INFLUENCES OF EXTERNAL FACTORS ON *ESCHERICHIA COLI* DISTRIBUTION, CONCENTRATION, SOURCES, AND FATE IN SECONDARY ENVIRONMENTS

A Dissertation

by

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ABSTRACT

E. coli contamination in surface waters is a universal issue that signifies increased risks to human health. Understanding *E. coli* fate, transport, sources, and distribution in watersheds is critical for reducing these risks. This study assessed *E. coli* fate in simulated mesocosms constructed using unaltered creek water and sediments with variable nutrient and flow treatments. *E. coli* concentrations in soil and runoff from small upland watersheds were used to assess transport and distribution while bacterial source tracking determined its sources.

Nutrient amendments and flow rate changes did not significantly alter *E. coli* fate in water or sediments but produced visible differences in some scenarios. Nutrient amendments representing irrigation runoff and wastewater spills did not produce discernable *E. coli* decay rate changes in water but marginally decreased observed decay rates in sediments. Alternatively, nutrient amendments affected heterotrophic bacteria decay and growth in water and sediments. Median heterotrophic bacteria decay and growth constant slopes were not significantly different between treatments and control, but were significantly different than median *E. coli* constant slopes during the initial growth phase suggesting that they outcompeted *E. coli* for available nutrient resources. *E. coli* concentrations were modeled with measured water quality parameters demonstrating that they could be predicted from independent variables including turbidity, specific conductivity, nitrate, ammonia, and orthophosphorus.

Watershed land use and cover significantly affected runoff and soil *E. coli* concentrations, runoff *E. coli* loads, and sediment concentrations and loads but not runoff volume. Within land uses, soil *E. coli* loads were significantly less than runoff *E. coli* loads suggesting that fecal deposition dominates loading in runoff. Wildlife contributed most runoff and soil *E. coli,* but livestock, humans and pets were also identified *E. coli* source contributors. Significant *E. coli* source composition differences were identified between watersheds in runoff but not soils. Grassed watersheds exhibited significant source composition differences between soil and runoff but this was not observed in cropland.

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CHAPTER I

INTRODUCTION

1.1 Problem Statement

Fecal contamination is a global water quality concern. It impacts people's ability to safely consume water and increases pathogen exposure risk during swimming or bathing. Waterborne illness causes an estimated 2 million deaths annually, and millions more suffer from pathogen infection [*WHO*, 2004]. Pathogen monitoring is tedious, difficult, and expensive, thus quantifying the presence of microorganisms associated with critical pathogens is common [*Gerba*, 2009]. Lake-based epidemiological studies conducted in the 1970s found sufficient correlations between swimming associated gastroenteritis, *Escherichia coli* (*E. coli*) and enterococcus occurrence for the U.S. Environmental Protection Agency (USEPA) to recommend them as appropriate fecal indicator bacteria (FIB) for surface water contamination [*Cabelli*, 1982; *USEPA*, 1986].

E. coli, enterococci, and fecal coliforms are the FIB used in Texas to determine if a waterbody supports designated contact recreation uses [*TCEQ*, 2010]. The Texas Administrative Code defines primary contact recreation as "water recreation activities, such as wading by children, swimming, water skiing, diving, tubing, surfing, whitewater kayaking, canoeing, and rafting, involving a significant risk of ingestion of water." It established numeric criteria for *E. coli*, enterococci, and fecal coliforms of 126, 35, and 200 colony forming units (cfu)/100 mL of water respectively to support this use [*TCEQ*,

2010]. According to the 2014 Texas Integrated Report, approximately 43% of impaired waterbody segments are caused by excessive bacteria concentrations [*TCEQ*, 2013].

Once impaired, Section 303(d) of the Federal Water Pollution Control Act, or Clean Water Act, requires that a total maximum daily load (TMDL) or TMDL alternative such as watershed protection plans (WPPs) be established and implemented to restore water quality. Regardless of strategy utilized to restore water quality, stakeholders are often engaged in their development. In watersheds where planning is underway, stakeholders often ask where bacteria in water originate, and what happens to them outside the host organism? While these questions seem simple, their answers are complicated and not clearly understood [*Byappanahalli et al.*, 2012b; *Yamahara et al.*, 2009]. Numerous attempts to provide answers have produced piecemeal information that partially addresses these questions; however, further developing sound scientific information is needed to effectively address excessive bacteria levels in surface waters.

1.2 Research Approach

Stakeholder questions regarding *E. coli* fate in water bodies and the need for scientific information regarding *E. coli* source contributions to overall loads spurred this research effort. To provide this information, three research objectives established were:

1. To assess the impacts of varying nutrient amendments and flow rates on culturable *E. coli* fate in simulated stream mesocosms,
2. To evaluate land use and land cover influence on culturable *E. coli* concentration in the upper 5 cm of soil and surface runoff from defined watersheds, and

3. To evaluate source composition and similarities in culturable soils and surface runoff *E. coli* populations from watersheds receiving only natural bacteria inputs.

Results from simulated instream mesocosm experiments are presented in Chapter II. *E. coli* concentrations in water and sediment were evaluated over time and compared to define differences in *E. coli* response due to treatment effects. Concentrations were compared to ambient water quality parameters, heterotrophic bacteria concentrations, and nutrient concentrations to define existing relationships within treatment scenarios.

An assessment of *E. coli* concentrations in soil and runoff compared to land use and land cover are presented in Chapter III. Findings are presented in relation to sediment concentration and volume of runoff. Differences in relationships between each land use and land cover were evaluated and discussed.

Results of bacterial source tracking (BST) analysis for soil and runoff *E. coli* isolates from small experimental watersheds with varying land use and land cover are presented in Chapter IV. Identified bacteria sources are compared between watersheds and sample media to determine land use and land cover effects on bacteria loading.

In Chapter V, project findings and watershed management implications are discussed. Information produced expands knowledge regarding *E. coli* fate relative to nutrient loading, and land use and land cover effects on watershed *E. coli* loading and sources. Limitations of the research and future assessment needs are also discussed.

CHAPTER II

NUTRIENT LOADING IMPACTS ON CULTURABLE *E. COLI* FATE IN SIMULATED STREAM MESOCOSMS

2.1 Overview

E. coli fate and transport in secondary environments has received substantial research attention. Temperature, moisture availability in soils, organic matter content, nutrient availability, salinity, radiation, and microbial competition and predation are commonly noted to influence its fate [*Ishii et al.,* 2010] and are at least partly responsible for its ability to persist and grow in secondary environments [*Byappanahalli and Fujioka,* 2004; *Byappanahalli et al.*, 2012a; *Garzio-Hadzick et al.,* 2010; *Habteselassie et al.,* 2008; *Haller et al.,* 2009; *Ishii et al.,* 2006; *Ishii et al.,* 2010; *Vital et al.,* 2008; *Vital et al.,* 2010]. Many previous investigations evaluating *E. coli* response to these environmental factors have not used aquatic ecosystems approaches to replicate instream environments. Instead, simplified systems utilizing sterile materials have been used. Information produced from such experiments improved *E. coli* fate understanding, but often translates poorly to real instream environments. Growth and decay constants developed and utilized in fate and transport models likely misrepresent *E. coli* life cycles in secondary environments. Temporal *E. coli* response to nutrient amendments in re-created natural stream mesocosms was monitored in this study to further understand *E. coli* fate instream.

2.2 Introduction

E. coli are found in bird and mammal feces and were originally thought to exist in the host's gastrointestinal tract or in freshly excreted feces [*Leclerc et al.*, 2001; *Savageau*, 1983]. Initially, rapid die-off shortly after excretion from the host was assumed [*Bolster et al.*, 2005; *Gerba and McLeod*, 1976; *Van Donsel and Geldreich*, 1971; *Van Donsel et al.*, 1967], and this and other factors led to the *E. coli's* common use as FIB for waterbodies. Alternatively, *E. coli* are known to persist and grow in some secondary environments such as sediment, soil, and water [*Bolster et al.*, 2005; *Garzio-Hadzick et al.*, 2010; *Habteselassie et al.*, 2008; *Harmel et al.*, 2010; *Ishii et al.*, 2006; *Ishii et al.*, 2010; *Van Donsel et al.*, 1967; *Vital et al.*, 2008; *Vital et al.*, 2010]. This potentially diminishes their effectiveness for identify recent fecal pollution. As a result, some environmental *E. coli* may be 'naturalized' instead of fecal derived. *Byappanahalli and Fujioka* [2004], *Ishii et al.* [2006] and others have found *E. coli* that are able to persist and grow in non-sterile, unfertilized soil.

E. coli fate in non-sterile water is not clear since most studies utilized sterilized media to evaluate their persistence [*Flint*, 1987; *Lim and Flint*, 1989; *McCrary et al.*, 2013; *Na et al.*, 2006]. Instream, *E. coli* experience many external stressors [*Savageau*, 1983; *Winfield and Groisman*, 2003] and their fate is not well understood. This thus diminishing the utility of existing fate information for watershed based modeling purposes and leads to considerable uncertainty in their results [*Harmel et al.*, 2010].

2.2.1 Factors Affecting the *E. coli* Life Cycle in Aquatic Environments

Warm-blooded animals' large intestine is *E. coli's* primary habitat [*Smith,* 1965]. *E. coli* are adapted to this consistently warm, moist, nutrient rich, an anaerobic environment which promotes rapid reproduction [*Savageau,* 1983]. Ambient conditions in secondary environments are quite different. Low nutrient availability, large temperature variability, microbial competition, and predation influence *E. coli* growth and persistence [*Ishii and Sadowsky,* 2008].

2.2.1.1 Abiotic Factors

Ambient conditions in secondary environments can exert considerable stress on *E. coli* compared to that experienced in its primary habitat [*Savageau,* 1983]. Stressors considered most influential include temperature variation [*Berry and Foegeding,* 1997; *Na et al.,* 2006; *Solo-Gabriele et al.,* 2000], solar radiation exposure [*Davies-Colley et al.,* 1994; *Fujioka et al.,* 1981; *Whitman et al.,* 2004], and nutrient limitation [*Barcina et al.,* 1997; *Byappanahalli et al.,* 2012a; *Ishii et al.,* 2010; *van Elsas et al.,* 2011; *Winfield and Groisman,* 2003].

Temperature is often considered the most critical factor influencing *E. coli* survival [*Flint,* 1987; *Ishii et al.,* 2010]. Secondary environment temperatures are typically lower than intestinal temperatures which can vary slightly, but are commonly around 37^{o}C [*Savageau,* 1983]. Secondary environments can reach this temperature but are commonly lower and exhibit considerable temporal variation [*van Elsas et al.,* 2011]. Temperatures lower than the primary environment exerts external stress on *E. coli* which

can decrease cell metabolic activity. Depending on the media utilized, temperatures at or near the internal body temperature of mammals may produce an initial growth response followed by rapid decay where low temperatures yield little or no growth response followed by a slight decay rate over [*Garzio-Hadzick et al.*, 2010; *Ishii et al.*, 2010; *Lim and Flint*, 1989; *Pachepsky et al.*, 2011]. *Craig et al.* [2004] compared *E. coli* survival in microcosms containing coastal water and intact sediment cores from multiple locations near Adelaide, Australia. Incubation temperatures significantly influenced *E. coli* survival in water and sediment. *E. coli* survival was greatest at low incubation temperatures (10^{o}C) as evidenced by lower decay rates than 20 and 30^{o}C incubations. Survival was also greater in sediment than water for all scenarios. *Flint* [1987] found similar results in untreated river water collected upstream and downstream of a wastewater treatment plant (WWTP) outfall. *E. coli* decay rates in both waters decreased incrementally when incubated at 37, 25, 15, and 4^{o}C.

Cattle and raccoon derived *E. coli* were subjected to incubation at 0, 10, 20, and 50 ^{o}C over 168 hours in sterile creek water to evaluate their growth and persistence response by *Padia et al.* [2012]. *E. coli* from both species grew at slightly increasing rates from 0 to 10 to 20^{o}C; however, at 50^{o}C no survival was noted after 24 hours incubation. *Gallagher et al.* [2012] subjected *E. coli* from white-tailed deer and feral hog feces to temperatures of 10, 25, and 30^{o}C over a 30 hour period. At 10^{o}C, net *E. coli* decay from both species occurred while growth rate increased from 25 to 30^{o}C. Higher *E. coli* decay rates were also noted at 4^{o}C than at 20^{o}C in sterile river water mesocosms inoculated with pig manure and incubated over 43 days [*Marti et al.*, 2011].

Nutrient availability is also considered an important influence on *E. coli* fate in secondary environments. Nutrients are often limited in soil and water when compared to intestinal environments [*Savageau*, 1983]. *E. coli* and all other heterotrophic bacteria require carbon, nitrogen, and phosphorus in approximately a 100:10:1 ratio [*LeChevallier et al.*, 1991] thus making nutrient availability important for their survival., One or more of these nutrients are often limiting in secondary environments and may suppress the ability of *E. coli* and other heterotrophs to grow.

In sterile environments, nutrient amendments have produced *E. coli* growth shortly after application. *Lim and Flint* [1989] applied various sources of nutrients to both sterile and non-sterile lake water. Carbon in the forms of glucose, lactose, and glycerol all produced *E. coli* growth in sterile water with no significant difference in growth response between the treatments and controls in non-sterile water. Synthetic sewage made up of peptone, yeast extract, urea, ammonium sulfate, potassium phosphate, and iron sulfate added at multiple percent concentrations produced *E. coli* growth in both sterile and non-sterile waters; however, rapid decay was observed in non-sterile waters. Larger synthetic sewage doses decreased observed decay. However, potassium phosphate additions that increased ambient phosphorus concentrations to 50 mg/L sterile and non-sterile lake water did not produce significant differences between the treatment and control. Increasing ammonium sulfate concentrations produced *E. coli* growth in sterile lake water incubated at both 15 and 37^{o}C while no growth was observed at any concentration in non-sterile waters. Decay rates observed in both temperature scenarios decreased with increasing ammonium sulfate concentrations.

Other experiments have also illustrated *E. coli* fate from complex nutrient amendments. *McCrary et al.* [2013] applied nutrient amendments from turfgrass and leaf litter leachate at varying concentrations to sterilized WWTP effluent. Under all treatment scenarios, *E. coli* growth was observed; however, responses varied considerably. Turfgrass extracts supplemented at low and medium rates yielded rapid *E. coli* growth while the high treatment concentration produced the slowest growth. Leaf extracts produced slower *E. coli* growth than grass extract treatments. Differences in microbially available dissolved organic carbon (DOC) were suggested as the cause of *E. coli* growth differences. Similarly, *Surbeck et al.* [2010] found apparent linkages between DOC and phosphorus concentrations in unfiltered creek water microcosms and between DOC concentrations and *E. coli* concentrations in runoff. They suggested minimum thresholds of 7 mg/L and 0.07 mg/L for DOC and phosphorus respectively to support net *E. coli* growth in non-sterile microcosms.

Solar radiation is known to directly effect *E. coli* survival in secondary environments. Sufficient solar radiation exposure can cause mortality through DNA damage or internal cellular component oxidation [*Whitman et al.*, 2004]. Exact cellular inactivation causes in water are often debated. Short-wave UV radiation and the amount of total solar insolation exposure have both been suggested as primary factors [*Davies-Colley et al.*, 1994; *Whitman et al.*, 2004]. Regardless of mechanism, numerous accounts note the effects of solar radiation on *E. coli* and other FIB. *Desai and Rifai* [2013] measured diurnal *E. coli* concentrations variations spanning several orders of magnitude in White Oak Bayou that appear correlated with solar radiation and water temperature.

Solar radiation was also shown to inactivate *E. coli* in soil [*Wu et al.*, 2009]; however, it is unclear whether radiation or other mechanisms caused inactivation.

In natural aquatic environments, water is underlain by sediment which has long been recognized as an *E. coli* reservoir [*Gerba and McLeod*, 1976; *Van Donsel and Geldreich*, 1971]. *E. coli* is commonly found in water attached to soil particles [*Bai and Lung*, 2005; *Davies et al.*, 1995; *Muirhead et al.*, 2004; *Rehmann and Soupir*, 2009]. Under normal or low flow conditions, many sediment particles settle to the bottom while fine particles remain suspended. Sediment provides a more hospitable environment for *E. coli* than water [*Garzio-Hadzick et al.*, 2010; *Pachepsky et al.*, 2011] and can enhance its survival. *Gerba and McLeod* [1976] found that increased organic matter concentration in sediments compared to overlying water allows longer *E. coli* survival. An improved ability to compete for nutrients in sediment has been suggested [*Davies et al.*, 1995]. Sediment also reduces UV light exposure and suppresses predation and allows extended *E. coli* survival compared to other media [*Jamieson et al.*, 2005; *Koirala et al.*, 2008]. These effects extend *E. coli* survival in sediment and provide a considerable *E. coli* reservoir that is routinely resuspended in overlying water.

Sediment *E. coli* concentrations are reported to be 10 to several thousand times larger than concentrations in overlying water [*Brinkmeyer et al.*, 2015; *Buckley et al.*, 1998; *Crabill et al.*, 1999; *Hartz et al.*, 2008]. Water and sediment interactions occur routinely, and the interplay between them can influence *E. coli* concentrations in overlying water [*Brinkmeyer et al.*, 2015; *Grimes*, 1975; *Jamieson et al.*, 2005]. Sediment disturbances from storm events [*Jamieson et al.*, 2005], simulated floods

[*Davies-Colley et al.*, 2004], wave action [*Hartz et al.*, 2008], tidal washing [*Solo-Gabriele et al.*, 2000], and mechanical disturbances such as dredging [*Grimes*, 1975] have produced significant *E. coli* concentration increases in overlying or downstream waters. Stream sediment has also been implicated as the primary source of *E. coli* found in stream water under normal, low flow conditions [*Brinkmeyer et al.*, 2015]. However, inconsistent reports of significant correlations between *E. coli* concentrations observed in sediment and overlying water are common [*Brinkmeyer et al.*, 2015; *Byappanahalli et al.*, 2003; *Crabill et al.*, 1999; *LaLiberte and Grimes*, 1982; *Savageau*, 1983]. An improved understanding of water and sediment *E. coli* interaction is needed as attempts to model their effects are often oversimplified [*Rehmann and Soupir*, 2009].

2.2.2.2 Biotic Factors

Antagonistic action within microbial communities through resource competition and predation impacts the *E. coli* life cycle in aquatic environments. *E. coli* is a member of the heterotrophic bacteria community and requires simple carbon, nitrogen, phosphorus, sulfur, and trace elements for growth and persistence [*Ishii and Sadowsky*, 2008]. *E. coli* density is commonly several orders of magnitude less than the total heterotrophic community [*Byappanahalli and Fujioka*, 2004].

Resource competition has been noted to significantly effect *E. coli* fate in fresh water and sediment filled mesocosms [*Wanjugi and Harwood*, 2013]. *Byappanahalli and Fujioka* [2004] found similar results in soil where inhibiting growth of competing microbes, adding nutrients, and combining these treatments all promoted *E. coli* growth.

Predation can also significantly effect *E. coli* survival and has been demonstrated through controlled presence and absence studies. *Wanjugi and Harwood* [2014] excluded competition from fresh water mesocosms and found increased *E. coli* decay due to predation alone in aquatic freshwater environments. *Enzinger and Cooper* [1976] found that presence and increasing populations of predatory protozoa produced corresponding declines in *E. coli* populations in estuarine waters. Similar results have been seen with other FIB as well [*Davies et al.*, 1995].

The objective of this study was to assess nutrient loading impacts on culturable *E. coli* fate in simulated stream mesocosms. The hypothesis for this assessment was that nutrient addition to mesocosms would significantly alter *E. coli* growth and decay responses in simulated stream mesocosms. *E. coli* fate parameters estimated from this study would improve transport models to simulate instream *E. coli* concentrations.

2.3 Materials and Methods

2.3.1 Mesocosm Design

Six repurposed algae raceways located inside the Hobgood Building at Texas A&M University were used to create laboratory scale simulated stream mesocosms. They were constructed of 1.11 cm thick, clear Plexiglass® to similar dimensions ranging from 30 to 30.95 cm and 120.5 to 121.1 cm long (Figure 2.1). Each mesocosms was equipped with a variable speed paddle wheel fitted with six fins measuring 12.7 cm wide by 20.3 cm long. When filled to the desired level, the paddles extended approximately 10.2 cm into the water. Carriages made from 5.08 cm square tubing housed two

mesocosms and created an exoskeleton that allowed enclosures to be affixed that prevented cross contamination. The laboratory space created a semi-climate controlled environment, but the presence of exterior walk doors and large garage doors allowed considerable ambient temperature fluctuations to occur.

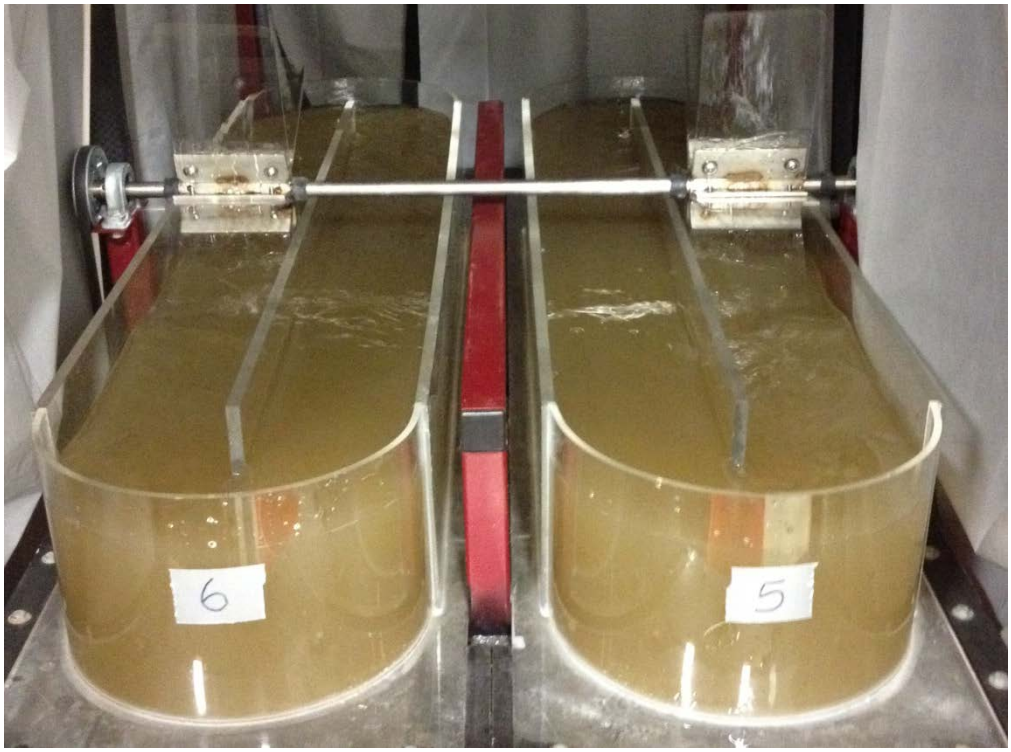

Figure 2.1. Simulated instream mesocosms

2.3.2 Mesocosm Establishment

Simulated stream mesocosms were established with unaltered water and sediments collected from Carters Creek approximately 75 m downstream of Briarcrest Dr. in Bryan, TX. Turbid creek water and sediment were transported directly to the laboratory for immediate mesocosm establishment. Water from multiple 18.9 L high-

density polyethylene (HDPE) transport containers was poured into the mesocosms and allowed to settle until sediment introduction was completed. Each mesocosm was filled with 45 L of water by volume determined from its internal dimensions. Sediment was added after water to minimize disturbance and resuspension into the water column. Approximately 1.5 L of saturated sediment by volume was added to one end of each mesocosm. Once water and sediment addition was completed, paddle wheels in each mesocosm were activated at the prescribed speed.

Treatments were applied to four of the six mesocosms with two receiving low nutrient doses, two receiving high nutrient doses, and two controls receiving no amendment. Low and high flow rates were applied to each treatment and control scenario producing six unique mesocosm conditions in each trial (Table 2.1). Trials were denoted by a dash (-) and trial number following mesocosm labels (e.g., Control – Low Speed treatment for trial 2 = C1-2). Water and sediment samples were collected directly from each mesocosm and processed to determine culturable *E. coli* and heterotrophic bacteria concentrations per 100 mL of water and gram of sediment. Biofilm formed in each mesocosm was sampled and processed to determine concentrations of *E. coli* present within sampled material.

Table 2.1. Mesocosm treatment labels

Treatment Scenario	Scenario Label	Treatment Scenario	Scenario Label
Control – Low Speed	C1	Control – High Speed	C2
High Nutrient – Low Speed	H1	High Nutrient – High Speed	H2
Low Nutrient – Low Speed	L1	Low Nutrient – High Speed	L2

Single ‘low dose’ and ‘high dose’ treatments were applied on day one of each trial to mimic one-time nutrient amendments that a stream may receive such as urban irrigation runoff (low dose) or a sanitary sewer overflow (high dose). Treatments were made using reagent grade laboratory chemicals. Stock solutions (1 M) of potassium phosphate (KH_2PO_4), potassium nitrate (KNO_3), and sucrose ($C_{12}H_{22}O_{11}$) were applied as nutrient amendments. Amendments were calculated from initial nutrient conditions measured in each mesocosm (Table 2.2). The low dose was produced a 10-fold nitrate ($N0_3$-N) and phosphate (PO_4-P) increase and a 2-fold dissolved organic carbon (DOC) increase while the high dose produced a 5-fold DOC increase and 50-fold NO_3-N and PO_4-P increases.

Table 2.2. Mesocosm establishment dates and initial nutrient conditions

Start Date & Trial	Mesocosm	Initial Parameter Concentrations (mg/L)						NTU	µS/cm
		NO_3-N	NH_4-N	PO_4-P	DOC	Total N	DON	Turbidity	Specific Conductance
Trial #1 12/1/2014	C1	0.22	0.15	0.27	43.04	1.25	0.88	258	327
	C2	0.17	0.16	0.25	43.68	1.31	0.99	535	327
	H1	0.13	0.16	0.29	42.98	1.30	1.02	347	324
	H2	0.17	0.16	0.23	43.72	1.36	1.02	604	322
	L1	0.13	0.15	0.28	43.80	1.33	1.05	307	323
	L2	0.15	0.15	0.28	44.15	1.25	0.95	449	323
Trial #2 2/09/2015	C1	0.12	0.11	0.08	13.09	0.51	0.29	90.2	464
	C2	0.11	0.12	0.08	13.13	0.51	0.28	146	466
	H1	0.12	0.11	0.09	13.06	0.52	0.28	111	466
	H2	0.12	0.11	0.09	13.07	0.52	0.29	190	466
	L1	0.13	0.11	0.09	12.69	0.52	0.28	107	466
	L2	0.13	0.11	0.09	12.66	0.56	0.32	124	467
Trial #3 4/06/2015	C1	0.17	0.23	0.12	16.89	0.90	0.50	150	486
	C2	0.12	0.28	0.07	15.43	0.85	0.46	556	483
	H1	0.12	0.23	0.12	15.20	0.82	0.48	135	485
	H2	0.13	0.33	0.10	15.50	0.86	0.40	382	483
	L1	0.12	0.24	0.11	15.26	0.87	0.52	164	482
	L2	0.12	0.23	0.10	15.34	0.85	0.50	290	485

2.3.3 Sampling Procedures

Mesocosm sampling began immediately following establishment (Day 0) and occurred at approximately the same time on days 1, 2, 3, 4, 7, 10, 14, 18, and 22. Water samples were collected directly from mesocosms into sterile 500 mL HDPE sample bottles placed into the flow of the mesocosm without disturbing underlying sediment. Approximately 30 g of sediments were collected from multiple locations in each mesocosm using disposable plastic spatulas and placed into 207 mL Whirl-Pak® bags. Biofilm was sampled from a 4 cm^2 area in each mesocosm on days 7, 14, and 22. Material was scraped with a disposable plastic spatula and placed directly into test tubes containing 9 mL of phosphate buffered saline (PBS) solution.

2.3.4 Analytical Methods

E. coli in water and sediment was enumerated using the USEPA Method 1603 [*USEPA*, 2006]. This method uses membrane filtration and a modified membrane-Thermotolerant *E. coli* agar (mTEC). Aliquots of appropriate volume were processed from water samples and results were reported as cfu/100 mL. Sediment samples were prepared for analysis by placing 10 g of sediment into sterile specimen cups containing 90 mL of PBS and shaking them vigorously. Appropriate size aliquots were processed in identical fashion as water samples. Results were reported as cfu/g_{wet} of sediment. Heterotrophic bacteria were enumerated similarly using Standard Method 9215D, a direct heterotrophic plate count methodology [*APHA*, 1997]. Results for water and sediment samples were reported as cfu/mL and cfu/ g_{wet} sediment respectively.

Samples were processed immediately following collection to determine ambient turbidity, temperature, pH, dissolved oxygen (DO), and specific conductivity concentrations. Turbidity was measured with a Hach 2100Q Portable Turbidimeter and reported in Nephelometric Turbidity Units. Temperature, pH, DO, and specific conductivity were measured with a VWR SB90M5 multi-parameter benchtop meter and were reported in ^{o}C, standard units, mg/L, and µS/cm respectively.

NO_3-N, ammonium (NH_4-N), PO_4-P, DOC, total dissolved nitrogen (TDN) were determined by the Nutrient and Water Analysis (NAWA) Laboratory at Texas A&M University. Water subsamples were filtered through 0.7µm glass fiber filters and placed in 100mL HDPE sample bottles for transport to the NAWA lab. NO3-N, NH4-N, and PO4-P were measured colorimetrically (USEPA methods 353.2, 350.1, and 365.1 respectively) using a Smartchem Discrete Analyzer. DOC and TDN were measured with Pt-catalyzed, high temperature combustion (USEPA methods 415.1) performed with Shimadzu TOC-VCSH with a TMN-1 unit. DOC was measured as non-purgeable DOC by addition of 2M HCl to acidify the sample and purging for 4 min to remove dissolved inorganic carbon. Dissolved organic nitrogen (DON) was calculated by deducting NO_3-N + NH_4-N from TDN.

