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FEASIBILITY OF USING ^{15}N-ENRICHED *ESCHERICHIA COLI* AS A BACTERIAL TRACER IN THE CANE RUN/ROYAL SPRING BASIN, KENTUCKY

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ABSTRACT OF THESIS

FEASIBILITY OF USING ^{15}N-ENRICHED *ESCHERICHIA COLI* AS A BACTERIAL TRACER IN THE CANE RUN/ROYAL SPRING BASIN, KENTUCKY

A novel tracer method has used ^{15}N to label *Escherichia coli* and track the transport of bacteria, a common contaminant, through karst aquifers. Use of this method could provide valuable insight into the movement of bacteria in aquifers, which would help improve remediation methods and strategies. A wild strain of *E. coli* was isolated from the Cane Run/Royal Spring basin in the Inner Bluegrass region of Kentucky. The strain was serotyped O^-:H^- and virulence testing showed the strain did not have virulence factors of *E. coli* commonly pathogenic to humans. Five karst microcosms were filled with sterilized water collected from Royal Spring in Georgetown, Kentucky. Each microcosm was inoculated with wild-type *E. coli*, enriched in ^{15}N, and incubated at 14° C for 130 days. The microcosms were periodically sampled for the concentration and nitrogen isotope composition of *E. coli* over 130 days. The *E. coli* survived at concentrations within one log of the average initial value of 5.62×10^{10} for the duration of the study. Statistical modeling showed no significant difference in δ^{15}N values from day 1 and day 130. This strain is therefore recommended for traces in the Cane Run/Royal Spring basin.

KEYWORDS: *E. coli*, environmental microbiology, groundwater tracers, karst, stable isotopes

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THESIS

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The Graduate School

University of Kentucky

2010

FEASIBILITY OF USING ^{15}N-ENRICHED *ESCHERICHIA COLI* AS A BACTERIAL TRACER IN THE CANE RUN/ROYAL SPRING BASIN, KENTUCKY

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
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By

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CHAPTER 1: INTRODUCTION

1.1 Introduction and justification

The city of Georgetown, located in the Inner Bluegrass region of Kentucky, relies primarily on Royal Spring for its water supply. Royal Spring is the outlet of a karst groundwater basin, delineated by qualitative dye tracing, which lies largely but not entirely within the watershed of Cane Run, an intermittent stream. The groundwater basin is expected to consist of a network of solution openings running under both urban and agricultural areas connected to the main conduit arriving at the spring. The watershed/groundwater basin supplying Royal Spring is thought to be contaminated. This is evidenced by segments of Cane Run having been placed on Kentucky's 303 (d) list. The 303 (d) list contains a listing of surface waters "not supporting one or more designated uses and requiring the development of a Total Maximum Daily Load (TMDL)" as determined by the Division of Water of the Kentucky Department for Environmental Protection after an assessment of water quality conditions (KEPPC Division of Water 2008).

Stable nitrogen isotopes have been used as a means of tracing viable *Escherichia coli (E. coli)* in karst environments (Ward 2008). This method, which tracks pathogen transport, is useful in determining the rates at which bacteria travel in groundwater and the remobilization capability of the bacteria within the subsurface. Application of this method within the Cane Run watershed and Royal Spring groundwater basin would provide valuable insight regarding the movement of bacteria to a spring used as a public water supply. However, before this method can be employed, careful consideration needs to be made regarding the safety of the microorganism used and its persistence in the environment. Additionally, information regarding the fate of a ^{15}N spike in *E. coli* over time is essential for accurate interpretation of trace results.

1.2 Hypothesis and approach

Hypothesis: The nitrogen isotope composition of ^{15}N-enriched *E. coli* is conserved over time as *E. coli* persists in sterilized karstic water.

Approach: Wild-type *E. coli* will be isolated from Royal Spring during low flow conditions. Serological analyses and virulence testing will be used to determine suitability for the strain's use as a groundwater tracer. Microcosms will be used to simulate the conditions of the Royal Spring groundwater basin under low flow. Water samples will be obtained from Royal Spring, sterilized, and placed in the microcosms. Wild-type *E. coli* will be cultured in ^{15}N-enriched medium to label the bacteria with the relatively rare isotope of nitrogen, and then used to inoculate the microcosms. The concentration and $\delta^{15}N$ value of the *E. coli* will be measured at daily to monthly intervals to determine the persistence of *E .coli* in the microcosms over time.

1.3 *Escherichia coli*

1.3.1 General characteristics

E. coli is a member of the family Enterobacteriaceae. Enterobacteriaceae, or enteric bacteria, are facultative anaerobic Gram-negative rods. Total coliforms are Enterobacteriaceae that use lactose to feed fermentation, producing gas. Fecal coliforms are total coliforms that produce gas and acid at higher temperatures (44.5 +/- 0.2 °C) (Hach 2000). *E. coli*, a fecal coliform, is further distinguished from other fecal coliforms by its ability to produce lactic, acetic, and succinic acid by a fermentation pathway (Chapelle 1993).

1.3.2 Nitrogen utilization

The basic nutrients required for growth in *E. coli* are glucose, NH_4^+, Mn^{2+}, Mg^{2+}, Fe^{2+}, K^+, Cl^-, SO_4^{2-}, and PO_4^{3-} (Moat et al. 2002). *E. coli,* a facultative anaerobic microorganism, uses both respiratory and fermentative pathways during carbohydrate metabolism (Balows et al. 1992a). *E. coli* uses oxygen as an electron acceptor in respiratory pathways to obtain energy. When oxygen is absent *E. coli* can use fermentation pathways to obtain energy, or they need another molecule to act as an electron acceptor in respiratory pathways. Nitrate (NO_3^-) is one of a number of

molecules that can fill this role, and when used the process is thus termed nitrate respiration (Moat et al. 2002).

In the process of nitrate respiration, respiratory nitrate reductase reduces nitrate to nitrite (NO_2^-), releasing a proton (H^+) to the outside of the cell membrane. The proton motive force created by this reaction is used to generate ATP and facilitate other processes within the cell. Respiratory nitrate reductase, an enzyme, is stimulated for synthesis in anaerobic environments when nitrate is present. Nitrate also decreases the activity of enzymes in fermentative pathways because energy can be generated through nitrate respiration. Therefore, fermentation does not need to play as large a role in energy production (Stewart 1988).

Nitrogen is not only used for the production of energy in *E. coli*, but also plays an important role in the structure of amino acids and other nitrogenous compounds in the cell (Moat et al. 2002). Because of this, *E. coli* need a way to incorporate nitrogen into cellular components. The process *E. coli* use to incorporate nitrogen into the cell is termed assimilation. Ammonium (NH_4^+) is the form of nitrogen needed for assimilation in *E. coli*. *E. coli* can either obtain ammonium from the environment, or reduce nitrite (created from the reduction of nitrate during nitrate respiration) to hydroxylamine and reduce hydroxylamine to ammonia (NH_3). Once in ammonium form (the ion form of ammonia), the nitrogen is assimilated when α-ketoglutarate (a compound formed during carbohydrate metabolism) is amidated to form glutamic acid. The nitrogen can then be transferred to other compounds in the cell through transamination reactions (Moat et al. 2002). Figure 1.1 shows a summary of the above utilizations of nitrogen by *E. coli*.

1.3.3 Serology and serotyping

Serotyping is a widely employed classification method used to subdivide *E. coli* into strains based on the properties of antigens that are present or absent on the cell surface (Buchanan and Gibbons 1974). Approximately 173 different O, 56 different H, and 80 different K antigens are identifiable in *E. coli* (Machado et al. 2000). The strain is classified by identifying its O, K, and H antigens, such as *E. coli* O111:K58:H2 (a strain associated with infantile diarrhea) (Buchanan and Gibbons 1974). The serotyping system developed through investigation of disease in the early and middle 1900s, when scientists

were seeking a way to associate diseases and outbreaks with specific strains of bacteria (Gyles 1994). O and H antigens are now more prevalent in the classification of *E. coli* into OH serotypes, with the K antigen being identified only in certain cases (Balows et al. 1992b, Gyles 1994).

Lipid A, core oligosaccharide, and O antigen together compose lipopolysaccharide (LPS), a compound in the outer membrane of *E. coli* (Gyles 1994). Antigens positioned on the surface of the cell are termed somatic antigens. O antigens are somatic and heat stable at 100-121°C. K antigens are also somatic and are termed capsular polysaccharides because they form capsules (i.e., another layer) on LPS, which can mask O antigen during serological analyses (Balows et al. 1992b, Gyles 1994). There are three types of K antigen: L, A, and B. The three types react differently to heating, which is important in serological analyses. H antigens are located on flagellin, the protein that composes flagella. The flagellum is the source of locomotion in motile microorganisms (Gyles 1994). H antigens are inactivated by 100° C heat (Balows et al. 1992b).

In general, and traditionally, serological analyses are performed by combining bacterial suspensions of the isolated strain with known antibodies for each of the different antigens. Agglutination, or clumping, of the antigen occurs when its specific antibody is present, indicating a positive result for that known antibody and thereby identifying the type of antigen (Gyles et al. 1994, Machado et al. 2000, Ørskov et al. 1977). For determination of O antigen, suspensions are first heated for 1 hr at 100° C to inactivate K antigens. In some instances the suspensions must be heated for 2 h at 120° C to inactivate heat resistant K antigens. The agglutination reactions for O antigens can be performed after inactivation of the K antigens (Gyles 1994, Ørskov et al. 1977).

Some *E. coli* strains do not respond to antibodies in the agglutination reactions; they are designated as O^- or H^- (or both). An O^- result can occur in two ways. First, an O^- result can be caused by the antigen not reacting with any of the antibodies used in the agglutination reactions. In other words, the strain has an O antigen, but the antigen is specific to an antibody that was not used in the agglutination reaction (DebRoy 2009). Second, mutations in genes used for the synthesis of O-specific polysaccharide chains can cause antigens to change from smooth (S) O^+ to rough (R) O^- forms, causing a loss in

antigen specificity. Smooth (S) strains of *E. coli* contain O antigen, while in rough (R) strains LPS is terminated after the core oligosaccharide. The smooth (S) and rough (R) terminology is a notation based on colony morphology in culture (Gyles 1994). H^- strains of *E. coli* are non-motile and do not produce flagella (DebRoy 2009)..

1.3.4 Virulence factors

Virulence factors are agents used by pathogenic strains of *E. coli* to attach to hosts, invade host cells, manipulate host defenses, and employ various strategies that strengthen their ability to colonize and reproduce in hosts (Donnenberg 2002, Groisman 2001). They are encoded by genes termed virulence genes, producing substances which either directly interact with host cells, or are used to transport other virulence factors to host cells (Alberts et al. 2002). While beneficial to an invading strain of pathogenic *E. coli*, virulence factors are harmful to the host and contribute to disease. Common commensal strains of *E. coli*, such as those typically found in the intestine of humans and animals, lack virulence factors (Donnenberg 2002). Table 1.1 shows a summary of the virulence factors discussed below.

Enterotoxins are proteins and peptides generated by *E. coli* that cause fluid loss in the host digestive system, resulting in watery diarrhea (Donnenberg 2002). Strains that possess these virulence factors are termed enterotoxigenic *E. coli* (ETEC), and infect both animals and humans (Gyles 1994). Enterotoxins are divided into two groups: heat labile toxin (LT) and heat stable toxin (ST) (Donnenberg 2002, Gyles 1994, DebRoy and Maddox 2001).

LT is secreted from *E. coli* into the host intestine, where it binds to the host cell membrane and undergoes endocytosis. Once in the cell, LT acts to increase the concentration of cAMP, which causes a change in the ion channels of the host cells that leads to increased Cl^- secretion and decreased Na^+ and Cl^- absorption. Ultimately, this causes water to be lost from the host cell into the intestinal cavity due to changes in the osmotic gradient of the cell (Donnenberg 2002).

Heat stable enterotoxins are divided into two unrelated groups: STa and STb. Both STa and STb are secreted from *E. coli* into the host intestine, and cause fluid loss from host cells. STa, once in the host intestine, binds to host cells and acts to increase the

concentration of cGMP (DebRoy and Maddox 2001, Donnenberg 2002, Gyles 1994). The increased cGMP levels lead to increased Cl^- secretion and decreased Na^+ and Cl^- absorption. Again, this causes water to be lost from host cells to the intestine (Donnenberg 2002). The mechanism of STb is less clear, but when present STb is associated with damage to host intestinal cells (Donnenberg 2002, Gyles 1994).

Verotoxins (VT), also called Shiga-like toxins (SLT), form another set of virulence factors. Strains possessing these factors are termed enterohemorrhagic (EHEC) strains, and infect both animals and humans. Serotype O157:H7 is a particularly pathogenic EHEC strain that is the source of many disease outbreaks in humans. Serotype O157:H7 is present in both healthy and diseased cattle, and thus has potential to contaminate meat products. Shiga-like toxins in *E. coli* are subdivided into two groups: Stx1 and Stx2. Both inhibit protein synthesis by preventing tRNA from binding to ribosomes (DebRoy and Maddox 2001). EHEC strains inhabit the colon, with SLTs causing damage to host colon cells (DebRoy and Maddox 2001, Gyles 1994).

Enteropathogenic strains of *E. coli* (EPEC) contain a virulence factor, among others, that allows the *E. coli* cells to closely adhere to host cells. The adhesion protein intimin is encoded by the *eae* gene and acts to facilitate the formation of A/E (attaching and effacing) lesions. The receptor protein Tir is injected into the host cell and binds to intimin, a key step in the formation of A/E lesions. The A/E lesions enable *E. coli* to adhere to and also interact closely with host cells (DebRoy and Maddox 2001, Donnenberg 2002). EPEC strains cause diarrhea not through toxins, but through mechanisms that decrease the absorptive surface area of intestinal cells (A/E lesion formation) and increase Cl^- secretion, causing water loss (DebRoy and Maddox 2001).

Uropathogenic (UPEC) strains of *E. coli* can contain cytotoxic necrotizing factor (CNF), a toxin that is divided into types: CNF1 and CNF2. Strains that have CNF are termed necrotoxigenic *E. coli* (NTEC). CNFs are linked genetically to other virulence factors, and act in numerous ways to promote pathogenesis, including an association with factors that promote adherence (DebRoy and Maddox 2001, Donnenberg 2002). CNF1 has been associated with human and domestic mammal isolates, while CNF2 has only been associated with ruminants (DebRoy and Maddox 2001).

1.3.5 Indicator microorganisms and waterborne disease

Bacteria, viruses, protozoa, and helminths are all types of water-associated microorganisms that have certain strains which cause disease. The diseases these organisms cause have a heavy burden on humans, both in terms of life lost and also in terms of time, energy, and economic loss due to illness. According to a World Health Organization (WHO) report, "one tenth of global disease burden could be prevented by improving water supply, sanitation, hygiene and management of water resources" (Prüss-Üstün et al. 2008). In addition, an economic benefit of an estimated 18.143 billion US dollars would result from decreasing the number of people without access to improved water resources in half by 2015 (Prüss-Üstün et al. 2008). This intervention only accounts for improvements in water quality. Additional benefits would result from improvements in water supply and sanitation practices. These statistics underscore the importance of water quality worldwide, and though not every country may have the needed access for these improvements, a system is still needed to quantify whether or not water is safe to consume and/or use for recreation.

Fecal indicator monitoring has been the system used to help protect humans from the burden of water-associated disease. Indicator organisms are used as a proxy to detect for the presence of pathogenic organisms. Bacterial pathogens are unlikely to be present when fecal indicator organisms are absent. This monitoring method is important due to the difficulty of detecting pathogens in the environment and the danger of routinely culturing them (Hach, 2000). According to Hach (2000), there are five key criteria that should be present for an indicator organism to be reliable. First, "[T]he organism must be exclusively of fecal origin and consistently present in fresh fecal waste." Second, the organism "must occur in greater numbers than the associated pathogen." Third, the organism "must be more resistant to environmental stresses and persist for a greater length of time than the pathogen." Fourth, the organism "must not proliferate to any great extent in the environment." Last, "simple, reliable, and inexpensive methods should exist for the detection, enumeration, and identification of the indicator organism." Examples of organisms that meet the indicator criteria include coliform bacteria, fecal streptococci, and *Clostridium perfringens* (Hach 2000). The most widely used indicator organisms, and the organisms on which most developed countries rely for developing

water quality standards, are from the total coliform group. *E.coli* is considered the best coliform indicator of fecal contamination because it is thermotolerant and can be presumed to come from warm environments (Hach 2000).

Standards have been developed by organizations to assess water quality based on concentrations of indicator microorganisms. The WHO has set a standard stating that *E. coli* "must not be detectable in any 100-ml sample" of water intended for drinking (WHO 2006). The United States Environmental Protection Agency (USEPA) has a "maximum contaminant level goal" set at zero for *E. coli* in its Primary Drinking Water Regulations (Federal Register 2002). The Commonwealth of Kentucky has monitoring regulations for public suppliers of water, but it should be noted that private wells in Kentucky are not regulated by either the EPA or the commonwealth. The regulations for public suppliers are somewhat complicated, but a brief synopsis is as follows. Routine monitoring for presence or absence of total coliforms is required, with number of samples collected proportional to population size. A positive sample is followed up with a series of repeat samples, and an additional positive sample is followed up by another series of repeat samples. Positive samples are also tested for either fecal coliforms or *E. coli.* A public water system collecting more than 40 samples monthly is compliant if "no more than five and zero-tenths (5.0) percent of the samples collected during a month are total coliform-positive", while a public water system collecting less than 40 samples monthly is compliant if "no more than one (1) sample collected during a month is total coliform-positive. A fecal coliform-positive repeat sample or E. coli-positive repeat sample, or a total coliform-positive repeat sample following a fecal coliform-positive or E. coli-positive routine sample constitutes a violation of the MCL [maximum contaminant level] for total coliforms" (Kentucky Legislature 2008b).

Standards have also been set for water used in recreation. The USEPA (1986) primary recreational contact recommendations for *E. coli* have a single sample maximum of 235 cfu per 100 mL and a geometric mean maximum for five or more samples in a 30-day period of 126 cfu per 100 mL. The Kentucky standard for primary contact recreational waters differs from that of the EPA. The Kentucky standard states that *E. coli* "shall not exceed […] 130 colonies per 100 ml […] as a geometric mean based on not less than five (5) samples taken during a thirty (30) day period", and that *E. coli*

should not exceed 240 colonies per 100 mL in 20% of samples in a 30-day evaluation period "during the recreation season of May 1 through October 31" (Kentucky Legislature 2008c).

1.4 Stable isotope tracers

Atoms are composed of protons, electrons, and neutrons; those having the same number of protons in the nucleus are grouped as elements. The isotopes of an element are atoms that have the same number of protons but differing numbers of neutrons. Isotopes can be stable or radioactive. Radioactive isotopes are inherently unstable and decay at a constant rate; they are used for age dating and in nuclear processes. Stable isotopes do not decay, and can be used as tracers in investigations of the environment, nutrient cycling, and molecular biology. Stable isotopes of the elements carbon (C), hydrogen (H), nitrogen (N), oxygen (O), and sulfur (S) are commonly used for such studies (Fry 2006).

The usage of stable isotopes as tracers is based upon the ratios of light (relatively low mass) to heavy (relatively high mass) isotopes of an element. Fundamental isotope abundances for elements on Earth were set in place during nucleosynthetic processes that formed the elements that comprise the solar system. Nevertheless, small differences in isotope abundances can be attributed to physical, chemical, and biological processes occurring in natural systems. These small differences provide insight into the origin and modification of natural and man-made materials (Fry 2006).

At present, isotope measurements are commonly made with an isotope ratio mass spectrometer (IRMS). Various devices are interfaced with the IRMS to convert samples into appropriate gases that can be ionized and measured by the IRMS. The ionized molecules are accelerated through a potential field and separated (based on inertia) by a magnetic sector. Separated ions of specific mass-to-charge ratios travel to collectors, which convert beam intensity to a current output that is then used to calculate isotope values (Fry 2006).

Delta (δ) notation is a convention used to compare isotope ratios in a sample to a standard. The equation for δ is

$$\delta = [((R_{SAMPLE}/R_{STANDARD}) - 1)] \times 1000$$

where

$$R = {}^{H}F/{}^{L}F$$

and

F = abundance of the rare (^{H}F) (usually heavy) isotope to the common (^{L}F) (usually light) isotope.

δ is described in units of ‰ or per mil as a result of the multiplication by 1000 in the formula. This allows small differences is isotope values to appear large, and also makes the numbers easy to work with. Natural samples have δ values that normally range between -100 and 50‰, depending on the element. For nitrogen, the international reference standard is air, with a value of 0.0036765 for $^{15}N/^{14}N$ (Fry 2006).

Isotope fractionation and mixing are two processes that cause isotope abundance to deviate slightly in natural materials. Fractionation occurs when the abundance of an isotope changes as a result of a chemical, physical, or biological process in which one isotope is preferentially utilized relative to another. The formula

$$\Delta = \delta_{SOURCE} - \delta_{PRODUCT}$$

describes the difference in isotope composition (in ‰) between source and product. Isotope mixing can be described by a mass-balance equation:

$$\Delta_{MIXTURE} = (\delta_{SOURCE1})\times f_1 + (\delta_{SOURCE2})\times f_2$$

where

δ = isotope value, f = fraction

and

$$f_1 + f_2 = 1.$$

The mixing equation is applicable in many research situations. As an example, isotope mixing is commonly investigated in studies involving nutrient cycling feeding habits for organisms. If two (or more) isotope sources can be identified for an organism, and if each source has a known isotope value, then mixing equations can be used to show how much each source is contributing to the organism's diet (Fry 2006).

Particular isotopes can also be enriched through manufacturing processes, and are available commercially for use in laboratory and field studies. Compounds enriched in a particular isotope can create a source signal that is outside the range observed in the natural world and so are useful as tracers (Fry 2006).

1.5 Karst

Karst is a terrane defined by the dissolution of soluble rock, predominantly carbonate, which results in specific hydrological and morphological features including sinkholes, sinking and underground streams, springs, and caverns (Field 2002). This morphology often complicates drainage divides because surface terrain does not necessarily coincide with underground features. Karst aquifers are characterized by the flow of water through any cavities, joints, faults, or bedding planes that have formed through dissolution (Field 2002). Karst occupies approximately 12% of Earth's land surface, and an estimated 25% of the world's population relies on karst aquifers for water supply (Ford and Williams 1989). In the United States, 20% of the land surface lies above karst aquifers, including 40% of the land surface east of the Mississippi River (Quinlan 1989).

Karst aquifers are particularly vulnerable to contamination. A variety of contaminants, ranging from solid wastes and chemicals in urban areas to fertilizers and pesticides in agricultural areas, can quickly and easily enter the aquifer via the features noted above. Septic tank runoff and sewage are of concern in both urban and agricultural areas due to the potential to contain pathogens capable of causing disease (Veni et al. 2001). The vulnerability of karst aquifers to contamination results in a need to identify potential contaminant sources and protect watersheds in such areas. Figure 1.2 shows a diagram of typical Inner Bluegrass Region karst.

1.6 Research area

The Inner Bluegrass Region of central Kentucky is approximately 5600 square kilometers in size and is in general characterized by gently rolling hills with 50 m or less relief (Thraillkill et al. 1982). This region is made of carbonate rocks exposed by erosion of the Cincinnati Arch, and as such is heavily karstified, with numerous sinkholes and some areas larger than 10 square kilometers lacking surface drainage. From the surface downward, the Clays Ferry Formation, Lexington Limestone, Tyrone Limestone, Oregon Formation, and Camp Nelson Limestone make up the stratigraphic units (Thraillkill et al. 1982). Although most major conduits and springs are located in the lower units of the

Lexington Limestone, Thraillkill et al. (1982) notes that this is likely caused by similarities in bedding and surface topography, not lithology. Thraillkill et al. (1982) also notes that groundwater basins and surface watersheds lack any consistent correlation, meaning karst conduits frequently cross surface-water divides.

The study area is located between the cities of Lexington and Georgetown and straddles the counties of Fayette and Scott (Figures 1.3, 1.4, and 1.5). Significant portions of the study area are within the Kentucky Horse Park, site of the 2010 World Equestrian Games (Figures 1.6 and 1.7). The city of Lexington, in Fayette County, has a population of 260,512, whereas the city of Georgetown, in Scott County, has a population of 18,080 (US Census Bureau 2000a, b). Royal Spring (Figures 1.8 and 1.9) is located within the city of Georgetown, and serves as the main source of the city's drinking water, with additional water available for purchase from the Frankfort Plant Board and Kentucky-American Water Company (Georgetown Municipal Water & Sewer Service 2007). Figure 1.10 displays a summary of the monthly discharges at Royal Spring from 1993-2002 and Cane Run (at Berea Road) from 1999 to 2002. Royal Spring had an annual mean discharge of 20.77 cubic feet per second (0.59 cubic meters per second), with discharge varying significantly during the course of the year (McClain et al. 2002). Figures 1.11, 1.12, and 1.13 display Royal Spring discharge, Cane Run discharge, and precipitation at Cane Run, respectively, for the 2008 calendar year.

The water supplied to the spring is classified as "Groundwater Under the Direct Influence of Surface Water", which is defined as "water beneath the surface of the ground with significant occurrence of insects or other macroorganisms, algae, large-diameter pathogens such as Giardia lamblia, or Cryptosporidium, or significant and relatively rapid shifts in water characteristics such as turbidity, temperature, conductivity, or pH, which closely correlate to climatological or surface water conditions" (Georgetown Municipal Water & Sewer Service 2007; Kentucky Legislature 2008a).

Cane Run is a stream, with headwaters in Lexington, which flows northward into North Elkhorn Creek approximately 7 miles (11.3 kilometers) downstream from Georgetown (Mull 1968). The upstream reach of Cane Run is located within an industrial and agricultural area (near New Circle and North Broadway). The stream enters a predominantly agricultural area as it flows northward and enters the Kentucky

Horse Park. Cane Run often runs dry from the middle of July to early October in the vicinity of Berea Road, as seen in Figures 1.10 and 1.12 (Mull 1968).

The Royal Spring groundwater basin drainage system is expected to consist of a network of solution openings connected to a main conduit that discharges at Royal Spring (Mull 1968). However, the precise route of the main conduit has not yet been determined. Sinkholes within the bed of Cane Run allow surface flow from Cane Run to directly enter the subsurface drainage system, connecting the Cane Run watershed and Royal Spring groundwater basin. Seepage runs also show that Cane Run is losing along these reaches (Mull 1968). Because of these characteristics, contamination from both urban and agricultural areas is a concern. Major transportation routes (I-64, I-75, and railroads) also cross over the area, adding to the contamination risk (Figure 1.14) (Thraillkill 1984). The discharge at Royal Spring is larger than what would be expected of recharge exclusively from Cane Run, so recharge from other sources is expected as well (Mull 1968).

1.7 Literature review

1.7.1 Bacterial survival

Bacterial survival studies have occurred in a wide range of environments (e.g. Allwood et al. 2003; Banning et al. 2002; Craig et al. 2004; Korhonen and Martikainen 1991; Schumacher 2002), but the study of bacterial survival in karst environments has been limited and has produced mixed results. In the mantled karst of the northwest Arkansas Ozarks, Davis et al. (2005) found that *E. coli* placed in "survival chambers" can survive for at least 75 days. Personné et al. (1998) found that after 54 hours in aerobic conditions and 47 hours in anaerobic conditions, fecal bacteria populations were down to zero in water sampled from a fissured karst environment.

Korhonen and Martikainen (1991) found that predation and competition for nutrients play an important role in *E. coli* survival in lake water. Allwood et al. (2003) demonstrated the importance of temperature on *E. coli* survival in dechlorinated and sterilized tap water. They found that a 90% reduction of *E. coli* population occurred after 7.7 days at 4°C, 5.7 days at 25°C, and 2-3 days at 37°C (Allwood et al. 2003). Banning et al. (2002) tagged *E. coli* with green fluorescent protein (GFP) and then measured

survival rate in microcosms containing effluent (Subiaco Wastewater Treatment Plant, Western Australia) and anaerobic groundwater (Swan Coastal Plain, Western Australia). They also examined the influence of sterilization of both the effluent and groundwater on *E. coli* survival. In the sterilized microcosms, they found a 90% reduction of population occurred after 65 days in the effluent and 82 days in the groundwater, respectively. A 90% reduction in population occurred after only 2 days in the nonsterilized microcosms for both effluent and groundwater (Banning et al. 2002). Schumacher (2002) estimated an *E. coli* die off rate of 0.084 h^{-1} at Shoal Creek in Missouri using *in situ* "diffusion chambers" designed to isolate bacteria but still allow for exchange of water and dissolved nutrients (method similar to that of Davis et al. 2005).

Studies have also examined the role sediment plays on *E. coli* survival. Gerba and McLeod (1975) found that *E. coli* survive longer in seawater with sediment than seawater without sediment, and attributed this to increased organic material available in the sediment. Craig et al. (2004) used microcosms to determine survival of *E. coli* in recreational coastal water and sediment. They found that *E. coli* persistence is greater in sediment (at least 28 days at 10°C) than in the overlying water, and that increasing temperature has a negative effect on survival in both sediment and overlying water (Craig et al. 2004).

The studies mentioned above give a general idea of the complexity of bacterial survival. According to Roszak and Colwell (1987), conditions in natural, nutrient-poor environments make growth difficult, and die off occurs when these conditions vary from optimal bacterial environments (nutrient rich). Faust et al. (1975) showed that 75.6% of *E. coli* die-off in estuarine waters is due to temperature, dissolved oxygen, and salinity, and also suggested that the most important factor in survival is temperature. A review by John and Rose (2005) downplayed the importance of temperature on coliform survival, saying that "little correlation between temperature and inactivation rate is apparent". They state that inactivation of fecal coliforms may be complex, with predation, competition, nutrient availability, and other factors playing a role. In addition, John and Rose (2005) state that temperature likely interacts with these variables, adding to the complexity. They conclude there is a need for studies with consistent methods and procedures to reduce variability among findings.

The studies above indicate that differing environments will have differing survival rates due to a variety of factors. Therefore, survival should be examined in each environment independently until consistent results are achieved. This is especially important for remediation of contaminated areas because survival rates will have an influence on the duration and spatial extent of contamination. The need for additional research is particularly evident in karst settings, which are an important source of water for humans and where only two documented studies were found. Karst is unique, and its morphology and hydrology will result in conditions different from lake, estuarine, marine, and other subsurface environments.

1.7.2 Isotope-enriched bacterial tracer studies

Tracers are used in hydrogeology to determine characteristics of groundwater flow. Goldscheider and Drew (2007) define a tracer as "any type of substance in the water or property of the water that can be used to obtain information on the groundwater flow and transport of matter". Fluorescent dyes and microspheres have been used as tracers to simulate bacterial contamination events. Despite the fact that fluorescent dyes and microspheres have been useful as proxies, methods have recently been developed to use bacteria themselves as a tracer, allowing additional insight into flow and transport (e.g. Holben and Ostrom 2000, Ting 2000, Ward 2008). Ting (2000) developed a method to tag indigenous *E. coli* with europium for use in tracing experiments. Holben and Ostrom (2000) cultured bacteria in ^{13}C-enriched medium to alter the bacteria's isotopic signature but conserve physical and genetic traits, and then used the bacteria as a tracer to examine bacterial transport on the Eastern Shore of Virginia. Ward (2008) recently developed a method to label wild strains of *E. coli* with ^{15}N for tracer applications, and found that ^{15}N labeled *E. coli* behaved differently than the solutes and microspheres often used to model bacterial transport.

Table 1.1 Selected virulence factors, the strains of *E. coli* with which they are associated, and clinical manifestations (adapted from *E. coli* Reference Center 2009).

