

Growth Kinetics of Wildlife *E. coli* Isolates in Soil and Water

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ABSTRACT

Bacteria are the major cause of surface water contamination in the United States. US Environmental Protection Agency (USEPA) uses the Total Maximum Daily Load (TMDL) process to regulate the *E. coli* loads from fecal sources in a watershed. Different point and non-point sources can contribute to the fecal contamination of a waterbody including municipal and on-site wastewater treatment plants, livestock, birds, and wildlife. Unfortunately, wildlife sources in many rural watersheds are poorly characterized. *E. coli* is also known to persist in waterbodies when no known fecal sources are present. In this study, *E. coli* from wildlife fecal material was enumerated and the fate of *E. coli* under different environmental factors was studied. No growth was observed in soil at 4% moisture content and in water at 10°C. The highest *E. coli* growth was recorded in water at 30°C. It can be seen from these results that there was variation in the fate of *E. coli* under different environmental conditions. The fate of *E. coli* in the environment is a complex process and is influenced by many factors and their interactions, making it difficult to predict. The findings from this study along with additional studies can be used to improve the accuracy of model predictions to estimate the *E. coli* loads in watersheds.

Keywords: Bacteria; Fecal Contamination; TMDL; Water Quality

1. Introduction

The leading cause of impairment for waterbodies in the United States is from bacteria [1]. Bacterial impairment of rivers and streams originates from fecal contamination. Wastewater effluents and fecal material from both livestock and wildlife are potential sources of fecal contamination in a watershed. Warm-blooded mammals shed pathogenic bacteria in their feces. Pathogenic bacteria such as *Salmonella typhi*, *Shigella*, *Campylobacter jejuni*, and *Escherichia coli* (*E. coli*) O157:H7 are responsible for waterborne diseases that include typhoid fever, dysentery, campylobacteriosis, and *E. coli* O157:H7 infection, respectively. These illnesses can include symptoms of diarrhea, fever, nausea, vomiting, and abdominal cramps. Few of these symptoms can last for days and even lead to death in immune-compromised individuals [2]. *E. coli* is the current indicator organism for fecal contamination and used by the Environmental Protection Agency (EPA) to assess bacterial impairment in waterbodies. In 2008, 405 streams in Texas were bacterially impaired according to the 303(d) list of impaired waterbodies in the United States [3].

Under sub-tropical and temperate environments *E. coli* has been observed to persist [4-8]. Sources of *E. coli* in a waterbody are not only external sources but also *in situ*. Specifically, sediments have been found to be reservoirs of enteric bacteria, including *E. coli*, and a potential source in waterbodies [4-13]. Environmental controls have been shown to have a role in sustaining *E. coli* populations in the environment [14-16].

The trends of *E. coli* survival in variety of waterbodies including lake, river, sea, and creek have been studied [17-24]. Temperature is suggested to be the most important factor that affects bacterial survival in water [18]. The survival and growth rate of *E. coli* in river water can be affected by temperature differently. Hendricks (1972) observed a higher growth rate of *E. coli* in river water at 30°C than at lower temperatures. However, Flint (1987) observed the survival of *E. coli* in river water was less at 30°C than at 4°C and 25°C. Filip *et al.* (1988) also observed longer survival of *E. coli* at lower temperatures. They reported *E. coli* survived for 100 days in groundwater at 10°C [19]. Additionally, Padia (2010) found that *E. coli* survival in creek water was the highest at 20°C compared to *E. coli* survival at 0°C, 10°C, and 50°C over a period of one-week.

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Growth and survival of *E. coli* in soil is affected by moisture content [6,8,14-16,25,26]. Change in soil moisture content from dry to saturated conditions was found to promote the growth of *E. coli* in soil [6,8]. At dry soil conditions, the *E. coli* die-off was observed to be faster than saturated soil moisture conditions [25]. Sjogren (1994) set-up laboratory soil microcosms and observed that the survival of *E. coli* was the longest when soil was under saturated moisture conditions, lasting up to 23.3 months.

