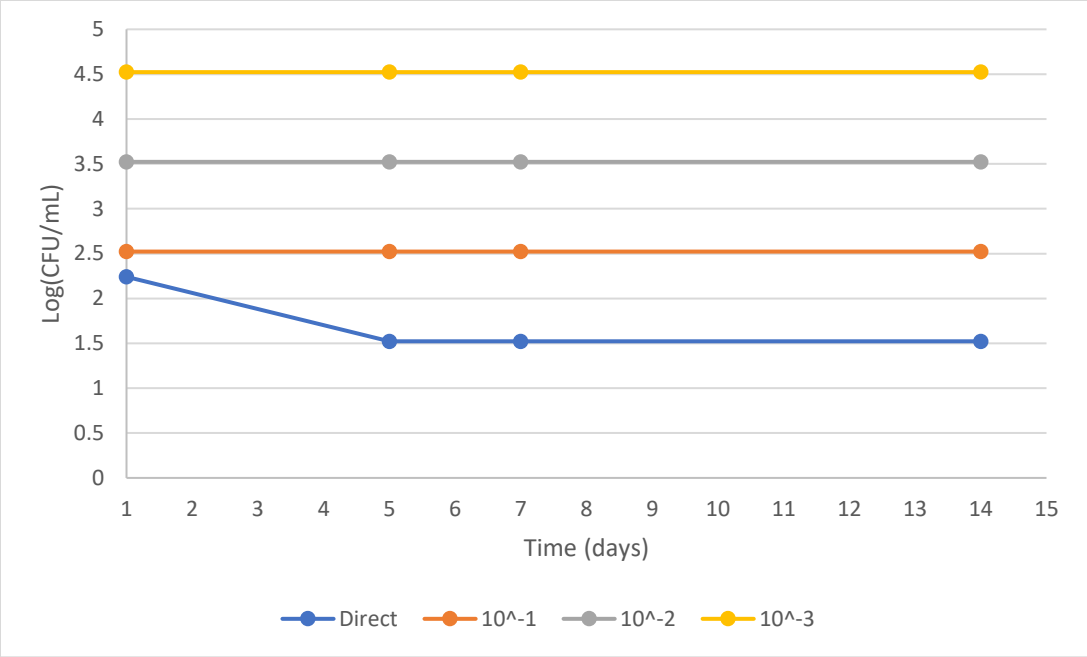

Figure 24: Growth Curve of *Salmonella* on XLD at 37°C (Temperature Treatment 4)