Virulence Factor	Name	Associated With	Clinical Signs
LT	Heat labile toxin	ETEC	Neonatal diarrhea
STa, STb	Heat stabile toxin a, b	ETEC	Neonatal diarrhea
Stx1, Stx2	Shiga-like toxin 1, 2	EHEC	Hemorrhagic colititis, hemolytic-uremic syndrome (HUS)
eae	Intimin	EPEC	Attaching and effacing lesions
CNF1, CNF2	Cytotoxic necrotizing factors 1, 2	NTEC	Diarrhea

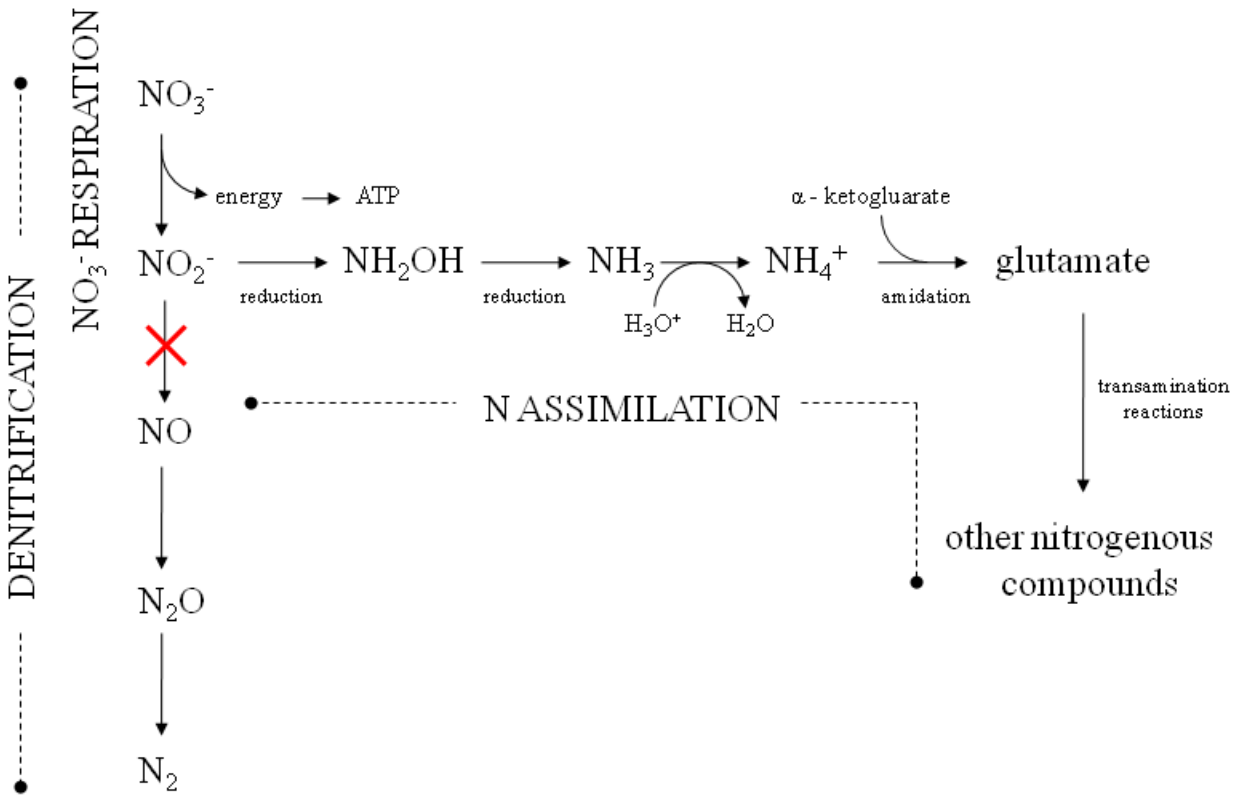


Figure 1.1 Utilization of nitrogen by *E. coli*.

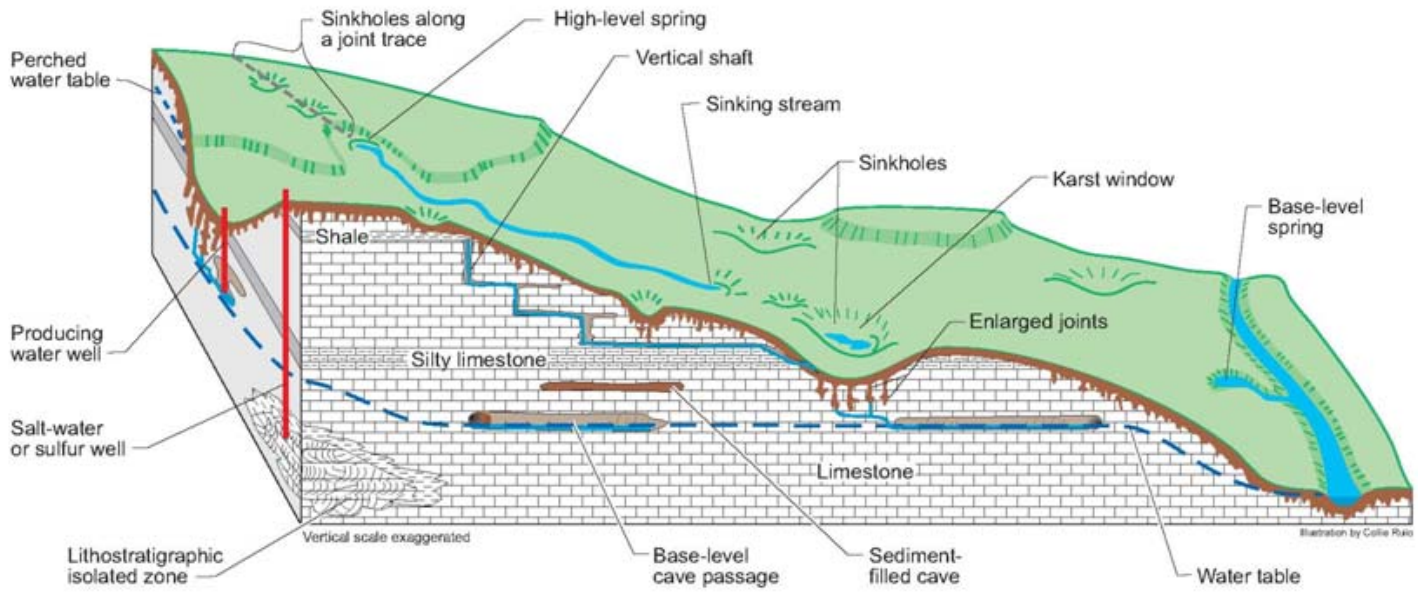


Figure 1.2 Generalized block diagram of Inner Bluegrass karst (Currens 1995).

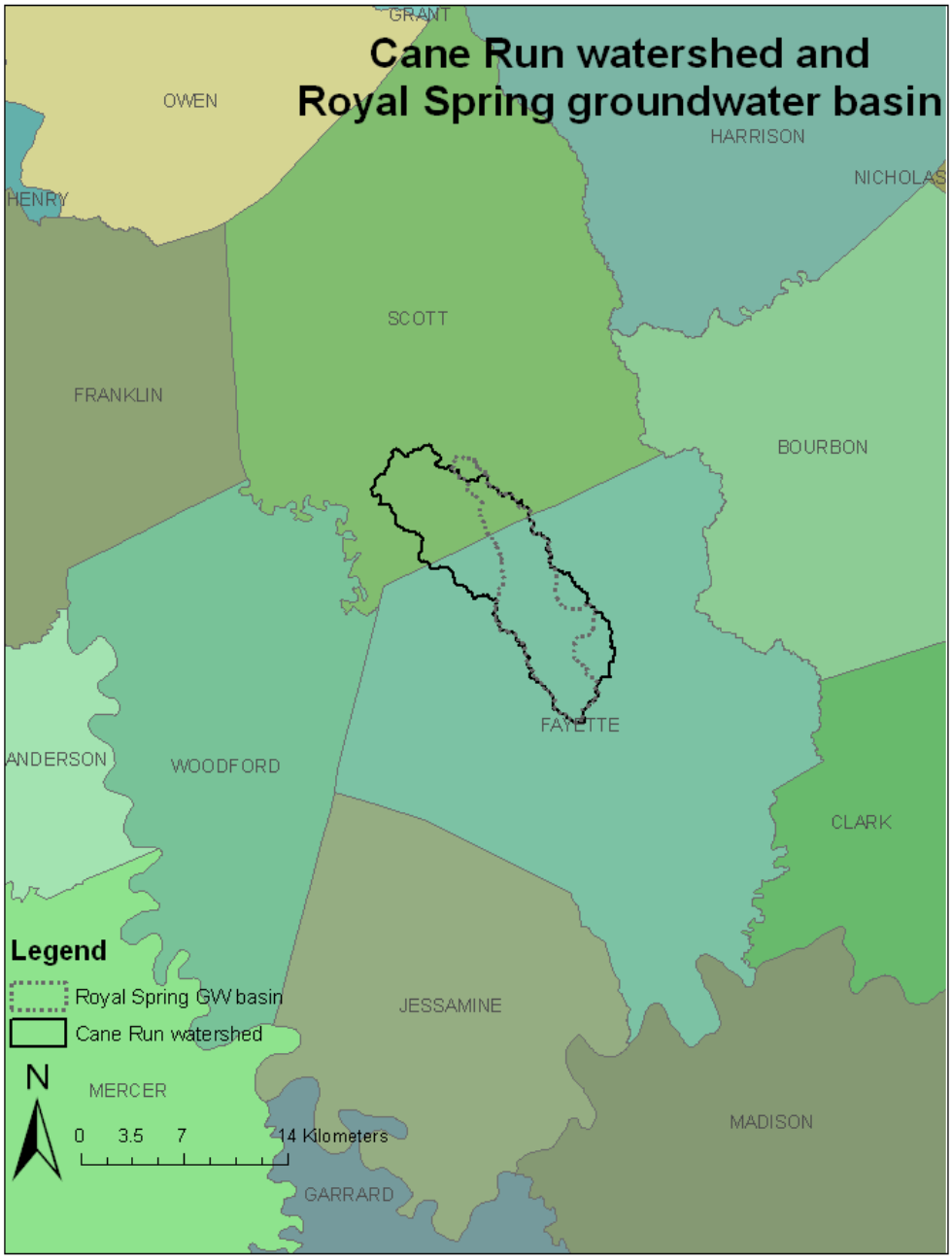


Figure 1.3 Cane Run watershed and Royal Spring groundwater basin relative to counties in Kentucky (compiled using GIS data from Commonwealth of Kentucky [2009] and Currens and Paylor [2004]).

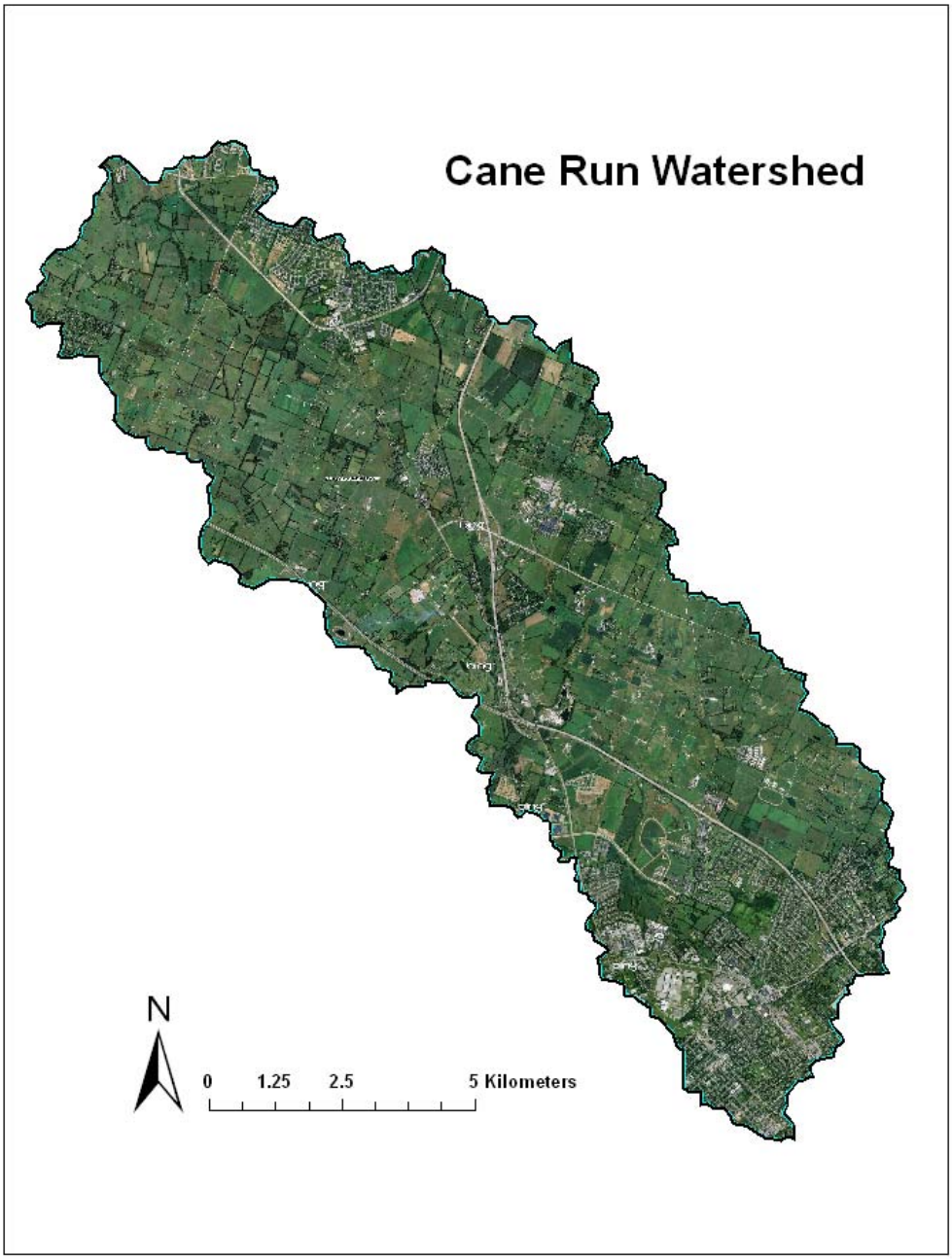


Figure 1.4 Satellite imagery of Cane Run watershed. The urban area in the northern portion of the figure (Georgetown) is separated from the urban area in the southern portion of the figure (Lexington) by agricultural areas and major transportation routes (compiled using GIS data from Commonwealth of Kentucky [2009] and imagery from Microsoft [2009]).

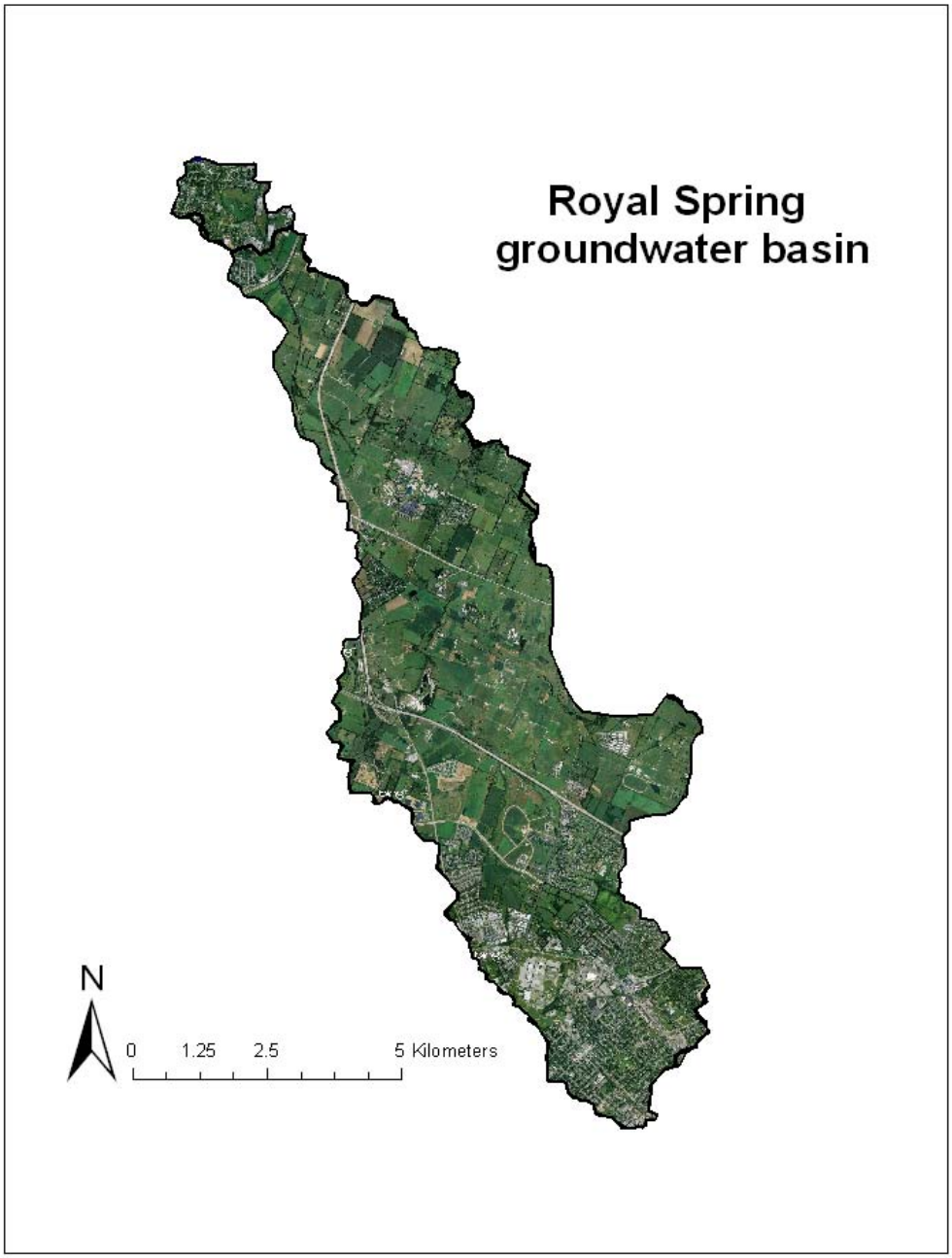


Figure 1.5 Satellite imagery of Royal Spring groundwater basin. As in Figure 1.4, note the major transportation routes, agricultural areas, and urban areas of Georgetown and Lexington (compiled using GIS data from Currens and Paylor [2004] and imagery from Microsoft [2009]).

Figure 1.6 Cane Run watershed within the Kentucky Horse Park (photograph taken summer 2008).

Figure 1.7 Drilling to locate the main karst conduit in the Kentucky Horse Park (photograph taken summer 2008).

Figure 1.8 Royal Spring, Georgetown, KY (photograph taken fall 2008).

Figure 1.9 Royal Spring impoundment, Georgetown, KY (photograph taken fall 2008).

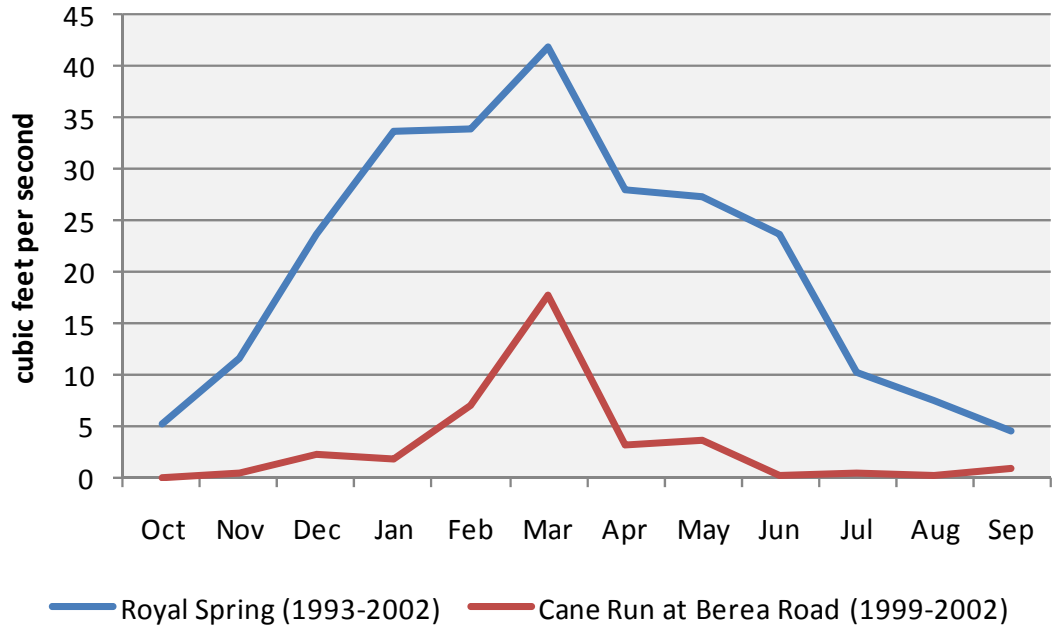


Figure 1.10 Summary of average monthly discharge at Cane Run (1999-2002) and Royal Spring (1993-2002) (compiled using data from McClain et al. [2002]). 1 $ft^3/sec = 0.023\ m^3/sec$.

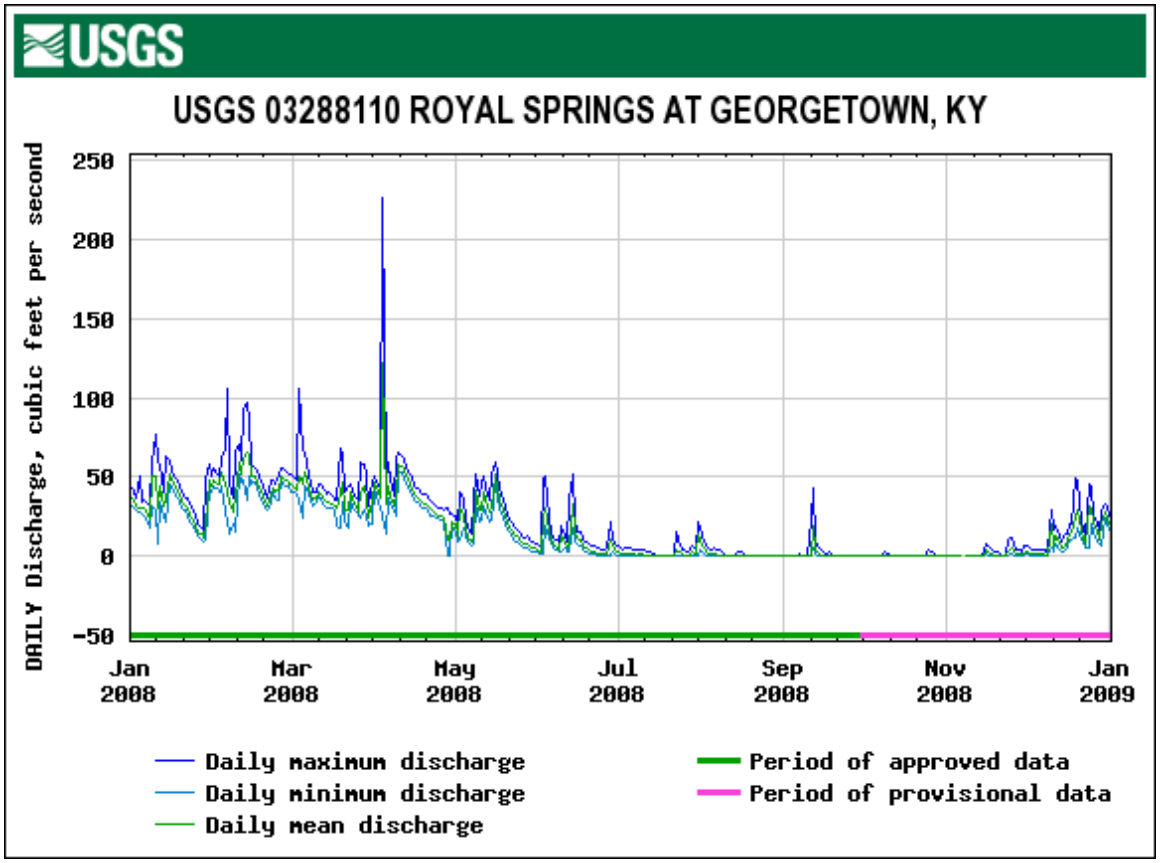


Figure 1.11 Royal Spring discharge for the 2008 calendar year (USGS 2009). 1 ft^3/sec = 0.023 m^3/sec.

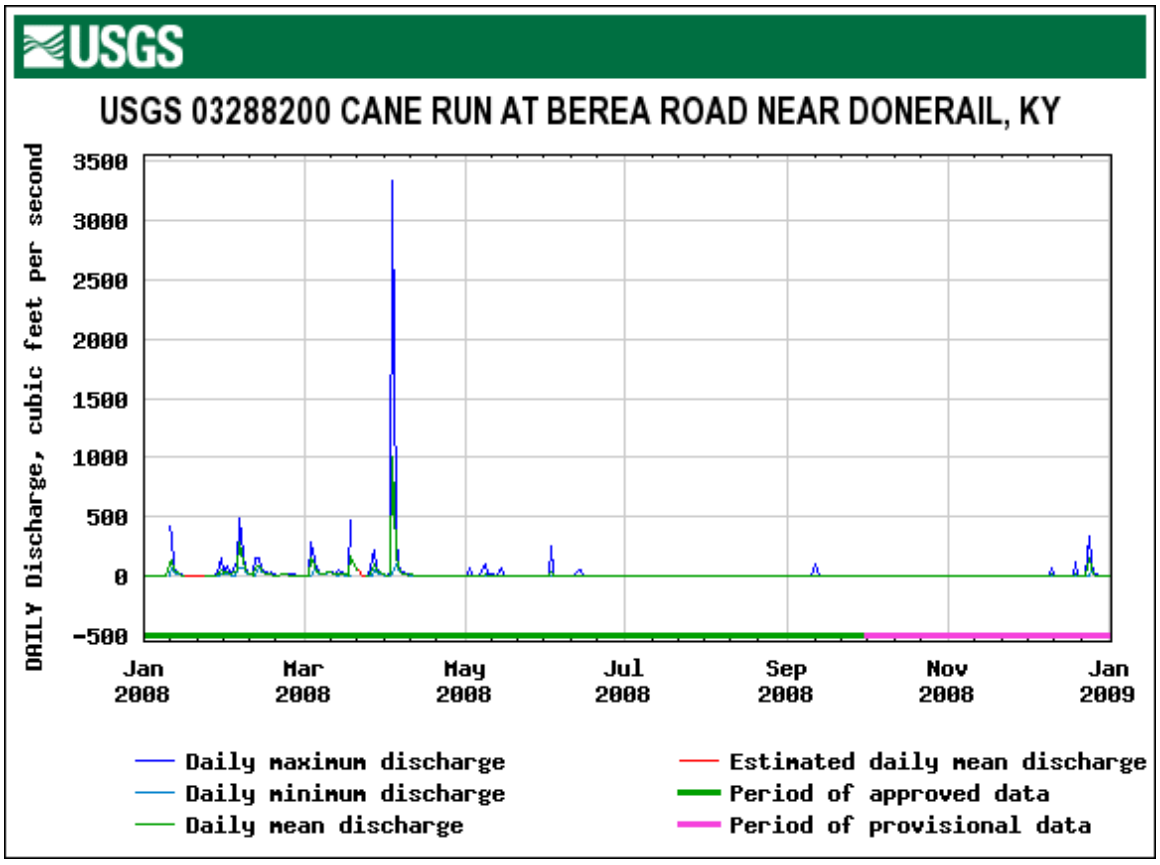


Figure 1.12 Cane Run discharge for the 2008 calendar year (USGS 2009). 1 ft^3/sec = 0.023 m^3/sec.

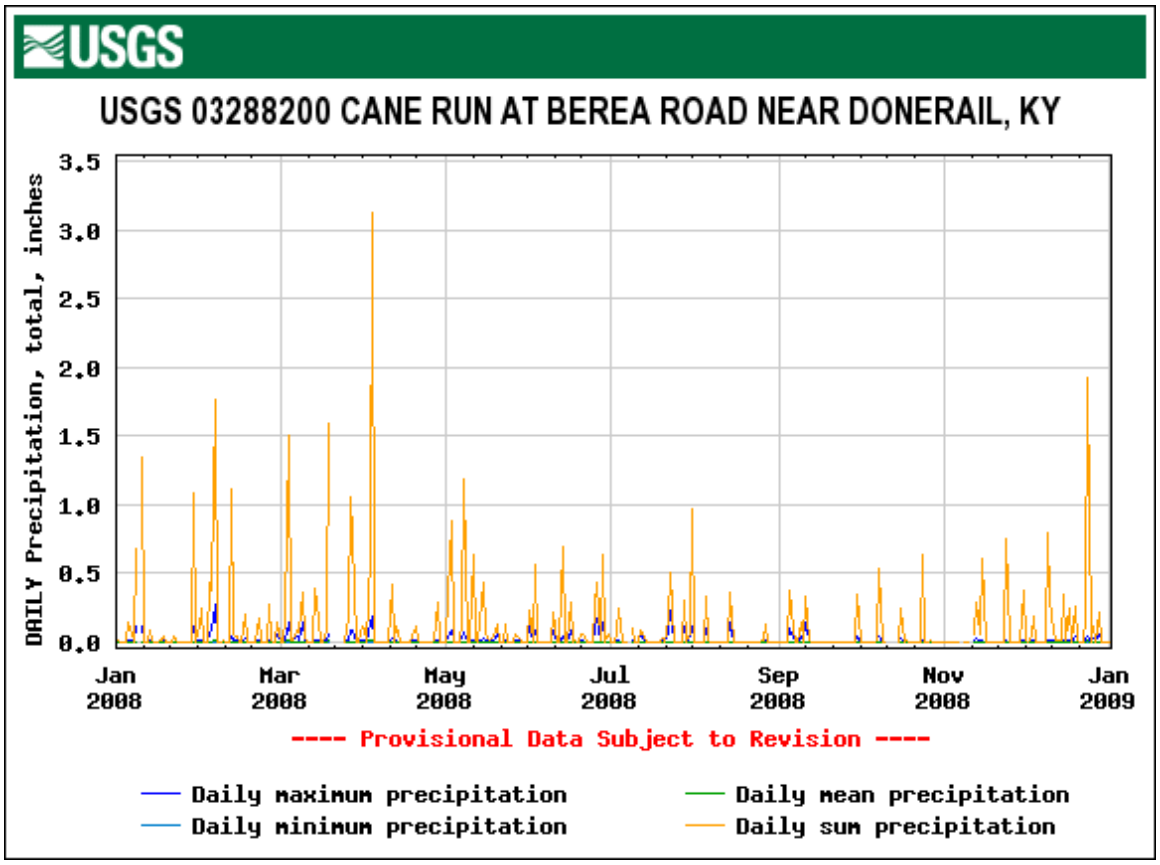


Figure 1.13 Precipitation at gauging station within Cane Run watershed for the 2008 calendar year (USGS 2009). 1.00 in = 2.54 cm.

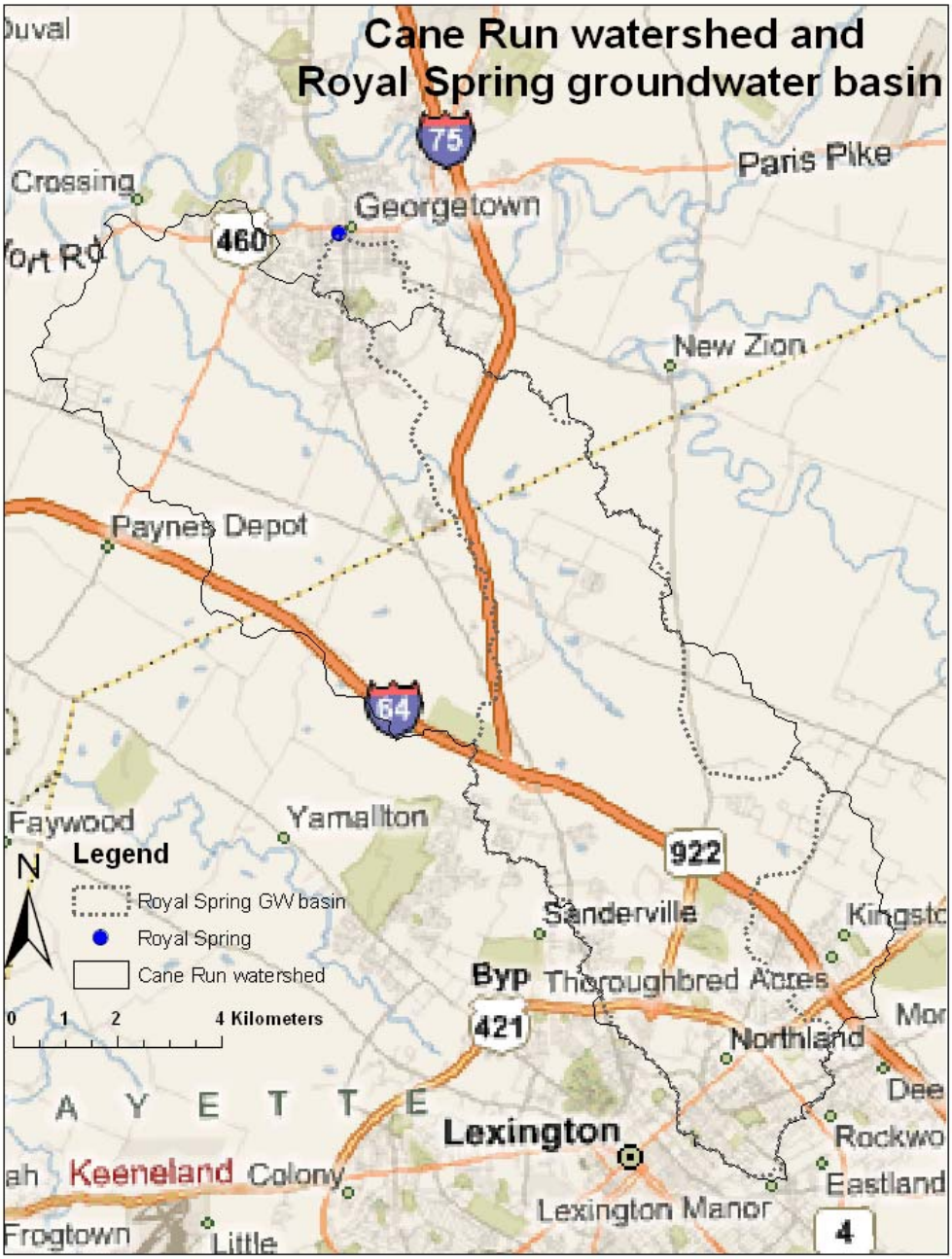


Figure 1.14 Major transportation routes overlying Cane Run watershed and Royal Spring groundwater basin (compiled using GIS data from Commonwealth of Kentucky [2009] and Currens and Paylor [2004], along with imagery from Microsoft [2009])

CHAPTER 2: METHODS

2.1 Method summary

The experimental workflow of this research is diagrammed in Figure 2.1. A strain of wild-type *E. coli* was isolated from Royal Spring. Serological analyses and virulence testing were performed on the strain to determine its potential for pathogenicity. The wild-type *E. coli* was grown in medium enriched in ^{15}N to incorporate an isotope label. The labeled *E. coli* were distributed into microcosms (including controls) containing sterilized Royal Spring water. On testing days, a series of dilutions were prepared from the microcosms to enumerate microbial populations. Two analyses were performed on the samples. First, IDEXX Colilert® and IDEXX Quanti-Tray/2000 (IDEXX Laboratories, Westbrook, ME) were used to determine the most probable number (MPN) of *E. coli* in each microcosm. Second, IRMS was used to determine the ^{15}N enrichment levels of the *E. coli*. Both of the analyses were performed on days 0, 1, 3, 8, 15, 28, 60, and 130, although the IRMS analyses occurred in two rounds of testing (a set of samples for range finding and then a set of samples suited for statistical analysis). Details of the methods and preparation of samples follow. Figures 2.2 to 2.9 show diagrams of the dilutions used and experimental set-up on testing days.