2.3.5 Microbial Growth and Decay Calculations

E. coli and heterotroph decay or growth was quantified by calculating kinetic decay or growth constants (k,d^{-1}), doubling time [$T_{d,}$ (day)], or half-life [$t_{1/2,}$ (day)]. *E. coli* concentrations plotted over time revealed distinct growth and decay phases in most

cases. As a result, trials were divided into two or three phases. In water, *E. coli* decay within each mesocosm was divided into two phases. For all other scenarios, three phases were utilized. Phase length varied dependent upon the observed changes in growth and decay within each mesocosm. First-order kinetics was used to describe *E. coli* and heterotroph decay and growth in water and sediment. The slope of a fitted regression line through the natural log (ln) of *E. coli* concentrations represents k,d^{-1}. Negative slopes are considered decay constants and positive slopes are growth constants. $T_{d,}$ (day) and $t_{1/2,}$ (day) were calculated by dividing ln(2) by calculated k,d^{-1} values.

2.3.6 Bacteria and Nutrient Mass Balance Calculations

Mass balance calculations identified net *E. coli* and heterotroph changes in water and sediment, and nutrients in water during each trial. Initial bacteria concentrations were multiplied by initial water volume and sediment mass to determine initial mass. Nutrient mass was calculated similarly. Nutrient concentration and water volume on day two (after nutrient amendment) were used in mass balance calculations. Final mass was calculated from final constituent concentrations and estimated water volume or sediment mass remaining. Precise amounts of sediment and water removed from each mesocosm during sampling were not measured. Evaporative losses were not quantified.

2.3.7 Statistical Analysis

Data analyses were conducted to identify statistically significant differences within mean and median decay constants slope values in sediment and water. Most data

were non-normally distributed according to Kolmogorov-Smirnov testing; therefore, the non-parametric Kruskal-Wallis test was used to identify the presence of statistically significant differences in median slopes between three or more data groups. The Mann-Whitney test was used to compare only two groups of data. In a few cases, the assumptions of normality were met and allowed use of a one-way analysis of variance (ANOVA) to test for significant differences in the means. However, data variances were often unequal and the number of samples within each group was small rendering a traditional ANOVA inappropriate. In these cases, a Welch's ANOVA was used as it does not assume equal variances. Linear and nonlinear regressions were used to describe relationships between monitored water quality parameters and $\log_{10}$ transformed *E. coli* concentrations. Regression model goodness of fit was evaluated using the standard error of regression (S) which measures the average distance that observed values fall from the regression line. Lastly, standard stepwise regression and best subsets regression were applied to evaluate potential relationships between $\log_{10}$ transformed *E. coli* concentrations and select measured water quality parameters. Predictors were standardized by subtracting the mean and dividing by the standard deviation in an effort to reduce multicollinearity. Reported p values, R^2, predicted R^2, Mallow's C_p, and S were all used to evaluate model appropriateness. Parameter variance inflation factors (VIF) were considered in an effort to exclude parameters with considerable multicollinearity. Significance for all analyses was determined using α=0.05, thus p values $\leq$0.05 were considered statistically significant. All statistical analyses were conducted using Minitab 17 software (Minitab Inc., State College, PA).

2.4 Results and Discussion

2.4.1 *E. coli* Persistence and Decay

Temporal *E. coli* concentrations measured within each mesocosm were used to evaluate changes in growth and decay to applied nutrient amendments and flow conditions. Decay and growth constants, doubling times, and half-lives were calculated from measured concentrations in each mesocosm.

2.4.1.1 *E. coli* Persistence and Decay in Water

No net *E. coli* growth was occurred in water during the mesocosms experiments. Instead, rapid *E. coli* decay occurred during the first four to seven days of each trial and was followed with gradual decay for the remainder of each trial (Appendix A). This response followed a biphasic die-off model similar to those observed in *E. coli* and other microbial and viral populations by *Petterson et al.* [2001] and *Seidu et al.* [2013]. Phase I represents a rapid decay phase and spanned zero to four or seven days. Phase II is characterized as a post-decay phase that was near stationary and lasted from either day 4 or 7 until the end of the experiment (day 22). In 8 of the 18 treatment scenarios, *E. coli* remained at non-detectable concentrations once they initially reached that point. In the other 10 treatment scenarios, small increases (≤5 cfu/100 mL) were observed after *E. coli* initially reached non-detectable concentrations. Only high flow, high nutrient treatment scenarios in two of the three trials did not reach non-detectable concentrations (3 and 6 cfu/100 mL respectively). The earliest that *E. coli* reached non-detectable concentrations in water was day four.

Treatment scenario decay constants (k,d^{-1}) were calculated for phases I and II within each trial (Table 2.3). Considerable variation in calculated decay constants exists; however, a Kruskal-Wallis test produced insufficient evidence to reject a null hypothesis of equal medians between treatment scenarios within phases I and II (p=0.22 and 0.64 respectively). *E. coli* $t_{1/2,}$ (day) values were also calculated for phase I and II of each treatment scenario (Table 2.4). No significant differences in $t_{1/2,}$ (day) medians within phase I or II were identified with the Kruskal-Wallis test (p=0.22 and 0.92 respectively).

Table 2.3. *E. coli* decay constants for varying treatment scenarios in water

	Calculated *E. coli* decay constants k,d^{-1}	
Treatment Scenario	**Phase I*†**	**Phase II*†**
Control - Low Flow (C1)	-0.919 to -0.822 (-0.879)	-0.035 to 0 (-0.012)
Control - High Flow (C2)	-0.96 to -0.402 (-0.744)	-0.125 to 0 (-0.078)
High Nutrient - Low Flow (H1)	-1.702 to -0.848 (-1.384)	-0.129 to 0 (-0.062)
High Nutrient - High Flow (H2)	-1.043 to -0.448 (-0.695)	-0.162 to 0 (-0.107)
Low Nutrient - Low Flow (L1)	-1.705 to -1.497 (-1.589)	-0.079 to 0 (-0.036)
Low Nutrient - High Flow (L2)	-1.656 to -0.47 (-0.95)	-0.208 to 0 (-0.112)

* range and (mean) of calculated values

† phase lengths vary within and between trials

Table 2.4. *E. coli* half-lives under varying treatment scenarios in water

	Calculated *E. coli* Half Life $t_{1/2,}$ (day)	
Treatment Scenario	**Phase I*†**	**Phase II*†**
Control - Low Flow (C1)	-0.844 to -0.754 (-0.79)	-20.087 to 0 (-6.696)
Control - High Flow (C2)	-1.725 to -0.722 (-1.081)	-5.556 to 0 (-2.762)
High Nutrient - Low Flow (H1)	-0.817 to -0.407 (-0.552)	-12.052 to 0 (-5.808)
High Nutrient - High Flow (H2)	-1.547 to -0.664 (-1.126)	-4.367 to 0 (-2.879)
Low Nutrient - Low Flow (L1)	-0.463 to -0.407 (-0.437)	-24.401 to 0 (-11.051)
Low Nutrient - High Flow (L2)	-1.475 to -0.419 (-0.951)	-5.496 to 0 (-2.94)

* range and (mean) of calculated values

† phase lengths vary within and between trials

Combined decay constants were also calculated for each mesocosm using the three treatment scenario replicates (Figure 2.2). Results were similar to individual constants; however, standardized lengths for Phases I (day 0 to 4) and II (day 4 to 22) were used for each mesocosm and led to combined constants calculated outside of the range reported in Table 2.3.

No significantly different *E. coli* growth or decay responses in water were observed for single nutrient additions (Figure 2.2; Appendix A). This suggests that a single addition of nutrient is not sufficient to alter the natural life cycle of *E. coli* in natural aquatic environments. This observation is logical as natural systems have many confounding factors that exert stress upon *E. coli* in this secondary environment. Additionally, competing microorganisms that are adapted to stream environments are likely to utilize the nutrients influx before *E. coli* thus suppressing or completely precluding any observable changes in the *E. coli* life cycle.

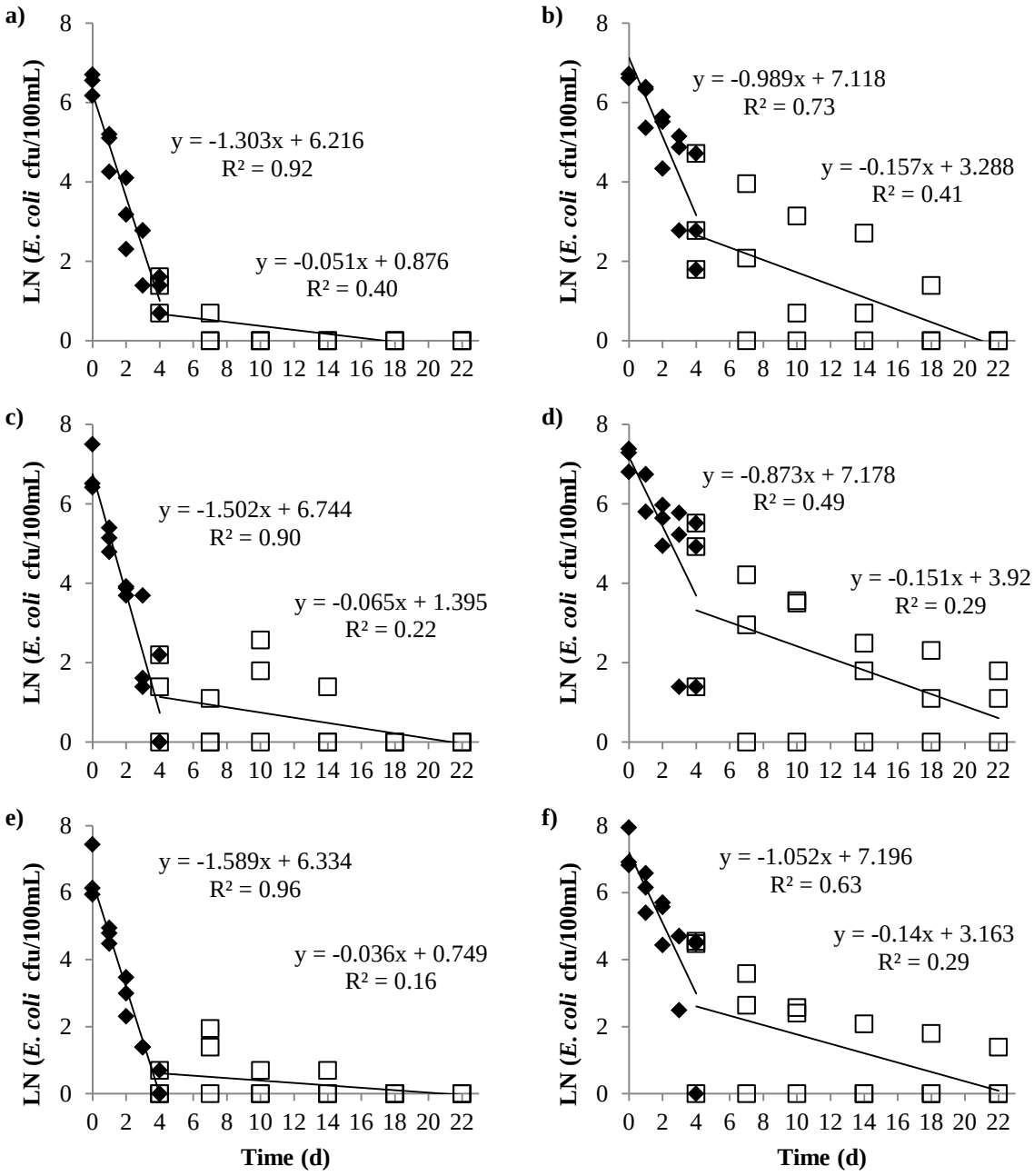

Figure 2.2. *E. coli* concentrations in water over time from all three trials combined. Graphics represent the mesocosm scenarios: a) C1; b) C2; c) H1; d) H2; e) L1; f) L2

2.4.1.2 *E. coli* Persistence and Decay in Sediment

E. coli persistence and decay in sediment within each mesocosm was highly variable. A slight growth response following application of nutrient amendment was identified in 10 of 12 treatment mesocosms. Four of the six controls also produced *E. coli* growth thus illustrating the effects other factors impart on *E. coli* growth and persistence in sediment. Variability in the distribution of *E. coli* in sediment may also lead to these measured differences. Overall, decay was observed in most cases; however, sustained growth over several days occurred at least once in all treatment scenarios.

A tri-phase *E. coli* growth and decay response was observed in sediments. Phase I began on day zero and ended from day two to seven. Phase II began from days two to seven and the ended from days seven to 14. Phase III began from days seven to 14 and ended on day 22. *E. coli* growth and decay were observed in all phases (Appendix A).

Calculated constants were found to be normally distributed by a Kolmogorov-Smirnov test but the variances of the data were not equal among groups. So, a Welch's ANOVA was used to test for the presence of significantly different means within each treatment scenario (Table 2.5). Insufficient evidence to reject the null hypothesis of equal means in phase I, II or III ($p=0.78$, 0.99, and 0.96) was produced. $T_{d,}$ (day) and $t_{1/2,}$ (day) values calculated for each trial and treatment scenario (Table 2.6) were non-normally distributed according to Kolmogorov-Smirnov testing. The Kruskal-Wallis test was used to determine the presence of significant differences in between decay constant slopes. The p-values for phase I, II and III (0.91, 0.90, and 0.80 respectively) suggest a lack of sufficient evidence to reject the null hypothesis of equal medians.

Data from all three trials within each treatment scenario were aggregated and average decay constants were calculated (Figure 2.3). Phase length was standardized causing observed growth within individual trials to be masked. In this aggregation, Phase I extended from day 0 to 3, Phase II spanned day 3 to day 10, and Phase III began on day 10 and ended on day 22. Statistical differences could not be evaluated as this approach produced a single decay constant value. However, subtle differences in observed decay rates in sediments within Phase I were observed. High nutrient mesocosms exhibited slowest decay rates and low nutrient mesocosms exhibited the next slowest decay rates. Control mesocosms decayed fastest suggesting that nutrient addition to the mesocosms may have altered the initial decay response.

Similar to *E. coli* in water, only net decay was observed within the six treatment scenarios; however, there were slight differences in *E. coli* decay observed between nutrient amendment scenarios. These differences suggest that single nutrient additions to mesocosms did influence the observed decay of *E. coli* within sediments. This finding is logical as sediment provides an environment for *E. coli* more similar to that of a large intestine. Sediments are often anaerobic, they contain more nutrients than water, and they provide protection from predatory organisms and shelter from sunlight. Therefore, *E. coli* in sediments are less likely to be stressed and may be better able to metabolize available nutrients faster than if they were suspended in water.

Table 2.5. *E. coli* growth and decay constants under varying treatment scenarios in sediments

	Calculated *E. coli* Decay and Growth Constants k,d^{-1}*		
Treatment Scenario	**Phase I*†‡**	**Phase II*†‡**	**Phase III*†‡**
Control - Low Flow	-0.48 to 0.347 (-0.192)	-0.328 to 0.034 (-0.154)	-0.168 to 0.052 (-0.051)
Control - High Flow	-0.551 to 0.406 (-0.17)	-0.251 to 0.12 (-0.092)	-0.337 to 0 (-0.127)
High Nutrient - Low Flow	-0.088 to 0.023 (-0.024)	-0.255 to 0.102 (-0.129)	-0.343 to -0.025 (-0.148)
High Nutrient - High Flow	-0.139 to 0.04 (-0.035)	-0.313 to 0.263 (-0.11)	-0.254 to -0.071 (-0.137)
Low Nutrient - Low Flow	-0.511 to 0.036 (-0.276)	-0.247 to 0.119 (-0.107)	-0.206 to 0 (-0.108)
Low Nutrient - High Flow	-1.017 to 0.25 (-0.32)	-0.505 to 0.242 (-0.196)	-0.22 to -0.028 (-0.119)

* negative values represent decay constants, positive values represent growth constants

† range and (mean) of calculated values

‡ phase lengths vary within and between trials

Table 2.6. *E. coli* half-lives and doubling times under varying treatment scenarios in sediments

	Calculated *E. coli* Half-Life $t_{1/2}$,(day) or Doubling Time T_d, (day)*		
Treatment Scenario	**Phase I*†‡**	**Phase II*†‡**	**Phase III*†‡**
Control - Low Flow	-1.571 to 1.999 (-0.339)*	-4.027 to 20.625 (4.817)**	-19.25 to 13.327 (-3.354)
Control - High Flow	-1.9 to 1.709 (-0.483)	-4.833 to 5.799 (-0.598)	-16.079 to 0 (-6.046)
High Nutrient - Low Flow	-81.529 to 29.743 (-19.902)	-2.97 to 6.808 (0.374)	-28.286 to -2.023 (-13.15)
High Nutrient - High Flow	-130.75 to 17.196 (-39.518)	-2.478 to 2.631 (-0.686)	-9.83 to -2.732 (-6.855)
Low Nutrient - Low Flow	-1.967 to 19.144 (5.274)	-3.602 to 5.809 (-0.2)	-5.868 to 0 (-3.077)
Low Nutrient - High Flow	-3.596 to 2.768 (-0.503)	-2.138 to 2.861 (-0.216)	-25.2 to -3.157 (-11.57)

* negative values represent half-lives, positive values represent doubling times

† range and (mean) of calculated values

‡ phase lengths vary within and between trials

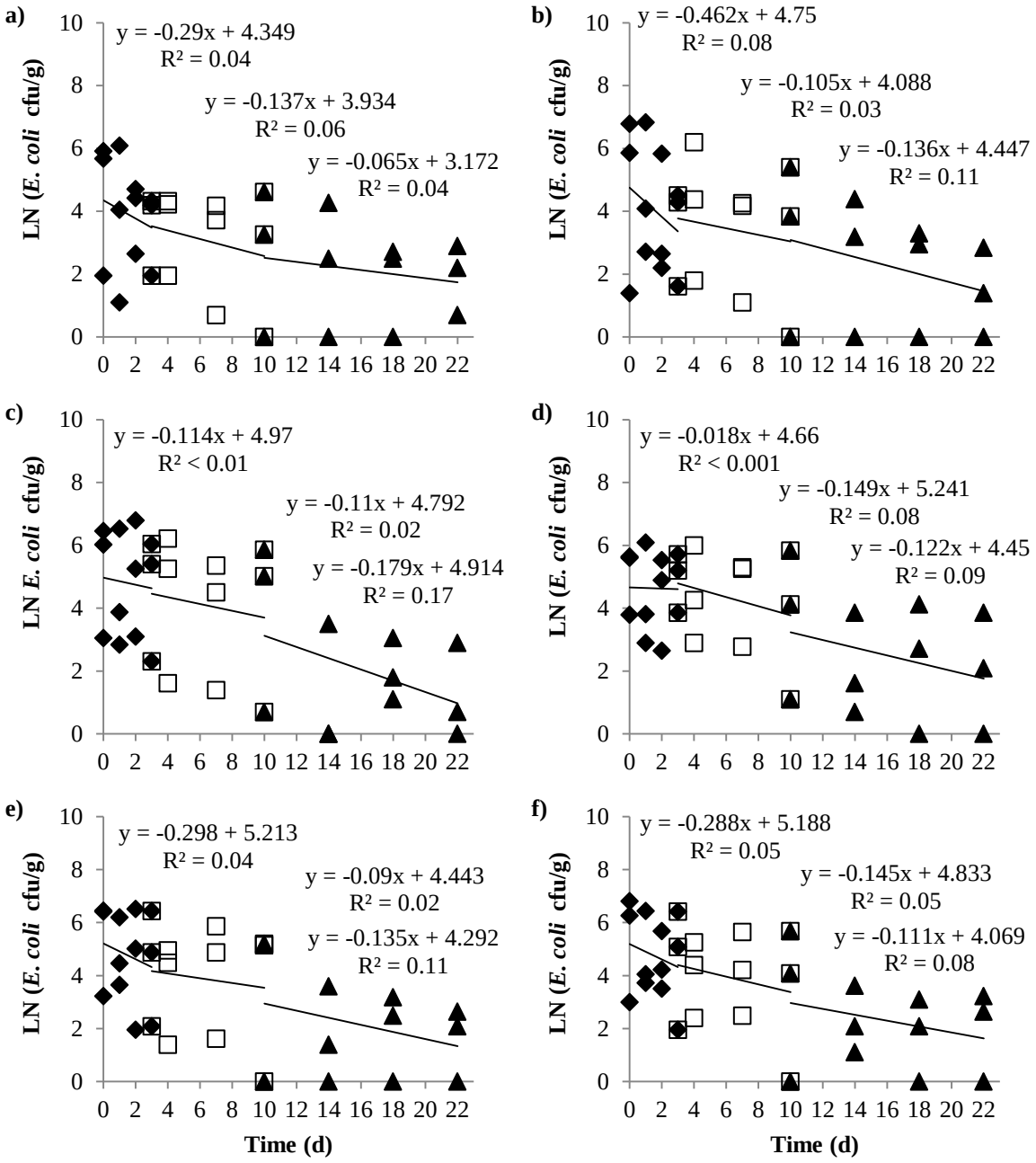

Figure 2.3. *E. coli* concentrations in sediment over time from all three trials combined. Graphics represent mesocosm scenarios: a) C1; b) C2; c) H1; d) H2; e) L1; f) L2

2.4.2 Heterotroph Persistence and Decay

Heterotrophic bacteria concentrations within stream mesocosms were measured over time to evaluate their response to nutrient amendments and flow conditions. Relationships and observed changes within heterotrophic bacteria populations in each mesocosm were used to calculate decay rates, growth rates, doubling times, and half-lives in each mesocosm.

2.4.2.1 Heterotrophic Bacteria Persistence and Decay in Water

Heterotrophic bacteria concentrations exhibited wide variability and fluctuating growth and decay during each trial. Unlike the biphasic *E. coli* decay response observed in water, heterotrophic bacteria growth and decay more closely resembled the tri-phasic growth and decay of sediment derived *E. coli* (Figure 2.4). Phase I began on day zero and ranged between two and seven days in length. Phase II began between days two and seven and ended between days seven and 14. Phase III subsequently began between days seven and 14 and ended on day 22.

Under nutrient treatment scenarios, heterotrophic bacteria concentrations increases occurred during phase I. In the first two trials, growth occurred rapidly until day four or seven and subsequently declined until trial completion. During the third trial, growth occurred until day three and was followed by a decline until day 10 or 14 when growth began to occur again (Appendix B). Growth and decay constants calculated for each phase within each treatment scenario exhibited considerable variability (Table 2.7). Constants calculated for each phase were grouped by treatment scenario and compared

using a Welch's ANOVA which produced respective p-values of 0.24, 0.70, and 1.00 for phases I, II, and III. Sufficient evidence was not produced to reject the null hypothesis of equal means. The assumption of normality was supported by Kolmogorov-Smirnov testing. Results should be interpreted with caution given the small sample size (n=3).

Doubling time and half-life were also calculated for each phase for all mesocosms during each trial and exhibited considerable variability (Table 2.8). Data grouped by treatment scenario were found to be non-normally distributed through application of Kolmogorov-Smirnov testing so the Kruskal-Wallis test was applied to determine the presence of significant differences in median values. Phases I, II, and III yielded p-values of 0.91, 0.35, and 0.5 respectively. Sufficient evidence was not produced to reject the null hypothesis of equal medians between the groups.

Nutrient amendments obviously impacted heterotroph growth and decay in the water column. Nutrient amendments produced slower decay rates than observed in the control mesocosms, they counteracted decay, and enhanced heterotroph growth. Nutrient addition produced larger net decay during phases II and III combined during trials one and two. The last trial produced a similar response in phase II; however, net heterotrophic bacteria growth was observed in phase III.

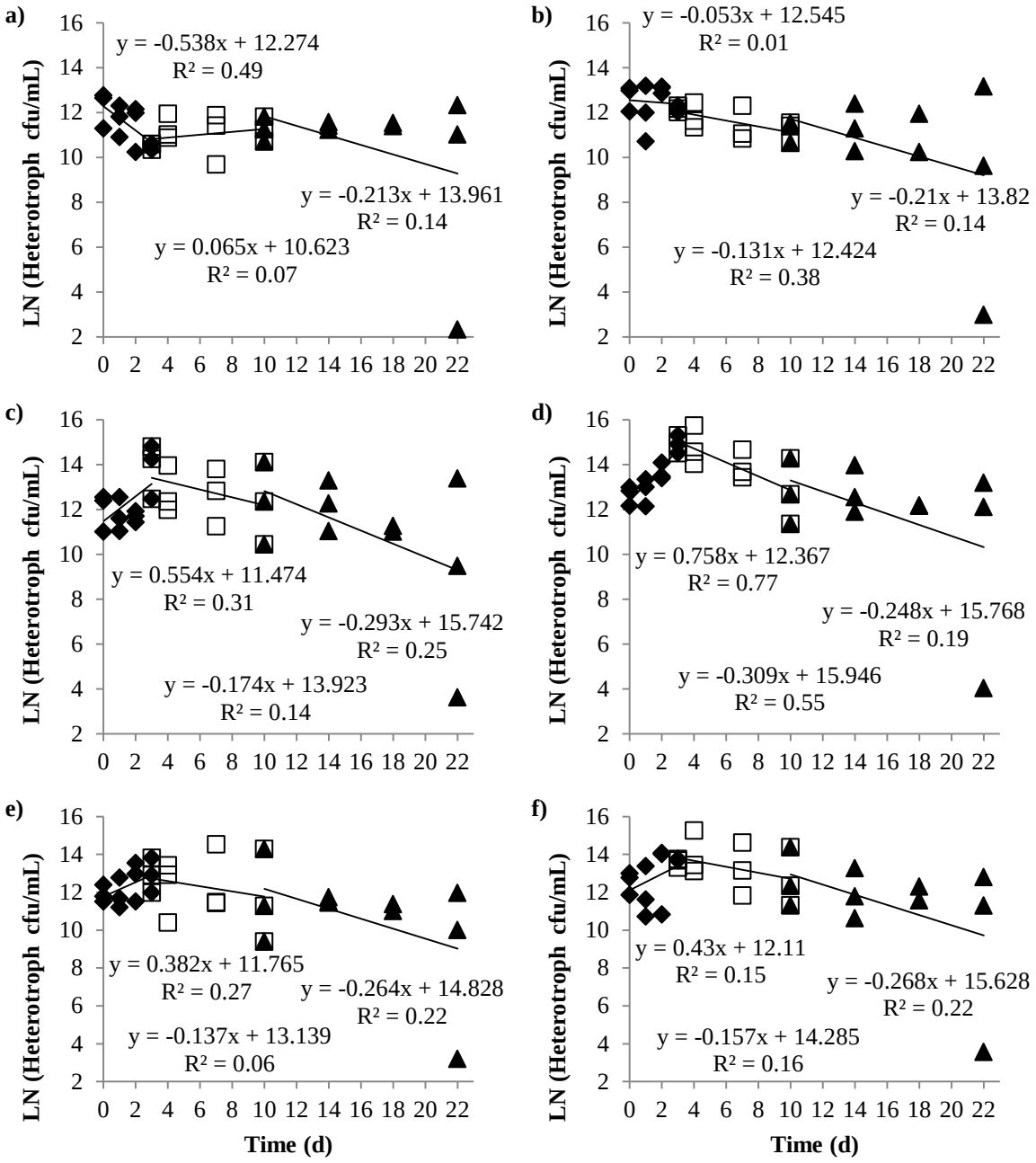

Figure 2.4. Heterotrophic bacteria concentrations in water over time from all three trials combined. Graphics represent the mesocosm scenarios: a) C1; b) C2; c) H1; d) H2; e) L1; f) L2

Table 2.7. Heterotrophic bacteria decay and growth constants under varying treatment scenarios in water

	Calculated Heterotroph Decay and Growth Constants (k,d^{-1})		
Treatment Scenario	**Phase I*†‡**	**Phase II*†‡**	**Phase III*†‡**
Control - Low Flow	-0.759 to 0.427 (-0.168)	-0.219 to -0.029 (-0.128)	-1.113 to 0.075 (-0.327)
Control - High Flow	-0.618 to 0.549 (0.166)	-0.371 to -0.056 (-0.187)	-1.038 to 0.097 (-0.346)
High Nutrient - Low Flow	0.09 to 1.122 (0.554)	-0.304 to -0.014 (-0.15)	-1.08 to 0.219 (-0.42)
High Nutrient - High Flow	0.57 to 1.032 (0.746)	0.332 to -0.07 (-0.212)	-1.064 to 0.161 (-0.37)
Low Nutrient - Low Flow	-0.05 to 0.741 (0.288)	-0.257 to 0.141 (-0.107)	-1.068 to 0.19 (-0.384)
Low Nutrient - High Flow	0.576 to 0.895 (0.709)	-0.163 to 0.424 (0.172)	-0.883 to 0.126 (-0.361)

* range and (mean) of calculated values

† negative values are decay constants, positive values are growth constants

‡ phase lengths vary within and between trials

Table 2.8. Heterotrophic bacteria half-lives and doubling times under varying treatment scenarios in water

	Calculated Heterotroph Half-Life $t_{1/2,}$(day) or Doubling Time $T_{d,}$ (day)*		
Treatment Scenario	**Phase I*†‡**	**Phase II*†‡**	**Phase III*†‡**
Control - Low Flow	-4.048 to 1.622 (-1.113)	-24.231 to -3.16 (-11.15)	-0.623 to 12.287 (6.952)
Control - High Flow	-11.214 to 67.282 (19.11)	-12.419 to -1.867 (-6.502)	-7.144 to 7.174 (-0.213)
High Nutrient - Low Flow	0.618 to 7.70 (3.287)	-49.149 to -2.283 (-18.881)	-1.74 to 3.16 (0.259)
High Nutrient - High Flow	0.672 to 1.217 (0.993)	-9.957 to -2.086 (-5.006)	-3.33 to 4.299 (0.106)
Low Nutrient - Low Flow	-13.972 to 4.017 (-3.007)	-3.405 to 4.922 (-0.393)	-2.528 to 3.64 (0.154)
Low Nutrient - High Flow	0.775 to 1.203 (1.011)	-4.244 to 2.703 (0.031)	-2.13 to 5.483 (0.856)

* range and (mean) of calculated values

† negative values are half-lives, positive values are doubling times

‡ phase lengths vary within and between trials

2.4.2.2 Heterotrophic Bacteria Persistence and Decay in Sediments

Heterotrophic bacteria growth and decay in sediment generally mirrored trends observed in overlying water within each mesocosm. Growth and decay were separated into three phases. Phase I began on day zero and ranged between two and seven days in length. Phase II subsequently began between days 2 and 7 and lasted through days 7 to 18. Phase III began between days 7 and 18 and ended on day 22 of each trial. Within each phase, growth and decay constants were developed and doubling time and half-lives were calculated.

