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Comparative Analysis of Survival and Decay of Fecal Indicator Bacteria in Bovine Feces and Freshwater Microcosms

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ABSTRACT

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Agricultural runoff can carry FIB that can pollute surface waters through the soil matrix. This study was designed to inspect the impact of temperature and matrix on the survival of FIB. The FIB were routinely enumerated over an 18-day period from fecal samples and freshwater microcosms maintained at 4^{o}C, 22^{o}C, and 35^{o}C. It was found that the FIB studied underwent a primary growth of up to 1-$\log_{10}$ to 3-$\log_{10}$, highlighting the weakness of conventional FIB as indicators of pathogen contamination. The concentrations of FIB in the water phase were found to be significantly greater than those observed in the fecal phase in all FIB and their associated survivals were found to be significantly different too. Similarly, temperature was also found to be a significant factor for the survival of FIB. While the differences in the survival were significant, there was a slight variation in the patterns regarding the differences.

DEDICATION

I dedicate this thesis to my late father, who I consider to be the bravest and the kindest person I have known. He was and continues to be my inspiration.

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CHAPTER 1
INTRODUCTION

The regulatory standards used to assess microbial quality of recreational waters require the use of fecal indicator organisms [1]. Elevated concentration of fecal indicator organisms leads to the impairment of surface waters due to the implied risk of waterborne disease outbreaks [2]. According to a survey conducted by the Center for Disease Control and Prevention, cause of waterborne disease outbreaks that occurred from 1986-1998, where the causative agent could be identified, were predominantly from agricultural sources that was linked to animal farming [3]. In the United States, about 25% of the impaired surface waters listed on the 303d list are impaired due to pathogen contamination [2].

Remediation of impaired waters depends on microbial inactivation rates which are influenced by environmental parameters such as temperature, exposure to solar radiation, salinity, predation, etc. Studies have predominantly focused on the decay and persistence of fecal indicator organisms in water and soil matrices; however, little data exists on the decay and persistence of indicator organisms in feces. Due to the distinct physicochemical environment, the survival of fecal indicators is suggested to be longer in feces compared to the survival of indicators in water or soil [4]. Very few studies have investigated the differential survival of fecal indicator bacteria in different matrices. Such measures will aid in the improvement of daily load programs to reduce pollutant loading to the nation's water bodies in an attempt to remediate impaired waters [5].

Public Health and Regulatory Significance

Agriculture is one of the leading sources of fecal contamination in water [6]. Notably, the waste from livestock feedlots can contribute substantially to the fecal pollution in the surrounding surface waters [4]. Runoff from agricultural waste can carry fecally-derived organisms that can potentially pollute surface waters and groundwater through the soil matrix [4]. Besides, the application of fecal waste on land in the form of manure or sludge can adversely affect the microbial populations in soil by altering the nutrient and decomposition cycle [7]. From a public health perspective, there is a substantial concern about disease transmission. Animals are known to be reservoirs for many enteric pathogens that are released into the environment in animal feces [8]. The presence of pathogenic bacteria of fecal origin in food and drinking water poses a public health hazard [9]. Ingestion of food or water tainted with fecal matter can cause a wide array of diseases such as gastroenteritis, hemorrhagic colitis, salmonellosis, shigellosis, etc. [10, 11]. Based on the intensity and the frequency of the exposure, these infections can range from mild to fatal [12]. Globally, diarrhea is considered the leading cause of enteric infections associated with poor sanitation and unclean water supply [13, 14].

On the other hand, there has been a steady increase in the incidence of foodborne illnesses associated with fresh produce in the past three decades [15]. Contamination occurs when the produce comes in contact with fecal deposits from farm animals, contaminated manure or with animal reservoirs [15]. The increase in contaminated produce places an enormous public health burden and an economic burden concerning morbidity and mortality. This study also has applications in the improvement of zoonotic risk assessments, since the emphasis is placed on animal waste.

Although pathogen contamination in surface waters results from the fecal waste of many domestic and wild animals, fecal waste from cows requires closer examination. According to the USDA, the United States is the largest producer of beef and cow milk [16]. Likewise, the 2015 World Food Statistics report compiled by the United Nations Food and Agriculture Organization (FAO) claimed that the United States ranked eighth with regards to the number of cattle per capita [17]. Given this, fecal pollution from cows ought to be scrutinized further based on its extensive impact.

The United States Environmental Protection Agency (US EPA) recommends the use of fecal indicator bacteria to determine the extent of fecal pollution in water [18]. Surface waters that do not meet the required water quality standards are deemed impaired as per the federal Clean Water Act [5]. According to the US EPA, the Clean Water Act was enacted to maintain and restore the chemical, physical and biological integrity of American surface waters that are impaired or in danger of impairment. Under section 303d of the Clean Water Act, each state is required to monitor and assess the water quality of the surface waters to identify waters that are not in compliance with water quality standards. In case of impairment, the states are required to establish priorities and implement improvements. This is done through a the following steps – 1) defining water quality goals to be adopted; 2) monitoring and assessing state-wide surface waters; 3) compiling a list of impaired waters through data consolidation and water-quality assessment; 4) developing effective Total Maximum Daily Load (TMDL) plans; 5) controlling point sources and managing the nonpoint sources of pollution to help achieve the water quality goals [19].

Remediation of impaired surface waters is accomplished using watershed implementation plans informed by the Total Maximum Daily Load (TMDL) and developed based on the fate and

transport of fecal bacteria. When establishing a criterion for water quality, it is important to be able to accurately and precisely determine the concentrations of bacteria within areas of fecal deposits or manure-treated soils that are proximal to impaired sources of water [20]. Based on the concentrations of bacteria within the fecal deposits or manure-amended soil, predictions about in-stream concentrations can be made [20]. Similarly, regulations regarding better management of animal fecal pollution will be made more efficient by studying the growth and survival of FIB in fecal matter used for land application [20]. According to Muirhead et al., there is a significant linear relationship between the concentration of *E. coli* in bovine feces and the concentration of *E. coli* in the run-off [21].

In addition to studying the fate and transport properties of fecally-derived bacteria, attempts ought to be made to comprehensively identify all sources of the fecal pollution. Identifying sources of pollution is an integral part of the Total Maximum Daily Load Plan - the primary goal of which is to determine the required pollutant reductions for the restoration of impaired surface waters [5]. To attain necessary water quality standards, an effective TMDL needs to account for all pollution sources, including point and non-point sources [22]. A point source is a singular, identifiable source of pollution that is actively engaged in discharging pollutants as part of its operations, such as a municipal sewage plant. Non-point sources are more diffuse and come from a variety of sources such as land runoff, farm fields, forests, etc. [22, 23].

Most TMDLs use a non-point source pollution model when determining the maximum allowable loading rate and loading quantity from identified sources [5]. A variety of different Microbial Source Tracking (MST) methods can be used within a TMDL framework to determine the major sources of pollution [23]. While the methods applied in this study do not aid with

source tracking, studying differential survival and inactivation rates of indicator organisms are integral to the establishment of an effective TMDL.

Data from distinct studies suggest that persistence of indicator organisms, in different matrices such as water or feces, is not consistent across the matrices [6, 24, 25, 26]. One objective of this study was to determine if the distribution and proportionality of fecal indicator bacteria (*E. coli* and enterococcus) are consistent in bovine feces and freshwater microcosms. Since the persistence of fecal indicator bacteria can also be affected by physical and chemical factors, a microcosm was constructed that simulates, to some extent, the physicochemical properties typically found in the environment.

Aim and Scope

The objective of this study was to – 1) determine the influence of temperature on the survival and differential decay of the following fecal indicators – fecal coliforms, *E. coli* and enterococci – in cow feces; and 2) compare the decay kinetics for fecal indicators in feces and freshwater microcosm. In addition to fecal coliforms, *E. coli*, and enterococci, this study also investigates the change in total bacterial loads over an 18-day period. To our knowledge, no study has addressed the differential survival of the FIB in the fecal matrix and compared it to survival in freshwater microcosms. This study aimed to address this knowledge gap, under varying temperature conditions

Research Questions and Hypotheses

In this study, the following questions were being examined –

1) How does temperature influence the survival and decay of fecal indicator bacteria (fecal coliforms, *E. coli*, enterococci, and total bacteria) in bovine feces?
2) How do the decay kinetics of fecal indicators compare in feces and freshwater microcosms?

We hypothesized that the concentrations of total bacteria would be higher than that of fecal coliforms, *E. coli* and enterococci. No bacterial growth was expected to occur in the manure samples. In addition, it was hypothesized that the samples maintained at warmer temperatures would exhibit a rapid die-off, in comparison to the samples maintained at lower temperatures such as 4^{o}C and 20^{o}C. Such a scenario was anticipated since the survival of fecal indicator bacteria is favored by cold temperatures [3]. With regards to the matrix type, survival rates in water phase were expected similar to the persistence in the fecal phase.

CHAPTER 2

LITERATURE REVIEW

Bacteriological Pollution Due To Agriculture

According to the Center for Disease Control and Prevention, livestock feeding operations are a primary source of surface and groundwater pollution [4, 27]. A report published by the US EPA estimated that fecal wastes from agricultural feedlots were responsible for the impairment of a staggering 173,629 river miles, 3,183,159 lake acres, and 2,971 estuary square miles [28]. Aside from feedlots, bacteriological pollution can also result from land application operations, pastures, manure stockpiles and treatment and storage lagoons. However, the most probable source of fecal pollution for the majority of the farms would be slurries and farmyard manure [29]. With the advent of intensive farming of animals, it has been estimated that cattle in the US produce about 1.2 billion tons of manure annually [30]. In traditional farming methods, composting destroyed the majority of the pathogens due to the high temperature and aerobic nature of the process. With agricultural intensification, waste is collected and stored in the form of semi-liquid slurries. The slurries form hot, anaerobic systems that fail to destroy pathogens [29]. Hence, these pathogens have the risk of being disseminated into the environment. When used in appropriate quantity as a fertilizer, manure can benefit the growth of crops. However, fecal wastes can become a hazard because of the release of nitrates and phosphates in an aqueous environment [29]. Improper management of fecal waste can have a dire impact on the environment. For instance, the runoff water from regions consisting of large quantities of fecal deposits will have high concentrations of nitrogen and phosphorus, causing a nutrient imbalance in nearby surface waters. This nutrient imbalance can lead to the formation of algal

blooms, causing the lakes to undergo eutrophication. Creating an imbalance in the nutrient will also exert a negative impact on the biodiversity of the aquatic life.

Fecal Indicator Concept

Given the extent of the fecal pollution and its associated human health hazards, it is important to monitor the surface waters for pathogens routinely. However, the direct testing of pathogens is difficult due to the following reasons: 1) pathogens are rare and sporadic; 2) pathogens are difficult to culture; and 3) working with pathogens poses a hazard due to their virulent and infectious nature to those assessing the water quality [31]. Arguments have been made in favor of using molecular techniques (microarrays, PCR, etc.) to assay pathogens, despite the concerns with sensitivity, specificity, and quantification [31]. Even in the case of molecular techniques, the risk of working with pathogens still exists. The indicator paradigm was introduced with the intent of minimizing risk to lab personnel and the associated costs of monitoring pathogens directly.

A fecal indicator is a group of organisms that indicate the presence of fecal contamination in surface waters, which implies pathogen presence [32]. Based on the pathogen of interest, different classes of indicators can be used, such as bacterial, viral, protozoan, etc. Based on adaptability to environmental conditions and environmental resistance, indicator systems differ regarding survival and persistence [32]. While the presence of fecally-derived microorganisms is an indicator of the poor water quality, it does not serve as a confirmation of the presence of fecal pathogens. Specifically, fecal indicators only act as a proxy in water quality assessment [33].

An organism is considered an ideal fecal indicator if it meets the following criteria: 1) it is a nonpathogenic organism typically found in the gut of humans and animals and is shed in fecal matter; 2) it is present in relatively higher concentration than the pathogen in the fecal matter; 3) it does not replicate in the environment; 4) it is easy to culture, isolate and quantify from all types of samples; 5) it is more susceptible to disinfection and environmental conditions; and 6) the density of the organism must correlate with the pathogenic microbial pollution or the potential health risks of the pathogen [34].

Fecal Indicators – Conventional and Alternative

Historically, total coliforms, fecal coliforms, *E. coli*, and enterococci have been used as fecal indicator organisms to monitor microbial impairment of aquatic systems [35, 36, 37]. Although waters could be impaired due to excessive concentrations of pathogenic bacteria, protozoa, and viruses, microbial water quality assessments are moreover based on bacterial concentrations of fecal indicators. However, there are many different types of fecal indicator organisms. The fecal indicators can broadly be classified as conventional and alternative.

Conventional indicators are bacterial – namely, total coliforms, fecal coliforms, *E. coli*, and enterococci [38]. These microorganisms are typically found in the gut microflora of higher mammals and birds; hence, they are associated with enteric pathogens. However, the predominant criteria for water quality assessment based on *E. coli* and enterococci are problematic and incomprehensive. This is due to the genetic diversity between isolates from the same host animal, ability to replicate outside of the host animal and variation between spatial and temporal stability [39]. Other limitations suggested by Savichtcheva et al. include the following: 1) their inability to identify the source of pollution; 2) low levels of correlation with the

pathogens; and 3) diminished sensitivity to detection methods [35]. Despite the limitations, they are widely used due to the following reasons – 1) easy to cultivate; 2) inexpensive quantification methods; and 3) low risk to human health [38].

Conversely, alternative indicators are often used in conjunction with conventional indicators, making for a more robust indicator system [34, 36, 39, 40]. Alternative indicators are broadly categorized as fecal anaerobes, viruses, and fecal organic compounds, where each category has its strengths and limitations [35, 36, 40]. Fecal anaerobes that qualify for the indicator criteria include *Bifidobacterium*, *Bacteroides* and *Clostridium perfringens* [35]. Bacteriophage that infects *Bacteroides fragilis* HSP40 and Coliphages (FRNA phage) are frequently used as viral indicators of fecal pollution in environmental samples [35, 41, 42]. Lastly, coprostanol is a fecal sterol that is used as a chemical indicator of fresh fecal contamination, since it has a relatively short half-life (<10 days at 20^{o}C) in aerobic aquatic conditions [35].

Molecular Markers as Indicator and Microbial Source Tracking

Although the use of conventional indicator organisms as a surrogate for pathogens is common for performing water quality assessment, these indicators cannot specify the source of the fecal pollution [4, 6]. The use of molecular markers as indicators for fecal pathogens has been adopted in the recent decades. Molecular markers are defined as host-specific (human, ruminant, avian, etc.) oligonucleotides that are adopted from the genetic information of indicator organisms, such as *Bacteroides*, *Bifidobacterium*, *E. coli*, enterococci, etc. The popularity of molecular markers in contemporary water quality research is due to its ability to identify agricultural sources of pollution (point and non-point) that will help develop an effective water

quality criteria. Approaches that use the library-based and culture-independent microbial source-tracking (MST) methods are being adopted at a rapid pace due to MSTs ability to identify the source of the fecal pollution [24]. MST is based on the assumption that the population distribution of fecal indicator bacteria and MST markers in the matrix is proportional to their distribution in the fecal sample [24].

Fecal Indicator Bacteria

The use of coliforms as indicators began in Britain in 1901 [32]. The term coliform was coined based on *Bacillus coli*, which was later named *Escherichia coli*. In 1880, Von Fritsch identified *Klebsiella pneumoniae* and *Klebsiella rhinoscleromatis* as microorganisms that are specifically found in human feces. Since then bacteria have been used as indicators of water quality. In 1885, Percy and Grace Frankland pioneered the use of bacteriological examination to test the quality of drinking water in London. However, the enumeration of coliform colonies did not occur until the early 1900s [32]. Also, the development of MacConkey Broth in 1905 aided in the identification of coliform bacteria based on it lactose-fermenting properties. The development of the IMViC test (indole, methyl red, Voges-Proskauer, Citrate) in the 1920s also helped in the identification and the characterization of the coliforms [32].

The conventional fecal indicators of contemporary times are fecal coliforms, *E. coli* and enterococci. These organisms are favored over other indicators because they are typically found in the gut microflora of higher mammals and birds, which explains their association with enteric pathogens. Also, these organisms are easy to cultivate, inexpensive and safe to handle [5]. Fecal coliforms used to be considered the predominant indicator, typically used to assess bacterial hazards in surface waters, but *E. coli* and enterococci assessments have been used more

frequently in the past decades since they are known to have a higher association with food-borne illness outbreaks [58]. Figure 1 shows a classification of the fecal indicators.

Total Coliform

The total coliforms (TC) are a group of aerobic and facultative anaerobic, gram-negative, non-spore-forming rod-shaped bacteria that produce gas upon lactose fermentation in the appropriate media at 35^{o}C after 48 hours [44]. The total coliform group includes many types of bacterial genera such as *Citrobacter, Enterobacter*, and *Klebsiella* [45]. The coliform bacteria were used to assess water quality for most of the 20th century. Membrane filtration (MF) and most probable number (MPN) were the most commonly used identification and quantification methods.

Despite their extensive use in the recent past, the use of total coliforms as fecal indicators has many limitations. All members of the coliform group have been known to regrow in the natural aquatic environment. The decay rate of TC depends on the ambient temperature and the amount of organic matter in the water. Bacterial concentrations of TC tend to be high when the temperatures and organic matter content are elevated [44, 46]. In the event of high regrowth rates due to favorable abiotic factors, there is an important concern regarding the false indication of fecal contamination [44].

Fecal Coliform

Fecal coliforms (FC) are a subset of the total coliform group. The fecal coliforms include *Escherichia* and *Klebsiella* genera. They are different from total coliforms due to their ability to ferment lactose at 44.5^{o}C within 24 hours [44]. As with total coliforms, fecal coliforms are

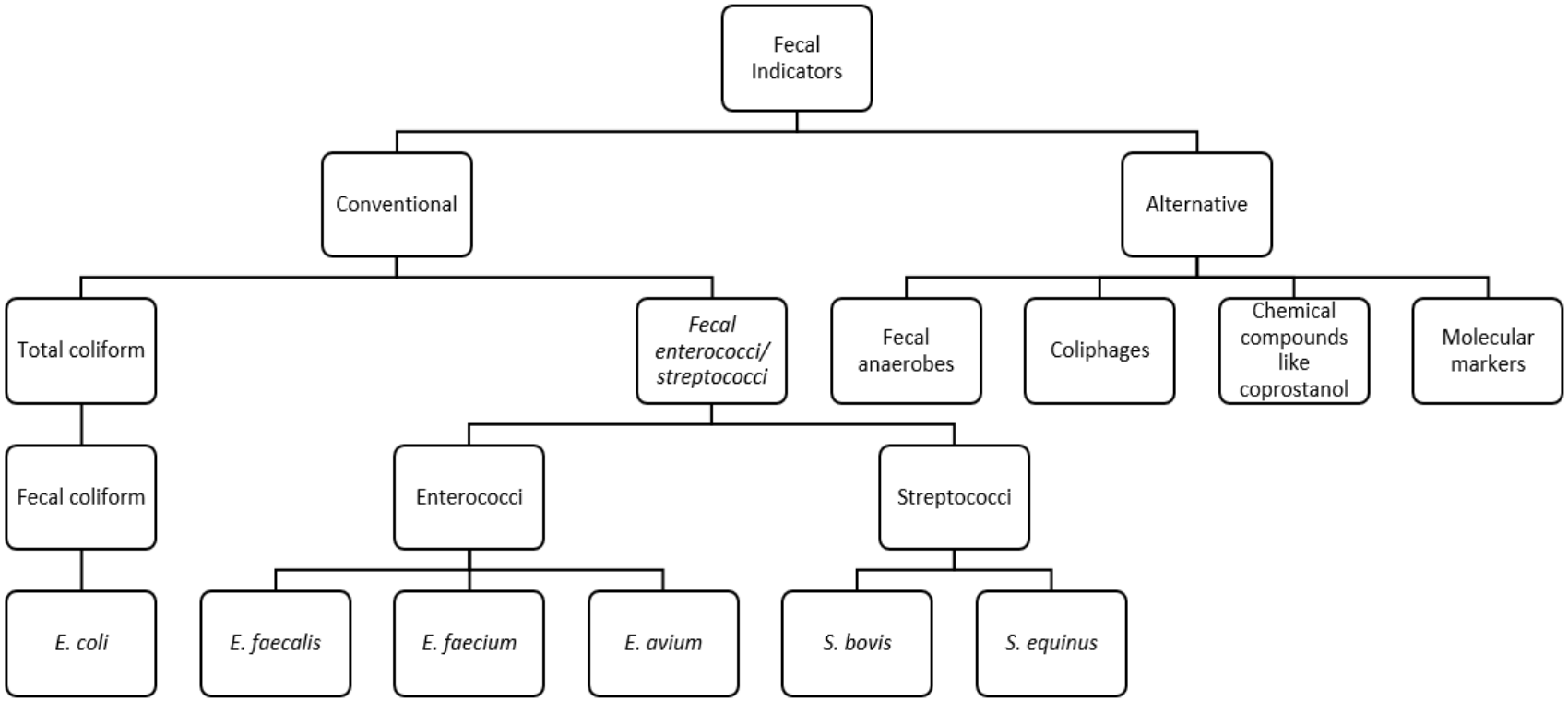

Figure 1. Classification of conventional and unconventional fecal indicators. (Adapted from Ashbolt *et al.*) [32]

limited in their indicative capacity for fecal pathogens. Fecal coliforms are known to frequently occur in unpolluted tropical and temperate waters/soils, which may lead to erroneous assumptions regarding contamination [47, 48].

Escherichia coli

Escherichia coli is a gram-negative bacillus that belongs to the Enterobacteriaceae family. *E. coli* is a type of a fecal coliform, which is distinguishable from other fecal coliforms based on the absence of urease and the presence of β-glucuronidase enzymes. *E. coli* occurs in several different strains that are broadly classified into 6 different subgroups; each strain occupies a certain ecological niche and can be broadly categorized as commensal or pathogenic [49]. The pathogenic strains are further classified as verotoxigenic, enterohemorrhagic, enteroinvasive and/or uropathogenic, which are all capable of causing harm to humans. It is noteworthy that the pathogenic strains of *E. coli* are responsible for causing deadly foodborne illness outbreaks based on their high pathogenicity and tolerance to low pH environment. The commensal form of *E. coli* is a harmless bacterium that is an important member of the human/ruminant gut microflora, which is known to assist in the breakdown of specific carbon compounds such as cellulose, pectin, hemicellulose etc. [50]

Due to its presence in the gut microflora, Theobald Smith first proposed the use of *E. coli* as a surrogate for pathogens in drinking water in the 1890s [36]. The earliest experiments developed to test for the presence of *E. coli* exploited its thermotolerant properties. *E. coli* is deemed a thermotolerant coliform based on its ability to ferment lactose at 44-45^{o}C [45]. Aside from its ability to thrive at higher temperatures, it is a non-spore forming facultative anaerobe that is able to ferment lactose along with the typical gas and acid production within 48-hours

[29]. Given its presence in the gut microflora of humans and ruminants, *E. coli* was considered a good indicator of fecal pollution due to the ease with which it can be cultured [36]. However, with the advent of sequencing technology, it is now known that *E. coli* makes up only 1% of the total intestinal bacterial population in cattle [36]. Also, some studies have shown that *E. coli* can replicate in the environment [20, 26]. Despite its weaknesses as an indicator, it is considered relevant in water quality studies due to its possible application as a surrogate for its well-known pathogenic strain, *E. coli* O157: H7 [55]. Although the *E. coli* species consists of biovars that are mostly harmless commensals, a few strains of *E. coli* have been involved in deadly outbreaks and are classified as the causative agents of diarrhea diseases, urinary infections and meningitis [25, 29, 52].

Specific Characteristics Affecting Fate and Transport. The fate and transport of *E. coli* are influenced by physiological properties that dictate aspects of bacterial attachment and inactivation. Bacterial attachment is a product of cell surface structures such as lipopolysaccharides (LPS), flagella, and fimbriae [45]. The composition of LPS, including the hydrophobic lipid A constituent, core oligosaccharide and O antigen, governs the bacterial attachment dynamics, uptake of metal ions and precipitation and dissolution reactions induced by microbial activity [45]. On the other hand, bacterial inactivation is caused by a multitude of environmental factors such as temperature, sunlight, predation etc. which will be further elaborated in the following sections.

Enterococcus

Enterococci are gram-positive spherical bacteria that commonly occur in pairs or a chain-like arrangement [37, 54]. These bacteria are catalase-negative, non-spore forming species that are categorized as obligate fermentative chemoorganotrophs [37]. While a few enterococcus species act as opportunistic pathogens (*E. faecalis* or *E. faecium*), most enterococci are commensal bacteria that are a constituent of the gut microflora in warm-blooded animals [37, 55]. Potential health hazards that are commonly associated with enterococcus include nosocomial infections, neonatal infections, bacteremia and central nervous system (CNS) infections [37]. Although pathogenic enterococci exhibit low virulence, the potential health hazards and the economic burden associated with it can be mitigated by using non-virulent strains as a surrogate for the pathogenic strains.

Currently, enterococci enumeration is a standard method to test for microbial pollution in marine waters [56]. A 1946 study conducted by Ostrolenk et al. was the first to consider enterococci as a potential indicator for fecal contamination [55]. Enterococcus was considered an ideal indicator based on its non-pathogenic, easy-to-culture characteristics and positive density correlation with high pathogen contamination [37]. However, there are some weaknesses that challenge its indicator value. Enterococci do not exclusively originate from fecal matter and can be found in soils and sediments as part of the endogenous microbial population [37]. Also, enterococci can be shed by a variety of host species (wild and domestic); but, the health risks associated with enterococci from different host species is highly variable [37].

Specific Characteristics Affecting Fate and Transport. Surface properties that are typical of enterococci, such as net charge and hydrophobicity, greatly influence the fate of the bacteria

[57]. Extracellular polymeric substances like peptidoglycans and polysaccharides create a negative net charge on the cell surface, thereby favoring bacterial attachment to a substance that has a positive charge [57]. Enterococci often attach to sediment and vegetation resulting in temporary sequestering [37]. Upon disturbance, however, these organisms can reenter the water column [37].

Generalized Fate and Transport Model for FIB

An important pathway of entry of fecal pollution into surface waters occurs when surface waters are in close proximity to pastures; the contaminated surface waters serve as a habitat for the fecally-derived microorganisms [33]. Watershed-modeling often includes fate and transport processes that begin with the fecal deposits on land in the form of defecation or land application [33, 58]. These fecal deposits introduce the fecally-derived microorganisms to the soil surface, which are then mobilized through rainfall or irrigation. The fecally-derived microorganisms are then carried through the soil matrix as particle-associated or soil-solution fractions [33]. Throughout this process, bacterial concentrations within the fecal deposits are changing due to growth or die-off resulting from abiotic and biotic factors. The remaining bacteria within the deposits partition into runoff pathways or an infiltration pathway. The run-off directly leads to the proximal water-source, whereas the infiltration transports the fecally-derived microorganism to the groundwater and/or artificial drainage. Fecally-derived microorganisms may get transported to the surface water from the groundwater as they get released due to resuspension caused by high flow. Figure 2 shows a graphic representation of the fate and transport pathway of fecally-derived microorganisms from fecal deposits to the water source [33].

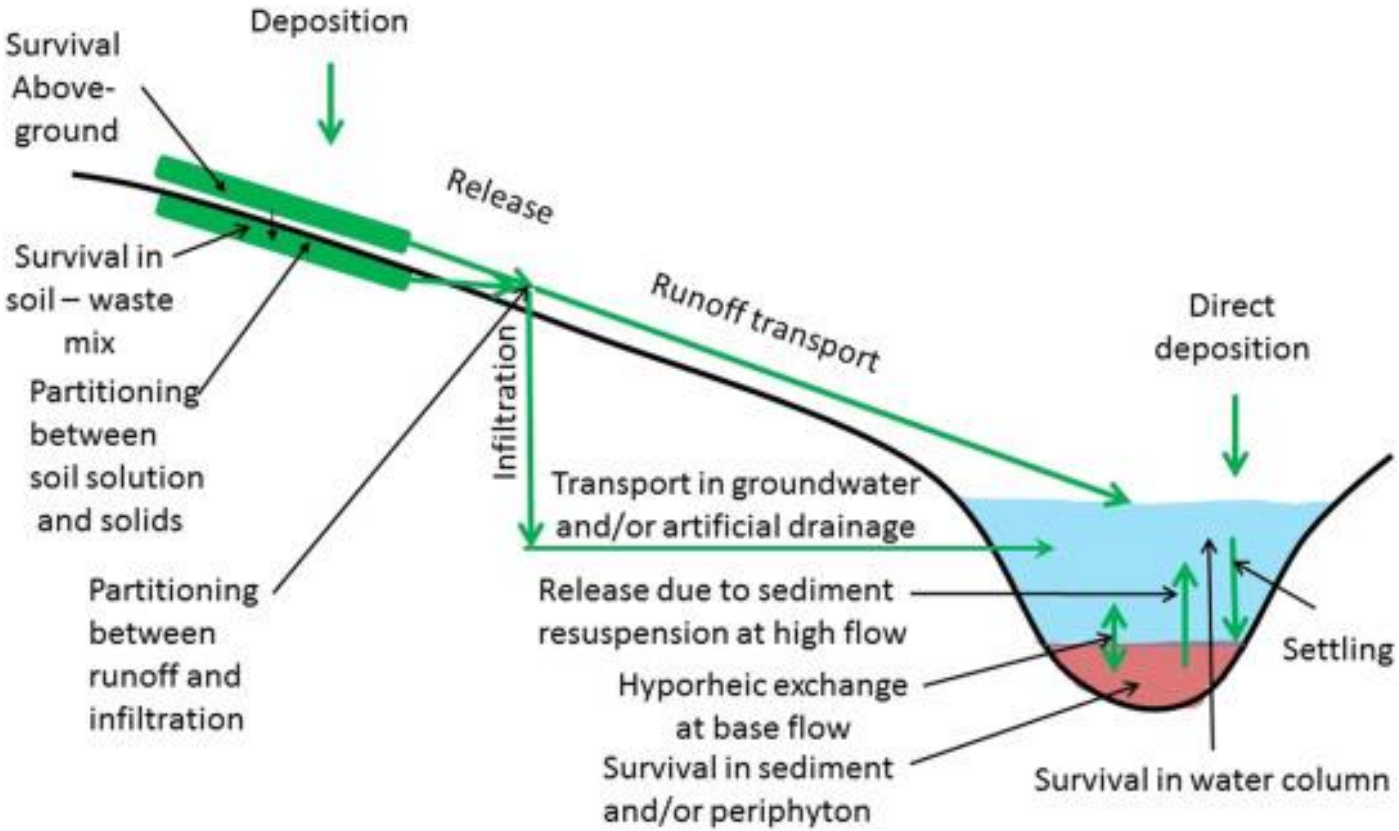

Figure 2. Fate and transport pathway of fecally-derived microorganisms from fecal deposits to the water source [33]. Printed with permission from Elsevier Science.