2.2 Isolation of wild-type *E. coli*

On 30 October 2008 a 1-L sample of water was obtained from Royal Spring. Sampling occurred on the southern side of the spring between the outlet and impounding wall (refer to Figures 1.8 and 1.9). The sample was put on ice and transferred to the Environmental Research Training Laboratories (ERTL) on the University of Kentucky campus. The sample was serially diluted to volumes of 10 mL, 1 mL, 0.1 mL, 0.01 mL, 0.001 mL, and 0.0001 mL using HACH dilution water. Each dilution was filtered through a 0.45-µm cellulose filter, along with a blank containing only HACH dilution water. The filters were plated with 2 mL of Difco EC medium with MUG and incubated at 44.5°C for 24 h. The incubated plates were placed under ultraviolet (UV) light to identify fluorescing wild-type *E. coli* colonies. A single, isolated, wild-type colony was transferred by sterile loop to 125 mL of Bacto tryptic soy broth and incubated at 44.5°C

for 24 h on a shaker table. The log-phase growth of wild-type *E. coli* was then used to inoculate previously prepared Difco tryptic soy agar slants. The slants were incubated at room temperature for 24 h and then placed in a refrigerator at 4°C for storage. For longer-term storage, the wild-type *E. coli* was transferred by sterile loop from the slant to 125 mL of Bacto tryptic soy broth, and incubated at 44.5°C for 24 h on a shaker table to log phase of mass growth. The mass growth was centrifuged at 3000g by a Thermo Scientific Sorvall Legend RT+ Centrifuge to separate cells from supernatant. The supernatant was removed by pipette and the cells were resuspended in 10% glycerol. Aliquots (1 mL) of this solution were frozen at -80°C.

Gram staining of the wild-type *E. coli* was performed to ensure that Gram-negative rods were isolated. A drop of mass growth was heat-fixed to a glass slide. The smear was stained with crystal violet for 1 min and gently washed with water. Gram's iodine was applied for 1 min followed by another gentle water wash. The smear was decolorized with 95% alcohol and again gently washed with water. Finally, the smear was counterstained for 45 sec with safranin, washed with water, and blotted dry. The slide was viewed under microscope, and digital photographs were obtained.

2.3 Serology and virulence testing

A slant of the wild-type *E. coli* was sent to the Pennsylvania State University *E. coli* Reference Center for serology and virulence testing. The Center performed tests to identify the O and H antigens, and also LT, STa, STb, Stx1, Stx2, *eae*, CNF1, and CNF2 virulence factors. The virulence factors chosen for analyses were based on suggested PCR screening isolates for E. coli provided by the Pennsylvania State University E. coli Reference Center (2009) (Table 2.1). The O antigen was identified using the method prescribed by Ørskov et al. (1977) and the H antigen was identified using the method prescribed by Machado et al. (2000). Virulence testing was conducted according to DebRoy and Maddox (2001). These methods are summarized below.

Determination of the O antigen was performed via bacterial agglutination reactions. Antisera were produced in rabbits using cultures heated at 100° C for 2 h. Cultures of the strain being serotyped were heated at 100° C for 1 h. Tubes or trays (or

automated methods) were then used to carry out the agglutination reactions. This method used 164 different O groups (∅rskov et al. 1977).

H antigen determination was performed by molecular biology methods. DNA of the strain being serotyped was extracted and purified. Primers were used to amplify the *fliC* flagellin gene using PCR. The restriction endonuclease *Hha*I was used to cleave the amplified sequence, and the resulting fragments were separated by gel electrophoresis. A computer package digitized the gel, and was then used to analyze patterns in the restriction fragments and identify the H group (Machado et al. 2000).

The virulence factors LT, STa, STb, Stx1, Stx2, *eae*, CNF1, and CNF2 were identified by PCR methods. Primers for the gene sequences encoding each of the virulence factors were used in PCR reactions. Gel electrophoresis was used to analyze the PCR reactions and the gels were digitized using Kodak gel scanners. Positive controls are used to identify samples that possess the respective virulence factors (DebRoy and Maddox 2001).

2.4 ^{15}N fate and *E. coli* survival

2.4.1 Microcosm creation

On 8 December 2008, 1-L samples of water were obtained from Royal Spring at the same location as previously sampled. The samples were placed on ice and transferred to the University of Kentucky Department of Earth and Environmental Sciences hydrogeology lab, where they were frozen until ready for use. On 14 April 2009 these samples were transferred to ERTL and autoclaved to kill any viable microorganisms. A 500-mL volume of the sterilized Royal Spring water was placed in each of five 1-L bottles (i.e. microcosms), and labeled A, B, C, D, and E. Additional volumes of the sterilized Royal Spring water were used as controls in 250-mL bottles: 125 mL for a room-temperature microcosm, 125 mL for a refrigerated microcosm, 200 mL for a sterile Royal Spring control, and 50 mL for a sterile control containing Royal Spring water and 50 mL of M9 medium. An additional sterile control labeled “media” was prepared by placing 200 mL of M9 medium in a 250-mL bottle. Table 2.2 shows a summary of the

microcosms and controls with their respective starting volumes of sterile Royal Spring water. All of the microcosms were stored in a wine chiller at 14°C until inoculation.

2.4.2 Enrichment of wild-type *E. coli* with ^{15}N

Bacto tryptic soy broth (100 mL) was inoculated on 14 April 2009 with two drops of log-phase mass growth culture of the isolated wild-type *E. coli*. The solution was incubated at 37°C for 24 h on a shaker table to log phase of mass growth.

^{15}N-enriched M9 medium was prepared by mixing 18.00 g Na_2HPO_4, 9.01 g KH_2PO_4, 1.53 g NaCl, 3.07 g $^{15}NH_4SO_4$ (98+ atom % ^{15}N) and 2 L of deionized (DI) water. This solution (part 1) was autoclaved for 20 min. A second solution (part 2) was prepared by mixing 30 mL glucose, 6 mL $MgSO_4$, 0.3 mL $CaCl_2$, and 963.7 mL of DI water. This solution was also autoclaved for 20 min. After autoclaving, parts 1 and 2 were combined to make 3 L of ^{15}N enriched M9 medium.

The M9 medium was placed in four 1-L bottles, minus two 250-mL increments which were dispersed in each of two 500-mL bottles. All of the bottles, except for one 250-mL bottle (sterile controls), were inoculated with 2 mL of the log-phase tryptic soy broth mass growth. The bottles were incubated at 37°C for 24 h on a shaker table to log phase of mass growth.

The mass growth was centrifuged at 3500g for 6 min using a Thermo Scientific Sorvall Legend RT+ Centrifuge to separate cells from supernatant. The supernatant was discarded and the cells were resuspended in sterilized Royal Spring water. The resuspended *E. coli* was used to inoculate microcosms A-E, each of which received 20 mL of resuspended *E. coli*, and the room temperature and refrigerated positive controls, each of which received 5 mL of resuspended *E. coli*. The sterile Royal Spring control, medium control, and half-and-half sterile Royal Spring and medium control were not inoculated. The medium control contained 200 mL of sterile M9 medium, and the half-and-half control contained 50 mL of M9 medium and 50 mL of sterile Royal Spring water (Table 2.2). It should be noted that positive controls were designed to have the same *E. coli* concentrations as microcosms A-E for consistency.

All of the microcosms and controls were placed in the dark in a wine chiller at 14°C and were removed only for testing and periodic shakings. This experiment was

designed to model low-flow conditions under which only a minimal amount of natural mixing would occur. Therefore, shaking occurred approximately twice per week for the first 60 days of the experiment, and only sporadically thereafter. Shaking was performed by rotating a microcosm upside down and right side up 10 times.

2.4.3 IDEXX

IDEXX Colilert® and IDEXX Quanti-Tray/2000 were used for quantification of *E. coli*. The IDEXX quantification method generates an MPN for the number of total coliforms and *E. coli* in a 100 mL sample. The MPN technique uses statistics to obtain an estimate of viable microorganisms in a sample. As with all viable microbial enumeration methods, the MPN technique must "recover live microorganisms from environmental samples, maintain the viability of those microbial populations to be enumerated, and permit their growth in the laboratory so that they can be detected and their numbers quantified" (Atlas and Bartha 1998).

The IDEXX method is approved by the US Environmental Protection Agency (EPA), AOAC, and other international organizations and accepted by *Standard Methods for the Examination of Water and Wastewater* (APHA 1998). Colilert® medium contains two enzymes, β-galactosidase and β-glucuronidase, that are used by coliforms and *E. coli* to metabolize nutrients. These enzymes cause changes in color and fluorescence when they metabolize nutrients. Coliforms use β-galactosidase to cleave *o*-nitrophenyl-β-D-galactopyranoside (ONPG), and the resulting *o*-nitrophenol group causes a change in color from colorless to yellow. *E. coli* use β-glucuronidase to cleave 4-methylumbelliferyl-β-D-glucuronide (MUG), and the resulting β-D-glucuronide group product fluoresces (IDEXX 2007).

Samples are combined with Colilert® medium, poured into a Quanti-Tray/2000 and incubated. Each Quanti-Tray/2000 contains 49 large cells and 48 small cells. Each of these cells will remain colorless if coliforms are absent, or turn yellow if one or more coliforms are present. A cell that turns yellow will then fluoresce under UV light if one of the coliforms in that cell is *E. coli* (IDEXX 2007).

The procedure to generate the MPN was as follows. Colilert® was first added to the sample and mixed. The mixture was poured into a Quanti-Tray/2000, which was then

sealed using the Quanti-Tray Sealer, and incubated at 35°C for 24 h. After incubation, the numbers of large yellow and small yellow cells were counted, along with the numbers of large fluorescent and small fluorescent cells. The IDEXX Quanti-Tray/2000 MPN table contains MPN values associated with the number of positive large and small cells (exhibiting color change or fluorescence), and is used with the observed counts to generate an MPN (IDEXX no date listed).

2.4.4 Sampling procedures

Samples for IDEXX and ^{15}N analyses took place on days 0, 1, 3, 8, 15, 28, 60, and 130 for microcosms A-E. In general, the procedure for an individual microcosm was as follows, though there was some modification in the dilutions used for the procedures based on the day of sampling (Figures 2.2, 2.3, and 2.4). On a sampling day, the microcosm was removed from the wine chiller and an aliquot was withdrawn to make three different series of serial dilutions (replicates) as follows. The microcosm was shaken 25 times. A 1-mL aliquot was taken from the microcosm and dispersed into HACH dilution-water concentrate to create the first serial dilution for replicate 1. A second 1-mL aliquot was taken and dispersed into HACH dilution water to create the first serial dilution for replicate 2, and a third 1-mL aliquot for replicate 3. Each of the replicates was then serially diluted. It was from dilutions in each of these series of replicates that samples were then taken for both IDEXX and $\delta^{15}N$ analyses.

Table 2.3 shows a summary of the dilutions used for measurement of *E. coli* concentration. IDEXX samples were taken from two different dilutions per replicate series for all of the sampling days except day 0. Thus, there were six total IDEXX tests for each microcosm on a sampling day (except for day 0). For an IDEXX test, a Colilert® packet was dissolved in an appropriate volume of DI water. An aliquot was taken from a serially diluted replicate (after shaking) and dispersed into the Colilert® solution. The mixture was shaken 25 times and poured into a Quanti-Tray/2000, which was then sealed using the Quanti-Tray Sealer and incubated at 35°C for 24 hr. The cells of the Quanti-Tray/2000 were examined for fluorescence, and counted to generate a most probable number (MPN) of *E. coli.* The number of cells exhibiting color change from clear to yellow was also counted to verify contamination from another coliform was not occurring.

Isotope ratio mass spectrometry was used to measure $\delta^{15}N$ values of the *E. coli* in each of the microcosms. Sample preparation occurred according to the method developed by Ward (2008), with slight modification. For each IRMS sample, a 1- or 10-mL (depending on the day) sample was taken from a dilution and filtered through a pre-ashed (20 min @ 550°C) 0.7-μm glass fiber filter (Whatman GF/F 1825 021 or Millipore APFF02500) under vacuum to capture the enriched *E. coli*. A small volume (<5 mL) of HACH dilution water was added to the filter before adding the sample. Each sample was washed with three small volumes (<5 mL) of HACH dilution water to wash sample residue from the sides of the filtering apparatus into the filter. Sample preparation was performed on three different dilutions from each replicate series, resulting in a total of 18 samples per microcosm, and this sampling was prepared in duplicate. Table 2.3 shows a summary of the dilutions used for $\delta^{15}N$ analyses of *E. coli* trapped on each filter, and Figures 2.3, 2.4, and 2.5 diagram the experimental set-up.

Each filter was packed in pre-cleaned aluminum foil and labeled following filtration. The filters were then placed in an oven at 30°C for 12-20 h of drying with the aluminum packing opened. After drying, the portion of each filter that was not exposed to sample was carefully removed to minimize the amount of glass fiber consumed in the isotope analyses. For example, a small ring was first cut around the outside of the filter, removing the clean portion of the filter. The filter was folded in half and a thin layer of glass fiber on the underside of each filter was scraped off each of the folded halves. The folded filter was then folded in half again two more times, and placed in a 7×10 mm tin capsule. The tin capsule was compressed around the filter, producing a small sphere. The tin-capsule packaged filters were placed in a 96-well plate and frozen until isotope ratio analyses.

One additional step was performed for samples analyzed by IRMS. Each packed sample was loaded into another tin capsule containing 10 μl of dried NH_4Cl solution (0.036M). This step was needed because the samples were highly enriched in ^{15}N but they were diluted.

One set of samples was analyzed at the University of Virginia Department of Environmental Sciences (UVA). For this set of analyses, samples were chosen to encompass the entire range of dilutions to ensure the isotope label was measurable (not

diluted too much or too little to be measured). From the initial results, a more targeted set of samples was chosen for IRMS analyses that would be valuable for statistical analyses. This second set of IRMS analyses was performed at the Woods Hole Marine Biological Laboratory (MBL) Stable Isotope Laboratory. The only difference in sample preparation between the two labs should be noted. The addition of the NH_4Cl solution for the UVA samples occurred at UVA, whereas the addition of the NH_4Cl solution for the MBL samples occurred at the University of Kentucky.

The analyzed samples are identified in Table 2.4. For sample identification, the first numbers signify the day that the sample was prepared. The following letter signifies the microcosm or control from which the sample was collected. The number following the letter signifies the replicate, and the final numbers identify the dilution. For example, sample 28A1 10^{-7} was prepared on day 28 from the 10^{-7} dilution of the first replicate from microcosm A. The samples labeled 'df' contained sample duplicates that were filtered though a second brand of filters (two filter brands with the same specifications were used in the experiment). MBL samples labeled 'd' are duplicates of UVA samples. Control samples are identified in the following section.

The data are reported using standard delta notation (Craig 1957):

$$\delta = [(R_{SAMPLE}/R_{STANDARD} - 1)]\times 1000$$

where

$$R = {}^{H}F/{}^{L}F$$

and

$${}^{H}F = \text{abundance of rare isotope and}$$

$${}^{L}F = \text{abundance of common isotope, respectively, of element } F.$$

Adding a second source of nitrogen allowed for the samples to be measured and the true $\delta^{15}N$ value to be calculated following the proportion equation:

$$\Delta_{MIXTURE} = (\delta_{SOURCE1})\times f_1 + (\delta_{SOURCE2})\times f_2$$

where

$$\delta = \text{isotope value, } f = \text{fraction}$$

and

$$f_1 + f_2 = 1.$$

For this experiment:

$\delta_{SOURCE1}$ = $\delta^{15}N$ of bacteria trapped on the filter, any particulates trapped on the filter, and the pre-ashed filter

and

f_1 = fraction of total N from source 1,

whereas

$\delta_{SOURCE2}$ = $\delta^{15}N$ of added NH_4Cl

and

f_2 = fraction of total N from source 2.

At low levels of f_1, f_2 will dominate, resulting in δ values reflective of the background. At a certain level of f_1, the enriched bacteria will rise above the background nitrogen, at which point trends in the isotope data can be identified. The filters were pre-ashed 20 min @ 550°C to mobilize as much ^{15}N from the filters as possible. Particulates from the Royal Spring water should be minimal since the sample was diluted in HACH dilution water. None of the constituents of the dilution water contained nitrogen.

2.4.5 Summary of controls

As mentioned previously, microcosm control experiments were also created for this experiment (see Table 2.2). Two positive (inoculated) microcosm controls were created and used to test for the effects of variation in temperature on the *E. coli* and ^{15}N label. These two controls contained the same ratio of sterilized Royal Spring water and ^{15}N-enriched *E. coli* as microcosms A-E. One microcosm was incubated at room temperature (~23°C) in the dark, while the other was incubated at refrigerator temperature (~2°C) in the dark.

Three negative (not inoculated) microcosm control experiments were performed to test for bacterial contamination and initial ^{15}N levels. The first negative control contained sterilized Royal Spring water, the second M9 medium, and the third a mixture of half sterilized Royal Spring water and half M9 medium. The replicates were not serially diluted for these experiments (Figure 2.9). Instead, each replicate was taken as an aliquot directly from the microcosm. This was done because there was no need to dilute the samples for nitrogen isotope analysis (assuming that the negative microcosm controls were not contaminated).

Additional controls were analyzed by IRMS to define the background level of nitrogen isotope composition for reagents and materials used in the experiments. Samples were prepared using 10 μL of dried NH_4Cl solution (0.036M) packed in tin capsules to determine the amount (and $\delta^{15}N$ value) of nitrogen added to each sample. Filters were analyzed using ashed filters packed in tin capsules to determine the amount (and $\delta^{15}N$ value) of nitrogen in the filters. Finally, HACH dilution water was run through ashed filters and those filters were analyzed to determine if the HACH dilution water contained nitrogen that was trapped in the filter.

Positive and negative control experiment sampling occurred on days 0, 28, and 132, but not all of the samples were analyzed by IRMS. The testing procedures for IDEXX and preparation of ^{15}N samples were the same as those for microcosms A-E, though the dilutions were modified for the day 132 IDEXX samples. Table 2.5 shows a summary of the analyses and dilutions performed for each microcosm control sample and Figures 2.5, 2.6, 2.7, 2.8, and 2.9 diagram the set-up of control experiments. Table 2.4 identifies those controls that were analyzed on the IRMS. For sample identification, the first numbers signify the day that the sample was prepared. The following letters signify the identity of the sample. Samples were labeled as follows: 'filter' contained the pre-ashed filters, 'HACH' contained filters through which dilution water had been run, 'RS' contained filters through which uninoculated Royal Spring water had been run, 'M' contained filters through which uninoculated medium had been run, and 'RSM' contained filters through which uninoculated Royal Spring water and medium had been run. Samples labeled 'NH_4Cl' contained the dried NH_4Cl solution. The number following signifies the replicate. The control samples were not diluted.

2.5 Statistical analyses

Statistical Analysis System (SAS) was used to analyze general linear models (GLMs) of the MPN and isotope data. Model 1 was used to compare microcosms. MPN values were the dependent variable in this model, with microcosms and time the independent variables. MPN values were normalized to make the data easier to manipulate. This model generated an analysis of variance (ANOVA) summary table with calculated *F* values for the full model, microcosm, time, and interaction of

time×microcosm. Each *F* value has an associated *p* value that states the certainty of the relationship. A *p* value ≤ 0.05 was considered significant in this study. The *p* value associated with time×microcosm shows the significance of the interaction of time and microcosm on MPN values. Interactions occur when an independent variable has effects on the dependent variable that vary according to a second independent variable (Hatcher and Stepanski 1994). Additionally, a matrix of *p* values was created to compare each microcosm with each of the other microcosms on each day of the study. Each matrix had five rows and five columns (one matrix for each day). The first row signified microcosm A, the second row microcosm B, etc. The first column signified microcosm A, the second column microcosm B, etc. At the intersection of each row and column in the matrix, a *p* value compares the microcosm in that row with the microcosm in that column. A *p* value ≤ 0.05 would show that the microcosm was significantly different than the microcosm to which it was being compared for that specific day.

Model 2 compared data collected at specific time points (or days). Normalized MPN values were the dependent variable in this model, with microcosms and time the independent variables. An ANOVA summary table was again generated. The *p* value matrix for this model compared data from each day with each of the other days in each microcosm. Therefore, there were eight rows and eight columns (each signifying a time point) in each of five matrices (one matrix for each microcosm). A significant difference between two time points was signified at $p \leq 0.05$.

Model 3 compared time. This model differed from the previous models in that all of the observations from each time point were compiled, regardless of the microcosm from which the observation originated. Only time was used as an independent variable, with MPN values again being dependent. The MPN values were transformed by natural log. This model also generated an ANOVA table with *F* statistics and *p* values, and a *p* value matrix for all pair-wise combinations of time points. In this matrix, a *p* value of less than 0.05 indicates a significant difference between two time points.

Model 4 compared microcosms. In this model, all of the observations from each microcosm were compiled, regardless of the day they were tested. MPN values were transformed by natural log and were the dependent variable, and microcosms were the independent variable. Again, an ANOVA table and *p* value matrix were generated. A *p*

value ≤ 0.05 indicated that a particular microcosm was significantly different than the microcosm with which it was being compared.

Model 5 compared microcosms. $\delta^{15}N$ values (MBL samples) were the dependent variable, with microcosms as the independent variable. An ANOVA table and *p* value matrix were generated. The *p* values in this matrix indicated whether $\delta^{15}N$ values from each microcosm were significantly different from the $\delta^{15}N$ values of another microcosm.

Model 6 compared time. $\delta^{15}N$ values (MBL samples) were the dependent variable, with time as the independent variable. Again, an ANOVA table and *p* value matrix were generated. The *p* values in this matrix indicated whether $\delta^{15}N$ values from each day were significantly different from the $\delta^{15}N$ values of another day.

The first and second models are two-way ANOVAs, while the third through sixth models are F tests. Interaction models could not be run using $\delta^{15}N$ values because data were not available from all of the replicates.

Lastly, a correlation coefficient was calculated in Excel to explore the relationship between MPN and $\delta^{15}N$ values. The correlation coefficient was calculated using samples for which both analyses were performed.

2.6 Supporting information

2.6.1 Isolation of wild-type *E. coli*

The inclusion of MUG in Difco EC Medium allows for the detection of *E. coli*. Most *E. coli* use an enzyme, β-D-glucuronidase, to cleave MUG into 4-methylumbelliferone. The latter compound fluoresces under UV light (Park et al. 1995) Some strains may fail to grow in this medium, or fail to produce fluorescence because they do not possess the necessary enzyme (Becton, Dickinson and Company 2009). *E. coli* O157:H7 is consistently MUG negative, that is, it does not fluoresce (Thompson et al. 1990). Therefore, this isolation methodology avoids selecting for one of the more dangerous strains of *E. coli*.

2.6.2 Gram's staining

Gram's staining, named after Dr. Christian Gram, is a cell-staining procedure used to differentiate two primary groups of bacteria. The staining procedure differentiates between the two groups of bacteria, termed gram-positive and gram-negative, on the basis of structural differences in bacterial cell walls. Gram-positive bacteria have a thick peptidoglycan layer, while gram-negative bacteria have a thin peptidoglycan layer. When a Gram's stain is completed, gram-positive bacteria will appear purple, while gram-negative bacteria will appear red (Cappuccino and Sherman 2005). *E. coli* is gram-negative.

Crystal violet, the primary stain, is used to stain all of the cells. Gram's iodine is then used to bind the crystal violet better and strengthen the stain's color. 95% alcohol is used as a decolorizer for gram-negative bacteria, removing the primary stain. In gram-positive bacteria, the thicker peptidoglycan layer prevents the decolorizing effect from occurring. Instead, alcohol dehydrates the peptidoglycan layer and more tightly binds the primary stain. Safranin is used as a counterstain to color the gram-negative bacteria, having previously been decolorized. Gram-positive bacteria do not absorb the safranin because they already have crystal violet bound in their cell walls (Cappuccino and Sherman 2005). The resulting color differences between the two groups can then be observed under microscope.

Table 2.1 Suggested PCR screening for virulence attributes in *E. coli* isolates (*E. coli* Reference Center 2009).

Host	LT	STa	STb	*stx* 1	*stx* II	*cnf* 1	*cnf*2	eae	K88	K99	987P	CS31A	F1845	F107	*bfp*
Bovine		x		x	x	x	x	x		x		x	x		
Porcine	x	x	x	x	x	x	x	x	x	x	x			x	
Equine	x	x	x	x	x	x	x	x							
Canine	x	x	x	x	x	x	x	x							
Feline	x	x	x	x	x	x	x	x							
Human	x	x	x	x	x	x	x	x							x

Table 2.2 Summary of starting volumes of sterilized Royal Spring water, M9 medium, and resuspended *E. coli* (inoculation solution) used in microcosms and controls.

All in mL	**Sterile Royal Spring**	**M9 medium**	**Resuspended *E. coli***
Microcosm A (14°C)	500	0	20
Microcosm B (14°C)	500	0	20
Microcosm C (14°C)	500	0	20
Microcosm D (14°C)	500	0	20
Microcosm E (14°C)	500	0	20
Room temp. microcosm (~25°C)	125	0	5
Refrigerated microcosm (~2°C)	125	0	5
Sterile Royal Spring	200	0	0
Media	0	200	0
50:50 Royal Spring:media	50	50	0

Table 2.3 Summary of testing days and dilutions used for sample analyses.

	Dilutions used	
Day	**IDEXX**	**^{15}N Filter**
0	10^{-8}	10^{-4}, 10^{-6}, 10^{-8}
1	10^{-8}, 10^{-10}	10^{-3}, 10^{-5}, 10^{-7}
3	10^{-8}, 10^{-10}	10^{-3}, 10^{-5}, 10^{-7}
8	10^{-8}, 10^{-10}	10^{-3}, 10^{-5}, 10^{-7}
15	10^{-8}, 10^{-10}	10^{-3}, 10^{-5}, 10^{-7}
28	10^{-8}, 10^{-10}	10^{-3}, 10^{-5}, 10^{-7}
60	10^{-8}, 10^{-10}	10^{-3}, 10^{-5}, 10^{-7}
130	10^{-7}, 10^{-9}	10^{-3}, 10^{-5}, 10^{-7}

Table 2.4 Identification of IRMS samples sent to University of Virginia Department of Environmental Sciences and Woods Hole MBL Stable Isotope Laboratory.

UVA samples	MBL samples
NH_4Cl	NH_4Cl
NH_4Cl	NH_4Cl
15Filter	130Filter
28HACH	130HACH
0RS1	0RS2
0M1	0M2
0RSM1	0RSM2
28A1 10^{-7}	0A1 10^{-4}d
15A1 10^{-7}	1A1 10^{-3}d
8A1 10^{-7}	3A1 10^{-3}d
3A1 10^{-7}	8A1 10^{-3}d
1A1 10^{-7}	15A1 10^{-3}d
0A1 10^{-8}	28A1 10^{-3}d
8A2 10^{-7}	60A3 10^{-3}d
28C3 10^{-7}df	130A3 10^{-3}d
28A1 10^{-5}	28R3 10^{-3}
15A1 10^{-5}	130R3 10^{-3}
8A1 10^{-5}	28F3 10^{-3}
3A1 10^{-5}	130F3 10^{-3}
1A1 10^{-5}	1A3 10^{-3}
0A1 10^{-6}	1B3 10^{-3}
8A2 10^{-5}	1C3 10^{-3}
28B2 10^{-5}df	1D3 10^{-3}
28A1 10^{-3}	1E3 10^{-3}
15A1 10^{-3}	60A3 10^{-3}
8A1 10^{-3}	60B3 10^{-3}
3A1 10^{-3}	60C3 10^{-3}
1A1 10^{-3}	60D3 10^{-3}
0A1 10^{-4}	60E3 10^{-3}
8A2 10^{-3}	28A3 10^{-3}
28A1 10^{-3}df	28B3 10^{-3}
	28C3 10^{-3}
	28D3 10^{-3}
	28E3 10^{-3}
	130A3 10^{-3}
	130B3 10^{-3}
	130C3 10^{-3}
	130D3 10^{-3}
	130E3 10^{-3}

Table 2.5 Summary of sampling days and dilutions used for analyses of controls.