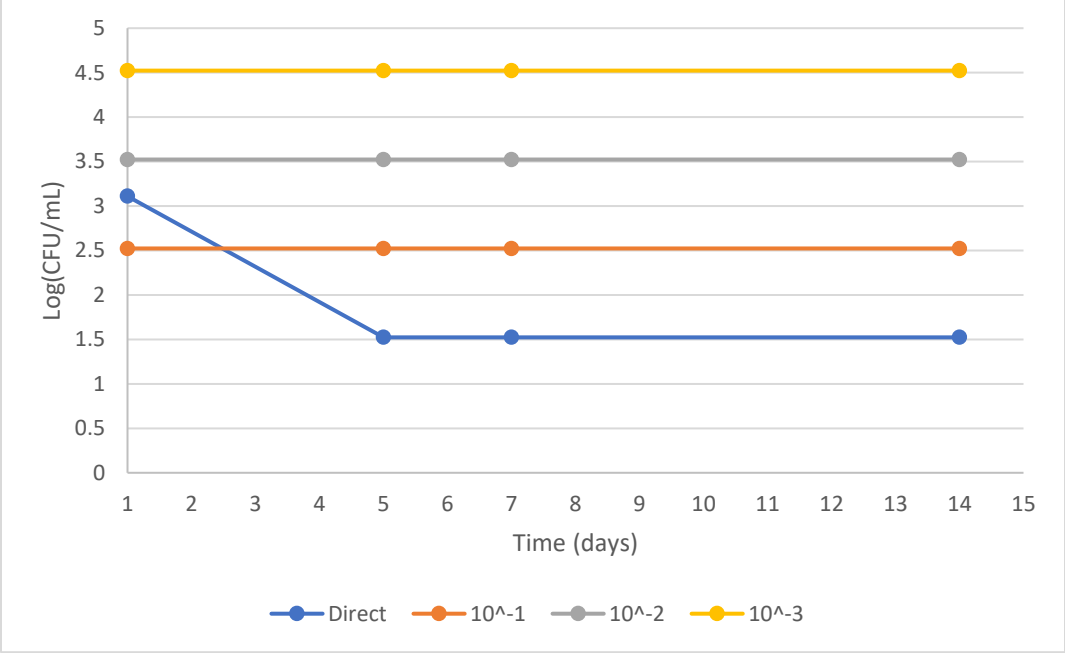

Figure 25: Growth Curve *E. coli* on MFC at 37°C (Temperature Treatment 4)

Meta-Analysis

The decay rates for *Salmonella* in the main experiment were negative. Decay rates for both *Salmonella* and *E. coli* generally followed the rates of other samples taken from water.

Table 5: Decay Rate of Select Microorganisms in Marine and Fresh Water at Various Temperatures Taken from Various Published Sources.

The purpose of this table is to associate the decay rate values to the appropriate published literature it was calculated from. Studies which did not test for *Salmonella*, *E. coli*, or other bacteria are labeled as N/A.

Decay Rate of Organisms in Marine & Fresh Water Between 1 and 10°C		
	Decay Coefficient k [hour^-1]	
Source	*Salmonella*	*E. coli*
Sampson et al. (2006)	N/A	0.0027
Flint (1987)	N/A	-0.033
Rhodes and Kator (1988)	-0.00667	0.019
Rhodes and Kator (1988)	0.00042	N/A
Rhodes and Kator (1988)	-0.2	N/A
Liner (2019)	-0.012	-0.084
Liner (2019)	-0.084	-0.03
Liner (2019)	-0.017	-0.02

Decay Rate of Organisms in Marine & Fresh Water Between 20 and 29°C		
	Decay Coefficient k [hour^-1]	
Source	*Salmonella*	*E. coli*
Scheuerman, Farrah, and Bitton (1987)	0.036	0.039
Sampson et al. (2006)	N/A	0.017
Flint (1987)	N/A	-0.103
Rhodes and Kator (1988)	0.02	0.0026
Rhodes and Kator (1988)	0.013	N/A
Liner (2019)	-0.078	-0.044
Liner (2019)	-0.054	-0.033
Liner (2019)	0.023	0.051

Decay Rate of Organisms in Marine & Fresh Water Between 30 and 40°C		
	Decay Coefficient k [hour^-1]	
Source	*Salmonella*	*E. coli*
Scheuerman, Schmidt, Alexander (1988)	N/A	0.01
Scheuerman, Schmidt, Alexander (1988)	N/A	0.005
Anderson et al. (2005)	N/A	-0.42
Anderson et al. (2005)	N/A	-0.27
Jimenez et al. (1989)	0.0064	0.0064
Jimenez et al. (1989)	0.013	N/A
Jimenez et al. (1989)	0.00	N/A
Flint (1987)	N/A	-0.14
Liner (2019)	-0.035	-0.36
Liner (2019)	-0.028	-0.28
Liner (2019)	-0.033	0.012

Table 6: Decay Rate of Select Microorganisms in Manure and Sediments at Various Temperatures Taken from Various Published Sources.

Studies which did not test for *Salmonella*, *E. coli*, or other bacteria are labeled as N/A. These results were later used in an ANOVA as a comparison to the main study. The main study is not included in this table as its data remained the same as Table 8.