Phase I generally supported net heterotroph growth in sediments (Figure 2.5); however, decay was observed in 5 of the 18 individual mesocosms (Appendix B). Phase II typically represented a stationary phase with slight decay and growth observed. In treatment mesocosms, this phase was shorter than or the same length as the same phase in the control mesocosms due to the nutrient application. Phase III exhibited increased decay rates compared to phase II.

Kruskal-Wallis testing was used to test for differences in median slopes of decay and growth constants, doubling time (Table 2.9), and half-life (Table 2.10) due to the non-normal distribution of the data. Respectively, p-values for phases I, II, and III for growth and decay constants were 0.92, 0.91, and 0.9, and were 0.23, 0.67, and 0.72 for doubling time and half-life. This did not provide sufficient evidence to reject the null hypothesis of equal medians within all groups.

Table 2.9. Heterotrophic bacteria decay and growth constants under varying treatment scenarios in sediments

	Calculated Heterotroph Decay and Growth Constants (k,d^{-1})		
Treatment Scenario	**Phase I*†‡**	**Phase II*†‡**	**Phase III*†‡**
Control - Low Flow	-0.444 to 0.779 (0.182)	-0.086 to 0.099 (-0.006)	-0.76 to -0.079 (-0.311)
Control - High Flow	-0.613 to 1.154 (0.096)	-0.157 to 0.149 (0.008)	-0.335 to -0.052 (-0.157)
High Nutrient - Low Flow	0.113 to 0.403 (0.238)	-0.09 to 0.102 (0.022)	-0.155 to -0.015 (-0.101)
High Nutrient - High Flow	-0.313 to 0.46 (0.364)	-0.109 to 0.037 (-0.045)	-0.226 to -0.037 (-0.105)
Low Nutrient - Low Flow	-0.085 to 0.617 (0.274)	-0.144 to 0.092 (-0.008)	-0.122 to 0.133 (-0.028)
Low Nutrient - High Flow	-0.052 to 0.57 (0.277)	-0.055 to 0.056 (0.018)	-0.165 to -0.017 (-0.073)

* range and (mean) of calculated values
† negative values are decay constants, positive values are growth constants
‡ phase lengths vary within and between trials

Table 2.10. Heterotrophic bacteria doubling times and half-lives under varying treatment scenarios in sediments

	Calculated Heterotroph Half-Life $t_{1/2,}$(day) or Doubling Time $T_{d,}$ (day)*		
Treatment Scenario	**Phase I*†‡**	**Phase II*†‡**	**Phase III*†‡**
Control - Low Flow	-1.562 to 3.305 (0.878)	-21.323 to 6.972 (-7.47)	-8.739 to -0.912 (-5.709)
Control - High Flow	-2.749 to 0.601 (-1.093)	-4.42 to 21.793 (7.343)	-13.251 to -2.069 (-7.896)
High Nutrient - Low Flow	1.719 to 6.133 (3.792)	-7.717 to 12.786 (3.948)	-47.793 to -4.465 (-19.156)
High Nutrient - High Flow	1.506 to 2.215 (1.962)	-11.07 to 18.986 (0.512)	-18.579 to -3.061 (-11.630)
Low Nutrient - Low Flow	-8.143 to 2.385 (-1.545)	-4.803 to 24.316 (9.01)	-7.3178 to 5.207 (-2.593)
Low Nutrient - High Flow	-13.327 to 2.216 (-3.299)	-12.623 to 12.978 (4.236)	-40.526 to -4.091 (-21.727)

* range and (mean) of calculated values
† negative values are half-lives, positive values are doubling times
‡ phase lengths vary within and between trials

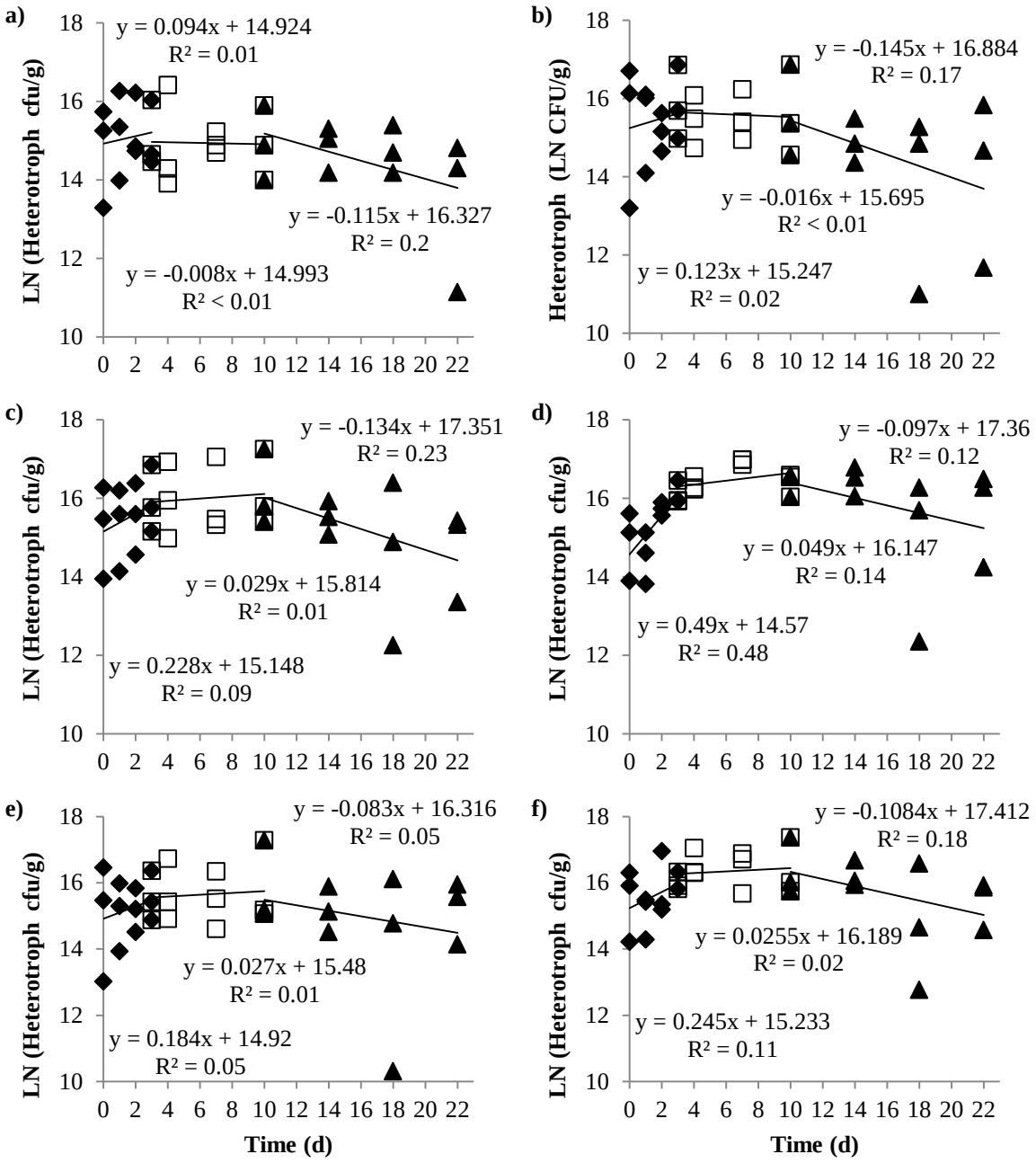

Figure 2.5. Heterotrophic bacteria concentrations in sediments over time from all three trials combined. Graphics represent the mesocosm scenarios: a) C1; b) C2; c) H1; d) H2; e) L1; f) L2

Nutrient amendments influenced heterotrophic bacteria growth and decay in sediment. Where growth occurred in control mesocosms, application of low and high nutrient doses to similar mesocosms increased the growth rate or extended its duration (Appendix B). When decay occurred in Phase I of the controls, nutrient amendment decreased decay rates or produced a growth response in the treatment mesocosms. These trends are further observed in the aggregated growth and decay constants (Figure 2.5). These results illustrate nutrient amendment influences on the heterotrophic bacteria community in sediments and supports earlier claims that their increased activity could be partly responsible for the lack of an *E. coli* growth response.

2.4.3 Comparison of *E. coli* and Heterotrophic Bacteria Kinetics Constants

E. coli and heterotrophic bacteria growth and decay constants, doubling times, and half-lives were compared between water and sediment within individual mesocosms to demonstrate differences in their metabolic activity. Differences in *E. coli* and heterotrophic bacteria growth or decay slope within mesocosms demonstrate the presence of resource competition and provide justification for suppressed *E. coli* growth. Graphical evidence suggests potential differences in growth and decay in similar phases between *E. coli* and heterotrophic bacteria in water (Figures 2.2 and 2.4; Appendices A and B) and sediment (Figure 2.3 and 2.5; Appendices A and B). In water, phase III of heterotrophic bacteria growth and decay was excluded from analysis because *E. coli* in water did not have a corresponding phase.

E. coli and heterotrophic bacteria decay and growth constant, doubling times, and half-lives in water were mostly non-normally distributed according to Kolmogorov-Smirnov testing, so a Kruskal-Wallis test was used to identify significant differences in median slopes within each phase. In water, p-values for phase I and II were <0.01 and 0.49 respectively. This provided evidence of significant differences between *E. coli* and heterotroph growth and decay constants in phase I but not phase II. Doubling time and half-life data were normally distributed and allowed use of a one-way ANOVA and Tukey pairwise comparison of *E. coli* and heterotrophic bacteria growth and decay constants within each mesocosm. Doubling times and half-lives produced p-values of 0.18 and 0.67 respectively which provided insufficient evidence to reject a null hypothesis of equal median slopes. Due to the small sample size within each group (n=3), results should be interpreted cautiously.

Growth and decay constants for sediment *E. coli* and heterotrophic bacteria in all phases were normally distributed while doubling times and half-lives were not according to a Kolmogorov-Smirnov testing. A Kruskal-Wallis test was applied to each phase within each mesocosm to identify significant differences in median slopes for growth and decay constants between *E. coli* and heterotrophic bacteria. Growth and decay constant p-values were 0.48, 0.92, and 0.99 while p-values for doubling times and half-lives were 0.83, 0.96, and 0.84 for phase I, II, and III respectively. These results do not provide sufficient evidence to reject the null hypothesis of equal medians between groups and should be interpreted cautiously due to the small sample size within each group (n=3).

These results provide evidence that heterotrophic bacteria are able to better utilize nutrient amendments than *E. coli* and may suppress *E. coli* growth following nutrient amendments through resource competition. This is particularly evident in water where significant differences in growth and decay were identified following nutrient additions to treatment mesocosms (phase I). Lack of significant differences in phase I constants within control mesocosms further supports this assertion. Further, evidence of statistically similar growth and decay constant slopes in subsequent phases demonstrate the return to return to relatively stationary *E. coli* and heterotrophic bacteria concentrations once nutrient amendments were fully metabolized.

2.4.4 Influence of Flow Velocity on *E. coli* in Water

Flow velocity in each mesocosm appeared to have greater influence on *E. coli* decay in water than nutrient amendments. High flow velocities delayed *E. coli* decay compared to low flow velocities. Initial *E. coli* concentrations were similar in all cases; however, *E. coli* concentrations in low and high flow velocity mesocosms diverge before converging at or near the end of the trial (Figure 2.6). Mean *E. coli* concentrations under low flow conditions were within the standard deviation of concentrations in high flow mesocosms suggesting that no significant differences between flow conditions exist.

The Mann-Whitney test was applied to mean $\log_{10}$ *E. coli* concentrations within each mesocosm type (control, high nutrient, low nutrient) to test for significant differences in median concentrations observed during each sampling day. No p-values produced indicated the presence of a significant difference in median values at $\alpha=0.05$

(Table 2.11). Results do suggest several strong, but not significant differences on days one and two in all mesocosms, day three in low nutrient mesocosms and day four of the control mesocosms. Based on graphical evidence (Figure 2.6), this finding was expected. However, results should be considered cautiously given the small sample size (n=3).

Table 2.11. Mann-Whitney test p-values test comparing mean *E. coli* concentrations within treatments and between flow conditions

	Time (Days)							
Treatment	**0**	**1**	**2**	**3**	**4**	**7**	**10**	**14**
Control	0.19	0.08	0.08	0.19	0.08	0.38	0.38	0.38
High Nutrient	0.66	0.08	0.08	0.51	0.18	0.51	0.51	0.35
Low Nutrient	0.38	0.08	0.08	0.08	0.35	0.51	0.35	1.00

2.4.5 Influences of Nutrient Amendments on *E. coli* in Water

E. coli concentrations in relation to nutrient amendments were further evaluated to investigate the potential for nutrient amendments to affect observed *E. coli* growth under different flow conditions (Figure 2.7). Visually, little difference exists in the mean *E. coli* concentrations between control, low nutrient, and high nutrient mesocosms on most sampling days. Kruskal-Wallis testing was used to evaluate daily mean concentrations among control, low, and high nutrient mesocosms under low and high flow conditions. Evidence provided by p-values (Table 2.12) was not sufficient to reject the null hypothesis of equal median *E. coli* concentrations. Further, these results do not suggest the presence of considerable differences in observed mean concentrations.

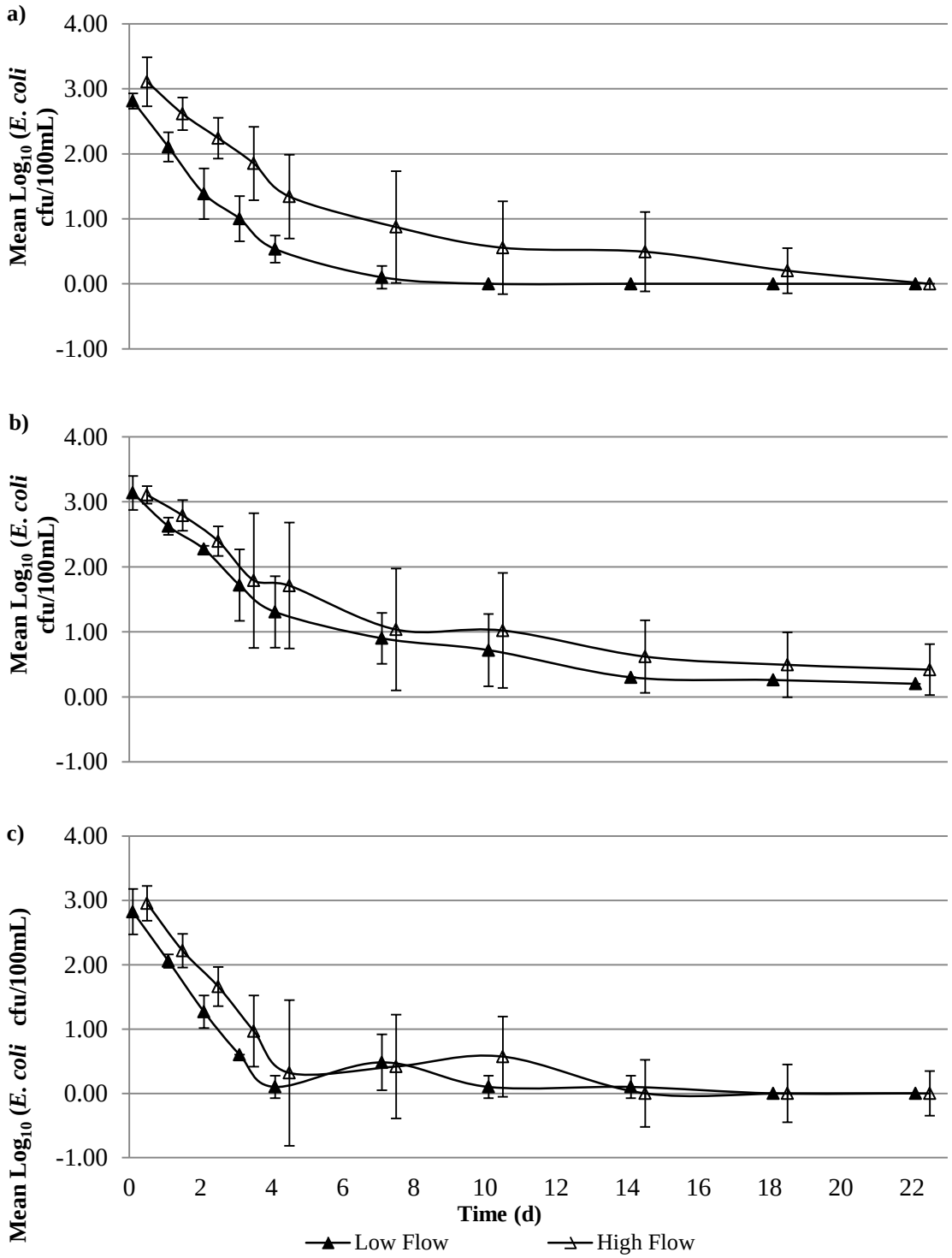

Figure 2.6. Mean and standard deviation of *E. coli* responses to varying flow velocities within treatments: a) control, b) high nutrient, c) low nutrient (note: data points are slightly offset horizontally to improve readability)

Table 2.12. Kruskal-Wallis test p-values comparing nutrient impacts on *E. coli* concentrations

	Time (Days)									
Flow Rate	**0**	**1**	**2**	**3**	**4**	**7**	**10**	**14**	**18**	**22**
Low Flow	0.56	0.40	0.23	0.23	0.28	0.38	0.20	0.37	1.00	1.00
High Flow	0.73	0.43	0.58	0.56	0.56	0.87	0.58	0.72	0.69	0.28

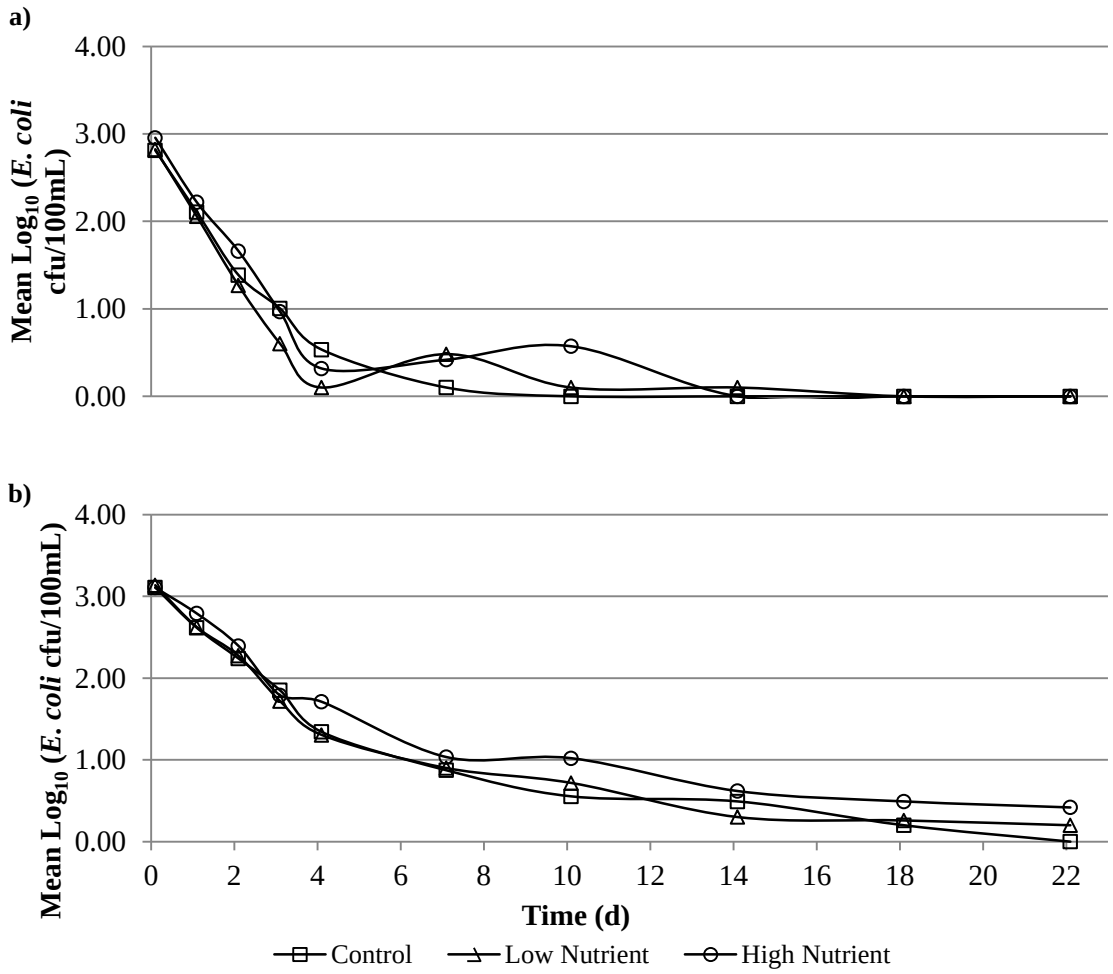

Figure 2.7. Mean *E. coli* concentrations from various nutrient treatments under two flow conditions within similar mesocosms: a) low flow, b) high flow. (note: mean *E. coli* concentrations are within the observed standard deviations of the other mesocosms and are not plotted to improve readability of this graph)

2.4.6 Relationships between *E. coli* and Measured Parameters

E. coli concentrations in water were evaluated compared to other measured parameters to identify potential *E. coli* relationships with these independent variables. Analysis revealed that *E. coli* are sometimes dependent upon individual and multiple parameters in these trials. Turbidity was most commonly related to *E. coli* concentrations; however, identified relationships were not consistent between individual treatments or trials. As a result, a number of monitored parameters were identified as significant predictors of *E. coli* concentrations in at least one instance.

2.4.6.1 Modeled Relationships between *E. coli* and Monitored Parameters

Nonlinear regression was used to model *E. coli* concentrations with measured parameters in all but one mesocosm where linear regression was appropriate. Potential predictor variables were evaluated individually for each of the 18 mesocosm scenarios. Evaluated variables included temperature, pH, specific conductance, DO, turbidity, NO_3-N, NH_4-N, PO_4-P, DOC, and TN. In most cases, a modeled relationship could not be established between potential predictor variables and *E. coli*. Specific conductance and turbidity were the most common predictors of *E. coli* concentration and were found to have reasonable relationships in 10 and 12 of the 18 mesocosms, respectively.

E. coli concentrations were modeled mathematically for 33 of the 198 possible predictor variables evaluated (Table 2.13; Appendix C). Most regression models fit the data well with 25 of the 33 models having standard error of regression (S) values <0.25. At that level, the 95% prediction interval for predicting another *E. coli* value using the

developed model is 0.5 $\log_{10}$ *E. coli* units away from the fitted model. The remaining eight regression equations had S values ranging from 0.26 to 0.46 which produces a prediction interval within an order of magnitude of the fitted regression line.

These results suggest that some relationships between monitored parameters and *E. coli* concentration in water can be modeled mathematically. Turbidity was most commonly related to *E. coli* concentrations through a positive relationship. Turbidity has been evaluated as a potential indicator of instream *E. coli* concentrations in numerous studies and has been closely related in many cases [*Muirhead et al.*, 2004; *Wittman et al.*, 2013] and poorly related in others [*McDonald et al.*, 1982; *Wagner et al.*, 2013]. Our findings agree with the contradictory body of research and support cautious use of turbidity as a surrogate for *E. coli* concentrations in water.

Specific conductance commonly exhibited a negative relationship with *E. coli* concentrations in water. This finding is not uncommon, as other works have found these relationships in small watershed monitoring [*Wittman et al.*, 2013] where rainfall dilution presumably causes negative relationships. Others relate specific conductance to salinity which has adverse effects on *E. coli* survival at high levels [*Ishii and Sadowsky*, 2008]. Contradictory findings also exist where observed *E. coli* concentrations decline along with specific conductance values [*McLellan et al.*, 2007]. Our findings seemingly concur with the work of *Wittman et al.* [2013]; however, dilution did not occur in the mesocosms. Instead, the observed increase in specific conductance is likely due to bacterial cell compound release upon cell death.

Table 2.13. Regression equations to predict log_{10} *E. coli* concentrations in water

Treatment Scenario	Predictor Variable	Regression Model Type	Model Equation	S – Standard Error of Regression
C1-1	Turbidity	Loglogistic	= 1.88051 + (- 0.0181005 - 1.88051) / (1 + exp(7.61108 * ln(Turbidity / 18.8655)))	0.0531
	Nitrate	Exponential	= 4.65779 * exp(-16.1632 * Nitrate)	0.1315
	Orthophosphate	Exponential	= 125.916 * exp(-19.5922 * Orthophosphate)	0.4523
	Specific Conductance	General Linear Model	= exp(79.6364 - 0.243853 * Specific Conductance)	0.0700
C1-2	Turbidity	Loglogistic	= 2.07907 + (- 0.0431143 - 2.07907) / (1 + exp(7.62642 * ln(Turbidity / 7.90589)))	0.1897
	Nitrate	Rational Polynomial	= (0.139218 - 0.490157 * Nitrate) / (1 - 13.0123 * Nitrate + 43.2772 * Nitrate ^2)	0.0506
	Specific Conductance	Exponential	= 1.38315e+006 * exp(-0.0283993 * Specific Conductance)	0.3157
C1-3	Turbidity	Loglogistic	= 2.19001 + (- 0.276102 - 2.19001) / (1 + exp(13.0982 * ln(Turbidity / 10.3007)))	0.2453
	Nitrate	Exponential	= 2.43913 * exp(-2.7276 * Nitrate)	0.1349
	Orthophosphate	Exponential	= 22.5933 * exp(-19.1118 * Orthophosphate)	0.1208
	Total Nitrogen	Rational Polynomial	= (0.52288 - 0.263582 * Total Nitrogen) / (1 - 2.19909 * Total Nitrogen + 1.39719 * Total Nitrogen ^ 2)	0.1073
C2-1	Turbidity	Loglogistic	= 3.12838 + (- 0.392477 - 3.12838) / (1 + exp(1.25903 * ln(Turbidity / 42.7145)))	0.1389
	Nitrate	Rational Polynomial	= (-0.175125 + 2.81029 * Nitrate) / (1 - 21.5521 * Nitrate + 120.191 * Nitrate ^2)	0.2395
	Specific Conductance	Exponential	= 2.09083e+007 * exp(-0.0480154 * Specific Conductance)	0.2212
C2-2	Turbidity	Weibull	= 2.19321 * exp(-exp(2.85267 - 0.289681 * Turbidity))	0.1278
	Orthophosphate	Exponential	= 26.6331 * exp(-18.5549 * Orthophosphate)	0.1994
	Specific Conductance	Exponential	= 39018.5 * exp(-0.0206143 * Specific Conductance)	0.2347
C2-3	Nitrate	Linear	= 2.663 - 1.431 * Nitrate	0.1564
	Orthophosphate	Linear	= 3.204 - 7.145 * Orthophosphate	0.1712
	Specific Conductance	Linear	= 8.305 - 0.01180 * Specific Conductance	0.2706
	Total Nitrogen	Linear	= 3.397 - 1.357 * Total Nitrogen	0.2498
H1-2	Turbidity	Logistic	= 2.34708 + (- 0.102263 - 2.34708) / (1 + exp((Turbidity - 10.1216) / 2.0652))	0.0351

Table 2.13. Continued

Treatment Scenario	Predictor Variable	Regression Model Type	Model Equation	S – Standard Error of Regression
H2-1	Turbidity	Logistic	= 3.06202 + (0.143865 - 3.06202) / (1 + exp((Turbidity - 176.028) / 58.6842))	0.0695
H2-2	Turbidity	Logistic	= 2.53736 + (- 0.000792206 - 2.53736) / (1 + exp((Turbidity - 26.6338) /3.54259))	0.4584
L1-1	Turbidity	Linear	= - 0.1580 + 0.06302 * Turbidity	0.2431
	Specific Conductance	Exponential	= 1.9189e+007 * exp(-0.0492147 * Specific Conductance)	0.2467
L1-2	Turbidity	Loglogistic	= 2.29929 + (- 0.113068 - 2.29929) / (1 + exp(2.51541 * ln(Turbidity / 8.91424)))	0.1256
	Specific Conductance	Exponential	= 478165 * exp(-0.0261412 * Specific Conductance)	0.3262
L1-3	Specific Conductance	Exponential	= 1.18992e+007 * exp(-0.0321242 * Specific Conductance)	0.2559
L2-1	Turbidity	Power	= 0.0929199 * Turbidity ^ 0.637819	0.3121
L2-1	Specific Conductance	Logistic	= 0.379062 + (2.90949 - 0.379062) / (1 + exp((Specific Conductance - 356.299) /4.54253))	0.3978
L2-2	Turbidity	Gompertz Growth	= 2.47352 * exp(-exp(2.33916 - 0.188898 * Turbidity))	0.0151
L2-2	Specific Conductance	Logistic	= -0.0106851 + (2.33246 + 0.0106851) / (1 + exp((Specific Conductance - 523.751) / 7.2335))	0.0315

Nutrient and *E. coli* relationships were found in control mesocosms only (Table 2.13) and generally exhibited a negative relationship when present. The negative relationship of PO_4-P and *E. coli* concentration identified in four of the six control mesocosms indicates that P may not be limiting *E. coli* growth in these mesocosms. Modeled relationships between *E. coli* and nitrogen concentrations (NO_3-N and total N) were found in eight instances within the six control mesocosm scenarios. In mesocosm treatment scenarios C1-2, C1-3, and C2-1, slight increases in NO_3-N and total N concentrations corresponded with higher *E. coli* concentrations (Appendix C). In all other cases, NO_3-N and total N concentrations were inversely related to *E. coli* concentrations. Relationships between nutrients and *E. coli* concentrations could not be modeled in any treatment mesocosm providing further evidence that nutrient amendments did not directly affect *E. coli* concentrations (Appendix D). This finding and the rapid decline of nutrient availability provides further evidence that heterotrophic bacteria were able to rapidly consume nutrient amendments in treatment mesocosms.