	IDEXX			**^{15}N Filter**		
	Testing days			*Testing days*		
	Dilutions used			Dilutions used		
Room temp. microcosm	*0*	*28*	*132*	*0*	*28*	*132*
	10^{-8}	10^{-8} 10^{-10}	10^{-7} 10^{-9}	10^{-4} 10^{-6} 10^{-8}	10^{-3} 10^{-5} 10^{-7}	10^{-3} 10^{-5} 10^{-7}
Refrigerated microcosm	*0*	*28*	*132*	*0*	*28*	*132*
	10^{-8}	10^{-8} 10^{-10}	10^{-8} 10^{-10}	10^{-4} 10^{-6} 10^{-8}	10^{-3} 10^{-5} 10^{-7}	10^{-3} 10^{-5} 10^{-7}
Sterile Royal Spring	*0*	*28*	*132*	*0*	*28*	*132*
	10^{-2}	10^{-2}	10^{-2}	10^{0}	10^{0}	10^{0}
Media	*0*	*28*	*132*	*0*	*28*	*132*
	10^{-2}	10^{-2}	10^{-2}	10^{0}	10^{0}	10^{0}
50:50 Royal Spring:media	*0*	*28*	*132*	*0*	*28*	*132*
	10^{-2}	10^{-2}	10^{-2}	10^{0}	10^{0}	10^{0}

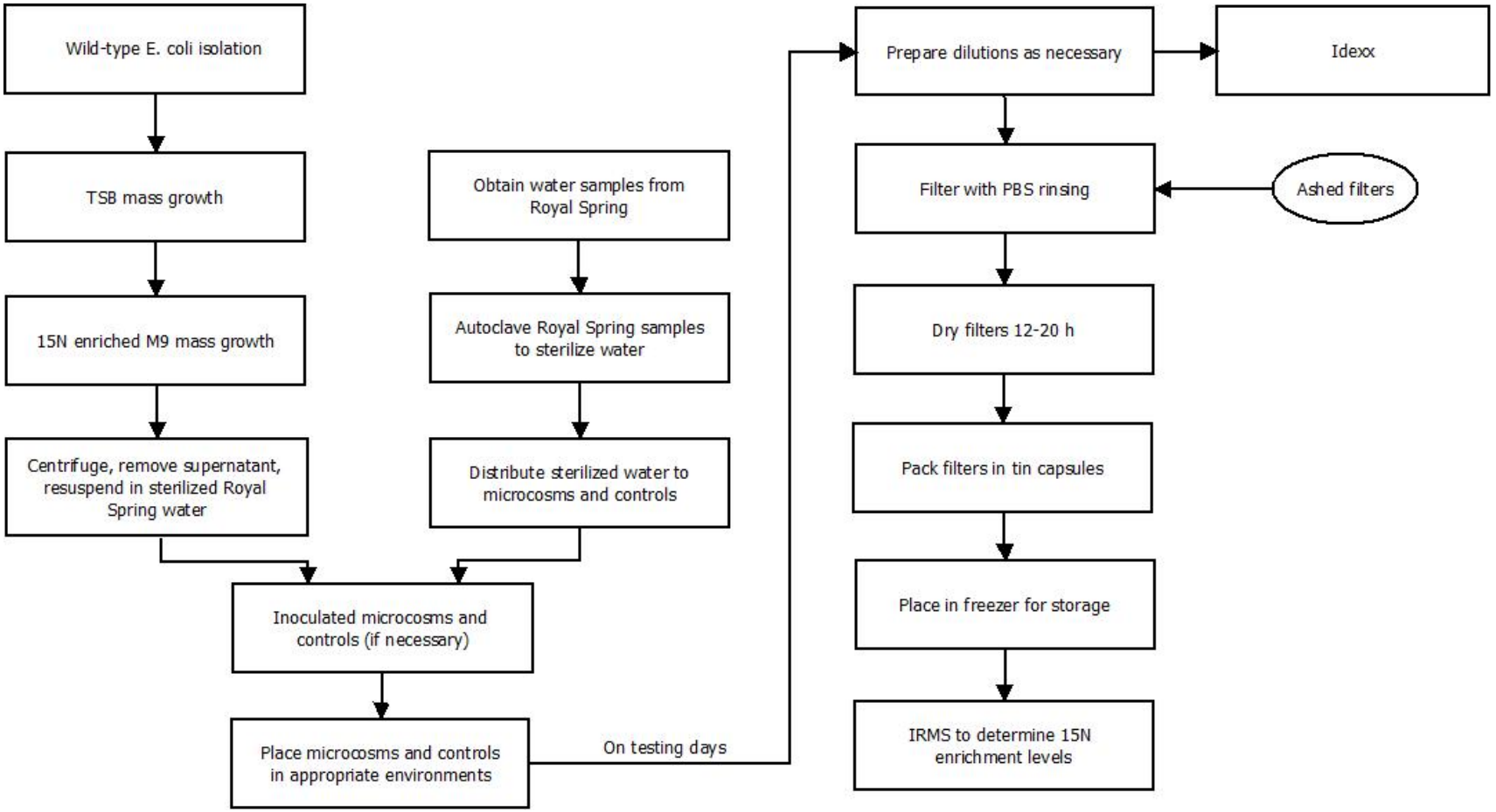
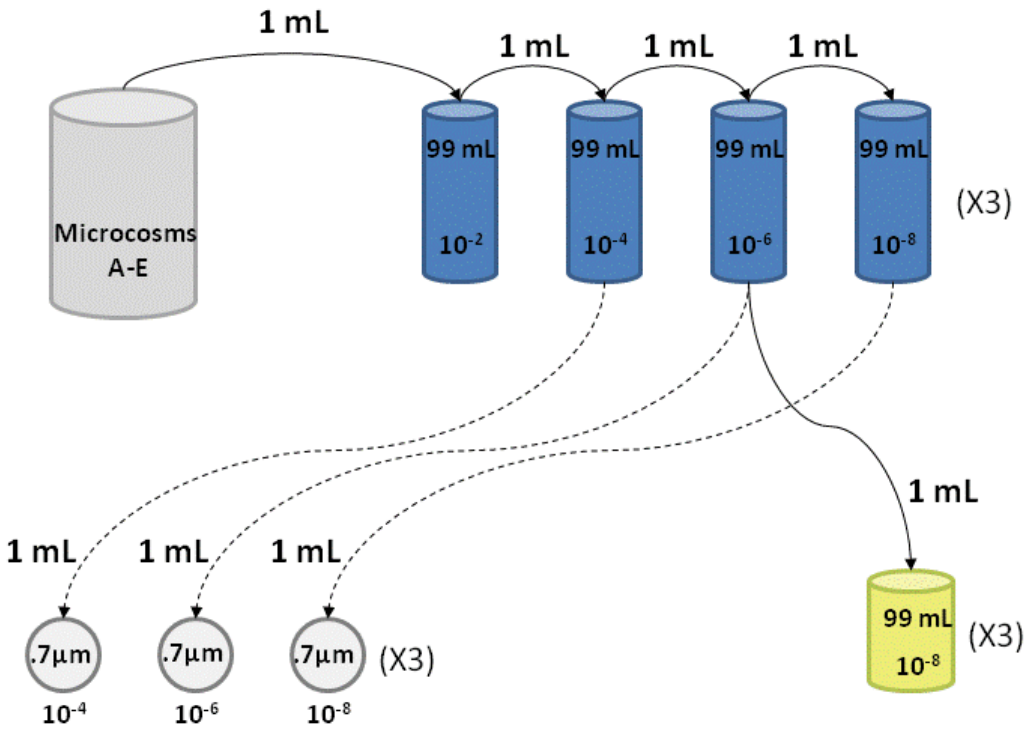


Figure 2.1 Experimental design workflow



Blue = HACH dilution water
Gray circles = ashed filters
Yellow = DI Water with Colilert® media for IDEXX
X3 = three sample replicates
10^{-n} (n = 2, 4, 6, 8) = dilutions

Day 0

Figure 2.2 Dilutions, IDEXX testing, and IRMS sample preparation for day 0.

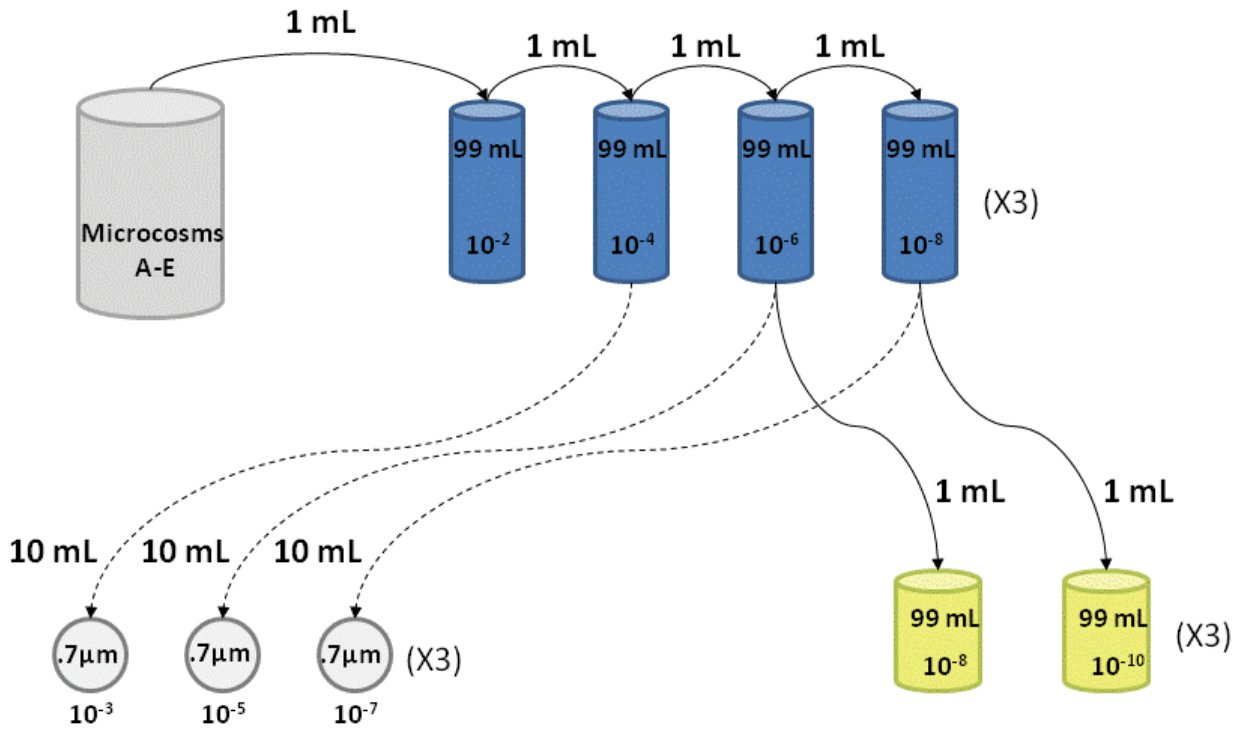


Figure 2.3 Dilutions, IDEXX testing, and IRMS sample preparation for days 1, 3, 8, 15, 28, and 60.

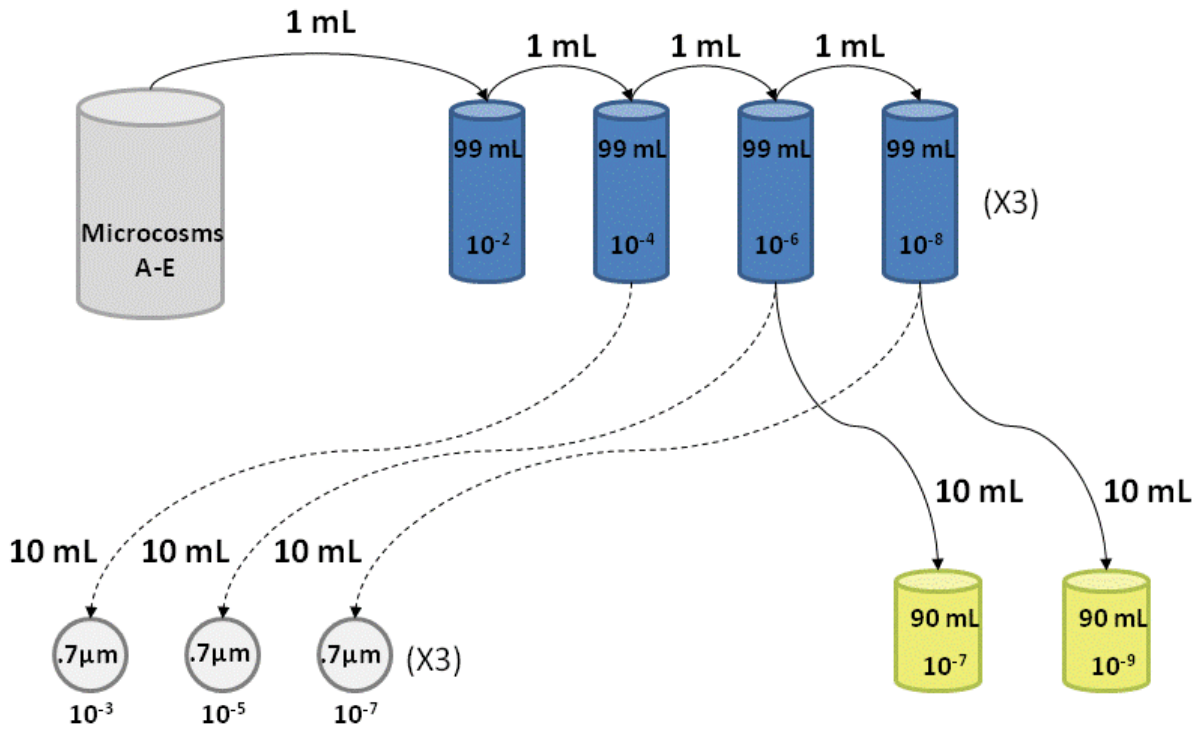


Figure 2.4 Dilutions, IDEXX testing, and IRMS sample preparation for day 130.

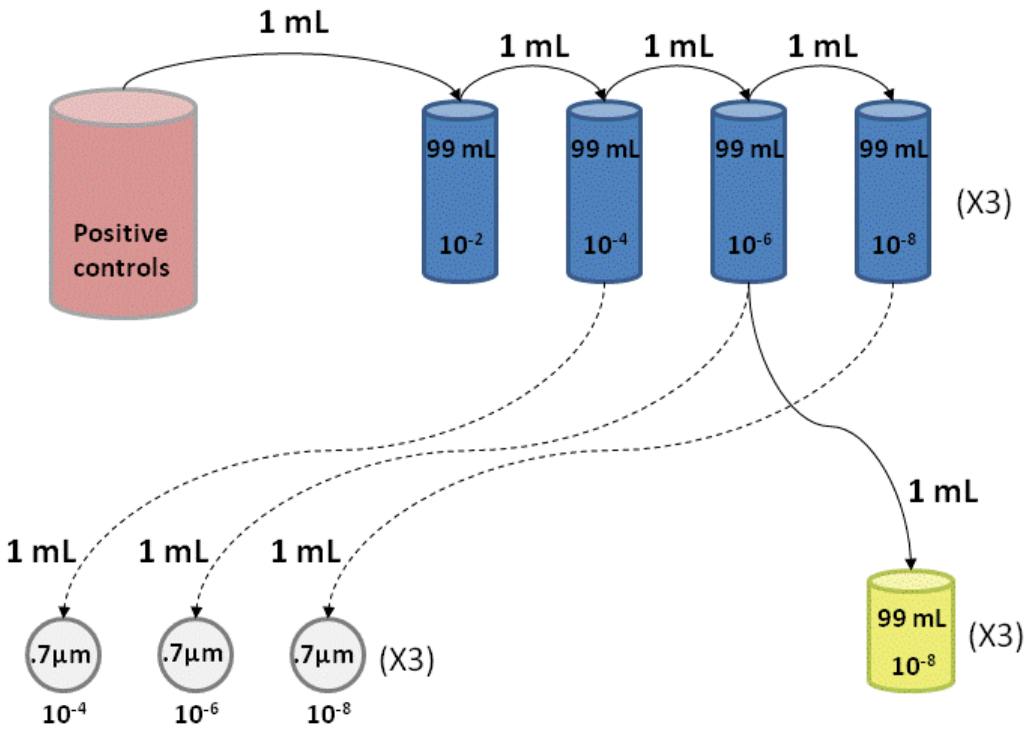


Figure 2.5 Dilutions, IDEXX testing, and IRMS sample preparation for day 0 positive controls.

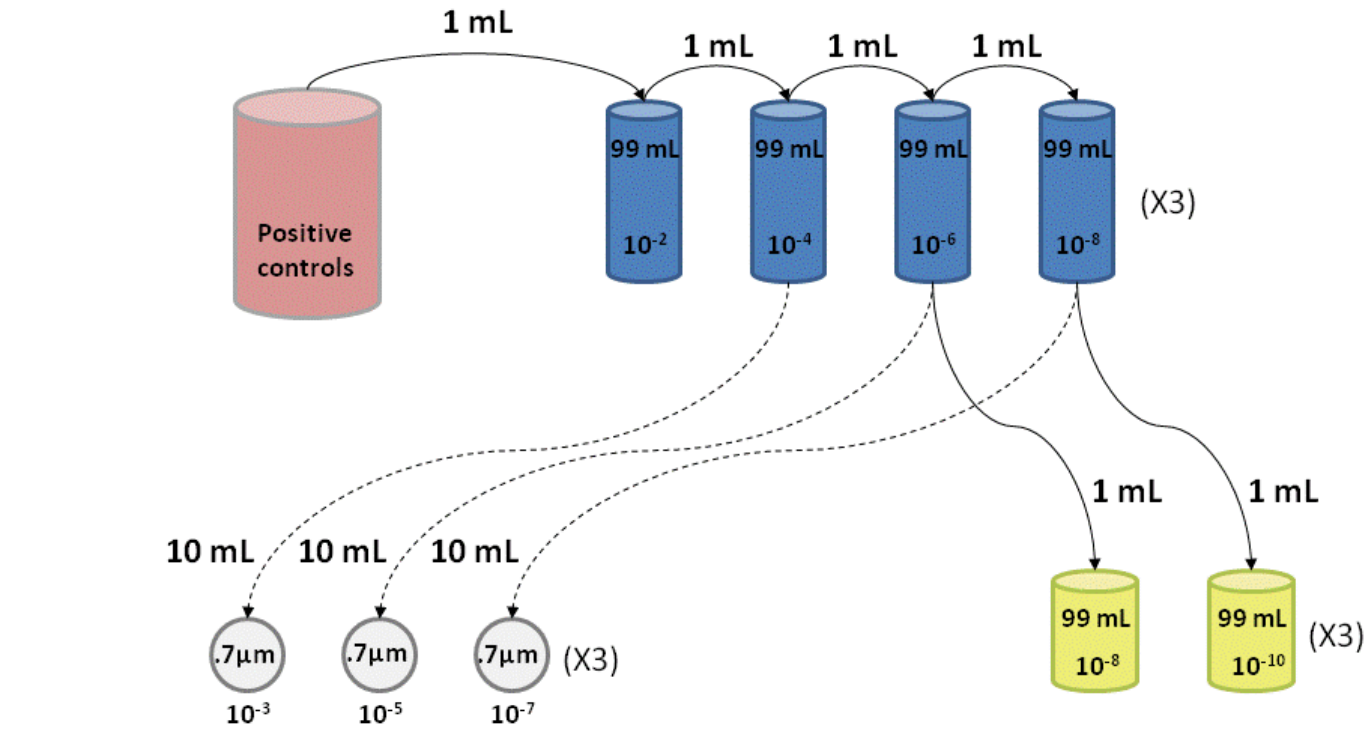


Figure 2.6 Dilutions, IDEXX testing, and IRMS sample preparation for day 28 positive controls.

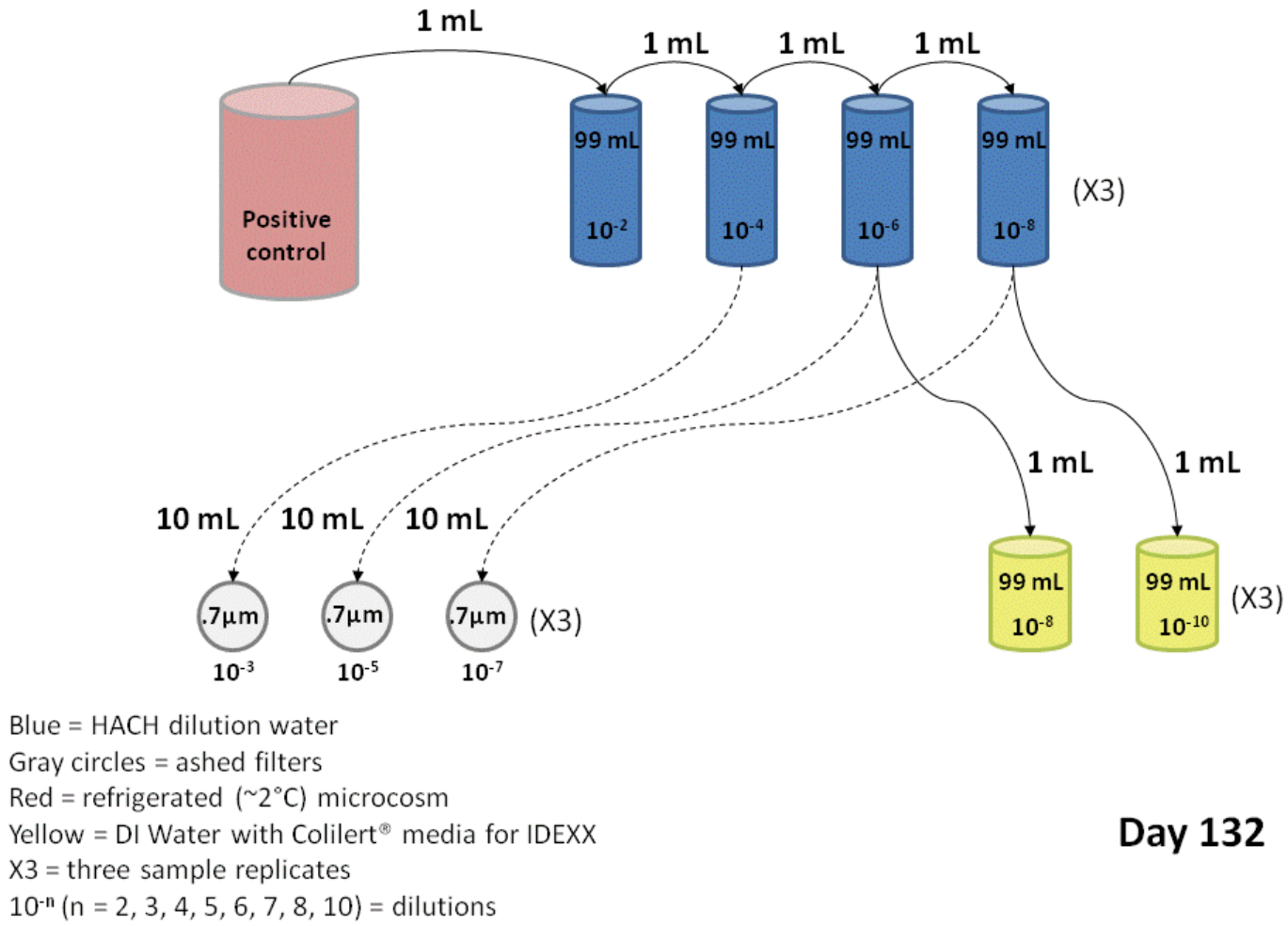


Figure 2.7 Dilutions, IDEXX testing, and IRMS sample preparation for day 132 refrigerated positive control.

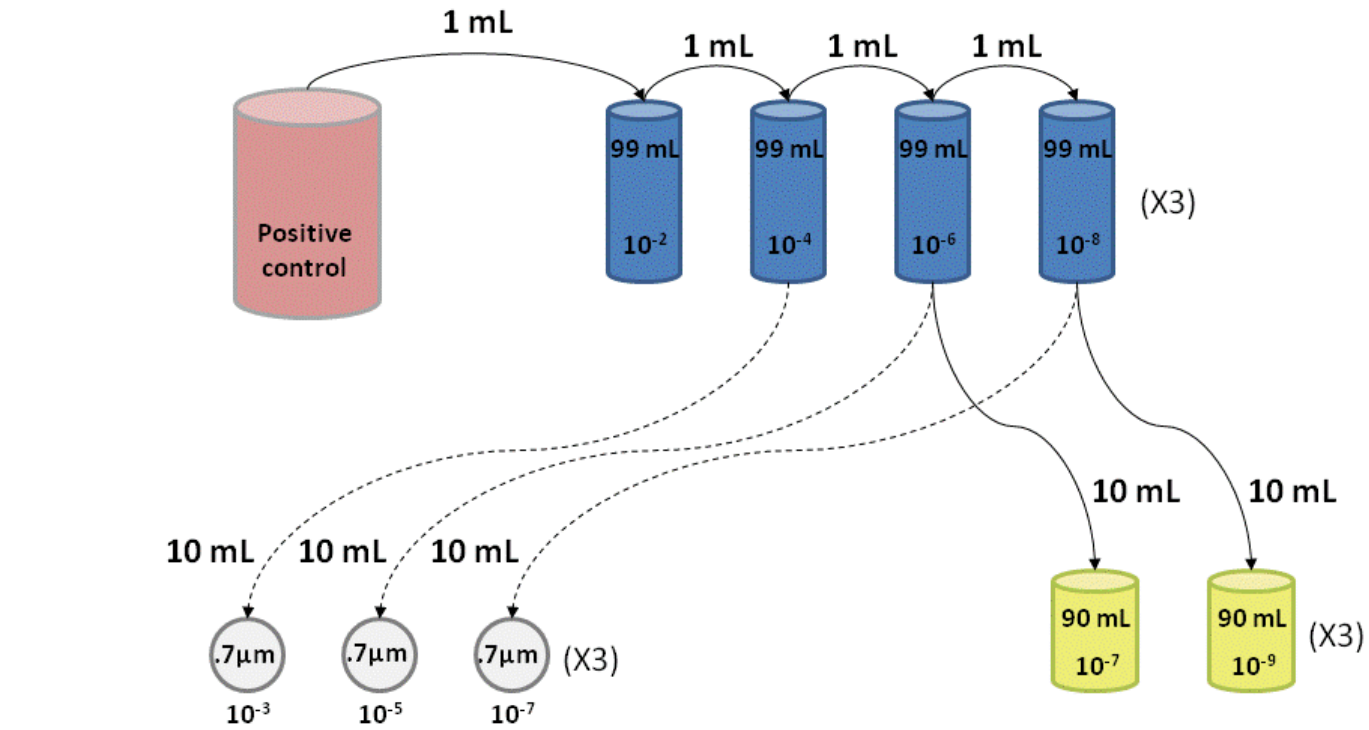
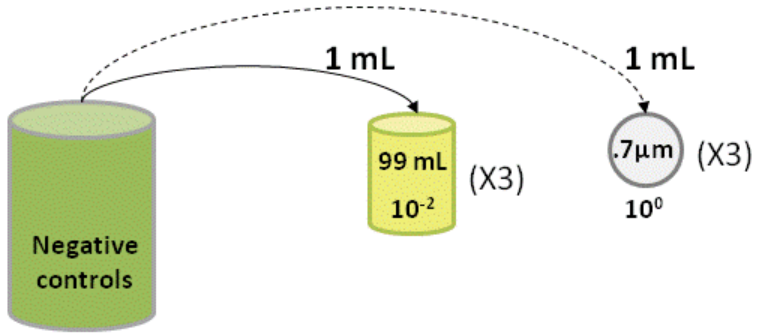


Figure 2.8 Dilutions, IDEXX testing, and IRMS sample preparation for day 132 room temperature positive control.



Green = Royal Spring, media, and 50:50 Royal Spring:media
Yellow = DI Water with Colilert® media for IDEXX
X3 = three sample replicates
10^{n} (n = -2, 0) = dilutions

Days 0, 28, 132

Figure 2.9 Dilutions, IDEXX testing, and IRMS sample preparation for negative controls on days 0, 28, and 132.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Isolation, serology, and virulence testing of wild-type *E. coli*

A single isolated fluorescing colony was chosen from the 10× dilution as the wild-type strain. The 10× dilution had 28 fluorescing colonies (Table 3.1). If the volume of sample and number of *E. coli* was multiplied by 10 (280 cfu per 100 mL), this sample would exceed the USEPA (1986) primary recreational contact recommendations for *E. coli* in a single sample (maximum 235 cfu per 100 mL). Figure 3.1 shows an image of the gram-stained wild-type *E. coli*. The wild-type *E. coli* were gram-negative, as expected.

Table 3.2 shows results of serological analyses and virulence testing. The isolated strain of wild-type *E. coli* was O^-:H^-. O^- strains are either rough (R) and lacking the O antigen portion of their lipopolysaccharide (LPS), or do not react in agglutination reactions because the antisera used do not match their O antigen. H^- strains are non-motile and do not produce flagella. The isolated strain was negative for all of the tested virulence factors, indicating that the strain is likely commensal to either humans or animals. It should be noted that although this wild-type strain does not possess any of the virulence factors used for testing, virulence factors are known to transfer horizontally between strains via genetic mechanisms (Donnenberg 2002).

3.2 *E. coli* survival

Figure 3.2 shows the average MPN for each microcosm (along with room and refrigerated controls) over the length of the study. The figure was created by averaging the IDEXX results of the three replicates for each microcosm on each day. *E. coli* had a mean starting concentration of 5.62×10^{10} with a standard error of 4.12×10^{9} and mean ending concentration of 5.88×10^{10} with a standard error of 7.53×10^{9}. All concentrations are shown in *E.* coli/100 mL. This indicated that the *E. coli* survived well over the course of the study, since the starting concentration was within the standard error of the ending concentration, and vice versa. The refrigerated (~2°C) control showed results similar to microcosms A-E, whereas the room temperature (~23°C) control showed more die-off after 132 days. Statistical models were used to further explore trends in the MPN data.

Model 1 in SAS was used to test the effect of microcosms, time, and their possible interaction on MPN. The model output shows that variation in the MPN data is largely a result of time, with an *F* value of 15.64 and *p* value of <0.0001. The microcosms, termed treatments in the models, had an *F* value of 2.52 and *p* value of 0.0478. The interaction term time×microcosm had an *F* value of 2.18 and *p* value of 0.0038. This showed that interaction of the two independent variables was significant. In other words, the variation in the MPN data from each microcosm is caused by the influence of time on MPN values. The model also produced a treatment comparison matrix with *p* values for the interaction of each microcosm with each of the other microcosms for each day of the experiment. The results of the treatment comparison matrix are summarized in Table 3.3. Output for Model 1 (and all models following) is reproduced in Appendix D. Based on the results of day 0, no correction was applied for variations in the original concentration of *E. coli* in each microcosm because the differences in day 0 concentration between microcosms were not statistically significant.

Noting that time played an important role in the variation of MPN data, the same statistical model was rerun to test for interactions between time points in each of the microcosms (Model 2). Multiple significant differences were found for each microcosm in the time comparison matrix produced by the model output, and these differences are summarized in Table 3.4. Only some of the significant differences are meaningful in terms of understanding the behavior of the *E. coli*. For example, day 3 might be significantly different than day 28, but if day 8 is also significantly different than day 15 the relationship between days 3 and 28 is less valuable for interpretation than the relationship between days 8 and 15. For ease of interpretation, additional models were run to determine significant differences in the dataset as a whole.

Model 3 was a marginal model used to compare time points with one another. In this model, the data from all of the microcosms were compiled for each time point. This model produced an *F* value of 9.56 and *p* value of <0.0001, indicating that there were significant differences between time points. The output matrix produced by the model is summarized in Table 3.5.

Model 4 was a marginal model used to compare microcosms with one another. This model was similar to the first, but instead compiled all MPN data for each

microcosm together, regardless of time. The model produced an *F* value of 1.21 and *p* value of 0.3085, meaning the microcosms were not significantly different from one another. Table 3.6 shows a summary of the comparisons between each microcosm, produced by analysis of *p* values in the model's output matrix. The only significant difference found was between microcosms A and E. The output of this model reinforced the interpretation of model 1, that time was playing a larger role in the variation of the data than differences between each of the microcosms.

Figure 3.3 displays the average MPN for all microcosms on each day after natural- log transformation. Model 3 (Table 3.3) examined the effect of time on MPN and can therefore be used to analyze Figure 3.3. There are no significant differences between days 0 and 1, 1 and 3, and 3 and 8. This shows that the concentration of the *E. coli* remained stable for the first eight days of the study.

Between days 8 and 15, there was a significant increase in the concentration of *E. coli*. This indicated increased reproduction of the bacteria, which could be due to the use of remnant (i.e. unspent) medium introduced into the microcosm during resuspension of the bacteria. As outlined in Section 2.4.2, the mass growth of *E. coli* was centrifuged and the supernatant (enriched M9 medium) removed to separate cells from the supernatant. The cells were then resuspended in sterilized Royal Spring water. It is possible (and likely) that a small amount of the supernatant was unable to be removed. Under this scenario, the *E. coli* would have been adjusting to their new environment between days 0 and 8. After adjustment, remnant medium would be used as the nutrient source and increase concentration between days 8 and 15. Alternatively, if no remnant medium existed, this increase in concentration could be explained by the use of nutrients in the sterilized spring water in the microcosms.

There was no significant difference between days 15 and 28, showing that the concentration remained stable over this time period. This indicates that whatever nutrient source the bacteria were using to increase concentration between days 8 and 15 had been consumed, or population effects had taken control. It is unclear if the bacteria were actively reproducing to maintain their concentration (and thus using more nutrients), or simply persisting in the microcosms (using fewer nutrients). The trends between days 8 and 15 and days 15 and 28 seem to indicate that at first the bacteria used whatever

nutrients were available to increase their concentration slightly, and then were simply persisting in the microcosms. Chemical analyses of nutrient levels could be used to delve deeper into these trends in future studies. The average MPN for day 28 is significantly greater than for day 60, indicating die-off. There is no significant difference between days 60 and 130, showing stability in concentration.

There are three additional relationships that should be discussed, even though their respective time points are not adjacent to one another. The relationship between days 15 and 60 shows a significant difference with a *p* value of <0.0001. The relationship between days 15 and 130 also shows a significant difference with a p value of <0.0001. Lastly, the relationship between days 28 and 130 shows a significant difference with a *p* value of <0.0001. The overall trend between days 15 and 130 is downward, indicating die-off, though there are stable concentrations between days 60 and 130. Table 3.7 shows a summary of *p* values used in this analysis.

Lastly, comparison of days 0 and 130 shows a *p* value of 0.8829, indicating there is no significant difference between the starting and ending concentrations of *E. coli*. *E. coli* therefore can persist for at least 130 days in sterilized Royal Spring water under simulated low-flow karst conditions with starting concentrations near 5×10^{10} *E. coli* per 100 mL.