Decay Rate of Organisms in Manure Between 1 and 10°C		
	Salmonella	*E. coli*
Jiang et al. (2001)	N/A	0.009
Arrus et al. (2006)	0.0055	N/A
Arrus et al. (2006)	0.0040	N/A
Sampson et al. (2006)	N/A	0.0018
Himathongkhorn et al. (1999)	0.0053	0.0067

Decay Rate of Organisms in Manure Between 20 and 29°C		
	Salmonella	*E. coli*
Arrus et al. (2006)	0.0126	N/A
Arrus et al. (2006)	0.004	N/A
Himathongkhorn et al. (1999)	0.034	0.00065
Sampson et al. (2006)	N/A	0.018
Kudva, Blanch, and Hovde (1998)	N/A	0.0008

Decay Rate of Organisms in Manure Between 30 and 40°C		
	Salmonella	*E. coli*
Jiang et al. (2001)	N/A	0.013
Arrus et al. (2006)	0.056	N/A
Himathongkhorn et al. (1999)	0.023	N/A
Sampson et al. (2006)	N/A	0.0082
Semenov et al. (2010)	0.0038	N/A
Kudva, Blanch, and Hovde (1998)	N/A	0.0045

The p-value for all ANOVA tests remained the same between 95 and 90% confidence intervals. *Salmonella* Growth between 20 and 29°C was the only ANOVA test that indicated a significance between manure and water samples (P = 0.010).

Table 7: A Single-Factor ANOVA Comparing the Decay Rate of *Salmonella* in Water and Manure Samples Taken from Various Published Sources

***Salmonella* Growth Between 1 and 10°C**			
Confidence Interval	*F*	*P-value*	*F crit*
95%	0.846	0.474	5.143
90%	0.846	0.474	3.463

***Salmonella* Growth Between 20 and 29°C**			
Confidence Interval	*F*	*P-value*	*F crit*
95%	3.031	0.123	5.143
90%	3.031	0.123	3.463

***Salmonella* Growth Between 30 and 40°C**			
Confidence Interval	*F*	*P-value*	*F crit*
95%	10.923	0.010	5.143
90%	10.923	0.010	3.463

Table 8: A Single-Factor ANOVA Comparing the Decay Rate of *E. coli* in Water and Manure Samples Taken from Various Published Sources.

The ANOVA tests for all temperature ranges indicated there was no difference in the growth of *E. coli* between water and manure.

***E. coli* Growth Between 1 and 10°C**			
Confidence Interval	*F*	*P-value*	*F crit*
95%	3.397	0.103	5.143
90%	3.397	0.103	3.463

***E. coli* Growth Between 20 and 29°C**			
Confidence Interval	*F*	*P-value*	*F crit*
95%	0.153	0.861	5.143
90%	0.153	0.861	3.463

***E. coli* Growth Between 30 and 40°C**			
Confidence Interval	*F*	*P-value*	*F crit*
95%	0.836	0.511	4.066
90%	0.836	0.511	2.924

Table 9: A Single-Factor ANOVA Comparing the Decay Rate of Enteric Pathogens in Water and Manure Samples Taken from Various Sources

The single factor ANOVA comparing data for both *Salmonella* and *E. coli* indicated a significance between the growth of the two pathogens in manure and water. The significance was determined at both 95 and 90% confidence intervals.

Decay Rate of Enteric Pathogens in Water and Manure Samples			
Confidence Interval	*F*	*P-value*	*F crit*
95%	3.218	0.048	3.168
90%	3.218	0.048	2.403

Table 10: A Two-Factor ANOVA Comparing the Decay Rate of Enteric Pathogens in Water and Manure Samples at Various Temperatures

Decay Rate of Enteric Pathogens in Water and Manure Samples at Low, Medium, and High Temperatures			
	F	*P-value*	*F crit*
Source of Sample	2.498	0.094	3.204
Temperature Range	4.387	0.018	3.204
Interaction	1.002	0.417	2.579