2.4.6.2 Modeled Multiple Parameter Relationships

Visual analysis of scatter plots comparing $\log_{10}$ transformed *E. coli* concentrations to measured parameters suggested potential relationships between specific conductance, turbidity, NO_3-N, NH_4-N, PO_4-P, and DOC. Stepwise multiple regression analysis performed and cross validated with best subsets regression produced models with high R^2 and predicted R^2 values (Table 2.14) in most cases. Attempts to include potential interaction effects in models where appropriate were made; however,

severe multicollinearity encountered between some model parameters and increased coefficient estimate variance and diminished predicted model appropriateness.

Table 2.14. Significant multiple regression models relating selected parameters to log_{10} *E. coli* concentration

Mesocosm	Model Equation	Model Statistics			
		P	R^2	R^2 (pred)*	S
C1-1	= -0.244 - 0.707 NO_3-N + 0.0495 DOC	<0.01	82.1	64.5	0.32
C1-2	= -0.312 + 0.1274 Turbidity	<0.01	83.9	54.7	0.38
C1-3	= -0.435 + 0.1567 Turbidity - 0.630 NO_3-N	0.00	93.2	36.9	0.24
C2-1	= 2.800 + 0.00973 Turbidity - 8.55 PO_4-P	0.00	95.6	81.6	0.26
C2-2	= 1.549 + 0.0399 Turbidity + 0.386 NH_4-N - 5.48 PO_4-P	<0.01	94.9	60.1	0.26
C2-3	= 2.287 + 0.00332 Turbidity - 5.470 PO_4-P	0.00	98.7	96.3	0.12
H1-1	= 4.83 - 0.00971 Specific Conductance - 0.1193 NO_3-N + 0.01260 DOC	<0.01	90.5	73.3	0.33
H1-2	= -0.6356 + 0.12264 Turbidity - 0.04387 NO_3-N + 0.02098 DOC	0.00	99.9	98.3	0.04
H1-3	= 8.49 - 0.01309 Specific Conductance	<0.01	73.1	64.2	0.42
H2-1	= -0.558 + 0.009739 Turbidity + 0.04298 PO_4-P	0.00	99.4	96.9	0.09
H2-2	= -2.094 + 0.08250 Turbidity + 0.3107 NO_3-N + 0.678 NH_4-N	0.00	96.8	86.9	0.23
H2-3	= 4.449 - 0.005736 Specific Conductance + 0.00636 Turbidity	0.00	96.4	90.8	0.16
L1-1	= -0.158 + 0.06302 Turbidity	0.00	89.3	85.4	0.24
L1-2	= -0.2695 + 0.1178 Turbidity	0.00	94.7	77.5	0.19
L1-3	= 0.087 + 0.1036 Turbidity - 0.383 NO_3-N	<0.01	87.7	63.5	0.28
L2-1	= 8.90 - 0.01992 Specific Conductance - 2.813 NH_4-N + 0.01531 DOC	0.00	98.5	93.7	0.16
L2-2	= -0.518 + 0.09576 Turbidity - 0.289 NH_4-N + 0.02185 DOC	0.00	99.8	98.8	0.06
L2-3	= 7.08 - 0.00913 Specific Conductance - 0.319 NO_3-N	0.00	94.9	83.7	0.23

*predicted R^2 indicates how well the model predicts responses for new observations

Turbidity was included as a significant parameter in 13 of the 18 mesocosm models predicting *E. coli* concentrations. This was similar to the single parameter relationships identified; however, mesocosms where these relationships occurred are not the same in three instances. It should also be noted that increased turbidity yielded

higher *E. coli* concentrations in these models which is consistent with findings of *Muirhead et al.* [2004] and *Wittman et al.* [2013]. However, the lack of turbidity in remaining models demonstrates that it is not always a reliable *E. coli* concentration predictor in water as suggested by *McDonald et al.* [1982] and *Wagner et al.* [2013].

At least one nutrient parameter was a significant model factor in 13 of the 18 mesocosms. NO_3-N was a significant factor in six models while NH_4-N, PO_4-P, and DOC were included in four, three and five models respectively. Within these models, DOC exhibited a positive relationship with *E. coli* while NO_3-N, NH_4-N, and PO_4-P did not demonstrate consistent relationships. This suggests that a single nutrient parameter is a poor predictor of *E. coli* concentration in water. Interactions between model factors or external influences to the system such as microbial competition or predation may potentially cause these inconsistent relationships.

Specific conductance was included as a significant model parameter in 5 of the 18 mesocosms where it exhibited a negative relationship with *E. coli*. This low inclusion rate was unexpected since modeled relationships were identified in twice as many individual parameter models. Each mesocosm where specific conductance was identified as a significant model parameter also received nutrient amendments. Possible correlation between specific conductance and other predictor variables such as nutrients provide some potential explanation for its lack of significance in developed models.

Single and multiple parameter models were found to effectively predict *E. coli* concentrations in these trials conducted to mimic single nutrient additions to ‘natural’ systems. Although these modeled relationships are not likely to be duplicated in other

settings, they demonstrate the potential diversity of relationships that exist in real stream environments between *E. coli* and external factors. Aquatic environments are complex systems that contain many influential factors. The ability to predict *E. coli* concentrations with other monitored parameters demonstrates the utility of water quality models to better understand fate processes in natural systems. These findings also illustrate the importance of developing a complete understanding of water quality when attempting to model these processes.

2.4.7 Bacteria and Nutrient Mesocosm Mass Balance

Changes in nutrient mass and number of *E. coli* and heterotrophs were calculated for each mesocosm treatment scenario to further understand their fate within each mesocosm. Calculated *E. coli* and heterotroph quantities (Table 2.15) in water and sediment at the end of each trial were subtracted from initial quantities to determine net loss of bacteria during each trial. Changes in nutrient mass were calculated similarly; however, final mass was subtracted from the mass calculated on day two (first sample after nutrient amendments applied) rather than day zero.

E. coli in water decreased consistently in all mesocosm treatment scenarios. Final *E. coli* concentrations ranged from 1 to 6 cfu/100 mL so changes in quantity were almost exclusively a function of differing initial concentrations which ranged from 380 to 3,500 cfu/100 mL. This effectively resulted in the complete loss of *E. coli* in water. Losses ranged from 99.66 to 100% or from 1.71×10^5 to 1.57×10^6 cfu during the 22 day trials. Similar results were observed in sediment *E. coli* with net losses occurring in all

mesocosms. Final *E. coli* concentrations ranged from 1 to 117 cfu/g while initial concentrations ranged from 4 to 1,670 cfu/g. *E. coli* in sediment decreased during the trials by 4.77 x 10^3 to 1.33 x 10^6 cfu or 76.57 to 100% of the initial amount. These results demonstrate that net *E. coli* death occurred in all mesocosms in each trial.

Table 2.15. Bacteria mass balance for each mesocosm scenario

Mesocosm	*E. coli* (cfu)		Heterotrophs (cfu)	
	Water	Sediment	Water	Sediment
C1-1	-3.64E+05	-4.75E+05	-1.33E+10	-2.98E+09
C1-2	-2.16E+05	-8.04E+03	-3.59E+09	-8.00E+08
C1-3	-3.15E+05	-4.24E+05	-4.88E+09	-8.21E+09
C2-1	-1.57E+06	-4.17E+05	-1.87E+10	-5.93E+09
C2-2	-3.33E+05	-4.77E+03	-7.61E+09	-6.65E+08
C2-3	-3.69E+05	-1.30E+06	-5.40E+08	-2.41E+10
H1-1	-8.10E+05	-8.01E+05	-1.21E+10	-1.19E+10
H1-2	-3.01E+05	-3.03E+04	-2.53E+09	-9.35E+08
H1-3	-2.74E+05	-5.93E+05	1.51E+10	-1.65E+09
H2-1	-6.56E+05	-3.22E+05	-9.36E+09	8.71E+09
H2-2	-4.05E+05	-6.48E+04	-8.12E+09	2.50E+08
H2-3	-7.18E+05	-3.71E+05	2.12E+09	8.72E+09
L1-1	-7.65E+05	-8.47E+05	-9.91E+09	2.53E+09
L1-2	-2.07E+05	-3.63E+04	-5.79E+09	1.02E+09
L1-3	-1.71E+05	-8.98E+05	1.82E+09	-1.39E+10
L2-1	-1.26E+06	-6.95E+05	-1.66E+10	-2.68E+09
L2-2	-4.14E+05	-2.88E+04	-6.15E+09	3.97E+08
L2-3	-4.50E+05	-1.33E+06	-1.17E+09	-7.91E+09

Note: negative values indicate a net loss of mass or count; positive values indicate a net gain in mass or count

Biofilms formed in mesocosms and were evaluated to estimate the potential to harbor *E. coli.* Biofilm sampling occurred on days 7, 14, and 22. Its growth was largely confined to paddle wheel fins and is where samples were taken. *E. coli* concentrations observed in all biofilm samples ranged from 0 to 6.5 cfu/cm^2 (mean = 1.7 cfu) indicating that they can harbor *E. coli*. Using the area of the paddle wheel fins and the highest *E.*

coli concentration measured, up to 1.02 x 10^4 cfu/cm^2 could have resided in the biofilm. However, conducting this calculation using the mean *E. coli* concentration indicates that only 6.67 x 10^2 cfu/cm^2 are expected to exist in biofilm within each mesocosm. Small *E. coli* concentrations and biofilm quantity in each mesocosm diminish potential for biofilm to serve as an *E. coli* reservoir in these mesocosms. Heterotrophs were not enumerated in biofilms.

Heterotrophic bacteria quantity in water generally changed in similar fashion to *E. coli.* Net reductions in each trial ranged from 5.40 x 10^8 to 1.87 x 10^{10} cfu (2.5 to 99.76%) in control mesocosms while net increases occurred in three treatment scenarios and net decreases occurred in the other nine. Decreases ranged from 1.17 x 10^9 to 1.66 x 10^{10} while increases ranged from 1.082 x 10^9 to 1.51 x 10^{10} cfu (Table 2.15). Percentage wise, losses and gains ranged from -98.92 to 140% of the initial population.

In sediment, changes were similar to those in overlying water. All control mesocosms experienced net losses ranging from 6.65 x 10^8 to 2.41 x 10^{10} cfu, or 39.11 to 90.41% of the initial amount. Net increases and decreases occurred in treatment mesocosms and increased from 2.50 x 10^8 to 8.72 x 10^9 cfu (15.41 to 157.08%) or decreased from 9.35 x 10^8 to 1.39 x 10^{10} cfu (21.15 to 68.19%). These findings demonstrate the variable nature of bacteria's growth or decay response in aquatic microbial environments and depict the influences of competition, predation, and nutrient availability within these systems.

Nutrient concentration changes in each trial were more variable than those of *E. coli* and heterotrophic bacteria. Nutrients additions in treatment mesocosms caused the

majority of this variability. Total change in mass (day 0 to 22) and the change in mass from the time of nutrient amendment to the end of the trial (day 2 to 22) were calculated for each mesocosm (Table 2.16). Changes observed in the mass of NO_3-N were most consistent between all treatment scenarios. Net NO_3-N mass increases between days 0 and 22 occurred in all mesocosms while only the high nutrient (H1 and H2) mesocosms yielded net NO_3-N losses between days 2 and 22. This is most likely a result of microbial nitrification of NH_4-N to NO_3-N by chemoautotrophs and select heterotrophs. This observation is indicative of systems that contain ample NH_4-N and should be mirrored by overall reductions in NH_4-N. As expected, NH_4-N concentrations and mass increased and decreased inversely to NO_3-N (Appendix D); however, timing is offset and is not clearly reflected in Table 2.16. *E. coli* and heterotroph die-off in water and sediment are likely responsible for the NH_4-N increases that lead to NO_3-N increases through nitrification. Changes in NO_3-N and NH_4-N suggest the presence of a nitrogen demand in the mesocosms; however, it is not the limiting nutrient.

Control mesocosms exhibited some variability in DOC utilization. Half of the control mesocosms had net losses while the other half had net DOC increases which occurred when mass decay of heterotrophic bacteria occurred in the last week of the trial (2.06×10^6 cfu). Net DOC decreases occurred in all treatment mesocosms following nutrient amendment application. This appears directly related to heterotrophic bacteria DOC utilization. DOC appears limited in treatment mesocosms as its concentration rapidly returns to pre-amendment levels. A considerable DOC decrease between day 0 and 22 was observed in trial one due to high initial ambient DOC concentrations (42.98

– 44.15 mg/L). This trial began December 1, 2014 at the height of deciduous plant abscission which provided an ample carbon source.

Compared to initial conditions, a net increase in PO_4-P occurred in each mesocosm. Increases in available PO_4-P from nutrient amendments were followed by decreases through the end of each trial. Two cases were exceptions where slight increases of 5.18 and 6.94 mg were observed. Similar to nitrogen, PO_4-P mass and concentration increased toward the end of trial two in mesocosms H1 and H2 mirroring rapid heterotrophic bacteria decay. The gradual decline of PO_4-P throughout the trials suggests that it was not a limiting nutrient in these mesocosms.

Mass balance results confirm that net *E. coli* and heterotrophic bacteria in water and sediments losses occurred during each trial despite nutrient amendments. Changes in nutrient mass (Appendix D) and the heterotrophic bacteria growth response (Appendix B) clearly show microbial ability to utilize nutrient additions and provide support for the assertion that competition precluded *E. coli* growth in response to single nutrient amendments in treatment mesocosms.

2.5 Summary and Conclusions

Use of re-created stream mesocosms proved effective for evaluating aquatic microbial community dynamics in response to water chemistry changes. They allowed relative control over mesocosm conditions yet were able to mimic a natural stream environment. Addition of known nutrient amendments and exclusion of unknown inputs greatly improved the ability to investigate *E. coli* fate in response to these changes.

Table 2.16. Nutrient mass balance in mesocosm treatment scenarios

Mesocosm	Nitrate (mg)		Ammonium (mg)		Orthophosphate (mg)		Dissolved Organic Carbon (mg)		Total Nitrogen (mg)	
	Day 0*	Day 2**	Day 0	Day 2	Day 0	Day 2	Day 0	Day 2	Day 0	Day 2
C1-1	29.87	34.98	-0.87	-0.60	23.33	24.23	-1291.39	-34.74	6.39	27.07
C1-2	24.43	24.19	1.64	2.43	11.01	8.34	0.98	-27.73	27.22	26.12
C1-3	63.56	62.49	-10.22	-7.76	10.82	9.98	-105.74	-11.10	54.75	75.63
C2-1	23.78	27.00	-1.35	-3.51	3.80	5.39	-1,298.04	5.85	-2.72	22.16
C2-2	31.27	30.80	2.00	1.73	13.55	10.62	77.62	58.51	36.36	32.61
C2-3	64.11	60.82	-12.07	-6.09	16.23	13.95	105.27	204.18	56.10	76.00
H1-1	189.57	-63.19	-1.01	0.27	464.61	-170.07	-1,195.40	-5,872.71	161.47	-77.60
H1-2	209.65	-49.09	1.07	2.42	207.70	-20.40	90.81	-1,596.01	200.65	-49.19
H1-3	193.10	-1.34	-9.53	0.55	163.57	-95.39	-34.04	-1,602.23	201.29	9.21
H2-1	176.84	-190.21	-0.87	-1.55	377.13	-174.31	-1,210.89	-6,433.61	139.64	-210.77
H2-2	204.29	-43.32	1.26	2.74	205.08	-19.04	-25.00	-1,952.82	192.38	-47.10
H2-3	199.14	-21.58	-14.37	-0.35	120.00	-88.50	76.45	-1,521.56	202.99	-3.45
L1-1	57.53	22.59	-1.06	-0.64	55.51	-48.76	-1,398.62	-2,082.22	24.25	8.58
L1-2	65.74	32.10	2.19	3.14	37.04	5.18	43.94	-361.53	64.05	28.34
L1-3	90.72	75.05	-10.34	-1.21	26.89	-2.51	-51.93	-262.54	74.09	83.59
L2-1	52.13	5.93	-1.23	-1.71	56.96	-53.88	-1,374.05	-2,248.61	23.95	-4.44
L2-2	75.21	15.28	1.23	1.97	33.92	-1.22	92.25	-469.39	73.93	28.61
L2-3	97.32	86.40	-10.09	-0.57	37.25	6.94	78.25	-97.22	90.67	107.18

*Day 0 column represents the net gain or loss of nutrient from the initial mass of nutrient present in each mesocosm
**Day 2 column represents the net gain or loss of nutrient from the mass of nutrient present in each mesocosm following application of nutrient amendment in the treatment mesocosms

Findings did not support the hypothesis that *E. coli* concentrations in water and sediment would be significantly altered by treatments that mimic single additions of nutrient from irrigation runoff or a pollution event. Instead, *E. coli* routinely declined to, or near, non-detectable concentrations with no significant differences in decay rate detected. Applied flow rate produced a visual difference in observed decay; however, the median decay rate slopes were not significantly different between treatment scenarios.

Competition from other members of the heterotrophic bacteria community was confirmed in these trials. Unlike *E. coli*, clear changes in heterotrophic bacteria growth kinetics occurred in water and sediment. Decay and growth constant slopes were not significantly different between treatments in water or sediment, but they were significantly different than water *E. coli* decay slopes in phase I. This clearly depicts their differing response to the nutrient amendments. The heterotroph growth response suggests that they were able to rapidly utilize nutrient amendments and outcompete *E. coli* for this resource. Predation cannot be precluded from this scenario and may have affected decay of all heterotrophic bacteria, including *E. coli*.

Mass balance calculations provided insight into biological and chemical changes in mesocosms over time. Net *E. coli* losses occurred in all scenarios in water and sediment supporting the presumption that *E. coli* died instead of moving to a different media. Biofilms in all mesocosms harbored *E. coli*; however, concentrations did not supply sufficient evidence to suggest that they moved into biofilms and persisted. Nutrient balances also illustrated that DOC was in high demand within each mesocosm.

Relating observed *E. coli* concentrations in water to measured parameters identified several significant factors that could be used to model observed *E. coli* concentrations. Limited consistency in parameters related to *E. coli* occurred but is expected when modeling dynamic aquatic ecosystems. Turbidity reasonably predicted *E. coli* concentration in most, but not all mesocosms. Nutrients parameters were not identified as individual *E. coli* predictors in most cases, but were significant factors in most multiple parameter regression equations developed. Variations in factors included in developed models illustrates natural aquatic system complexity and the difficulty in modeling *E. coli* concentrations in the environment.

Using unaltered creek water and sediments provided insights into the microbial response in natural aquatic ecosystems to nutrient amendments but also introduced considerable variability. Results provide useful information that can and should further the understanding of *E. coli* fate in natural systems and will improve watershed scale modeling efforts if utilized. Inherent uncertainties in water quality monitoring should be accounted for when using these *E. coli* fate parameters for modeling purposes [*Harmel et al.*, 2010; *Harmel et al.*, 2016]. The experimental design did not allow for true replicates of each treatment scenario thus statistics performed were based on small data sets. Larger scale experiments with multiple replicates are needed to improve the study design and produce additional information on *E. coli* fate in aquatic environments.

CHAPTER III

LAND USE AND LAND COVER IMPACTS ON CULTURABLE *E. COLI* IN RUNOFF AND SOIL

3.1 Overview

Land cover and land use can impact runoff production and soil health. The presence, type, and quantity of vegetation may also influence the presence and amount of *E. coli* by directly impacting soil temperatures, ultraviolet light transmittance [*Fujioka et al.*, 1981], moisture content, nutrient concentrations, soil organic matter content [*Ishii et al.*, 2006], and animal utilization of the site. Further, land use and land cover also influence soil porosity, runoff production and erosive potential [*Ward and Elliot*, 1995] which impact bacterial transport. These factors also influence the ability of *E. coli* to survive, persist, and be transported from the site [*Roodsari et al.*, 2005].

Land use evaluations have determined its effects and demonstrate its potential for exerting significant influences on water quality. Transition from natural or unimpacted uses to more developed use generally causes water quality declines [*Goto and Yan*, 2011b; *Harmel et al.*, 2010; *Larned et al.*, 2004; *Liang et al.*, 2013]. Research conducted compared influences of differing land uses including urban and forests [*Goto and Yan*, 2011b], grazed pasture and forests [*Donnison et al.*, 2004], and grazed and ungrazed agricultural landscapes [*Harmel et al.*, 2010; *Wagner et al.*, 2012]. Watersheds with more intensive uses routinely yield higher *E. coli* loads; however, *E. coli* concentrations from areas impacted by natural sources often exceed instream water quality standards for

bacteria and sometimes exceed those from more intensively utilized areas [*Donnison et al.*, 2004; *Harmel et al.*, 2010; *Liang et al.*, 2013; *Wagner et al.*, 2012].

3.2 Introduction

A number of factors influence runoff production from a watershed. These include soil type, porosity, organic matter content, slope, rainfall intensity, and land cover [*Ward and Elliot*, 1995]. Once runoff begins, *E. coli* is transported offsite and into surface waters [*Tyrrel and Quinton*, 2003].

Runoff is water that flows over land or through shallow soils and resurfaces down gradient [*Ward and Elliot*, 1995]. At small scales, runoff is strongly influenced by the soil's infiltration capacity [*Horton*, 1933] which is a complex process that is highly variable depending on site specific conditions and soil heterogeneity. Soil texture, particle size, bulk density, and the presence of preferential flow paths all impact water's ability to move into soils and they collectively determine available pore space in soil that water can fill. Antecedent moisture conditions influence water infiltration [*Dugas et al.*, 1998] by altering the amount of available pore space soil suction forces [*Ward and Elliot*, 1995]. When rainfall continues long enough to saturate soil, water ponds on or resurfaces and begins to run over the land as saturated overland flow [*Hewlett and Hibbert*, 1967]. Preferential flow paths can significantly impact runoff by rapidly transporting large volumes of water deep into the soil profile [*Allen et al.*, 2005]. Their presence can greatly alter local hydrology as realized infiltration rates often exceeds that of the soil and they may delay or preclude runoff production [*Harmel et al.*, 2006a].

Land use has considerable influence on runoff production since it determines the amount and type of cover present. Bare ground or impermeable surfaces generally produce large runoff volume compared to vegetated areas through reduced infiltration rates. Alternatively, vegetated areas produce less runoff due to improved soil structure, soil moisture utilization by plants, and the increased presence of macropores [*Pan et al.*, 2006]. Data collected and presented by *Bhark and Small* [2003] illustrate the enhanced effects that vegetation presence can have on rainfall infiltration into the soil profile.

Plant canopies can reduce infiltration in some rainfall scenarios. Some or all of a rainfall event can be intercepted by the canopy depending on its density and the rainfall intensity. In work evaluating interception losses in *Juniperus ashei, Owens et al.* [2004] found that up to 40% of annual rainfall was intercepted by the tree canopy and litter. Similarly, *Thurow et al.* [1987] determined annual interception rates of 25.4%, 18.1%, and 10.8% for Live oak mottes, midgrass, and shortgrass respectively. Rainfall intensity was a critical factor affecting rainfall interception by the plant community.

E. coli fate and transport from upland landscapes via surface runoff is largely driven by site specific characteristics. Primary factors controlling its loss are adhesion, filtration, physiological state of the cell, soil characteristics, water flow rate, predation, and cell motility [*Newby et al.*, 2009a]. Soil type and organic matter content impact cell adsorption, mechanical filtration, and subsequent bacteria transport [*Ferguson et al.*, 2003; *Newby et al.*, 2009a]. Clay particles and organic matter provide adsorption sites where *E. coli* adhere [*Maier and Pepper*, 2009] once they interact. Interaction occurs through diffusion (random interaction), active movement, or by active transport (cell

moves in solution). Following initial contact or when proximity is small enough, cell adhesion to particles can occur through electrostatic interactions, van der Waals forces, or hydrophobic interactions [*Newby et al.*, 2009a].

Soil particle size influences bacterial transport through pore space sizes. Soils with smaller pore spaces physically retain bacteria cells whose size exceeds pore space dimensions [*Newby et al.*, 2009a]. Cell health influences physical straining by altering cell size and shape. Ionic strength of the soil solution also impacts bacterial filtration through cell size alterations and soil pore space availability [*Newby et al.*, 2009a].

Hydrology is perhaps the most influential factor influencing bacteria transport from landscapes. Rainfall can dislodge soil and organic matter particles upon impact and translocates those particles when runoff occurs. Soil particle type influences dislodgement and transport as smaller tightly bound particles (clays) are not easily detached but are easily transported while larger, loosely bound particles (sands) are the opposite. Temperature, solar radiation, humidity and wind also impact erosion by changing evapotranspiration rates, soil moisture, and soil particle dislodgement. Vegetative cover influences erosion by diffusing rainfall impact, slowing runoff velocity, holding soil in place, improving soil health, and increasing transpiration rates. Slope also influences erosive potential [*Ward and Elliot*, 1995]. Bacterial adhesion to eroded particles allows them to remain attached and subsequently be transported off site.

Bacterial survival in soil is largely driven by the same suite of factors discussed previously for aquatic and sediment environments. Light, temperature, moisture, soil texture, nutrient availability, and pH all influence bacteria survival in soil along with

competition and predation from other soil microbiota [*Ishii and Sadowsky*, 2008]. Moisture content in soil can be quite different than large intestine or a saturated environment. In feces, common moisture contents range from 50 to 90% by weight [*Abe et al.*, 1999; *Graham et al.*, 1982; *Weber et al.*, 2002] while they are typically much less in soil thus exerting an external stress on *E. coli*. Low moisture conditions depress *E. coli* growth and result in net decay [*Berry and Miller*, 2005; *Gallagher et al.*, 2012; *Ishii et al.*, 2010; *Padia et al.*, 2012]; however, survival over relatively short periods of time (several days to months) seems less effected by dry conditions [*Ishii et al.*, 2010]. Frequent wetting and drying may actually enhance *E. coli* growth once more favorable moisture conditions return [*Solo-Gabriele et al.*, 2000].

Sunlight influences on soil and bacteria can drastically affect its survival. Sunlight affects soil temperature which can vary widely within single days and between seasons. Temperature variations can stress certain bacteria, and sunlight transmits ultraviolet radiation into the upper few centimeters of the soil [*Maier and Pepper*, 2009] which is detrimental to bacteria cells.

Nutrient availability in soils contributes to the harshness of soil environments for enteric bacteria. Fewer nutrients are available in soil than in feces thus growth potential is greatly reduced. This can cause cellular inactivation or prolonged starvation. Soil texture and organic matter content also influence nutrient availability. Soil pH can also influence bacterial survival; however, typical soil pH values range between 6 and 8 which are similar to some enteric environments [*Maier and Pepper*, 2009].

Land resource utilization by avian and mammalian wildlife is strongly related to the type, quantity, and quality of habitat present relative to species needs. *Morrison et al.* [2012] describes habitat as an area with a combination of suitable resources and environmental conditions that promotes species occupancy and allows for their survival and reproduction. Vegetation presence, type, and quantity plays a critical role in meeting the food and shelter needs of wildlife and considerably influences wildlife utilization. Human or seasonal vegetation alterations directly affect wildlife utilization [*Morrison et al.*, 2012]. This indirectly influences the type and quantity of fecal matter deposited in that area and may partly explain the differences in observed runoff *E. coli* concentrations from watersheds with varying land covers.

The objectives of this research were to evaluate the influences of land use and land cover on culturable *E. coli* generated in runoff and the upper 5 cm of soil from defined experimental watersheds. We hypothesized that differences in land use and land cover would result in significant differences in measured *E. coli* present in surface soils and transported off-site via runoff.

3.3 Materials and Methods

3.3.1 Experimental Design

Land use and land cover effects on culturable *E. coli* concentrations in upland soils and runoff from defined watersheds were evaluated on three field-scale watersheds at the U.S. Department of Agriculture, Agricultural Research Service (USDA-ARS)

Grassland, Soil, and Water Research Laboratory near Riesel, Texas. Land uses evaluated included remnant native prairie, managed hay pasture and cultivated cropland.

3.3.2 Watershed Descriptions

Watersheds are located approximately 3.2 km East of Riesel, TX on the border of Falls and McLennan counties. This facility was established in the late 1930s within the Brushy Creek watershed of the larger Brazos River basin. It is located in the Texas Blackland Prairie, and its soils consist solely of Houston Black clays. When wet, these soils are very slowly permeable but extensive crack formation that creates preferential flow paths under dry conditions occurs. Reported mean annual rainfall ranges from 850 to 910 mm [*Allen et al.*, 2005; *Arnold et al.*, 2005; *Harmel et al.*, 2006a].

3.3.2.1Remnant Native Prairie (SW12)

SW12 is a 1.2 ha remnant native prairie watershed with 3.8% average slope that is located within a larger 9 ha remnant prairie pasture. Management has been consistent since 1948 [*Harmel et al.*, 2006a] and includes annual mowing or haying interspersed with intermittent herbicide treatments (management data available online at: www.ars.usda.gov/spa/hydro-data).

3.3.2.2 Managed Hay Pasture (SW17)

SW17 is a 1.2 ha managed hay pasture with 1.8% slope [*Harmel et al.*, 2006a] situated within a larger 1.72 ha hay pasture. The pasture has been Coastal bermudagrass

for more than 50 years and has predominantly been hayed. Cattle were grazed on the site from 2000 to 2010, and poultry litter applications at a rate of 6.8 Mg/ha (3 tons/ac) occurred in 2011 and 2012 (management data available online at: www.ars.usda.gov/spa/hydro-data).

3.3.2.3 Cultivated Cropland (Y6)

Y6 is a 6.6 ha, terraced, conventionally cultivated cropland watershed with a 3.2% average slope (Harmel et al., 2006) that has been continuously cropped since 1943. Crops produced include clover, cotton, corn, hay grazer, oats, sorghum, sudangrass, and wheat. The plot also received intermittent fertilizer and herbicide treatments as needed (management data available online at: www.ars.usda.gov/spa/hydro-data).