Water quality and watershed modeling tools such as Spatially Explicit Load Enrichment Calculation Tool (SELECT), Hydrological Simulation Program-Fortran (HSPF), and Soil and Water Assessment Tool (SWAT) are used in the bacterial Total Maximum Daily Load (TMDL) process to characterize *E. coli* sources in a watershed and estimate the required reductions in *E. coli* loads [27-30]. In rural watersheds, wildlife can contribute a majority of the fecal pollution and should be considered [31]. Unfortunately, *E. coli* concentrations present in wildlife fecal material are not well documented. Watershed modeling tools incorporate environmental factors to estimate *E. coli* loads in a watershed but more data are needed [32].

The main objective of this research was to study the fate of *E. coli* isolates from wildlife fecal material in water at different temperatures and in soil at different moisture conditions. The specific objectives were to 1) enu-

merate and obtain isolates from wildlife species' fecal material; 2) determine kinetic characteristics of *E. coli* isolates enumerated from feral hog and deer fecal material in water at different temperatures; and 3) determine kinetic characteristics of *E. coli* isolates enumerated from feral hog and deer fecal material in soil at different soil moisture conditions.

2. Study Area

Cedar Creek watershed is located in East Central Texas, USA, within both Brazos and Robertson County (**Figure 1**).

Cedar Creek is one of the 405 impaired water bodies in Texas that does not meet the bacteria criteria for the state [3]. It also is categorized as 5c which requires additional data and information for a TMDL to be scheduled by the Texas Commission on Environmental Quality (TCEQ). There is very little urban influence in Cedar Creek. The land use is mainly rangelands and forested areas (**Table 1**). Direct fecal deposition from cattle, wildlife, and birds along with other non- point sources contribute to the fecal contamination of the creek.

3. Methodology

3.1. Sampling Protocol

Two sub-watersheds within Cedar Creek watershed were

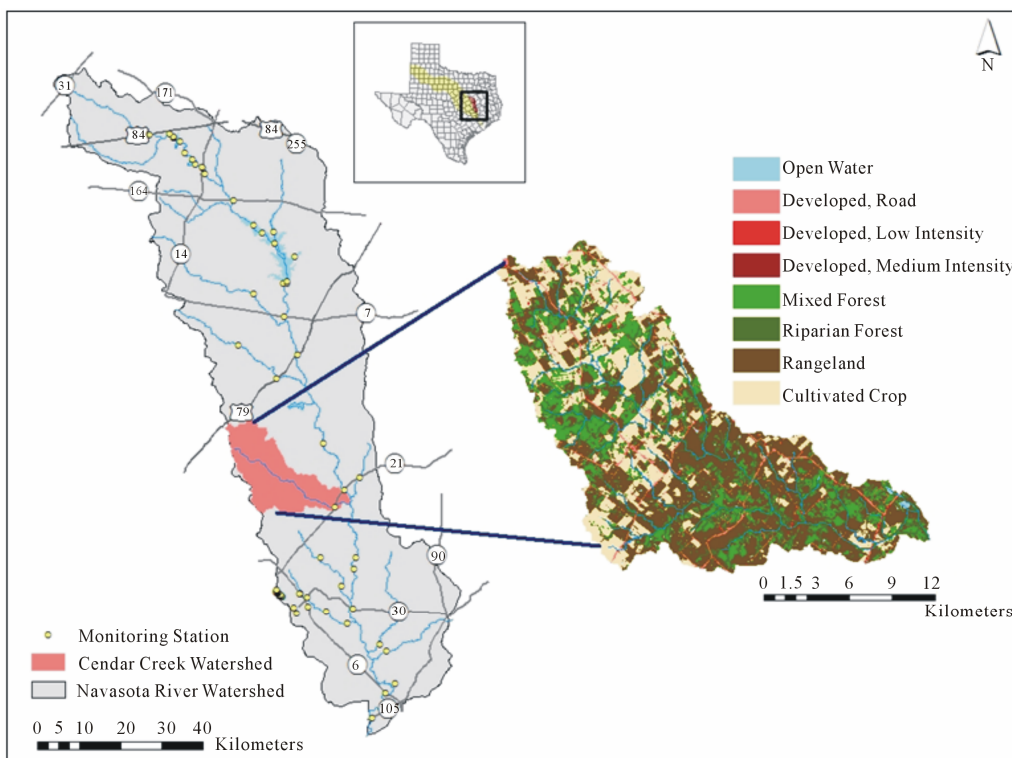


Figure 1. Location of Cedar Creek Watershed in central Texas.

Table 1. Cedar creek watershed characteristics.