Discussion

Physical Assessment of Sinking Creek

Fluctuation of the environmental parameters from Sinking Creek could indicate the presence of stressors which impact the overall health of the creek. Factors such as low dissolved oxygen and low pH, affect microbial growth. *E. coli* and *Salmonella* prefer a neutral pH (CDC, 2014; Winfield and Groisman, 2003). The pH remained neutral to alkaline for the entirety of the sampling period. October 16th indicated the highest recorded pH within the creek. Higher pH of water in the creek could be from anthropogenic sources such as urban runoff or over use of fertilizer (TDEC, 2017). The high pH range may have contributed to the lack of *Salmonella* and *E. coli* for that sampling period. Dissolved oxygen concentrations increased as sampling continued. Interestingly, the extremely low dissolved oxygen levels recorded on October 29th did not affect the growth of *Salmonella* and *E. coli.* It is known that *Salmonella* and *E. coli* bacteria can grow in anerobic conditions (CDC, 2014; Winfield and Groisman, 2003). The general colony forming units on R2A were also not affected by the low dissolved oxygen; indicating there is a likely high concentration of anerobic bacteria. Low levels of dissolved oxygen could also indicate the presence of fertilizer runoff and other anthropogenic influences. The high pH values and low dissolved oxygen support the claim that Sinking Creek is highly impaired.

Pilot Study

The pilot study was conducted to determine the general presence of microorganisms in Sinking Creek. High concentrations of growth on R2A indicated the water samples supported a large microbiota. Two replications of the pilot study decreased in growth between one and seven days of incubation. This is consistent with the hypothesis; as It was predicted that the results of the

pilot study would decrease in growth over time. Pilot study replication #3's increase in growth of dilutions $10^{-1} – 10^{-3}$ could have been the result of competition between microorganisms in the direct sample; and therefore, as the dilutions increased, other microorganisms could proliferate (Farovitch, 2016) (Figure 7). Contamination of the Pilot Study #1 control is likely the result of unsterile deionized water or improper aseptic technique. Contamination of the control could have been prevented by autoclaving the deionized water prior to plating. As a result of the pilot study, all control water was autoclaved for the main experiment.

The Main Experiment

The results of the main experiment indicated that *Salmonella* was present in the Sinking Creek water samples; and thus, disproves the hypothesis that the pathogen would not be present in the creek. The presence of *Salmonella* in Sinking Creek could be due to agricultural runoff or potential sewage contamination (TDEC, 2017). As predicted *E. coli* bacteria was also present in Sinking Creek. This was assumed because the creek is listed on the USEPA's 303(d) as impaired due to *E. coli* contamination (TDEC, 2017). Both *Salmonella* and *E. coli* grew best at temperatures of 4 and 28°C. This does not support the hypothesis; which stated the pathogens would grow best at 37°C. *Salmonella* and *E. coli* can grow at temperatures as low as 4°C; however, it is proven the microorganisms prefer the warmer temperatures of their host (Arrus et al., 2006). It is possible the bacteria in Sinking Creek have adapted to colder temperatures (Arrus et al., 2006). To support this, more replications of the temperature treatments should be conducted. Furthermore, studies comparing native *Salmonella* and *E. coli* in the creek to *Salmonella* and *E. coli* inoculated in the samples could further support the claim that the native pathogens have adapted to colder temperatures. Bacterial growth usually peaked between five to

seven days of incubation. This data is consistent with a typical bacterial growth curve (Farovitch, 2016).