3.3 Fate of ^{15}N in isotope labeled *E. coli*

Results from the first set of isotope analyses (UVA) are reported in Table 3.8. The first set of isotope samples was used to determine the appropriate dilution to use for the second set of samples (MBL). All of the samples came from microcosm A for the first set. Performing this range-finding analysis was necessary because it was uncertain which dilution would show δ^{15}N values above background. Again, the concern was that the heavily diluted samples would not have a measurable amount of the rare isotope. Were this to occur, there would be difficulty in performing the IRMS measurement of a sample so highly enriched, and the data would not be useful for analysis. Table 3.8 shows that the 10^{-3} dilution was appropriate for the second set of isotope samples because it had δ^{15}N values well above background. The values from day 0 had different dilutions from the rest of the dataset and therefore were not directly comparable. Ignoring day 0

and replicate samples, the 10^{-7} dilution had a mean $\delta^{15}N$ value of 2.02 ‰ with a standard error of 0.02 ‰, while the 10^{-5} dilution had a mean of 5.42 ‰ with a standard error of 0.30 ‰. The 10^{-3} dilution sample set had a mean $\delta^{15}N$ value of 152.03 ‰ with a standard error of 11.14 ‰. The coefficient of variation (CV) for the 10^{-7}, 10^{-5}, and 10^{-3} dilutions was 0.02, 0.13, and 0.16, respectively. On day 8, replicates were run to obtain an estimate for the precision of isotope data from similar samples. The 10^{-7} dilution had a difference of 0.02 ‰ between the first and second replicate, while the 10^{-5} dilution had a difference of 1.00 ‰ between the first and second replicate. The 10^{-3} dilution had a difference of 15.58 ‰, indicating less precision in the more highly enriched samples. Slight variation in f_1 (the fraction of enriched bacteria) would result in more variation in δ of the sample once above background levels, simply because the *E. coli* were so highly enriched. Figure 3.4 displays time series trends from 0 to 28 days for the first set of isotope samples.

The second set of samples, which consisted of samples of the third replicate of the 10^{-3} dilution from each microcosm (on days 1, 28, 60, and 130), was used for statistical analyses and to extend the isotope data to 130 days. Table 3.9 shows a side-by-side comparison of duplicate samples run at both the University of Virginia and Woods Hole MBL Stable Isotope Lab. The NH_4Cl background solution was prepared and dispersed at the University of Virginia for the first set of isotope samples and at the University of Kentucky for the second set of samples. The second sample set had lower $\delta^{15}N$ values for the background solution, indicating possible differences in the $\delta^{15}N$ value of reagents or inter-lab variability.

The data displayed in Table 3.9 showed that duplicate samples had different $\delta^{15}N$ values depending on the lab performing analyses. Omitting day 0 samples, the UVA duplicate samples had a mean of 152.03 ‰ with a standard error of 11.14 ‰ and CV of 0.16 for the 10^{-3} dilution, whereas the MBL duplicate samples had a mean of 913.00 ‰ with a standard error of 66.44 ‰ and CV of 0.16 for the 10^{-3} dilution. The discrepancy between means of the UVA and MBL duplicate samples was further investigated by determining the ratio of the MBL duplicate samples to the UVA duplicate samples. This ratio had a mean of 6.02 with a standard error of 0.23. The small standard error indicates that the ratio between samples was fairly constant. A large standard error would indicate

greater variation in the ratio between duplicates, meaning that sometimes both duplicates would have high values and other times both duplicates would have low values, and still other times there would be a combination of high and low values. The fairly constant ratio seen in the data indicates that one of the duplicates was consistently high while the other was consistently low. This suggests that the discrepancy was likely a result of a difference in some sample preparatory procedure. The only such procedure differing between the sample sets was preparation and dispersal of the NH_4Cl background.

The isotope proportionation equation

$$\Delta_{MIXTURE} = (\delta_{SOURCE1})\times f_1 + (\delta_{SOURCE2})\times f_2$$

can be used to explain this discrepancy. The δ of an individual sample in this experiment was $\Delta_{MIXTURE}$ and $\delta_{SOURCE1}$ was the highly enriched bacteria trapped on the filter. $\delta_{SOURCE2}$ was the NH_4Cl background added to each sample. Since $\delta_{SOURCE1}$ is highly enriched, a small change in the fraction of $\delta_{SOURCE2}$ in the sample will have a large effect on $\Delta_{MIXTURE}$. In this case, the second set of samples had higher δ values likely as a result of less NH_4Cl background added relative to the first set of samples. This could be explained by small differences in concentration of nitrogen in the background solution or small differences in the amount of background solution added to the sample. The first set of samples could have had a higher concentration or volume of background solution, and/or the second set of samples could have had a lower concentration or volume of background solution. A difference in background concentration could result from solution preparation or from differences in the product used to create the solution. One or more of the above possibilities probably caused the differences between sample sets.

The second set of isotope samples was analyzed statistically using SAS to look for trends in the $\delta^{15}N$ data. First, the microcosms were compared to one another using a GLM (Model 5). This model resulted in an *F* value of 0.72 and *p* value of 0.5944, meaning that as a whole the microcosms were not significantly different from one another in $\delta^{15}N$ values, as with the MPN models. In addition, the *p* value matrix showed that none of the individual microcosms had a significant difference from any other microcosm. Second, time was analyzed as a source of variation in the data using a GLM (Model 6). This model resulted in an *F* value of 3.10 and *p* value of 0.0562. The *p* value matrix showed that day 1 was significantly different than days 28 and 60. Figure 3.5

displays $\delta^{15}N$ values for the second set of samples. $\delta^{15}N$ values increase between days 1 and 28, and as noted above, this trend was statistically significant. The trend in $\delta^{15}N$ values can be explained by the upward trend in MPN between days 8 and 15, which suggests that remnant medium was the nutrient source for the *E. coli* concentration uptick. If the *E. coli* were using ^{15}N-enriched remnant medium, f_1 would increase with bacterial concentration while $\delta_{SOURCE1}$ would remain constant, resulting in higher δ values. If the *E. coli* were using nutrients inherent to the spring water to reproduce, f_1 would again increase as the concentration of bacteria increased. However, $\delta_{SOURCE1}$ would decrease because the bacteria reproducing using spring nutrients would be less enriched, and thus would either maintain or decrease sample δ values from previous measurements.

3.4 Correlation between *E. coli* MPN and $\delta^{15}N$

A scatterplot for MPN and $\delta^{15}N$ values was created using MBL samples that had undergone both analyses and is presented in Figure 3.6. The scatterplot indicates that there is no relationship between $\delta^{15}N$ and MPN values. That is, as MPN increases, $\delta^{15}N$ appears to stay the same. A correlation coefficient of -0.02 was computed using Excel, indicating there is not a linear relationship between $\delta^{15}N$ and MPN values.

According to the interpretation of the preceding section, there should be some correlation between $\delta^{15}N$ and MPN values. Figure 3.6 contains samples from days 1, 28, 60, and 130. Samples from day 60 and 130 could be hiding a correlation between $\delta^{15}N$ and MPN values on days 1 and 28. To investigate this further, another scatterplot was created using only the data from days 1 and 28 (Figure 3.7). The correlation coefficient calculated using Excel was 0.41421, indicating there is a slight positive linear relationship between the $\delta^{15}N$ and MPN values on days 1 and 28, as would be expected according to the previous interpretation.

Table 3.1 Results of isolation procedure.

Dilution (mL)	10	1	.1	.01	.001	.0001
Count (fluorescing)	28	1	0	0	0	0

Table 3.2 Results of serological analyses and virulence testing.

	O type	H type	LT	STa	STb	Stx1	Stx2	*eae*	CNF1	CNF2
WT-*E. coli*	-	-	-	-	-	-	-	-	-	-

Table 3.3 Summarized results of treatment comparison matrix (Model 1). It should be noted that the relationships in this table are reciprocal. For example, if microcosm D is significantly different than microcosm A, microcosm A is significantly different than microcosm D.

Day	Interpretation
0	No significant differences between A, B, C, D, and E
1	No significant differences between A, B, C, D, and E
3	D significantly different than A, B, and C
8	No significant differences between A, B, C, D, and E
15	C significantly different than B and E
28	No significant differences between A, B, C, D, and E
60	E significantly different than A and B
130	A significantly different than B, C, D, and E

Table 3.4 Summarized results of time comparison matrix (Model 2). It should be noted that the relationships in this table are reciprocal. For example, if day 15 is significantly different than day 0, day 0 is significantly different than day 15.

Microcosm	Interpretation
[A-E]	Day 15 significantly different than days 0, 1, 3, 8, and 60 Day 28 significantly different than days 0, 1, 3, 8, and 60 Day 60 significantly different than day 130 Day 130 significantly different than days 0, 1, 3, 8, and 60

Table 3.5 Summarized results of marginal model comparing significant differences between time points (Model 3).

Day	Interpretation
0	Significantly different than days 15 and 28
1	Significantly different than days 15 and 28
3	Significantly different than days 15 and 28
8	Significantly different than days 15 and 28
15	Significantly different than days 0, 1, 3, 8, 60, and 130
28	Significantly different than days 0, 1, 3, 8, 60, and 130
60	Significantly different than days 15 and 28
130	Significantly different than days 15 and 28

Table 3.6 Summarized results of marginal model comparing significant differences between microcosms (Model 4).

Microcosm	Interpretation
A	Significantly different than E
B	No significant differences between A, B, C, D, and E
C	No significant differences between A, B, C, D, and E
D	No significant differences between A, B, C, D, and E
E	Significantly different than A

Table 3.7 Summary of *p* values used for analysis of time series MPN data.

Relationship between	p value	Interpretation
Days 0 and 1	0.6011	No significant difference
Days 1 and 3	0.7438	No significant difference
Days 3 and 8	0.1732	No significant difference
Days 8 and 15	<0.0001	Significant difference
Days 15 and 28	0.095	No significant difference
Days 28 and 60	0.0077	Significant difference
Days 60 and 130	0.1568	No significant difference
Days 15 and 60	<0.0001	Significant difference
Days 15 and 130	<0.0001	Significant difference
Days 28 and 130	<0.0001	Significant difference

Table 3.8 Isotope results from first set of samples with analyses performed at the University of Virginia Department of Environmental Sciences. Samples marked 'df' indicate that the sample is a replicate of a similar sample using an alternate filter brand.

Sample	$\delta^{15}N$ (‰)
0A1 10^{-8}	2.05
1A1 10^{-7}	1.98
3A1 10^{-7}	1.97
8A1 10^{-7}	2.08
8A2 10^{-7}	2.06
15A1 10^{-7}	2.03
28A1 10^{-7}	2.02
28C3 10^{-7} df	1.99
0A1 10^{-6}	3.08
1A1 10^{-5}	4.89
3A1 10^{-5}	6.02
8A1 10^{-5}	6.11
8A2 10^{-5}	5.11
15A1 10^{-5}	5.04
28A1 10^{-5}	4.86
28B2 10^{-5} df	4.99
0A1 10^{-4}	154.05
1A1 10^{-3}	142.11
3A1 10^{-3}	129.39
8A1 10^{-3}	133.10
8A2 10^{-3}	148.68
15A1 10^{-3}	168.08
28A1 10^{-3}	187.45
28A1 10^{-3} df	248.76

Table 3.9 Control and duplicate sample analyses performed at University of Virginia and Woods Hole.

	UVA samples	MBL samples
Sample	$\delta^{15}N$ (‰)	$\delta^{15}N$ (‰)
NH_4Cl	1.50	-5.1
NH_4Cl	1.44	-3.2
15Filter / 130Filter	2.72	NA
28HACH / 130HACH	1.55	NA
0RS1 / 0RS2	1.87	0.7
0RSM1 / 0RSM2	1.66	13.1
0M1 / 0M2	1.75	18.7
0A1 10^{-4}	154.05	75.3
1A1 10^{-3}	142.11	789.8
3A1 10^{-3}	129.39	767.9
8A1 10^{-3}	133.10	870.8
15A1 10^{-3}	168.08	1100.4
28A1 10^{-3}	187.45	1036.1

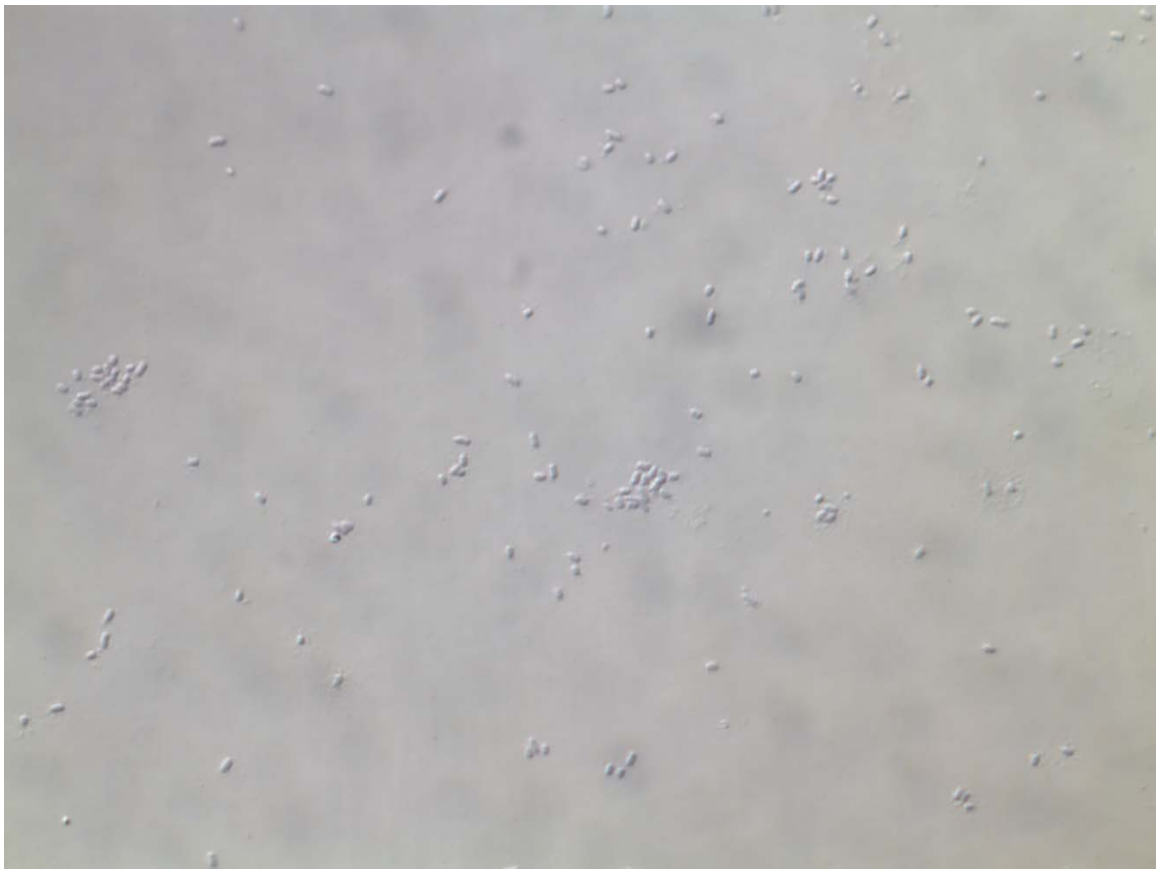


Figure 3.1 DIC (Differential Interference Contrast) image of gram-stained wild-type *E. coli* (1000× multiplication)

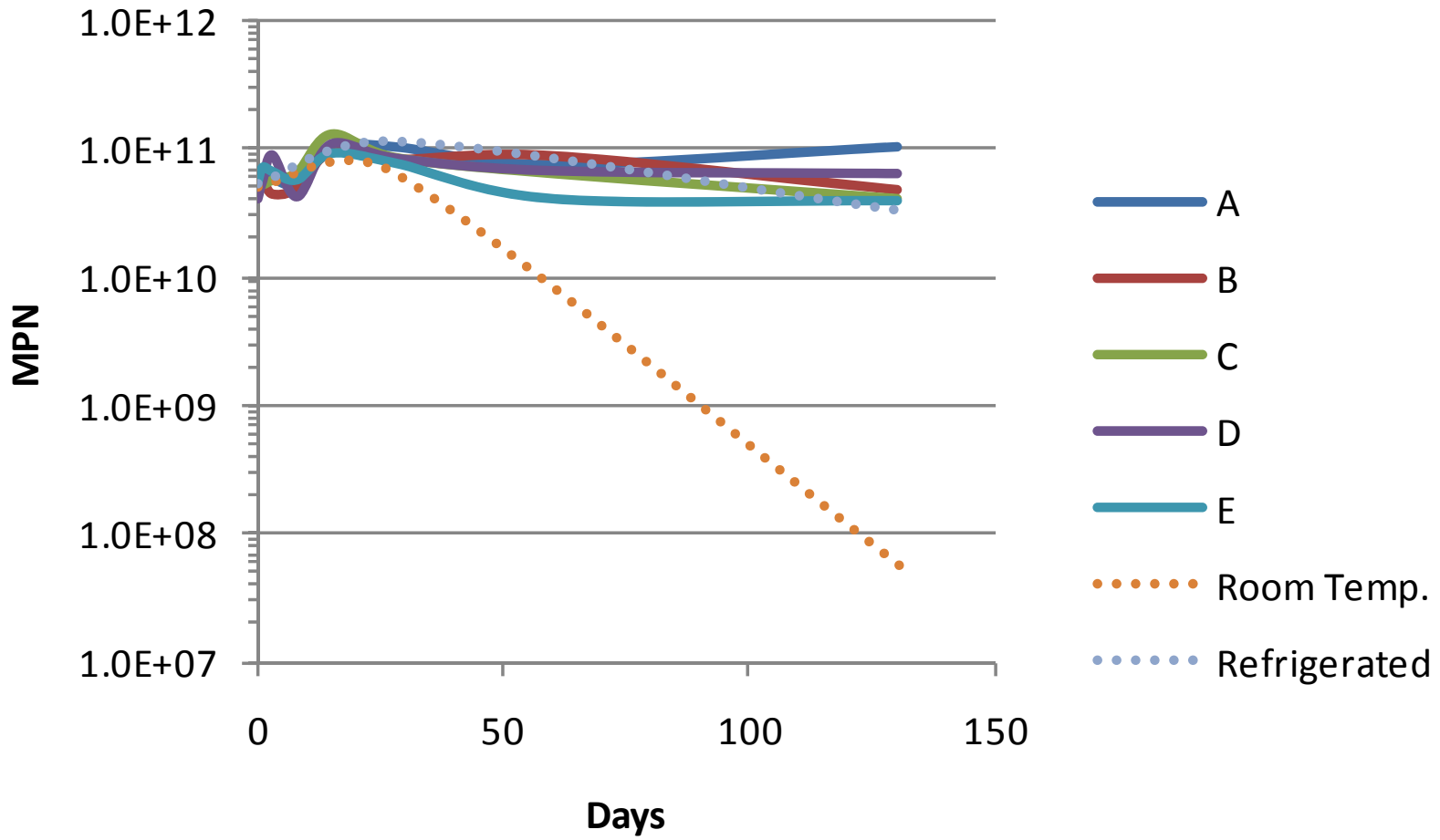


Figure 3.2 Average MPN for microcosms A, B, C, D, and E at days 0, 1, 3, 8, 15, 28, 60, and 130; and room temperature (~23°C) and refrigerated (~2°C) controls at days 0, 28, and 132.

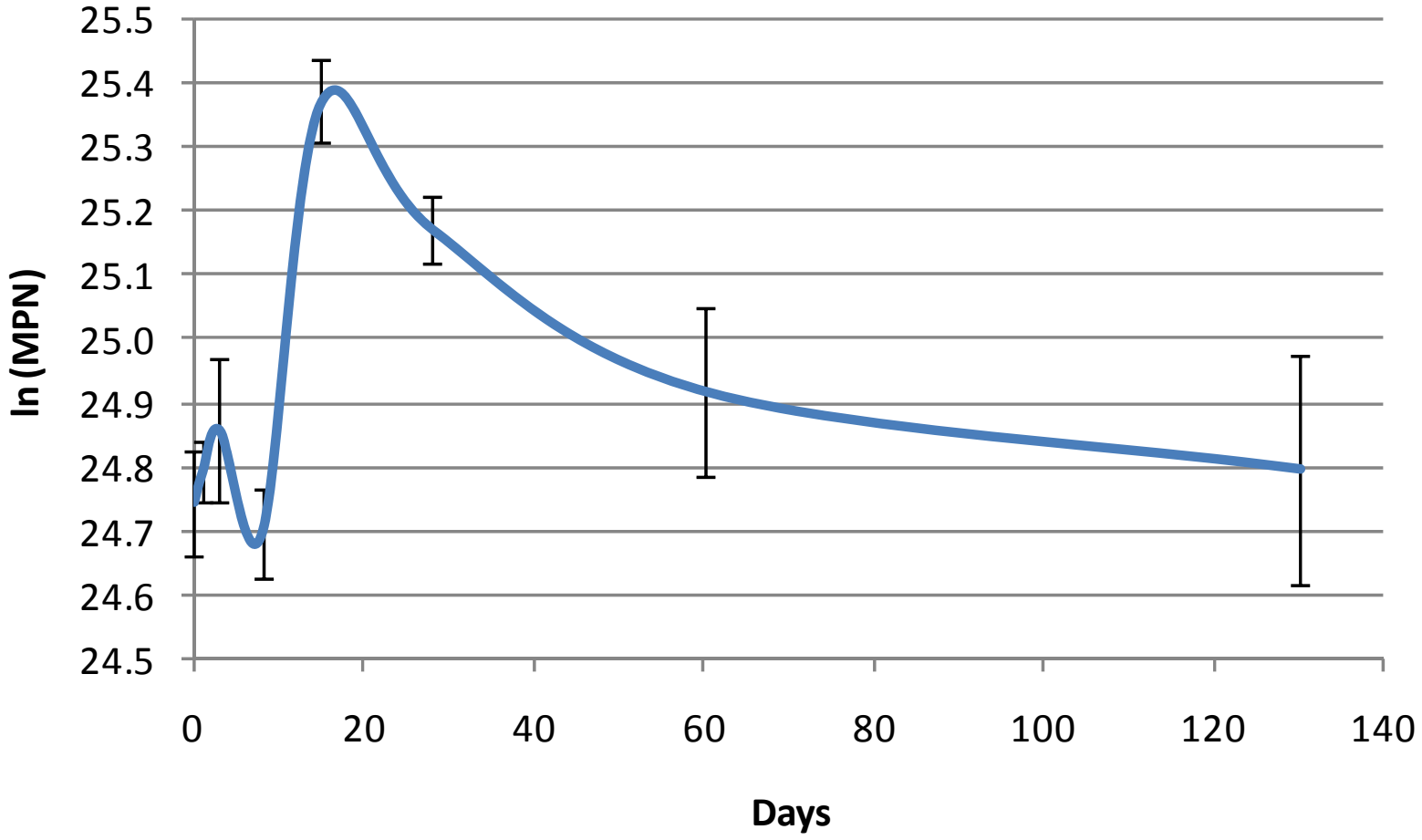


Figure 3.3 Average MPN of all replicates from microcosms A-E after natural-log transformation. Error bars represent standard error of the mean.

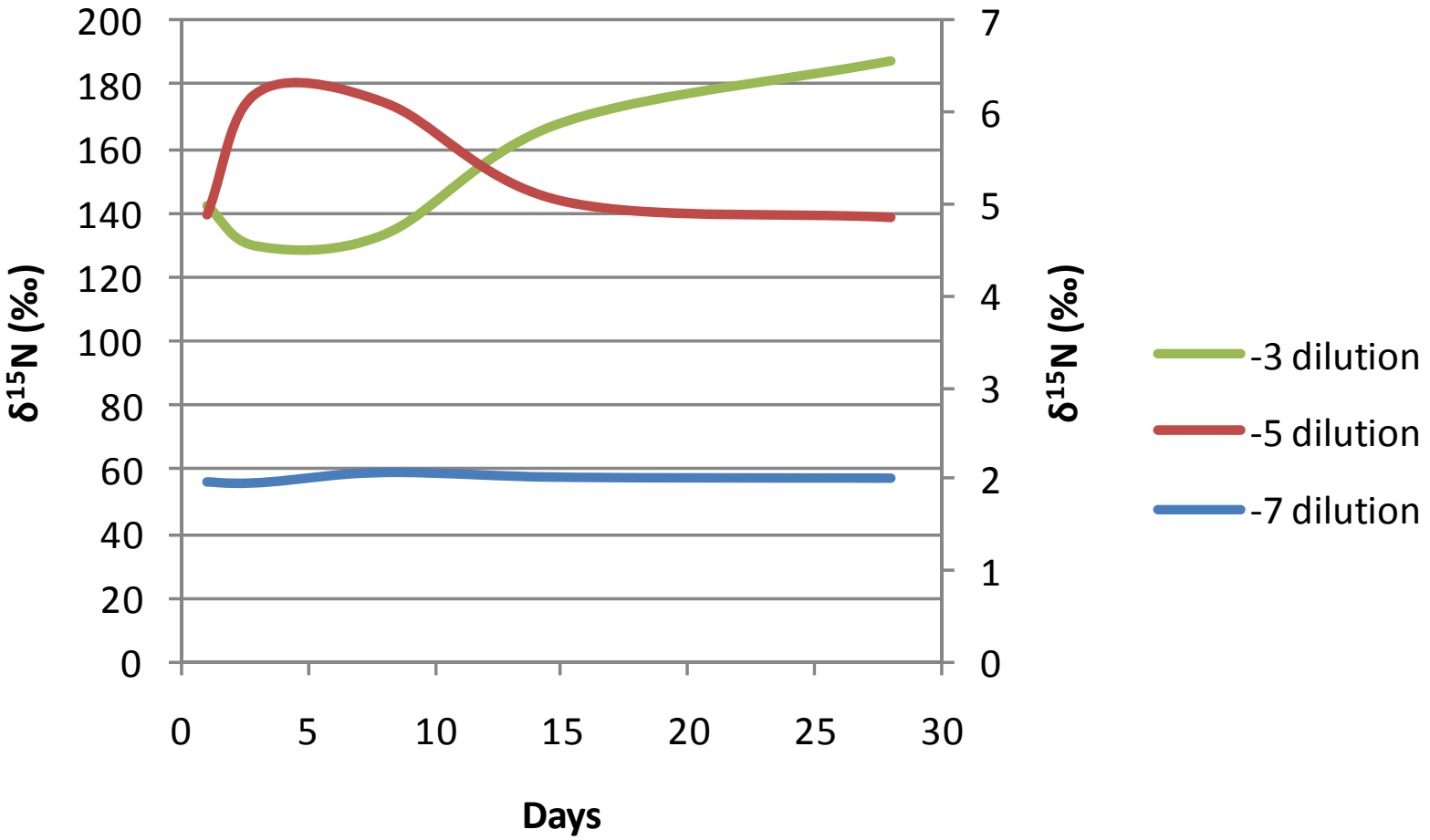


Figure 3.4 $\delta^{15}N$ values, grouped by log units of dilution, for first set of samples analyzed at the University of Virginia. The 10^{-3} dilution is plotted on the left vertical axis, whereas the 10^{-5} and 10^{-7} dilutions are plotted on the right vertical axis.

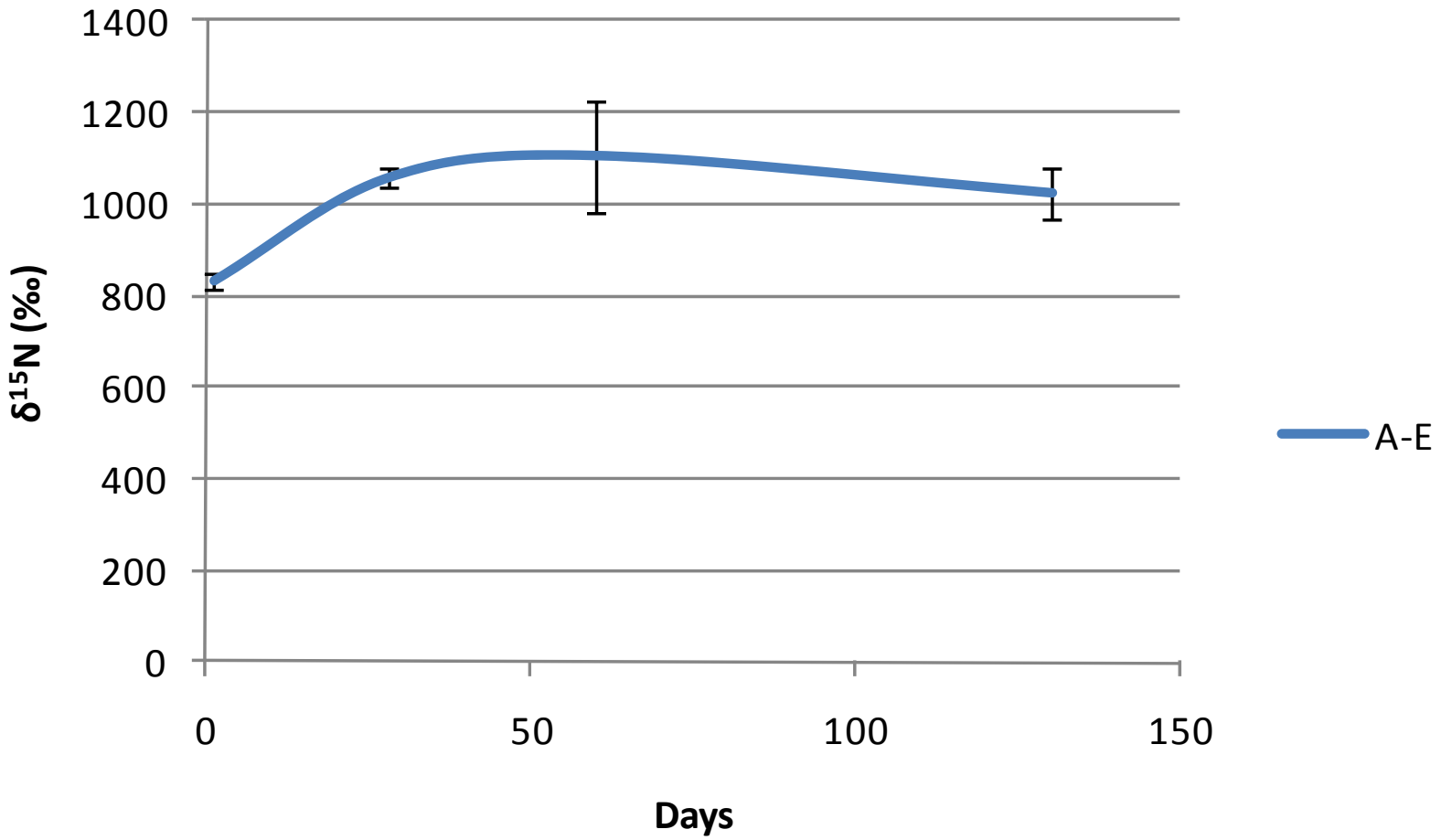


Figure 3.5 $\delta^{15}N$ data for second set of samples analyzed at the Woods Hole MBL Stable Isotope Laboratory. Data shown represent averages of microcosms A-E for days 1, 28, 60, and 130. Error bars represent standard error of the mean.