Total Area	340.54 km ²
Land Use	95.3% undeveloped forest 3.9% developed area 0.82% open waters
Climate	subtropical
Rainfall (Annual)	810 - 1220 mm
Soil*	sandy loam (66% sand, 18% silt, and 16% clay) 1.2% organic matter strongly acidic (pH 5.2)

used for sampling with landowner co-operation. The land use of the sub-watersheds is mainly rangeland. The sampling protocol for obtaining the fecal material included a grid-design for trapping the wildlife. A wildlife expert designed the protocol for trapping and collecting the fecal material. The wildlife species trapped included raccoon, opossum, feral hog, deer, skunk, and armadillo. A more detailed description of sampling protocol is discussed in [24]. Briefly, once the wildlife species were trapped the fecal material was collected using sterile Whirl-Pak® bags while wearing latex gloves. The sex, age, date of trapping, and location were recorded. The samples were transported in an insulated cooler on ice at 5°C to the Water Quality Engineering Laboratory at Texas A & M University.

3.2. Isolating *E. coli* from Wildlife Fecal Samples

The fecal samples were brought to the lab and kept frozen at -20°C until processed. When processed, the fecal material was defrosted and one gram was measured out with sterile scoop. The samples were serially diluted in de-ionized (DI) water. The diluted samples were run through a membrane filtration system, following the EPA method 1603 [33]. The membrane of 0.45 µm pore size was removed from the filtration system with sterile forceps and placed on modified Thermo-tolerant *E. coli*, mTEC, (Difco®) agar plates. The plates were inverted and placed in an incubator at 35.5°C ± 0.5°C for two hours to revive the cells. Then the plates were sealed in Whirl-Pak® bags and placed in a water bath at 44.5°C ± 0.5°C for 22 hrs to select for thermo-tolerant *E. coli*. The plates were counted and values of 30 to 300 CFUs (colony forming units) were recorded.

Randomly selected isolates from each plate were streaked on Nutrient agar with, 4-methylumbelliferyl-β-D-glucuronide (MUG), (Difco®) and placed inverted in an incubator at 35.5°C for 24 hrs. MUG is a colorless substrate that is hydrolyzed by an enzyme present in *E. coli*, to a fluorescent product, 4-methylumbelliferone. *E. coli* was confirmed if the MUG plates fluoresced. Confirmed isolates were stored in labeled centrifuge tubes

with 1 mL of Luria-Bertani (LB) broth (Difco®) and 10% glycerol in -20°C freezer.

3.3. Growth Kinetics of *E. coli* in Water under Different Temperatures

Three *E. coli* isolates from each feral hog (H1-3, H4-1, and H7-1) and deer (D1-c, D2-c, and D2-d) fecal sample were enriched in 100 mL of LB broth at 35.5°C for 24 hrs. The enriched LB broth was diluted to 10⁻⁴ by adding 1 mL of LB broth into 100 mL sterile DI water, stirred, and then 1 mL of the diluted LB broth was added to 100 mL of sterile DI water. Finally, creek water microcosms were made with 1 mL of the 10⁻⁴ dilution of LB broth is added to 100 mL of sterile Cedar Creek water (autoclaved three times at 121°C for 15 min). The creek water microcosms were triplicated and kept in an incubator set according to the experimental temperatures (10°C, 25°C, and 30°C). Over 30 hrs, 0.1 mL of each water microcosm were spread plated onto MacConkey agar (Difco®) plate at different sampling times. The *E. coli* concentrations (CFU/mL) were recorded at each sampling time.

The kinetic characteristics of *E. coli* strains in water at different temperatures were determined using first order kinetics. The natural log of the bacterial counts was plotted against time to obtain the rate constant, k. A trend line was fitted to the data to determine the k-value from the slope of the line. If the k-value was positive, then the doubling time (t_d) was calculated. If the k-value was negative, then the half-life (t_{1/2}) was calculated.