Many processes can limit bacterial movement within the soil matrix. Of all process, physical filtration is considered the most dominant process that limits bacterial mobility through the soil matrix into the water systems [25]. Based on the size of a typical fecal indicator bacteria (0.2 to 5 μm), many of the microbes are subject to straining, which ultimately impacts the overall bacterial load in the water systems [44]. In addition to filtration, adsorption can mitigate bacterial movement. In a typical porous media, bacteria transport is normally calculated using low water content and high clay content, are much more likely to filter out fecally-derived microorganisms [59]. Conversely, high porosity soils with high water content can filter out the advection-dispersion-sorption equation [45]. According to Reddy et al., soils with low porosity, bacteria, since the bacteria do not occur as free cells in the liquid phase; instead, they adhere to the clay particles via adsorption and may be filtered out in the process [25].

The study of fate and transport characteristics of fecally-derived microorganisms are integral to developing effective criteria for water quality because it improves the accuracy and precision of estimation of in-stream bacterial concentration for modeling purposes. Estimations of in-stream bacterial concentrations can be based on information from the following – parameters of bacterial survival in fecal/soil matrix, the rate of release from fecal deposits or sediment/soil, parameters of bacterial survival in aquatic systems etc.

In sum, the fecal bacteria fate and transport are dependent upon water and sediment transport processes. Models that apply current hydrologic and erosion approaches are increasingly used in watershed modeling studies, because of the models' accuracy. For instance, Hydrological Simulation Program-FORTRAN and Soil and Water Assessment Tool are the most commonly used watershed models in the U.S. that include the aforementioned approaches [43].

Factors Influencing Survival of FIB

There are several factors that affect the survival of fecal indicator bacteria in different matrices [25]. These factors can broadly be classified as abiotic and biotic factors [56]. Also, it is noteworthy that while all the following factors affect the survival of fecal indicator bacteria, the degree to which they impact the die-off depends on the geochemical properties of the residential matrix [60]. Some factors exert a greater influence on microbial persistence than others, based on lack of uniform exposure to the factor impacting the die-off. Also, the influence of the factors affecting survival tends to differ depending on spatial and temporal variability [60]. Although the role of each factor can be tested in a microcosm or mesocosm in a controlled laboratory setting, multiple factors simultaneously impact the survival of fecally-derived microorganism in the natural environment [24]. The following discussion further expounds on the abiotic and biotic factors and their respective influences on the survival and decay of fecal indicator bacteria.

Abiotic Factors

Abiotic factors are those that impact the survival of fecally-derived microbes based on physicochemical properties of the environment. These include factors like temperature, UV light, the degree of acidity or alkalinity (pH), salinity, the presence of oxygen, moisture content, suspended solids, nutrient availability and matrix composition etc. [58, 60, 61]. The atmospheric conditions dictate the solar radiation (UV light), temperature and moisture (precipitation), whereas the physicochemical properties of the environment affect the pH, nutrient availability, organic matter content and elemental composition. In the natural environment, a combination of abiotic factors can largely influence the survival and persistence of fecal indicator bacteria.

Furthermore, many of these factors, such as suboptimal temperatures, prolonged exposure to solar radiation, and limitation of nutrients can lead to loss of culturability [47].

Temperature. Temperature is considered one of the most critical and well-studied of the abiotic factors that impact survival of fecal indicator bacteria [49]. However, different research groups have adopted a variety of unique conditions to study the effect of temperature on the decay rate of FIB, which renders the data difficult to compare. For instance, researchers have chosen to study the survival of FIB in different residential matrices like sediment, water etc. Despite the heterogeneous conditions, a trend collectively emerges from the research data across the board, in which temperature is observed to share a decreasing exponential relationship with the survival rate of bacteria [62]. Also, research data suggests that survival of FIB tends to higher in stable and constant temperature scenarios compared to fluctuating temperature, with survival being lower when temperate fluctuation was larger (greater than 7^{o}C) than smaller fluctuations (less than 4^{o}C) [49]. Temperatures below 5^{o}C can trigger fecal indicator bacteria to enter a dormant state, where the bacteria are considered viable but not culturable. Alternatively, high temperatures of greater than 35^{o}C can rapidly increase the die-off of fecal indicator organisms due to thermal stress [56]. The link between thermal stress and gene expression patterns offers an explanation to why high temperatures and temperature fluctuations can impede bacterial survival, particularly in *E coli*. A recent study showed that many *E. coli* genes that are thermoregulated at 20^{o}C and 35^{o}C are controlled by the same histone-like nucleoid structuring protein; hence, differential gene regulation in response to temperature fluctuations occurs in close proximity to genes responsible for regulation that occurs at a single temperature [49].

However, the current understanding of the relationship between temperature and heat and cold shock proteins is still limited.

A study carried out by Howell et al. focused on the survival of fecal coliforms and fecal streptococci at three temperatures (4^{o}C, 25^{o}C, and 35^{o}C) in bovine feces-amended sediments of varying particle size [63]. The experiments were carried out under controlled laboratory conditions using physiological saline to decrease cell death due to osmotic shock. The results revealed that temperature significantly affected the mortality rates of fecal coliforms and fecal streptococci. In the same vein, Guber et al. studied the effect of temperature on the survival of *E. coli* in white-tailed deer feces [26]. A series of deer pellets were collected and incubated at 4^{o}C, 25^{o}C, and 35^{o}C respectively. Survival of *E. coli* was determined on various days over a 32-day period using membrane filtration technique, followed by incubation on mTEC media. The results showed that the bacteria initially underwent a short growth phase, followed by a relatively long die-off phase. While the growth rates were found to be different for each temperature, the die-off rates were observed to be quite similar at 4^{o}C, 20^{o}C, and 35^{o}C. The computed values of the inactivation rates of *E. coli* maintained at 4^{o}C, 20^{o}C, and 35^{o}C were 0.158, 0.175 and 0.194 per day respectively. The growth rate lasted much longer for the organisms that were maintained at 20^{o}C compared to the samples maintained at 4^{o}C and 35^{o}C. In addition to undergoing long-lasting growth, the fecal indicator bacteria maintained at 20^{o}C exhibited the fastest growth and the die-off phase did not commence until the eighth day of the survival study.

Sunlight and Solar Radiation. In addition to temperature, insolation also plays an integral and a potent role in the inactivation of fecal indicator bacteria in the natural environment [64, 65, 66]. When subjected to sunlight, the bacterial populations are affected based on the exposure to

UV radiation from the sun. Inactivation by UV is primarily caused by genetic damage, due to the formation of pyridine dimers and other nucleic acid lesions as byproducts of photoreactions [65]. These byproducts ultimately obstruct DNA replication and transcription, which in turn inhibits cell division. Hence, solar radiation is inversely proportional to the survival of fecal indicator bacteria [62].

Many attempts have been made to study the effect of irradiation on the survival of fecal indicators. One such study was carried out by Sinton et al., in which the researchers aimed at estimating inactivation rates of fecal indicators, fecal coliforms, *E. coli*, enterococci, and coliphages, due to exposure to sunlight [64]. Ten outdoor experiments were conducted in multiple 300-litre chambers, containing effluents from waste stabilization pond mixed with fresh or saline waters that were incubated at 14^{o}C. The inactivation rates for the fecal indicators were found to be higher in the presence of sunlight compared to inactivation rates estimated for containers placed in the absence of sunlight ($K_S > K_D$) [64]. Furthermore, the decay was found to be the fastest in enterococci, followed by fecal coliform, *E. coli*, and the coliphages respectively. Seasonal differences were also considered, and it was found that enterococci were inactivated much faster in summer than the winter [64]. Other studies that focused on marine and freshwater environments determined that survival tends to be lower in marine waters compared to freshwaters when exposed to solar radiation [66].

A study carried out by Korajkic focused on the survival of fecal indicator bacteria (*E. coli* and enterococci) in bovine manure and sewage fecal source exposed to insolation [66]. Submersible mesocosms were set up and deployed for 7-days in a freshwater site and marine water site. The results revealed that sunlight played a significant role in lowering the survival of enterococci from cattle waste [66].

pH. The pH, which is the measure of acidity or basicity of a solution, can impact the survival and persistence of bacteria. Although there are some bacteria that thrive in highly acidic conditions, most bacteria tend to grow best in the pH range of 6.0 – 7.5. The same is true for fecal indicators. According to a study conducted by Šolić et al., the optimum pH for fecal coliforms ranges from 6 to 7, with a rapid decline in survival for pH conditions not within the given range [62]. The time taken for 90% reduction in fecal coliform (T_{90}) was found to be 40% less under acidic conditions compared to basic conditions [62].

Pearson et al. studied the effect of pH on the survival of fecal coliforms in waste stabilization ponds in Portugal [67]. The physicochemical properties of the pond were monitored and fecal coliforms were enumerated using the membrane filtration method. Inactivation rate increased above pH of 8.5, the inactivation rate was greater in nutrient-poor conditions. The largest die-off was observed when the pH was in the range of 8.5 to 9.0 [67]. The bacterial populations were observed to undergo bacterial regrowth when the pH was adjusted and brought closer to 7, which is considered more favorable. A similar die-off pattern was observed in nutrient-rich conditions, followed by the bacterial regrowth that was seen upon the adjustment of pH to more favorable conditions [67].

Oxygen. While the effect of most abiotic factors on the survival of fecal indicator bacteria are well studied, the same cannot be said about the effect of dissolved oxygen. With the rise in concentrations of organic carbon in a water source, anaerobic or microaerophilic conditions may develop as a consequence. These conditions, in turn, impact the survival of the bacterial load within those waters.

Since fecal indicator bacteria originate from a source where they are adapted to low oxygen concentrations (i.e., the lower intestine of warm-blooded animals and birds), Roslev et al. hypothesized that the high concentrations of dissolved oxygen will adversely impact the survival of fecal indicators in nondisinfected drinking water [68]. To study the effect, aerobic and anaerobic drinking water microcosms were constructed, which were inoculated with certain strains of *E. coli* or raw sewage. Survival of *E. coli* was measured using membrane filtration and *in situ* hybridization with 16S rRNA-targeted fluorescent oligonucleotide probes. Anaerobic conditions increased survival of both inoculated and indigenous sewage *E. coli* [68]. Under aerobic conditions, *E. coli* underwent a biphasic decrease in survival, where the initial first-order decay rate was found to be -0.11 day^{-1}. The initial phase was followed by a faster decay rate of -0.35 day^{-1} [68]. Also, enterococci were enumerated from the sewage samples using Slanetz-Bartley agar followed by transfer to bile-aesculin-azide agar. Similar to *E. coli*, aerobic conditions also decreased survival of enterococci.

Pearson et al. also reported a similar impact of dissolved oxygen on the survival of fecal coliforms in waste stabilization ponds [67]. It was found that fecal coliform concentrations were much higher at lower depths in the ponds, where the oxygen concertation was depleted. Overall, these results indicate a negative relationship between survival of fecal indicators and dissolved oxygen [67, 68].

Salinity. High salinity in the matrix is known to lower the survival of the fecal indicator bacteria [64, 69, 70]. Bordalo et al. conducted a study to investigate the survival of fecal coliform and enterococci in a tropical estuary in Eastern Thailand under varying salinities and sunlight conditions [69]. Three salinity conditions were set up in water microcosms spiked with raw

urban sewage – low salinity, progressive mixing with brackish water and fast mixing with high salinity. Enterococci had shown a higher resistance to salinity compared to fecal coliforms. Survival of FIB was found to be lowest in the fast mixing, high salinity environment, followed by progressive mixing with moderate salinity. The highest survival rates were observed for the low salinity environment.

Similarly, Sinton et al. performed an experiment that studied the impact of salinity, in conjunction with sunlight inactivation, on the survival of the fecal indicator bacteria in waste stabilization ponds [64]. It was observed that sunlight inactivation was rapid in high salinity conditions. The sunlight inactivation coefficients for fecal coliforms were 0.622 in seawater, 0.452 in 50:50 freshwater and seawater mixture, and 0.281 in freshwater conditions. Similar coefficients were observed for *E. coli*. As for enterococci, the sunlight inactivation coefficients were 0.197 in seawater, 0.155 in 50:50 freshwater and seawater mixture, and 0.133 in freshwater conditions.

While high salinity in conjunction with sunlight expedites inactivation in an aquatic environment, bacterial survival increases in the sediments despite unfavorable conditions like high salinity and the presence of UV radiation [70]. This is because bacteria get adsorbed to sediment particles, which in turn protects the microbes from decay and inactivation. Also, adsorbed bacteria are able to feed on nutrients associated with the sediment particles, which allows for bacterial growth [70].

Nutrient Availability and Moisture. Since bacteria depend on organic matter for nutrition, nutrient availability within the matrix promotes the survival of fecal indicators and may aid in the regrowth of enteric bacteria. The impact of nutrient availability on the survival of fecal

indicators differs based on the type of matrix in which the fecal indicators are thriving. Access to nutrients is considered an integral abiotic factor for survival of microbes in sediment/soil. Once enteric bacteria are removed from their primary habitat through fecal deposits, their survival depends on the nutrient supply in the secondary habitat, which can range from eutrotrophic to oligotrophic levels [71]. If the secondary habitat has a high concentration of organic material, the decay rate for fecal indicators is low. This is because access to a high concentration of organic matter leads to retention of nutrients, thereby promoting the growth of bacteria. However, in the aquatic environment, available organic carbon becomes less accessible since the water dilutes the mixture. Additionally, the survival rates of fecal indicator bacteria in the soil surface tends to be higher than the survival rates in the subsurface levels due to the lack of nitrogen in the lower levels [25].

The level of moisture is an important survival factor for fecally derived microorganisms present in the soil and fecal matrix [71]. In addition to intrinsic soil properties, soil moisture content depends on characteristics such as vegetation cover, land use, soil type, slope gradient etc. [72]. For solid phase matrix, elevated moisture content results in enhanced survival. Survival of conventional indicators have been found to be elevated after high rainfall event or in flooded conditions [25]. This may be due to increased release and mobilization of fecal indicator bacteria in high moisture conditions. The relationship between increased flooding due to rainfall and increased incidence of diarrheal diseases have been observed in several epidemiological studies. Based on enhanced dispersion and dissemination of fecal indicators, it is fitting that hydrology plays an important role in the survival of fecal indicators [72].

Biotic Factors

Fecally-derived microorganisms and other autochthonous organisms influence the survival of fecal indicator bacteria in the secondary habitat. Their influence collectively manifests in the form of biotic factors. Biotic factors include predation and competition [49, 61]. In the natural environment, the population of fecal indicator bacteria will interact with other fecally-derived microorganisms, which can result in all microbes competing for the same resources. Additionally, predation from protozoan species can cause a sharp decline in the bacterial concentrations within a sample [49]. These interactions can be loose or intricate and the cumulative effect of these interactions negatively impacts fecal indicator bacteria. Finally, as seen in abiotic factors, a combination of biotic factors can lead to loss of culturability for the fecal indicator bacteria.

Predation. Predation is one of the chief biotic factors that affect the survival of fecal indicator bacteria in aquatic systems [57, 58]. Studies that focused on the impact of autochthonous microbiota on the survival of enteric bacteria in natural waters suggest that the autochthonous organisms cause a sharp decline in the viable FIB populations [57]. These autochthonous organisms include protozoa, parasitic bacteria (such as *Bdellovibrio sp.*) and bacteriophages. However, protozoan predation has been shown to negatively affect FIB survival in natural waters more than other autochthonous organisms [58]. A study conducted by Enzinger and Cooper studied the decay of *E. coli* (introduced using fecal samples) in estuarine waters that contained protozoa and lytic bacteria [73]. The results revealed that the selective filtration of protozoa from the estuarine water caused a negligible decline in FIB populations. Whereas, the addition of excess protozoa into estuarine water caused a rapid decline in FIB survival. This

implies that the protozoan grazing of FIB impacts enteric bacterial populations far more than other predatory organisms included in the autochthonous microbiota. In the same vein, Davies et al. studied the impact of cycloheximide on the survival of fecal coliform, fecal streptococci and *Clostridium perfringens* in marine and freshwater sediments [70]. Cycloheximide is a chemical agent that inhibits the growth of flagellate and ciliate protozoa. In this study, the addition of cycloheximide inhibited the growth of predatory protozoa, which ultimately led to a surge in the survival of fecal coliform and streptococci. In the absence of cycloheximide, the protozoan grazing led to a net FIB die-off [70].

Competition. The presence of heterotrophic bacteria among the autochthonous microbiota causes the FIB to compete for nutrients, which in turn negatively impacts the survival of FIB. In addition to competing for limited resources, the autochthonous bacteria may also produce antagonistic compounds, causing cellular injury to the fecally-derived microorganisms leading to an enhanced FIB die-off rate. However, research shows that the impact of competition on the survival of fecal indicators varies based the type of FIB. For instance, Wanjugi and Harwood conducted a study in which the influence of competition from indigenous on FIB survival was measured using kanamycin treatment [74]. The addition of kanamycin was meant to reduce the numbers of indigenous bacteria, thereby limiting competition. The results of this experiment showed that the survival of *E. coli* improved greatly upon the addition of kanamycin, whereas the kanamycin did not affect the survival of enterococci sp. [74]. Similarly, a study carried out by Korajkic specifically focused on the survival of fecal indicator bacteria (*E. coli* and enterococci) in bovine manure and sewage fecal source in the presence of indigenous microbiota population [66]. Submersible mesocosms were set up and deployed for 7-days in a freshwater site and marine water site. According to the results, the presence of indigenous microorganisms

significantly impacted the survival of *E. coli* and enterococci. The presence of indigenous microbiota caused a total variation of 21.2% (p-value – 0.0048) for enterococci and a total variation of 23.4% (p-value – 0.0031) for *E. coli* [66].

Viability and Dormancy

Fecal indicator bacteria occur in different physiological states. When under stress due to biotic and abiotic factors, the fecally-derived bacteria may enter a viable-but-non-culturable (VBNC) state [75]. Coined by Rita Colwell and her coworkers in 1985, the term VBNC is defined as a dormant state in which a bacterium is metabolically active but unable to multiply on a medium that normally supports its growth due to specific physicochemical and biological environmental parameters [76, 77]. These parameters can include low temperature, lack of nutrients, unfavorable pH etc. [77].

Bacterial cells are considered viable if they are metabolically active [77]. According to Kell et al., the concept of culturability can be operationally defined as the state of being immediately culturable using conventional or unconventional culture-based techniques [78]. Non-culturable cells are capable of shrinking significantly in size to the extent that they escape detection using traditional methods such as collection by filtration followed by microscopy [76].

Survival in Fecal Matrix

To construct a material budget for pathogen contamination in aquatic systems, it is important to estimate the potential impact of animal fecal deposits on water quality of proximal surface waters [79]. The fecal deposits on pastures are subject to a variety of factors that impacts the environmental survival of bacteria. Based on environmental factors (such as rainfall,

exposure to sunlight, and high temperatures), the fecal deposits may disintegrate, blend with the soil systems, and eventually get washed into nearby surface waters. However, the survival of fecal indicator bacteria is governed by a specific set of factors before they are transported into aquatic systems. Survival of FIB in fecal or soil systems is chiefly affected by moisture content, temperature, organic matter, and antagonism. According to Gerba et al., the survival of FIB and pathogens in soils is less than 2-3 months [80]. Few studies have investigated the survival of FIB in the fecal matrix. Once such study performed by Oladeinde et al. investigated the survival of FIB and *Bacteroides*-associated MST markers in cow pats that were subject to various sunlight and temperature conditions [6]. The decay rate of *E. coli* was found to be -0.176 day^{-1} in the shaded treatment.

Decay Models

Modeling the survival of fecal indicator bacteria is integral to the development of deterministic models that calculate the total maximum input of fecal deposits into the environmental systems without eroding its ecological integrity. Inactivation or decay of fecal indicator bacteria is defined as reduction of FIB concentration (N) due to exposure to a disinfectant (concentration C) during time *t* [83]. In the context of viability, inactivation may also be deemed as the loss of culturability of FIB [61]. However, under favorable conditions, VBNC bacteria may revert back to their culturable status [81]. The variation in decay rates of fecal indicator bacteria are dependent on the biotic and abiotic factors. In addition to offering quantitative and qualitative information on bacterial mortality, bacterial kinetics can distinguish between environmental factors that impede survival and those that promote growth [59]. Factors

that modulate rapid inactivation in fecal indicator bacteria include exposure to sunlight, fluctuating ambient temperatures, low moisture content, and lack of nutrients, etc. [81].

First Order Kinetic Model

Although inactivation of fecal indicator bacteria can be described in terms of a second-order decay kinetics, a first-order rate expression is used more frequently in FIB inactivation studies [81, 82, 87]. However, historically, inactivation was assumed to exclusively follow first-order kinetics as postulated by Chick et al. [83, 84]. Chick's first order kinetics model postulated that bacterial inactivation can be represented as a straight line on a semi-logarithmic graph, and is represented as follows:

$$\frac{dN(t)}{dt} = -k * N(t) \quad \text{..... (1)}$$

where *dN(t)* is the change in concentration of FIB over time *t* and *k* is the first-order inactivation rate [81, 84]. Notably, the application of other parameters that impact inactivation rates renders the log-linear assumption weak, since the mechanism of inactivation is far more complex than the one proposed by Chick, which essentially mirrors a unimolecular reaction [72, 73]. Watson refined Chick's model by using *k* as a function where its magnitude is controlled by the factors that impede bacterial survival [81]. The Chick-Watson first-order decay equation is written as follows:

$$\ln(\frac{N}{N_0}) = -\frac{kC_0^n}{nk}[1 - \exp(-nk * t)] \quad \text{..... (2)}$$

where N/N_0 is the change in concentration of FIB over time *t*, *k* is the first-order inactivation rate, C is the concentration of the disinfectant/inactivation parameter and n is a constant of dilution [85, 86]. The first order decay model does not account for inactivation resistant subpopulation. In an attempt to circumvent this drawback, many researchers have applied a biphasic decay model

for a better fit. Hellweger et al. applied biphasic kinetics while investigating the decay of fecal bacteria in surface waters that flowed a biphasic pattern of decay. The initial decay was found to be much higher than the latter due to change in cell density [88].

CHAPTER 3

MATERIALS AND METHODS

Manure Collection

Freshly voided cow feces were collected from three farms in East Tennessee. Figure 3 shows the geographical locations of these farms. The manure samples were collected three times during February-March 2017. Of the three farms from which samples were collected, one specialized in beef production, and the other two were dairy farms. Each farm housed about 10-50 cattle.

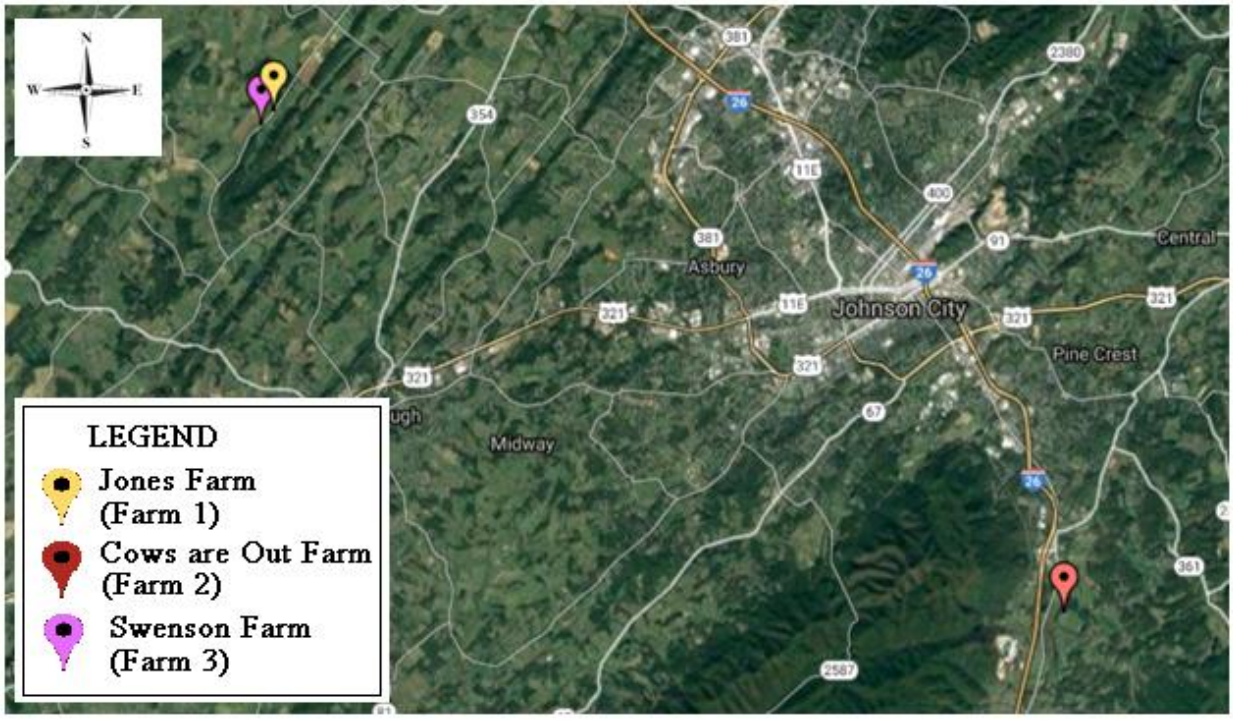

Figure 3. Locations of the three farms that provided the samples for this study. Yellow – Jones Farm (Farm 1); Red – *Cows Are Out* Farm (Farm 2); purple – Swenson Farm (Farm 3). Image taken from Google Maps.

The freshly excreted feces from three farms were collected in large sterile Whirl-Pak (Nasco, WI) bags and stored on ice with no exposure to light. The bags were then rapidly

transported to the study site. The study was conducted in the Environmental Health Sciences Laboratory housed in the Department of Environmental Health at East Tennessee State University (ETSU).

Preparation of Manure for Bacteriological Analysis

After collection, the manure samples were homogenized thoroughly using high-speed mechanical blending. While processing the samples, care was taken to reduce the light exposure as much as possible. The homogenized samples were then split into two parts: the fecal phase and the freshwater microcosms.

Temperature and Matrix Variation

For the fecal phase, about 150g of homogenized manure sample was split into three portions for the temperature variation. Each portion was stored in a container fitted with a lid to minimize loss of moisture due to evaporation. Additionally, the containers used for storing fecal samples were covered with aluminum foil to minimize exposure to light. For each homogenized manure sample, 50g of fecal sample was weighed using a calibrated digital balance (Scout Pro Model; Ohaus Corp., Pine Brook, NJ) and stored in three containers that were incubated at 4^{o}C, 22^{o}C, and 35^{o}C respectively.

For the water phase, three foil-covered containers, fitted with lids, were used to setup microcosms. The water for the microcosm was taken from Sinking Creek (Site 1). Located in Johnson City, Sinking Creek is listed as an impaired surface water on the 303d list due to pathogen contamination. The collected water sample was then triple-filtered through 47mm

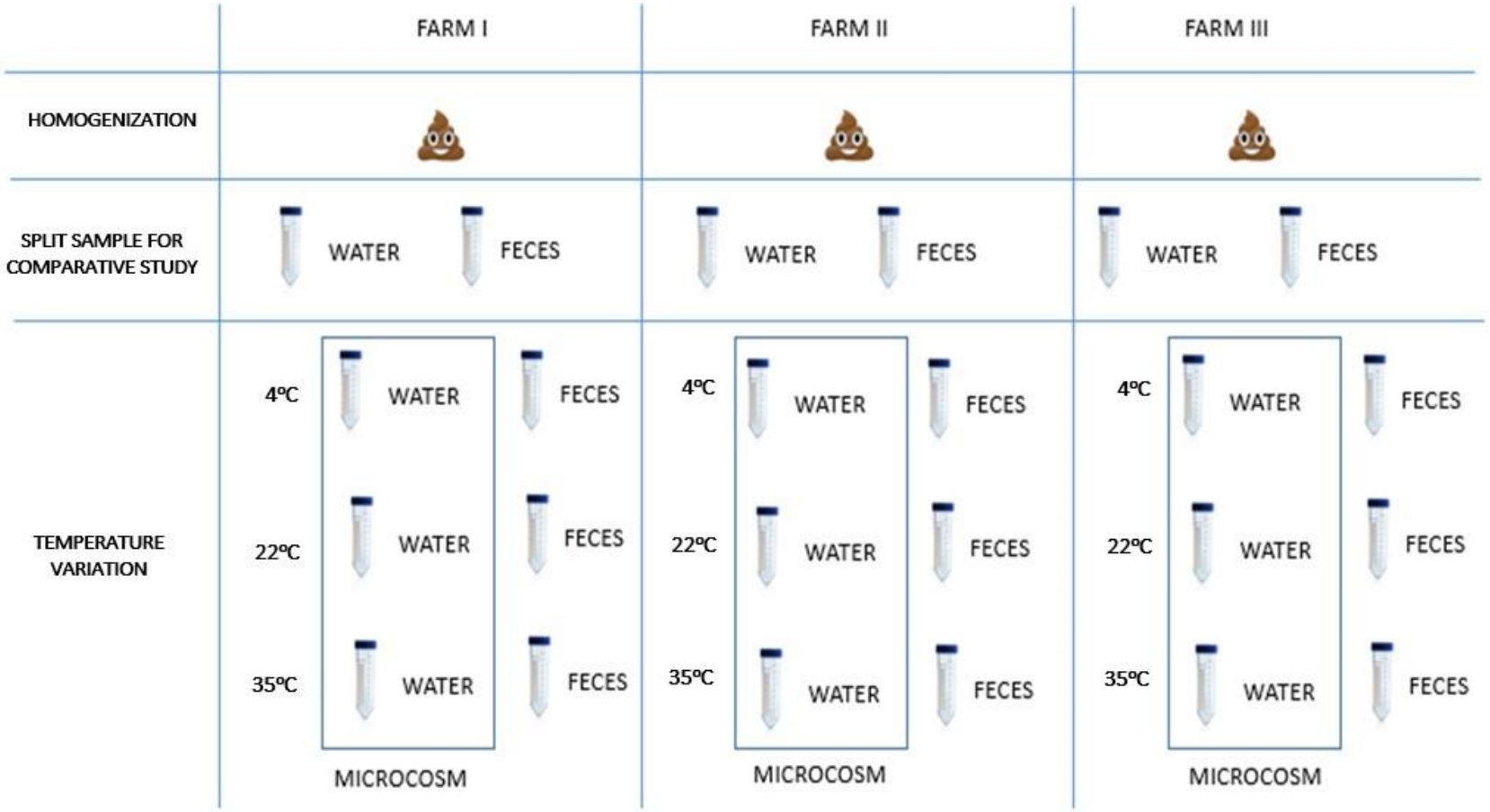

Figure 4. Experimental setup of manure collection and processing

0.2µm hydrophilic polypropylene membrane filters (Gelman Sciences, Ann Arbor, MI) using a filtering apparatus. This was done to filter out the autochthonous microorganisms that might be present in the water while maintaining its physicochemical nature. The microorganisms are removed from the filtrate to avoid competition for resources within the microcosm. The filtrate was then used to setup 100ml microcosms that were inoculated with 1g aliquot of homogenized fecal material. Each container was thoroughly mixed for 60s to release the bacteria from the fecal material. The three microcosms were stored in incubators set at 4ºC, 22ºC, and 35ºC respectively. Figure 4 illustrates the preparation and sample variation used in the experiments.