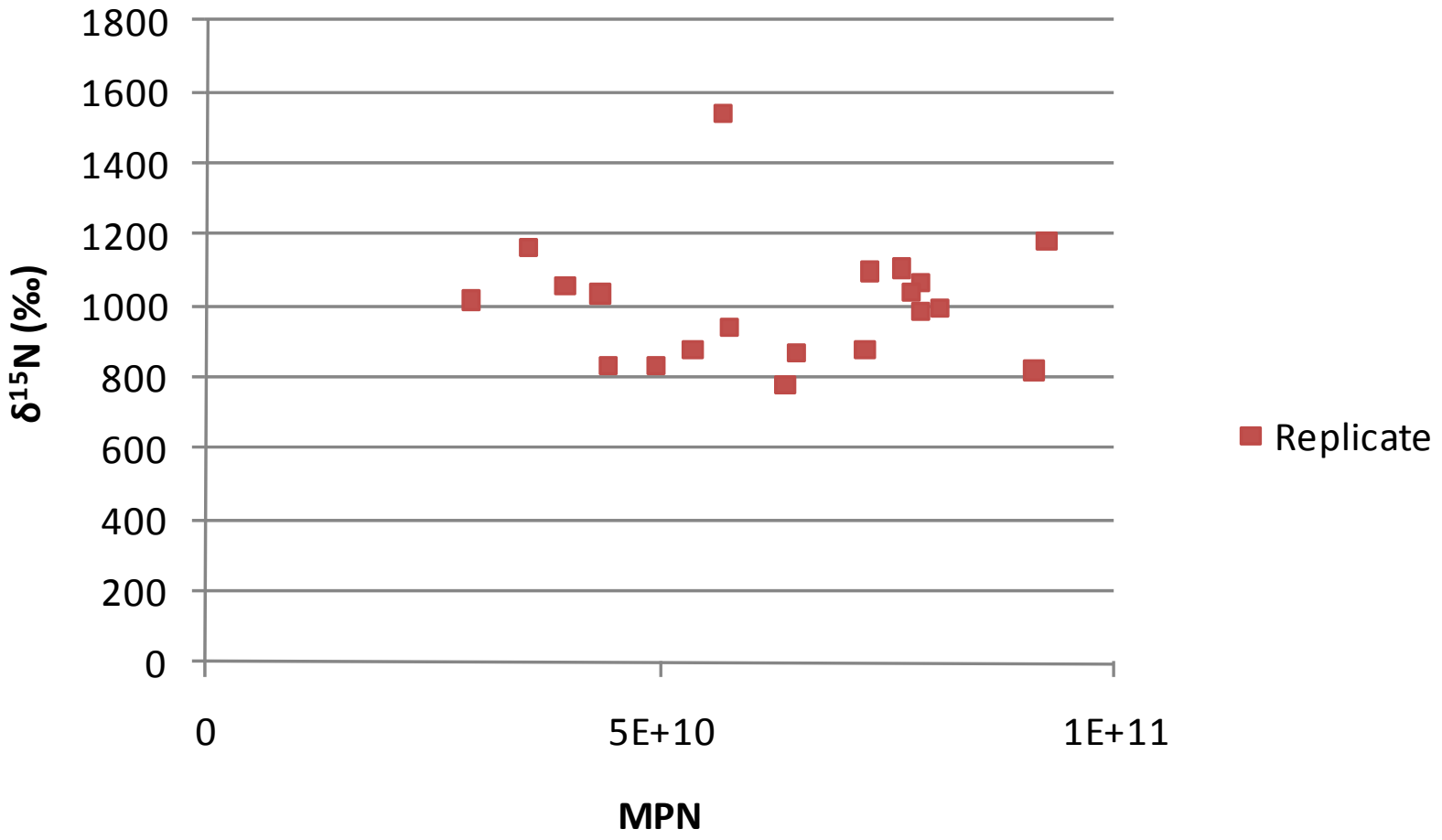


Figure 3.6 Scatterplot of $\delta^{15}N$ versus MPN using replicates containing both MPN and isotope data (MBL samples). Correlation coefficient equals -0.02.

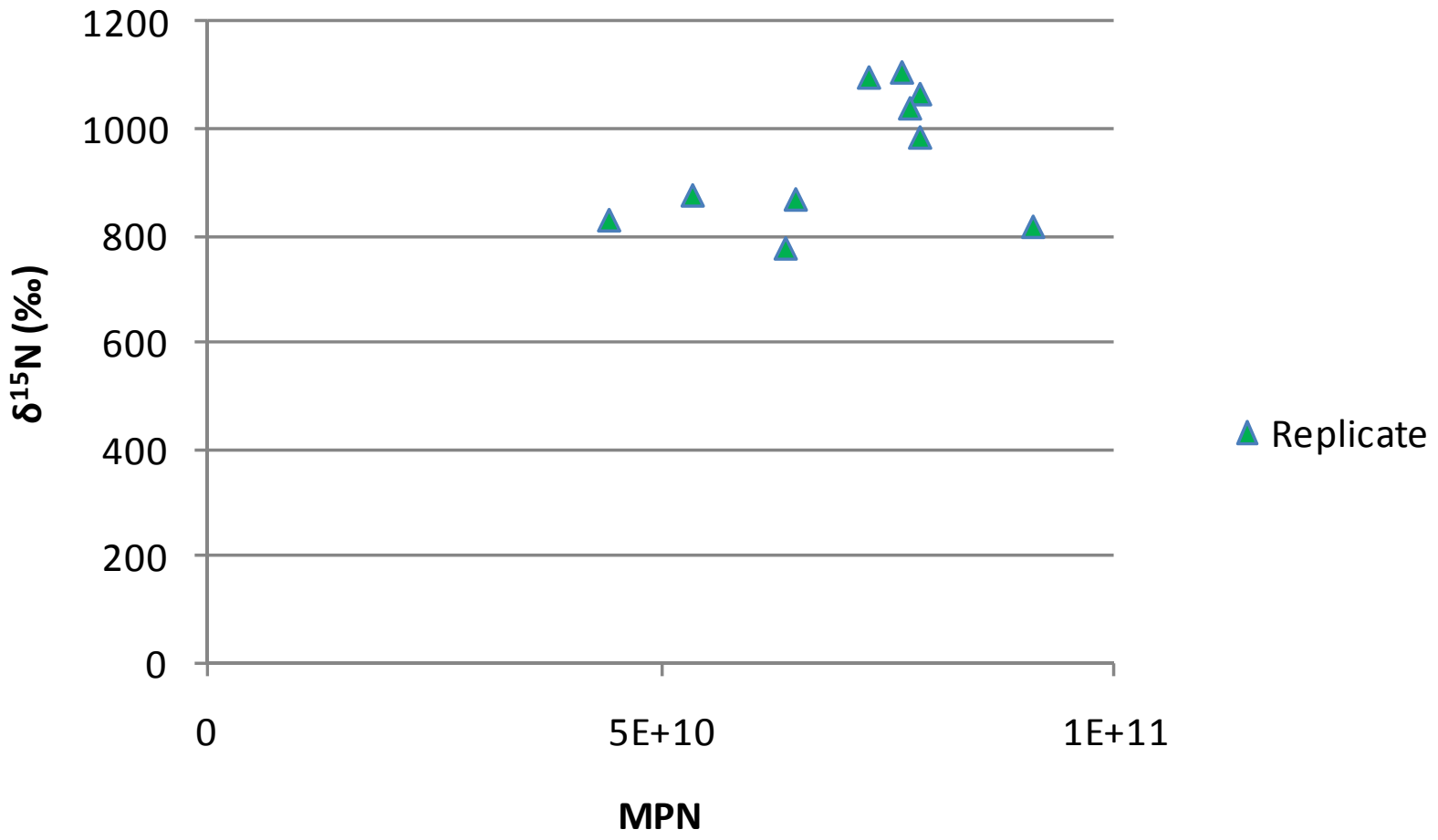


Figure 3.7 Scatterplot of $\delta^{15}N$ versus MPN using only days 1 and 28 replicates containing both MPN and isotope data (MBL samples). Correlation coefficient equals 0.41421.

CHAPTER 4: CONCLUSIONS

4.1 Conclusions

A wild-type strain of *E. coli* was isolated from Royal Spring during low-flow conditions in late October 2008. Serological analyses identified the strain as O^-:H^-, meaning the organism either is rough and lacks an O antigen or had an O antigen that did not react with any of the standard antisera used. The wild-type strain does not carry flagella. Virulence testing for factors LT, STa, STb, Stx1, Stx2, *eae*, CNF1, and CNF2 showed negative results.

Virulence factors can transfer horizontally between strains via genetic mechanisms, an important consideration before introduction of a ^{15}N-labeled strain into the environment. Knowing this, the isolated strain is likely as good a candidate as any to study bacterial (and by proxy pathogen) transport in the Cane Run watershed and Royal Spring groundwater basin. First, the strain was isolated from the environment to which it would be reintroduced, and would not have the possible detrimental effect of introducing a non-indigenous organism to the groundwater. Second, the isolated strain is MUG positive, indicating that it is not the pathogenic organism *E. coli* O157:H7. Third, virulence testing indicated the isolated strain is likely commensal to humans and/or animals. Fourth, the isolated strain has been typed serologically. If there are suspected illnesses after a trace, the organism can be isolated from infected individuals and serotyped to show if a horizontal transfer of virulence factors occurred causing the ^{15}N labeled strain to become pathogenic, or if it is another strain causing the illness. Lastly, the water arriving at Royal Spring is treated by the Georgetown Municipal Water & Sewer Service. Any ^{15}N-labeled *E. coli* arriving at Royal Spring after a trace should therefore be eliminated through water treatment. Still, care should be taken before introducing any cultured microorganism to the environment. It is recommended that a viable trace be coordinated with the Georgetown Municipal Water & Sewer Service to occur when the treatment plant is not drawing water from Royal Spring. Downstream implications should also be considered depending on the concentration and quantity of labeled bacteria used in a trace. However, after enough distance the labeled bacteria should approach natural concentration levels.

The isolated strain of *E. coli* was shown to survive for 130 days in sterilized Royal Spring water under simulated karst conditions. The concentration at day 0 was within the standard error of the concentration at day 130, and vice versa. The *E. coli* had a mean starting concentration of 5.62×10^{10} with a standard error of 4.12×10^{9} and a mean ending concentration of 5.88×10^{10} with a standard error of 7.53×10^{9}. It is expected that this strain would survive well beyond 130 days under the same conditions. Although there was statistically significant die-off from the maximum of 1.04×10^{11} on day 15, the day 130 concentration was different by less than one order of magnitude, indicating slow rates of die-off.

Similarly, the ^{15}N label was shown to be conserved over the course of the study. Using the second sample set, there was no significant difference in $\delta^{15}N$ values from day 1 and day 130. There was a statistical significance between days 1 and 28, but this is likely explained by a statistically significant trend in MPN data between days 8 and 15 due to the use of remnant enriched medium by the *E. coli*. No linear correlation was seen between MPN and $\delta^{15}N$ values using the data from days 1, 28, 60, and 130. A weak positive linear correlation was seen only using data from days 1 and 60.

A point of emphasis identified in this study is the importance of the isotope proportionation equation when working with highly enriched samples. Care must be taken first to ensure that a suitable background level of nitrogen is available to allow samples to be measureable. Second, if a background solution of nitrogen must be added to the samples, care must be taken to use the same product and preparatory procedure if the samples are to be directly compared. Slight changes in the fractions of either enriched bacteria or added background will have noticeable effect on the overall δ of the sample.

4.2 Suggestions for future research

Supplements to this study that could provide additional insights are as follows. First, chemical analysis of nutrient levels throughout the course of the study would have allowed determination of the nutrients the *E. coli* were using, the rates at which they were being used, and whether any nutrients were limiting. More conclusions about the population dynamics of the system could have been drawn if these data were available.

Second, analysis of samples passing through the filter for $\delta^{15}N$ would have enabled a comparison between the δ of the enriched bacteria and the δ value of the spring water. Analysis of these δ values over time could identify trends in how the bacteria are or are not partitioning the stable isotope label. Lastly, a comparison of the bacterial survival and fate of ^{15}N in a live microcosm would gain valuable information regarding behavior of these variables in a bioactive environment. This behavior first needed to be examined in a stable environment to gain a frame of reference.

Bacterial traces using this method are recommended to model pathogen transport in the Cane Run watershed and Royal Spring groundwater basin. Use of this method could gain valuable insight into the movement of bacterial contaminants in the already contaminated system, which would help improve remediation methods and strategies.

APPENDICES

Appendix A: Compilation of MPN and isotope data for the duration of the study

Table A.1 MPN data and Woods Hole data for all replicate samples with mean, standard deviation, and standard error of the mean calculated using all samples for each day.

Sample	MPN	Dilution Factor	10^-8 Count	MPN	Dilution Factor	10^-10 Count	Final Count (*E. coli*/100mL)	δ_{15N} (‰)	δ_{13C} (‰)
0A1	549.30	1.00E+08	5.49E+10				5.49E+10		
0A2	629.40	1.00E+08	6.29E+10				6.29E+10		
0A3	629.40	1.00E+08	6.29E+10				6.29E+10		
0B1	658.60	1.00E+08	6.59E+10				6.59E+10		
0B2	829.70	1.00E+08	8.30E+10				8.30E+10		
0B3	248.90	1.00E+08	2.49E+10				2.49E+10		
0C1	>1011.2	1.00E+08							
0C2	689.30	1.00E+08	6.89E+10				6.89E+10		
0C3	629.40	1.00E+08	6.29E+10				6.29E+10		
0D1	>1011.2	1.00E+08							
0D2	416.00	1.00E+08	4.16E+10				4.16E+10		
0D3	396.80	1.00E+08	3.97E+10				3.97E+10		
0E1	501.20	1.00E+08	5.01E+10				5.01E+10		
0E2	601.50	1.00E+08	6.02E+10				6.02E+10		
0E3	524.70	1.00E+08	5.25E+10				5.25E+10		
Day 0									
Mean							5.62E+10		
Standard Deviation							1.48E+10		
Standard Error of the Mean							4.12E+09		

Table A.1 continued

Sample	MPN	Dilution Factor	10^-8 Count	MPN	Dilution Factor	10^-10 Count	Final Count (*E. coli*/100mL)	δ_{15N} (‰)	δ_{13C} (‰)
1A1	416.00	1.00E+08	4.16E+10	6.3	1.00E+10	6.30E+10	5.23E+10		
1A2	396.80	1.00E+08	3.97E+10	6.3	1.00E+10	6.30E+10	5.13E+10		
1A3	524.70	1.00E+08	5.25E+10	7.5	1.00E+10	7.50E+10	6.37E+10	778.2	-29.2
1B1	658.60	1.00E+08	6.59E+10	7.5	1.00E+10	7.50E+10	7.04E+10		
1B2	344.10	1.00E+08	3.44E+10	6.3	1.00E+10	6.30E+10	4.87E+10		
1B3	549.30	1.00E+08	5.49E+10	5.2	1.00E+10	5.20E+10	5.35E+10	876.6	-29.3
1C1	574.80	1.00E+08	5.75E+10	4.1	1.00E+10	4.10E+10	4.92E+10		
1C2	478.60	1.00E+08	4.79E+10	4.1	1.00E+10	4.10E+10	4.44E+10		
1C3	456.90	1.00E+08	4.57E+10	8.4	1.00E+10	8.40E+10	6.48E+10	869.2	-25.8
1D1	436.00	1.00E+08	4.36E+10	8.4	1.00E+10	8.40E+10	6.38E+10		
1D2	501.20	1.00E+08	5.01E+10	7.3	1.00E+10	7.30E+10	6.16E+10		
1D3	574.80	1.00E+08	5.75E+10	3.1	1.00E+10	3.10E+10	4.42E+10	830.7	-25.7
1E1	416.00	1.00E+08	4.16E+10	6.3	1.00E+10	6.30E+10	5.23E+10		
1E2	574.80	1.00E+08	5.75E+10	7.5	1.00E+10	7.50E+10	6.62E+10		
1E3	501.20	1.00E+08	5.01E+10	13.2	1.00E+10	1.32E+11	9.11E+10	818.4	-27.9
Day 1									
Mean							5.85E+10	834.6	-27.6
Standard Deviation							1.23E+10	40.1	1.8
Standard Error of the Mean							3.17E+09	17.9	0.8

Table A.1 continued

Sample	MPN	Dilution Factor	10^-8 Count	MPN	Dilution Factor	10^-10 Count	Final Count (*E. coli*/100mL)	δ_{15N} (‰)	δ_{13C} (‰)
3A1	549.30	1.00E+08	5.49E+10	6.3	1.00E+10	6.30E+10	5.90E+10		
3A2	501.20	1.00E+08	5.01E+10	2.0	1.00E+10	2.00E+10	3.51E+10		
3A3	721.50	1.00E+08	7.22E+10	8.6	1.00E+10	8.60E+10	7.91E+10		
3B1	524.70	1.00E+08	5.25E+10	3.1	1.00E+10	3.10E+10	4.17E+10		
3B2	378.40	1.00E+08	3.78E+10	4.1	1.00E+10	4.10E+10	3.94E+10		
3B3	436.00	1.00E+08	4.36E+10	6.3	1.00E+10	6.30E+10	5.33E+10		
3C1	574.80	1.00E+08	5.75E+10	6.3	1.00E+10	6.30E+10	6.02E+10		
3C2	549.30	1.00E+08	5.49E+10	5.2	1.00E+10	5.20E+10	5.35E+10		
3C3	601.50	1.00E+08	6.02E+10	5.2	1.00E+10	5.20E+10	5.61E+10		
3D1	416.00	1.00E+08	4.16E+10	8.4	1.00E+10	8.40E+10	6.28E+10		
3D2	501.20	1.00E+08	5.01E+10	11.0	1.00E+10	1.10E+11	8.01E+10		
3D3	870.40	1.00E+08	8.70E+10	15.8	1.00E+10	1.58E+11	1.23E+11		
3E1	436.00	1.00E+08	4.36E+10	7.5	1.00E+10	7.50E+10	5.93E+10		
3E2	689.30	1.00E+08	6.89E+10	5.2	1.00E+10	5.20E+10	6.05E+10		
3E3	629.40	1.00E+08	6.29E+10	8.5	1.00E+10	8.50E+10	7.40E+10		
Day 3									
Mean							6.24E+10		
Standard Deviation							2.12E+10		
Standard Error of the Mean							5.47E+09		

Table A.1 continued

Sample	MPN	Dilution Factor	10^-8 Count	MPN	Dilution Factor	10^-10 Count	Final Count (*E. coli*/100mL)	δ_{15N} (‰)	δ_{13C} (‰)
8A1	416.00	1.00E+08	4.16E+10	6.3	1.00E+10	6.30E+10	5.23E+10		
8A2	574.80	1.00E+08	5.75E+10	2.0	1.00E+10	2.00E+10	3.87E+10		
8A3	601.50	1.00E+08	6.02E+10	7.5	1.00E+10	7.50E+10	6.76E+10		
8B1	378.40	1.00E+08	3.78E+10	2.0	1.00E+10	2.00E+10	2.89E+10		
8B2	629.40	1.00E+08	6.29E+10	7.4	1.00E+10	7.40E+10	6.85E+10		
8B3	416.00	1.00E+08	4.16E+10	6.3	1.00E+10	6.30E+10	5.23E+10		
8C1	328.20	1.00E+08	3.28E+10	9.6	1.00E+10	9.60E+10	6.44E+10		
8C2	478.60	1.00E+08	4.79E+10	7.5	1.00E+10	7.50E+10	6.14E+10		
8C3	549.30	1.00E+08	5.49E+10	8.4	1.00E+10	8.40E+10	6.95E+10		
8D1	456.90	1.00E+08	4.57E+10	6.3	1.00E+10	6.30E+10	5.43E+10		
8D2	360.90	1.00E+08	3.61E+10	2.0	1.00E+10	2.00E+10	2.80E+10		
8D3	360.90	1.00E+08	3.61E+10	5.2	1.00E+10	5.20E+10	4.40E+10		
8E1	629.40	1.00E+08	6.29E+10	3.1	1.00E+10	3.10E+10	4.70E+10		
8E2	416.00	1.00E+08	4.16E+10	<1.0	1.00E+10				
8E3	549.30	1.00E+08	5.49E+10	7.5	1.00E+10	7.50E+10	6.50E+10		
Day 8									
Mean							5.30E+10		
Standard Deviation							1.41E+10		
Standard Error of the Mean							3.77E+09		

Table A.1 continued

Sample	MPN	Dilution Factor	10^-8 Count	MPN	Dilution Factor	10^-10 Count	Final Count (*E. coli*/100mL)	δ_{15N} (‰)	δ_{13C} (‰)
15A1	721.50	1.00E+08	7.22E+10	14.6	1.00E+10	1.46E+11	1.09E+11		
15A2	721.50	1.00E+08	7.22E+10	13.5	1.00E+10	1.35E+11	1.04E+11		
15A3	574.80	1.00E+08	5.75E+10	13.4	1.00E+10	1.34E+11	9.57E+10		
15B1	689.30	1.00E+08	6.89E+10	7.5	1.00E+10	7.50E+10	7.20E+10		
15B2	658.60	1.00E+08	6.59E+10	13.1	1.00E+10	1.31E+11	9.84E+10		
15B3	829.70	1.00E+08	8.30E+10	13.5	1.00E+10	1.35E+11	1.09E+11		
15C1	658.60	1.00E+08	6.59E+10	14.8	1.00E+10	1.48E+11	1.07E+11		
15C2	721.50	1.00E+08	7.22E+10	27.2	1.00E+10	2.72E+11	1.72E+11		
15C3	755.60	1.00E+08	7.56E+10	13.4	1.00E+10	1.34E+11	1.05E+11		
15D1	913.90	1.00E+08	9.14E+10	13.4	1.00E+10	1.34E+11	1.13E+11		
15D2	791.50	1.00E+08	7.92E+10	18.3	1.00E+10	1.83E+11	1.31E+11		
15D3	549.30	1.00E+08	5.49E+10	10.8	1.00E+10	1.08E+11	8.15E+10		
15E1	574.80	1.00E+08	5.75E+10	15.5	1.00E+10	1.55E+11	1.06E+11		
15E2	755.60	1.00E+08	7.56E+10	11.0	1.00E+10	1.10E+11	9.28E+10		
15E3	658.60	1.00E+08	6.59E+10	7.4	1.00E+10	7.40E+10	6.99E+10		
Day 15									
Mean							1.04E+11		
Standard Deviation							2.45E+10		
Standard Error of the Mean							6.33E+09		

Table A.1 continued

Sample	MPN	Dilution Factor	10^-8 Count	MPN	Dilution Factor	10^-10 Count	Final Count (*E. coli*/100mL)	δ_{15N} (‰)	δ_{13C} (‰)
28A1	755.60	1.00E+08	7.56E+10	16.1	1.00E+10	1.61E+11	1.18E+11		
28A2	629.40	1.00E+08	6.29E+10	15.8	1.00E+10	1.58E+11	1.10E+11		
28A3	721.50	1.00E+08	7.22E+10	8.5	1.00E+10	8.50E+10	7.86E+10	1064.7	-24.7
28B1	791.50	1.00E+08	7.92E+10	9.8	1.00E+10	9.80E+10	8.86E+10		
28B2	755.60	1.00E+08	7.56E+10	8.4	1.00E+10	8.40E+10	7.98E+10		
28B3	791.50	1.00E+08	7.92E+10	7.4	1.00E+10	7.40E+10	7.66E+10	1105.0	-26.3
28C1	721.50	1.00E+08	7.22E+10	7.5	1.00E+10	7.50E+10	7.36E+10		
28C2	658.60	1.00E+08	6.59E+10	13.5	1.00E+10	1.35E+11	1.00E+11		
28C3	721.50	1.00E+08	7.22E+10	8.5	1.00E+10	8.50E+10	7.86E+10	984.2	-23.4
28D1	601.50	1.00E+08	6.02E+10	12.2	1.00E+10	1.22E+11	9.11E+10		
28D2	689.30	1.00E+08	6.89E+10	9.7	1.00E+10	9.70E+10	8.30E+10		
28D3	689.30	1.00E+08	6.89E+10	8.6	1.00E+10	8.60E+10	7.75E+10	1038.5	-26.6
28E1	601.50	1.00E+08	6.02E+10	7.5	1.00E+10	7.50E+10	6.76E+10		
28E2	629.40	1.00E+08	6.29E+10	10.7	1.00E+10	1.07E+11	8.50E+10		
28E3	478.60	1.00E+08	4.79E+10	9.8	1.00E+10	9.80E+10	7.29E+10	1095.6	-28.5
Day 28									
Mean							8.55E+10	1057.6	-25.9
Standard Deviation							1.43E+10	48.7	1.9
Standard Error of the Mean							3.69E+09	21.8	0.9

Table A.1 continued

Sample	MPN	Dilution Factor	10^-8 Count	MPN	Dilution Factor	10^-10 Count	Final Count (*E. coli*/100mL)	δ_{15N} (‰)	δ_{13C} (‰)
60A1	574.80	1.00E+08	5.75E+10	6.3	1.00E+10	6.30E+10	6.02E+10		
60A2	549.30	1.00E+08	5.49E+10	11.0	1.00E+10	1.10E+11	8.25E+10		
60A3	524.70	1.00E+08	5.25E+10	10.9	1.00E+10	1.09E+11	8.07E+10	996.4	-24.0
60B1	436.00	1.00E+08	4.36E+10	19.9	1.00E+10	1.99E+11	1.21E+11		
60B2	549.30	1.00E+08	5.49E+10	11.0	1.00E+10	1.10E+11	8.25E+10		
60B3	629.40	1.00E+08	6.29E+10	5.2	1.00E+10	5.20E+10	5.75E+10	940.4	-20.6
60C1	416.00	1.00E+08	4.16E+10	9.8	1.00E+10	9.80E+10	6.98E+10		
60C2	298.70	1.00E+08	2.99E+10	9.8	1.00E+10	9.80E+10	6.39E+10		
60C3	396.80	1.00E+08	3.97E+10	7.4	1.00E+10	7.40E+10	5.68E+10	1541.1	-24.4
60D1	574.80	1.00E+08	5.75E+10	9.7	1.00E+10	9.70E+10	7.72E+10		
60D2	501.20	1.00E+08	5.01E+10	5.2	1.00E+10	5.20E+10	5.11E+10		
60D3	478.60	1.00E+08	4.79E+10	9.7	1.00E+10	9.70E+10	7.24E+10	879.3	-24.8
60E1	378.40	1.00E+08	3.78E+10	8.4	1.00E+10	8.40E+10	6.09E+10		
60E2	360.90	1.00E+08	3.61E+10	1.0	1.00E+10	1.00E+10	2.30E+10		
60E3	396.80	1.00E+08	3.97E+10	3.1	1.00E+10	3.10E+10	3.53E+10	1163.6	-23.3
Day 60									
Mean							6.64E+10	1104.2	-23.4
Standard Deviation							2.27E+10	266.2	1.7
Standard Error of the Mean							5.87E+09	119.0	0.7

Table A.1 continued

Sample	MPN	Dilution Factor	10^-8 Count	MPN	Dilution Factor	10^-10 Count	Final Count (*E. coli*/100mL)	δ_{15N} (‰)	δ_{13C} (‰)
130A1	549.30	1.00E+08	5.49E+10	20.3	1.00E+10	2.03E+11	1.29E+11		
130A2	658.60	1.00E+08	6.59E+10	10.7	1.00E+10	1.07E+11	8.64E+10		
130A3	629.40	1.00E+08	6.29E+10	12.2	1.00E+10	1.22E+11	9.25E+10	1179.8	-24.2
130B1	396.80	1.00E+08	3.97E+10	9.7	1.00E+10	9.70E+10	6.83E+10		
130B2	436.00	1.00E+08	4.36E+10	3.1	1.00E+10	3.10E+10	3.73E+10		
130B3	478.60	1.00E+08	4.79E+10	3.1	1.00E+10	3.10E+10	3.94E+10	1054.8	-22.4
130C1	272.30	1.00E+08	2.72E+10	7.4	1.00E+10	7.40E+10	5.06E+10		
130C2	360.90	1.00E+08	3.61E+10	2.0	1.00E+10	2.00E+10	2.80E+10		
130C3	456.90	1.00E+08	4.57E+10	4.1	1.00E+10	4.10E+10	4.33E+10	1033.6	-25.8
130D1	456.90	1.00E+08	4.57E+10	14.4	1.00E+10	1.44E+11	9.48E+10		
130D2	416.00	1.00E+08	4.16E+10	5.2	1.00E+10	5.20E+10	4.68E+10		
130D3	360.90	1.00E+08	3.61E+10	6.3	1.00E+10	6.30E+10	4.95E+10	833.7	-22.1
130E1	378.40	1.00E+08	3.78E+10	5.2	1.00E+10	5.20E+10	4.49E+10		
130E2	416.00	1.00E+08	4.16E+10	4.1	1.00E+10	4.10E+10	4.13E+10		
130E3	272.30	1.00E+08	2.72E+10	3.1	1.00E+10	3.10E+10	2.91E+10	1017.8	-24.1
Day 130									
Mean							5.88E+10	1023.9	-23.7
Standard Deviation							2.92E+10	124.1	1.5
Standard Error of the Mean							7.53E+09	55.5	0.7

Table A.2 Mean, standard deviation, and standard error of the mean for MPN data (*E. coli*/100 mL) calculated using the replicate samples from each microcosm on each day.

	Microcosm	Day 0	Day 1	Day 3	Day 8	Day 15	Day 28	Day 60	Day 130
Mean	**A**	**6.03E+10**	**5.58E+10**	**5.77E+10**	**5.29E+10**	**1.03E+11**	**1.02E+11**	**7.45E+10**	**1.03E+11**
Standard Deviation	**A**	**4.62E+09**	**6.9E+09**	**2.2E+10**	**1.44E+10**	**6.7E+09**	**2.1E+10**	**1.24E+10**	**2.3E+10**
Standard Error of the Mean	**A**	**2.67E+09**	**3.98E+09**	**1.27E+10**	**8.33E+09**	**3.87E+09**	**1.21E+10**	**7.14E+09**	**1.33E+10**
Mean	**B**	**5.79E+10**	**5.75E+10**	**4.48E+10**	**4.99E+10**	**9.31E+10**	**8.16E+10**	**8.71E+10**	**4.84E+10**
Standard Deviation	**B**	**2.98E+10**	**1.14E+10**	**7.44E+09**	**1.99E+10**	**1.91E+10**	**6.21E+09**	**3.22E+10**	**1.73E+10**
Standard Error of the Mean	**B**	**1.72E+10**	**6.59E+09**	**4.29E+09**	**1.15E+10**	**1.1E+10**	**3.59E+09**	**1.86E+10**	**1E+10**
Mean	**C**	**6.59E+10**	**5.28E+10**	**5.66E+10**	**6.51E+10**	**1.28E+11**	**8.42E+10**	**6.35E+10**	**4.07E+10**
Standard Deviation	**C**	**4.24E+09**	**1.07E+10**	**3.42E+09**	**4.06E+09**	**3.82E+10**	**1.43E+10**	**6.49E+09**	**1.15E+10**
Standard Error of the Mean	**C**	**2.45E+09**	**6.16E+09**	**1.97E+09**	**2.35E+09**	**2.21E+10**	**8.25E+09**	**3.75E+09**	**6.65E+09**
Mean	**D**	**4.06E+10**	**5.65E+10**	**8.85E+10**	**4.21E+10**	**1.08E+11**	**8.38E+10**	**6.69E+10**	**6.37E+10**
Standard Deviation	**D**	**1.36E+09**	**1.07E+10**	**3.07E+10**	**1.33E+10**	**2.51E+10**	**6.85E+09**	**1.39E+10**	**2.7E+10**
Standard Error of the Mean	**D**	**7.84E+08**	**6.18E+09**	**1.77E+10**	**7.65E+09**	**1.45E+10**	**3.95E+09**	**8.05E+09**	**1.56E+10**
Mean	**E**	**5.42E+10**	**6.99E+10**	**6.46E+10**	**5.6E+10**	**8.97E+10**	**7.52E+10**	**3.98E+10**	**3.84E+10**
Standard Deviation	**E**	**5.25E+09**	**1.96E+10**	**8.15E+09**	**1.27E+10**	**1.84E+10**	**8.91E+09**	**1.93E+10**	**8.28E+09**
Standard Error of the Mean	**E**	**3.03E+09**	**1.13E+10**	**4.71E+09**	**7.35E+09**	**1.06E+10**	**5.14E+09**	**1.12E+10**	**4.78E+09**

Table A.3 Isotope data from first set of samples (UVA).

	Sample	δ15N (‰)	δ13C (‰)
	OA1 10^-8	2.05	-21.82
	1A1 10^-7	1.98	-21.14
	3A1 10^-7	1.97	-21.88
	8A1 10^-7	2.08	-21.85
	15A1 10^-7	2.03	-22.02
	28A1 10^-7	2.02	-22.03
10^-7 Mean		2.02	-21.78
10^-7 Standard Deviation		0.04	0.37
10^-7 Standard Error of the Mean		0.02	0.17
	OA1 10^-6	3.08	-21.99
	1A1 10^-5	4.89	-22.09
	3A1 10^-5	6.22	-21.93
	8A1 10^-5	6.11	-21.89
	15A1 10^-5	5.04	-21.93
	28A1 10^-5	4.86	-21.75
10^-5 Mean		5.42	-21.92
10^-5 Standard Deviation		0.68	0.12
10^-5 Standard Error of the Mean		0.30	0.05
	OA1 10^-4	154.05	-21.58
	1A1 10^-3	142.11	-21.47
	3A1 10^-3	129.39	-21.45
	8A1 10^-3	133.10	-21.46
	15A1 10^-3	168.08	-21.66
	28A1 10^-3	187.45	-21.54
10^-3 Mean		152.03	-21.52
10^-3 Standard Deviation		24.91	0.09
10^-3 Standard Error of the Mean		11.14	0.04

Table A.4 Isotope data from second set of samples.