3.3.3 Sampling Procedures

3.3.3.1 Soil Sampling Technique

Soil samples were collected from each watershed during four sampling events. Collection occurred along transects within each watershed extending upslope from the flow control structure inlet to the watershed border. Sampling locations were selected to capture the variability of conditions within each watershed (e.g., under grasses, between bunch grasses, atop terraces, within terrace benches, within the grassed waterway, etc.).

Leaf litter or crop residue was removed from the soil surface when present. Soil samples were taken to a depth of approximately 5 cm with a 7.62 cm diameter soil sampling probe. Between individual sample collections, residual soil was removed from

the soil probe, sprayed with 200-proof ethanol, and flared with a propane torch. Latex gloves were worn to remove collected samples from the probe and placed into sterile 710 mL Whirl-Pak® bags (Nasco, Fort Atkinson, WI). Gloves were changed between samples to prevent cross contamination. Sample bags were labeled with the watershed and sample number then placed in an iced cooler and transported to the Soil and Aquatic Microbiology Laboratory (SAML) at Texas A&M University for immediate analysis.

3.3.3.2 Runoff Sample Collection

Overland flow from each watershed was collected using ISCO Avalanche refrigerated auto-samplers (Teledyne-ISCO, Inc., Lincoln, NE) maintained and operated by USDA-ARS personnel. Samplers were co-located with appropriately sized flow control structures and set to collect a 200 mL sample for each 1.32 mm of runoff from each watershed (calculated volumetrically). Samples were composited into sterile, 16L HDPE bottles to produce flow–weighted, composite samples for each event. Upon cessation of flow, or before sample holding times were approached, sample bottles were retrieved from the field. Subsamples were poured into labeled 532 mL Whirl-Pak® bags from the composite bottles following thorough mixing. Samples were held in a refrigerator until delivery on ice to SAML for analysis. Runoff volume was determined by recording water depth in flow control structures using ISCO 730 Bubbler Flow Modules and established stage-discharge relationships [*Harmel et al.,* 2014]. SW12 and 17 are equipped with 0.91 m (3 ft.) H-flumes, and Y6 is fitted with a combination v-notch weir and Parshall flume that allows small and large runoff event measurements.

3.3.4 Analytical Methods

Runoff and soil samples were processed at SAML to enumerate and isolate *E. coli* using USEPA Method 1603. This membrane filtration method utilizes modified membrane-Thermotolerant *E. coli* agar (mTEC) and a 24±2 hour incubation period [*USEPA*, 2006]. Aliquots of appropriate volume were processed from water samples, and results were reported as cfu/100 mL. Soil samples were prepared for analysis by placing 10g of soil into sterile specimen cups containing 90 mL of PBS. Aliquots of appropriate size were processed, and results were reported as cfu/g_{wet} of soil.

3.3.5 Statistical Methods

Data analyses were conducted to identify statistically significant differences within mean and median *E. coli* concentrations and loads in water and soil, sediment concentrations and loads, and runoff volumes between watersheds. Data were non-normally distributed based on Kolmogorov-Smirnov test results; therefore, the non-parametric Kruskal-Wallis test was used to identify the presence of statistically significant differences in median values between the three watersheds. Mann-Whitney tests were identified specific watershed pairs with significantly different medians. Regression analysis R^2 values and p-values produced with a one-way ANOVA depicted how well *E. coli* correlated to other measures. To determine significance, α=0.05 was used, thus p values $\leq$0.05 were considered statistically significant. All statistical analyses were conducted using Minitab 17 software (Minitab Inc., State College, PA). Boxplots were created using SigmaPlot 13 software (Systat Software Inc., San Jose, CA).

3.4 Results and Discussion

Runoff *E. coli* concentrations varied widely as observed in similar studies [*Harmel et al.*, 2013; *Harmel et al.*, 2010; *Wagner et al.*, 2012]. Most measured concentrations were relatively low compared to observed maximums (Table 3.1). Standard deviations were at least 50% larger than means, and the range of concentrations spanned three orders of magnitude or more. Soil *E. coli* (cfu/g_{wet}) results contained similar variation with standard deviations at least 75% larger than mean concentrations and a two to three order of magnitude data range (Table 3.2). One considerable finding is the quantity of soil samples where *E. coli* were not detected. Only 15.6 to 33.3% of the samples collected in each watershed (n=51) contained culturable *E. coli*.

Table 3.1. Descriptive statistics for *E. coli* in runoff (cfu/100 mL)

Statistics	SW12	SW17	Y6
n	26	15	22
Mean	8,719	14,252	14,920
Median*	960[a]	5,950[b]	5,400[b]
StDev	31,069	21,141	31,283
Minimum	160	20	80
Maximum	160,000	80,000	150,000

***** values sharing the same letter are not significantly different (α=0.05)

Table 3.2. Descriptive statistics of *E. coli* in soil samples (cfu/g_{wet})

Statistics	SW12	SW17	Y6
n	51	51	51
n with Culturable *E. coli*	14	17	8
Mean	22.8	50.1	13.6
Geometric Mean	13.9	15.7	10.8
Median*†	10[a]	10[a]	10[b]
StDev	47.5	156.2	23.8
Minimum	10	10	10
Maximum	335	1,065	180

***** values sharing the same letter are not significantly different (α=0.05)
† significant differences on data adjusted for ties; medians not adjusted

3.4.1 Land Use and Land Cover Effects on *E. coli* Concentration in Runoff and Soil

Descriptive statistics presented and visual analysis (Table 3.1, Figure 3.1, a) suggest the presence of different median runoff *E. coli* concentrations among watersheds. Mann-Whitney testing provides significant evidence that the median *E. coli* concentration from SW12 is different from SW17 and Y6 ($p=0.05$ and <0.01 respectively) while SW17 and Y6 are statistically similar ($p=0.9$).

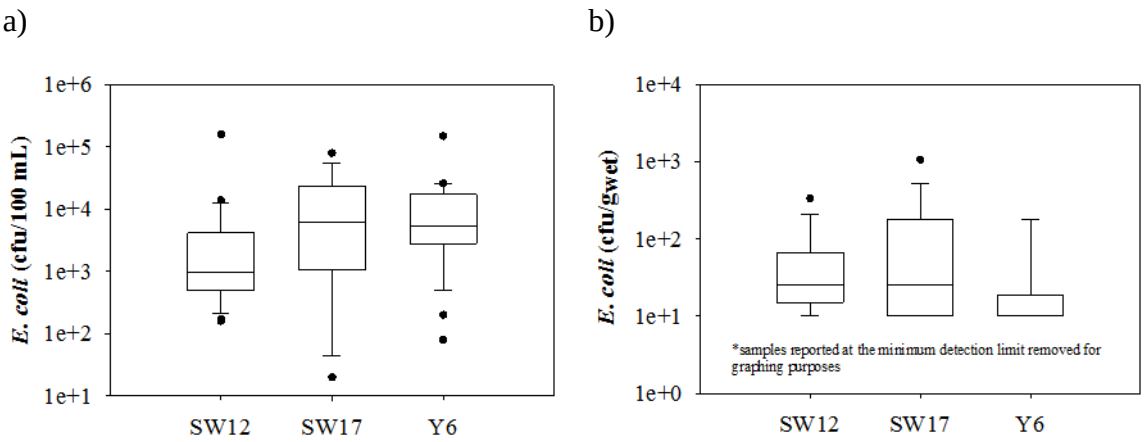

Figure 3.1. *E. coli* concentrations from evaluated watersheds: a) runoff; b) soil

Similar to runoff, potential differences in soil *E. coli* concentrations are depicted through data summary statistics and graphical evidence (Table 3.2, Figure 3.1, a). However, the large number of samples yielding no culturable *E. coli* were reported at the detection limit (10 cfu/g_{wet}) and complicated analysis. These samples were removed for graphing purposes and were accounted for during statistical analysis. Kruskal-Wallis testing did not suggest significant differences in median *E. coli* concentrations ($p=0.27$); however, when results were adjusted for tied values, a potential difference was identified

(p=0.04). Mann-Whitney testing produced similar results with insufficient evidence to reject the null hypothesis of equal median values (p=0.87, 0.12, 0.22). Adjusted for ties, the presence of significant differences in median values was noted for the SW12-Y6 (p=0.01) and SW17-Y6 (p=0.03) pairs while SW12 and SW17 remained similar (p=0.82). Given the large number of minimum reporting limit values included and small sample size, these results should be interpreted cautiously.

Land use and land cover was found to significantly effect runoff *E. coli* concentrations in other studies. *Harmel et al.* [2013] found significant differences between three of the same watershed land uses. Cultivated fields (some receiving composted poultry litter) and hayed pastures (with intermittent rotational grazing) were found to have significantly lower median *E. coli* concentrations than the ungrazed, native prairie (SW12). These finding contradict results from this study and illustrate spatial and temporal variability of runoff *E. coli* concentrations. Other similar studies found that forested land use produced significantly lower concentrations than urban land uses [*Goto and Yan*, 2011b; *Meneses et al.*, 2015]. *Strauch et al.* [2014] also evaluated forest, agriculture and urban land uses in Hawaii and found that forested watersheds produced lower *E. coli* concentrations than agricultural (cultivated) or urban watersheds. In work that incorporated livestock grazing, *Harmel et al.* [2010] found significantly lower *E. coli* concentrations in cultivated cropland runoff than pastures alone or pastures and cropland combined. However, the effects of grazing seem to outweigh those of land use since no significant differences in *E. coli* concentration were found in runoff from the grazed pasture or pasture and cropland.

In soils, research shows considerable *E. coli* variation but suggests the influences of land use on concentrations. In Hawaii, *Goto and Yan* [2011b] reported *E. coli* concentrations in soil ranging from 6 to 18,200 (cfu/g_{dry}) and suggested that soils from urban land uses have less *E. coli* than forested soils despite the lack of significant differences identified. Alternatively, *Byappanahalli and Fujioka* [2004] found *E. coli* concentrations ranging from 24 to 294 (cfu/g_{dry}) in upland, ungrazed grassland. In temperate environments with moisture conditions similar to those in this study, *E. coli* concentrations reportedly ranged from 1 to 20,800 (cfu/g_{dry}) with reported mean concentration of 25 (cfu/g_{dry}) [*Byappanahalli et al.*, 2006]. Despite differences in site specific conditions and maximum *E. coli* concentrations reported, results from this study exhibit similar trends in variability and also demonstrate an overall lack of significant differences due to land use. Only when tied values within the dataset were adjusted, did potential significant differences surface.

While this study compared different land uses than many of the referenced studies, results were generally similar. Land use intensity in referenced studies (grazing pressure or human use) was commonly identified as a significant factor in observed runoff *E. coli* concentration differences. Results suggest that *E. coli* contributions from wildlife, or background sources, are lowest in SW12 and approximately equal in Y6 and SW17. Wildlife occurrence data collected during the runoff sampling period and reported by *Gregory et al.* [2015] refutes this suggestion. Passive infrared cameras recorded approximately twice as many wildlife occurrences at SW12 (n=920) than SW17 (n=420) or Y6 (n=526). This assessment is not a perfect representation of actual

wildlife usage because the number of individual animals using each watershed was not recorded. However, photos do provide evidence indicating the usage level of each watershed. This suggests the presence of other factors that influence *E. coli* concentrations in runoff from these watersheds.

3.4.2 Effects of Land Use and Land Cover on Runoff Generation and *E. coli* Loads

Runoff volume and *E. coli* concentration where combined to generate an *E. coli* load. As a result, differences in runoff production and *E. coli* concentration between watersheds can influence *E. coli* loads generated from each watershed. To compare runoff produced from each watershed on equal terms, total runoff volume collected was converted to a volumetric depth (mm) over the watershed area. Visual analysis and descriptive runoff volume statistics from each watershed illustrate variability in the data (Figure 3.2, a; Table 3.3); however, obvious differences in means and medians not are discernable. The Kruskall-Wallis test did not provide sufficient evidence to reject the null hypothesis of equal medians between watersheds ($p=0.4$), and the Mann-Whitney test confirmed equal medians between each group with p-values of 0.36, 0.91, and 0.14 respectively for watershed pairs SW12-SW17, SW12-Y6, and SW17-Y6.

Despite statistically similar runoff volumes between land uses not agreeing with other findings [*Kosmas et al.*, 1997; *Pan and Shangguan*, 2006], abnormally wet conditions for a portion of the study period justify this disagreement. Beginning in December 2014, frequent rain events occurred and continued through the end of data collection in May 2015. As a result >65% of runoff events in each watershed occurred in

the last six months of data collection when soil moisture content was well above normal. In early work at the Riesel watersheds conducted by *Baird* [1948], a similar lack of difference in runoff production between land uses was noted during above average moisture conditions. *Harmel et al.* [2006a] also reported similar findings in a long-term soil loss and runoff evaluation during above average moisture conditions.

Table 3.3. Descriptive statistics for runoff event volume (mm) and *E. coli* loads (cfu/ha) produced from each watershed

	Statistics	SW12	SW17	Y6
Runoff Volume (mm)	n	26	15	22
	Mean	22.4	39.4	18.7
	Median*	10.6[a]	15.1[a]	9.3[a]
	StDev	38.0	17.3	23.3
	Minimum	1.3	1.3	1.3
	Maximum	190.5	314.9	82.0
***E. coli* Load (cfu/ha)**	Mean	7.21E+09	2.93E+10	1.80E+10
	Median*	1.47E+09[a]	5.50E+09[b]	5.71E+09[b]
	StDev	1.44E+10	3.75E+10	3.16E+10
	Minimum	4.93E+07	7.66E+07	1.59E+08
	Maximum	6.28E+10	1.06E+11	1.36E+11

***** values sharing the same letter are not significantly different (α=0.05)

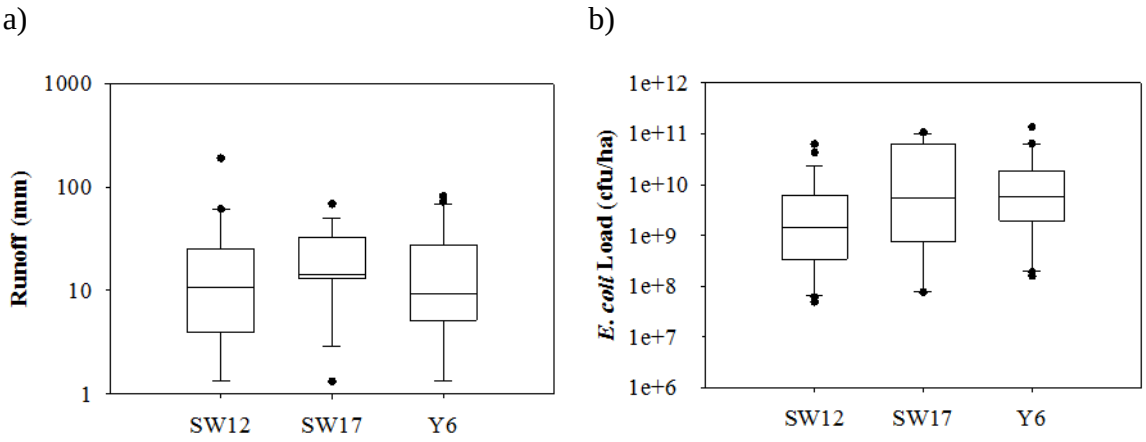

Figure 3.2. Runoff event volume and *E. coli* loads from each watershed. a) runoff volume (mm) and b) *E. coli* load (cfu/ha)

Calculated *E. coli* loads were evaluated to identify potential differences between watersheds. Descriptive statistics and visual *E. coli* loads analysis suggests potential differences in median loads (Figure 3.2, b; Table 3.3). Significantly different median *E. coli* loads were found between the watersheds based on Kruskal-Wallis testing ($p=0.04$). Mann-Whitney testing produced sufficient evidence to reject the null hypothesis of equal medians between SW12 and SW17 ($p=0.05$), and SW12 and Y6 ($p=0.03$), but not SW17 and Y6 ($p=0.79$). These findings and identified differences in *E. coli* concentrations suggest that *E. coli* concentrations have a stronger influence than runoff volume on *E. coli* loads. Graphic evidence supports this statement as the largest runoff volumes do not necessarily produce the largest *E. coli* loads (Figure 3.3). Alternatively, the highest observed *E. coli* concentrations do yield some of the largest observed loads.

In these same watersheds, *Harmel et al.* [2013] identified conflicting results. In their findings, cultivated land uses (inclusive of watershed Y6) and hayed pastures produced significantly lower *E. coli* loads than native prairie (SW12). Inter-annual variability was observed between mean and median *E. coli* loads. Disparate *E. coli* loads were reported by *Wagner et al.* [2012] on watersheds SW12 and SW17 between 2008 and 2010 during prescribed grazing trials. Median *E. coli* loads for SW12 (ungrazed control) were significantly larger than those of SW17 (treatment); however, median loads at SW12 were found to be statistically similar, lower, and higher than those from SW17 within the three monitoring years.

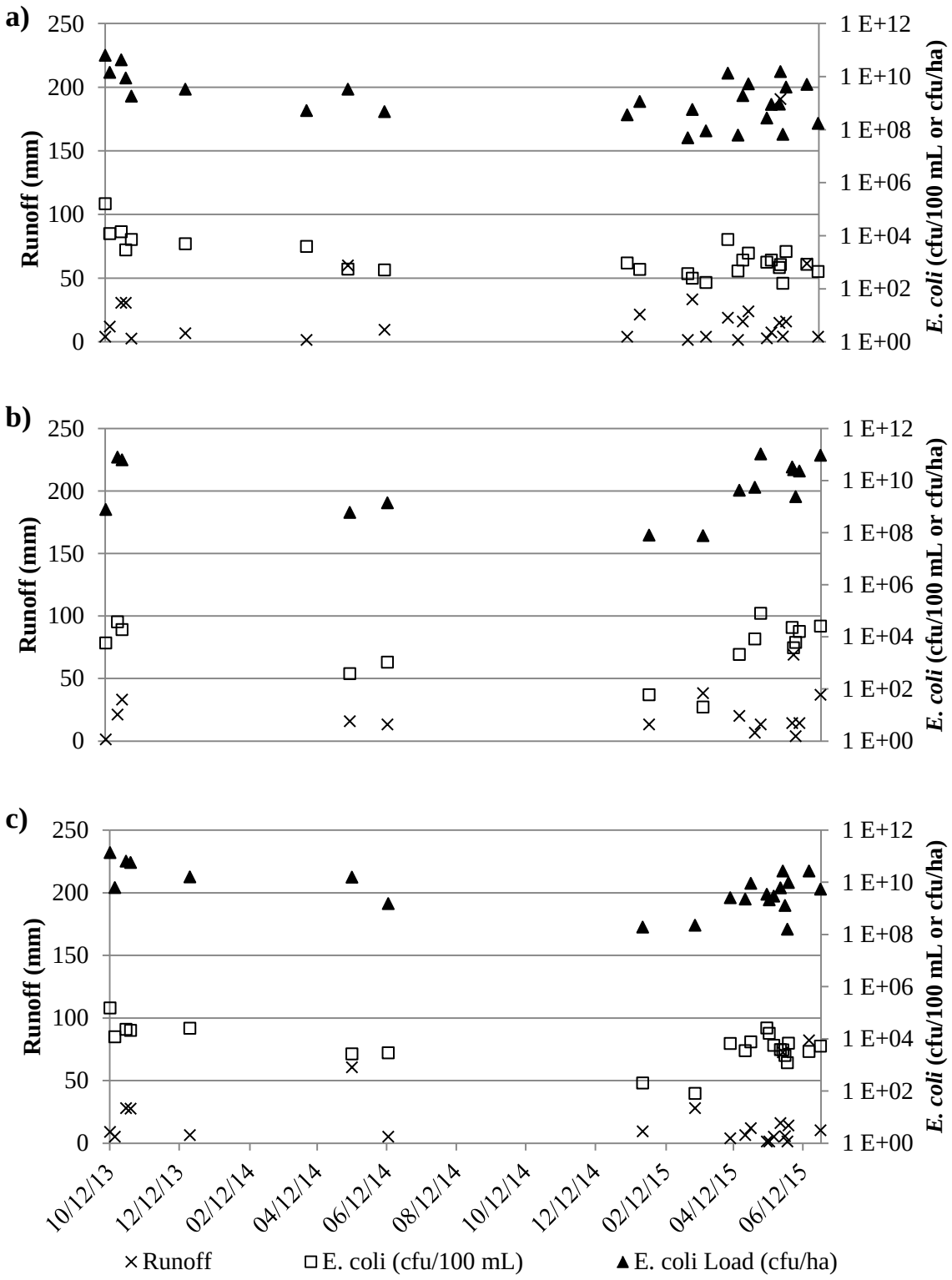

Figure 3.3. Runoff volume, *E. coli* concentrations, and loads from each watershed. a) SW12, b) SW17, c) Y6

3.4.3 Land Use and Land Cover Effects on Sediment Concentration and Loading and Its Relationship to *E. coli* Concentration in Runoff

Sediment in runoff has been implicated as a possible contributor to runoff *E. coli* concentrations because *E. coli* readily attaches to sediment particles [*Ferguson et al.*, 2003; *Muirhead et al.*, 2006]. Sediment concentrations and loads were evaluated to determine the presence of differences between evaluated watersheds. Visual data analysis (Figure 3.4) and descriptive statistics (Table 3.4) suggest the presence of differences in mean and median sediment concentrations and loads between watersheds. Stark temporal differences in ground cover in these watersheds produced an expectation that differences in sediment loading and concentrations did exist.

Table 3.4. Descriptive statistics for sediment concentration and loads from each watershed

	Statistics	SW12	SW17	Y6
Sediment Concentration (mg/L)	n	26	15	22
	Mean	100.5	108.9	357.8
	Median*	54.1[a]	82.2[a]	269.5[b]
	StDev	114.1	72.9	278.3
	Minimum	6.8	28.9	37.0
	Maximum	435.5	224.2	1,017.8
Sediment Loads (kg/ha)	Mean	47.4	18.0	76.0
	Median*	4.9[a]	14.4[b]	22.3[b]
	StDev	160.7	15.2	157.6
	Minimum	0.1	2.7	1.9
	Maximum	818.3	56.7	738.0

***** values sharing the same letter are not significantly different (α=0.05)

Data were non-normally distributed according to Kolmogorov-Smirnov testing, so the Kruskal-Wallis test was applied to test for differences in median sediment loads and concentrations. Sufficient evidence to reject the null hypothesis of equal medians

(p=0.00) was found for sediment concentrations. Median sediment concentrations from Y6 were significantly higher than SW12 (p=0.00) and SW17 (p<0.01) as indicated by Mann-Whitney testing. Concentrations for SW12 and SW17 were found to be statistically similar (p=0.23). Sediment loads produced different results. A Kruskal-Wallis test provided evidence of significantly different median sediment loads (p<0.01); however, Mann-Whitney testing indicated that median sediment loads from SW12 was significantly lower than that of SW17 (p=0.04) and Y6 (p<0.01) while SW17 and Y6 were statistically similar (p=0.1).

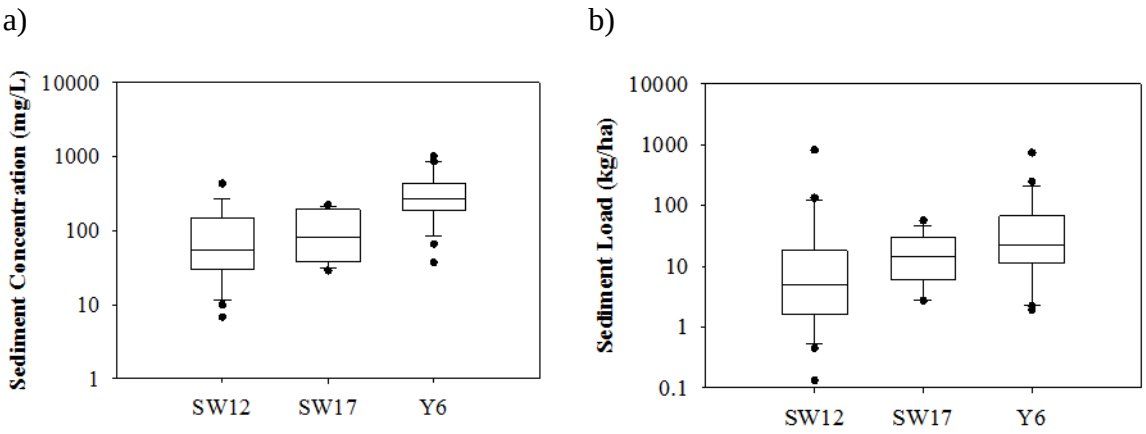

Figure 3.4. Sediment concentration and load in runoff from each watershed. a) sediment concentration and b) sediment load

Relationships between *E. coli* and sediment concentrations and loads were explored to reveal potential connections. Scatter plots of sediment concentration and loads (Figure 3.5, a and b) illustrated the presence of a potential relationship between *E. coli* and sediment concentrations. *E. coli* concentrations in SW12 and Y6 appear to have a positive relationship with sediment concentration while SW17 does not exhibit an

obvious connection. Data were normally distributed according to Kolmogorov-Smirnov testing, so an ANOVA was applied to $\log_{10}$ transformed *E. coli* and non-transformed sediment concentrations and suggested a significant association for SW12 ($p=0.05$) but not SW17 ($p=0.96$) or Y6 ($p=0.85$). Relationships between sediment loads and *E. coli* concentration were variable. SW12 exhibited a positive relationship between the two while those at Y6 and SW17 were negative and neutral respectively. An ANOVA performed on $\log_{10}$ transformed *E. coli* and sediment loads concentrations yielded p-values of 0.15, 0.81, and 0.46 respectively for SW12, SW17, and Y6 and did not provide sufficient evidence to suggest the presence of significant relationships.

Relationships between sediment and runoff volume were explored. Visual analysis demonstrates the variable sediment concentration response to runoff volume while sediment loads are positively affected by runoff volume in all watersheds (Figure 3.5, c and d). Log_{10} transformed runoff volume, sediment concentrations and loads were normally distributed according to Kolmogorov-Smirnov tests. ANOVA results comparing sediment concentrations to runoff volume did not provide sufficient evidence to suggest significant relationships at SW12 ($p=0.24$), SW17 ($p=0.09$), or Y6 ($p=0.81$). However, substantial evidence was provided for relationships between sediment load and runoff from SW12 ($p=0.00$), SW17 ($p<0.01$), and Y6 ($p=0.00$) to be considered significant.

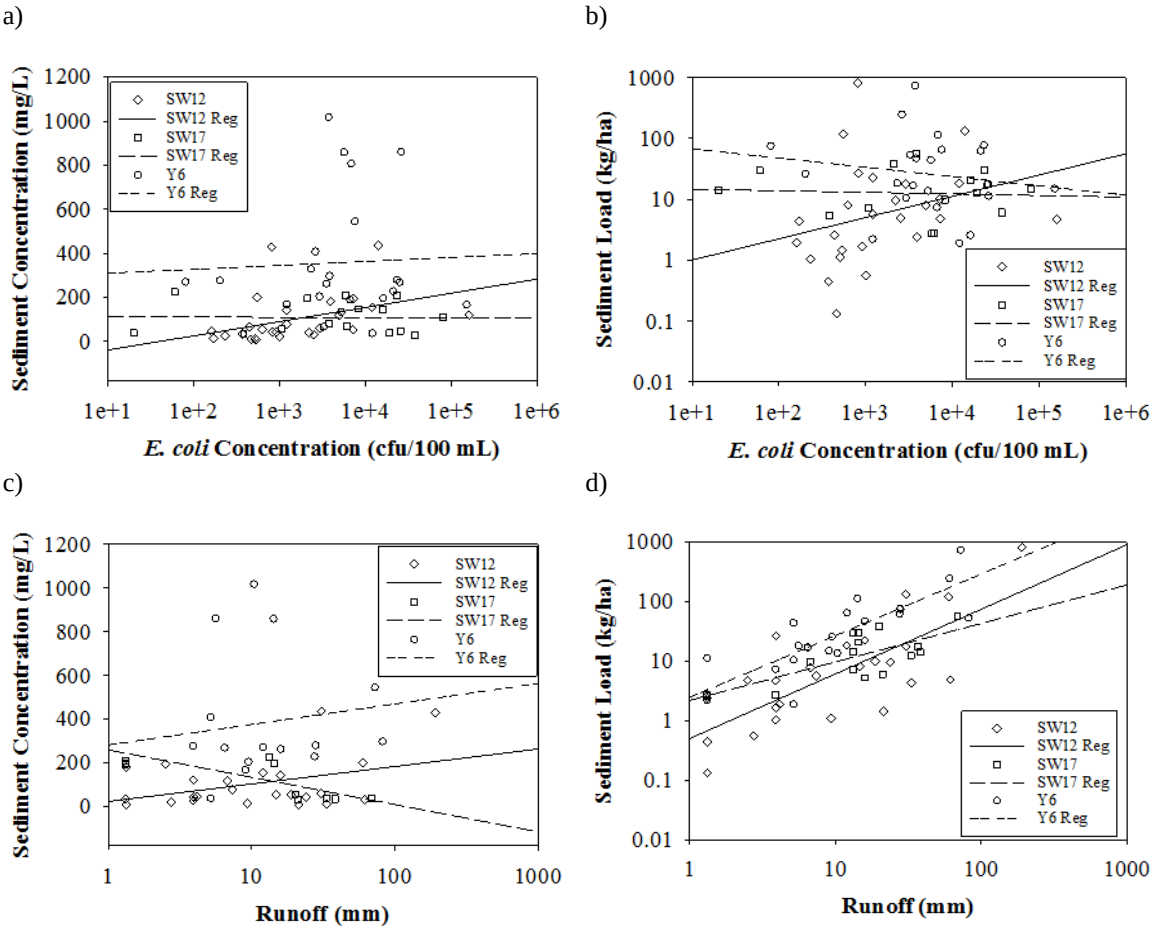

Figure 3.5. Relationships among sediment concentration and loads, *E. coli* concentration and runoff volume. a) sediment concentration vs. *E. coli* concentration by site; b) sediment loads vs. *E. coli* concentration by site; c) sediment concentration vs. runoff by site; d) sediment load vs. runoff by site

Median sediment concentrations from Y6 were significantly higher than those from continually vegetated watersheds (SW12 and SW17) as expected due to temporal variations in ground cover and intermittent soil disturbances. Sediment loads followed similar trends with the median values from Y6 being larger than both SW12 and SW17, but they deviated in that SW17 produced a median load that was statistically similar to Y6. This is possibly due to differences in crops on Y6 (oats vs corn) alone or the

combination of runoff volume, runoff event timing, and rainfall intensity in relation to applied management that temporarily modified land cover density or presence during the evaluation period as suggested by *Harmel et al.* [2006a]. Runoff volume provides evidence to justify this similarity in median sediment loads. Lower mean and median runoff volume occurred at Y6 than other watersheds despite the statistical similarity. *Miller et al.* [2015] found similar results in runoff from native Canadian prairie plots. They demonstrated that the presence and quantity of vegetation did not significantly affect sediment or *E. coli* concentrations leaving the plots but did significantly reduce the loads of each constituent.