Sampling and Enumeration of Bacteria for Survival Analysis

The samples from each matrix and temperature variant were collected on the following days: 0, 2, 4, 6, 8, 10, 14, and 18. Day 0 is the day on which the manure samples were collected. For all fecal phase samples, 1g aliquot of fecal material was collected from each temperature variant using sterile spatulas and suspended in a sterilized reagent grade water in a 1:10 ratio. To aid in releasing the bacteria from the fecal material, the solution was thoroughly vortexed for 90s (Vortex-Genie; Scientific Industries, New York, NY). Dilution series of 10-fold (10^{-1} – 10^{-6}) for each suspension were prepared for drop plating.

For the freshwater microcosms, each container was swirled for 30s before sampling. A 1ml aliquot of water sample was pipetted from each temperature variant into 9ml of reagent-grade water. The solution was thoroughly vortexed for 30s. Serial dilutions of 10-fold (10^{-1} – 10^{-6}) for each suspension were prepared for drop plating. Fecal coliforms, *E. coli*, enterococci and total bacteria were enumerated using drop-plate technique as described by Alam et al. [89]. Figure 5 shows a schematic representation of the drop-plate setup.

1) Total bacteria – 10 µl of each suspended sample and its dilutions were plated onto tryptic soy agar. After plating each sample, the plates were left undisturbed for 30 min to allow for the plated samples to be absorbed into the media. Once the samples were absorbed, the plates were inverted and incubated at 35^{o}C for 24 hrs. The white colonies were counted using a dissecting microscope.

2) Fecal coliforms – 10 µl of each suspended sample and its dilutions were plated onto m-fecal coliform (FC) agar. After plating each sample, the plates were left undisturbed for 30 min to allow for the plated samples to be absorbed into the media. Once the samples were absorbed, the plates were inverted and incubated at 44^{o}C for 24 hrs. The blue colonies were counted using a dissecting microscope.

3) *E. coli* – 10 µl of each suspended sample and its dilutions were plated onto eosin methylene blue (EMB) agar. After plating each sample, the plates were left undisturbed for 30 min to allow for the plated samples to be absorbed into the media. Once the samples were absorbed, the plates were inverted and incubated at 35^{o}C for 24 hrs. The colonies with dark centers and metallic green sheen were counted using a dissecting microscope.

4) Enterococci – 10 µl of each suspended sample and its dilutions were plated onto m-enterococcusal agar. After plating each sample, the plates were left undisturbed for 30 min to allow for the plated samples to be absorbed into the media. Once the samples were

absorbed, the plates were inverted and incubated at 44°C for 24 hrs. The dark pink colonies were counted using a dissecting microscope.

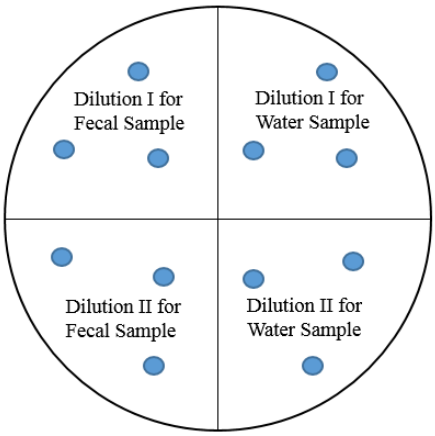

Figure 5. Drop-plate setup. Two dilutions from each matrix type of a temperature-variant were plated as illustrated.

Quality Assurance and Quality Control

Field procedures. The information regarding the samples collected were recorded at the time of collection. Information that was recorded included the date and time of sampling, sample type, number of cattle from which the fecal samples were collected, farming practices from which the sample was collected, and sample storage conditions. Also, equipment blanks were used to ensure that samples were not contaminated via sampling.

Laboratory procedures. For each of the five media used, a blank sterile sample (sterile milli-Q water) was used as the negative control. Additionally, positive controls of *Escherichia coli* (ATCC#25922) was used on EMB agar and m-FC agar. Similarly, a positive control of *Enterococcus faecium* (ATCC#667) was used on m-enterococcusal agar. The results were accepted only if the negative controls indicated no colonies. The positive controls were expected

to show the presence of organisms that were used in the inoculation. The samples were rejected if the positive controls were found to be negative.

Analytical procedures. A qualitative assessment of the data was performed initially to observe the trends of decay and persistence of the bacteria. Data resulting from samples not meeting the data quality objectives was excluded in the analysis. A confirmation of consistency was determined based on the observable patterns displayed by each manure sample. The confirmation was based on statistical analysis that are further expounded on in the next section.

Analysis of Data

The number of colony forming units (CFUs) were determined *per gram* of wet weight of manure for each matrix and temperature variant using Microsoft Excel 2013. The number of colony forming units were calculated using the following formula –

$$\text{CFU g } ww^{-1} = \frac{Colony\ Counts}{Dilution\ Plated} * Dilution\ Factor \quad \text{.. (3)}$$

The colony counts were then $\log_{10}$ transformed to meet the normality assumption. Geometric means were calculated for each treatment condition. The survival data for each bacterial group based on temperature and matrix variants were plotted against time using SigmaPlot Version 13 (Systat Software Inc., San Jose, CA). Furthermore, regression analyses were performed to determine the inactivation rates for each treatment condition. The time taken for 90% reduction in bacterial concentrations (T_{90}) were estimated by taking the reciprocal of the decay rate. All regression analyses were performed on SigmaPlot. The effects of temperature, matrix type and time were assessed using a three-way analysis of variance (ANOVA), followed by Bonferroni *post hoc* tests. A Bonferroni *post hoc* test were preferred over other post hoc tests such as Tukey,

Scheffe, Newman-Keuls etc. because it preserves the alpha while performing both simple and complex contrasts. The statistical analyses were performed on SAS 9.4 (SAS Institute Inc., Cary, NC). Statistical significance was set at $\alpha = 0.05$.

CHAPTER 4

RESULTS

Survival was evaluated for total bacteria, fecal coliforms, *E. coli* and enterococci in fecal and water phases incubated at 4^{o}C, 22^{o}C, and 35^{o}C respectively. Figure 7 depicts the *E. coli*, enterococci, fecal coliform and total bacterial colonies on EMB, m-enterococcusal, m-FC agar and TSA respectively. The survival was measured on days 0, 2, 4, 6, 8, 10, 14, and 18. The first factor (matrix type) has 2 levels; hence a = 2. The second factor is the temperature used, which has 3 levels; hence b = 3. The third factor is the number of days on which the survival was measured, which is eight; hence, c = 8. So, there are abc = 48 possible treatment combinations. Each bacterial group had 1296 observations based on 9 samples and three replicate data points. The survival plots for each bacterial indicator group are illustrated in the following section, in addition to decay rates and results from the ANOVAs.

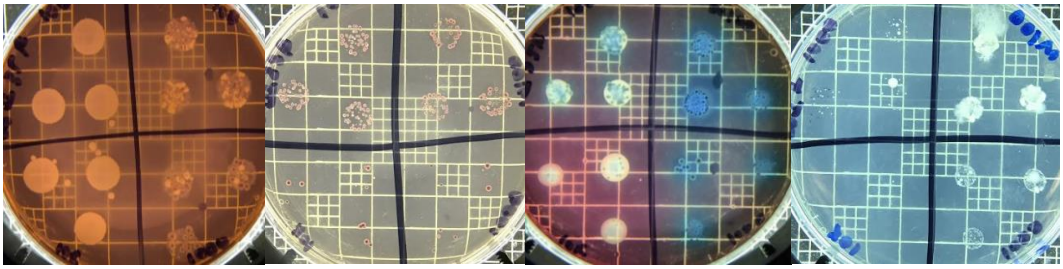

Figure 6. *E. coli*, enterococci, fecal coliform and total bacterial colonies on Eosin Methylene Blue (EMB), m-enterococcusal, m-Fecal Coliform agar and Tryptic Soy Agar (TSA) respectively.

Total Bacteria

The rate of decline of total bacteria was determined in fecal and water matrices at varying temperatures of 4ºC, 22ºC, and 35ºC. The decay rate was measured in terms of a $\log_{10}$ reduction over an eighteen-day period. The survival of total bacteria in fecal and water matrices at varying temperatures are illustrated in Figure 7 and Figure 8 respectively. As illustrated by the survival boxplots, the bacteria underwent an initial growth phase, which was followed by a long die-off phase. For the fecal matrix, the growth phase seemed to have lasted from the day that the sample was collected up to day 4 – 6. The total bacterial growth for the water matrix apparently peaked at day 4. Overall, the concentrations of total bacteria were found to be higher in the water phase, compared to the fecal phase. Furthermore, the concentrations of total bacteria were found to be higher in samples that were maintained at 22ºC, compared to samples maintained at 4ºC and 35ºC.

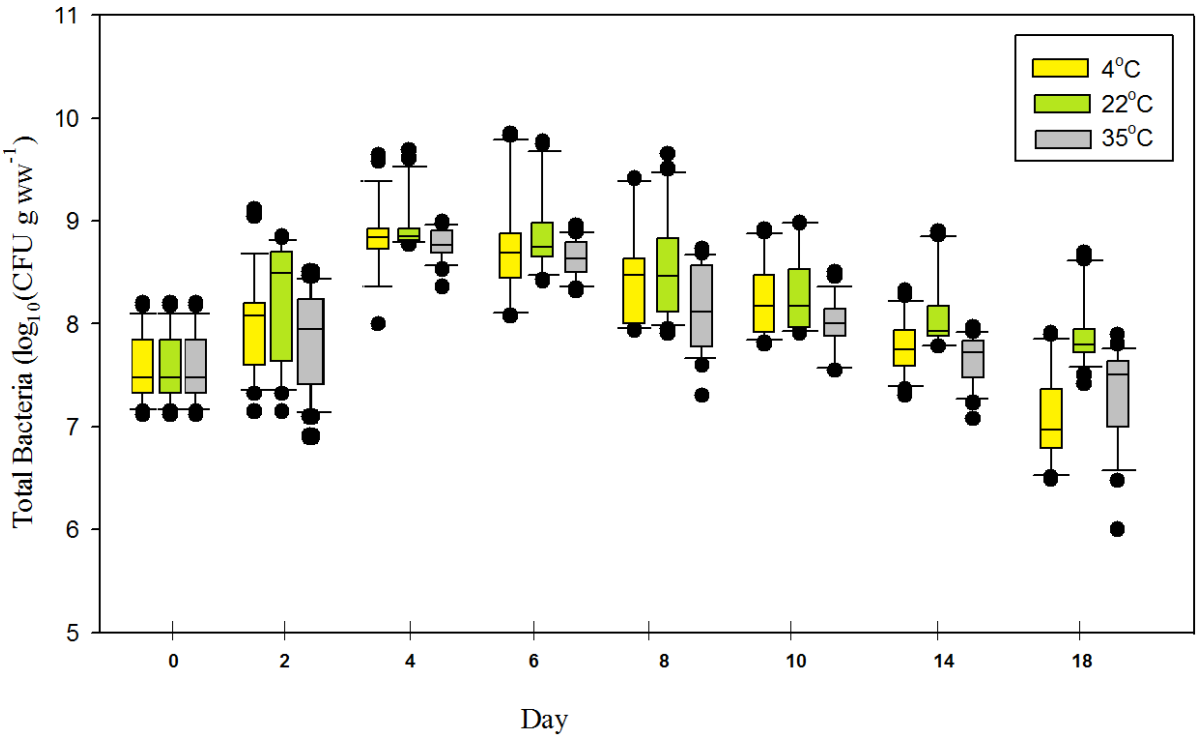

Figure 7. Survival of total bacteria in the fecal phase at 4ºC, 22ºC, and 35ºC.

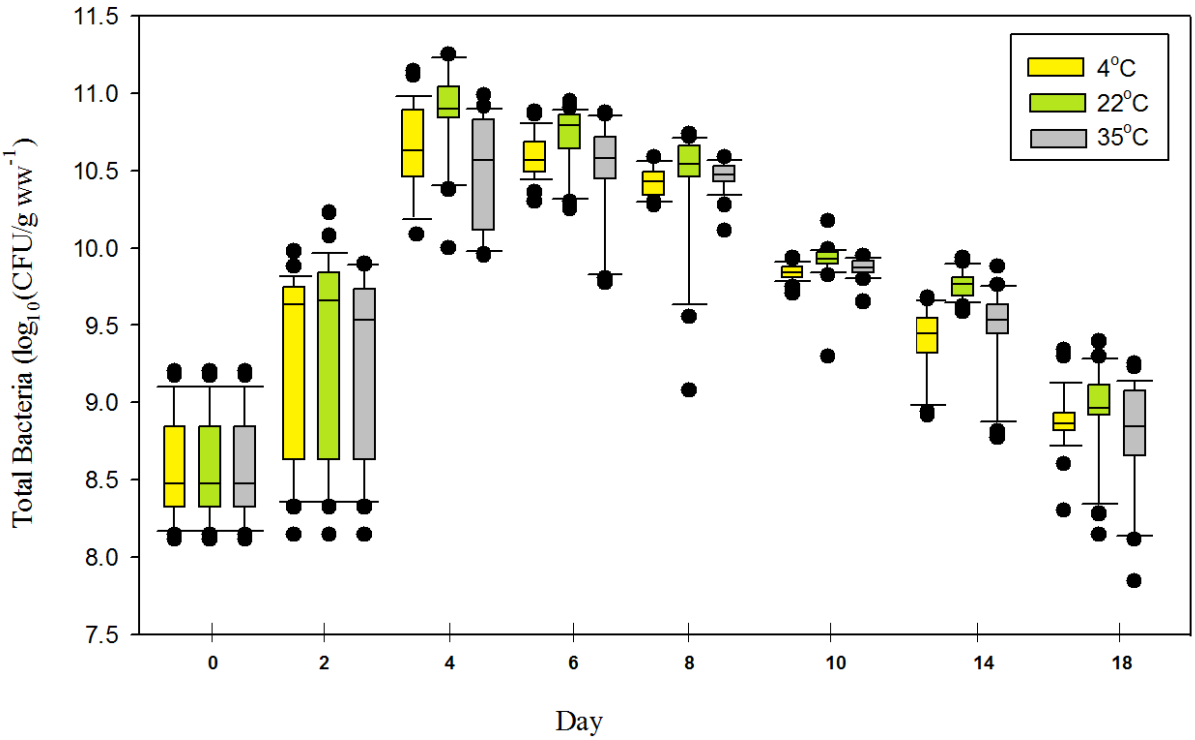

Figure 8. Survival of total bacteria in the water phase at 4ºC, 22ºC, and 35ºC.

Regression equations were determined using the geometric means of the $\log_{10}$ (CFU/ g wet weight) of total bacteria observed on days 0, 2, 4, 6, 8, 10, 14, and 18. Table 1 lists the regression equations, decay rates and the T_{90} values for each matrix and temperature combination. There are two regression equations listed for each treatment type – overall and post-growth. Overall decay signifies the decay observed from day 0 to day 18, whereas post-growth decay only accounts for the observed die-off that takes place after the initial growth. The coefficient of determination was found to be higher for the post-growth regression equations, making for a better fit compared to the overall model. Figure 9 and 10 display the post-growth decay rates and T_{90} values for each matrix and temperature combination. The decay rates were higher for the samples maintained in the water phase compared to the fecal phase. Conversely, the T_{90} values were found to be higher for samples maintained in the fecal phase. The highest T_{90} value of 12.76 days was observed for fecal samples incubated at 22 ºC.

Table 1. Regression equations, coefficient of determinations, decay rates and T_{90} values for total bacteria at varying treatment conditions based on temperature and matrix for overall and post-growth phases. The decay rates and the T_{90} values are denoted with an identifying letter to demarcate significance patterns on the following post-growth die-off rates and T_{90} values for the bar graph

Treatment/Condition	Regression Equation	R^2 Value	Decay Rate	T_{90} (Days)
Matrix – Fecal Temperature - 4	Overall - y = -0.0547x + 8.5564	0.3536	-0.0547	18.2815
	Post-growth – y = -0.1243x + 9.4271	0.9866	-0.1243 a	8.04505 a
Matrix – Water Temperature - 4	Overall - y = -0.0281x + 8.9777	0.0566	-0.0281	35.5872
	Post-growth – y = -0.1361x + 10.323	0.9702	-0.1361 d	7.34754 d
Matrix – Fecal Temperature - 22	Overall – y = -0.024x + 8.537	0.3536	-0.024	41.6667
	Post-growth – y = -0.0784x + 9.2128	0.9485	-0.0784 b	12.7551 b
Matrix – Water Temperature - 22	Overall – y = -0.0203x + 9.0304	0.0274	-0.0203	49.2611
	Post-growth – y = -0.1338x + 10.445	0.9644	-0.1338 e	7.47384 e
Matrix – Fecal Temperature - 35	Overall – y = -0.0501x + 8.4544	0.4143	-0.0501	19.9601
	Post-growth – y = -0.1052x + 9.1424	0.9655	-0.1052 c	9.5057 c
Matrix – Water Temperature - 35	Overall - y = -0.0258x + 8.9346	0.0496	-0.0258	38.7597
	Post-growth – y = -0.1309x + 10.245	0.9419	-0.1309 f	7.63942 f

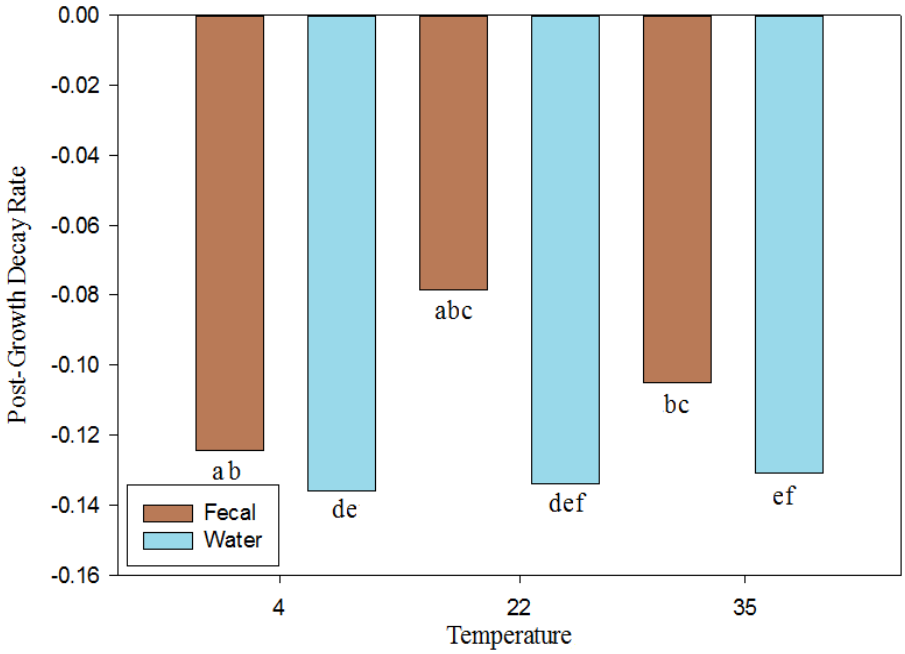

Figure 9. Post-growth decay rates for total bacteria in treatments based on matrix and temperature. The combination of letters indicate significance at $\alpha = 0.05$, relative to other temperature groups within each matrix.

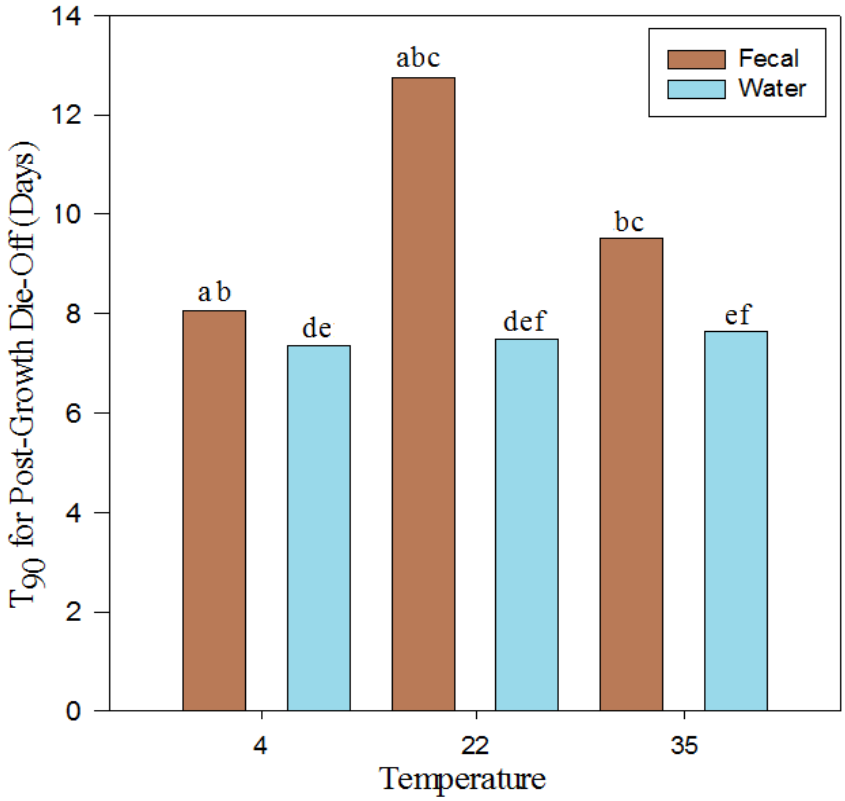

Figure 10. T_{90} for total bacteria in different treatment based on matrix and temperature. T_{90} was calculated based on post-growth die-off rates. The combination of letters indicate significance at $\alpha = 0.05$, relative to other temperature groups within each matrix.

In order to compare the different treatments of temperature and matrix over time, a three-way ANOVA was performed (Appendix C). The data was $\log_{10}$ transformed to meet the parametric assumption of normality and variance homogeneity. The results of the ANOVA showed that the model was significant ($F = 242.99$; $P < 0.0001$) as shown in Table 2. The interaction effects were found to be significant for each factor, temperature, day and matrix, in addition to the following combinations - temperature*matrix, day*matrix, temperature*day and temperature*matrix*day (Table 3).

Table 2. Three-way ANOVA analysis for survival of total bacteria in fecal and water matrices at three temperatures of 4°C, 22°C, and 35°C over an 18-day period. The significance level was set to 0.05.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	47	1363.787829	29.016762	242.99	<.0001
Error	1248	149.030219	0.119415		
Corrected Total	1295	1512.818048			

Table 3. Three-way ANOVA showing the interactions between temperature, day and matrix for the survival of total bacteria in fecal and water matrices at three temperatures of 4°C, 22°C, and 35°C over an 18-day period. The significance level was set to 0.05. * indicates significance.

Variable	DF	SS	Mean Square	F Value	Pr > F
Temperature	2	10.7478745	5.3739372	45.00	<.0001*
Day	7	474.7112786	67.8158969	567.90	<.0001*
Temperature*Day	14	5.3144285	0.3796020	3.18	<.0001*
Matrix	1	831.7769436	831.7769436	6965.42	<.0001*
Temperature*Matrix	2	1.2194795	0.6097398	5.11	0.0062*
Day*Matrix	7	35.1235347	5.0176478	42.02	<.0001*
Temperature*Day*Matrix	14	4.8942896	0.3495921	2.93	0.0002*

To understand the differences among the various treatment conditions, Bonferroni *post hoc* tests were performed. Tables 4 and 5 indicate which temperature comparisons and matrix comparisons were found to be significant. For the total bacteria, all temperature comparisons were deemed significant with the exception of comparisons between 4°C and 35°C. Likewise, the

differences between the fecal and water matrices were also found to be significant. Results indicating differences between days are included in the Appendix C.

Table 4. Bonferroni test indicating which temperature comparisons are significant. Comparisons significant at the 0.05 level are included.

Temperature Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		Significance
22 - 4	0.16415	0.10897	0.21932	Yes
22 - 35	0.21288	0.15771	0.26806	Yes
4 - 35	0.04874	-0.00644	0.10391	No

Table 5. Bonferroni test indicating which temperature comparisons are significant. Comparisons significant at the 0.05 level are included.

Matrix Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		Significance
2 - 1	1.60225	1.56459	1.63992	Yes

To test if there were any differences among the results based on the farms from which the samples were collected, an additional three-way ANOVA was performed within each matrix type with temperature, farm and day as three independent variables. For the survival of total bacteria in the fecal phase, the differences between samples collected from the three farms were found to be significant (P = 0.0036). Upon performing the Bonferroni test, it was found that the besides the differences between Farm 1 and 3, all other differences are signifinant (Table 6) .

Table 6. Bonferroni test for the survival of total bacteria in fecal matrix indicating which farm comparisons are significant. Comparisons significant at the 0.05 level are included.

Farm Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		Significance
2 - 3	0.08655	0.00186	0.17125	Yes
2 - 1	0.11390	0.02920	0.19859	Yes
3 - 1	0.02734	-0.05735	0.11204	No

Conversly, for the water matrix, the differences between samples collected from the three farms were found to be insignificant (P = 0.1163). As expected, the Bonferroni test showed no significant differences in any of the pairwise comparisons (Table 7).

Table 7. Bonferroni test for the survival of total bacteria in water matrix indicating which farm comparisons are significant. Comparisons significant at the 0.05 level are included.

Farm Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		Significance
3 - 2	0.00525	-0.06767	0.07816	No
3 - 1	0.05709	-0.01582	0.13001	No
2 - 1	0.05185	-0.02107	0.12476	No

Fecal coliforms

Using a similar comparison as used for total bacteria, the decay of fecal coliforms was determined in fecal and water matrices at varying temperatures of 4^{o}C, 22^{o}C, and 35^{o}C. The decay rate was measured in terms of a $\log_{10}$ reduction over an eighteen-day period. The survival of fecal coliforms in fecal and water matrices at varying temperatures are illustrated in Figure 11 and 12 respectively. According to the survival boxplots, the fecal coliforms underwent an initial growth phase, which was followed by a long die-off phase. Overall, the growth phase in water and fecal matrices seemed to have lasted from the day that the sample was collected up to day 4. Furthermore, the concentrations of fecal coliforms were found to be higher in the water phase, compared to the fecal phase. However, regarding the temperature variation, the concentrations of fecal coliforms were found to be higher in samples that were maintained at 22^{o}C, compared to samples maintained at 4^{o}C and 35^{o}C.

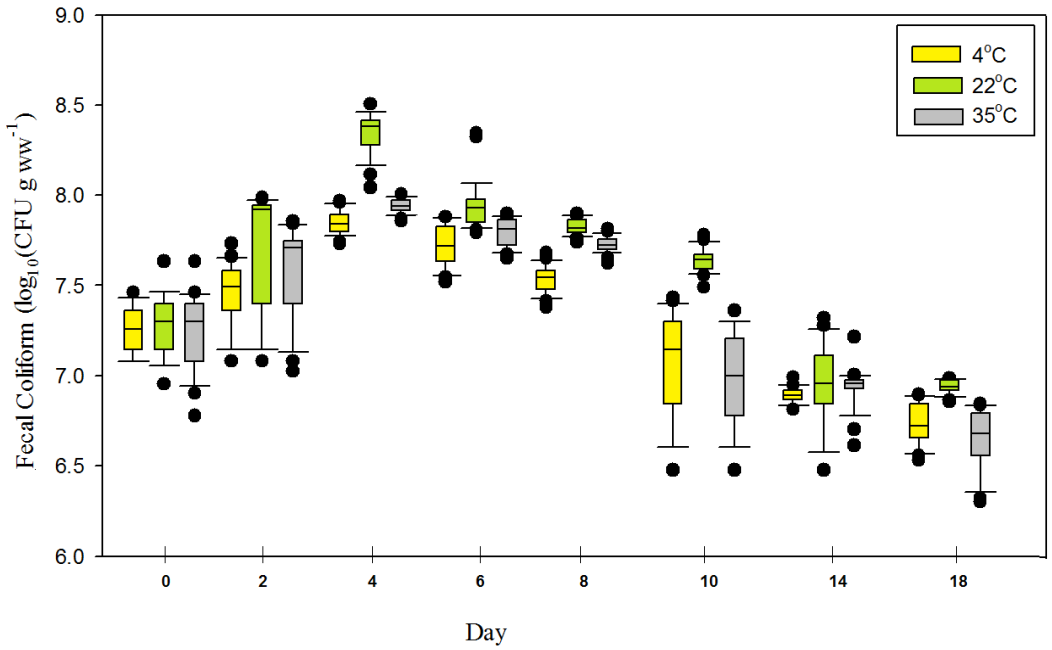

Figure 11. Survival of fecal coliforms in the fecal phase at 4ºC, 22ºC, and 35ºC.

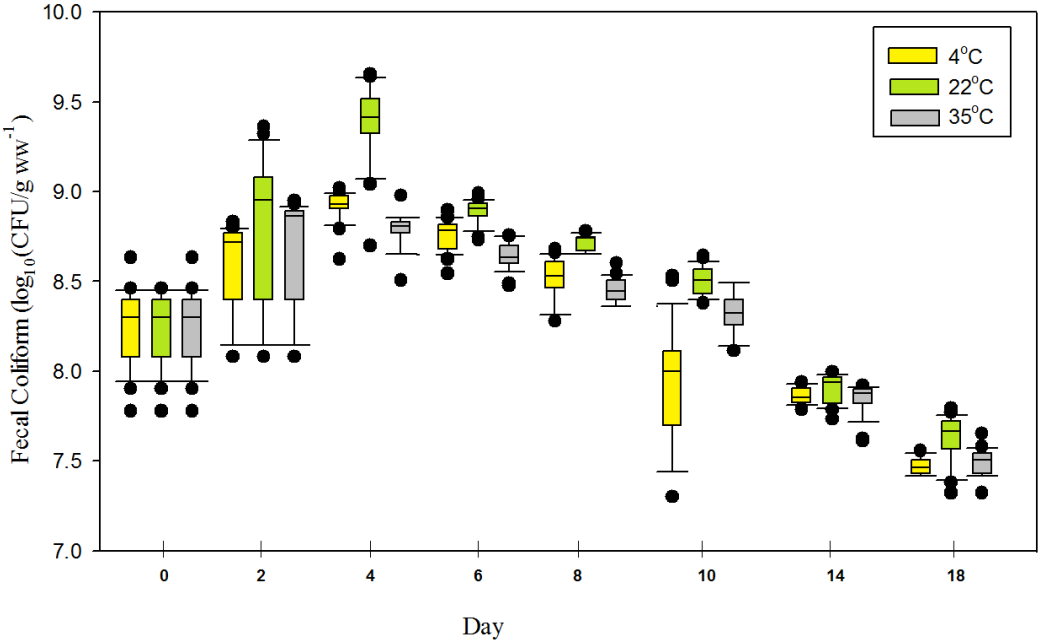

Figure 12. Survival of fecal coliforms in the water phase at 4ºC, 22ºC, and 35ºC.

Regression equations were determined based on the geometric means of the $\log_{10}$ (CFU/ g wet weight) of fecal coliforms observed on days 0, 2, 4, 6, 8, 10, 14, and 18. Table 8 lists the regression equations, decay rates and the T_{90} values for each matrix and temperature combination. There are two regression equations listed for each treatment type – overall and

post-growth. Overall decay signifies the decay observed from day 0 to day 18, whereas post-growth decay only accounts for the observed die-off that takes place after the initial growth. The coefficient of determination was found to be higher for the post-growth regression equations, making for a better fit compared to the overall model. Figure 13 and 14 display the post-growth decay rates and T_{90} values for each matrix and temperature combination. The decay rates were highest for the samples maintained at 22ºC, followed by the samples incubated at 35ºC and 4ºC respectively. The highest T_{90} value of 11.86 days was observed for water samples incubated at 4ºC.