	Sample	δ15N (‰)
	1A3	778.17
	1B3	876.63
	1C3	869.15
	1D3	830.69
	1E3	818.38
Day 1 Mean		834.60
Day 1 Standard Deviation		40.07
Day 1 Standard Error of the Mean		17.92
	28A3	1064.69
	28B3	1104.96
	28C3	984.24
	28D3	1038.47
	28E3	1095.61
Day 28 Mean		1057.59
Day 28 Standard Deviation		48.70
Day 28 Standard Error of the Mean		21.78
	60A3	996.45
	60B3	940.38
	60C3	1541.09
	60D3	879.31
	60E3	1163.56
Day 60 Mean		1104.16
Day 60 Standard Deviation		266.18
Day 60 Standard Error of the Mean		119.04
	130A3	1179.76
	130B3	1054.84
	130C3	1033.59
	130D3	833.71
	130E3	1017.78
Day 130 Mean		1023.94
Day 130 Standard Deviation		124.05
Day 130 Standard Error of the Mean		55.48

Appendix B: Composition of microbiological media and chemistry of water samples

Difco EC Medium with MUG

Approximate formula per liter

20.0 g	Tryptose
5.0 g	Lactose
1.5 g	Bile Salts No. 3
4.0 g	Dipotassium Phosphate
1.5 g	Monopotassium Phosphate
5.0 g	Sodium Chloride
0.05 g	MUG

(Becton, Dickinson and Company 2009)

Bacto Tryptic Soy Broth

Approximate formula per liter

17.0 g	Pancreatic Digest of Casein
3.0 g	Enzymatic Digest of Soybean Meal
5.0 g	Sodium Chloride
2.5 g	Dipotassium Phosphate
2.5 g	Dextrose

(Becton, Dickinson and Company 2009)

Difco Tryptic Soy Agar

Approximate formula per liter

15.0 g	Pancreatic Digest of Casein
5.0 g	Enzymatic Digest of Soybean Meal
5.0 g	Sodium Chloride
15.0 g	Agar

(Becton, Dickinson and Company 2009)

^{15}N Enriched M9 Medium

Approximate formula per liter

Part 1

6.0 g	Na_2HPO_4
3.0 g	KH_2PO_4
0.50 g	NaCl
1.0 g	$^{15}NH_4SO_4$, 98+ Atom % ^{15}N

Part 2

10.0 mL	20% Glucose
2.0 mL	1M $MgSO_4$
0.1 mL	1M $CaCl_2$

HACH Dilution Water

Formula per liter

1	Magnesium Chloride Pillow
1	Potassium Dihydrogen Phosphate Pillow

Water chemistry of Royal Spring samples prior to autoclaving

Samples collected on 8 December 2008

13.7° C	Temperature
7.377	pH
780.7 μS/cm	Conductivity
0.37 ppm	Fluoride
56.52 ppm	Chloride
91.00 ppm	Sulfate
1.72 ppm	Nitrate – N
103.27 ppm	Calcium
14.59 ppm	Magnesium
36.96 ppm	Sodium
212.0 mg/L	Bicarbonate as HCO_3^-

Appendix C: Input codes used for statistical analyses in SAS

Model 1

```
title 'interaction model with treatment comparison diffMatrix
(blocks in time)';
proc glm data=warden;
class timeLex treat;
model smallcount = timeLex treat timeLex*treat / solution;
lsmeans timeLex*treat / pdiff;
run;
```

Model 2

```
title 'interaction model with time comparison diffMatrix (blocks
in treat)';
proc glm data=warden;
class treat timeLex;
model smallcount = timeLex treat treat*timeLex / solution;
lsmeans timeLex*treat / pdiff;
run;
```

Natural log data transformation

```
data warden;
modify warden;
logCount = log(Final_Count);
run;
quit;
```

Model 3

```
title 'marginal model for time';
proc glm data=warden2;
class timeLex;
model logCount = timeLex/ solution;
lsmeans timeLex / pdiff;
run;
```

Model 4

```
title 'marginal model for treat';
proc glm data=warden2;
class treat;
model logCount = treat/ solution;
lsmeans treat / pdiff;
run;
```

Model 5

```
title 'marginal model for microcosm';
proc glm data=Jw;
class Microcosm;
model delta15N = Microcosm/ solution;
lsmeans Microcosm / pdiff;
run;
quit;
```

Model 6

```
title 'marginal model for time';
proc glm data=Jw;
class time;
model delta15N = time/ solution;
lsmeans time / pdiff;
run;
quit;
```

Appendix D: SAS output

Model 1

interaction model with treatment comparison diffMatrix (blocks in time)

The GLM Procedure

Class Level Information

Class	Levels	Values
timeLex	8	000 001 003 008 015 028 060 130
treat	5	A B C D E

Number of Observations Read	120
Number of Observations Used	117

Dependent Variable: smallCount smallCount

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	39	53762720399	1378531292	4.66	<.0001
Error	77	22773733046	295762767		
Corrected Total	116	76536453445			

R-Square	Coeff Var	Root MSE	smallCount Mean
0.702446	25.11767	17197.75	68468.76

Source	DF	Type I SS	Mean Square	F Value	Pr > F
timeLex	7	32500245200	4642892171	15.70	<.0001
treat	4	3171841564	792960391	2.68	0.0377
timeLex*treat	28	18090633636	646094058	2.18	0.0038

Source	DF	Type III SS	Mean Square	F Value	Pr > F
timeLex	7	32374556001	4624936572	15.64	<.0001
treat	4	2982054848	745513712	2.52	0.0478
timeLex*treat	28	18090633636	646094058	2.18	0.0038

Parameter		Estimate	Standard Error	t Value	Pr > |t|
Intercept		38445.00000 B	9929.12831	3.87	0.0002
timeLex	000	15801.66667 B	14041.90791	1.13	0.2639
timeLex	001	31421.66667 B	14041.90791	2.24	0.0281
timeLex	003	26133.33333 B	14041.90791	1.86	0.0665
timeLex	008	17522.50000 B	15699.33032	1.12	0.2678

timeLex	015	51205.00000 B	14041.90791	3.65	0.0005
timeLex	028	36713.33333 B	14041.90791	2.61	0.0107
timeLex	060	1323.33333 B	14041.90791	0.09	0.9252
timeLex	130	0.00000 B	.	.	.
treat	A	64176.66667 B	14041.90791	4.57	<.0001
treat	B	9911.66667 B	14041.90791	0.71	0.4824
treat	C	2223.33333 B	14041.90791	0.16	0.8746
treat	D	25285.00000 B	14041.90791	1.80	0.0757
treat	E	0.00000 B	.	.	.
timeLex*treat	000 A	-58153.33333 B	19858.25661	-2.93	0.0045
timeLex*treat	000 B	-6251.66667 B	19858.25661	-0.31	0.7538

Parameter		Estimate	Standard Error	t Value	Pr > |t|
timeLex*treat	000 C	9465.00000 B	21062.86187	0.45	0.6544
timeLex*treat	000 D	-38891.66667 B	21062.86187	-1.85	0.0687
timeLex*treat	000 E	0.00000 B	.	.	.
timeLex*treat	001 A	-78251.66667 B	19858.25661	-3.94	0.0002
timeLex*treat	001 B	-22245.00000 B	19858.25661	-1.12	0.2661
timeLex*treat	001 C	-19251.66667 B	19858.25661	-0.97	0.3354
timeLex*treat	001 D	-38618.33333 B	19858.25661	-1.94	0.0555
timeLex*treat	001 E	0.00000 B	.	.	.
timeLex*treat	003 A	-71055.00000 B	19858.25661	-3.58	0.0006
timeLex*treat	003 B	-29671.66667 B	19858.25661	-1.49	0.1392
timeLex*treat	003 C	-10208.33333 B	19858.25661	-0.51	0.6087
timeLex*treat	003 D	-1403.33333 B	19858.25661	-0.07	0.9438
timeLex*treat	003 E	0.00000 B	.	.	.
timeLex*treat	008 A	-67272.50000 B	21062.86187	-3.19	0.0020
timeLex*treat	008 B	-15982.50000 B	21062.86187	-0.76	0.4503
timeLex*treat	008 C	6910.83333 B	21062.86187	0.33	0.7437
timeLex*treat	008 D	-39107.50000 B	21062.86187	-1.86	0.0672
timeLex*treat	008 E	0.00000 B	.	.	.
timeLex*treat	015 A	-51030.00000 B	19858.25661	-2.57	0.0121
timeLex*treat	015 B	-6435.00000 B	19858.25661	-0.32	0.7468
timeLex*treat	015 C	36055.00000 B	19858.25661	1.82	0.0733
timeLex*treat	015 D	-6523.33333 B	19858.25661	-0.33	0.7434
timeLex*treat	015 E	0.00000 B	.	.	.
timeLex*treat	028 A	-36893.33333 B	19858.25661	-1.86	0.0670
timeLex*treat	028 B	-3426.66667 B	19858.25661	-0.17	0.8635
timeLex*treat	028 C	6811.66667 B	19858.25661	0.34	0.7325
timeLex*treat	028 D	-16608.33333 B	19858.25661	-0.84	0.4055
timeLex*treat	028 E	0.00000 B	.	.	.
timeLex*treat	060 A	-29465.00000 B	19858.25661	-1.48	0.1420

timeLex*treat	060 B	37398.33333 B	19858.25661	1.88	0.0634
timeLex*treat	060 C	21533.33333 B	19858.25661	1.08	0.2816
timeLex*treat	060 D	1856.66667 B	19858.25661	0.09	0.9258
timeLex*treat	060 E	0.00000 B	.	.	.
timeLex*treat	130 A	0.00000 B	.	.	.
timeLex*treat	130 B	0.00000 B	.	.	.
timeLex*treat	130 C	0.00000 B	.	.	.
timeLex*treat	130 D	0.00000 B	.	.	.
timeLex*treat	130 E	0.00000 B	.	.	.

NOTE: The X'X matrix has been found to be singular, and a generalized inverse was used to solve the normal equations. Terms whose estimates are followed by the letter 'B' are not uniquely estimable

Least Squares Means

time Lex	treat	smallCount LSMEAN	LSMEAN Number
000	A	60270.000	1
000	B	57906.667	2
000	C	65935.000	3
000	D	40640.000	4
000	E	54246.667	5
001	A	55791.667	6
001	B	57533.333	7
001	C	52838.333	8
001	D	56533.333	9
001	E	69866.667	10
003	A	57700.000	11
003	B	44818.333	12
003	C	56593.333	13
003	D	88460.000	14
003	E	64578.333	15
008	A	52871.667	16
008	B	49896.667	17
008	C	65101.667	18
008	D	42145.000	19
008	E	55967.500	20
015	A	102796.667	21
015	B	93126.667	22
015	C	127928.333	23
015	D	108411.667	24
015	E	89650.000	25

028	A	102441.667	26
028	B	81643.333	27
028	C	84193.333	28
028	D	83835.000	29
028	E	75158.333	30
060	A	74480.000	31
060	B	87078.333	32
060	C	63525.000	33
060	D	66910.000	34
060	E	39768.333	35
130	A	102621.667	36
130	B	48356.667	37
130	C	40668.333	38
130	D	63730.000	39
130	E	38445.000	40

Least Squares Means

Least Squares Means for effect timeLex*treat
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: smallCount

i/j	1	2	3	4	5
1		0.8668	0.7192	0.215	0.6692
2	0.8668		0.6105	0.2748	0.7951
3	0.7192	0.6105		0.1454	0.4588
4	0.215	0.2748	0.1454		0.3888
5	0.6692	0.7951	0.4588	0.3888	
6	0.7506	0.8807	0.5201	0.3375	0.9127
7	0.846	0.9789	0.5941	0.2853	0.8156
8	0.5982	0.7191	0.4067	0.4395	0.9204
9	0.7909	0.9223	0.551	0.3145	0.8711
10	0.4964	0.397	0.8029	0.0665	0.2694
11	0.8553	0.9883	0.6014	0.2806	0.8064
12	0.2746	0.3542	0.1826	0.7908	0.5039
13	0.7941	0.9257	0.5536	0.3127	0.8677
14	0.0482	0.0326	0.1554	0.0032	0.0171
15	0.7598	0.636	0.9314	0.1314	0.4641
16	0.5998	0.7209	0.4079	0.4383	0.9222
17	0.4623	0.57	0.3102	0.5572	0.7576
18	0.7317	0.6098	0.9578	0.1233	0.4419
19	0.2006	0.2651	0.1338	0.9239	0.3915
20	0.7848	0.902	0.5639	0.3756	0.913
21	0.0033	0.002	0.0214	0.0002	0.0009
22	0.0219	0.0142	0.0873	0.0013	0.007
23	<.0001	<.0001	0.0002	<.0001	<.0001
24	0.001	0.0006	0.0084	<.0001	0.0002
25	0.0397	0.0266	0.135	0.0025	0.0138
26	0.0036	0.0022	0.0227	0.0002	0.001
27	0.1321	0.095	0.3202	0.0108	0.0547
28	0.0925	0.065	0.2484	0.0069	0.0361
29	0.0974	0.0687	0.2577	0.0074	0.0384
30	0.2923	0.223	0.5586	0.0309	0.1405
31	0.3147	0.2415	0.5878	0.0342	0.1537
32	0.06	0.0411	0.182	0.0041	0.022
33	0.8173	0.6902	0.8784	0.149	0.5107
34	0.6376	0.5233	0.9506	0.0983	0.37
35	0.1483	0.2003	0.0996	0.9559	0.3057
36	0.0035	0.0021	0.022	0.0002	0.0009
37	0.3988	0.4985	0.2663	0.6245	0.676
38	0.1667	0.2233	0.1116	0.9986	0.3366
39	0.806	0.6795	0.8887	0.1454	0.5015
40	0.1242	0.1698	0.0839	0.8892	0.2639

i/j	6	7	8	9	10
1	0.7506	0.846	0.5982	0.7909	0.4964
2	0.8807	0.9789	0.7191	0.9223	0.397
3	0.5201	0.5941	0.4067	0.551	0.8029
4	0.3375	0.2853	0.4395	0.3145	0.0665
5	0.9127	0.8156	0.9204	0.8711	0.2694
6		0.9016	0.834	0.958	0.3193
7	0.9016		0.739	0.9434	0.3825
8	0.834	0.739		0.7931	0.229
9	0.958	0.9434	0.7931		0.3453
10	0.3193	0.3825	0.229	0.3453	
11	0.8923	0.9906	0.7301	0.934	0.3889
12	0.4369	0.368	0.5696	0.4067	0.0784
13	0.9546	0.9468	0.7899	0.9966	0.3475
14	0.0226	0.0306	0.0132	0.0258	0.1894
15	0.5333	0.6173	0.4057	0.5684	0.7075
16	0.8358	0.7408	0.9981	0.795	0.2299
17	0.6758	0.5881	0.8346	0.6378	0.159
18	0.5093	0.5915	0.3852	0.5435	0.7353
19	0.3342	0.2765	0.4487	0.3087	0.0519
20	0.9911	0.9208	0.8425	0.9713	0.3787
21	0.0013	0.0019	0.0006	0.0015	0.0216
22	0.0095	0.0133	0.0053	0.011	0.1017
23	<.0001	<.0001	<.0001	<.0001	<.0001
24	0.0003	0.0005	0.0002	0.0004	0.0075
25	0.0183	0.0249	0.0105	0.0209	0.1629
26	0.0014	0.002	0.0007	0.0016	0.023
27	0.0695	0.09	0.0436	0.0777	0.4042
28	0.0466	0.0614	0.0285	0.0525	0.3108
29	0.0493	0.0649	0.0303	0.0555	0.323
30	0.1718	0.2132	0.116	0.1886	0.7073
31	0.1872	0.2312	0.1274	0.2051	0.7434
32	0.0288	0.0386	0.0171	0.0327	0.224
33	0.5834	0.6708	0.4489	0.62	0.6528
34	0.4309	0.5063	0.3194	0.4622	0.8338
35	0.2574	0.2096	0.3549	0.2362	0.0352
36	0.0013	0.0019	0.0007	0.0015	0.0223
37	0.598	0.5154	0.7505	0.5621	0.1297
38	0.2848	0.2334	0.3888	0.2621	0.0409
39	0.5735	0.6602	0.4403	0.6098	0.6633
40	0.2205	0.178	0.3086	0.2015	0.0281

i/j	11	12	13	14	15
1	0.8553	0.2746	0.7941	0.0482	0.7598
2	0.9883	0.3542	0.9257	0.0326	0.636
3	0.6014	0.1826	0.5536	0.1554	0.9314
4	0.2806	0.7908	0.3127	0.0032	0.1314
5	0.8064	0.5039	0.8677	0.0171	0.4641
6	0.8923	0.4369	0.9546	0.0226	0.5333
7	0.9906	0.368	0.9468	0.0306	0.6173
8	0.7301	0.5696	0.7899	0.0132	0.4057
9	0.934	0.4067	0.9966	0.0258	0.5684
10	0.3889	0.0784	0.3475	0.1894	0.7075
11		0.3618	0.9374	0.0315	0.6256
12	0.3618		0.4043	0.0026	0.1634
13	0.9374	0.4043		0.026	0.5712
14	0.0315	0.0026	0.026		0.093
15	0.6256	0.1634	0.5712	0.093	
16	0.7319	0.568	0.7917	0.0133	0.407
17	0.58	0.7186	0.6348	0.0075	0.299
18	0.5996	0.1527	0.5463	0.1003	0.9704
19	0.2714	0.8495	0.3067	0.0015	0.1142
20	0.9124	0.4797	0.9683	0.0418	0.5849
21	0.0019	<.0001	0.0015	0.3105	0.008
22	0.0137	0.0009	0.0111	0.7405	0.0455
23	<.0001	<.0001	<.0001	0.0063	<.0001
24	0.0005	<.0001	0.0004	0.1594	0.0025
25	0.0257	0.002	0.0211	0.9327	0.0781
26	0.0021	0.0001	0.0016	0.3225	0.0086
27	0.0922	0.0105	0.0784	0.6287	0.228
28	0.063	0.0064	0.053	0.7621	0.1665
29	0.0665	0.0069	0.056	0.7428	0.1742
30	0.2175	0.0338	0.19	0.3465	0.4535
31	0.2358	0.0379	0.2066	0.3226	0.4828
32	0.0397	0.0035	0.033	0.9219	0.1132
33	0.6794	0.1867	0.623	0.0797	0.9404
34	0.5138	0.1198	0.4648	0.129	0.8686
35	0.2054	0.7201	0.2345	0.0009	0.0812
36	0.002	<.0001	0.0016	0.3164	0.0083
37	0.5078	0.8017	0.5592	0.0055	0.2516
38	0.2289	0.7684	0.2603	0.0011	0.0926
39	0.6688	0.182	0.6127	0.0822	0.952
40	0.1743	0.6512	0.2001	0.0006	0.0665

i/j	16	17	18	19	20
1	0.5998	0.4623	0.7317	0.2006	0.7848
2	0.7209	0.57	0.6098	0.2651	0.902
3	0.4079	0.3102	0.9578	0.1338	0.5639
4	0.4383	0.5572	0.1233	0.9239	0.3756
5	0.9222	0.7576	0.4419	0.3915	0.913
6	0.8358	0.6758	0.5093	0.3342	0.9911
7	0.7408	0.5881	0.5915	0.2765	0.9208
8	0.9981	0.8346	0.3852	0.4487	0.8425
9	0.795	0.6378	0.5435	0.3087	0.9713
10	0.2299	0.159	0.7353	0.0519	0.3787
11	0.7319	0.58	0.5996	0.2714	0.9124
12	0.568	0.7186	0.1527	0.8495	0.4797
13	0.7917	0.6348	0.5463	0.3067	0.9683
14	0.0133	0.0075	0.1003	0.0015	0.0418
15	0.407	0.299	0.9704	0.1142	0.5849
16		0.8328	0.3865	0.4473	0.8442
17	0.8328		0.2823	0.5825	0.7
18	0.3865	0.2823		0.1062	0.5624
19	0.4473	0.5825	0.1062		0.3814
20	0.8442	0.7	0.5624	0.3814	
21	0.0006	0.0003	0.0089	<.0001	0.0038
22	0.0053	0.0029	0.0495	0.0005	0.0204
23	<.0001	<.0001	<.0001	<.0001	<.0001
24	0.0002	<.0001	0.0028	<.0001	0.0013
25	0.0106	0.0059	0.0844	0.0011	0.0351
26	0.0007	0.0003	0.0095	<.0001	0.0041
27	0.0439	0.0266	0.2424	0.0062	0.106
28	0.0286	0.0169	0.1779	0.0037	0.0761
29	0.0304	0.018	0.1861	0.004	0.0798
30	0.1166	0.0759	0.476	0.0213	0.2253
31	0.1279	0.084	0.5062	0.024	0.242
32	0.0172	0.0098	0.1217	0.002	0.0511
33	0.4504	0.3348	0.9109	0.132	0.6316
34	0.3206	0.2294	0.8979	0.0818	0.4879
35	0.3537	0.4729	0.0751	0.866	0.3054
36	0.0007	0.0003	0.0092	<.0001	0.0039
37	0.7487	0.913	0.2367	0.6595	0.6292
38	0.3875	0.513	0.0858	0.9165	0.3329
39	0.4417	0.3276	0.9224	0.1283	0.6224
40	0.3074	0.4173	0.0614	0.7929	0.2678

i/j	21	22	23	24	25
1	0.0033	0.0219	<.0001	0.001	0.0397
2	0.002	0.0142	<.0001	0.0006	0.0266
3	0.0214	0.0873	0.0002	0.0084	0.135
4	0.0002	0.0013	<.0001	<.0001	0.0025
5	0.0009	0.007	<.0001	0.0002	0.0138
6	0.0013	0.0095	<.0001	0.0003	0.0183
7	0.0019	0.0133	<.0001	0.0005	0.0249
8	0.0006	0.0053	<.0001	0.0002	0.0105
9	0.0015	0.011	<.0001	0.0004	0.0209
10	0.0216	0.1017	<.0001	0.0075	0.1629
11	0.0019	0.0137	<.0001	0.0005	0.0257
12	<.0001	0.0009	<.0001	<.0001	0.002
13	0.0015	0.0111	<.0001	0.0004	0.0211
14	0.3105	0.7405	0.0063	0.1594	0.9327
15	0.008	0.0455	<.0001	0.0025	0.0781
16	0.0006	0.0053	<.0001	0.0002	0.0106
17	0.0003	0.0029	<.0001	<.0001	0.0059
18	0.0089	0.0495	<.0001	0.0028	0.0844
19	<.0001	0.0005	<.0001	<.0001	0.0011
20	0.0038	0.0204	<.0001	0.0013	0.0351
21		0.4931	0.0774	0.6904	0.3521
22	0.4931		0.0154	0.2798	0.8051
23	0.0774	0.0154		0.1686	0.0079
24	0.6904	0.2798	0.1686		0.1854
25	0.3521	0.8051	0.0079	0.1854	
26	0.9799	0.5091	0.0734	0.6719	0.3652
27	0.136	0.416	0.0015	0.0603	0.5702
28	0.1891	0.5265	0.0026	0.0886	0.6986
29	0.1809	0.5101	0.0024	0.0841	0.6799
30	0.0526	0.2045	0.0003	0.0204	0.3053
31	0.0472	0.1881	0.0003	0.018	0.2834
32	0.2665	0.6679	0.0047	0.1328	0.8552
33	0.0065	0.0383	<.0001	0.002	0.0666
34	0.0126	0.0657	<.0001	0.0041	0.1094
35	<.0001	0.0003	<.0001	<.0001	0.0007
36	0.9901	0.5009	0.0754	0.6812	0.3585
37	0.0002	0.0021	<.0001	<.0001	0.0043
38	<.0001	0.0004	<.0001	<.0001	0.0008
39	0.0068	0.0396	<.0001	0.0021	0.0687
40	<.0001	0.0002	<.0001	<.0001	0.0005

i/j	26	27	28	29	30
1	0.0036	0.1321	0.0925	0.0974	0.2923
2	0.0022	0.095	0.065	0.0687	0.223
3	0.0227	0.3202	0.2484	0.2577	0.5586
4	0.0002	0.0108	0.0069	0.0074	0.0309
5	0.001	0.0547	0.0361	0.0384	0.1405
6	0.0014	0.0695	0.0466	0.0493	0.1718
7	0.002	0.09	0.0614	0.0649	0.2132
8	0.0007	0.0436	0.0285	0.0303	0.116
9	0.0016	0.0777	0.0525	0.0555	0.1886
10	0.023	0.4042	0.3108	0.323	0.7073
11	0.0021	0.0922	0.063	0.0665	0.2175
12	0.0001	0.0105	0.0064	0.0069	0.0338
13	0.0016	0.0784	0.053	0.056	0.19
14	0.3225	0.6287	0.7621	0.7428	0.3465
15	0.0086	0.228	0.1665	0.1742	0.4535
16	0.0007	0.0439	0.0286	0.0304	0.1166
17	0.0003	0.0266	0.0169	0.018	0.0759
18	0.0095	0.2424	0.1779	0.1861	0.476
19	<.0001	0.0062	0.0037	0.004	0.0213
20	0.0041	0.106	0.0761	0.0798	0.2253
21	0.9799	0.136	0.1891	0.1809	0.0526
22	0.5091	0.416	0.5265	0.5101	0.2045
23	0.0734	0.0015	0.0026	0.0024	0.0003
24	0.6719	0.0603	0.0886	0.0841	0.0204
25	0.3652	0.5702	0.6986	0.6799	0.3053
26		0.1426	0.1976	0.1891	0.0557
27	0.1426		0.8564	0.8764	0.6455
28	0.1976	0.8564		0.9797	0.5219
29	0.1891	0.8764	0.9797		0.5385
30	0.0557	0.6455	0.5219	0.5385	
31	0.05	0.6114	0.4912	0.5073	0.9616
32	0.2773	0.6998	0.8378	0.8179	0.3986
33	0.007	0.2008	0.1451	0.1521	0.41
34	0.0134	0.2973	0.2221	0.2318	0.5586
35	<.0001	0.0038	0.0022	0.0024	0.0138
36	0.9898	0.1393	0.1933	0.1849	0.0541
37	0.0002	0.0203	0.0127	0.0136	0.06
38	<.0001	0.0046	0.0027	0.0029	0.0163
39	0.0073	0.2059	0.1491	0.1563	0.4182
40	<.0001	0.0029	0.0017	0.0018	0.0107

i/j	31	32	33	34	35
1	0.3147	0.06	0.8173	0.6376	0.1483
2	0.2415	0.0411	0.6902	0.5233	0.2003
3	0.5878	0.182	0.8784	0.9506	0.0996
4	0.0342	0.0041	0.149	0.0983	0.9559
5	0.1537	0.022	0.5107	0.37	0.3057
6	0.1872	0.0288	0.5834	0.4309	0.2574
7	0.2312	0.0386	0.6708	0.5063	0.2096
8	0.1274	0.0171	0.4489	0.3194	0.3549
9	0.2051	0.0327	0.62	0.4622	0.2362
10	0.7434	0.224	0.6528	0.8338	0.0352
11	0.2358	0.0397	0.6794	0.5138	0.2054
12	0.0379	0.0035	0.1867	0.1198	0.7201
13	0.2066	0.033	0.623	0.4648	0.2345
14	0.3226	0.9219	0.0797	0.129	0.0009
15	0.4828	0.1132	0.9404	0.8686	0.0812
16	0.1279	0.0172	0.4504	0.3206	0.3537
17	0.084	0.0098	0.3348	0.2294	0.4729
18	0.5062	0.1217	0.9109	0.8979	0.0751
19	0.024	0.002	0.132	0.0818	0.866
20	0.242	0.0511	0.6316	0.4879	0.3054
21	0.0472	0.2665	0.0065	0.0126	<.0001
22	0.1881	0.6679	0.0383	0.0657	0.0003
23	0.0003	0.0047	<.0001	<.0001	<.0001
24	0.018	0.1328	0.002	0.0041	<.0001
25	0.2834	0.8552	0.0666	0.1094	0.0007
26	0.05	0.2773	0.007	0.0134	<.0001
27	0.6114	0.6998	0.2008	0.2973	0.0038
28	0.4912	0.8378	0.1451	0.2221	0.0022
29	0.5073	0.8179	0.1521	0.2318	0.0024
30	0.9616	0.3986	0.41	0.5586	0.0138
31		0.3724	0.4377	0.5914	0.0156
32	0.3724		0.0975	0.155	0.0012
33	0.4377	0.0975		0.8101	0.0947
34	0.5914	0.155	0.8101		0.0569
35	0.0156	0.0012	0.0947	0.0569	
36	0.0486	0.2718	0.0067	0.013	<.0001
37	0.0666	0.0073	0.2834	0.1903	0.5426
38	0.0184	0.0014	0.1077	0.0655	0.9491
39	0.4463	0.1004	0.9884	0.8214	0.092
40	0.0122	0.0009	0.078	0.0461	0.9252

i/j	36	37	38	39	40
1	0.0035	0.3988	0.1667	0.806	0.1242
2	0.0021	0.4985	0.2233	0.6795	0.1698
3	0.022	0.2663	0.1116	0.8887	0.0839
4	0.0002	0.6245	0.9986	0.1454	0.8892
5	0.0009	0.676	0.3366	0.5015	0.2639
6	0.0013	0.598	0.2848	0.5735	0.2205
7	0.0019	0.5154	0.2334	0.6602	0.178
8	0.0007	0.7505	0.3888	0.4403	0.3086
9	0.0015	0.5621	0.2621	0.6098	0.2015
10	0.0223	0.1297	0.0409	0.6633	0.0281
11	0.002	0.5078	0.2289	0.6688	0.1743
12	<.0001	0.8017	0.7684	0.182	0.6512
13	0.0016	0.5592	0.2603	0.6127	0.2001
14	0.3164	0.0055	0.0011	0.0822	0.0006
15	0.0083	0.2516	0.0926	0.952	0.0665
16	0.0007	0.7487	0.3875	0.4417	0.3074
17	0.0003	0.913	0.513	0.3276	0.4173
18	0.0092	0.2367	0.0858	0.9224	0.0614
19	<.0001	0.6595	0.9165	0.1283	0.7929
20	0.0039	0.6292	0.3329	0.6224	0.2678
21	0.9901	0.0002	<.0001	0.0068	<.0001
22	0.5009	0.0021	0.0004	0.0396	0.0002
23	0.0754	<.0001	<.0001	<.0001	<.0001
24	0.6812	<.0001	<.0001	0.0021	<.0001
25	0.3585	0.0043	0.0008	0.0687	0.0005
26	0.9898	0.0002	<.0001	0.0073	<.0001
27	0.1393	0.0203	0.0046	0.2059	0.0029
28	0.1933	0.0127	0.0027	0.1491	0.0017
29	0.1849	0.0136	0.0029	0.1563	0.0018
30	0.0541	0.06	0.0163	0.4182	0.0107
31	0.0486	0.0666	0.0184	0.4463	0.0122
32	0.2718	0.0073	0.0014	0.1004	0.0009
33	0.0067	0.2834	0.1077	0.9884	0.078
34	0.013	0.1903	0.0655	0.8214	0.0461
35	<.0001	0.5426	0.9491	0.092	0.9252
36		0.0002	<.0001	0.007	<.0001
37	0.0002		0.5856	0.277	0.4824
38	<.0001	0.5856		0.1046	0.8746
39	0.007	0.277	0.1046		0.0757
40	<.0001	0.4824	0.8746	0.0757	

Model 2

interaction model with time comparison diffMatrix (blocks in treat)

The GLM Procedure

Class Level Information

Class	Levels	Values
treat	5	A B C D E
timeLex	8	000 001 003 008 015 028 060 130

Number of Observations Read 120
Number of Observations Used 117

Dependent Variable: smallCount smallCount

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	39	53762720399	1378531292	4.66	<.0001
Error	77	22773733046	295762767		
Corrected Total	116	76536453445			

R-Square	Coeff Var	Root MSE	smallCount Mean
0.702446	25.11767	17197.75	68468.76

Source	DF	Type I SS	Mean Square	F Value	Pr > F
timeLex	7	32500245200	4642892171	15.70	<.0001
treat	4	3171841564	792960391	2.68	0.0377
treat*timeLex	28	18090633636	646094058	2.18	0.0038

Source	DF	Type III SS	Mean Square	F Value	Pr > F
timeLex	7	32374556001	4624936572	15.64	<.0001
treat	4	2982054848	745513712	2.52	0.0478
treat*timeLex	28	18090633636	646094058	2.18	0.0038

Parameter		Estimate	Standard Error	t Value	Pr > |t|
Intercept		38445.00000 B	9929.12831	3.87	0.0002
timeLex	000	15801.66667 B	14041.90791	1.13	0.2639
timeLex	001	31421.66667 B	14041.90791	2.24	0.0281

timeLex	003	26133.33333 B	14041.90791	1.86	0.0665
timeLex	008	17522.50000 B	15699.33032	1.12	0.2678
timeLex	015	51205.00000 B	14041.90791	3.65	0.0005
timeLex	028	36713.33333 B	14041.90791	2.61	0.0107
timeLex	060	1323.33333 B	14041.90791	0.09	0.9252
timeLex	130	0.00000 B	.	.	.
treat	A	64176.66667 B	14041.90791	4.57	<.0001
treat	B	9911.66667 B	14041.90791	0.71	0.4824
treat	C	2223.33333 B	14041.90791	0.16	0.8746
treat	D	25285.00000 B	14041.90791	1.80	0.0757
treat	E	0.00000 B	.	.	.
treat*timeLex	A 000	-58153.33333 B	19858.25661	-2.93	0.0045
treat*timeLex	A 001	-78251.66667 B	19858.25661	-3.94	0.0002

Dependent Variable: smallCount smallCount

Parameter	Estimate	Standard Error	t Value	Pr > |t|
treat*timeLex A 003	-71055.00000 B	19858.25661	-3.58	0.0006
treat*timeLex A 008	-67272.50000 B	21062.86187	-3.19	0.0020
treat*timeLex A 015	-51030.00000 B	19858.25661	-2.57	0.0121
treat*timeLex A 028	-36893.33333 B	19858.25661	-1.86	0.0670
treat*timeLex A 060	-29465.00000 B	19858.25661	-1.48	0.1420
treat*timeLex A 130	0.00000 B	.	.	.
treat*timeLex B 000	-6251.66667 B	19858.25661	-0.31	0.7538
treat*timeLex B 001	-22245.00000 B	19858.25661	-1.12	0.2661
treat*timeLex B 003	-29671.66667 B	19858.25661	-1.49	0.1392
treat*timeLex B 008	-15982.50000 B	21062.86187	-0.76	0.4503
treat*timeLex B 015	-6435.00000 B	19858.25661	-0.32	0.7468
treat*timeLex B 028	-3426.66667 B	19858.25661	-0.17	0.8635
treat*timeLex B 060	37398.33333 B	19858.25661	1.88	0.0634
treat*timeLex B 130	0.00000 B	.	.	.
treat*timeLex C 000	9465.00000 B	21062.86187	0.45	0.6544
treat*timeLex C 001	-19251.66667 B	19858.25661	-0.97	0.3354
treat*timeLex C 003	-10208.33333 B	19858.25661	-0.51	0.6087
treat*timeLex C 008	6910.83333 B	21062.86187	0.33	0.7437
treat*timeLex C 015	36055.00000 B	19858.25661	1.82	0.0733
treat*timeLex C 028	6811.66667 B	19858.25661	0.34	0.7325
treat*timeLex C 060	21533.33333 B	19858.25661	1.08	0.2816
treat*timeLex C 130	0.00000 B	.	.	.
treat*timeLex D 000	-38891.66667 B	21062.86187	-1.85	0.0687
treat*timeLex D 001	-38618.33333 B	19858.25661	-1.94	0.0555
treat*timeLex D 003	-1403.33333 B	19858.25661	-0.07	0.9438

treat*timeLex D 008	-39107.50000 B	21062.86187	-1.86	0.0672
treat*timeLex D 015	-6523.33333 B	19858.25661	-0.33	0.7434
treat*timeLex D 028	-16608.33333 B	19858.25661	-0.84	0.4055
treat*timeLex D 060	1856.66667 B	19858.25661	0.09	0.9258
treat*timeLex D 130	0.00000 B	.	.	.
treat*timeLex E 000	0.00000 B	.	.	.
treat*timeLex E 001	0.00000 B	.	.	.
treat*timeLex E 003	0.00000 B	.	.	.
treat*timeLex E 008	0.00000 B	.	.	.
treat*timeLex E 015	0.00000 B	.	.	.
treat*timeLex E 028	0.00000 B	.	.	.
treat*timeLex E 060	0.00000 B	.	.	.
treat*timeLex E 130	0.00000 B	.	.	.