The Meta-Evaluation

The goal of the meta-evaluation was to determine a significance in the decay rates of enteric pathogens in samples taken from manure and water. Manure was chosen as a comparison to water because of the abundance of published literature which tested the survival of *E. coli* and *Salmonella* at various temperatures in manure. It was predicted that manure would yield lower decay rates than samples taken from water. This was determined because of manure's high nutrient availability; however, other factors such as water activity and sunlight exposure were not considered in the formation of the hypothesis. The results of the meta-evaluation concluded that there was no significance between samples taken from manure or water; thus, the results of the meta-evaluation disprove the original hypothesis. However, a larger ANOVA was conducted using both *Salmonella* and *E. coli* and resulted in a significance between samples taken from manure and water (Table7). A two-factor ANOVA was conducted to find a significance between temperature range and the source of the sample (Table 8). The ANOVA revealed there was no significance in the decay rate of enteric pathogens between temperature and the source of the sample. The results of the meta-evaluation could be more representative with a larger sample size. It is recommended that more sources are used in future experiments. The meta-evaluation resulted in mostly negative decay rates for the main experiment. The negative decay rates indicated the microorganisms in Sinking Creek were not dying at the same rate as the comparison literature. Sinking Creek has demonstrated visible nutrient overloading; because of this, it is possible the microorganisms in the main experiment were able to survive longer than the comparison literature (Croxen et al., 2013; Farovitch, 2016).

Limitations of the Study

Limitations of the main experiment included environmental change and seasonal variability. The varied environmental parameters and changing seasons could have affected the growth of *Salmonella* and *E. coli*. Though factors such as percent oxygen did not seem to have an observed effect on the growth of *Salmonella* and *E. coli*, other factors that were not measured could have affected growth. Nutrient availability and competition among other microorganisms could have impacted the growth *Salmonella* and *E. coli* in the main experiment. Another limitation of the main experiment was occasional fungal contamination. Fungal growth on R2A and MFC agar could have resulted in lower direct counts of the microorganisms. Furthermore, contamination could also have occurred through improper aseptic technique or contaminated lab equipment. To limit the risk of contamination, glassware and pipette tips were autoclaved before use; however, it is still possible contamination could have occurred. Contamination due to improper aseptic technique could also result in lower direct counts. To count colony forming units as accurately as possible, microorganisms were enumerated using a plate count microscope. However, given the plates were left to incubate for up to 48 hours, there was a chance that larger colonies had formed over smaller ones. Limitations of the meta-evaluation were primarily due to a small sample size. A larger sample size could have yielded more representative results for the meta-evaluation.

Recommendations for Further Research

The results of this study indicate further research is needed. Based off the results of the main experiment, it is recommended that continuing replications of the temperature treatments are implemented. Studies comparing the survival of indigenous *Salmonella* and *E. coli* to that of inoculated pathogens could be done to determine the survival mechanisms developed by the native species. The results of these further studies could prove the ingenious pathogens of

Sinking Creek have developed such survival mechanisms to grow at lower temperatures. Additionally, sampling farther downstream of the below wetlands site is recommended to further understand the effectiveness of the wetlands. Due to lack of statistical significance, a larger sample size is needed to determine a potential significance between manure and water samples. Furthermore, statistical test could be run using other microorganisms in manure and water samples.

Conclusion

The main goal of this experiment was to determine the presence of *Salmonella* and *E. coli* in Sinking Creek and understand how temperature effects the survival of these organisms. *Salmonella* was found to be present in water samples taken from Sinking Creek; which disproves the hypothesis that *Salmonella* would not be present in the Creek. The finding of *Salmonella* and *E. coli* bacteria in Sinking Creek could have potential human health implications. Those in contact with the creek are at risk of developing infection. However, replication studies may yield different results. The presence of *Salmonella* and *E. coli* at the below wetlands site could imply the wetlands are not preventing pathogens from moving downstream. The survival experiment resulted in the highest growth of *Salmonella* and *E. coli* at 4 and 28°C; which disproves the prediction that the highest growth would occur at 37°C.

The purpose of the meta-analysis was to compare data from the main experiment to published literature. The goal of the meta-evaluation was to determine a significance between the decay rates of *Salmonella* and *E. coli* in manure vs. water samples. It was hypothesized that samples taken from manure would result in lower decay rates than water samples. The meta-evaluation concluded that there was no visible significance between manure and water samples. However, a larger sample size could yield different results.