Table 8. Decay rates for fecal coliforms at varying treatment conditions based on temperature and matrix. The decay rates and the T_{90} values are denoted with an identifying letter to demarcate significance patterns on the following post-growth die-off rates and T_{90} values for the bar graph.

Treatment/ Condition	Regression Equation	Coefficient of Determination	Decay Rate	T_{90} (Days)
Matrix – Fecal Temperature - 4	Overall - y = -0.0553x + 7.7849	0.685	-0.0553	18.083
	Post-growth – y = -0.0857x + 8.1658	0.911	-0.0857 a	11.669 a
Matrix – Water Temperature - 4	Overall - y = -0.0556x + 7.7796	0.7161	-0.0556	17.986
	Post-growth – y = -0.0843x + 8.1377	0.9208	-0.0843 d	11.862 d
Matrix – Fecal Temperature - 22	Overall - y = -0.0559x + 8.0475	0.5038	-0.0559	17.889
	Post-growth – y = -0.1047x + 8.654	0.9356	-0.1047 b	9.551 b
Matrix – Water Temperature - 22	Overall - y = -0.0556x + 8.9116	0.3272	-0.0556	17.985
	Post-growth – y = -0.1231x + 9.7394	0.9721	-0.1231 e	8.123 e
Matrix – Fecal Temperature - 35	Overall - y = -0.0544x + 7.7891	0.4806	-0.0544	18.382
	Post-growth – y = -0.0989x + 8.3384	0.8724	-0.0989 c	10.111 c
Matrix – Water Temperature - 35	Overall - y = -0.0587x + 8.7627	0.6456	-0.0587	17.036
	Post-growth – y = -0.0949x + 9.2062	0.9944	-0.0949 f	10.537 f

To compare the effect of temperature and matrix over time, a three-way ANOVA was performed (Appendix C). The data was $\log_{10}$ transformed to meet the parametric assumption of normality and variance homogeneity. The results of the ANOVA showed that the model was significant ($F = 500.63$; $P < 0.0001$) as shown in Table 9. The differences within each factor were found to be significant, in addition to the following combinations - temperature*matrix, day*matrix, temperature*day and temperature*matrix*day (Table 10). Bonferroni post hoc tests were performed to ascertain which pairwise comparisons were significantly different.

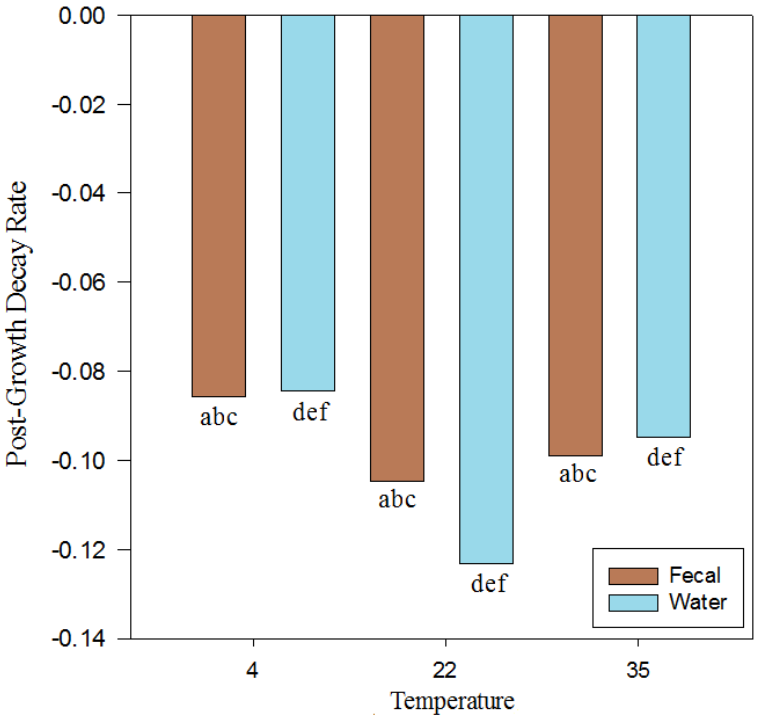

Figure 13. Post-growth die-off rates for fecal coliform in different treatments based on matrix and temperature. The combination of letters indicate significance at $\alpha = 0.05$, relative to other temperature groups within each matrix.

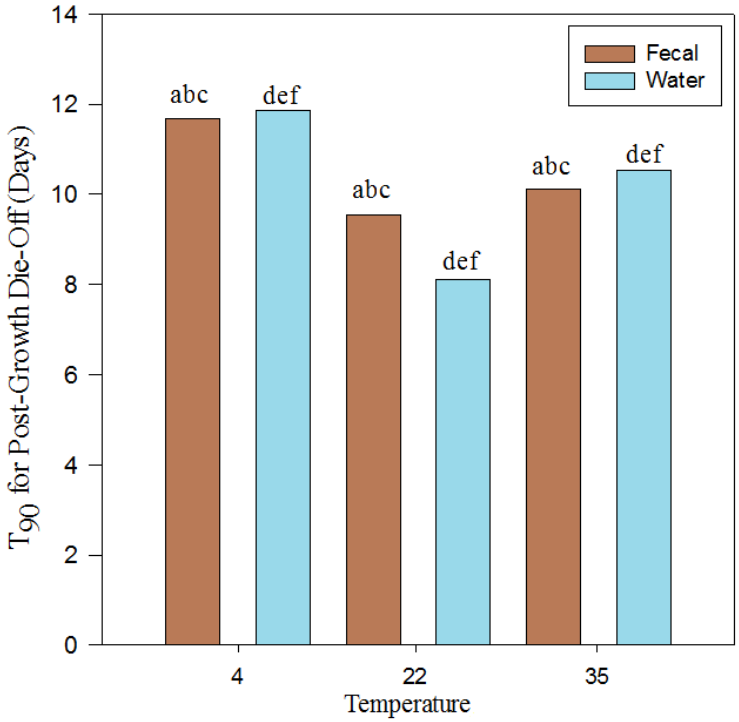

Figure 14. T90 for fecal coliforms in different treatment based on matrix and temperature. T90 was calculated based on post-growth die-off rates. The combination of letters indicate significance at $\alpha = 0.05$, relative to other temperature groups within each matrix.

Table 9. Three-way ANOVA analysis for survival of fecal coliforms in fecal and water matrices at three temperatures of 4ºC, 22ºC, and 35ºC over an 18-day period. The significance level was set to 0.05. * indicates significance.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	47	616.1548567	13.1096778	500.63	<.0001*
Error	1248	32.6806888	0.0261864		
Corrected Total	1295	648.8355455			

Table 10. Three-way ANOVA showing the interactions between temperature, day and matrix for the survival of fecal coliforms in fecal and water matrices at three temperatures of 4ºC, 22ºC, and 35ºC over an 18-day period. The significance level was set to 0.05. * indicates significance.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
temp	2	14.7033712	7.3516856	280.74	<.0001*
day	7	202.9582549	28.9940364	1107.22	<.0001*
temp*day	14	28.1933941	2.0138139	76.90	<.0001*
matrix	1	324.3713763	324.3713763	12387.0	<.0001*
temp*matrix	2	3.3326064	1.6663032	63.63	<.0001*
day*matrix	7	24.5679086	3.5097012	134.03	<.0001*
temp*day*matrix	14	18.0279451	1.2877104	49.17	<.0001*

The results for the Bonferroni multiple comparison tests are displayed in Tables 4 and 5, indicating which temperature comparisons and matrix comparisons were found to be significant. For the fecal coliforms, all temperature comparisons were significant. Likewise, the differences between the fecal and water matrices were also found to be significant. Results indicating differences between days are included in the Appendix C.

Table 11. Bonferroni test for the survival of fecal coliforms indicating which temperature comparisons are significant. Comparisons significant at the 0.05 level are included.

Temperature Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		Significance
22 - 35	0.20857	0.18273	0.23441	Yes
22 - 4	0.24003	0.21420	0.26587	Yes
35 - 4	0.03146	0.00563	0.05730	Yes

Table 12. Bonferroni test for the survival of fecal coliforms indicating which matrix comparisons are significant. Comparisons significant at the 0.05 level are included.

Matrix Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		Significance
1 - 2	1.000573	0.982936	1.018210	Yes

To test if there were any differences among the results based on the farms from which the samples were collected, an additional three-way ANOVA was performed within each matrix type with temperature, farm and day as three independent variables. For the survival of fecal coliforms in the fecal phase, the differences between samples collected from the three farms were found to be insignificant ($P = 0.3605$). Similarly, for the water matrix, the differences between samples collected from the three farms were also found to be insignificant ($P = 0.7375$). As expected, the Bonferroni tests showed no significant differences in any of the pairwise comparisons (Table 13 and 14).

Table 13. Bonferroni test for the survival of fecal coliforms in fecal matrix indicating which farm comparisons are significant. Comparisons significant at the 0.05 level are included.

Farm Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		Significance
1 - 3	0.01768	-0.01949	0.05485	No
1 - 2	0.02038	-0.01679	0.05754	No
3 - 2	0.00270	-0.03447	0.03986	No

Table 14. Bonferroni test for the survival of total bacteria in water matrix indicating which farm comparisons are significant. Comparisons significant at the 0.05 level are included.

Farm Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		Significance
3 - 2	0.00519	-0.03254	0.04293	No
3 - 1	0.01222	-0.02551	0.04996	No
2 - 1	0.00703	-0.03071	0.04477	No

Escherichia coli

E. coli is frequently used as a fecal indicator for pathogen contamination. Using a similar comparison as used for coliforms and total bacteria, the *E. coli* die-off rate was determined in fecal and water matrices at varying temperatures of 4^{o}C, 22^{o}C, and 35^{o}C. The decay rate was measured in terms of a $\log_{10}$ reduction over an eighteen-day period. The survival of *E. coli* in fecal and water matrices at varying temperatures are illustrated in Figure 15 and 16 respectively. As shown in Figures 15 and 16, the *E. coli* underwent an initial growth phase, which was followed by a long die-off phase. Overall, the growth phase in water and fecal matrices seemed to have lasted from the day that the sample was collected up to day 4. Furthermore, the concentrations of *E. coli* were found to be higher in the water phase, compared to the fecal phase. However, regarding the temperature variation, the concentrations of *E. coli* for the fecal phase were found to be higher in samples that were maintained at 22^{o}C, compared to samples maintained at 4^{o}C and 35^{o}C. For the water phase, some fluctuations were observed.

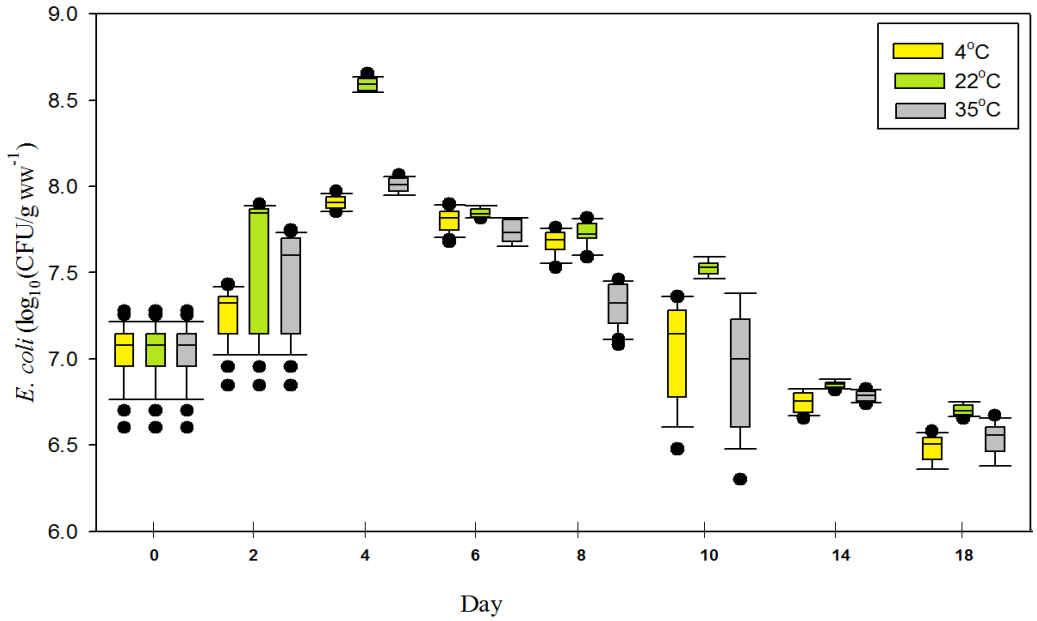

Figure 15. Survival of *E. coli* in the fecal phase at 4ºC, 22ºC, and 35ºC.

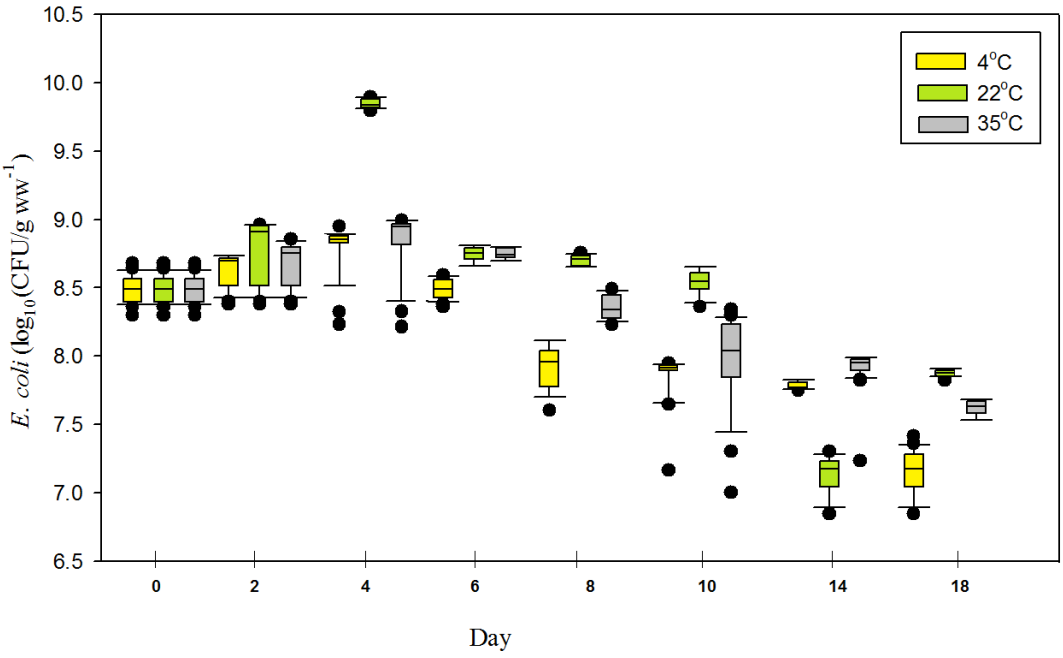

Figure 16. Survival of *E. coli* in the water phase at 4ºC, 22ºC, and 35ºC.

Regression equations were determined using the geometric means of the $\log_{10}$ (CFU/ g wet weight) of fecal coliforms observed on days 0, 2, 4, 6, 8, 10, 14, and 18. Table 8 lists the regression equations, decay rates and the T_{90} values for each matrix and temperature combination. There are two regression equations listed for each treatment type – overall and

post-growth. Overall decay signifies the decay observed from day 0 to day 18, whereas post-growth decay only accounts for the observed die-off that takes place after the initial growth. The coefficient of determination was found to be higher for the post-growth regression equations, making for a better fit compared to the overall model. Figure 13 and 14 display the post-growth decay rates and T_{90} values for each matrix and temperature combination. The decay rates were highest for the samples maintained at 22ºC, whereas the decay rates for samples incubated at 35ºC and 4ºC were quite similar. The highest T_{90} value of 10.78 days was observed for water samples incubated at 35ºC.

Table 15. Decay rates for *E. coli* at varying treatment conditions based on temperature and matrix. The decay rates and the T_{90} values are denoted with an identifying letter to demarcate significance patterns on the following post-growth die-off rates and T_{90} values for the bar graph

Treatment/Condition	Regression equation	R^2 value	Decay rate	T_{90} (days)
Matrix – Fecal Temperature - 4	Overall - y = -0.067x + 7.8253	0.6134	-0.067	14.93
	Post-growth – y = -0.1119x + 8.3909	0.9334	-0.1119 a	8.94 a
Matrix – Water Temperature - 4	Overall - y = -0.0842x + 8.8061	0.8264	-0.0842	11.88
	Post-growth – y = -0.1076x + 9.093	0.8904	-0.1076 d	9.29 d
Matrix – Fecal Temperature - 22	Overall - y = -0.0687x + 8.0817	0.5066	-0.0687	14.56
	Post-growth – y = -0.1277x + 8.8179	0.9198	-0.1277 b	7.83 b
Matrix – Water Temperature - 22	Overall - y = -0.0841x + 9.1662	0.4318	-0.0841	11.89
	Post-growth – y = -0.1466x + 9.94	0.7015	-0.1466 e	6.82 e
Matrix – Fecal Temperature - 35	Overall – y = -0.0546x + 7.6475	0.444	-0.0546	18.32
	Post-growth – y = -0.1049x + 8.267	0.9088	-0.1049 c	9.53 c
Matrix – Water Temperature - 35	Overall - y = -0.0648x + 8.848	0.7205	-0.0648	15.43
	Post-growth – y = -0.0928x + 9.1926	0.8792	-0.0928 f	10.78 f

To compare the effect of temperature and matrix over time, a three-way ANOVA was performed (Appendix C). The data was $\log_{10}$ transformed to meet the parametric assumption of normality and variance homogeneity. The results of the ANOVA showed that the model was significant ($F = 725.83$; $P < 0.0001$) as shown in Table 16. The differences within each factor were found to be significant, in addition to the following combinations - temperature*matrix, day*matrix, temperature*day and temperature*matrix*day (Table 17). Bonferroni post hoc tests were performed to ascertain which pairwise comparisons were significantly different.

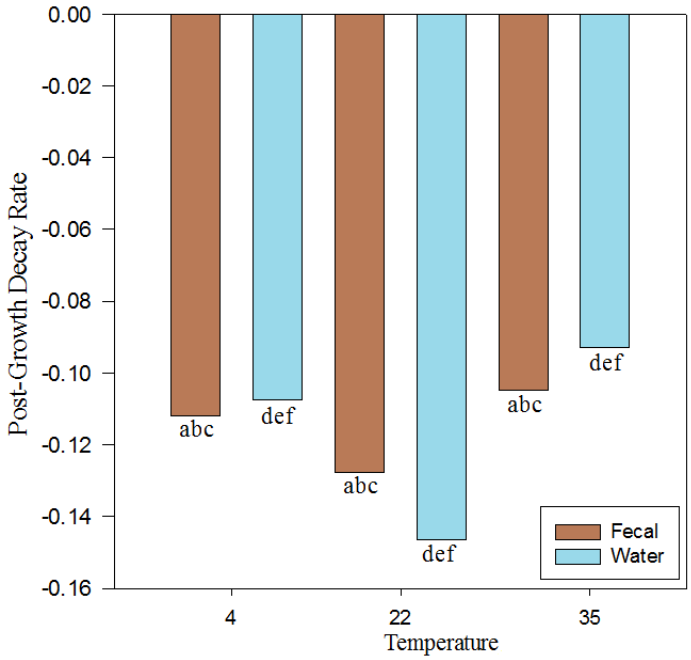

Figure 17. Post-growth die-off rates for *E. coli* in different treatments based on matrix and temperature. The combination of letters indicate significance at $\alpha = 0.05$, relative to other temperature groups within each matrix.

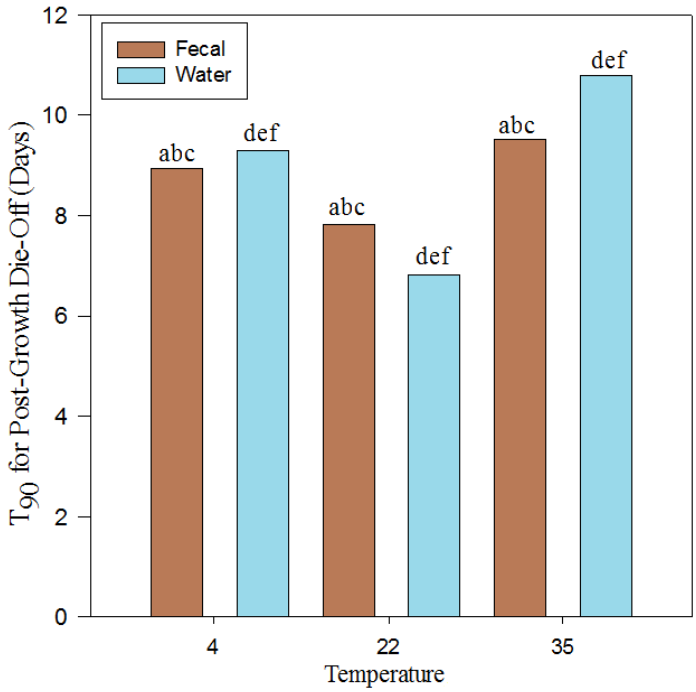

Figure 18. T90 for *E. coli* in different treatment based on matrix and temperature. T90 was calculated based on post-growth die-off rates. The combination of letters indicate significance at $\alpha = 0.05$, relative to other temperature groups within each matrix.

Table 16. Three-way ANOVA analysis for survival of *E. coli* in fecal and water matrices at three temperatures of 4ºC, 22ºC, and 35ºC over an 18-day period. The significance level was set to 0.05. * indicates significance.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	47	735.2120750	15.6428101	725.83	<.0001
Error	1248	26.8966026	0.0215518		
Corrected Total	1295	762.1086775			

Table 17. Three-way ANOVA showing the interactions between temperature, day and matrix for the survival of *E. coli* in fecal and water matrices at three temperatures of 4ºC, 22ºC, and 35ºC over an 18-day period. The significance level was set to 0.05. * indicates significance.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Temperature	2	22.1789312	11.0894656	514.55	<.0001*
Day	7	318.0227172	45.4318167	2108.03	<.0001*
Temperature*Day	14	33.1878412	2.3705601	109.99	<.0001*
Matrix	1	329.8102331	329.8102331	15303.2	<.0001*
Temperature*Matrix	2	2.3068450	1.1534225	53.52	<.0001*
Day*Matrix	7	16.1075241	2.3010749	106.77	<.0001*
Temperature*Day*Matrix	14	13.5979832	0.9712845	45.07	<.0001*

The results for the Bonferroni multiple comparison tests are displayed in Tables 18 and 19, indicating which temperature comparisons and matrix comparisons were found to be significant. For the *E. coli*, all temperature comparisons were significant. Likewise, the differences between the fecal and water matrices were also found to be significant. Results indicating differences between days are included in the Appendix C.

Table 18. Bonferroni test for the survival of *E. coli* indicating which temperature comparisons are significant. Comparisons significant at the 0.05 level are indicated.

Temperature Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		Significance
22 - 35	0.223555	0.199609	0.247500	Yes
22 - 4	0.310593	0.286648	0.334538	Yes
35 - 4	0.087038	0.063093	0.110984	Yes

Table 19. Bonferroni test for the survival of *E. coli* indicating which temperature comparisons are significant. Comparisons significant at the 0.05 level are indicated.

Matrix Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		Significance
2 - 1	1.008927	0.992926	1.024927	Yes

To test if there were any differences among the results based on the farms from which the samples were collected, an additional three-way ANOVA was performed within each matrix type with temperature, farm and day as three independent variables. For the survival of *E. coli* in the fecal phase, the differences between samples collected from the three farms were found to be insignificant (P = 0.0776). Similarly, for the water matrix, the differences between samples collected from the three farms were also found to be insignificant (P = 0.7844). As expected, the Bonferroni tests showed no significant differences in any of the pairwise comparisons (Table 20 and 21).

Table 20. Bonferroni test for the survival of *E. coli* in fecal matrix indicating which farm comparisons are significant. Comparisons significant at the 0.05 level are indicated.

Farm Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		Significance
2 - 3	0.02296	-0.01302	0.05894	No
2 - 1	0.03315	-0.00283	0.06912	No
3 - 1	0.01018	-0.02579	0.04616	No

Table 21. Bonferroni test for the survival of *E. coli* in water matrix indicating which farm comparisons are significant. Comparisons significant at the 0.05 level are indicated.

Farm Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		Significance
3 - 1	0.006337	-0.025408	0.038082	No
3 - 2	0.008965	-0.022780	0.040709	No
1 - 2	0.002627	-0.029118	0.034372	No

Enterococci

The decay of enterococci was determined in fecal and water matrices at varying temperatures of 4^{o}C, 22^{o}C, and 35^{o}C. The decay rate was measured in terms of a $\log_{10}$ reduction over an eighteen-day period. The survival of enterococci in fecal and water matrices at varying temperatures are illustrated in Figure 19 and 20 respectively. Similar to bacterial subgroups, the enterococci also underwent an initial growth phase, which was followed by a long die-off phase. Overall, the growth phase in water and fecal matrices seemed to have lasted from the day that the sample was collected up to day 6-8. Furthermore, the concentrations of enterococci were found to be higher in the water phase, compared to the fecal phase. However, regarding the temperature variation, the concentrations of enterococci were found to be slightly higher in samples that were maintained at 22^{o}C, compared to samples maintained at 4^{o}C and 35^{o}C.

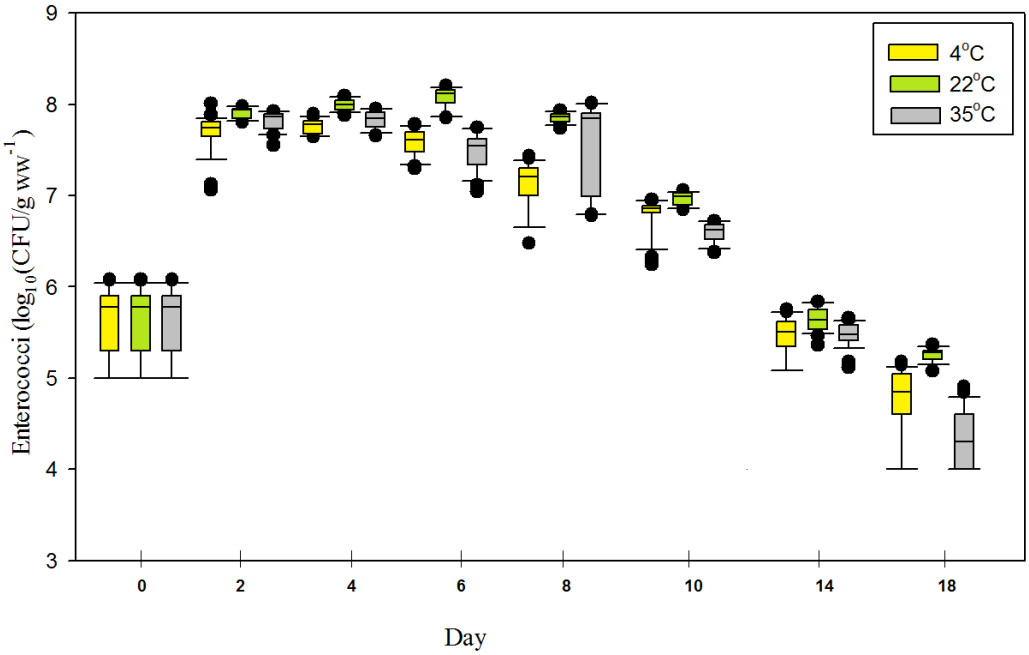

Figure 19. Survival of enterococci in the fecal phase at 4°C, 22°C, and 35°C.

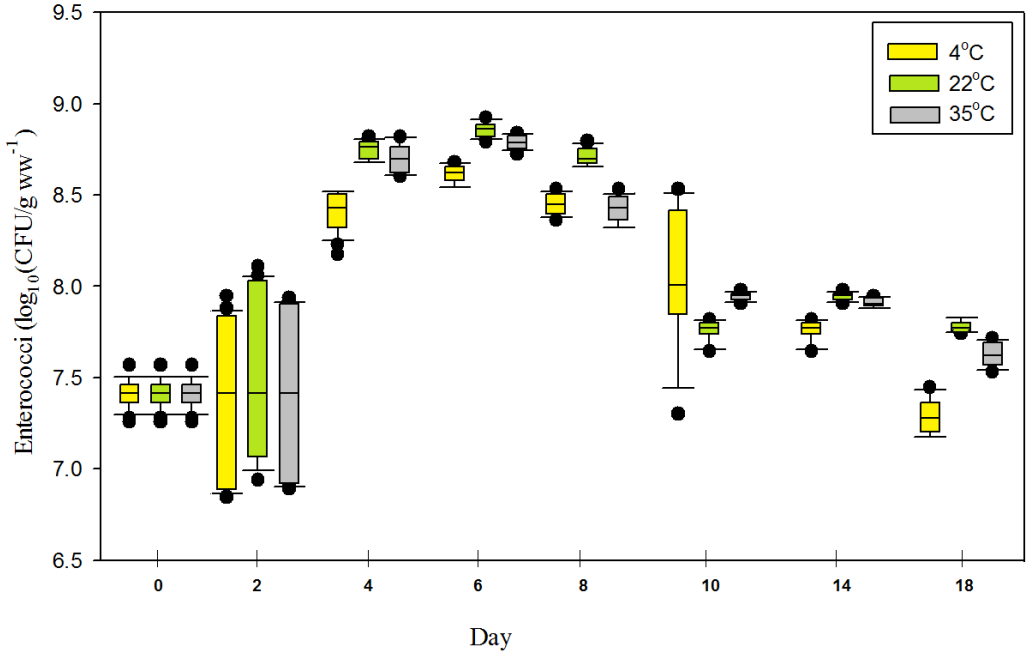

Figure 20. Survival of enterococci in the water phase at 4°C, 22°C, and 35°C.

Regression equations were determined using the geometric means of the $\log_{10}$ (CFU/ g wet weight) of enterococci observed on days 0, 2, 4, 6, 8, 10, 14, and 18. Table 8 lists the regression equations, decay rates and the T_{90} values for each matrix and temperature combination. There are two regression equations listed for each treatment type – overall and

post-growth. Overall decay signifies the decay observed from day 0 to day 18, whereas post-growth decay only accounts for the observed die-off that takes place after the initial growth. The coefficient of determination was found to be higher for the post-growth regression equations, making for a better fit compared to the overall model. Figure 21 and 22 display the post-growth decay rates and T_{90} values for each matrix and temperature combination. The decay rates were highest for the samples maintained in the fecal phase compared to samples maintained in the water phase. For the fecal phase, the decay rate was highest at 35°C (T_{90} = 11.68), followed by 22°C and 4°C respectively. Conversely, the highest decay rates were observed in samples maintained at 4°C, followed by 22°C and 35°C respectively.

Table 22. Decay rates for enterococci at varying treatment conditions based on temperature and matrix. The decay rates and the T_{90} values are denoted with an identifying letter to demarcate significance patterns on the following post-growth die-off rates and T_{90} values for the bar graph.