3.4. Growth Kinetics of *E. coli* in Soil under Different Moisture Conditions

One *E. coli* isolate from each feral hog (H1-3) and deer (D2-c) fecal sample was enriched in 100 mL of LB broth. Cedar Creek soil (Table 1; autoclaved three times at 121°C for 15 min and dried in oven for 10 hrs) was used in soil microcosms with 1 mL of enriched LB broth. An estimated amount of sterile DI water (0, 6, and 15 mL) was added to 30 g of soil to obtain experimental moisture contents (4%, 25%, and 57%). Three replicated soil microcosms were placed on a rotary shaker at 200 rpm at room temperature (22°C). Over one week, *E. coli* was enumerated by serially diluting one gram soil from each microcosm in DI water and 0.1 mL of the last three dilutions was spread-plated onto MacConkey agar plates. The *E. coli* bacterial counts (CFU/g) were recorded at five to seven different sampling times for the duration of a week. One isolate from each MacConkey plate with growth was randomly selected and streaked onto nutrient agar with MUG and grown at 35.5°C for 24 hrs to confirm the isolate was still *E. coli* and not contamination.

The kinetic characteristics of *E. coli* strains in soil at different soil moisture conditions were determined in the

same manner as described in Section C.

4. Results and Discussion

4.1. Growth of *E. coli* in Water at Different Temperatures

The change in concentration of *E. coli* isolates from feral hog and deer fecal material in sterilized Cedar Creek water was observed at different temperatures. Preliminary eight hour studies were conducted and it was found that water temperature affected the *E. coli* concentration over time. Concentration of *E. coli* increased in water at 25°C and at 30°C over time. At 10°C, decrease in *E. coli* concentration was observed (data not shown).

These findings were further tested with one isolate from feral hog fecal material (H1-3) and one isolate from deer fecal material (D2-c) over 30 hours. *E. coli* concentrations for H1-3 decreased in water at 10°C and increased in water at 25°C and 30°C (**Figure 2**). The change in *E. coli* concentrations for D2-c was the same for all three temperatures (**Figure 2**). Padia (2010) and Hendricks *et al.* (1972) observed increase in *E. coli* concentration in water at 30°C over time. However, [34] and [35] reported *E. coli* concentrations in water decreased at higher temperatures, such as 30°C.

These contradicting observations in the fate of *E. coli* in water at 30°C might be due to the difference in *E. coli* isolates used in these studies. In this study and the study by [24], thermo-tolerant *E. coli* was enumerated directly from the fecal material of potential sources, whereas laboratory *E. coli* strains that did not originate from fecal material were used in other studies [34,35]. The high temperature of the intestinal tract of mammals (~30°C) promotes growth of thermo-tolerant enteric bacteria, such as *E. coli*. For this reason, the EPA method to enumerate *E. coli* in water uses a temperature of 44.5°C to select for thermo-tolerant *E. coli* [33]. The results from this study using thermo-tolerant *E. coli* give a more accurate depiction of the fate of *E. coli* from fecal deposition compared to studies using laboratory *E. coli* strains.

The kinetic constant (*k*), doubling time (*t_d*), and half-life (*t_{1/2}*) were determined assuming first order kinetics.

The kinetic characteristics for *E. coli* isolates, H1-3 and D2-c, at each water temperature are shown in **Table 2**. The kinetic constants for both H1-3 and D2-c were the lowest at 10°C and the highest at 30°C in water (**Table 2**). The kinetic constants for H1-3 and D2-c were negative, indicating the *k*-value was a decay rate (**Table 2**). The growth rate of *E. coli* varies with temperature and is crucial in determining total maximum daily loads (TMDLs) for *E. coli*. The duration of this study was 30 hours in the laboratory but considered a day, 24 hours, in the aquatic environment. The doubling time/half-life, *t_d*/*t_{1/2}*, along with the *k*-value, can be used to quantify the amount of *E. coli* present after a period of time.

4.2. Growth of *E. coli* in Soil under Different Moisture Conditions

Concentrations of *E. coli* isolates from feral hog and deer fecal material in sterilized Cedar Creek soil (**Table 1**) at different moisture conditions were measured over time (**Figure 3**). Both *E. coli* isolates from feral hog (H1-3) and deer (D2-c) decreased in concentrations over 168 hours for 4%, 25%, and 57% moisture content, with the exception of H1-3 at 57% with a slight increase in concentration. At 4% moisture content, the *E. coli* concentrations for both isolates (H1-3 and D2-c) decreased to zero after only 50 hours. These observations were explained by first order kinetic constant (*k*), doubling time (*t_d*), and half-life (*t_{1/2}*). Kinetic study results for *E. coli* isolates, H1-3 and D2-c, in soil at different moisture contents are presented in **Table 3**.