Research describing forces and factors controlling runoff production and sediment yield at the field scale [*Lane et al.*, 1997] illustrates the significant effects of grass cover on sediment yield compared to non-vegetated areas [*Pan et al.*, 2006] or decreased vegetative density [*Allen et al.*, 2011; *Pan and Shangguan*, 2006] and provides support for results at this site. Measured sediment concentration and runoff volume from each watershed suggest that runoff production has a stronger influence on sediment load than sediment concentration. This finding is dissimilar to *E. coli* where concentration was a more important factor in total loading. Combined, these findings suggest a weak relationship between sediment and *E. coli* concentrations in the generation of *E. coli* loads. Results collected largely support this suggestion; however, a significant relationship between the two at SW12 does not. This further demonstrates the variability of *E. coli* in the environment.

3.4.4 Runoff and Soil *E. coli* Relationships

E. coli are known to attach to sediment particles in runoff [*Ferguson et al.*, 2003; *Muirhead et al.*, 2006] yet many remain unattached [*Tyrrel and Quinton*, 2003]. This suggests that soil bound *E. coli* may represent a sizable portion of the overall runoff *E. coli* load [*Solo-Gabriele et al.*, 2000]. Data collected were used to estimate the potential for soil derived *E. coli* to contribute to runoff *E. coli* loads. Direct comparisons were not possible since soil samples were only collected during four events while runoff was collected between 15 and 26 times. Therefore, sediment loads from individual runoff events were multiplied by the geometric mean of soil *E. coli* concentrations within each watershed to generate an indirect sediment *E. coli* load estimate. Descriptive statistics for measured runoff *E. coli* loads and those estimated in soil show clear differences in the magnitude of the respective loads among watersheds (Table 3.5). Mann-Whitney testing provided strong evidence that soil *E. coli* loads are significantly lower than runoff loads ($p<0.01$ within all watersheds).

Table 3.5. Estimated soil and measured runoff *E. coli* loading statistics

Statistics	**SW12**		**SW17**		**Y6**	
(cfu/ha)	**Runoff**	**Soil***	**Runoff**	**Soil***	**Runoff**	**Soil***
n	26	51	15	51	22	51
Mean	7.21E+09	6.56E+05	2.93E+10	2.83E+05	1.80E+10	8.22E+05
Median**	1.47E+09[a]	6.78E+04[b]	5.50E+09[a]	2.26E+05[b]	5.71E+09[a]	2.41E+05[b]
StDev	1.44E+10	2.23E+06	3.75E+10	2.39E+05	3.16E+10	1.70E+06
Minimum	4.93E+07	1.85E+03	7.76E+07	4.31E+04	1.59E+08	2.07E+04
Maximum	6.28E+10	1.13E+07	1.06E+11	8.91E+05	1.36E+11	7.98E+06

* soil loads calculated using geometric mean of soil *E. coli* from all samples within each site and the measured sediment load from each runoff event

****** values sharing the same letter within each watershed are not significantly different ($\alpha=0.05$)

Results suggest that soil within watersheds was not a major runoff *E. coli* source. This contradicts previous work where soil was found to contain considerably larger quantities of *E. coli* than water [*Fujioka et al.*, 1998; *Goto and Yan*, 2011b]. Instead, results suggest that recent fecal depositions are the primary *E. coli* contributor. This casts some doubt on the theory that fecal derived *E. coli* can become naturalized inhabitants of the soil community in these watersheds. Instead, *E. coli* naturalization into the soil suggested by others [*Byappanahalli et al.*, 2012a; *Byappanahalli et al.*, 2012b; *Ishii et al.*, 2006] may be a site specific phenomenon. Variations in environmental conditions (temperature and moisture content) in evaluated watersheds most likely influenced *E. coli* survival. Timing between soil and runoff sample collections complicates this assessment and adds to the considerable uncertainty present in runoff sampling [*Harmel et al.*, 2006b; *McCarthy et al.*, 2008] and soil samples [*Maier and Pepper*, 2009]. Additional data collection and analyses to support this claim are needed.

3.5 Summary and Conclusions

Results demonstrate that land use and land cover can affect runoff *E. coli* concentration and loads. Ungrazed native prairie (SW12) exhibited a significantly lower median *E. coli* concentration than managed hay pasture (SW17) and cultivated cropland (Y6). SW12 typically had more ground cover and a vibrant plant community which suggests that it can naturally attenuate more pollutant load than other watersheds. This contrasts earlier work conducted at this site used additional land uses and attributed high runoff *E. coli* concentrations and loads to increased wildlife populations [*Harmel et al.*,

2013]. Similar data variability in each study emphasizes the common difficulty of identifying a consistent *E. coli* response to environmental factors in natural systems.

Determining reasons for *E. coli* concentration variations between watersheds has proven difficult for researchers, and this project was no different. Multiple factors influence *E. coli* and other pollutants transport off-site during runoff events [*Blaustein et al.*, 2016; *Cardoso et al.*, 2012; *Collins et al.*, 2005; *Harmel et al.*, 2010; *Oliver et al.*, 2015; *Wagner et al.*, 2012]. In this evaluation, differences in *E. coli* loadings appear most related to runoff *E. coli* concentration and watershed land use and land cover.

Soil did not harbor a large number of *E. coli* in any watershed. Sediment loads and soil *E. coli* concentrations enabled rough soil borne *E. coli* contributions to runoff *E. coli* loads to be estimated. Calculated median *E. coli* contributions accounted for only 0.0041 to 0.0046% of the total runoff *E. coli* load providing strong evidence that soil is not a major source of runoff *E. coli* from these watersheds. Rather, this suggests that fecal deposition occurring in each watershed is largely responsible for *E. coli* loading.

Collectively, these results further illustrate *E. coli* variability in the environment and demonstrate the challenges faced in managing its loading to downstream waters. Land use and land cover effects influence *E. coli* delivery off-site; however, other factors also contribute to *E. coli* loading. These findings demonstrate that background sources of *E. coli* can contribute sizable loads and in some cases, astounding *E. coli* concentrations in runoff. However, managing these sources of *E. coli* to reduce instream loads remains challenging. Applying management practices to retain rainfall onsite present a viable suite of tools for reducing *E. coli* loading but are certainly not a ubiquitous solution.

CHAPTER IV

DIFFERENCES IN *E. COLI* SOURCE COMPOSITION OF RUNOFF AND SOIL FROM MULTIPLE WATERSHEDS

4.1 Overview

Fecal contamination sources were historically identified through sanitary source surveys, but this approach does not yield accurate information regarding fecal loading distribution to waterbodies. More recently, bacterial source tracking (BST) has been used to identify bacteria sources in surface water. It provides sound evidence that illustrates *E. coli* source categories, but it too fails to describe fecal loading distributions. BST results produced at sub-watershed scales improve source distribution information, but remain incomplete. Variation in source distribution across landscapes and within land cover types is not clearly understood. This research expands BST application to upland soils and edge of field runoff to evaluate its ability to identify *E. coli* sources at the micro-watershed scale. Connections between source and quantity of *E. coli* in soil and runoff were evaluated and the differences between these associations of various land cover types were explored.

4.2 Introduction

Fecal indicator bacteria are the largest single cause of water body impairments in the United States [*USEPA*, 2015]. In accordance with Section 303(d) of the Clean Water Act, States are required to identify impaired waters and establish management strategies

such as total maximum daily loads (TMDL) to restore water quality [*USEPA*, 2009]. TMDLs define the maximum amount of pollutant that a waterbody can receive and still meet its designated water quality standard. This ultimately drives management decisions in the watershed [*USEPA*, 1991] that focus on preventing pollutant ingress to the waterbody. Generally, bacteria loads are differentiated between point and nonpoint sources with relative ease. Identifying specific bacteria sources and load contributions is more challenging and often poorly understood [*He et al.*, 2007]. Thus, other methods are required to determine *E. coli* source contributions in environmental samples.

Local knowledge of contributing sources and conducting sanitary source surveys are a common first step in source identification and are recommended to develop a basic understanding of *E. coli* contributors in a watershed [*Jones et al.*, 2009]. Population estimates available for livestock and some wildlife allow reasonable estimates of their respective *E. coli* contributions to be developed. However; many species in a watershed that contribute to the overall *E. coli* load are not surveyed nor are their fecal loading rates well understood. Larger animal species are assumed to contribute the bulk of *E. coli* in the overall observed load due to fecal production volume; however, sufficient data do not exist to support this claim and relative *E. coli* contributions to the overall load from species present remain unknown. Solely applying a source characterization approach leaves watershed managers with knowledge gaps regarding *E. coli* loading and caused the use of other source identification efforts and loading quantification methods.

Edge-of-field runoff studies targeting specific source contributions are one approach utilized to provide needed loading information. *Harmel et al.* [2010] and

Wagner et al. [2012] evaluated runoff *E. coli* loads from small watersheds with and without cattle grazing. Findings confirmed significantly higher runoff *E. coli* loading from stocked fields and illustrate the loading influence of this known source. *E. coli* loads from control sites with no grazing were still large and demonstrate the potential effects of *E. coli* loading from unidentified sources. *Harmel et al.* [2010] noted the need for improved *E. coli* source understanding to improve management and *Wagner et al.* [2012] deduced that contributing sources at ungrazed sites include rodents, birds, other wildlife, and naturalized *E. coli* present in the soil.

BST is an alternate source identification approach that provides the ability to identify *E. coli* sources with increased specificity. BST encompasses a suite of methods that identify specific characteristics of target organisms within environmental samples. These characteristics are assumed to directly relate to a host species or category (e.g., livestock, wildlife, etc.) [*Field and Samadpour*, 2007]. Multiple methods exist and no single approach is viewed as the best; however, the science continues to evolve and improve [*Dick et al.*, 2010; *Field and Samadpour*, 2007]. Generally, BST methods are divided into genotypic or phenotypic approaches. Genotypic methods utilize molecular techniques to create DNA fingerprints based on organism-specific DNA sequences while phenotypic approaches measure an expressed trait of the organism [*USEPA*, 2005]. These techniques are further divided into library-dependent and library-independent approaches.

Library-dependent techniques require development of a database consisting of bacteria DNA fingerprints from known hosts [*Stoeckel and Harwood*, 2007; *USEPA*,

2005]. To identify bacteria sources in an environmental isolate, bacteria are cultured, isolated, and fingerprinted. DNA fingerprints from environmental isolates and known sources are then compared to identify sources [*Stoeckel and Harwood*, 2007]. Library-dependent methods can identify specific source categories and sometimes specific source species which can produce quantitative results; however, caution is necessary when interpreting results due to potential classification inaccuracies. Improvements in classification validation, such as jackknife analysis and challenge sampling improve predictive capabilities, but these approaches must be rigorously utilized to ensure the effective use of this approach [*Stoeckel and Harwood*, 2007]. These issues can be compounded by sampling design, representativeness of the fecal loading pool, and inclusion of sufficient temporal and spatial variability within the library. Cost constraints typically restrict the number of known sources and environmental isolates collected and processed. This can induce selective pressure on bacteria present, thus the potential for species represented in an environmental sample to not be identified is considerable [*Field and Samadpour*, 2007].

Alternatively, library-independent methods do not require known-source library development. These methods detect genetic markers associated with known fecal contamination sources [*Stoeckel and Harwood*, 2007]. Numerous markers exist and others are in development; however, not all species are represented by available markers. Library-independent approaches detect the presence or absence of genetic markers in a sample, and do not discriminate between live or dead cells. Results are not quantitative. Analysis speed is greater than library-dependent methods since no culturing is required.

This prevents unintentional inclusion of selection pressure from the culturing process. Confidence in the ability to detect pollution source presence in a sample is high if marker specificity and sensitivity are high; however, false positives and negatives do occur. Additionally, the correlation between developed markers, FIB, and pathogens is not well established and diminishes the utility of these markers to relate detected fecal pollution presence to human health risk [*Field and Samadpour*, 2007].

No BST method is superior to all other methods [*USEPA*, 2005] and no single method is preferred by regulators since questions remain regarding potential temporal or spatial variations in genetic diversity [*Gerba*, 2009]. Thus the decision to utilize a specific method, or suite of methods, is often dependent upon project specific factors. A decision tree to aid in selecting the appropriate type of BST approach presented in *USEPA* [2005] proposes asking the following questions:

- Is the problem adequately defined?
- Has an adequate sanitary survey been conducted?
- How many sources were identified in the sanitary survey?
- Is the watershed/study area of manageable size?
- What is the desired level of discrimination?

When identification of specific hosts is the goal, application of library-dependent, genotypic methods is recommended [*USEPA*, 2005]. A two-method combination such as enterobacterial repetitive intergenic consensus sequence polymerase chain reaction (ERIC-PCR) and RiboPrinting approach termed (ERIC-RP) has been recommended as a cost effective approach for conducting BST in Texas [*Jones et al.*, 2009]. ERIC-PCR is a repetitive element polymerase chain reaction (PCR) that

identifies repeated sequences in DNA between genes through the use of oligonucleotide primers and repeated DNA strand probing [*de Bruijn*, 1992]. The location and number of 126 base-pair DNA sequences varies by strain of bacteria [*Versalovic et al.*, 1991] and produces distinct banding patterns or fingerprints [*de Bruijn*, 1992]. PCR amplifies target DNA by generating copies of a specific DNA sequence within the sample. Briefly, PCR is a three-step process that consists of denaturing double-stranded DNA, primer annealing, and extension. Denaturation melts and separates the DNA strand allowing oligonucleotide primers with DNA sequences complimentary to the denatured DNA to anneal, or hybridize with separated DNA strands. Forward and reverse primers isolate specific areas of DNA for amplification. Polymerase enzymes add bases to primer ends to replicate the DNA sequence of interest through extension [*Newby et al.*, 2009b].

DNA fingerprints are compared to determine differences between samples. Visual analysis of fingerprints is possible for rudimentary assessments; however, refined approaches that indicate how similar fingerprints are require advanced statistical analysis techniques [*Versalovic et al.*, 1994]. Differences in screening approaches, image processing and PCR protocols can lead to varying results thus degrading fingerprint reproduction accuracy between laboratories [*Jones et al.*, 2009].

Riboprinting is a type of genetic fingerprinting known as ribotyping. It utilizes restriction enzymes such as *Hin*dIII to selectively cut DNA at specified points to produce variable size DNA fragments that are sorted by length during gel electrophoresis. Selected DNA probes hybridize to ribosomal RNA and produce distinct banding patterns to create DNA fingerprints [*Clark*, 1997; *Jones et al.*, 2009]. DuPont™ commercialized

an automated unit (Riboprinter®) that performs this task and reduces potential sample processing error. The workstation captures DNA fingerprint images and compares them to stored images from previously samples or reference libraries [*DuPont*, 2013].

The objectives of this evaluation were to identify *E. coli* source composition in soil and runoff collected from three land use types, and to explore differences in source composition between land uses within sampling media (e.g., soil and water) and between sampling media within each land use. We hypothesize that *E. coli* source composition would be different within sampling media from the multiple watersheds and that it would be similar between sampling media within each watershed.

4.3 Materials and Methods

4.3.1 Experimental Design

Soil and runoff *E. coli* sources from watersheds without direct inputs of non-natural fecal material for an extended time were evaluated. Soil and runoff samples were collected from three field-scale experimental watersheds at the USDA-ARS Grassland, Soil, and Water Research Laboratory near Riesel, Texas. Confirmed *E. coli* isolates were subjected to BST to determine the source category most likely contributing that isolate to the watershed. Isolate sources identified in soil and runoff samples collected from each watershed were compared within and between watersheds to determine similarities in *E. coli* source composition between sample media and watershed.

4.3.2 Watershed Description

Watersheds are located approximately 3.2 km East of Riesel, TX on the border of Falls and McLennan counties. This facility was established in the late 1930s within the Brushy Creek watershed of the larger Brazos River basin. It is located in the Texas Blackland Prairie, and its soils consist solely of Houston Black clays. When wet, these soils are very slowly permeable but experience extensive crack formation that creates preferential flow paths under dry conditions. Reported mean annual rainfall at ranges from 850 to 910 mm [*Allen et al.*, 2005; *Arnold et al.*, 2005; *Harmel et al.*, 2006a].

4.3.2.1 Remnant Native Prairie (SW12)

SW12 is a 1.2 ha remnant native prairie watershed with 3.8% average slope that is located within a larger 9 ha remnant prairie pasture. Management has been consistent since 1948 [*Harmel et al.*, 2006a] and includes mowing or haying interspersed with intermittent herbicide treatments (management data available online at: www.ars.usda.gov/spa/hydro-data).

4.3.2.2 Managed Hay Pasture (SW17)

SW17 is a 1.2 ha managed hay pasture with 1.8% slope [*Harmel et al.*, 2006a] situated within a larger 1.72 ha pasture. The pasture has been Coastal bermudagrass for more than 50 years and has predominantly been hayed. Cattle were grazed on the site from 2000 to 2010 and poultry litter applications at a rate of 6.8 Mg/ha (3 tons/ac)

occurred in 2011 and 2012 (management data available online at: www.ars.usda.gov/spa/hydro-data).

4.3.2.3 Cultivated Cropland (Y6)

Y6 is a 6.6 ha, terraced, conventionally cultivated cropland site with 3.2% average slope (Harmel et al., 2006) that has been continuously cropped since 1943. Crops produced include clover, cotton, corn, hay grazer, oats, sorghum, sudangrass, and wheat. The plot also received intermittent fertilizer and herbicide treatments as needed (management data available online at: www.ars.usda.gov/spa/hydro-data).

4.3.3 Sampling Procedures

4.3.3.1 Soil Sampling Technique

Soil samples were collected from each watershed during four sampling events. Collection occurred along transects within each watershed extending upslope from the flow control structure inlet to the watershed border. Sampling locations were selected to capture the variability of conditions within each watershed (e.g., under grasses, between bunch grasses, atop terraces, within terrace benches, within the grassed waterway, etc.).

Leaf litter or crop residue was removed from the soil surface when present. Soil samples were taken to a depth of approximately 5 cm with a 7.62 cm diameter soil sampling probe. Between individual sample collections, residual soil was removed from the soil probe, sprayed with 200-proof ethanol, and flared with a propane torch. Latex gloves were worn to remove collected samples from the probe and placed into sterile

710 mL Whirl-Pak® bags (Nasco, Fort Atkinson, WI). Gloves were changed between samples. Sample bags were labeled with the watershed and sample number then placed in an iced cooler and transported to the Soil and Aquatic Microbiology Laboratory (SAML) at Texas A&M University for immediate analysis.

4.3.3.2 Runoff Sample Collection

Overland flow from each watershed was collected using ISCO Avalanche refrigerated auto-samplers (Teledyne-ISCO, Inc., Lincoln, NE) maintained and operated by USDA-ARS personnel. Samplers were co-located with appropriately sized flow control structures and set to collect a 200 mL sample for each 1.32 mm of runoff from each watershed (calculated volumetrically). Samples were composited into sterile, 16L HDPE bottles to produce flow–weighted, composite samples for each event. Upon cessation of flow, or before sample holding times were approached, sample bottles were retrieved from the field. Subsamples were poured into labeled 532 mL Whirl-Pak® bags from the composite bottles following thorough mixing. Samples were held in a refrigerator until delivery on ice to SAML for analysis. Runoff volume was determined by recording water depth in flow control structures using ISCO 730 Bubbler Flow Modules and established stage-discharge relationships [*Harmel et al.*, 2014]. SW12 and 17 are equipped with 0.91 m (3 ft.) H-flumes, and Y6 is fitted with a combination v-notch weir and Parshall flume that allows small and large runoff event measurements.

4.3.4 Analytical Methods

Runoff and soil samples were processed at SAML to enumerate and isolate *E. coli* using USEPA Method 1603. This membrane filtration method utilizes modified membrane-Thermotolerant *E. coli* agar (mTEC) and a 24±2 hour incubation period [*USEPA*, 2006]. Aliquots of appropriate volume were processed from water samples, and results were reported as cfu/100 mL. Soil samples were prepared for analysis by placing 10g of soil into sterile specimen cups containing 90 mL of PBS. Aliquots of appropriate size were processed, and results were reported as cfu/g_{wet} of soil.

Selected *E. coli* enumerated from soil and runoff were picked and streaked onto nutrient agar with 4-methylumbelliferyl-β-D-glucuronide (NA-MUG) to confirm culture purity through glucuronidase activity. Selecting five confirmed *E. coli* isolates per water sample and four per soil sample was the target for BST archival and analysis. BST was conducted using the combined ERIC-PCR and RiboPrinting method, ERIC-RP.

ERIC-PCR utilizes PCR to amplify repetitive DNA sequences within *E. coli* genomes to create DNA fingerprints specific to each *E. coli* isolate. RiboPrinting is similar to ERIC-PCR in that it produces genetic fingerprints, but they are produced by endonuclease enzymes (e.g., *Hin*dIII) that cut select DNA sequences from cell genomes. Sequences are arranged by size and probed for specific conserved ribosomal RNA gene sequences to produce *E. coli* strain specific DNA fingerprints [*Jones et al.*, 2009].

DNA fingerprints from soil and water *E. coli* isolates produced by each method were compared to known *E. coli* source DNA fingerprints to identify statistically similar matches. Unknown isolates matching a known isolate with at least 80% similarity were

considered a positive match. Isolates were categorized by 3-way split (livestock and domestic animals, humans, wildlife, unidentified) to bolster the number of isolates within each category and by 7-way split (avian wildlife, non-avian wildlife, cattle, other avian livestock, other non-avian livestock, pets, human, unidentified) to better illustrate the breadth of contributing *E. coli* sources.

4.3.5 Statistical Analysis

Data analyses were conducted using Pearson's Chi-square testing to identify associations between watershed and sampling media for the categorical *E. coli* source identification data. An $\alpha=0.05$ was used to denote significance of test results. All statistical analyses were conducted using Minitab 17 software (Minitab Inc., State College, PA).

4.4 Results and Discussion

4.4.1 Runoff *E. coli* Sources

Visual analysis of BST results (Figure 4.1 and Table 4.1) reveals differences in *E. coli* source composition between watersheds; however, they are not drastic in most cases. Pearson's Chi-square testing was used to identify significant differences, or associations, between categorical *E. coli* isolate classifications and watersheds. Results did not supply sufficient evidence to identify statistically significant associations between *E. coli* categories and watershed at $\alpha=0.05$ level using 3-way ($p=0.07$) or 7-way ($p=0.2$) splits. A weak association between source category and watershed is suggested

in the 3-way split (Figure 4.1). The small number of identifications in some source categories likely diminished assessment power, and differences in sample size from each watershed probably contributed to muted source contribution variations identified. Additionally, similar source identifications within samples indicate that closely related *E. coli* were selected from individual water samples in some cases. This adds further uncertainty to assessment results and likely produced source identification results that deviate from the real *E. coli* source distributions in each watershed.

Table 4.1. Number of *E. coli* isolates identified by source category in runoff

Source Categories		SW12		SW17		Y6	
3-way	7-way	3-way	7-way	3-way	7-way	3-way	7-way
Wildlife	Avian	100	19	34	8	56	11
	Non-Avian		81		26		45
Livestock and Domesticated	Cattle	38	7	23	7	14	5
	Pets		15		4		1
	Other Avian		4		3		2
	Other Non-Avian		12		9		6
Human	Human	8	8	0	0	2	2
Unidentified	Unidentified	14	14	3	3	8	8

Wildlife sources were identified as the predominant runoff *E. coli* contributor in all watersheds. Between 56 and 70% of analyzed isolates were identified as wildlife derived *E. coli.* Avian wildlife identifications made up 19 to 24% of total wildlife derived *E. coli* identified suggesting that three to four times more *E. coli* are contributed to these watersheds by mammalian wildlife than avian. Overall, wildlife contributions were expected to constitute a larger portion of the contributing sources since each watershed is managed to exclude anthropogenic *E. coli* contributions.

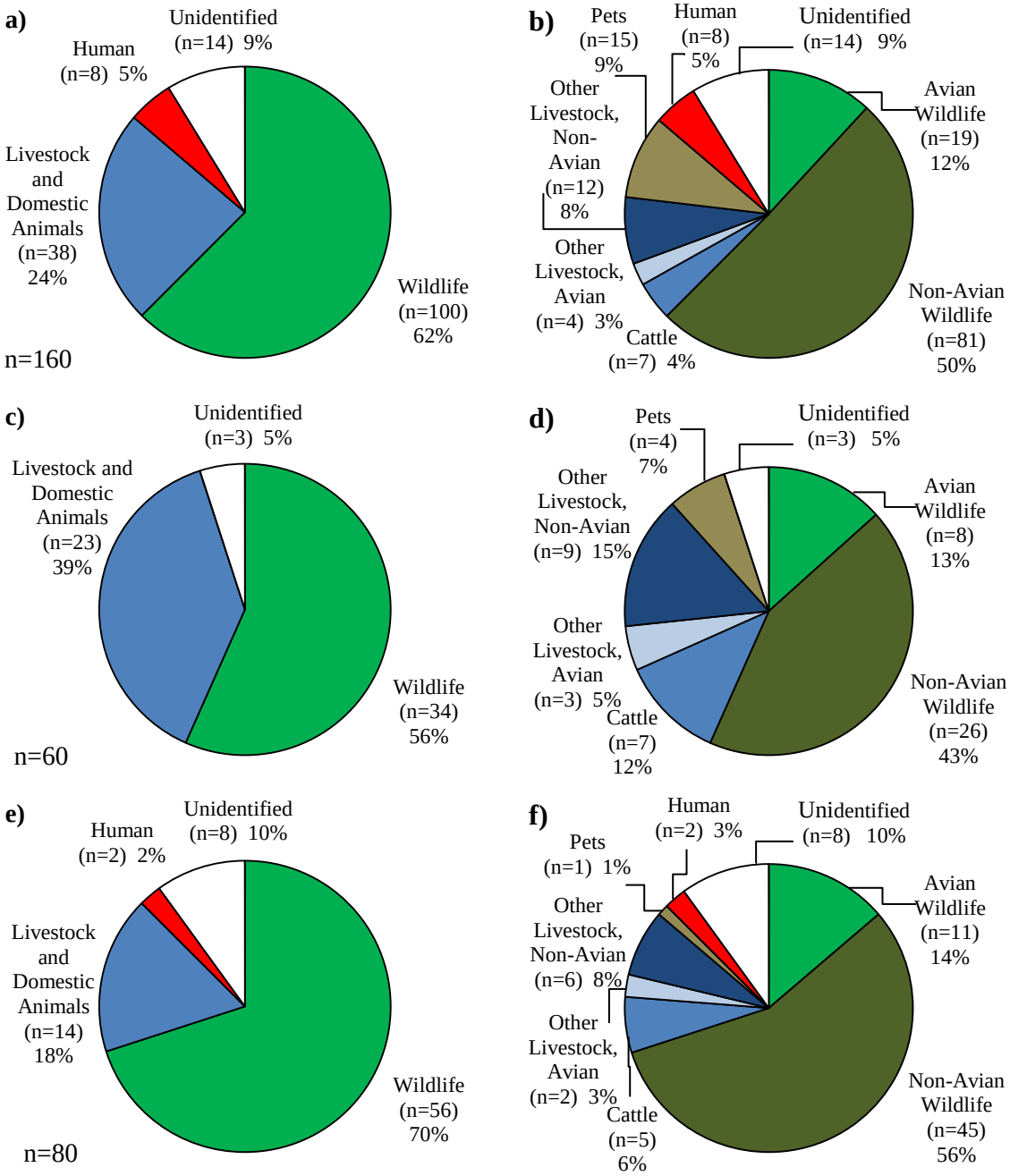

Figure 4.1. Runoff BST results for each watershed. a) SW12, 3-way; b) SW12, 7-way; c) SW17, 3-way; d) SW17, 7-way; e) Y6, 3-way; f) Y6, 7-way

Livestock and other domesticated animal identifications as contributing *E. coli* sources in 18 – 39% of the isolates are unexpected. Documented cattle and pet occurrences in the watersheds provide some support for these identifications [*Gregory et al.*, 2015], but the noted level of occurrence does not seem to justify their relatively large contribution. Infrequent human identifications were surprising since no known contributions of human fecal matter exist in the watersheds. Unidentified *E. coli* sources were implicated in ≤10% of evaluated isolates from each watershed thus suggesting that the Texas *E. coli* BST Library supplemented with locally collected known source isolates performed well for runoff samples.

Human derived *E. coli* identification raises questions regarding result accuracy; however, several explanations make their presence plausible. Some *E. coli* isolates identify as matches to multiple sources and are deemed cosmopolitan *E. coli* [*Dick et al.*, 2005] and could have resulted in this classification. Transmission vectors can also deliver unexpected sources of *E. coli* into the watershed. Some animals observed on site [*Gregory et al.*, 2015] including coyotes, opossums, dogs, and vultures are known to consume fecal matter of other species or human wastewater effluent on occasion. This constitutes a plausible pathway for human derived *E. coli* from nearby locations to enter the watersheds. A number of houses are located near these watersheds and utilize on-site sewage facilities to dispose of their waste. If failing, untreated wastewater can pond on the surface and providing a source of human *E. coli* for transmission vector ingestion. Transmission vectors may also be responsible for other unexpected *E. coli* being identified.