Treatment/Condition	Regression equation	R^2 value	Decay rate	T_{90} (days)
Matrix – Fecal Temperature - 4	Overall – y = -0.1595x + 7.9859	0.7846	-0.1595	6.27
	Post-growth – y = -0.2328x + 8.9087	0.9767	-0.2328 a	4.30 a
Matrix – Water Temperature - 4	Overall - y = -0.0298x + 8.2155	0.0192	-0.0122	81.97
	Post-growth – y = -0.1097x + 9.2485	0.9701	-0.1097 d	9.12 d
Matrix – Fecal Temperature - 22	Overall - y = -0.141x + 8.1431	0.6405	-0.1410	7.09
	Post-growth – y = -0.2578x + 9.643	0.9525	-0.2578 b	3.88 b
Matrix – Water Temperature - 22	Overall – y = -0.007x + 8.1188	0.0052	-0.007	142.86
	Post-growth – y = -0.0964x + 9.4275	0.9677	-0.0964 e	10.37 e
Matrix – Fecal Temperature - 35	Overall - y = -0.1612x + 7.9089	0.6775	-0.1612	6.20
	Post-growth – y = -0.2611x + 9.1537	0.9636	-0.2611 c	3.83 c
Matrix – Water Temperature - 35	Overall – y = -0.0032x + 8.0253	0.0012	-0.0032	312.5
	Post-growth – y = -0.0856x + 9.0574	0.7051	-0.0856 f	11.68 f

To compare the effect of temperature and matrix over time, a three-way ANOVA was performed (Appendix C). The data was $\log_{10}$ transformed to meet the parametric assumption of normality and variance homogeneity. The results of the ANOVA showed that the model was significant ($F = 865.56$; $P < 0.0001$) as shown in Table 23. The differences within each factor were found to be significant, in addition to the following combinations - temperature*matrix, day*matrix, temperature*day and temperature*matrix*day (Table 24). Bonferroni post hoc tests were performed to ascertain which pairwise comparisons were significantly different.

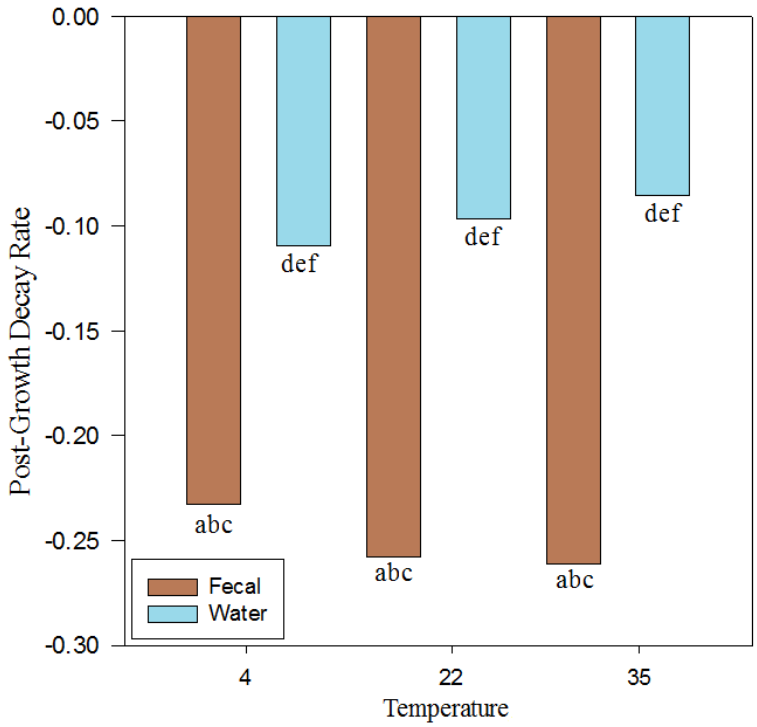

Figure 21. Post-growth die-off rates for enterococci in different treatments based on matrix and temperature. The combination of letters indicate significance at $\alpha = 0.05$, relative to other temperature groups within each matrix.

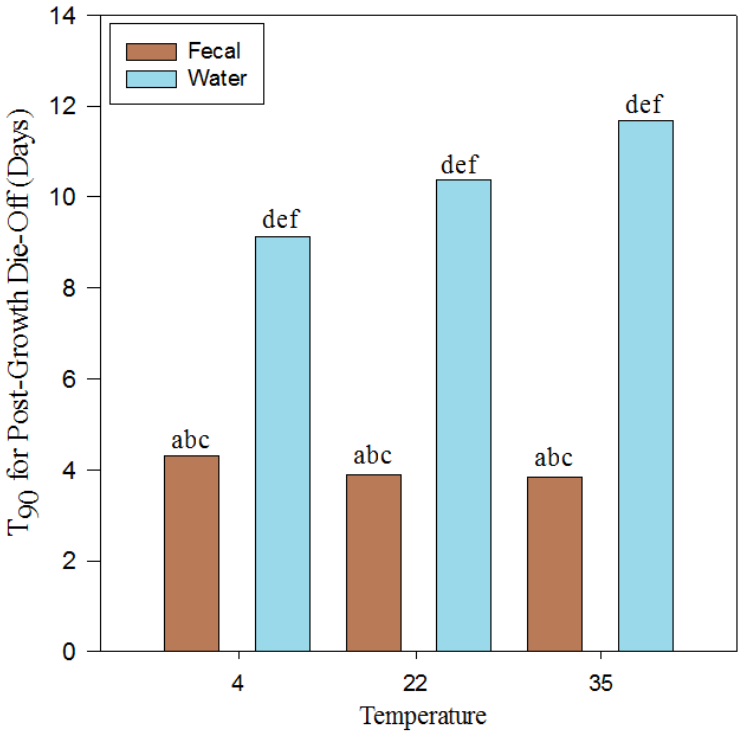

Figure 22. T90 for enterococci in different treatment based on matrix and temperature. T90 was calculated based on post-growth die-off rates. The combination of letters indicate significance at $\alpha = 0.05$, relative to other temperature groups within each matrix.

Table 23. Three-way ANOVA analysis for survival of fecal coliforms in fecal and water matrices at three temperatures of 4°C, 22°C, and 35°C over an 18-day period. The significance level was set to 0.05. * indicates significance.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	47	1605.636924	34.162488	865.56	<.0001*
Error	1248	49.256707	0.039469		
Corrected Total	1295	1654.893631			

Table 24. Three-way ANOVA showing the interactions between temperature, day and matrix for the survival of enterococci in fecal and water matrices at three temperatures of 4°C, 22°C, and 35°C over an 18-day period. The significance level was set to 0.05. * indicates significance.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Temperature	2	14.2506414	7.1253207	180.53	<.0001
Day	7	741.0996953	105.8713850	2682.43	<.0001
Temperature*Day	14	11.3115200	0.8079657	20.47	<.0001
Matrix	1	551.6360772	551.6360772	13976.6	<.0001
Temperature*Matrix	2	3.6550282	1.8275141	46.30	<.0001
Day*Matrix	7	275.3839328	39.3405618	996.76	<.0001
Temperature*Day*Matrix	14	8.3000288	0.5928592	15.02	<.0001

The results for the Bonferroni multiple comparison tests are displayed in Tables 23 and 24, indicating which temperature comparisons and matrix comparisons were found to be significant. For the enterococci, all temperature comparisons were significant. Likewise, the differences between the fecal and water matrices were also found to be significant. Results indicating differences between days are included in the Appendix C.

Table 23. Bonferroni test indicating which temperature comparisons are significant. Comparisons significant at the 0.05 level are indicated.

Temperature Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		Significance
22 - 35	0.19165	0.15924	0.22405	Yes
22 - 4	0.24393	0.21152	0.27633	Yes
35 - 4	0.05228	0.01988	0.08469	Yes

Table 24. Bonferroni test indicating which temperature comparisons are significant. Comparisons significant at the 0.05 level are indicated.

Matrix Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		Significance
2 - 1	1.30483	1.28318	1.32648	Yes

To test if there were any differences among the results based on the farms from which the samples were collected, an additional three-way ANOVA was performed within each matrix type with temperature, farm and day as three independent variables. For the survival of enterococci in the fecal phase, the differences between samples collected from the three farms were found to be significant (P = 0.0004). Table 25 indicates which pairwise comparisons are significant. The differences were signifincat for all comparisosns with the exception for Farms 2 and 3. For the water matrix, the differences between samples collected from the three farms were found to be insignificant (P = 0.7591). As expected, the Bonferroni tests showed no significant differences in any of the pairwise comparisons (Table 26).

Table 25. Bonferroni test for the survival of enterococci in fecal matrix indicating which farm comparisons are significant. Comparisons significant at the 0.05 level are included.

Farm Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		Significance
2 - 3	0.00538	-0.04514	0.05590	No
2 - 1	0.07532	0.02480	0.12585	Yes
3 - 1	0.06994	0.01942	0.12046	Yes

Table 26. Bonferroni test for the survival of enterococci in water matrix indicating which farm comparisons are significant. Comparisons significant at the 0.05 level are included.

Farm Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		Significance
1 - 3	0.00584	-0.03450	0.04618	No
1 - 2	0.01247	-0.02787	0.05281	No
3 - 2	0.00663	-0.03371	0.04696	No

CHAPTER 5

DISCUSSION

This study was designed to inspect the impact of temperature and matrix on the temporal fate (growth, survival and die-off) of fecal indicators bacteria. Total bacteria, fecal coliform, *E. coli*, and enterococci were routinely enumerated over an 18-day period from fecal samples and freshwater microcosms maintained at three different temperatures of 4^{o}C, 22^{o}C, and 35^{o}C. The experiments were performed to examine the extent of bacterial pollution due to runoff from pastures, since runoff from pasturelands is considered one of the key polluters of streams and rivers. The decay rates of the fecal indicator bacteria were characterized to represent two agricultural scenarios for comparison purpose. In the first scenario, bovine feces are surface deposited on pasturelands, without being amended into the soil. Alternatively, in the second scenario, the fecal matter is washed into a proximal water source. This study was executed in a laboratory setting since examining the differential survival of FIB in cow pats and natural waters would be difficult to carry out and unethical as it would further compromise the environmental integrity of soil and water. Surface deposited fecal matter can be a significant source of FIB for proximal waters [6]. Therefore, it is critical to examine the comparative survival of FIB in dry fecal matter and stream water inoculated with fecal matter.

Several studies have examined the survival and fate of fecal indicators in soil and water matrices [4, 5, 6, 8, 9 15, 18, 20]. However, very few studies have placed the emphasis on the persistence of FIB in fecal matrix. In addition to being a key contributor of FIB to proximal waters, manure also provides a distinct biological and physico-chemical environment for fecal indicator bacteria, which can collectively impact the survival and release of bacteria from the

fecal matter [4]. Hence, comparative studies that examine differential survival of FIB are critical for the determination of the total maximum bacterial inputs into an impaired stream for remediation purposes.

Fecal coliform, *E. coli* and enterococci are routinely used as bacterial fecal indicators for pathogen contamination in surface waters, hence they were used as the primary FIB in this study [36, 47, 48, 56]. Historically, total bacterial load was used as an indicator for fecal pollution, which is why it was included in this study as a potential fecal indicator [32]. Although the impact of abiotic factors on the survival of FIB is well studied, to our knowledge, no study has examined the differential decay of FIB derived from bovine feces in fecal and water phase simultaneously.

Initial Growth

A major finding in this study is that all bacterial groups studied underwent a primary growth of up to 1-$\log_{10}$ to 3-$\log_{10}$ compared to the initial concertation, regardless of the matrix and temperature variation. Many studies have reported similar findings regarding initial increase in bacterial concentrations in fecal matter, manure-amended soils, sediments and surface waters [26, 61, 88, 91]. The results of the survival analysis for all bacterial subgroups displayed an initial growth that last from day 0 up to day 2-8. Overall, the concentrations of FIB increased by 1 to 3 orders of magnitude for all the bacterial groups. Also, the growth was found to be higher in water phase compared to the fecal phase. Muirhead and Little John arrived at a similar conclusion while studying the *E. coli* die-off in intact and disrupted cow pats [92].

In addition to moisture content, FIB survival was also influenced by temperature [92]. Furthermore, initial increase appeared to be the highest at 22^{o}C, followed by 4^{o}C and 35^{o}C. This

finding is in agreement with the study conducted by Guber et al., in which the researchers were examining the effect of temperature on survival of *E. coli* derived from White-Tailed Deer feces. The growth detected at 20^{o}C was much higher than the FIB growth observed at 4^{o}C and 35^{o}C. The observed growth at 22^{o}C surpassing the growth at 35^{o}C could be explained by the apparent dominance of cold-adapted fecally-derived organisms. Since the manure samples were collected during February and March, the organisms seemed to have acclimated to the cold temperatures, resulting in an increased growth at cooler temperatures such as 22^{o}C. Growth at 4^{o}C was observed to be fairly low since the incubation temperature was far too low to favor high growth rates [93]. The bacterial growth observed in this study has serious implications for the use of FIB concentrations derived from freshly voided feces, since these concentrations do not account for the growth that subsequently occurs and cannot be used to inform the TMDL models. Using FIB concentrations of freshly voided feces for TMDLs could lead to considerable underestimation of the bacteria inputs.

It was understood that the conventional FIB would not be able to survive in a secondary habitat since they have been evolved to occupy a niche in lower intestines of warm-blooded animals. However, several studies have shown that FIB can flourish in extraintestinal environments. Many factors collectively aid in the initial increase of FIB concentrations in the secondary habitat. These factors include oxygen, nutrient availability, and moisture content. All these three factors could have potentially played a role in the subsequent growth observed in the water matrix. However, the extraintestinal growth of FIB in secondary habitats does to some degree compromise its indicator status since it could lead to false indication of pathogen contamination [44, 81].

Effect of temperature on survival

The temperature was found to be a significant factor for the survival of all bacterial subgroups over an 18-day period. Other studies have also found temperature to be an integral factor that drives survival patterns of fecal indicator bacteria [4, 26, 52, 62, 63, 93, 96]. However, the patterns of survival were found to be different across the bacterial subgroups that were analyzed. For the survival of total bacteria, the observed decay rate patterns were different for the two matrices. In the fecal phase, the decay rate was found to be the highest in samples maintained at 4^{o}C, followed by 35^{o}C and 22^{o}C respectively. Similarly, the decay rates for the water phase were found to be the highest at 4^{o}C. However, the decay rates in water phase observed at 22^{o}C were greater than those at 35^{o}C, whereas the opposite was found to be true for the fecal phase. This could be attributed to the interaction between matrix type and temperature. The survival was found to be the lowest for samples maintained at 4^{o}C possibly due to loss of culturability. Furthermore, the results for the total bacteria survival indicate that the FIB that were isolated were cold-adapted, which is reasonable since the manure samples were collected during the February and March. This finding is significant since it has important implications for altering TMDLs based on seasonal variations.

As for the survival of fecal coliform and *E. coli*, the patterns for both matrices were similar, based on temperature. For both matrices, the decay rates were the highest for samples maintained at 22^{o}C followed by 35^{o}C and 4^{o}C. All comparisons were significant. However, the decay rates for samples maintained at 4^{o}C were found to be higher in *E. coli* than those maintained at 35^{o}C, instead of the opposite observed in the fecal coliforms. A similar pattern was reported by Guber and colleagues, where the decay rate for samples maintained at 20^{o}C was found to be greater than those maintained at 4^{o}C. However, decay rate for samples maintained at

35ºC was found to be the highest in Guber et al., which is distinct from our findings. This finding refutes the hypothesis that expected a rapid die-off for samples maintained at relatively higher temperature of 35ºC, compared to 4ºC and 22ºC. This finding is significant since it insinuates that a temperature of 35ºC is conducive to longer survival.

Lastly, there was a clear pattern detected for the decay rates for enterococci in fecal phase – 4ºC > 22ºC > 35ºC. Interestingly, the opposite was true for samples maintained in the water phase, hence the decays rates were as follows - 35ºC > 22ºC > 4ºC. The results for the water phase are in line with other studies that reported a similar pattern of more rapid die-off with increasing temperatures [5, 6]. However, the observed pattern for the fecal phase is unique to our study. It is more likely that the die-off appears to be more rapid, but the cells probably changed states from being culturable to viable but non-culturable for the colder temperatures.

Overall, the results for the temperature analysis reported in this study have identified certain unique survival patterns, which are critical for the establishment of an effective TMDL plan. However, since this study exhibited a high degree of deviation in the survival patterns of each bacterial subgroup in response to matrix and temperature variation, this study also highlights the complexity of extrapolating the laboratory-derived findings to field conditions [51]. One standard regulation cannot be used to regulate diverse types of pasturelands and water bodies.

Effect of matrix type on survival

The concentrations of FIB in the water phase were significantly greater than the concentrations observed in the fecal phase in all bacterial subgroups. However, the difference between survival of FIB in samples maintained in fecal and water matrices were found to be

significantly contrasting for all bacterial subgroups. While the differences in the decay rates in the two matrices were identified as significant, there was a slight variation in the patterns regarding the differences among various bacterial subgroups. For the survival of total bacteria, the observed decay rates were significantly higher in the water phase compared to the fecal phase. Anderson et al. reached a similar conclusion when testing for survival of fecal coliforms and enterococci in freshwater microcosms and sediments [24]. According to their findings, fecal coliform decay rates in sediments were significantly lower than decay rates observed in the water column. Likewise, Oladeinde and colleagues also reported longer persistence of fecal indicators in the fecal matrix [6]. However, no comparative analysis was performed by Oladeinde et al. to compare the survival of FIB in fecal and water matrices. Wang et al. surveyed the effect of temperature on the survival of FIB derived from bovine feces, in addition to studying the effect of moisture content on persistence [4]. However, no microcosms were used in the experiment and the fecal samples were maintained at a maximum moisture level of 83%. Notably, temperature was found to produce a significant effect on survival of FIB, whereas moisture content was found to play a significant role in the survival of enterococci only. The evidence presented in our study is at odds with the findings produced by Wang et al. However, the moisture levels used in our study were significantly higher than those used in the Wang's research.

The survival of fecal coliforms in different matrices were significantly different without a specific pattern. The FC survival was much higher at 22^{o}C in water samples but lower for 4^{o}C and 35^{o}C. The same is true for the *E. coli* survival, where *E. coli* survival was higher for the water phase at 22^{o}C but lower for 4^{o}C and 35^{o}C. It makes sense that the survival patterns match for these two bacterial groups, since *E. coli* is a subset of the fecal coliform group. Although the

differences between the matrices were found to be significant, the survival of fecal coliforms and *E. coli* seem to be more responsive to temperature than matrix variation due to the lack of pattern between the two matrices. Other studies have reported similar results were temperature played a more integral role in determining the survival pattern in fecal coliforms [6, 94, 95].

A specific pattern was detected in the decay rates of enterococci based on matrix variation. The enterococci were detected to be significantly more persistent in the water phase compared to the fecal phase. Kibbey et al. conducted a study to examine the effect of temperature and moisture on enterococci in soils [96]. Their study revealed results that are in line with our finding; i.e., T_{95} values for soils saturated with moisture were much higher in comparison to 50% field capacity moisture levels or air-dried soils. This can be explained due to drought stress which can significantly impact the survival rate of fecal indicators.

The results regarding the matrix type refute the hypothesis that decay rates are similar in water and fecal matrices. The decay rates based on the matrix type varied on the for each bacteria group. Fecal coliform and *E. coli* exhibited no specific pattern regarding matrix type, perhaps due to the greater influence of temperature. Great decay rates were observed in enterococci in the fecal phase, as opposed to the water phase. Alternatively, the decay rates of total bacteria were found to be higher in water phase, when compared to fecal phase. These results signify the weakness of the common fecal indicator bacteria, since they are leading to conflicting results with regards to the role of matrix in the survival of fecally-derived microorganisms.

Effect of farming practices on survival

Samples for this study were collected from three farms in East Tennessee. The designated Farm 1 (Jones Farm) and Farm 3 (Swenson Farm) specialized in beef-production, whereas the Farm 2 (The Cows Are Out Farm) specialized in dairy farming. According to the results from the multivariate ANOVAs, the differences between samples collected from each farm were not significant with a few exceptions. The differences were significant for the survival of total bacteria in fecal phase between Farm 1: Farm 2 and Farm 2: Farm 3. The differences between Farm 1 and Farm 3 were deemed not significant. Since both Farm 1 and Farm 3 specialize in beef production and are fairly close to each other, variables such as diet and environmental factors are much similar for cattle that are housed in those farms. This could explain the significant differences between samples collected from Farm 2, compared to Farms 1 and 3. Similarly, the pairwise comparisons performed to test for significant differences between farms for the survival of enterococci in the fecal phase was found to be significantly different between Farms 1 and 2. The difference in the mean concentrations of fecal indicator bacteria based on farming practices and land use is in agreement with the findings of Crowther et al. [90]. According to Crowther et al., the mean concentrations of fecal indicator bacteria (*E. coli*) were significantly different based on livestock type – dairy and beef cattle. However, the difference between samples collected from Farms 1 and 3 were also found to be significantly different for the survival of enterococci in fecal phase. This difference could be attributed to the introduced artifacts during manure sample collection. Care was taken to collect the freshly voided, undisturbed cow feces, however, on a few occasions, the cows pats were trampled on by the

cattle which could have slightly altered the bacterial composition with regards to enterococci [91].

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

Two different matrices were used to measure the survival of fecal indicator bacteria - fecal and water. The survival was measured at three different temperatures – 4^{o}C, 22^{o}C, and 35^{o}C. The survival of the FIB was determined based on cultivation-based methods. Fecal coliform, *E. coli*, enterococci and total bacterial load were used as bacterial fecal indicators for pathogen contamination in surface waters. Although the impact of abiotic factors on the survival of FIB is well studied, to our knowledge, no study has examined the differential decay of FIB derived from bovine feces in fecal and water phase simultaneously. Initial growth was observed in all bacterial subgroups indicating the weakness of these fecal indicator bacteria, since ideal indicators ought not to exhibit extraintestinal growth.

The results indicated matrix type and temperature to be a significant factor for all bacterial subgroups. However, Bonferroni tests were applied to perform pairwise comparisons to indicate which comparisons were significant. While many of the survival patterns reported in this study are in agreement with previously published work, certain patterns were found to be unique to our study. For instance, decay rates for enterococci in fecal phase displayed the following patterns – 4^{o}C > 22^{o}C > 35^{o}C, whereas the decay rates for the water phase followed the opposite pattern as shown - 35^{o}C > 22^{o}C > 4^{o}C. These findings highlight the complications in extrapolating laboratory-derived data to field conditions that might apply to pasturelands [51].

Overall, this study provides important information about the survival of typical FIB in fecal and water matrix under the influence of varying temperatures. This study also highlights the weakness of using conventionally used FIB as indicators of pathogen contamination. Future

studies that examine the survival of genetic markers and alternative indicators of fecal contamination are required to further bolster the establishment of effective TMDL plans.

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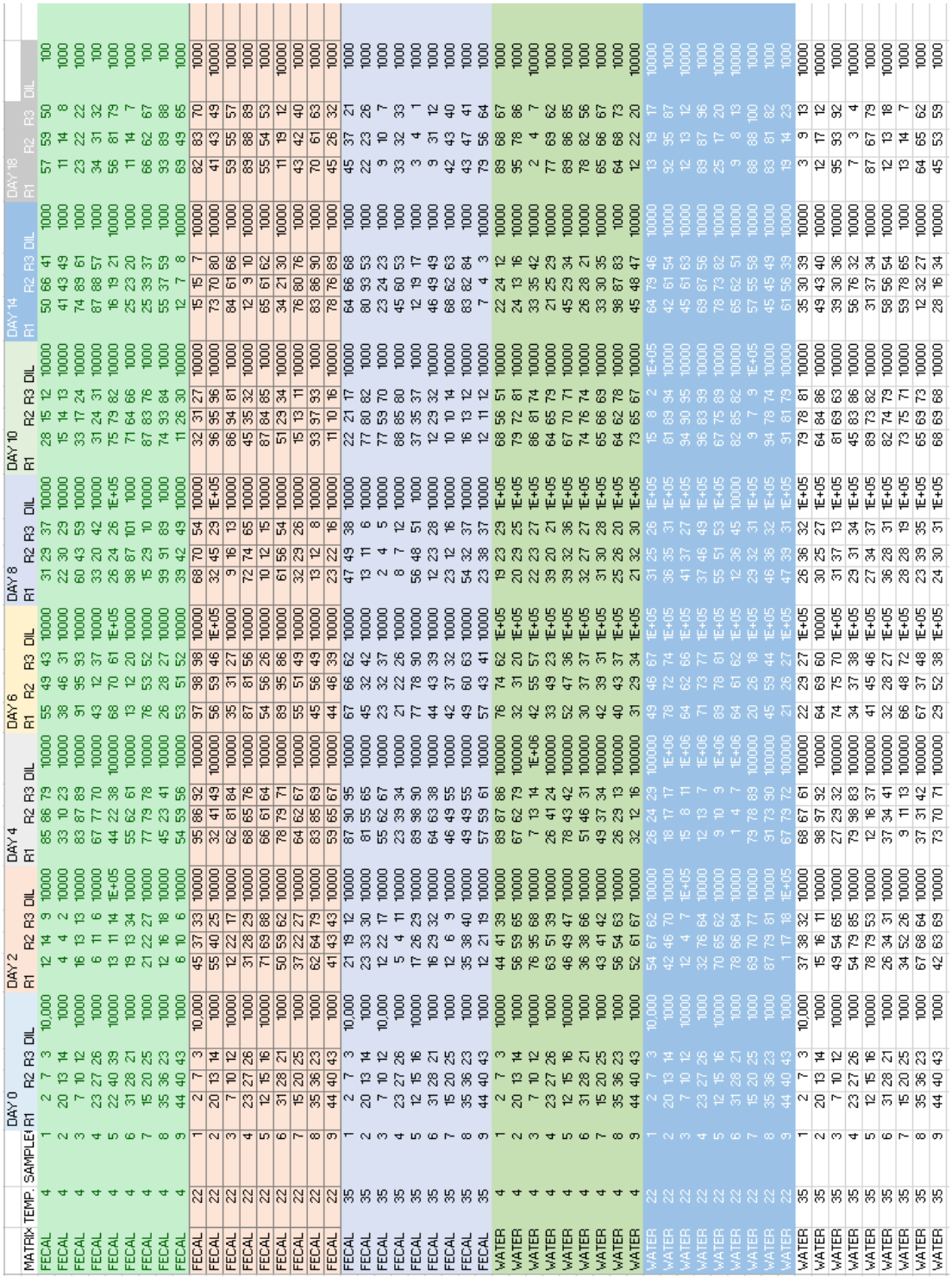
APPENDICES