In this study, a greater rate of decay was observed for *E. coli* in soil at 4% moisture content compared to soil at 25% or 57% moisture content (**Table 3**). Previous studies reported higher die-off rate for *E. coli* in dry soil than in saturated soil [14,15,25,36]. Based on the results from this study, *E. coli* concentration in dry soil with 4% moisture content will decrease to half the initial concentration after approximately three to four hours (**Table 3**). This rapid decay of *E. coli* concentration in dry soil would continue to occur until the *E. coli* concentrations becomes zero.

Table 2. Kinetic characteristics of *E. coli* isolates from feral hog and deer feces in sterilized Cedar Creek water at different temperatures.

Temperature	Feral hog (H1-3)			Deer (D2-c)		
	<i>k</i> (hr ⁻¹)	<i>t_{1/2}</i> (hr)	<i>t_d</i> (hr)	<i>k</i> (hr ⁻¹)	<i>t_{1/2}</i> (hr)	<i>t_d</i> (hr)
10°C	-0.0261 (<i>R</i> ² = 0.89)	26.6	-	-0.0286 (<i>R</i> ² = 0.35)	24.2	-
25°C	0.0812 (<i>R</i> ² = 0.86)	-	8.5	0.0985 (<i>R</i> ² = 0.86)	-	7.0
30°C	0.109 (<i>R</i> ² = 0.91)	-	6.4	0.198 (<i>R</i> ² = 0.97)	-	3.5