4.4.2 Soil *E. coli* Sources

Soil *E. coli* identification results suggest considerable differences in source category composition between sites (Figure 4.2 and Table 4.2). Most soil samples failed to yield culturable *E. coli* colonies which caused most isolated colonies to be analyzed. In total, only 19 *E. coli* isolates were analyzed with BST from Y6 soil samples compared to 63 from SW12 and 113 from SW17. Specific isolate match information reveals that a number of *E. coli* isolates within individual samples were identified as matches to the same known source isolate. This occurrence likely caused deviations in source identification results from real *E. coli* source distributions in each watershed. Further, the disparity in isolate numbers analyzed between watersheds adds uncertainty to the analysis.

Table 4.2. Number of *E. coli* isolates identified by source category in soil

Source Categories		SW12		SW17		Y6	
3-way	7-way	3-way	7-way	3-way	7-way	3-way	7-way
Wildlife	Avian	51	10	86	11	10	2
	Non-Avian		41		75		8
Livestock and Domesticated	Cattle	6	3	25	21	3	0
	Pets		0		0		0
	Other Avian		1		0		0
	Other Non-Avian		2		4		3
Human	Human	3	3	0	0	0	0
Unidentified	Unidentified	3	3	2	2	6	6

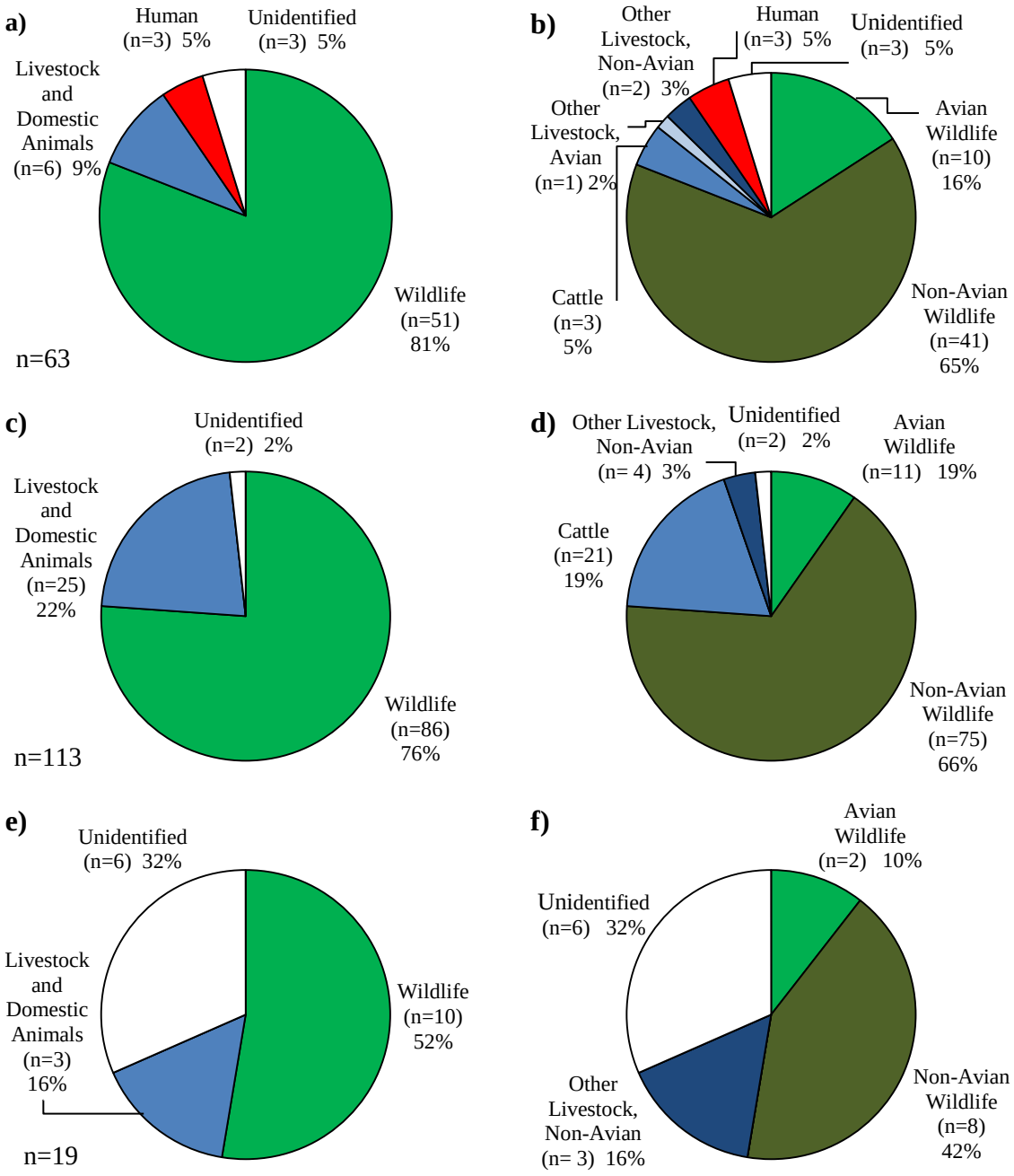

Figure 4.2. Soil BST results for each watershed. a) SW12, 3-way; b) SW12, 7-way; c) SW17, 3-way; d) SW17, 7-way; e) Y6, 3-way; f) Y6, 7-way

Pearson's Chi-squared testing was applied to test for possible associations between watersheds and *E. coli* sources identified. Initially the test failed to produce a valid Chi-square approximation using 3-way analysis results due to the small number of human identified *E. coli* in SW12 soils. Excluding human isolates from the analysis produced valid results that suggested the presence of statistically different associations between watersheds and sources identified (p=0.00). Similarly, an invalid approximation was produced using 7-way identification results. Removing human (n=3) and other livestock avian (n=1) sources from SW12 produced valid test results and suggested significant associations between watershed and sources identified (p=0.00). Visual analysis supports these findings.

E. coli categorized as wildlife were dominated soil source identifications and ranged from 52 to 81% of contributions in the watersheds. This finding was expected due to applied land management; however, the percentage of wildlife identified *E. coli* at Y6 was surprisingly low. This is likely due to the low number of *E. coli* isolates produced and the larger relative contribution of unidentified sources. Avian wildlife contributed only 13 to 20% of the overall wildlife identifications. Livestock and domestic animals (including cattle and pets) were identified more often than expected (9-22%). Photo documentation of these species in and near the watersheds somewhat justifies this finding [*Gregory et al.*, 2015]; however, other factors may contribute to these observations as well. Only three *E. coli* isolates in SW12 were categorized as human derived; however, this finding is unexpected due to the lack of human influence at this site. The number of unidentified *E. coli* isolates was somewhat larger than

expected from Y6 (32%, n=6) while only 5% of isolates from SW12 and 2% from SW17 were identified as unidentified. The small number of isolates produced from Y6 as compared to SW12 and SW17 likely contributes to this finding as one of the few soil samples containing *E. coli* that were cultured could have contributed a large number of isolates from the same unknown source.

Human, livestock, and pet sources of *E. coli* in the watershed were unexpected. Photo evidence recorded cattle in SW17 and Y6 on one occasion and dogs in SW12 on several occasions [*Gregory et al.,* 2015]. This provides a plausible explanation for identification of these *E. coli* sources. Cattle were also grazed on SW17 three years prior to sampling, thus residual *E. coli* from cattle could exist as a naturalized soil inhabitants; however, the low number of *E. coli* cultured from soil samples add question to this possibility. Human identifications were low (n=3); however, no known contributions of human fecal matter have occurred in these watersheds. Cosmopolitan *E. coli* strains that match more than one known source may cause this observation. Alternatively, a transmission vector such as a coyote, dog, or opossum could have consumed human *E. coli* and translocated it to the watershed. The percentage of unidentified *E. coli* isolate classifications was quite low for SW12 and SW17, but not Y6. This is likely a function of sample size at Y6; but could stem from the presence of underrepresented species in the Texas *E. coli* BST Library. *E. coli* from avian species not represented in the library are the likely source of these unidentified *E. coli* since mammalian species observed in the watershed are currently represented. *E. coli* naturalized into the soil could also

contribute to this finding; however, the small number of culturable *E. coli* identified in soils suggests that this is not likely.

4.4.3 Comparison of *E. coli* Sources within Watersheds and Sampling Media

E. coli isolate classifications from runoff and soil samples within each watershed were compared to determine if associations between sampling media exist. Both 3-way and 7-way identification splits were evaluated in each watershed (Figures 4.1 and 4.2, Tables 4.1 and 4.2). Visual analysis suggests the presence of differences in *E. coli* source categories between soil and runoff within each watershed and provides some evidence that soil *E. coli* in a watershed may not be from the same source as runoff *E. coli*.

Pearson's Chi-square testing was used to identify significant associations between sampling median within each watershed. Sufficient evidence was present to suggest a significant association between *E. coli* source category and sampling media in 3-way ($p=0.05$) but not 7-way splits ($p=0.15$) for SW12. Significant associations for the 3-way ($p=0.03$) and 7-way ($p=0.00$) identifications are suggested in SW17; however, no significant associations were suggested for 3-way or 7-way identifications in Y6 ($p=0.06$ and 0.08 respectively). Human identified isolates were removed from both analyses while pets and other livestock, avian were removed from 7-way analyses so valid Chi-square approximates could be produced.

Collectively, results suggest that runoff *E. coli* sources are not necessarily similar to soil *E. coli* sources. Instead, recent fecal loading to a watershed is more likely to contribute *E. coli* to runoff than soil. Visual analysis reveals subtle differences in source

category composition between soil and runoff within all watersheds; however, the mixture of significant and non-significant associations is not surprising given the low occurrence of *E. coli* isolates within some source categories.

4.5 Summary and Conclusions

Investigating potential *E. coli* sources differences between watersheds and sampling media using BST provided useful insights regarding background source contributions. Local watershed knowledge and applied management records allowed sound assumptions regarding *E. coli* source contributors to be established, but were not sufficient to fully describe actual source contributions. BST was able to identify influences of unexpected source categories responsible for a portion of the *E. coli* load in these watersheds. Site specific source characterization remained important for reconciling differences between known sources and those identified through BST. Findings provide further support for the use of multiple techniques to identify contributing bacteria sources to any watershed. The three-tier approach described by *Jones et al.* [2009] that combines source surveys, watershed inventories, targeted monitoring, and BST remains appropriate and should be employed to develop a broad understanding of bacteria contributions in a watershed.

BST results indicate subtle *E. coli* source composition differences between watersheds; however, they were not strongly pronounced. This lack of difference between watersheds could be real, or it may be due in part to the disparity in sample size between watersheds and within source categories. Despite this, results provide useful

insight to *E. coli* sources in these watersheds. Land use and associated wildlife presence appear to influence *E. coli* sources present; however, differences in source composition between watersheds are not strongly defined. *E. coli* source composition between sampling media within a watershed revealed some statistically significant differences. Land use, land cover and associated animal use likely contribute to these differences; however, heterogeneity in samples analyzed may contribute as well. Expanded sampling would improve the ability to identify differences between sites and within sampling media by increasing testing power and allow stronger relationships to be established.

Findings demonstrate that *E. coli* in watersheds occur due to natural processes that cannot be managed. Land use and land cover differences appear to influence *E. coli* source composition in soil and runoff thus changes to land use through habitat modification present potential tools for managing wildlife *E. coli* loading but will likely not produce drastic changes. Despite exclusion of anthropogenic bacteria source contributions, *E. coli* derived from these sources were still identified. While this could be a function of cosmopolitan *E. coli* occurrence, it also suggests that wildlife may serve as transmission vectors thus extending their influence on observed *E. coli* loads. Combined, these results demonstrate the challenges faced when managing *E. coli* loading in a watershed and highlight the need to account for background sources in water quality management efforts.

CHAPTER V

SUMMARY

5.1 *E. coli* Response to Nutrient Amendment in a Re-Created Stream Mesocosm

Understanding *E. coli* fate in secondary environments is crucial to develop effective management strategies that reduce *E. coli* loading to surface waters. This study advanced the state of knowledge regarding *E. coli* fate in stream mesocosms created using unsterilized water and sediment. Single nutrient doses representing loads observed in urban irrigation runoff events or a wastewater discharge failed to produce an instream *E. coli* growth response and did not yield significant differences in calculated decay constants. *E. coli* decay in water was biphasic with rapid decay occurring within one week (Phase I) followed by an extended stationary phase of relatively stable concentration (Phase II). *E. coli* half-life ranged from 0.41 to 1.72 days in Phase I and between 0 and 24.4 days in Phase II. In sediment, variable rates of *E. coli* growth, decay, and persistence were observed in three phases of variable length. Half-life and doubling time (negative and positive values respectively) ranged from -130.75 to 29.73 days in Phase I, -4.83 to 20.65 days in Phase II and from -28.29 to 13.33 days in Phase III. These findings demonstrate that *E. coli* persistence in sediment is prolonged compared to overlying water. This suggests that sediment provides a more suitable habitat by providing shelter from predators and improving nutrient availability. *Craig et al.* [2004] and *Shelton et al.* [2014] demonstrated similar *E. coli* growth and persistence trends using smaller microcosms with inoculated water and sediment to produce simulated

natural systems. However other work demonstrated recovery of disinfectant treated *E. coli* in sterilized water as a result of nutrient amendments [*Bolster et al.*, 2005; *McCrary et al.*, 2013] suggesting that even damaged *E. coli* may be able to proliferate given favorable conditions. These situations do not consider the antagonistic effects of predatory and competing microbes on *E. coli* growth potential thus the applicability to instream environments is limited. *Lim and Flint* [1989], *Ishii et al.* [2010], *Wanjugi and Harwood* [2013] and others have noted the complexity of microbial competition and predation and suggest it as a limiting, or dominant factor controlling *E. coli* fate in secondary environments. Heterotrophic bacteria response measured in this study verified their ability to rapidly utilize and exhaust nutrient additions and verified these claims. Predation was not evaluated; however, competition appears sufficient to suppress *E. coli* growth in natural stream mesocosms when nutrient amendments were provided.

Relationships between nutrients and other monitored water quality measures explored with non-linear regression were inconsistently present in treatment and control mesocosms. This suggests that no single parameter, or suite of parameters, provides a consistent estimate of *E. coli* in simulated natural systems. Turbidity was most commonly related to *E. coli* concentrations; however, observed relationships were not consistent across all trials. Therefore, turbidity should be used cautiously to predict *E. coli* as suggested by others [*McDonald et al.*, 2006; *Wagner et al.*, 2013]. Standard stepwise multiple regression and best subsets regression analysis identified various significant predictor variable combinations that could be used to describe *E. coli* concentrations measured. Different models were developed for each mesocosm

demonstrating the lack of consistency in predicting instream *E. coli* concentrations. However, the ability to model *E. coli* concentrations with various independent variables in these complex mesocosms provides justification for continued use of models to estimate *E. coli* fate and transport processes at watershed scales. Results demonstrate the variable nature of *E. coli* in secondary environments and reinforce the need to develop a sound understanding of site specific conditions when working to address *E. coli* loading concerns.

Results highlight the complex nature of natural systems and the dynamic response of microbes to system changes. In this case, nutrient amendments did not produce *E. coli* growth in water and only resulted in minor growth in sediments. This suggests that waters receiving brief nutrient addition do not adversely affect *E. coli* concentrations. Instead, this implies that direct deposition, instream sediment resuspension, and nonpoint source contributions are significantly larger influences to *E. coli* loadings in surface waters than instream regrowth. Therefore, management to reduce *E. coli* concentrations in streams should focus on preventing *E. coli* from entering the stream rather than attempting to limit its source of nutrition. However, further work is justified to extend knowledge regarding the influence of sustained nutrient loading in natural environments on *E. coli* regrowth potential.

5.2 Land Use and Land Cover Effects on *E. coli* in Runoff and Soil

In watersheds where anthropogenic sources of *E. coli* were excluded, land use and land cover significantly affected runoff quality and quantity. *E. coli* concentrations

exhibited significant spatial and temporal variability among watersheds. Runoff *E. coli* concentrations generated in the fall were typically higher than those from other seasons; however, this observation varied by watershed. Variations in wildlife use by watershed are suspected as the primary cause of *E. coli* concentration differences but sufficient site-specific evidence to support this theory was not collected. However, animal usage patterns change based on food and shelter availability; therefore, their fecal deposition patterns also change. Thus timing between rainfall and fecal deposition from background sources on measured loading should be considered similar to those noted in grazing systems [*Wagner et al.*, 2012]. Soil *E. coli* concentrations also differed between watersheds and exhibited considerable spatial and temporal variability. However, concentrations were much lower than runoff *E. coli* suggesting that soil is not a sizable source of *E. coli* in runoff. Further, estimated median sediment borne *E. coli* loads during each runoff event account for only 0.0041 to 0.0046% of the total runoff *E. coli* load during that event. As a result, recent fecal deposition in a watershed should be considered the primary runoff *E. coli* source.

This study demonstrated that background sources contribute considerable quantities of *E. coli* in various watershed types. Median *E. coli* concentrations in runoff from all watersheds were more than seven times greater than current Texas water quality standards for primary contact recreation. Thus runoff *E. coli* derived from unmanageable sources alone can produce runoff that does not meet instream water quality standards. However, these standards do not apply to edge-of-field runoff nor should they. Further, findings support the need to consider stormwater exemptions to current water quality

standards. *E. coli* concentrations measured are similar to those observed from intensive land uses such as grazing or urbanization in many cases [*Desai and Rifai*, 2010; *Goto and Yan*, 2011b; *Harmel et al.*, 2013; *Wagner et al.*, 2012] clearly demonstrating the magnitude of background *E. coli* effects. This illustrates the need to account for background *E. coli* sources in watershed loading assessments. In most cases, background sources are not considered and all measured loads are attributed to known contributors. While this does not inflate the overall reduction needed to meet water quality standards, it does over-allocate *E. coli* loads to known sources imparts an excessive reduction burden upon those sources.

5.3 Differences in *E. coli* Sources Between Watersheds and Sampling Media

Library-dependent BST identified *E. coli* source category contributors to runoff and soil in each watershed. No significant differences in runoff *E. coli* sources were identified between watersheds, but they were found in soil *E. coli* sources. Differences in *E. coli* source composition identified between soil and water within each watershed were both significant and non-significant depending on watershed. Sample size within some source categories were extremely small and diminished the power of statistical testing. However, visual observation of results suggests that statistical analysis results were appropriate. Findings did not provide clear support for upland temperate soils to be considered important runoff *E. coli* contributors like they have in other locations [*Fujioka et al.*, 1988; *Goto and Yan*, 2011a; *Ishii et al.*, 2006].

As expected, wildlife derived *E. coli* was most common in soil and water from each watershed. However, the presence of *E. coli* matching known anthropogenic sources including livestock, pets, and humans in some runoff and soil samples was surprising. Errant occurrences of cattle and pets noted provide justification for some of the unexpected classifications [*Gregory et al.*, 2015] but do not explain all results. This suggests the occurrence of cosmopolitan *E. coli*, or the influences of transmission vectors. Pets that live closely with humans may develop *E. coli* of similar DNA signature due to similarities in diet thus producing cosmopolitan *E. coli*. Alternatively, pets could ingest and translocate human derived *E. coli* into the watershed. A number of animals including opossums, dogs, and coyotes are known to consume fecal matter of other species and may also translocate it into the watershed.

Despite these surprise findings, BST results demonstrate the dynamic nature of *E. coli* loading to the environment and highlight challenges faced by those charged with managing these loads. The influence of background *E. coli* sources is further highlighted and supports the claim that they should be better accounted for when allocating watershed loads and determining needed management measures to restore water quality. Additional work to evaluate *E. coli* source composition in other watershed types (forests, urban, etc.) is also warranted and would further demonstrate the breadth of *E. coli* sources contributing to downstream waterbodies.

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APPENDIX A

E. COLI DECAY AND GROWTH CONSTANTS IN WATER AND SEDIMENT

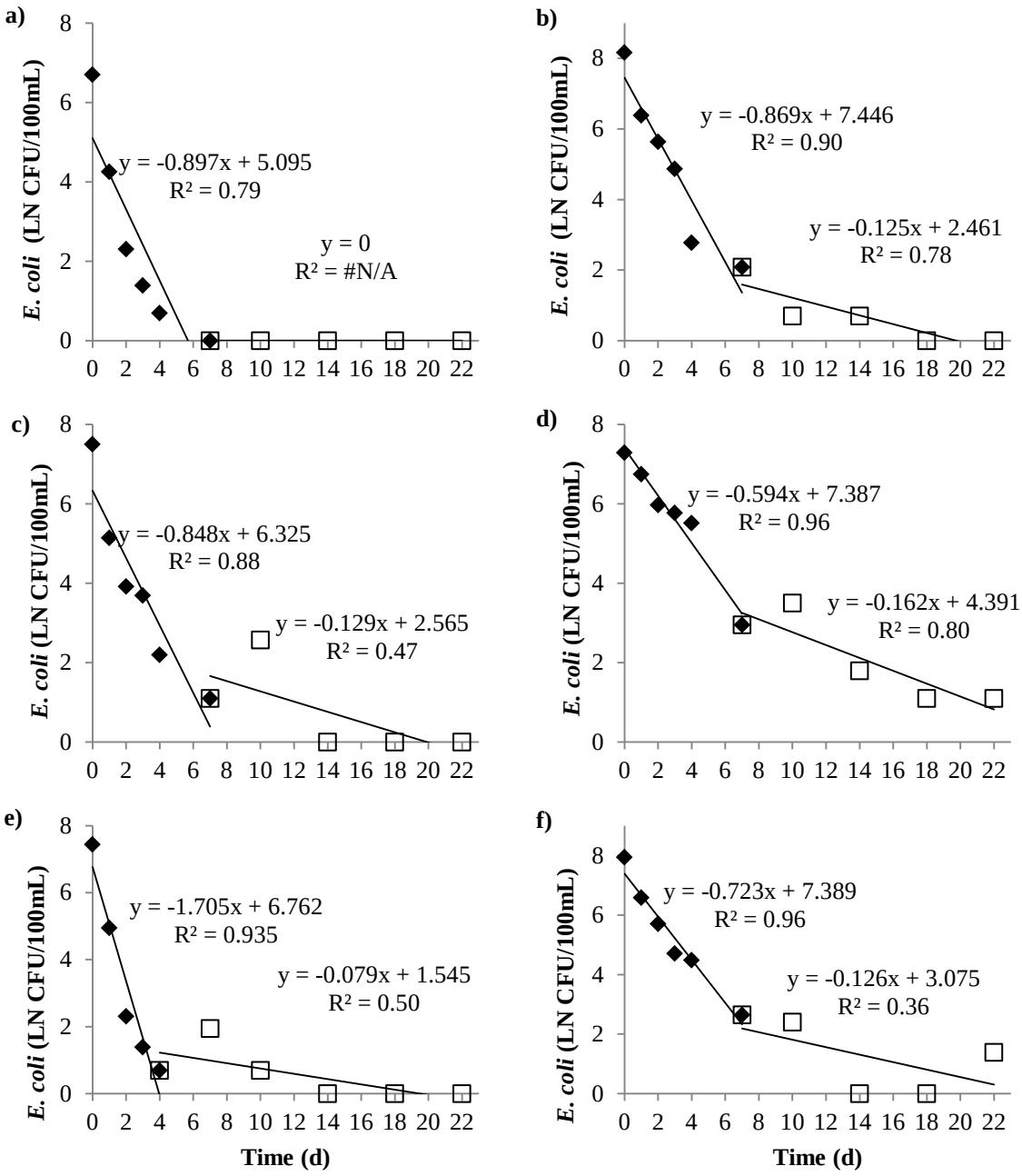

Figure A-1. LN of *E. coli* concentrations in water recorded in trial 1 plotted over time to produce decay and growth constants. Graphics depict results for mesocosm: a) C1; b) C2; c) H1; d) H2; e) L1; f) L2

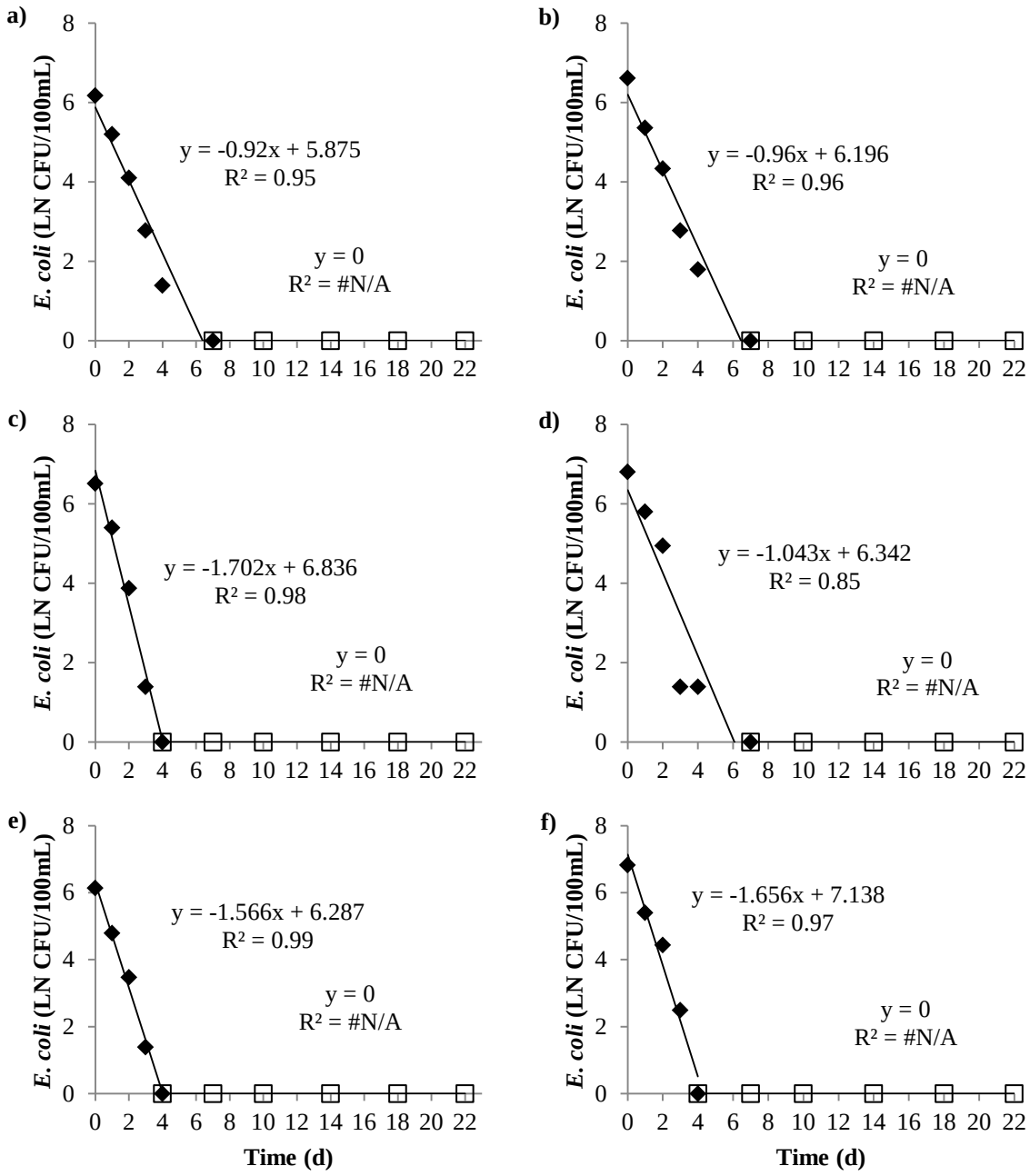

Figure A-2. LN of *E. coli* concentrations in water recorded in trial 2 plotted over time to produce decay and growth constants. Graphics depict results for mesocosm: a) C1; b) C2; c) H1; d) H2; e) L1; f) L2

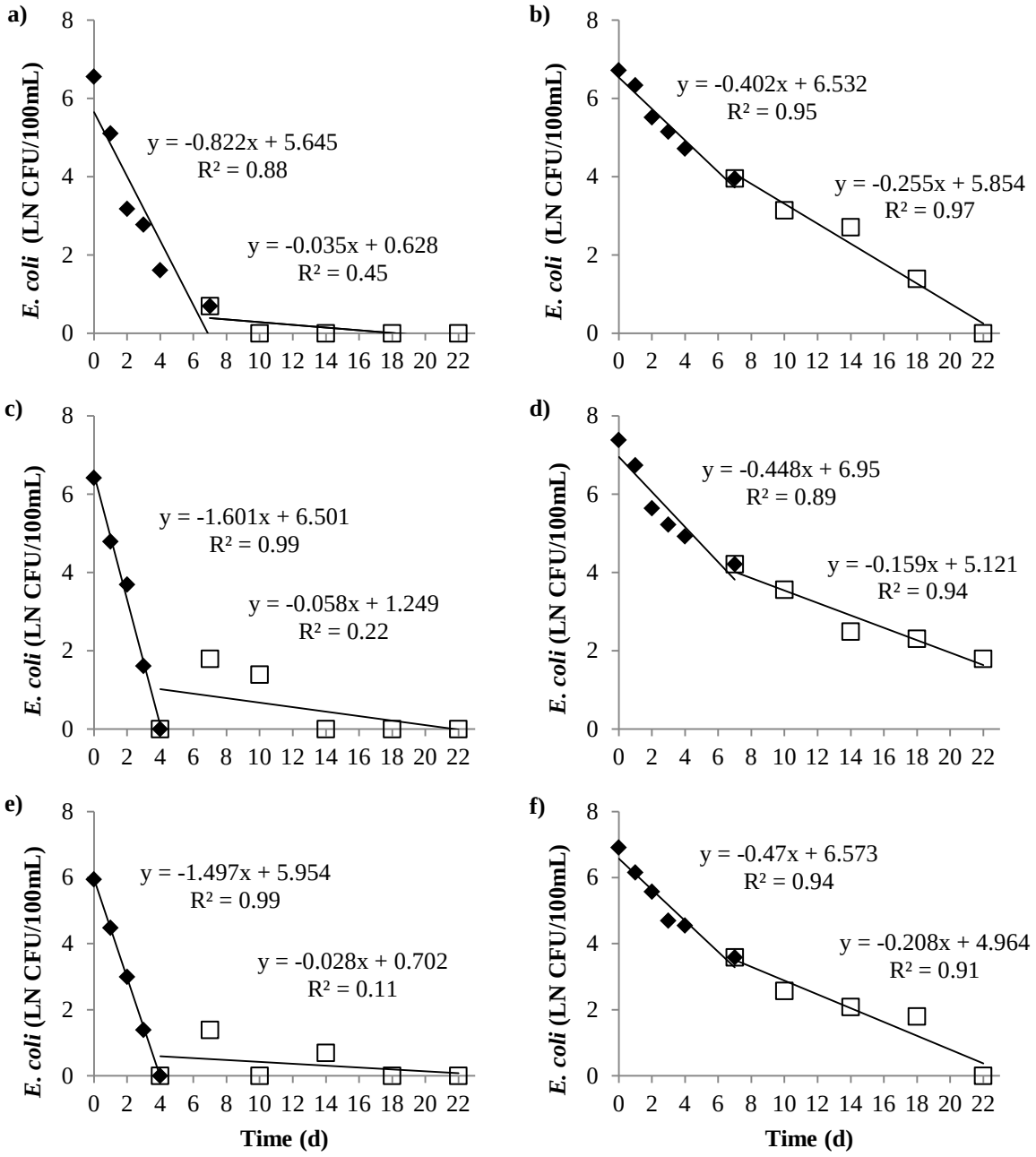

Figure A-3. LN of *E. coli* concentrations in water recorded in trial 3 plotted over time to produce decay and growth constants. Graphics depict results for mesocosm: a) C1; b) C2; c) H1; d) H2; e) L1; f) L2