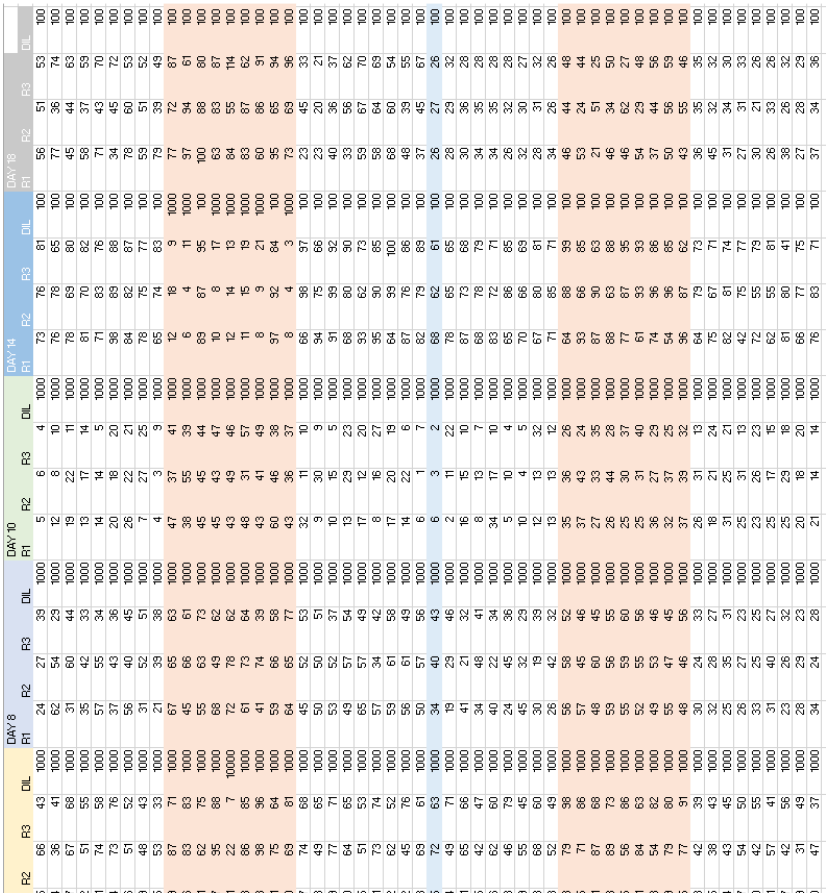
Appendix A: Raw Data

1) Total bacteria



MATRIX	TEMP.	SAMPLE#	DAY 0				DAY 2				DAY 4				DAY 6				DAY 8				DAY 10				DAY 14				DAY 18			
			R1	R2	R3	DIL	R1	R2	R3	DIL	R1	R2	R3	DIL	R1	R2	R3	DIL	R1	R2	R3	DIL	R1	R2	R3	DIL	R1	R2	R3	DIL	R1	R2	R3	DIL
FECAL	4	1	2	7	3	10,000	12	14	9	10000	85	86	79	10000	55	49	43	10000	31	29	37	10000	28	15	12	10000	50	66	41	1000	57	59	50	100
FECAL	4	2	20	13	14	1000	4	4	2	10000	33	10	23	10000	38	46	31	10000	22	30	29	10000	15	14	13	10000	41	43	49	1000	11	14	8	1000
FECAL	4	3	7	10	12	10000	16	13	13	10000	83	87	89	10000	91	95	93	10000	60	43	53	10000	33	17	24	10000	74	89	61	1000	23	22	22	1000
FECAL	4	4	23	27	26	1000	6	11	6	10000	67	77	70	10000	43	12	37	10000	33	20	42	10000	31	24	31	10000	87	88	57	1000	34	31	32	100
FECAL	4	5	22	40	39	10000	13	11	14	1E+05	44	22	38	100000	68	70	61	1E+05	26	24	26	1E+05	75	79	82	10000	16	19	21	10000	56	81	79	1000
FECAL	4	6	31	28	21	1000	19	13	34	10000	55	62	61	10000	13	12	20	10000	98	87	101	1000	71	64	66	1000	25	23	20	1000	11	14	7	1000
FECAL	4	7	15	20	25	1000	21	22	27	10000	77	79	78	10000	76	53	52	10000	15	29	10	10000	87	83	76	1000	25	39	37	1000	66	62	67	100
FECAL	4	8	35	36	23	1000	12	16	18	10000	45	23	41	10000	26	28	27	10000	99	91	89	1000	74	93	84	1000	55	37	59	1000	93	89	88	100
FECAL	4	9	44	40	43	1000	6	10	6	10000	54	59	56	10000	53	51	52	10000	39	42	49	10000	11	26	30	10000	12	7	8	10000	69	49	65	1000
FECAL	22	1	2	7	3	10,000	45	37	33	10000	95	86	92	10000	97	98	98	10000	68	70	54	10000	32	31	27	10000	15	15	7	10000	82	83	70	1000
FECAL	22	2	20	13	14	1000	55	40	25	10000	32	41	49	100000	56	59	46	1E+05	32	45	29	1E+05	96	95	96	10000	73	70	80	10000	41	43	49	10000
FECAL	22	3	7	10	12	10000	12	22	17	10000	62	81	84	10000	35	31	27	10000	9	16	13	10000	86	94	81	1000	84	61	66	1000	59	55	57	1000
FECAL	22	4	23	27	26	1000	31	28	29	10000	68	65	76	10000	87	81	56	10000	72	74	65	10000	45	35	32	10000	12	9	10	10000	89	88	89	1000
FECAL	22	5	12	15	16	10000	71	69	88	10000	66	61	64	10000	54	56	26	10000	10	12	15	10000	87	84	85	1000	65	61	62	1000	55	54	53	1000
FECAL	22	6	31	28	21	1000	50	53	62	10000	78	79	71	10000	89	95	86	10000	61	56	54	10000	51	29	34	10000	34	21	30	10000	11	19	12	10000
FECAL	22	7	15	20	25	1000	37	22	27	10000	64	62	67	10000	55	51	49	10000	32	29	26	10000	15	13	11	10000	76	80	76	1000	43	42	40	1000
FECAL	22	8	35	36	23	1000	62	64	79	10000	83	85	69	10000	45	56	49	10000	13	12	8	10000	93	97	93	1000	83	86	90	1000	70	61	63	1000
FECAL	22	9	44	40	43	1000	41	43	43	10000	53	65	67	10000	44	46	39	10000	23	22	16	10000	11	10	16	10000	78	76	89	1000	45	26	32	1000
FECAL	35	1	2	7	3	10,000	21	19	12	10000	87	90	95	10000	67	66	62	10000	47	49	38	10000	22	21	17	10000	64	66	68	1000	45	37	21	1000
FECAL	35	2	20	13	14	1000	23	33	30	10000	81	55	65	10000	45	32	42	10000	13	11	6	10000	80	80	82	1000	80	93	53	1000	22	23	26	1000
FECAL	35	3	7	10	12	10,000	12	22	17	10000	55	62	67	10000	23	32	37	10000	2	4	5	10000	77	59	70	1000	23	24	23	1000	9	10	7	1000
FECAL	35	4	23	27	26	1000	5	4	11	10000	23	39	34	10000	21	22	26	10000	8	7	12	10000	88	85	80	1000	45	60	53	1000	33	32	33	1000
FECAL	35	5	12	15	16	10000	17	26	29	10000	89	98	90	10000	77	78	90	10000	56	48	51	10000	37	35	37	1000	12	19	17	1000	3	4	1	1000
FECAL	35	6	31	28	21	1000	16	29	32	10000	64	63	38	10000	44	43	39	10000	12	23	28	10000	12	29	32	10000	46	49	49	1000	9	31	12	1000
FECAL	35	7	15	20	25	1000	12	6	9	10000	46	49	55	10000	42	37	32	10000	23	12	16	10000	10	10	14	10000	68	62	63	1000	42	43	40	1000
FECAL	35	8	35	36	23	1000	35	38	40	10000	49	49	55	10000	49	60	63	10000	54	32	37	10000	16	13	12	10000	83	82	84	1000	43	47	41	1000
FECAL	35	9	44	40	43	1000	12	21	19	10000	57	59	61	10000	57	43	41	10000	23	38	37	10000	12	11	12	10000	7	4	3	10000	79	56	64	1000
WATER	4	1	2	7	3	10000	44	41	39	10000	89	87	86	100000	76	74	62	1E+05	19	23	29	1E+05	68	56	51	10000	22	24	12	10000	89	68	67	1000
WATER	4	2	20	13	14	1000	56	59	65	10000	67	62	79	100000	32	31	20	1E+05	20	29	25	1E+05	79	72	81	10000	24	13	16	10000	95	78	86	1000
WATER	4	3	7	10	12	10000	76	95	68	10000	7	13	14	1E+06	42	55	57	1E+05	22	23	27	1E+05	86	81	74	10000	33	35	42	10000	2	4	7	10000
WATER	4	4	23	27	26	1000	63	51	39	10000	26	41	24	100000	33	49	23	1E+05	39	20	21	1E+05	64	65	79	10000	21	25	29	10000	77	69	62	1000
WATER	4	5	12	15	16	10000	46	49	47	10000	78	43	42	100000	52	47	36	1E+05	39	32	36	1E+05	67	70	71	10000	45	29	34	10000	89	86	85	1000
WATER	4	6	31	28	21	1000	36	36	66	10000	51	46	31	100000	30	37	37	1E+05	32	27	27	1E+05	74	76	74	10000	26	28	21	10000	78	82	56	1000
WATER	4	7	15	20	25	1000	43	41	42	10000	49	37	34	100000	42	39	31	1E+05	31	30	28	1E+05	65	66	69	10000	33	30	35	10000	65	66	67	1000
WATER	4	8	35	36	23	1000	56	54	63	10000	26	29	13	100000	40	43	37	1E+05	25	26	20	1E+05	64	62	78	10000	38	87	83	1000	64	68	73	1000
WATER	4	9	44	40	43	1000	52	61	67	10000	32	12	16	100000	31	29	34	1E+05	21	32	30	1E+05	73	65	67	10000	45	48	47	10000	12	22	20	10000
WATER	22	1	2	7	3	10,000	54	67	62	10000	26	24	29	100000	49	46	67	1E+05	31	25	26	1E+05	15	8	2	1E+05	64	79	46	10000	13	19	17	10000
WATER	22	2	20	13	14	1000	42	46	70	10000	18	17	17	1E+06	78	72	74	1E+05	36	35	31	1E+05	61	89	94	10000	42	61	54	10000	32	35	87	1000
WATER	22	3	7	10	12	10000	12	4	7	1E+05	15	8	11	1E+06	64	62	66	1E+05	41	37	27	1E+05	94	90	95	10000	45	61	63	10000	12	13	12	10000
WATER	22	4	23	27	26	1000	32	76	64	10000	12	13	7	1E+06	71	73	77	1E+05	37	46	49	1E+05	96	83	99	10000	69	87	56	10000	89	87	96	1000
WATER	22	5	12	15	16	10000	70	65	62	10000	9	10	9	1E+06	89	78	61	1E+05	55	51	53	1E+05	67	75	89	10000	78	73	82	10000	25	17	20	10000
WATER	22	6	31	28	21	1000	78	66	64	10000	1	4	7	1E+06	64	61	62	1E+05	12	36	45	10000	82	85	82	10000	65	62	51	10000	9	8	13	10000
WATER	22	7	15	20	25	1000	69	70	77	10000	73	78	89	100000	20	26	18	1E+05	29	32	31	1E+05	9	7	9	1E+05	57	55	58	10000	88	88	100	1000
WATER	22	8	35	36	23	1000	87	79	81	10000	91	73	90	100000	45	59	44	1E+05	46	36	32	1E+05	94	78	74	10000	45	45	49	10000	83	81	82	1000
WATER	22	9	44	40	43	1000	1	17	18	1E+05	67	79	72	100000	21	26	27	1E+05	47	39	31	1E+05	31	81	79	10000	61	56	39	10000	19	14	23	1000
WATER	35	1	2	7	3	10,000	37	38	32	10000	68	67	61	100000	22	29	27	1E+06	26	36	32	1E+05	79	78	81	10000	35	30	39	10000	3	9	13	10000
WATER	35	2	20	13	14	1000	15	16	11	10000	38	37	32	10000	64	69	60	10000	30	25	27	1E+05	64	84	86	10000	49	43	40	10000	12	17	12	10000
WATER	35	3	7	10	12	10000	43	54	65	10000	27	23	32	100000	74	75	70	1E+05	31	37	13	1E+05	81	69	63	10000	39	30	36	10000	95	93	92	1000
WATER	35	4	23	27	26	1000	54	79	85	10000	79	98	83	100000	34	37	38	1E+05	29	31	34	1E+05	45	83	86	10000	56	76	32	10000	7	3	4	10000
WATER	35	5	12	15	16	10000	78	79	53	10000	12	16	37	100000	41	45	46	1E+05	27	34	37	1E+05	89	73	82	10000	31	37	34	10000	87	67	79	1000
WATER	35	6	31	28	21	1000	26	34	31	10000	37	34	41	100000	32	28	27	1E+05	36	28	31	1E+05	82	74	79	10000	58	56	54	10000	12	13	18	10000
WATER	35	7	15	20	25	1000	34	52	26	10000	9	11	13	100000	66	48	72	1E+05	28	28	19	1E+05	73	75	71	10000	53	78	65	1000	13	14	7	1000
WATER	35	8	35	36	23	1000	67	68	64	10000	37	31	42	100000	67	37	48	1E+05	23	39	35	1E+05	65	69	73	10000	12	32	27	10000	64	65	62	1000
WATER	35	9	44	40	43	1000	42	63	69	10000	73	70	71	100000	29	52	38	1E+05	24	30	31	1E+05	68	69	68	10000	28	16	34	10000	45	53	59	1000

2) Fecal coliform



3) *E. coli*

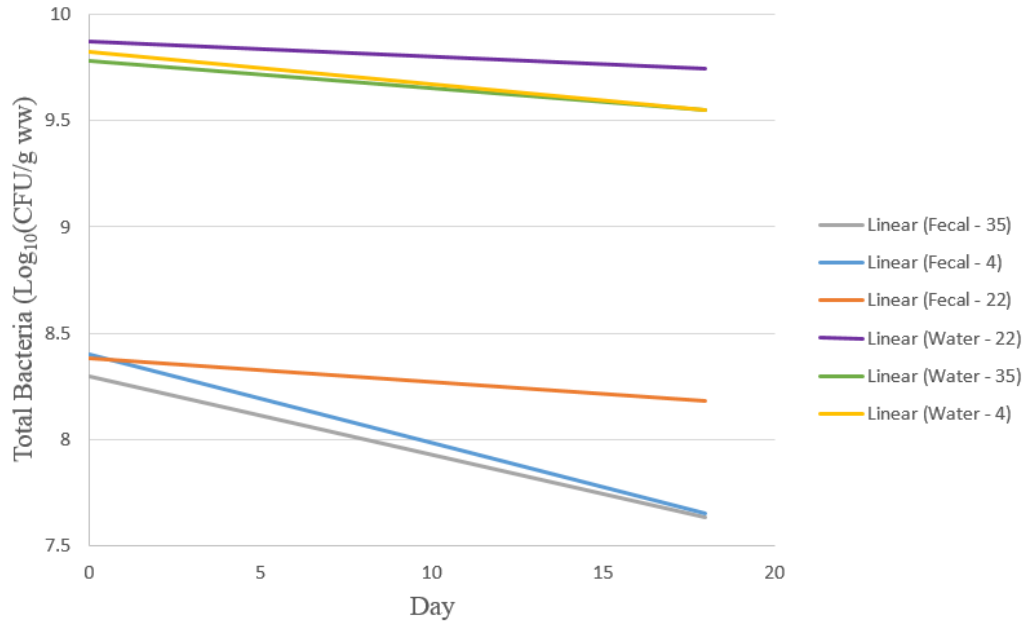
MATRIX	TEMP.	SAMPLE	DAY 0				DAY 2				DAY 4				DAY 6				DAY 8				DAY 10				DAY 14				DAY 18			
			R1	R2	R3	DIL	R1	R2	R3	DIL	R1	R2	R3	DIL	R1	R2	R3	DIL	R1	R2	R3	DIL	R1	R2	R3	DIL	R1	R2	R3	DIL	R1	R2	R3	DIL
FECAL	4	1	3	11	11	1,000	22	19	20	1000	85	85	88	1000	72	69	48	1000	49	39	52	1000	13	23	7	1000	49	51	53	100	34	23	26	100
FECAL	4	2	19	13	16	1,000	21	22	23	1000	94	88	72	1000	71	56	57	1000	56	52	36	1000	16	19	18	1000	55	53	67	100	35	27	33	100
FECAL	4	3	13	16	14	1,000	21	25	23	1000	86	73	87	1000	78	66	51	1000	48	56	51	1000	4	6	8	1000	49	58	67	100	38	35	37	100
FECAL	4	4	9	12	14	1,000	23	27	20	1000	86	79	90	1000	72	71	78	1000	36	48	34	1000	20	4	3	1000	65	57	45	100	24	26	32	100
FECAL	4	5	13	12	18	1,000	27	19	21	1000	77	72	87	1000	70	56	49	1000	57	58	51	1000	16	21	16	1000	47	63	50	100	38	32	37	100
FECAL	4	6	14	6	12	1,000	23	26	23	1000	78	77	71	1000	63	59	53	1000	47	57	51	1000	23	23	4	1000	60	49	54	100	37	29	37	100
FECAL	4	7	5	6	13	1,000	26	22	23	1000	88	77	75	1000	51	54	78	1000	38	46	42	1000	5	15	6	1000	49	67	57	100	23	32	29	100
FECAL	4	8	4	11	9	1,000	24	21	26	1000	81	73	94	1000	70	56	66	1000	54	57	45	1000	14	8	21	1000	63	65	52	100	23	32	26	100
FECAL	4	9	12	8	7	1,000	19	19	20	1000	86	76	74	1000	57	79	75	1000	43	43	47	1000	10	4	18	1000	58	61	46	100	26	27	25	100
FECAL	22	1	9	11	11	1,000	75	76	70	1000	40	42	41	10000	77	69	63	1000	52	53	51	1000	32	36	33	1000	73	70	76	100	54	48	53	100
FECAL	22	2	19	13	16	1,000	71	79	67	1000	36	42	35	10000	67	66	66	1000	63	66	44	1000	36	30	36	1000	73	75	76	100	52	54	56	100
FECAL	22	3	13	16	14	1,000	68	77	67	1000	35	37	37	10000	75	74	67	1000	50	58	65	1000	39	36	30	1000	66	71	71	100	56	51	56	100
FECAL	22	4	9	12	14	1,000	72	70	78	1000	43	36	38	10000	65	67	77	1000	53	39	59	1000	39	35	38	1000	73	74	67	100	56	48	49	100
FECAL	22	5	13	12	18	1,000	73	74	71	1000	37	38	39	10000	77	68	67	1000	65	57	52	1000	36	37	29	1000	67	73	68	100	52	48	48	100
FECAL	22	6	14	6	12	1,000	75	67	70	1000	36	45	35	10000	66	74	71	1000	47	55	39	1000	31	34	34	1000	68	66	69	100	50	47	56	100
FECAL	22	7	5	6	13	1,000	69	72	74	1000	39	36	38	10000	74	75	71	1000	51	42	66	1000	30	37	33	1000	69	76	71	100	48	47	47	100
FECAL	22	8	4	11	9	1,000	70	74	71	1000	41	45	42	10000	70	72	68	1000	57	62	40	1000	33	39	34	1000	71	69	69	100	47	46	51	100
FECAL	22	9	12	8	7	1,000	71	77	72	1000	42	41	40	10000	65	67	76	1000	51	61	56	1000	29	29	32	1000	75	68	69	100	50	49	45	100
FECAL	35	1	9	11	11	1,000	55	49	52	1000	117	112	114	1000	66	45	48	1000	21	26	28	1000	9	6	16	1000	60	59	61	100	40	31	26	100
FECAL	35	2	19	13	16	1,000	46	54	57	1000	83	102	112	1000	51	57	53	1000	16	13	23	1000	3	13	3	1000	56	55	66	100	45	45	23	100
FECAL	35	3	13	16	14	1,000	42	36	42	1000	110	105	114	1000	46	66	49	1000	17	17	22	1000	18	7	10	1000	67	57	64	100	25	37	43	100
FECAL	35	4	9	12	14	1,000	53	39	40	1000	114	112	96	1000	46	45	64	1000	19	22	13	1000	7	4	24	1000	62	64	63	100	24	33	24	100
FECAL	35	5	13	12	18	1,000	36	41	37	1000	95	99	98	1000	60	63	60	1000	15	22	29	1000	17	2	24	1000	56	57	66	100	40	37	36	100
FECAL	35	6	14	6	12	1,000	52	53	43	1000	114	32	113	1000	54	45	55	1000	27	19	28	1000	3	13	8	1000	56	66	67	100	36	37	44	100
FECAL	35	7	5	6	13	1,000	42	36	49	1000	112	89	93	1000	64	43	66	1000	12	19	21	1000	17	15	4	1000	65	62	57	100	23	24	31	100
FECAL	35	8	4	11	9	1,000	40	50	51	1000	94	105	96	1000	53	65	64	1000	27	16	27	1000	17	7	15	1000	66	65	59	100	47	40	30	100
FECAL	35	9	12	8	7	1,000	44	56	42	1000	89	95	90	1000	52	60	48	1000	16	21	26	1000	14	24	3	1000	56	55	61	100	27	44	29	100
WATER	4	1	26	35	44	1,000	53	52	48	1000	68	76	72	1000	33	27	38	1000	9	12	7	1000	78	81	80	100	67	58	56	100	15	9	19	100
WATER	4	2	23	24	25	1,000	54	49	50	1000	78	67	78	1000	30	27	26	1000	5	12	7	1000	88	83	79	100	65	67	59	100	12	15	8	100
WATER	4	3	41	39	33	1,000	54	48	51	1000	71	77	78	1000	31	30	28	1000	4	6	6	1000	82	87	82	100	62	67	59	100	5	20	15	100
WATER	4	4	42	48	37	1,000	52	53	49	1000	63	75	75	1000	32	29	36	1000	6	10	6	1000	87	87	82	100	58	60	58	100	21	7	11	100
WATER	4	5	38	35	33	1,000	52	49	53	1000	74	76	72	1000	24	38	35	1000	13	8	6	1000	83	82	85	100	58	57	57	100	14	13	12	100
WATER	4	6	31	23	32	1,000	53	52	53	1000	74	67	75	1000	31	28	33	1000	13	13	6	1000	84	80	88	100	58	61	64	100	10	21	18	100
WATER	4	7	30	31	30	1,000	53	51	49	1000	69	70	66	1000	39	39	26	1000	9	5	11	1000	80	83	87	100	59	64	64	100	22	17	18	100
WATER	4	8	20	26	27	1,000	50	51	50	1000	77	66	76	1000	25	33	36	1000	9	9	9	1000	87	79	79	100	59	57	66	100	9	13	23	100
WATER	4	9	25	24	24	1,000	49	51	49	1000	70	73	69	1000	38	23	23	1000	9	8	9	1000	84	82	83	100	57	58	65	100	16	15	15	100
WATER	22	1	26	35	35	1,000	30	80	84	1000	68	72	68	10000	49	46	63	1000	54	50	58	1000	41	41	38	1000	16	13	19	100	74	72	81	100
WATER	22	2	23	24	25	1,000	30	91	83	1000	67	68	78	10000	55	47	65	1000	45	52	51	1000	38	32	35	1000	16	15	12	100	78	67	72	100
WATER	22	3	41	39	33	1,000	61	85	90	1000	76	71	65	10000	53	65	63	1000	46	47	52	1000	32	43	36	1000	15	18	11	100	68	73	68	100
WATER	22	4	42	48	37	1,000	67	79	87	1000	64	73	67	10000	51	57	61	1000	53	56	45	1000	23	36	45	1000	17	18	16	100	74	74	77	100
WATER	22	5	38	35	33	1,000	83	92	91	1000	77	76	76	10000	63	62	55	1000	46	46	58	1000	34	35	51	1000	10	19	20	100	72	78	75	100
WATER	22	6	31	23	32	1,000	73	86	73	1000	65	66	67	10000	69	46	54	1000	54	50	55	1000	44	25	23	1000	14	12	20	100	74	76	79	100
WATER	22	7	30	31	30	1,000	84	90	90	1000	78	70	63	10000	59	46	46	1000	50	45	53	1000	36	28	34	1000	17	7	7	100	79	71	61	100
WATER	22	8	20	26	27	1,000	92	91	79	1000	79	70	66	10000	55	57	56	1000	53	57	54	1000	27	45	41	1000	15	10	13	100	71	74	71	100
WATER	22	9	25	24	24	1,000	81	70	86	1000	73	69	69	10000	61	65	62	1000	47	46	46	1000	38	45	33	1000	11	16	8	100	74	79	81	100
WATER	35	1	26	35	44	1,000	65	70	56	1000	87	87	88	1000	62	55	51	1000	20	24	23	1000	16	15	2	1000	90	97	89	100	46	38	34	100
WATER	35	2	23	24	25	1,000	66	63	68	1000	91	89	93	1000	50	56	62	1000	20	27	23	1000	18	22	17	1000	94	97	95	100	46	36	43	100
WATER	35	3	41	39	33	1,000	70	57	63	1000	99	97	95	1000	57	52	55	1000	17	17	19	1000	3	18	11	1000	91	93	97	100	36	46	40	100
WATER	35	4	42	48	37	1,000	61	72	72	1000	89	98	87	1000	53	63	55	1000	22	23	23	1000	7	7	5	1000	94	95	89	100	47	39	47	100
WATER	35	5	38	35	33	1,000	57	60	72	1000	90	92	93	1000	57	63	53	1000	21	19	31	1000	12	12	7	1000	89	88	90	100	41	34	42	100
WATER	35	6	31	23	32	1,000	61	63	66	1000	85	86	85	1000	53	61	51	1000	18	18	18	1000	3	1	7	1000	92	95	90	100	37	46	43	100
WATER	35	7	30	31	30	1,000	56	56	72	1000	89	92	90	1000	63	63	55	1000	28	30	19	1000	10	10	12	1000	94	92	96	100	38	47	44	100
WATER	35	8	20	26	27	1,000	60	63	68	1000	91	87	88	1000	63	63	55	1000	28	30	19	1000	10	10	20	1000	94	97	87	100	41	43	47	100
WATER	35	9	25	24	24	1,000	54	62	53	1000	99	98	92	1000	62	56	50	1000	24	28	19	1000	19	14	18	1000	89	90	95	100	34	46	45	100

4) Enterococci

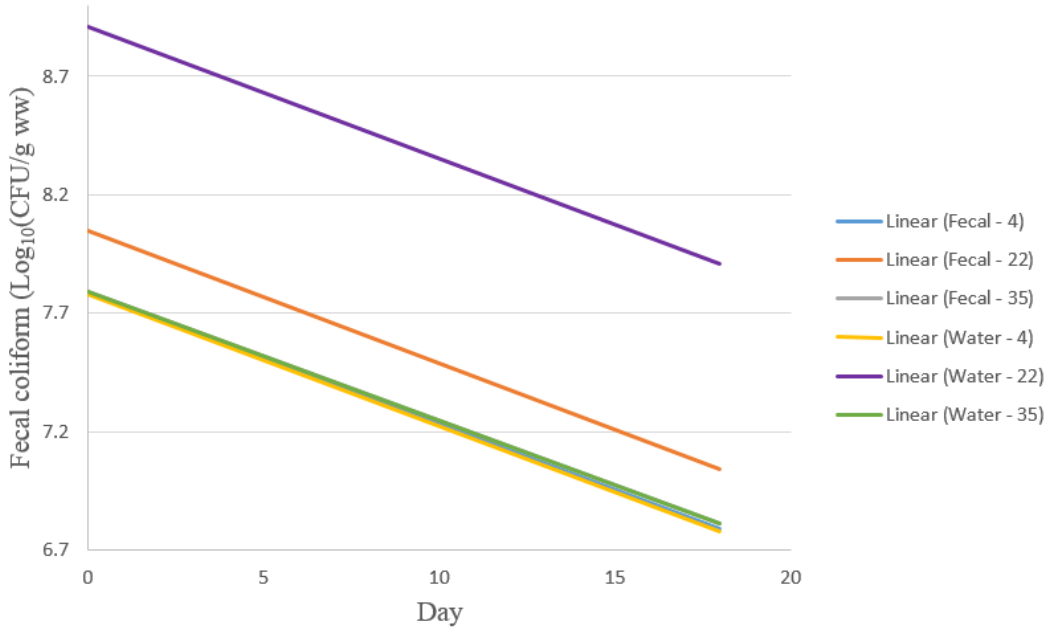
MATRIX	TEMP	SAMPLE	DAY 0				DAY 2				DAY 4				DAY 6				DAY 8				DAY 10				DAY 14				DAY 16			
			R1	R2	R3	DIL	R1	R2	R3	DIL	R1	R2	R3	DIL	R1	R2	R3	DIL	R1	R2	R3	DIL	R1	R2	R3	DIL	R1	R2	R3	DIL	R1	R2	R3	DIL
FECAL	4	1	6	2	1	100	47	45	43	1000	62	47	63	1000	39	43	22	1000	10	26	3	1000	100	73	72	100	18	26	23	10	6	3	13	10
FECAL	4	2	3	4	5	100	37	53	60	1000	66	57	66	1000	44	20	23	1000	9	24	22	1000	88	90	88	100	24	14	32	10	10	5	11	10
FECAL	4	3	2	8	9	100	23	67	65	1000	45	61	45	1000	45	36	52	1000	6	24	12	1000	76	90	77	100	57	46	42	10	13	7	5	10
FECAL	4	4	1	1	5	100	37	38	51	1000	56	46	65	1000	21	50	45	1000	19	27	5	1000	72	73	86	100	36	53	45	10	14	6	4	10
FECAL	4	5	8	8	2	100	55	63	53	1000	68	78	60	1000	60	27	30	1000	18	22	5	1000	91	79	67	100	33	22	31	10	3	10	5	10
FECAL	4	6	11	12	8	100	68	77	59	1000	60	73	44	1000	45	55	57	1000	18	11	11	1000	62	60	65	100	54	35	40	10	4	5	1	10
FECAL	4	7	2	6	6	100	56	69	63	1000	47	59	52	1000	60	37	30	1000	16	20	11	1000	73	60	77	100	47	15	31	10	1	7	7	10
FECAL	4	8	8	7	10	100	66	46	46	1000	70	64	75	1000	35	56	45	1000	14	18	17	1000	70	65	76	100	12	33	38	10	7	1	13	10
FECAL	4	9	11	7	4	100	67	64	66	1000	48	68	55	1000	41	34	33	1000	17	19	3	1000	70	60	67	100	26	12	12	10	15	7	8	10
FECAL	22	1	6	2	1	100	82	64	84	1000	89	99	87	1000	154	132	104	1000	76	68	76	1000	72	104	93	100	36	43	33	10	20	17	16	10
FECAL	22	2	3	4	5	100	88	89	84	1000	93	117	86	1000	104	78	134	1000	83	63	75	1000	115	75	79	100	67	57	55	10	21	20	15	10
FECAL	22	3	2	8	9	100	72	68	82	1000	100	103	105	1000	122	72	133	1000	73	60	58	1000	71	73	109	100	34	46	58	10	20	12	19	10
FECAL	22	4	1	1	5	100	66	71	81	1000	106	87	124	1000	122	135	107	1000	79	83	65	1000	93	98	106	100	31	36	33	10	19	15	18	10
FECAL	22	5	8	8	2	100	87	90	63	1000	96	110	107	1000	73	161	145	1000	77	59	79	1000	108	87	98	100	23	54	69	10	22	23	18	10
FECAL	22	6	11	12	8	100	72	94	95	1000	121	118	33	1000	155	126	150	1000	82	79	55	1000	75	85	99	100	57	36	55	10	18	19	14	10
FECAL	22	7	2	6	6	100	88	87	74	1000	90	79	122	1000	133	143	130	1000	68	84	86	1000	76	94	102	100	58	35	41	10	22	19	18	10
FECAL	22	8	8	7	10	100	91	90	92	1000	106	91	88	1000	108	142	88	1000	69	53	66	1000	87	109	102	100	29	67	50	10	14	19	22	10
FECAL	22	9	11	7	4	100	72	95	88	1000	83	76	116	1000	99	141	73	1000	64	69	74	1000	108	111	101	100	36	46	31	10	17	15	22	10
FECAL	35	1	6	2	1	100	50	74	43	1000	63	50	76	1000	16	54	38	1000	101	97	62	100	35	37	43	100	44	13	41	10	1	1	1	10
FECAL	35	2	3	4	5	100	72	73	46	1000	53	71	60	1000	42	54	27	1000	63	99	61	100	39	45	46	100	27	24	27	10	1	2	4	10
FECAL	35	3	2	8	9	100	46	47	47	1000	73	50	57	1000	46	35	19	1000	95	102	71	1000	50	27	26	100	34	15	32	10	3	1	5	10
FECAL	35	4	1	1	5	100	73	83	34	1000	61	75	46	1000	21	40	24	1000	71	61	76	1000	53	53	41	100	41	29	42	10	1	1	1	10
FECAL	35	5	8	8	2	100	77	78	47	1000	64	61	88	1000	56	37	34	1000	69	76	104	1000	26	27	40	100	42	38	36	10	2	2	4	10
FECAL	35	6	11	12	8	100	62	83	88	1000	75	83	89	1000	15	39	11	1000	74	101	32	100	27	45	24	100	23	23	26	10	1	3	2	10
FECAL	35	7	2	6	6	100	61	84	46	1000	79	79	90	1000	24	22	23	1000	67	80	77	1000	52	43	44	100	23	33	32	10	5	6	1	10
FECAL	35	8	8	7	10	100	36	78	57	1000	49	90	67	1000	56	13	42	1000	102	72	98	1000	42	33	45	100	27	30	26	10	3	8	7	10
FECAL	35	9	11	7	4	100	61	54	55	1000	83	81	45	1000	34	47	36	1000	97	73	65	1000	40	42	48	100	34	30	46	10	1	6	4	10
WATER	4	1	28	32	29	100	70	77	69	100	28	21	24	1000	35	35	43	1000	27	32	32	1000	97	101	103	100	59	64	45	100	19	23	15	100
WATER	4	2	23	18	26	100	89	73	73	100	23	31	32	1000	37	44	45	1000	32	33	29	1000	102	92	92	100	53	62	64	100	24	17	18	100
WATER	4	3	29	32	25	100	69	76	76	100	25	33	32	1000	38	45	40	1000	27	25	32	1000	105	95	102	100	65	61	55	100	25	15	15	100
WATER	4	4	27	37	22	100	68	88	54	100	21	33	27	1000	39	47	38	1000	23	32	28	1000	92	101	98	100	65	62	64	100	17	23	19	100
WATER	4	5	21	25	26	100	69	70	70	100	23	32	28	1000	35	47	35	1000	32	24	25	1000	104	91	90	100	48	61	58	100	15	17	19	100
WATER	4	6	24	20	23	100	73	75	69	100	28	15	21	1000	40	44	42	1000	27	29	24	1000	104	91	98	100	55	58	44	100	16	20	28	100
WATER	4	7	29	29	28	100	56	91	73	100	33	23	18	1000	44	38	45	1000	23	33	24	1000	103	90	98	100	57	59	54	100	15	27	17	100
WATER	4	8	20	19	27	100	70	75	55	100	27	20	28	1000	48	43	40	1000	28	26	29	1000	104	99	104	100	57	66	44	100	16	27	21	100
WATER	4	9	28	32	25	100	76	73	77	100	27	32	17	1000	45	46	40	1000	34	33	25	1000	93	96	102	100	62	61	48	100	19	22	20	100
WATER	22	1	28	32	29	100	112	110	117	100	54	59	60	1000	63	81	75	1000	52	56	45	1000	26	32	23	1000	89	91	86	100	61	65	56	100
WATER	22	2	23	18	26	100	110	87	110	100	49	57	54	1000	64	76	76	1000	45	48	55	1000	32	23	33	1000	82	89	93	100	57	67	60	100
WATER	22	3	29	32	25	100	106	103	98	100	56	50	62	1000	69	73	66	1000	59	59	47	1000	28	36	26	1000	81	90	86	100	62	67	58	100
WATER	22	4	27	37	22	100	95	104	123	100	61	60	62	1000	73	72	74	1000	45	63	49	1000	31	27	27	1000	89	91	87	100	58	57	61	100
WATER	22	5	21	25	26	100	112	98	108	100	49	58	48	1000	84	62	82	1000	50	45	60	1000	24	25	23	1000	93	89	86	100	59	63	67	100
WATER	22	6	24	20	23	100	113	98	102	100	60	60	48	1000	75	84	66	1000	55	59	45	1000	34	26	25	1000	91	95	85	100	59	65	55	100
WATER	22	7	29	29	28	100	115	117	97	100	62	49	66	1000	65	81	86	1000	45	53	49	1000	25	25	30	1000	88	90	86	100	59	63	63	100
WATER	22	8	20	19	27	100	129	132	100	100	48	62	65	1000	65	72	67	1000	47	54	49	1000	34	35	34	1000	83	94	91	100	57	58	58	100
WATER	22	9	28	32	25	100	107	116	345	100	50	56	64	1000	79	71	77	1000	46	57	54	1000	29	27	24	1000	65	82	85	100	56	61	58	100
WATER	35	1	28	32	29	100	77	83	79	100	58	40	42	1000	67	68	56	1000	26	21	31	1000	3	4	10	1000	63	89	87	100	51	34	52	100
WATER	35	2	23	18	26	100	82	83	69	100	64	55	42	1000	65	59	57	1000	25	32	31	1000	5	7	12	1000	76	77	86	100	35	40	34	100
WATER	35	3	29	32	25	100	81	80	88	100	42	65	65	1000	57	67	60	1000	27	23	30	1000	2	11	5	1000	78	79	85	100	38	49	39	100
WATER	35	4	27	37	22	100	81	88	80	100	51	48	41	1000	57	63	63	1000	21	24	34	1000	7	8	6	1000	83	79	77	100	49	45	36	100
WATER	35	5	21	25	26	100	77	79	86	100	66	52	43	1000	64	61	63	1000	21	30	27	1000	2	2	3	1000	77	87	76	100	43	48	42	100
WATER	35	6	24	20	23	100	80	83	83	100	41	42	55	1000	59	69	63	1000	29	28	23	1000	9	12	12	1000	79	83	86	100	35	42	51	100
WATER	35	7	29	29	28	100	83	78	91	100	40	43	49	1000	55	58	61	1000	21	32	23	1000	10	12	2	1000	79	78	80	100	36	43	46	100
WATER	35	8	20	19	27	100	87	89	78	100	65	53	46	1000	66	53	68	1000	21	23	29	1000	5	8	11	1000	85	76	86	100	43	37	51	100
WATER	35	9	28	32	25	100	81	81	91	100	50	61	53	1000	68	58	56	1000	27	31	31	1000	3	4	5	1000	81	85	79	100	42	39	44	100