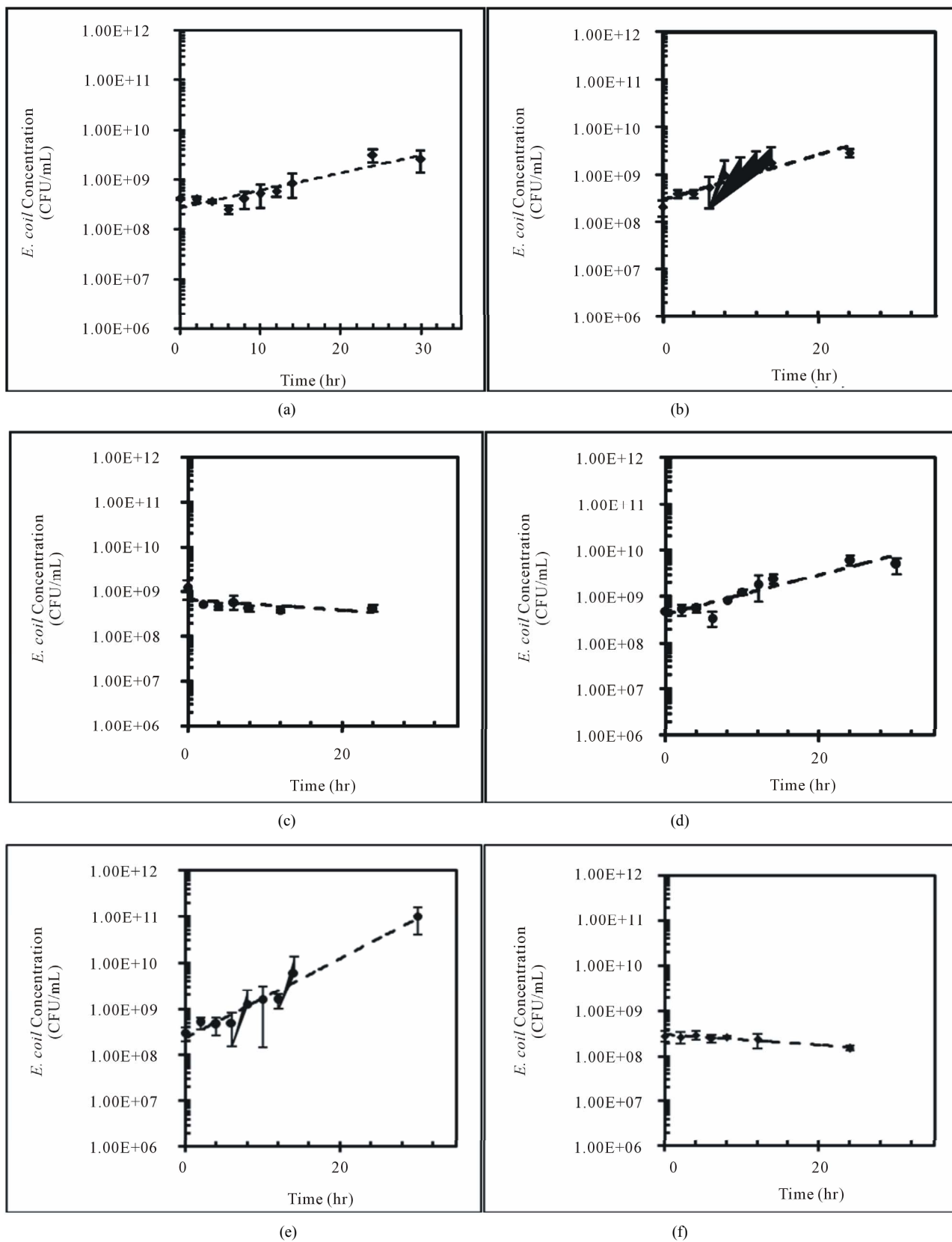


Figure 2. Concentrations (CFU/mL) of *E. coli* isolates, H1-3 and D2-c, in sterilized Cedar Creek water at different temperatures over time, (a) H1-3 at 10°C; (b) H1-3 at 25°C; (c) H1-3 at 30°C; (d) D2-c at 10°C; (e) D2-c at 25°C; (f) D2-c at 30°C.