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Appendices

Appendix A 1: Table Indicating the Raw Data for the Average Log (CFU/ml) of Temperature Treatment 2

Growth curves were not generated for Temperature Treatments 2 and 3.

Time (days)	Direct	10^-1	10^-2	10^-3
4°C R2A				
1	3.162 Log (CFU/ml)	2.582 Log (CFU/ml)	3.233 Log (CFU/ml)	2.719 Log (CFU/ml)
5	2.914 Log (CFU/ml)	3.481 Log (CFU/ml)	3.976 Log (CFU/ml)	3.242 Log (CFU/ml)
7	3.523 Log (CFU/ml)	4.000 Log (CFU/ml)	4.388 Log (CFU/ml)	3.623 Log (CFU/ml)
14	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.522 Log (CFU/ml)	4.522 Log (CFU/ml)
4°C MFC				
1	1.523 Log (CFU/ml)	1.623 Log (CFU/ml)	2.708 Log (CFU/ml)	1.523 Log (CFU/ml)
5	2.856 Log (CFU/ml)	2.623 Log (CFU/ml)	3.349 Log (CFU/ml)	2.523 Log (CFU/ml)
7	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)
14	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)
4°C XLD				
1	1.523 Log (CFU/ml)	1.856 Log (CFU/ml)	1.623 Log (CFU/ml)	1.523 Log (CFU/ml)
5	2.523 Log (CFU/ml)	2.623 Log (CFU/ml)	2.682 Log (CFU/ml)	2.523 Log (CFU/ml)
7	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)
14	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)

Time (days)	Direct	10^-1	10^-2	10^-3
28°C R2A				
1	3.110 Log (CFU/ml)	2.686 Log (CFU/ml)	3.078 Log (CFU/ml)	3.167 Log (CFU/ml)
5	3.428 Log (CFU/ml)	3.342 Log (CFU/ml)	2.964 Log (CFU/ml)	3.311 Log (CFU/ml)
7	3.523 Log (CFU/ml)	3.623 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)
14	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)
28°C MFC				
1	1.523 Log (CFU/ml)	1.523 Log (CFU/ml)	1.523 Log (CFU/ml)	1.523 Log (CFU/ml)
5	2.523 Log (CFU/ml)	2.724 Log (CFU/ml)	2.523 Log (CFU/ml)	2.523 Log (CFU/ml)
7	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)
14	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)
28°C XLD				
1	1.523 Log (CFU/ml)	1.523 Log (CFU/ml)	2.015 Log (CFU/ml)	2.230 Log (CFU/ml)
5	2.523 Log (CFU/ml)	2.523 Log (CFU/ml)	2.523 Log (CFU/ml)	3.020 Log (CFU/ml)
7	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)
14	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)

Time (days)	Direct	10^-1	10^-2	10^-3
37°C R2A				

1	2.843 Log (CFU/ml)	2.010 Log (CFU/ml)	2.964 Log (CFU/ml)	3.244 Log (CFU/ml)
5	3.313 Log (CFU/ml)	3.057 Log (CFU/ml)	3.057 Log (CFU/ml)	2.523 Log (CFU/ml)
7	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)
14	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)
37°C MFC				
1	1.523 Log (CFU/ml)	2.025 Log (CFU/ml)	2.015 Log (CFU/ml)	2.015 Log (CFU/ml)
5	2.523 Log (CFU/ml)	2.523 Log (CFU/ml)	2.523 Log (CFU/ml)	2.523 Log (CFU/ml)
7	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)
14	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)
37°C XLD				
1	1.523 Log (CFU/ml)	1.523 Log (CFU/ml)	3.401 Log (CFU/ml)	2.015 Log (CFU/ml)
5	2.523 Log (CFU/ml)	2.523 Log (CFU/ml)	2.523 Log (CFU/ml)	2.523 Log (CFU/ml)
7	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)
14	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)