Appendix B: Regression Graphs

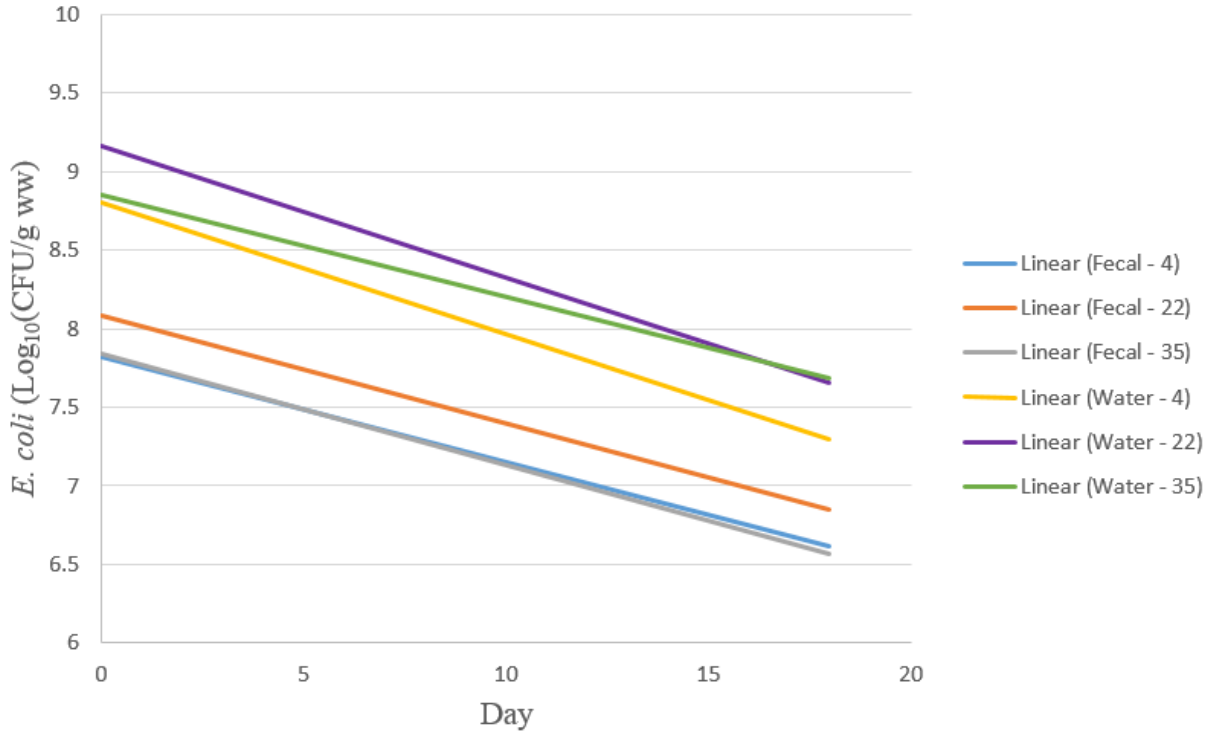
1) Total bacteria



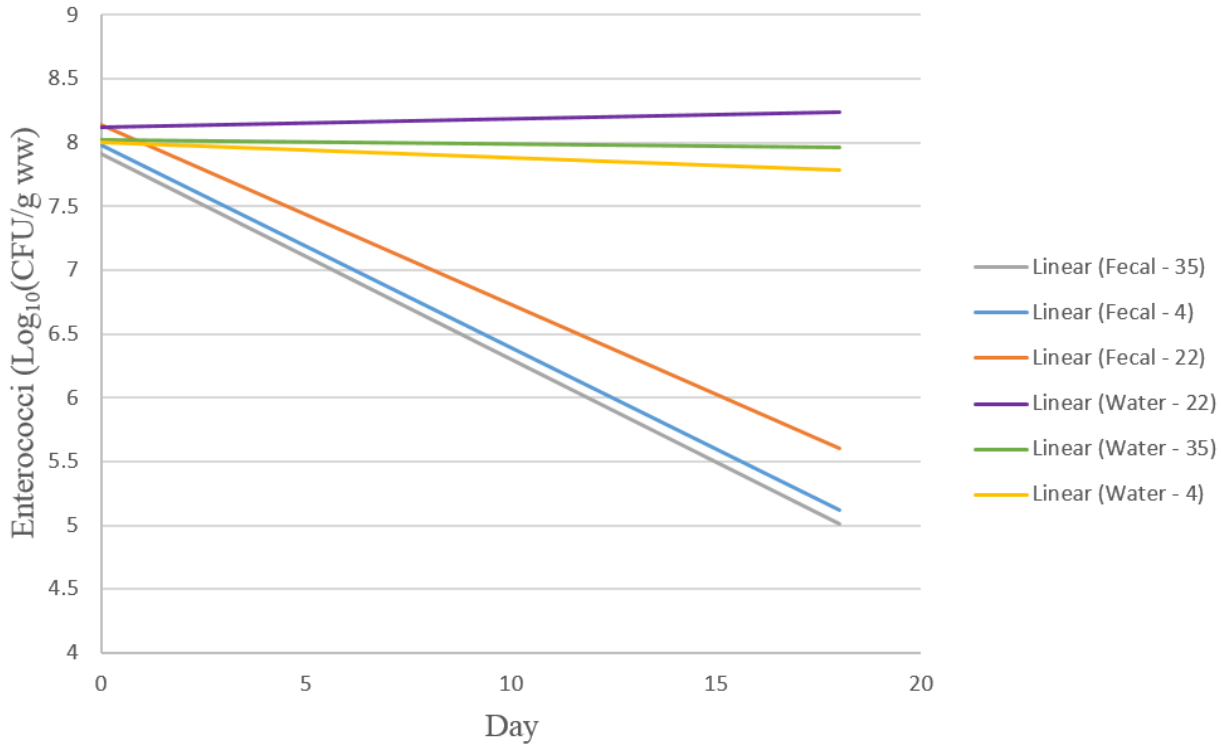
2) Fecal Coliform



3) E. coli



4) Enterococci



Appendix C: SAS Code and Comprehensive Outputs

1) Total bacteria

```
Proc import datafile = "E:\Survivaltest.xlsx"
out = Survivaltest DBMS = excel2000 REPLACE;
SHEET = "Sheet1";
RANGE = "A1:G";
run;
DATA Survivaltest2;
set survivaltest;
run;
Proc print data = Survivaltest2;
run;
proc sort DATA=survivaltest2;
by matrix;
run;
proc glm data = survivaltest2;
   class Temp day matrix;
   model log10CFUgww = Temp|day|matrix;
  OUTPUT out=pred p=ybar r=resid;
run;
proc glm data=Survivaltest2;
   class Temp day matrix;
   model log10CFUgww = Temp|day|matrix;
   means temp day matrix /Bon alpha=.05 cldiff;
  OUTPUT out=pred p=ybar r=resid;
run;
proc glm data=Survivaltest2;
   class Temp day Farm;
   by matrix;
   model log10CFUgww = temp day farm temp*day temp*farm;
   means temp day farm /Bon alpha=.05 cldiff;
  OUTPUT out=pred p=ybar r=resid;
run;
symbol1 v=circle l=32  c = black;
PROC GPLOT data=pred;
 PLOT resid*ybar/vref=0;
By matrix;
run;
PROC UNIVARIATE DATA=survivaltest2 normal;
  QQPLOT resid / normal;
  VAR resid;
By matrix;
run;
```

OUTPUT FOR TOTAL BACTERIA

Comprehensive results of the GLM Procedure

Class Level Information		
Class	Levels	Values
temp	3	4 22 35
day	8	0 2 4 6 8 10 14 18
matrix	2	1 2

Number of Observations Read		1296			
Number of Observations Used		1296			
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	47	1363.787829	29.016762	242.99	<.0001
Error	1248	149.030219	0.119415		

Corrected Total	1295	1512.818048			

R-Square	Coeff Var	Root MSE	log10CFUgww Mean
0.901488	3.865844	0.345565	8.938931

Source	DF	Type I SS	Mean Square	F Value	Pr > F
temp	2	10.7478745	5.3739372	45.00	<.0001
day	7	474.7112786	67.8158969	567.90	<.0001
temp*day	14	5.3144285	0.3796020	3.18	<.0001
matrix	1	831.7769436	831.7769436	6965.42	<.0001
temp*matrix	2	1.2194795	0.6097398	5.11	0.0062
day*matrix	7	35.1235347	5.0176478	42.02	<.0001
temp*day*matrix	14	4.8942896	0.3495921	2.93	0.0002

Source	DF	Type III SS	Mean Square	F Value	Pr > F
temp	2	10.7478745	5.3739372	45.00	<.0001
day	7	474.7112786	67.8158969	567.90	<.0001
temp*day	14	5.3144285	0.3796020	3.18	<.0001
matrix	1	831.7769436	831.7769436	6965.42	<.0001
temp*matrix	2	1.2194795	0.6097398	5.11	0.0062
day*matrix	7	35.1235347	5.0176478	42.02	<.0001
temp*day*matrix	14	4.8942896	0.3495921	2.93	0.0002

Bonferroni (Dunn) t Tests for log10CFUgww – temperature comparisons:

Alpha	0.05
Error Degrees of Freedom	1248
Error Mean Square	0.119415
Critical Value of t	2.39721
Minimum Significant Difference	0.0564

Comparisons significant at the 0.05 level are indicated by ***.				
temp Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
22 - 4	0.16415	0.10778	0.22051	***
22 - 35	0.21288	0.15652	0.26925	***
4 - 22	-0.16415	-0.22051	-0.10778	***
4 - 35	0.04874	-0.00763	0.10510	
35 - 22	-0.21288	-0.26925	-0.15652	***
35 - 4	-0.04874	-0.10510	0.00763	

Bonferroni (Dunn) t Tests for log10CFUgww – day comparisons:

Alpha	0.05
Error Degrees of Freedom	1248
Error Mean Square	0.119415
Critical Value of t	3.13048
Minimum Significant Difference	0.1202

Comparisons significant at the 0.05 level are indicated by ***.				
day Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
4 - 6	0.08265	-0.03755	0.20284	
4 - 8	0.34148	0.22128	0.46168	***
4 - 10	0.73340	0.61320	0.85360	***
4 - 2	1.05828	0.93808	1.17848	***

4 - 14	1.06882	0.94862	1.18902	***
4 - 18	1.59744	1.47724	1.71764	***
4 - 0	1.68871	1.56851	1.80891	***
6 - 4	-0.08265	-0.20284	0.03755	
6 - 8	0.25883	0.13863	0.37903	***
6 - 10	0.65075	0.53055	0.77095	***
6 - 2	0.97563	0.85544	1.09583	***
6 - 14	0.98617	0.86597	1.10637	***
6 - 18	1.51479	1.39460	1.63499	***
6 - 0	1.60606	1.48587	1.72626	***
8 - 4	-0.34148	-0.46168	-0.22128	***
8 - 6	-0.25883	-0.37903	-0.13863	***
8 - 10	0.39192	0.27172	0.51212	***
8 - 2	0.71680	0.59660	0.83700	***
8 - 14	0.72734	0.60714	0.84754	***
8 - 18	1.25596	1.13576	1.37616	***
8 - 0	1.34723	1.22703	1.46743	***
10 - 4	-0.73340	-0.85360	-0.61320	***
10 - 6	-0.65075	-0.77095	-0.53055	***
10 - 8	-0.39192	-0.51212	-0.27172	***
10 - 2	0.32488	0.20468	0.44508	***
10 - 14	0.33542	0.21522	0.45562	***
10 - 18	0.86404	0.74384	0.98424	***
10 - 0	0.95531	0.83511	1.07551	***
2 - 4	-1.05828	-1.17848	-0.93808	***
2 - 6	-0.97563	-1.09583	-0.85544	***
2 - 8	-0.71680	-0.83700	-0.59660	***
2 - 10	-0.32488	-0.44508	-0.20468	***
2 - 14	0.01054	-0.10966	0.13074	
2 - 18	0.53916	0.41896	0.65936	***
2 - 0	0.63043	0.51023	0.75063	***
14 - 4	-1.06882	-1.18902	-0.94862	***
14 - 6	-0.98617	-1.10637	-0.86597	***
14 - 8	-0.72734	-0.84754	-0.60714	***
14 - 10	-0.33542	-0.45562	-0.21522	***
14 - 2	-0.01054	-0.13074	0.10966	
14 - 18	0.52862	0.40842	0.64882	***
14 - 0	0.61989	0.49969	0.74009	***
18 - 4	-1.59744	-1.71764	-1.47724	***
18 - 6	-1.51479	-1.63499	-1.39460	***
18 - 8	-1.25596	-1.37616	-1.13576	***
18 - 10	-0.86404	-0.98424	-0.74384	***
18 - 2	-0.53916	-0.65936	-0.41896	***
18 - 14	-0.52862	-0.64882	-0.40842	***
18 - 0	0.09127	-0.02893	0.21147	
0 - 4	-1.68871	-1.80891	-1.56851	***
0 - 6	-1.60606	-1.72626	-1.48587	***
0 - 8	-1.34723	-1.46743	-1.22703	***
0 - 10	-0.95531	-1.07551	-0.83511	***
0 - 2	-0.63043	-0.75063	-0.51023	***
0 - 14	-0.61989	-0.74009	-0.49969	***
0 - 18	-0.09127	-0.21147	0.02893	

Bonferroni (Dunn) t Tests for log10CFUgww – matrix comparisons:

Alpha	0.05
Error Degrees of Freedom	1248
Error Mean Square	0.119415
Critical Value of t	1.96187
Minimum Significant Difference	0.0377

Comparisons significant at the 0.05 level are indicated by ***.				
matrix Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
2 - 1	1.60225	1.56459	1.63992	***
1 - 2	-1.60225	-1.63992	-1.56459	***

Differences between farms –

Matrix 1 (Fecal) –

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	29	167.6423881	5.7807720	43.00	<.0001
Error	618	83.0865702	0.1344443		
Corrected Total	647	250.7289583			

R-Square	Coeff Var	Root MSE	log10CFUgww Mean
0.668620	4.505717	0.366666	8.137805

Source	DF	Type I SS	Mean Square	F Value	Pr > F
temp	2	9.5775877	4.7887939	35.62	<.0001
day	7	147.5323754	21.0760536	156.76	<.0001
Farm	2	1.5272405	0.7636203	5.68	0.0036
temp*day	14	7.6450235	0.5460731	4.06	<.0001
temp*Farm	4	1.3601609	0.3400402	2.53	0.0396

Source	DF	Type III SS	Mean Square	F Value	Pr > F
temp	2	9.5775877	4.7887939	35.62	<.0001
day	7	147.5323754	21.0760536	156.76	<.0001
Farm	2	1.5272405	0.7636203	5.68	0.0036
temp*day	14	7.6450235	0.5460731	4.06	<.0001
temp*Farm	4	1.3601609	0.3400402	2.53	0.0396

Bonferroni (Dunn) t Tests for log10CFUgww for differences between farms - Matrix = 1 (Fecal)

Alpha	0.05
Error Degrees of Freedom	618
Error Mean Square	0.134444
Critical Value of t	2.40052
Minimum Significant Difference	0.0847

Comparisons significant at the 0.05 level are indicated by ***.				
Farm Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
2 - 3	0.08655	0.00186	0.17125	***
2 - 1	0.11390	0.02920	0.19859	***
3 - 2	-0.08655	-0.17125	-0.00186	***
3 - 1	0.02734	-0.05735	0.11204	
1 - 2	-0.11390	-0.19859	-0.02920	***
1 - 3	-0.02734	-0.11204	0.05735	

Matrix 2 (Water) –

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	29	368.7338553	12.7149605	127.61	<.0001

Error	618	61.5782912	0.0996412		
Corrected Total	647	430.3121465			

R-Square	Coeff Var	Root MSE	log10CFUgww Mean
0.856899	3.240844	0.315660	9.740057

Source	DF	Type I SS	Mean Square	F Value	Pr > F
temp	2	2.3897663	1.1948832	11.99	<.0001
day	7	362.3024380	51.7574911	519.44	<.0001
Farm	2	0.4302055	0.2151027	2.16	0.1163
temp*day	14	2.5636946	0.1831210	1.84	0.0304
temp*Farm	4	1.0477509	0.2619377	2.63	0.0336

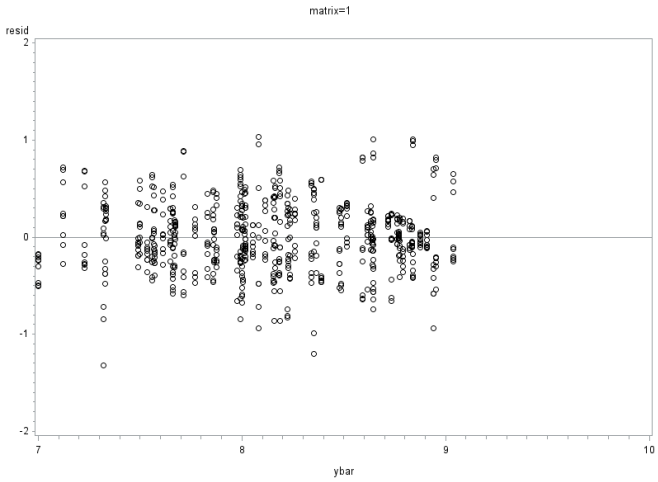
Source	DF	Type III SS	Mean Square	F Value	Pr > F
temp	2	2.3897663	1.1948832	11.99	<.0001
day	7	362.3024380	51.7574911	519.44	<.0001
Farm	2	0.4302055	0.2151027	2.16	0.1163
temp*day	14	2.5636946	0.1831210	1.84	0.0304
temp*Farm	4	1.0477509	0.2619377	2.63	0.0336

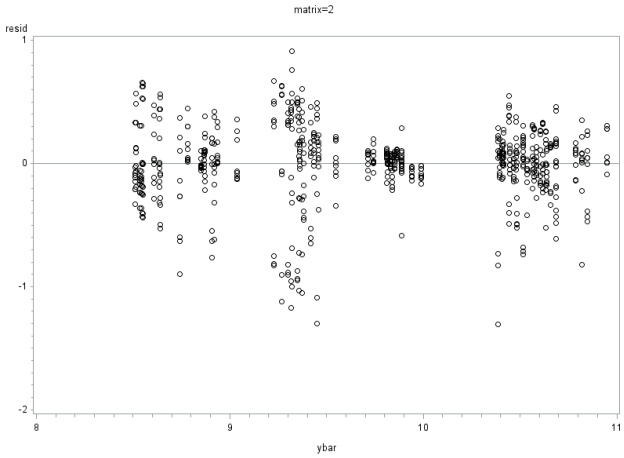
Bonferroni (Dunn) t Tests for log10CFUgww for differences between farms - Matrix = 2 (Water)

Alpha	0.05
Error Degrees of Freedom	618
Error Mean Square	0.099641
Critical Value of t	2.40052
Minimum Significant Difference	0.0729

Comparisons significant at the 0.05 level are indicated by ***.				
Farm Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
3 - 2	0.00525	-0.06767	0.07816	
3 - 1	0.05709	-0.01582	0.13001	
2 - 3	-0.00525	-0.07816	0.06767	
2 - 1	0.05185	-0.02107	0.12476	
1 - 3	-0.05709	-0.13001	0.01582	
1 - 2	-0.05185	-0.12476	0.02107	

QQ Plots - residuals





2) Fecal coliform

```
Proc import datafile = "E:\fcsurvival.xlsx"
out = fcsurvival DBMS = excel2000 REPLACE;
SHEET = "Sheet1";
RANGE = "A1:G";
run;
DATA fcsurvival2;
set fcsurvival;
run;
Proc print data = fcsurvival2;
run;
proc glm data = fcsurvival2;
   class Temp day matrix;
   model log10CFUgww = Temp|day|matrix;
  OUTPUT out=pred p=ybar r=resid;
run;
proc glm data=fcsurvival2;
   class Temp day matrix;
   model log10CFUgww = Temp|day|matrix;
   means temp day matrix /Bon alpha=.05 cldiff;
  OUTPUT out=pred p=ybar r=resid;
run;
proc glm data=fcsurvival2;
```

```
   class Temp day Farm;
   by matrix;
   model log10CFUgww = temp day farm temp*day temp*farm;
   means temp day farm /Bon alpha=.05 cldiff;
  OUTPUT out=pred p=ybar r=resid;
run;
symbol1 v=circle l=32  c = black;
PROC GPLOT data=pred;
 PLOT resid*ybar/vref=0;
By matrix;
run;
PROC UNIVARIATE DATA=fcsurvival2 normal;
  QQPLOT resid / normal;
  VAR resid;
By matrix;
run;
```

Comprehensive results of the GLM Procedure

Class Level Information		
Class	Levels	Values
temp	3	4 22 35
day	8	0 2 4 6 8 10 14 18
matrix	2	1 2

Number of Observations Read	1296
Number of Observations Used	1296

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	47	616.1548567	13.1096778	500.63	<.0001
Error	1248	32.6806888	0.0261864		
Corrected Total	1295	648.8355455			

R-Square	Coeff Var	Root MSE	log10CFUgww Mean
0.949632	2.033307	0.161822	7.958575

Source	DF	Type I SS	Mean Square	F Value	Pr > F
temp	2	14.7033712	7.3516856	280.74	<.0001
day	7	202.9582549	28.9940364	1107.22	<.0001
temp*day	14	28.1933941	2.0138139	76.90	<.0001
matrix	1	324.3713763	324.3713763	12387.0	<.0001
temp*matrix	2	3.3326064	1.6663032	63.63	<.0001
day*matrix	7	24.5679086	3.5097012	134.03	<.0001
temp*day*matrix	14	18.0279451	1.2877104	49.17	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
temp	2	14.7033712	7.3516856	280.74	<.0001
day	7	202.9582549	28.9940364	1107.22	<.0001
temp*day	14	28.1933941	2.0138139	76.90	<.0001
matrix	1	324.3713763	324.3713763	12387.0	<.0001
temp*matrix	2	3.3326064	1.6663032	63.63	<.0001
day*matrix	7	24.5679086	3.5097012	134.03	<.0001
temp*day*matrix	14	18.0279451	1.2877104	49.17	<.0001

Bonferroni (Dunn) t Tests for log10CFUgww – temperature comparisons:

Alpha	0.05
Error Degrees of Freedom	1248
Error Mean Square	0.026186
Critical Value of t	2.39721

Minimum Significant Difference	0.0264

Comparisons significant at the 0.05 level are indicated by ***.				
temp Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
22 - 35	0.20857	0.18218	0.23496	***
22 - 4	0.24003	0.21364	0.26643	***
35 - 22	-0.20857	-0.23496	-0.18218	***
35 - 4	0.03146	0.00507	0.05786	***
4 - 22	-0.24003	-0.26643	-0.21364	***
4 - 35	-0.03146	-0.05786	-0.00507	***

Bonferroni (Dunn) t Tests for log10CFUgww – day comparisons:

Alpha	0.05
Error Degrees of Freedom	1248
Error Mean Square	0.026186
Critical Value of t	3.13048
Minimum Significant Difference	0.0563

Comparisons significant at the 0.05 level are indicated by ***.				
day Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
4 - 6	0.22942	0.17313	0.28571	***
4 - 2	0.39231	0.33602	0.44860	***
4 - 8	0.40387	0.34758	0.46016	***
4 - 0	0.61578	0.55950	0.67207	***
4 - 14	0.78710	0.73081	0.84339	***
4 - 10	0.78824	0.73195	0.84453	***
4 - 18	1.37998	1.32370	1.43627	***
6 - 4	-0.22942	-0.28571	-0.17313	***
6 - 2	0.16289	0.10660	0.21917	***
6 - 8	0.17445	0.11816	0.23074	***
6 - 0	0.38636	0.33008	0.44265	***
6 - 14	0.55768	0.50139	0.61397	***
6 - 10	0.55882	0.50253	0.61511	***
6 - 18	1.15056	1.09428	1.20685	***
2 - 4	-0.39231	-0.44860	-0.33602	***
2 - 6	-0.16289	-0.21917	-0.10660	***
2 - 8	0.01156	-0.04472	0.06785	
2 - 0	0.22347	0.16719	0.27976	***
2 - 14	0.39479	0.33851	0.45108	***
2 - 10	0.39593	0.33964	0.45222	***
2 - 18	0.98767	0.93139	1.04396	***
8 - 4	-0.40387	-0.46016	-0.34758	***
8 - 6	-0.17445	-0.23074	-0.11816	***
8 - 2	-0.01156	-0.06785	0.04472	
8 - 0	0.21191	0.15563	0.26820	***
8 - 14	0.38323	0.32694	0.43952	***
8 - 10	0.38437	0.32808	0.44065	***
8 - 18	0.97611	0.91982	1.03240	***
0 - 4	-0.61578	-0.67207	-0.55950	***
0 - 6	-0.38636	-0.44265	-0.33008	***
0 - 2	-0.22347	-0.27976	-0.16719	***
0 - 8	-0.21191	-0.26820	-0.15563	***
0 - 14	0.17132	0.11503	0.22760	***
0 - 10	0.17246	0.11617	0.22874	***

0 - 18	0.76420	0.70791	0.82049	***
14 - 4	-0.78710	-0.84339	-0.73081	***
14 - 6	-0.55768	-0.61397	-0.50139	***
14 - 2	-0.39479	-0.45108	-0.33851	***
14 - 8	-0.38323	-0.43952	-0.32694	***
14 - 0	-0.17132	-0.22760	-0.11503	***
14 - 10	0.00114	-0.05515	0.05743	
14 - 18	0.59288	0.53660	0.64917	***
10 - 4	-0.78824	-0.84453	-0.73195	***
10 - 6	-0.55882	-0.61511	-0.50253	***
10 - 2	-0.39593	-0.45222	-0.33964	***
10 - 8	-0.38437	-0.44065	-0.32808	***
10 - 0	-0.17246	-0.22874	-0.11617	***
10 - 14	-0.00114	-0.05743	0.05515	
10 - 18	0.59174	0.53546	0.64803	***
18 - 4	-1.37998	-1.43627	-1.32370	***
18 - 6	-1.15056	-1.20685	-1.09428	***
18 - 2	-0.98767	-1.04396	-0.93139	***
18 - 8	-0.97611	-1.03240	-0.91982	***
18 - 0	-0.76420	-0.82049	-0.70791	***
18 - 14	-0.59288	-0.64917	-0.53660	***
18 - 10	-0.59174	-0.64803	-0.53546	***

Bonferroni (Dunn) t Tests for log10CFUgww – matrix comparisons:

Alpha	0.05
Error Degrees of Freedom	1248
Error Mean Square	0.026186
Critical Value of t	1.96187
Minimum Significant Difference	0.0176

Comparisons significant at the 0.05 level are indicated by ***.				
matrix Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
2 - 1	1.000573	0.982936	1.018210	***
1 - 2	-1.000573	-1.018210	-0.982936	***

Differences between farms –

Matrix 1 (Fecal) –

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	29	143.8955079	4.9619141	191.66	<.0001
Error	618	15.9994650	0.0258891		
Corrected Total	647	159.8949729			

Square	Coeff Var	Root MSE	log10CFUgww Mean
0.899938	2.157344	0.160901	7.458288

Source	DF	Type I SS	Mean Square	F Value	Pr > F
temp	2	5.3184298	2.6592149	102.72	<.0001
day	7	110.9550319	15.8507188	612.25	<.0001
Farm	2	0.0529218	0.0264609	1.02	0.3605
temp*day	14	27.5058209	1.9647015	75.89	<.0001
temp*Farm	4	0.0633035	0.0158259	0.61	0.6546

Source	DF	Type III SS	Mean Square	F Value	Pr > F
temp	2	5.3184298	2.6592149	102.72	<.0001

day	7	110.9550319	15.8507188	612.25	<.0001
Farm	2	0.0529218	0.0264609	1.02	0.3605
temp*day	14	27.5058209	1.9647015	75.89	<.0001
temp*Farm	4	0.0633035	0.0158259	0.61	0.6546