NOTE: The X'X matrix has been found to be singular, and a generalized inverse was used to solve the normal equations. Terms whose estimates are followed by the letter 'B' are not uniquely estimable.

Least Squares Means

	time	smallCount	LSMEAN
treat	Lex	LSMEAN	Number
A	000	60270.000	1
A	001	55791.667	2
A	003	57700.000	3
A	008	52871.667	4
A	015	102796.667	5
A	028	102441.667	6
A	060	74480.000	7
A	130	102621.667	8
B	000	57906.667	9
B	001	57533.333	10
B	003	44818.333	11
B	008	49896.667	12
B	015	93126.667	13
B	028	81643.333	14
B	060	87078.333	15
B	130	48356.667	16
C	000	65935.000	17
C	001	52838.333	18
C	003	56593.333	19
C	008	65101.667	20
C	015	127928.333	21

C	028	84193.333	22
C	060	63525.000	23
C	130	40668.333	24
D	000	40640.000	25
D	001	56533.333	26
D	003	88460.000	27
D	008	42145.000	28
D	015	108411.667	29
D	028	83835.000	30
D	060	66910.000	31
D	130	63730.000	32
E	000	54246.667	33
E	001	69866.667	34
E	003	64578.333	35
E	008	55967.500	36
E	015	89650.000	37
E	028	75158.333	38
E	060	39768.333	39
E	130	38445.000	40

Least Squares Means

Least Squares Means for effect treat*timeLex
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: smallCount

i/j	1	2	3	4	5	6	7	8
1		0.7506	0.8553	0.5998	0.0033	0.0036	0.3147	0.0035
2	0.7506		0.8923	0.8358	0.0013	0.0014	0.1872	0.0013
3	0.8553	0.8923		0.7319	0.0019	0.0021	0.2358	0.002
4	0.5998	0.8358	0.7319		0.0006	0.0007	0.1279	0.0007
5	0.0033	0.0013	0.0019	0.0006		0.9799	0.0472	0.9901
6	0.0036	0.0014	0.0021	0.0007	0.9799		0.05	0.9898
7	0.3147	0.1872	0.2358	0.1279	0.0472	0.05		0.0486
8	0.0035	0.0013	0.002	0.0007	0.9901	0.9898	0.0486	
9	0.8668	0.8807	0.9883	0.7209	0.002	0.0022	0.2415	0.0021
10	0.846	0.9016	0.9906	0.7408	0.0019	0.002	0.2312	0.0019
11	0.2746	0.4369	0.3618	0.568	<.0001	0.0001	0.0379	<.0001
12	0.4623	0.6758	0.58	0.8328	0.0003	0.0003	0.084	0.0003
13	0.0219	0.0095	0.0137	0.0053	0.4931	0.5091	0.1881	0.5009
14	0.1321	0.0695	0.0922	0.0439	0.136	0.1426	0.6114	0.1393
15	0.06	0.0288	0.0397	0.0172	0.2665	0.2773	0.3724	0.2718
16	0.3988	0.598	0.5078	0.7487	0.0002	0.0002	0.0666	0.0002
17	0.7192	0.5201	0.6014	0.4079	0.0214	0.0227	0.5878	0.022
18	0.5982	0.834	0.7301	0.9981	0.0006	0.0007	0.1274	0.0007
19	0.7941	0.9546	0.9374	0.7917	0.0015	0.0016	0.2066	0.0016
20	0.7317	0.5093	0.5996	0.3865	0.0089	0.0095	0.5062	0.0092
21	<.0001	<.0001	<.0001	<.0001	0.0774	0.0734	0.0003	0.0754
22	0.0925	0.0466	0.063	0.0286	0.1891	0.1976	0.4912	0.1933
23	0.8173	0.5834	0.6794	0.4504	0.0065	0.007	0.4377	0.0067
24	0.1667	0.2848	0.2289	0.3875	<.0001	<.0001	0.0184	<.0001
25	0.215	0.3375	0.2806	0.4383	0.0002	0.0002	0.0342	0.0002
26	0.7909	0.958	0.934	0.795	0.0015	0.0016	0.2051	0.0015
27	0.0482	0.0226	0.0315	0.0133	0.3105	0.3225	0.3226	0.3164
28	0.2006	0.3342	0.2714	0.4473	<.0001	<.0001	0.024	<.0001
29	0.001	0.0003	0.0005	0.0002	0.6904	0.6719	0.018	0.6812
30	0.0974	0.0493	0.0665	0.0304	0.1809	0.1891	0.5073	0.1849
31	0.6376	0.4309	0.5138	0.3206	0.0126	0.0134	0.5914	0.013
32	0.806	0.5735	0.6688	0.4417	0.0068	0.0073	0.4463	0.007
33	0.6692	0.9127	0.8064	0.9222	0.0009	0.001	0.1537	0.0009
34	0.4964	0.3193	0.3889	0.2299	0.0216	0.023	0.7434	0.0223
35	0.7598	0.5333	0.6256	0.407	0.008	0.0086	0.4828	0.0083
36	0.7848	0.9911	0.9124	0.8442	0.0038	0.0041	0.242	0.0039
37	0.0397	0.0183	0.0257	0.0106	0.3521	0.3652	0.2834	0.3585
38	0.2923	0.1718	0.2175	0.1166	0.0526	0.0557	0.9616	0.0541
39	0.1483	0.2574	0.2054	0.3537	<.0001	<.0001	0.0156	<.0001
40	0.1242	0.2205	0.1743	0.3074	<.0001	<.0001	0.0122	<.0001

i/j	9	10	11	12	13	14	15	16
1	0.8668	0.846	0.2746	0.4623	0.0219	0.1321	0.06	0.3988
2	0.8807	0.9016	0.4369	0.6758	0.0095	0.0695	0.0288	0.598
3	0.9883	0.9906	0.3618	0.58	0.0137	0.0922	0.0397	0.5078
4	0.7209	0.7408	0.568	0.8328	0.0053	0.0439	0.0172	0.7487
5	0.002	0.0019	<.0001	0.0003	0.4931	0.136	0.2665	0.0002
6	0.0022	0.002	0.0001	0.0003	0.5091	0.1426	0.2773	0.0002
7	0.2415	0.2312	0.0379	0.084	0.1881	0.6114	0.3724	0.0666
8	0.0021	0.0019	<.0001	0.0003	0.5009	0.1393	0.2718	0.0002
9		0.9789	0.3542	0.57	0.0142	0.095	0.0411	0.4985
10	0.9789		0.368	0.5881	0.0133	0.09	0.0386	0.5154
11	0.3542	0.368		0.7186	0.0009	0.0105	0.0035	0.8017
12	0.57	0.5881	0.7186		0.0029	0.0266	0.0098	0.913
13	0.0142	0.0133	0.0009	0.0029		0.416	0.6679	0.0021
14	0.095	0.09	0.0105	0.0266	0.416		0.6998	0.0203
15	0.0411	0.0386	0.0035	0.0098	0.6679	0.6998		0.0073
16	0.4985	0.5154	0.8017	0.913	0.0021	0.0203	0.0073	
17	0.6105	0.5941	0.1826	0.3102	0.0873	0.3202	0.182	0.2663
18	0.7191	0.739	0.5696	0.8346	0.0053	0.0436	0.0171	0.7505
19	0.9257	0.9468	0.4043	0.6348	0.0111	0.0784	0.033	0.5592
20	0.6098	0.5915	0.1527	0.2823	0.0495	0.2424	0.1217	0.2367
21	<.0001	<.0001	<.0001	<.0001	0.0154	0.0015	0.0047	<.0001
22	0.065	0.0614	0.0064	0.0169	0.5265	0.8564	0.8378	0.0127
23	0.6902	0.6708	0.1867	0.3348	0.0383	0.2008	0.0975	0.2834
24	0.2233	0.2334	0.7684	0.513	0.0004	0.0046	0.0014	0.5856
25	0.2748	0.2853	0.7908	0.5572	0.0013	0.0108	0.0041	0.6245
26	0.9223	0.9434	0.4067	0.6378	0.011	0.0777	0.0327	0.5621
27	0.0326	0.0306	0.0026	0.0075	0.7405	0.6287	0.9219	0.0055
28	0.2651	0.2765	0.8495	0.5825	0.0005	0.0062	0.002	0.6595
29	0.0006	0.0005	<.0001	<.0001	0.2798	0.0603	0.1328	<.0001
30	0.0687	0.0649	0.0069	0.018	0.5101	0.8764	0.8179	0.0136
31	0.5233	0.5063	0.1198	0.2294	0.0657	0.2973	0.155	0.1903
32	0.6795	0.6602	0.182	0.3276	0.0396	0.2059	0.1004	0.277
33	0.7951	0.8156	0.5039	0.7576	0.007	0.0547	0.022	0.676
34	0.397	0.3825	0.0784	0.159	0.1017	0.4042	0.224	0.1297
35	0.636	0.6173	0.1634	0.299	0.0455	0.228	0.1132	0.2516
36	0.902	0.9208	0.4797	0.7	0.0204	0.106	0.0511	0.6292
37	0.0266	0.0249	0.002	0.0059	0.8051	0.5702	0.8552	0.0043
38	0.223	0.2132	0.0338	0.0759	0.2045	0.6455	0.3986	0.06
39	0.2003	0.2096	0.7201	0.4729	0.0003	0.0038	0.0012	0.5426
40	0.1698	0.178	0.6512	0.4173	0.0002	0.0029	0.0009	0.4824

i/j	17	18	19	20	21	22	23	24
1	0.7192	0.5982	0.7941	0.7317	<.0001	0.0925	0.8173	0.1667
2	0.5201	0.834	0.9546	0.5093	<.0001	0.0466	0.5834	0.2848
3	0.6014	0.7301	0.9374	0.5996	<.0001	0.063	0.6794	0.2289
4	0.4079	0.9981	0.7917	0.3865	<.0001	0.0286	0.4504	0.3875
5	0.0214	0.0006	0.0015	0.0089	0.0774	0.1891	0.0065	<.0001
6	0.0227	0.0007	0.0016	0.0095	0.0734	0.1976	0.007	<.0001
7	0.5878	0.1274	0.2066	0.5062	0.0003	0.4912	0.4377	0.0184
8	0.022	0.0007	0.0016	0.0092	0.0754	0.1933	0.0067	<.0001
9	0.6105	0.7191	0.9257	0.6098	<.0001	0.065	0.6902	0.2233
10	0.5941	0.739	0.9468	0.5915	<.0001	0.0614	0.6708	0.2334
11	0.1826	0.5696	0.4043	0.1527	<.0001	0.0064	0.1867	0.7684
12	0.3102	0.8346	0.6348	0.2823	<.0001	0.0169	0.3348	0.513
13	0.0873	0.0053	0.0111	0.0495	0.0154	0.5265	0.0383	0.0004
14	0.3202	0.0436	0.0784	0.2424	0.0015	0.8564	0.2008	0.0046
15	0.182	0.0171	0.033	0.1217	0.0047	0.8378	0.0975	0.0014
16	0.2663	0.7505	0.5592	0.2367	<.0001	0.0127	0.2834	0.5856
17		0.4067	0.5536	0.9578	0.0002	0.2484	0.8784	0.1116
18	0.4067		0.7899	0.3852	<.0001	0.0285	0.4489	0.3888
19	0.5536	0.7899		0.5463	<.0001	0.053	0.623	0.2603
20	0.9578	0.3852	0.5463		<.0001	0.1779	0.9109	0.0858
21	0.0002	<.0001	<.0001	<.0001		0.0026	<.0001	<.0001
22	0.2484	0.0285	0.053	0.1779	0.0026		0.1451	0.0027
23	0.8784	0.4489	0.623	0.9109	<.0001	0.1451		0.1077
24	0.1116	0.3888	0.2603	0.0858	<.0001	0.0027	0.1077	
25	0.1454	0.4395	0.3127	0.1233	<.0001	0.0069	0.149	0.9986
26	0.551	0.7931	0.9966	0.5435	<.0001	0.0525	0.62	0.2621
27	0.1554	0.0132	0.026	0.1003	0.0063	0.7621	0.0797	0.0011
28	0.1338	0.4487	0.3067	0.1062	<.0001	0.0037	0.132	0.9165
29	0.0084	0.0002	0.0004	0.0028	0.1686	0.0886	0.002	<.0001
30	0.2577	0.0303	0.056	0.1861	0.0024	0.9797	0.1521	0.0029
31	0.9506	0.3194	0.4648	0.8979	<.0001	0.2221	0.8101	0.0655
32	0.8887	0.4403	0.6127	0.9224	<.0001	0.1491	0.9884	0.1046
33	0.4588	0.9204	0.8677	0.4419	<.0001	0.0361	0.5107	0.3366
34	0.8029	0.229	0.3475	0.7353	<.0001	0.3108	0.6528	0.0409
35	0.9314	0.4057	0.5712	0.9704	<.0001	0.1665	0.9404	0.0926
36	0.5639	0.8425	0.9683	0.5624	<.0001	0.0761	0.6316	0.3329
37	0.135	0.0105	0.0211	0.0844	0.0079	0.6986	0.0666	0.0008
38	0.5586	0.116	0.19	0.476	0.0003	0.5219	0.41	0.0163
39	0.0996	0.3549	0.2345	0.0751	<.0001	0.0022	0.0947	0.9491
40	0.0839	0.3086	0.2001	0.0614	<.0001	0.0017	0.078	0.8746

i/j	25	26	27	28	29	30	31	32
1	0.215	0.7909	0.0482	0.2006	0.001	0.0974	0.6376	0.806
2	0.3375	0.958	0.0226	0.3342	0.0003	0.0493	0.4309	0.5735
3	0.2806	0.934	0.0315	0.2714	0.0005	0.0665	0.5138	0.6688
4	0.4383	0.795	0.0133	0.4473	0.0002	0.0304	0.3206	0.4417
5	0.0002	0.0015	0.3105	<.0001	0.6904	0.1809	0.0126	0.0068
6	0.0002	0.0016	0.3225	<.0001	0.6719	0.1891	0.0134	0.0073
7	0.0342	0.2051	0.3226	0.024	0.018	0.5073	0.5914	0.4463
8	0.0002	0.0015	0.3164	<.0001	0.6812	0.1849	0.013	0.007
9	0.2748	0.9223	0.0326	0.2651	0.0006	0.0687	0.5233	0.6795
10	0.2853	0.9434	0.0306	0.2765	0.0005	0.0649	0.5063	0.6602
11	0.7908	0.4067	0.0026	0.8495	<.0001	0.0069	0.1198	0.182
12	0.5572	0.6378	0.0075	0.5825	<.0001	0.018	0.2294	0.3276
13	0.0013	0.011	0.7405	0.0005	0.2798	0.5101	0.0657	0.0396
14	0.0108	0.0777	0.6287	0.0062	0.0603	0.8764	0.2973	0.2059
15	0.0041	0.0327	0.9219	0.002	0.1328	0.8179	0.155	0.1004
16	0.6245	0.5621	0.0055	0.6595	<.0001	0.0136	0.1903	0.277
17	0.1454	0.551	0.1554	0.1338	0.0084	0.2577	0.9506	0.8887
18	0.4395	0.7931	0.0132	0.4487	0.0002	0.0303	0.3194	0.4403
19	0.3127	0.9966	0.026	0.3067	0.0004	0.056	0.4648	0.6127
20	0.1233	0.5435	0.1003	0.1062	0.0028	0.1861	0.8979	0.9224
21	<.0001	<.0001	0.0063	<.0001	0.1686	0.0024	<.0001	<.0001
22	0.0069	0.0525	0.7621	0.0037	0.0886	0.9797	0.2221	0.1491
23	0.149	0.62	0.0797	0.132	0.002	0.1521	0.8101	0.9884
24	0.9986	0.2621	0.0011	0.9165	<.0001	0.0029	0.0655	0.1046
25		0.3145	0.0032	0.9239	<.0001	0.0074	0.0983	0.1454
26	0.3145		0.0258	0.3087	0.0004	0.0555	0.4622	0.6098
27	0.0032	0.0258		0.0015	0.1594	0.7428	0.129	0.0822
28	0.9239	0.3087	0.0015		<.0001	0.004	0.0818	0.1283
29	<.0001	0.0004	0.1594	<.0001		0.0841	0.0041	0.0021
30	0.0074	0.0555	0.7428	0.004	0.0841		0.2318	0.1563
31	0.0983	0.4622	0.129	0.0818	0.0041	0.2318		0.8214
32	0.1454	0.6098	0.0822	0.1283	0.0021	0.1563	0.8214	
33	0.3888	0.8711	0.0171	0.3915	0.0002	0.0384	0.37	0.5015
34	0.0665	0.3453	0.1894	0.0519	0.0075	0.323	0.8338	0.6633
35	0.1314	0.5684	0.093	0.1142	0.0025	0.1742	0.8686	0.952
36	0.3756	0.9713	0.0418	0.3814	0.0013	0.0798	0.4879	0.6224
37	0.0025	0.0209	0.9327	0.0011	0.1854	0.6799	0.1094	0.0687
38	0.0309	0.1886	0.3465	0.0213	0.0204	0.5385	0.5586	0.4182
39	0.9559	0.2362	0.0009	0.866	<.0001	0.0024	0.0569	0.092
40	0.8892	0.2015	0.0006	0.7929	<.0001	0.0018	0.0461	0.0757

i/j	33	34	35	36	37	38	39	40
1	0.6692	0.4964	0.7598	0.7848	0.0397	0.2923	0.1483	0.1242
2	0.9127	0.3193	0.5333	0.9911	0.0183	0.1718	0.2574	0.2205
3	0.8064	0.3889	0.6256	0.9124	0.0257	0.2175	0.2054	0.1743
4	0.9222	0.2299	0.407	0.8442	0.0106	0.1166	0.3537	0.3074
5	0.0009	0.0216	0.008	0.0038	0.3521	0.0526	<.0001	<.0001
6	0.001	0.023	0.0086	0.0041	0.3652	0.0557	<.0001	<.0001
7	0.1537	0.7434	0.4828	0.242	0.2834	0.9616	0.0156	0.0122
8	0.0009	0.0223	0.0083	0.0039	0.3585	0.0541	<.0001	<.0001
9	0.7951	0.397	0.636	0.902	0.0266	0.223	0.2003	0.1698
10	0.8156	0.3825	0.6173	0.9208	0.0249	0.2132	0.2096	0.178
11	0.5039	0.0784	0.1634	0.4797	0.002	0.0338	0.7201	0.6512
12	0.7576	0.159	0.299	0.7	0.0059	0.0759	0.4729	0.4173
13	0.007	0.1017	0.0455	0.0204	0.8051	0.2045	0.0003	0.0002
14	0.0547	0.4042	0.228	0.106	0.5702	0.6455	0.0038	0.0029
15	0.022	0.224	0.1132	0.0511	0.8552	0.3986	0.0012	0.0009
16	0.676	0.1297	0.2516	0.6292	0.0043	0.06	0.5426	0.4824
17	0.4588	0.8029	0.9314	0.5639	0.135	0.5586	0.0996	0.0839
18	0.9204	0.229	0.4057	0.8425	0.0105	0.116	0.3549	0.3086
19	0.8677	0.3475	0.5712	0.9683	0.0211	0.19	0.2345	0.2001
20	0.4419	0.7353	0.9704	0.5624	0.0844	0.476	0.0751	0.0614
21	<.0001	<.0001	<.0001	<.0001	0.0079	0.0003	<.0001	<.0001
22	0.0361	0.3108	0.1665	0.0761	0.6986	0.5219	0.0022	0.0017
23	0.5107	0.6528	0.9404	0.6316	0.0666	0.41	0.0947	0.078
24	0.3366	0.0409	0.0926	0.3329	0.0008	0.0163	0.9491	0.8746
25	0.3888	0.0665	0.1314	0.3756	0.0025	0.0309	0.9559	0.8892
26	0.8711	0.3453	0.5684	0.9713	0.0209	0.1886	0.2362	0.2015
27	0.0171	0.1894	0.093	0.0418	0.9327	0.3465	0.0009	0.0006
28	0.3915	0.0519	0.1142	0.3814	0.0011	0.0213	0.866	0.7929
29	0.0002	0.0075	0.0025	0.0013	0.1854	0.0204	<.0001	<.0001
30	0.0384	0.323	0.1742	0.0798	0.6799	0.5385	0.0024	0.0018
31	0.37	0.8338	0.8686	0.4879	0.1094	0.5586	0.0569	0.0461
32	0.5015	0.6633	0.952	0.6224	0.0687	0.4182	0.092	0.0757
33		0.2694	0.4641	0.913	0.0138	0.1405	0.3057	0.2639
34	0.2694		0.7075	0.3787	0.1629	0.7073	0.0352	0.0281
35	0.4641	0.7075		0.5849	0.0781	0.4535	0.0812	0.0665
36	0.913	0.3787	0.5849		0.0351	0.2253	0.3054	0.2678
37	0.0138	0.1629	0.0781	0.0351		0.3053	0.0007	0.0005
38	0.1405	0.7073	0.4535	0.2253	0.3053		0.0138	0.0107
39	0.3057	0.0352	0.0812	0.3054	0.0007	0.0138		0.9252
40	0.2639	0.0281	0.0665	0.2678	0.0005	0.0107	0.9252	

Model 3

marginal model for time

The GLM Procedure

Class Level Information

Class	Levels	Values
timeLex	8	000 001 003 008 015 028 060 130

Number of Observations Read	120
Number of Observations Used	117

Dependent Variable: logCount

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	6.28462227	0.89780318	9.56	<.0001
Error	109	10.23366083	0.09388680		
Corrected Total	116	16.51828310			

R-Square	Coeff Var	Root MSE	logCount Mean
0.380465	1.231502	0.306410	24.88096

Source	DF	Type I SS	Mean Square	F Value	Pr > F
timeLex	7	6.28462227	0.89780318	9.56	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
timeLex	7	6.28462227	0.89780318	9.56	<.0001

Parameter		Estimate	Standard Error	t Value	Pr > |t|
Intercept		24.69586804 B	0.07911460	312.15	<.0001
timeLex	000	0.01713810 B	0.11610849	0.15	0.8829
timeLex	001	0.07802740 B	0.11188494	0.70	0.4870
timeLex	003	0.11468744 B	0.11188494	1.03	0.3076
timeLex	008	-0.04140539 B	0.11386536	-0.36	0.7168
timeLex	015	0.65184621 B	0.11188494	5.83	<.0001
timeLex	028	0.46339980 B	0.11188494	4.14	<.0001
timeLex	060	0.15953734 B	0.11188494	1.43	0.1568
timeLex	130	0.00000000 B	.	.	.

NOTE: The X'X matrix has been found to be singular, and a generalized inverse was used to solve the normal equations. Terms whose estimates are followed by the letter 'B' are not uniquely estimable.

Least Squares Means

time Lex	logCount LSMEAN	LSMEAN Number
000	24.7130061	1
001	24.7738954	2
003	24.8105555	3
008	24.6544627	4
015	25.3477142	5
028	25.1592678	6
060	24.8554054	7
130	24.6958680	8

Least Squares Means for effect timeLex
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: logCount

A-E	0	1	3	8	15	28	60	130
0		0.6011	0.4027	0.6209	<.0001	0.0002	0.2227	0.8829
1	0.6011		0.7438	0.2965	<.0001	0.0008	0.4679	0.487
3	0.4027	0.7438		0.1732	<.0001	0.0023	0.6893	0.3076
8	0.6209	0.2965	0.1732		<.0001	<.0001	0.0804	0.7168
15	<.0001	<.0001	<.0001	<.0001		0.095	<.0001	<.0001
28	0.0002	0.0008	0.0023	<.0001	0.095		0.0077	<.0001
60	0.2227	0.4679	0.6893	0.0804	<.0001	0.0077		0.1568
130	0.8829	0.487	0.3076	0.7168	<.0001	<.0001	0.1568	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Model 4

marginal model for treat

The GLM Procedure

Class Level Information

Class	Levels	Values
treat	5	A B C D E

Number of Observations Read 120

Number of Observations Used 117

Dependent Variable: logCount

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.68691656	0.17172914	1.21	0.3085
Error	112	15.83136654	0.14135149		
Corrected Total	116	16.51828310			

R-Square	Coeff Var	Root MSE	logCount Mean
0.041585	1.511065	0.375967	24.88096

Source	DF	Type I SS	Mean Square	F Value	Pr > F
treat	4	0.68691656	0.17172914	1.21	0.3085

Source	DF	Type III SS	Mean Square	F Value	Pr > F
treat	4	0.68691656	0.17172914	1.21	0.3085

Parameter		Estimate	Standard Error	t Value	Pr > |t|
Intercept		24.77728679 B	0.07839462	316.06	<.0001
treat	A	0.22329553 B	0.10970580	2.04	0.0442
treat	B	0.04628856 B	0.10970580	0.42	0.6739
treat	C	0.12340153 B	0.11086674	1.11	0.2681
treat	D	0.12266720 B	0.11086674	1.11	0.2709
treat	E	0.00000000 B	.	.	.

NOTE: The X'X matrix has been found to be singular, and a generalized inverse was used to solve the normal equations. Terms whose estimates are followed by the letter 'B' are not uniquely estimable.

Least Squares Means

treat	logCount LSMEAN	LSMEAN Number
A	25.0005823	1
B	24.8235753	2
C	24.9006883	3
D	24.8999540	4
E	24.7772868	5

Least Squares Means for effect treat

Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: logCount

i/j	1	2	3	4	5
1		0.1057	0.3645	0.361	0.0442
2	0.1057		0.4836	0.4877	0.6739
3	0.3645	0.4836		0.9947	0.2681
4	0.361	0.4877	0.9947		0.2709
5	0.0442	0.6739	0.2681	0.2709	

Model 5

The GLM Procedure

Class Level Information

Class	Levels	Values
Microcosm	5	A B C D E

Number of Observations Read	20
Number of Observations Used	20

Dependent Variable: delta15N delta15N

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	91435.8477	22858.9619	0.72	0.5944
Error	15	479405.3503	31960.3567		
Corrected Total	19	570841.1981			

R-Square	Coeff Var	Root MSE	delta15N Mean
0.160177	17.78721	178.7746	1005.074

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Microcosm	4	91435.84771	22858.96193	0.72	0.5944

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Microcosm	4	91435.84771	22858.96193	0.72	0.5944

Parameter	Estimate	Standard Error	t Value	Pr > |t|
Intercept	1023.834319 B	89.3872987	11.45	<.0001
Microcosm A	-19.067912 B	126.4127302	-0.15	0.8821
Microcosm B	-29.628668 B	126.4127302	-0.23	0.8179

Microcosm C	83.182648 B	126.4127302	0.66	0.5205
Microcosm D	-128.288526 B	126.4127302	-1.01	0.3263
Microcosm E	0.000000 B	.	.	.

NOTE: The X'X matrix has been found to be singular, and a generalized inverse was used to solve the normal equations. Terms whose estimates are followed by the letter 'B' are not uniquely estimable.

Least Squares Means

Microcosm	delta15N LSMEAN	LSMEAN Number
A	1004.76641	1
B	994.20565	2
C	1107.01697	3
D	895.54579	4
E	1023.83432	5

Least Squares Means for effect Microcosm
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: delta15N

i/j	1	2	3	4	5
1		0.9345	0.4312	0.4012	0.8821
2	0.9345		0.3863	0.4473	0.8179
3	0.4312	0.3863		0.1151	0.5205
4	0.4012	0.4473	0.1151		0.3263
5	0.8821	0.8179	0.5205	0.3263	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Model 6

The GLM Procedure

Class Level Information

Class	Levels	Values
Time	4	001 028 060 130

Number of Observations Read	20
Number of Observations Used	20

Dependent Variable: delta15N delta15N

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	209959.3918	69986.4639	3.10	0.0562
Error	16	360881.8062	22555.1129		
Corrected Total	19	570841.1981			

R-Square	Coeff Var	Root MSE	delta15N Mean
0.367807	14.94254	150.1836	1005.074

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Time	3	209959.3918	69986.4639	3.10	0.0562

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Time	3	209959.3918	69986.4639	3.10	0.0562

Parameter		Estimate	Standard Error	t Value	Pr > |t|
Intercept		1023.937884 B	67.16414652	15.25	<.0001
Time	001	-189.333572 B	94.98444691	-1.99	0.0636
Time	028	33.657110 B	94.98444691	0.35	0.7277
Time	060	80.220236 B	94.98444691	0.84	0.4108
Time	130	0.000000 B	.	.	.

NOTE: The X'X matrix has been found to be singular, and a generalized inverse was used to solve the normal equations. Terms whose estimates are followed by the letter 'B' are not uniquely estimable

The GLM Procedure
Least Squares Means

Time	delta15N LSMEAN	Number
001	834.60431	1
028	1057.59499	2
060	1104.15812	3
130	1023.93788	4

Least Squares Means for effect Time
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: delta15N

i/j	1	2	3	4
1		0.0321	0.0119	0.0636
2	0.0321		0.6306	0.7277
3	0.0119	0.6306		0.4108
4	0.0636	0.7277	0.4108	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

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