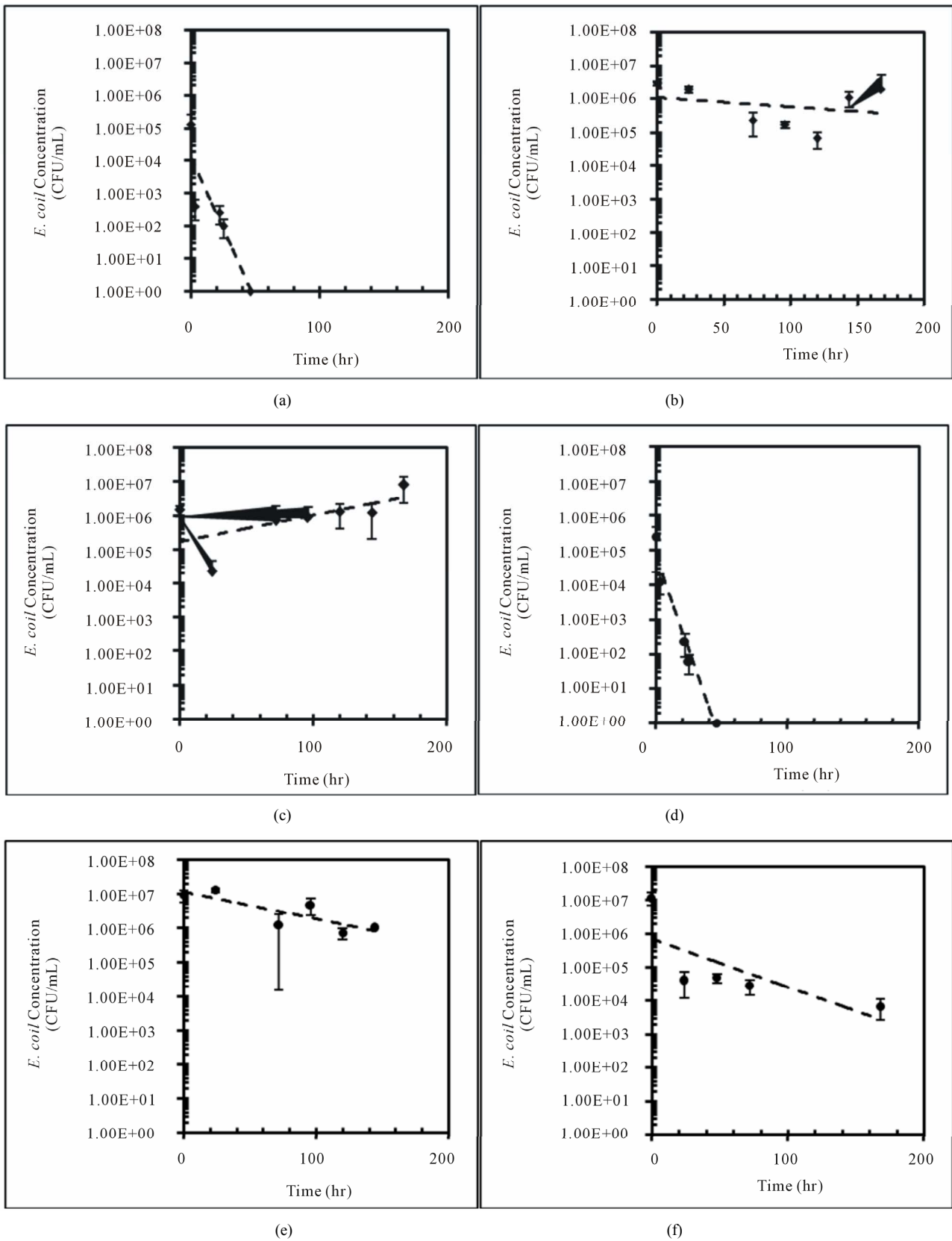


Figure 3. Concentrations (CFU/mL) of *E. coli* isolates, H1-3 and D2-c, in sterilized Cedar Creek soil at different moisture content over time, (a) H1-3 at 4%; (b) H1-3 at 25%; (c) H1-3 at 57%; (d) D2-c at 4%; (e) D2-c at 25% (f) D2-c at 57%.

Table 3. Kinetic characteristics of *E. coli* isolates from feral hog and deer feces in sterilized Cedar Creek soil at different moisture contents.

Volumetric Moisture Content	Feral hog (H1-3)			Deer (D2-c)		
	k (hr ⁻¹)	t _{1/2} (hr)	t _d (hr)	k (hr ⁻¹)	t _{1/2} (hr)	t _d (hr)
4%	-0.2016 (R ² = 0.81)	3.4	-	-0.2539 (R ² = 0.96)	2.7	-
25%	-0.0065 (R ² = 0.08)	106.6	-	-0.0187 (R ² = 0.69)	37.1	-
57%	0.0177 (R ² = 0.38)	-	39.2	-0.0326 (R ² = 0.55)	21.3	-

Several previous studies showed *E. coli* growth in saturated soils [14,15,25]. At 57% soil moisture content, growth was observed in *E. coli* isolated from feral hog feces (H1-3) while decay was observed in *E. coli* isolated from deer feces (D2-c). This difference may be due to the difference in moisture content of feral hog and deer fecal material. While processing the samples, it was observed that feral hog feces had more moisture than deer feces. Previous study showed the die-off of *E. coli* varied with the moisture condition of the fecal material [37].

It should be noted that in this study the effect of soil moisture content on the growth of *E. coli* was conducted in sterile soil microcosms, keeping all other environmental variables constant. Non-sterile soil might have resulted in lower *E. coli* concentrations due to competition for nutrients by the other microorganisms present in the soil [11,38]. The level of competition from other microorganisms is variable from soil to soil and would be difficult to predict. This study was designed to gain understanding of the fate of *E. coli* under different moisture contents with little to no competition within the soil.

5. Conclusions

Six different wildlife sources that could potentially contribute to *E. coli* contamination in Cedar Creek watershed, Texas were identified. The wildlife sources included feces from raccoon, opossum, feral hog, deer, skunk, and armadillo. The fecal material of each wildlife species was enumerated for *E. coli* and reported as CFU per g wet basis. The large range and variability in *E. coli* concentrations among few of the wildlife feces could be because of diverse dietary habits. There was a statistically significant difference in *E. coli* concentration in fecal samples collected in the summer and winter.

The growth of *E. coli* in sterilized Cedar Creek water at different temperatures varied depending on water temperature. Both deer and feral hog fecal *E. coli* isolates had the highest growth rate in water at 30°C and both isolates died off in water at 10°C. At 4% soil moisture content, both isolates died off rapidly. This suggests that under dry soil conditions *E. coli* will not survive for a

longer duration and will not pose a threat to nearby waterbodies. The *E. coli* isolate from feral hog fecal sample persisted in soil at 25% moisture content and slightly grew at 57% moisture content. However it should be noted that the correlation between *E. coli* concentration and these moisture contents was very low. Results from this study show the high variability in *E. coli* persistence, survival, and decay in terrestrial environment. It is evident that fate of *E. coli* in the environment is a complex process and governed by various factors. The interacting effects of these environmental factors add varying degrees of complexity to model and predict fecal contamination in watersheds.

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