Table Indicating the Raw Data for the Average Log (CFU/ml) of Temperature Treatment 3

Time (days)	Direct	10^-1	10^-2	10^-3
4°C R2A				
1	3.039 Log (CFU/ml)	3.240 Log (CFU/ml)	3.297 Log (CFU/ml)	3.083 Log (CFU/ml)
5	3.622 Log (CFU/ml)	3.728 Log (CFU/ml)	4.036 Log (CFU/ml)	3.116 Log (CFU/ml)
7	3.523 Log (CFU/ml)	3.724 Log (CFU/ml)	4.184 Log (CFU/ml)	3.724 Log (CFU/ml)
14	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)
4°C MFC				
1	2.512 Log (CFU/ml)	2.190 Log (CFU/ml)	2.353 Log (CFU/ml)	1.523 Log (CFU/ml)
5	2.856 Log (CFU/ml)	2.856 Log (CFU/ml)	2.856 Log (CFU/ml)	2.523 Log (CFU/ml)
7	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)
14	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)
4°C XLD				
1	2.914 Log (CFU/ml)	3.261 Log (CFU/ml)	3.285 Log (CFU/ml)	1.682 Log (CFU/ml)
5	2.523 Log (CFU/ml)	3.134 Log (CFU/ml)	3.534 Log (CFU/ml)	2.523 Log (CFU/ml)
7	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.623 Log (CFU/ml)	3.523 Log (CFU/ml)
14	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)

Time (days)	Direct	10^-1	10^-2	10^-3
28°C R2A				
1	3.308 Log (CFU/ml)	3.083 Log (CFU/ml)	3.139 Log (CFU/ml)	2.661 Log (CFU/ml)
5	4.108 Log (CFU/ml)	3.339 Log (CFU/ml)	3.407 Log (CFU/ml)	3.029 Log (CFU/ml)
7	4.265 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)
14	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)
28°C MFC				
1	2.652 Log (CFU/ml)	1.856 Log (CFU/ml)	1.957 Log (CFU/ml)	1.957 Log (CFU/ml)
5	3.116 Log (CFU/ml)	2.523 Log (CFU/ml)	2.523 Log (CFU/ml)	2.883 Log (CFU/ml)
7	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)

14	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)
28°C XLD				
1	3.462 Log (CFU/ml)	1.856 Log (CFU/ml)	1.523 Log (CFU/ml)	1.523 Log (CFU/ml)
5	3.650 Log (CFU/ml)	2.523 Log (CFU/ml)	2.523 Log (CFU/ml)	2.523 Log (CFU/ml)
7	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)
14	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)

Time (days)	**Direct**	**10^-1**	**10^-2**	**10^-3**
37°C R2A				
1	3.299 Log (CFU/ml)	2.589 Log (CFU/ml)	2.752 Log (CFU/ml)	2.450 Log (CFU/ml)
5	4.056 Log (CFU/ml)	2.682 Log (CFU/ml)	2.983 Log (CFU/ml)	2.883 Log (CFU/ml)
7	4.305 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)
14	4.724 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)
37°C MFC				
1	1.523 Log (CFU/ml)	1.523 Log (CFU/ml)	1.523 Log (CFU/ml)	1.523 Log (CFU/ml)
5	2.523 Log (CFU/ml)	2.523 Log (CFU/ml)	2.523 Log (CFU/ml)	2.523 Log (CFU/ml)
7	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)
14	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)
37°C XLD				
1	2.710 Log (CFU/ml)	1.523 Log (CFU/ml)	1.523 Log (CFU/ml)	1.523 Log (CFU/ml)
5	2.523 Log (CFU/ml)	2.523 Log (CFU/ml)	2.523 Log (CFU/ml)	2.523 Log (CFU/ml)
7	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)	3.523 Log (CFU/ml)
14	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)	4.523 Log (CFU/ml)