Bonferroni (Dunn) t Tests for log10CFUgww for differences between farms - Matrix = 1 (Fecal)

Alpha	0.05
Error Degrees of Freedom	618
Error Mean Square	0.025889
Critical Value of t	2.40052
Minimum Significant Difference	0.0372

Comparisons significant at the 0.05 level are indicated by ***.				
Farm Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
1 - 3	0.01768	-0.01949	0.05485	
1 - 2	0.02038	-0.01679	0.05754	
3 - 1	-0.01768	-0.05485	0.01949	
3 - 2	0.00270	-0.03447	0.03986	
2 - 1	-0.02038	-0.05754	0.01679	
2 - 3	-0.00270	-0.03986	0.03447	

Matrix 2 (Water) –

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	29	148.0750432	5.1060360	191.31	<.0001
Error	618	16.4941531	0.0266896		
Corrected Total	647	164.5691963			

R-Square	Coeff Var	Root MSE	log10CFUgww Mean
0.899774	1.931341	0.163369	8.458861

Source	DF	Type I SS	Mean Square	F Value	Pr > F
temp	2	12.7175478	6.3587739	238.25	<.0001
day	7	116.5711317	16.6530188	623.95	<.0001
Farm	2	0.0162610	0.0081305	0.30	0.7375
temp*day	14	18.7155183	1.3368227	50.09	<.0001
temp*Farm	4	0.0545843	0.0136461	0.51	0.7275

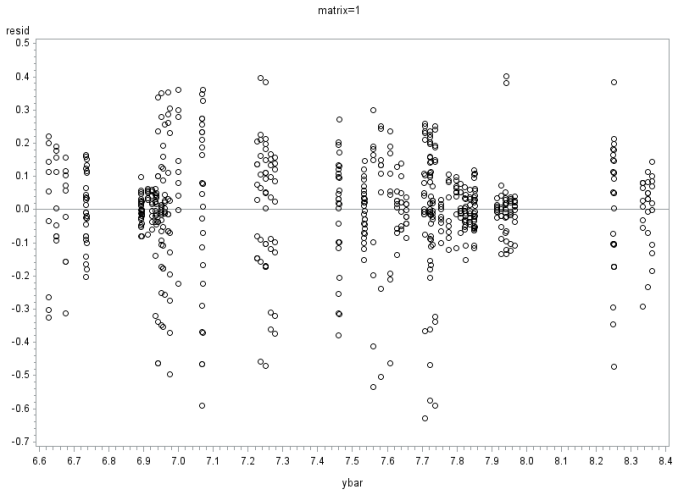
Source	DF	Type III SS	Mean Square	F Value	Pr > F
temp	2	12.7175478	6.3587739	238.25	<.0001
day	7	116.5711317	16.6530188	623.95	<.0001
Farm	2	0.0162610	0.0081305	0.30	0.7375
temp*day	14	18.7155183	1.3368227	50.09	<.0001
temp*Farm	4	0.0545843	0.0136461	0.51	0.7275

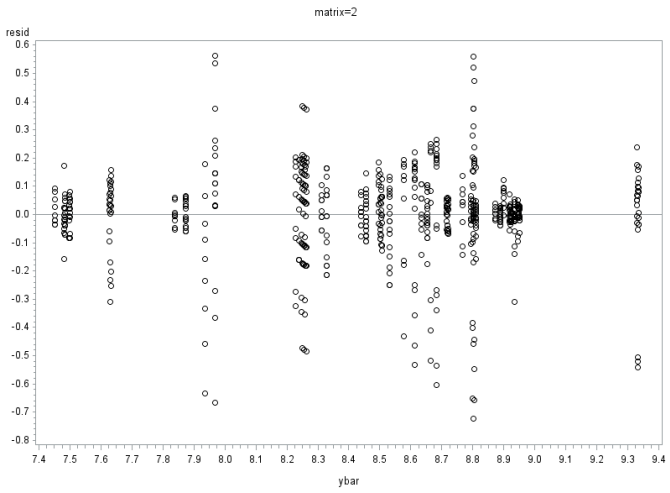
Bonferroni (Dunn) t Tests for log10CFUgww for differences between farms - Matrix = 2 (Water)

Alpha	0.05
Error Degrees of Freedom	618
Error Mean Square	0.02669
Critical Value of t	2.40052
Minimum Significant Difference	0.0377

Comparisons significant at the 0.05 level are indicated by ***.				
Farm Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
3 - 2	0.00519	-0.03254	0.04293	
3 - 1	0.01222	-0.02551	0.04996	
2 - 3	-0.00519	-0.04293	0.03254	
2 - 1	0.00703	-0.03071	0.04477	
1 - 3	-0.01222	-0.04996	0.02551	
1 - 2	-0.00703	-0.04477	0.03071	

QQ Plots – residuals





3) *E. coli*

```
Proc import datafile = "E:\ecsurvival.xlsx"
out = ecsurvival DBMS = excel2000 REPLACE;
SHEET = "Sheet1";
```

```
RANGE = "A1:G";
run;
DATA ecsurvival2;
set ecsurvival;
run;
Proc print data = ecsurvival2;
run;
proc glm data = ecsurvival2;
   class Temperature Day Matrix;
   model log10CFUgww = Temperature|Day|Matrix;
  OUTPUT out=pred p=ybar r=resid;
run;
proc glm data=ecsurvival2;
   class Temperature Day Matrix;
   model log10CFUgww = Temperature|Day|Matrix;
   means Temperature Day Matrix /Bon alpha=.05 cldiff;
  OUTPUT out=pred p=ybar r=resid;
run;
proc glm data=ecsurvival2;
   class Temperature Day Farm;
   by matrix;
   model log10CFUgww = Temperature Day Farm Temperature*Day Temperature*Farm;
   means Temperature Day Farm /Bon alpha=.05 cldiff;
  OUTPUT out=pred p=ybar r=resid;
run;
symbol1 v=circle l=32  c = black;
PROC GPLOT data=pred;
 PLOT resid*ybar/vref=0;
By matrix;
run;
PROC UNIVARIATE DATA=ecsurvival2 normal;
  QQPLOT resid / normal;
  VAR resid;
By matrix;
run;
```

Comprehensive results of the GLM Procedure

Class Level Information		
Class	Levels	Values
Temperature	3	4 22 35
Day	8	0 2 4 6 8 10 14 18
Matrix	2	1 2

Number of Observations Read	1296
Number of Observations Used	1296

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	47	735.2120750	15.6428101	725.83	<.0001
Error	1248	26.8966026	0.0215518		
Corrected Total	1295	762.1086775			

R-Square	Coeff Var	Root MSE	log10CFUgww Mean
0.964708	1.876870	0.146805	7.821808

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Temperature	2	22.1789312	11.0894656	514.55	<.0001
Day	7	318.0227172	45.4318167	2108.03	<.0001
Temperature*Day	14	33.1878412	2.3705601	109.99	<.0001
Matrix	1	329.8102331	329.8102331	15303.2	<.0001
Temperature*Matrix	2	2.3068450	1.1534225	53.52	<.0001

Day*Matrix	7	16.1075241	2.3010749	106.77	<.0001
Temperatu*Day*Matrix	14	13.5979832	0.9712845	45.07	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Temperature	2	22.1789312	11.0894656	514.55	<.0001
Day	7	318.0227172	45.4318167	2108.03	<.0001
Temperature*Day	14	33.1878412	2.3705601	109.99	<.0001
Matrix	1	329.8102331	329.8102331	15303.2	<.0001
Temperature*Matrix	2	2.3068450	1.1534225	53.52	<.0001
Day*Matrix	7	16.1075241	2.3010749	106.77	<.0001
Temperatu*Day*Matrix	14	13.5979832	0.9712845	45.07	<.0001

Bonferroni (Dunn) t Tests for log10CFUgww – temperature comparisons:

Alpha	0.05
Error Degrees of Freedom	1248
Error Mean Square	0.021552
Critical Value of t	2.39721
Minimum Significant Difference	0.0239

Comparisons significant at the 0.05 level are indicated by ***.				
Temperature Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
22 - 35	0.223555	0.199609	0.247500	***
22 - 4	0.310593	0.286648	0.334538	***
35 - 22	-0.223555	-0.247500	-0.199609	***
35 - 4	0.087038	0.063093	0.110984	***
4 - 22	-0.310593	-0.334538	-0.286648	***
4 - 35	-0.087038	-0.110984	-0.063093	***

Bonferroni (Dunn) t Tests for log10CFUgww – day comparisons:

Alpha	0.05
Error Degrees of Freedom	1248
Error Mean Square	0.021552
Critical Value of t	3.13048
Minimum Significant Difference	0.0511

Comparisons significant at the 0.05 level are indicated by ***.				
Day Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
4 - 6	0.43691	0.38585	0.48798	***
4 - 2	0.59133	0.54027	0.64239	***
4 - 8	0.72025	0.66918	0.77131	***
4 - 0	0.90827	0.85721	0.95934	***
4 - 10	1.02735	0.97628	1.07841	***
4 - 14	1.46459	1.41353	1.51566	***
4 - 18	1.60755	1.55649	1.65861	***
6 - 4	-0.43691	-0.48798	-0.38585	***
6 - 2	0.15442	0.10335	0.20548	***
6 - 8	0.28333	0.23227	0.33440	***
6 - 0	0.47136	0.42030	0.52242	***
6 - 10	0.59043	0.53937	0.64150	***
6 - 14	1.02768	0.97662	1.07874	***
6 - 18	1.17064	1.11957	1.22170	***
2 - 4	-0.59133	-0.64239	-0.54027	***
2 - 6	-0.15442	-0.20548	-0.10335	***
2 - 8	0.12892	0.07785	0.17998	***
2 - 0	0.31694	0.26588	0.36801	***
2 - 10	0.43602	0.38495	0.48708	***

2 - 14	0.87326	0.82220	0.92433	***
2 - 18	1.01622	0.96516	1.06728	***
8 - 4	-0.72025	-0.77131	-0.66918	***
8 - 6	-0.28333	-0.33440	-0.23227	***
8 - 2	-0.12892	-0.17998	-0.07785	***
8 - 0	0.18803	0.13696	0.23909	***
8 - 10	0.30710	0.25604	0.35816	***
8 - 14	0.74435	0.69328	0.79541	***
8 - 18	0.88730	0.83624	0.93837	***
0 - 4	-0.90827	-0.95934	-0.85721	***
0 - 6	-0.47136	-0.52242	-0.42030	***
0 - 2	-0.31694	-0.36801	-0.26588	***
0 - 8	-0.18803	-0.23909	-0.13696	***
0 - 10	0.11907	0.06801	0.17014	***
0 - 14	0.55632	0.50526	0.60738	***
0 - 18	0.69928	0.64821	0.75034	***
10 - 4	-1.02735	-1.07841	-0.97628	***
10 - 6	-0.59043	-0.64150	-0.53937	***
10 - 2	-0.43602	-0.48708	-0.38495	***
10 - 8	-0.30710	-0.35816	-0.25604	***
10 - 0	-0.11907	-0.17014	-0.06801	***
10 - 14	0.43725	0.38618	0.48831	***
10 - 18	0.58020	0.52914	0.63127	***
14 - 4	-1.46459	-1.51566	-1.41353	***
14 - 6	-1.02768	-1.07874	-0.97662	***
14 - 2	-0.87326	-0.92433	-0.82220	***
14 - 8	-0.74435	-0.79541	-0.69328	***
14 - 0	-0.55632	-0.60738	-0.50526	***
14 - 10	-0.43725	-0.48831	-0.38618	***
14 - 18	0.14296	0.09189	0.19402	***
18 - 4	-1.60755	-1.65861	-1.55649	***
18 - 6	-1.17064	-1.22170	-1.11957	***
18 - 2	-1.01622	-1.06728	-0.96516	***
18 - 8	-0.88730	-0.93837	-0.83624	***
18 - 0	-0.69928	-0.75034	-0.64821	***
18 - 10	-0.58020	-0.63127	-0.52914	***
18 - 14	-0.14296	-0.19402	-0.09189	***

Bonferroni (Dunn) t Tests for log10CFUgww – matrix comparisons:

Alpha	0.05
Error Degrees of Freedom	1248
Error Mean Square	0.021552
Critical Value of t	1.96187
Minimum Significant Difference	0.016

Comparisons significant at the 0.05 level are indicated by ***.				
Matrix Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
2 - 1	1.008927	0.992926	1.024927	***
1 - 2	-1.008927	-1.024927	-0.992926	***

Differences between farms –

Matrix 1 (Fecal) –

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	29	178.4433144	6.1532177	253.63	<.0001

Error	618	14.9931787	0.0242608		
Corrected Total	647	193.4364932			

R-Square	Coeff Var	Root MSE	log10CFUgww Mean
0.922490	2.128625	0.155759	7.317345

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Temperature	2	9.0703841	4.5351921	186.93	<.0001
Day	7	160.1174236	22.8739177	942.83	<.0001
Farm	2	0.1245302	0.0622651	2.57	0.0776
Temperature*Day	14	9.0844117	0.6488866	26.75	<.0001
Temperature*Farm	4	0.0465648	0.0116412	0.48	0.7506

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Temperature	2	9.0703841	4.5351921	186.93	<.0001
Day	7	160.1174236	22.8739177	942.83	<.0001
Farm	2	0.1245302	0.0622651	2.57	0.0776
Temperature*Day	14	9.0844117	0.6488866	26.75	<.0001
Temperature*Farm	4	0.0465648	0.0116412	0.48	0.7506

Bonferroni (Dunn) t Tests for log10CFUgww for differences between farms - Matrix = 1 (Fecal)

Alpha	0.05
Error Degrees of Freedom	618
Error Mean Square	0.024261
Critical Value of t	2.40052
Minimum Significant Difference	0.036

Comparisons significant at the 0.05 level are indicated by ***.				
Farm Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
2 - 3	0.02296	-0.01302	0.05894	
2 - 1	0.03315	-0.00283	0.06912	
3 - 2	-0.02296	-0.05894	0.01302	
3 - 1	0.01018	-0.02579	0.04616	
1 - 2	-0.03315	-0.06912	0.00283	
1 - 3	-0.01018	-0.04616	0.02579	

Matrix 2 (Water) –

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	29	227.1897793	7.8341303	414.79	<.0001
Error	618	11.6721719	0.0188870		
Corrected Total	647	238.8619513			

R-Square	Coeff Var	Root MSE	log10CFUgww Mean
0.951134	1.650559	0.137430	8.326272

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Temperature	2	15.4153921	7.7076961	408.10	<.0001
Day	7	174.0128177	24.8589740	1316.19	<.0001
Farm	2	0.0091746	0.0045873	0.24	0.7844
Temperature*Day	14	37.7014126	2.6929580	142.58	<.0001
Temperature*Farm	4	0.0509823	0.0127456	0.67	0.6096

Source	DF	Type III SS	Mean Square	F Value	Pr > F

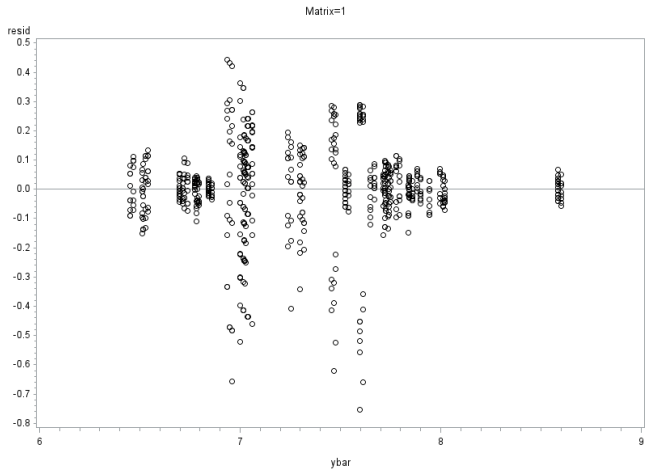
Temperature	2	15.4153921	7.7076961	408.10	<.0001
Day	7	174.0128177	24.8589740	1316.19	<.0001
Farm	2	0.0091746	0.0045873	0.24	0.7844
Temperature*Day	14	37.7014126	2.6929580	142.58	<.0001
Temperature*Farm	4	0.0509823	0.0127456	0.67	0.6096

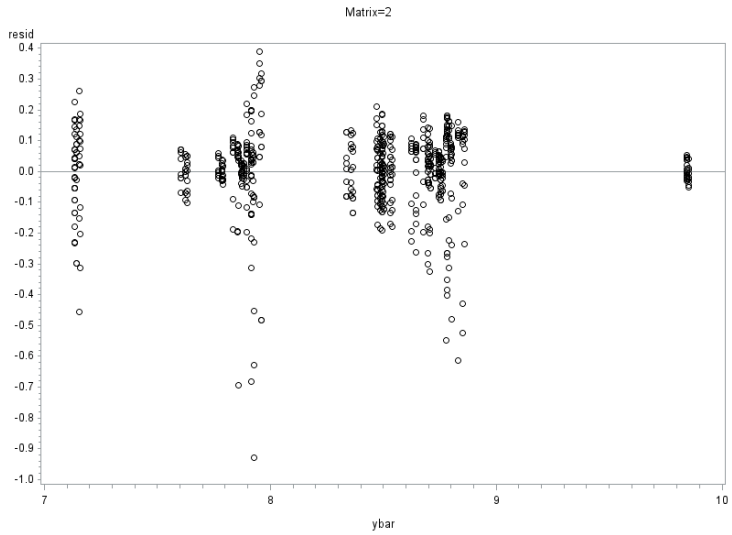
Bonferroni (Dunn) t Tests for log10CFUgww for differences between farms - Matrix = 2 (Water)

Alpha	0.05
Error Degrees of Freedom	618
Error Mean Square	0.018887
Critical Value of t	2.40052
Minimum Significant Difference	0.0317

Comparisons significant at the 0.05 level are indicated by ***.				
Farm Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
3 - 1	0.006337	-0.025408	0.038082	
3 - 2	0.008965	-0.022780	0.040709	
1 - 3	-0.006337	-0.038082	0.025408	
1 - 2	0.002627	-0.029118	0.034372	
2 - 3	-0.008965	-0.040709	0.022780	
2 - 1	-0.002627	-0.034372	0.029118	

QQ Plots - residuals





4) Enterococci

```
Proc import datafile = "E:\entsurvival.xlsx"
out = entsurvival DBMS = excel2000 REPLACE;
SHEET = "Sheet1";
RANGE = "A1:G";
run;
DATA entsurvival2;
set entsurvival;
run;
proc glm data = entsurvival2;
   class Temperature Day Matrix;
   model log10CFUgww = Temperature|Day|Matrix;
  OUTPUT out=pred p=ybar r=resid;
run;
proc glm data=entsurvival2;
   class Temperature Day Matrix;
   model log10CFUgww = Temperature|Day|Matrix;
   means Temperature Day Matrix /Bon alpha=.05 cldiff;
  OUTPUT out=pred p=ybar r=resid;
run;
proc glm data=entsurvival2;
   class Temperature Day Farm;
   by matrix;
   model log10CFUgww = Temperature Day Farm Temperature*Day Temperature*Farm;
   means Temperature Day Farm /Bon alpha=.05 cldiff;
  OUTPUT out=pred p=ybar r=resid;
run;
symbol1 v=circle l=32  c = black;
PROC GPLOT data=pred;
 PLOT resid*ybar/vref=0;
By matrix;
run;
PROC UNIVARIATE DATA=entsurvival2 normal;
  QQPLOT resid / normal;
  VAR resid;
By matrix;
run;
```

Comprehensive results of the GLM Procedure

Class Level Information		
Class	Levels	Values

Temperature	3	4 22 35
Day	8	0 2 4 6 8 10 14 18
Matrix	2	1 2

Number of Observations Read	1296
Number of Observations Used	1296

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	47	1605.636924	34.162488	865.56	<.0001
Error	1248	49.256707	0.039469		
Corrected Total	1295	1654.893631			

R-Square	Coeff Var	Root MSE	log10CFUgww Mean
0.970236	2.699892	0.198667	7.358325

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Temperature	2	14.2506414	7.1253207	180.53	<.0001
Day	7	741.0996953	105.8713850	2682.43	<.0001
Temperature*Day	14	11.3115200	0.8079657	20.47	<.0001
Matrix	1	551.6360772	551.6360772	13976.6	<.0001
Temperature*Matrix	2	3.6550282	1.8275141	46.30	<.0001
Day*Matrix	7	275.3839328	39.3405618	996.76	<.0001
Temperatu*Day*Matrix	14	8.3000288	0.5928592	15.02	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Temperature	2	14.2506414	7.1253207	180.53	<.0001
Day	7	741.0996953	105.8713850	2682.43	<.0001
Temperature*Day	14	11.3115200	0.8079657	20.47	<.0001
Matrix	1	551.6360772	551.6360772	13976.6	<.0001
Temperature*Matrix	2	3.6550282	1.8275141	46.30	<.0001
Day*Matrix	7	275.3839328	39.3405618	996.76	<.0001
Temperatu*Day*Matrix	14	8.3000288	0.5928592	15.02	<.0001

Bonferroni (Dunn) t Tests for log10CFUgww – temperature comparisons:

Alpha	0.05
Error Degrees of Freedom	1248
Error Mean Square	0.039469
Critical Value of t	2.39721
Minimum Significant Difference	0.0324

Comparisons significant at the 0.05 level are indicated by ***.				
Temperature Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
22 - 35	0.19165	0.15924	0.22405	***
22 - 4	0.24393	0.21152	0.27633	***
35 - 22	-0.19165	-0.22405	-0.15924	***
35 - 4	0.05228	0.01988	0.08469	***
4 - 22	-0.24393	-0.27633	-0.21152	***
4 - 35	-0.05228	-0.08469	-0.01988	***

Bonferroni (Dunn) t Tests for log10CFUgww – day comparisons:

Alpha	0.05
Error Degrees of Freedom	1248
Error Mean Square	0.039469
Critical Value of t	3.13048
Minimum Significant Difference	0.0691

Comparisons significant at the 0.05 level are indicated by ***.

Day Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
4 - 6	0.00691	-0.06219	0.07601	
4 - 8	0.22146	0.15236	0.29056	***
4 - 2	0.62268	0.55357	0.69178	***
4 - 10	0.89021	0.82111	0.95932	***
4 - 14	1.54385	1.47475	1.61295	***
4 - 0	1.70369	1.63459	1.77279	***
4 - 18	2.07118	2.00207	2.14028	***
6 - 4	-0.00691	-0.07601	0.06219	
6 - 8	0.21455	0.14545	0.28365	***
6 - 2	0.61577	0.54666	0.68487	***
6 - 10	0.88330	0.81420	0.95241	***
6 - 14	1.53694	1.46784	1.60604	***
6 - 0	1.69678	1.62768	1.76588	***
6 - 18	2.06427	1.99516	2.13337	***
8 - 4	-0.22146	-0.29056	-0.15236	***
8 - 6	-0.21455	-0.28365	-0.14545	***
8 - 2	0.40122	0.33211	0.47032	***
8 - 10	0.66875	0.59965	0.73786	***
8 - 14	1.32239	1.25329	1.39149	***
8 - 0	1.48223	1.41313	1.55133	***
8 - 18	1.84972	1.78061	1.91882	***
2 - 4	-0.62268	-0.69178	-0.55357	***
2 - 6	-0.61577	-0.68487	-0.54666	***
2 - 8	-0.40122	-0.47032	-0.33211	***
2 - 10	0.26754	0.19844	0.33664	***
2 - 14	0.92117	0.85207	0.99028	***
2 - 0	1.08101	1.01191	1.15011	***
2 - 18	1.44850	1.37940	1.51760	***
10 - 4	-0.89021	-0.95932	-0.82111	***
10 - 6	-0.88330	-0.95241	-0.81420	***
10 - 8	-0.66875	-0.73786	-0.59965	***
10 - 2	-0.26754	-0.33664	-0.19844	***
10 - 14	0.65364	0.58453	0.72274	***
10 - 0	0.81347	0.74437	0.88258	***
10 - 18	1.18096	1.11186	1.25006	***
14 - 4	-1.54385	-1.61295	-1.47475	***
14 - 6	-1.53694	-1.60604	-1.46784	***
14 - 8	-1.32239	-1.39149	-1.25329	***
14 - 2	-0.92117	-0.99028	-0.85207	***
14 - 10	-0.65364	-0.72274	-0.58453	***
14 - 0	0.15984	0.09074	0.22894	***
14 - 18	0.52733	0.45822	0.59643	***
0 - 4	-1.70369	-1.77279	-1.63459	***
0 - 6	-1.69678	-1.76588	-1.62768	***
0 - 8	-1.48223	-1.55133	-1.41313	***
0 - 2	-1.08101	-1.15011	-1.01191	***
0 - 10	-0.81347	-0.88258	-0.74437	***
0 - 14	-0.15984	-0.22894	-0.09074	***
0 - 18	0.36749	0.29838	0.43659	***
18 - 4	-2.07118	-2.14028	-2.00207	***
18 - 6	-2.06427	-2.13337	-1.99516	***
18 - 8	-1.84972	-1.91882	-1.78061	***
18 - 2	-1.44850	-1.51760	-1.37940	***
18 - 10	-1.18096	-1.25006	-1.11186	***
18 - 14	-0.52733	-0.59643	-0.45822	***
18 - 0	-0.36749	-0.43659	-0.29838	***

Bonferroni (Dunn) t Tests for log10CFUgww – matrix comparisons:

Alpha	0.05
Error Degrees of Freedom	1248
Error Mean Square	0.039469
Critical Value of t	1.96187
Minimum Significant Difference	0.0217

Comparisons significant at the 0.05 level are indicated by ***.				
Matrix Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
2 - 1	1.30483	1.28318	1.32648	***
1 - 2	-1.30483	-1.32648	-1.28318	***

Differences between farms –

Matrix 1 (Fecal) –

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	29	873.7603552	30.1296674	629.78	<.0001
Error	618	29.5660019	0.0478414		
Corrected Total	647	903.3263571			

R-Square	Coeff Var	Root MSE	log10CFUgww Mean
0.967270	3.261702	0.218727	6.705910

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Temperature	2	14.6706696	7.3353348	153.33	<.0001
Day	7	844.7651416	120.6807345	2522.52	<.0001
Farm	2	0.7627828	0.3813914	7.97	0.0004
Temperature*Day	14	13.5011540	0.9643681	20.16	<.0001
Temperature*Farm	4	0.0606071	0.0151518	0.32	0.8669

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Temperature	2	14.6706696	7.3353348	153.33	<.0001
Day	7	844.7651416	120.6807345	2522.52	<.0001
Farm	2	0.7627828	0.3813914	7.97	0.0004
Temperature*Day	14	13.5011540	0.9643681	20.16	<.0001
Temperature*Farm	4	0.0606071	0.0151518	0.32	0.8669

Bonferroni (Dunn) t Tests for log10CFUgww for differences between farms - Matrix = 1 (Fecal)

Alpha	0.05
Error Degrees of Freedom	618
Error Mean Square	0.047841
Critical Value of t	2.40052
Minimum Significant Difference	0.0505

Comparisons significant at the 0.05 level are indicated by ***.				
Farm Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
2 - 3	0.00538	-0.04514	0.05590	
2 - 1	0.07532	0.02480	0.12585	***
3 - 2	-0.00538	-0.05590	0.04514	

3 - 1	0.06994	0.01942	0.12046	***
1 - 2	-0.07532	-0.12585	-0.02480	***
1 - 3	-0.06994	-0.12046	-0.01942	***

Matrix 2 (Water) –

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	29	181.0855932	6.2443308	204.77	<.0001
Error	618	18.8456030	0.0304945		
Corrected Total	647	199.9311962			

R-Square	Coeff Var	Root MSE	log10CFUgww Mean
0.905740	2.179908	0.174627	8.010740

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Temperature	2	3.2350001	1.6175000	53.04	<.0001
Day	7	171.7184865	24.5312124	804.45	<.0001
Farm	2	0.0168146	0.0084073	0.28	0.7591
Temperature*Day	14	6.1103947	0.4364568	14.31	<.0001
Temperature*Farm	4	0.0048974	0.0012243	0.04	0.9969

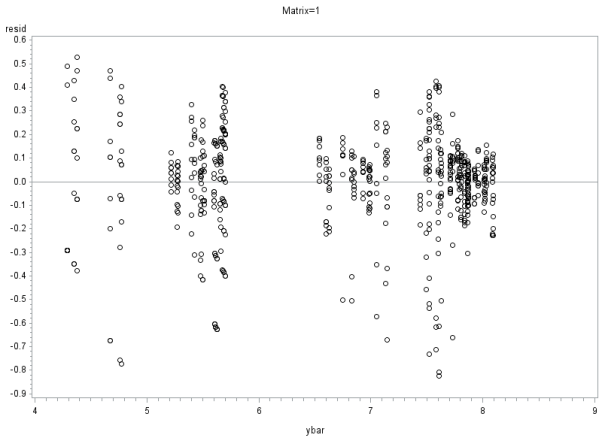
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Temperature	2	3.2350001	1.6175000	53.04	<.0001
Day	7	171.7184865	24.5312124	804.45	<.0001
Farm	2	0.0168146	0.0084073	0.28	0.7591
Temperature*Day	14	6.1103947	0.4364568	14.31	<.0001
Temperature*Farm	4	0.0048974	0.0012243	0.04	0.9969

Bonferroni (Dunn) t Tests for log10CFUgww for differences between farms - Matrix = 2 (Water)

Alpha	0.05
Error Degrees of Freedom	618
Error Mean Square	0.030495
Critical Value of t	2.40052
Minimum Significant Difference	0.0403

Comparisons significant at the 0.05 level are indicated by ***.				
Farm Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
1 - 3	0.00584	-0.03450	0.04618	
1 - 2	0.01247	-0.02787	0.05281	
3 - 1	-0.00584	-0.04618	0.03450	
3 - 2	0.00663	-0.03371	0.04696	
2 - 1	-0.01247	-0.05281	0.02787	
2 - 3	-0.00663	-0.04696	0.03371	

QQ Plots - residuals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0.4
0.3
0.2
0.1
0.0
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-0.2
-0.3
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-0.5
-0.6
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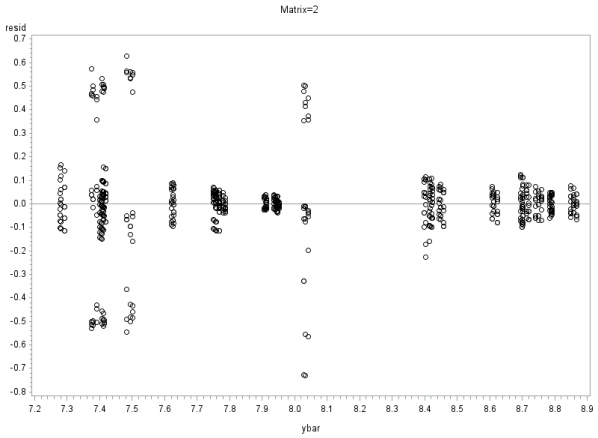
-0.8
-0.9
4
5
6
7
8
9
ybar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0.4
0.3
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0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
7.2
7.3
7.4
7.5
7.6
7.7
7.8
7.9
8.0
8.1
8.2
8.3
8.4
8.5
8.6
8.7
8.8
8.9
ybar

VITA

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	Selected for the National Environmental Public Health Internship Program (NEPHIP)