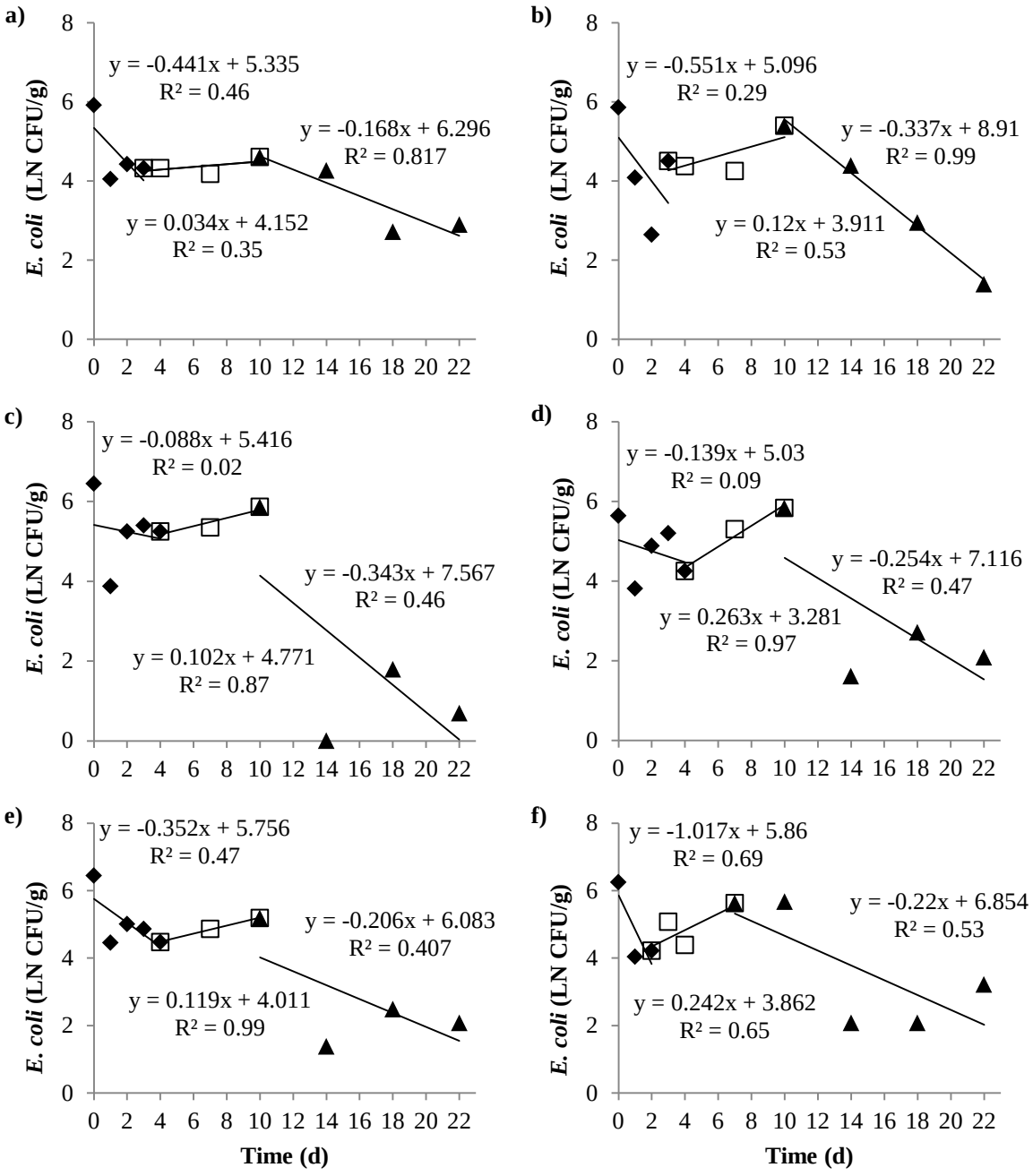

Figure A-4. LN of *E. coli* concentrations in sediment recorded in trial 1 plotted over time to produce decay and growth constants. Graphics depict results for mesocosm: a) C1; b) C2; c) H1; d) H2; e) L1; f) L2

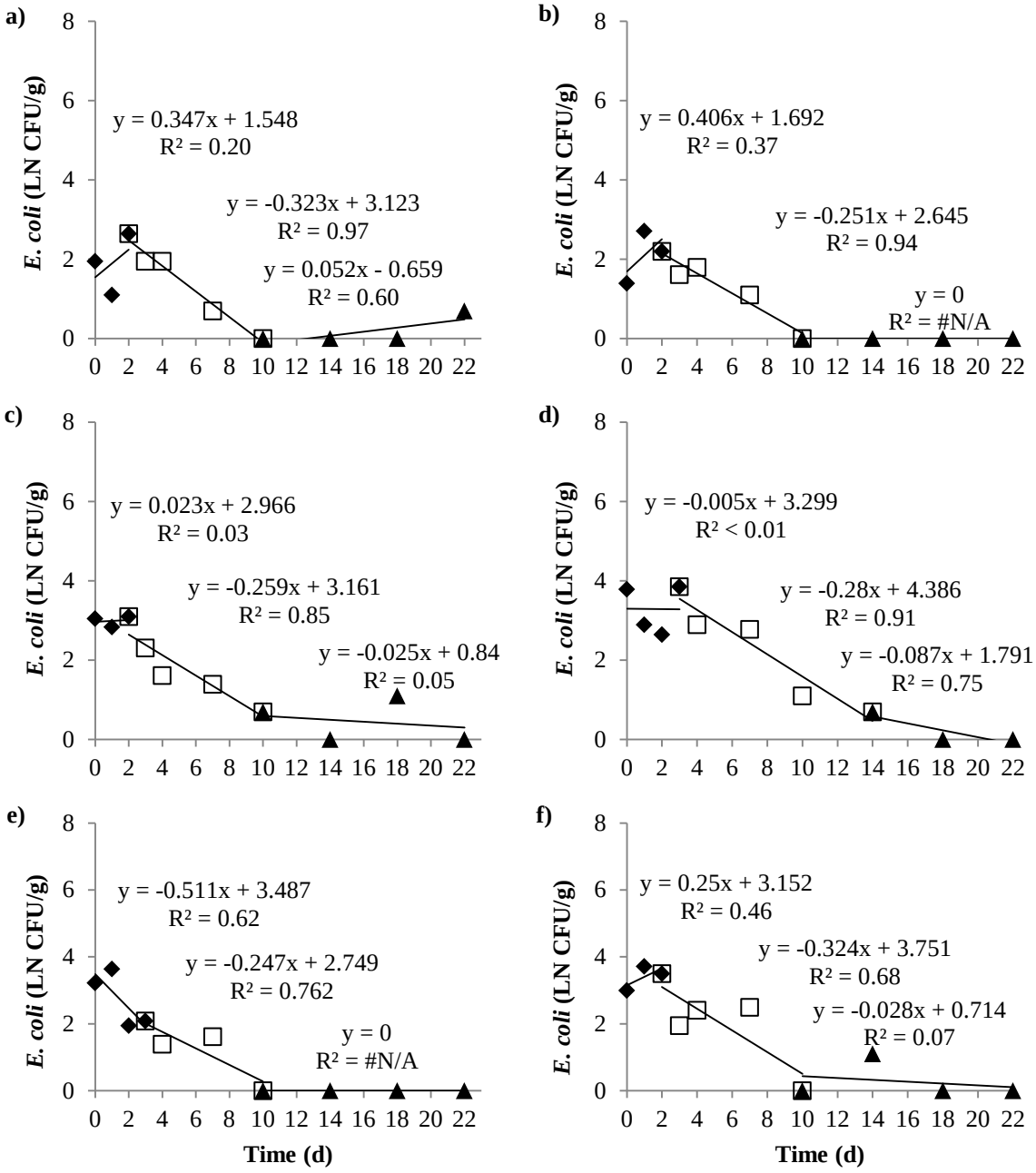

Figure A-5. LN of *E. coli* concentrations in sediment recorded in trial 2 plotted over time to produce decay and growth constants. Graphics depict results for mesocosm: a) C1; b) C2; c) H1; d) H2; e) L1; f) L2

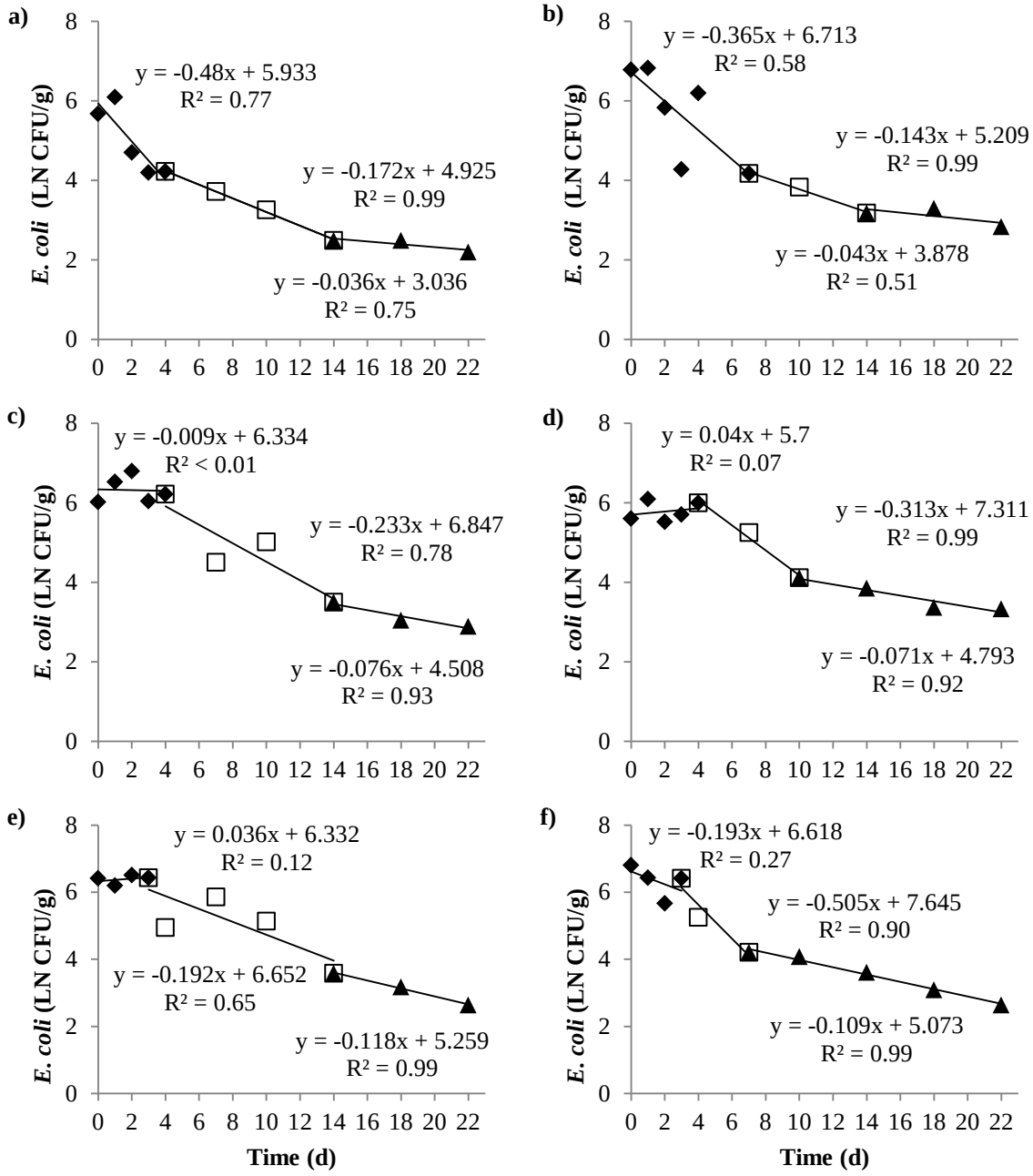

Figure A-6. LN of *E. coli* concentrations in sediment recorded in trial 3 plotted over time to produce decay and growth constants. Graphics depict results for mesocosm: a) C1; b) C2; c) H1; d) H2; e) L1; f) L2

APPENDIX B

WATER AND SEDIMENT HETEROTROPHIC BACTERIA DECAY AND GROWTH CONSTANTS

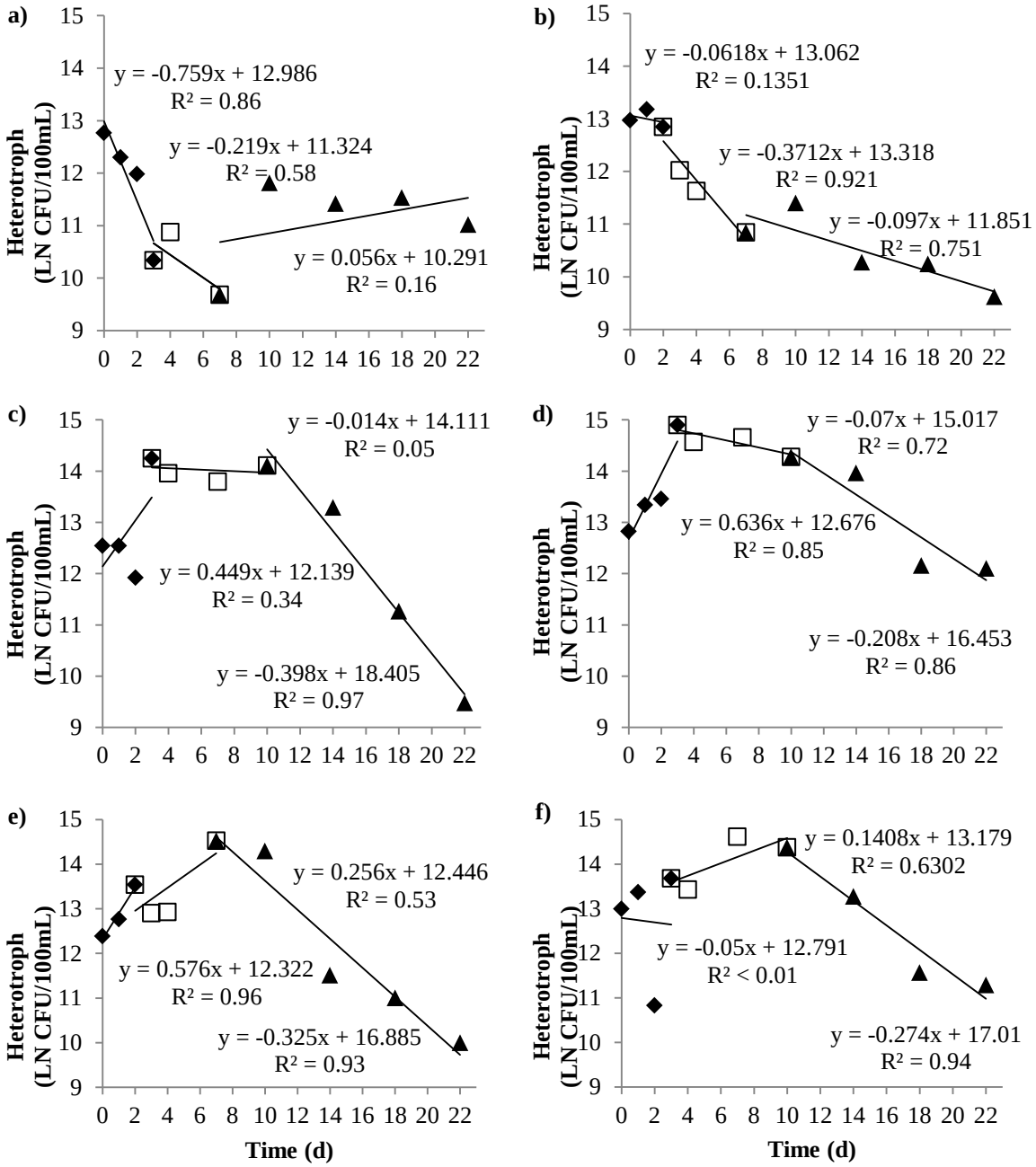

Figure B-1. LN of heterotroph concentrations in water recorded in trial 1 plotted over time to produce decay and growth constants. Graphics depict results for mesocosm: a) C1; b) C2; c) H1; d) H2; e) L1; f) L2

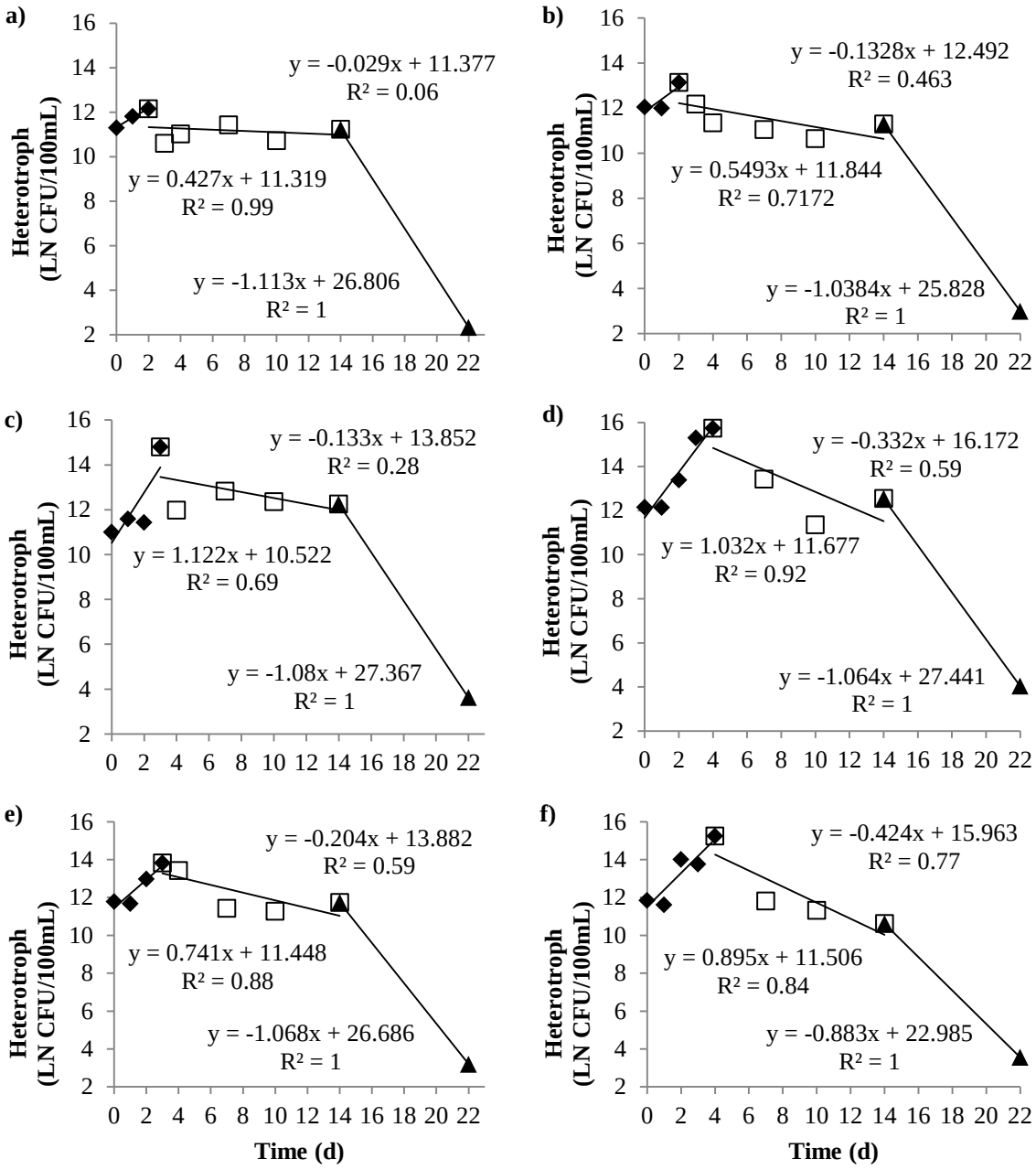

Figure B-2. LN of heterotroph concentrations in water recorded in trial 2 plotted over time to produce decay and growth constants. Graphics depict results for mesocosm: a) C1; b) C2; c) H1; d) H2; e) L1; f) L2

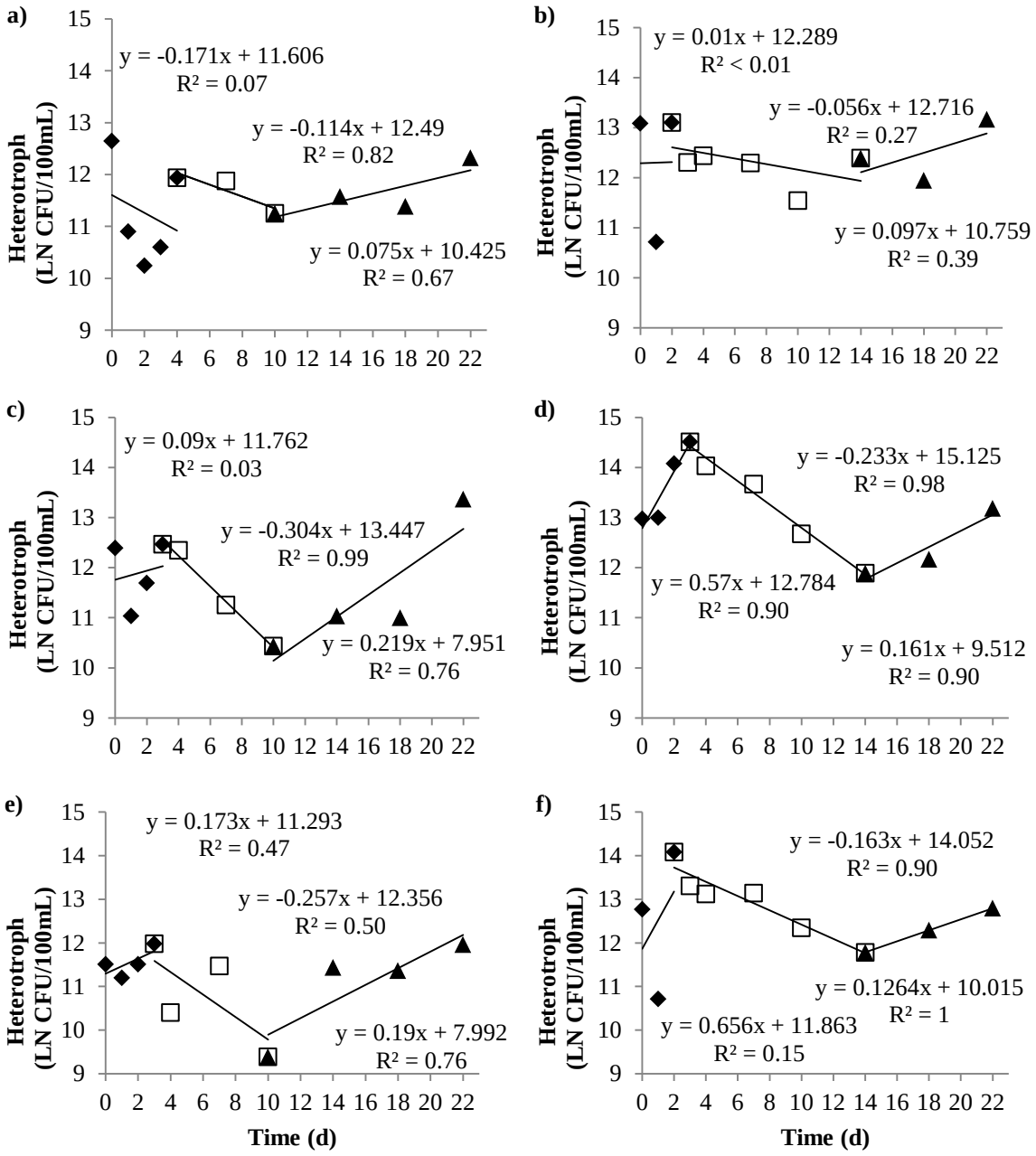

Figure B-3. LN of heterotroph concentrations in water recorded in trial 3 plotted over time to produce decay and growth constants. Graphics depict results for mesocosm: a) C1; b) C2; c) H1; d) H2; e) L1; f) L2

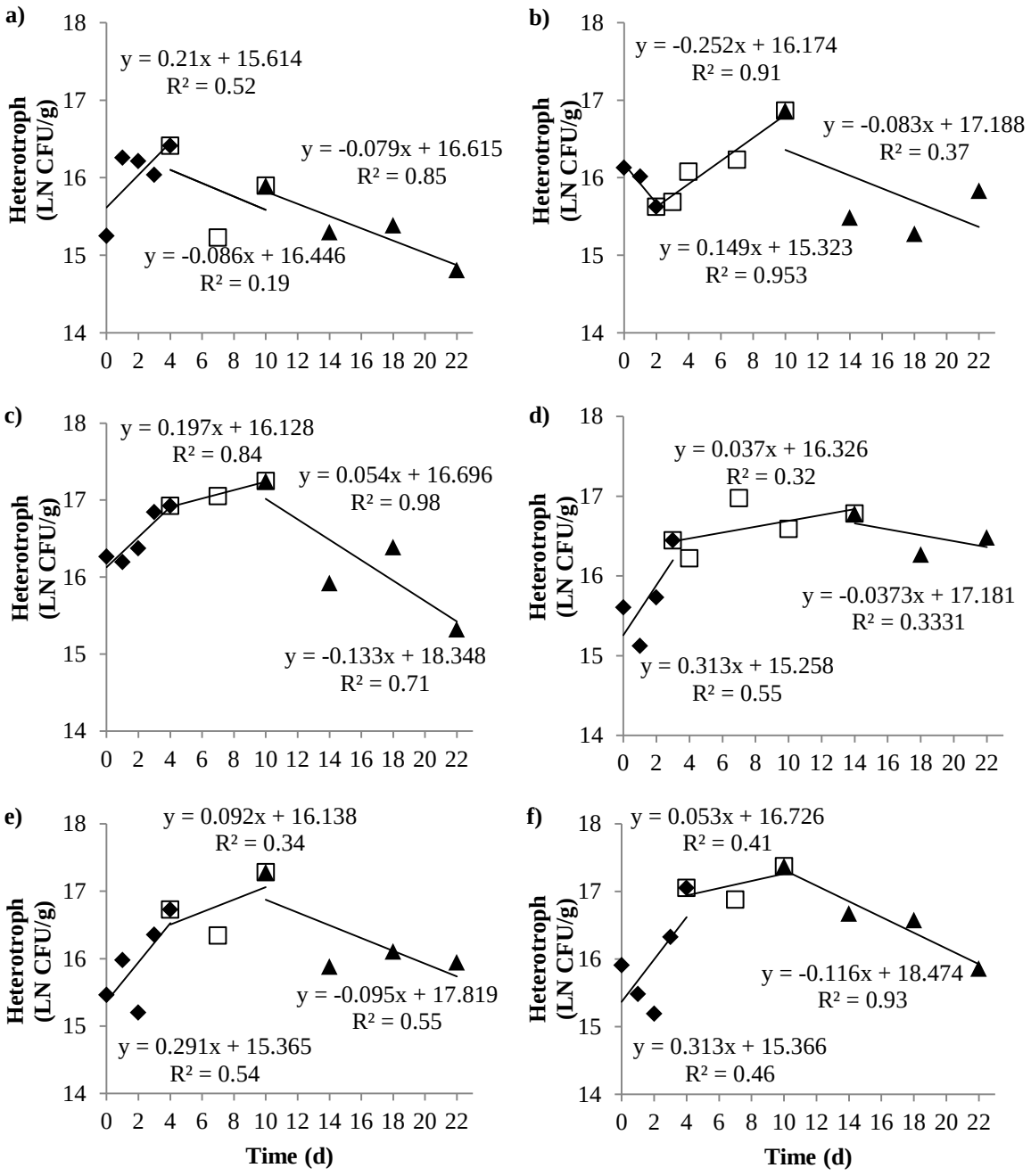

Figure B-4. LN of heterotroph concentrations in sediment recorded in trial 1 plotted over time to produce decay and growth constants. Graphics depict results for mesocosm: a) C1; b) C2; c) H1; d) H2; e) L1; f) L2

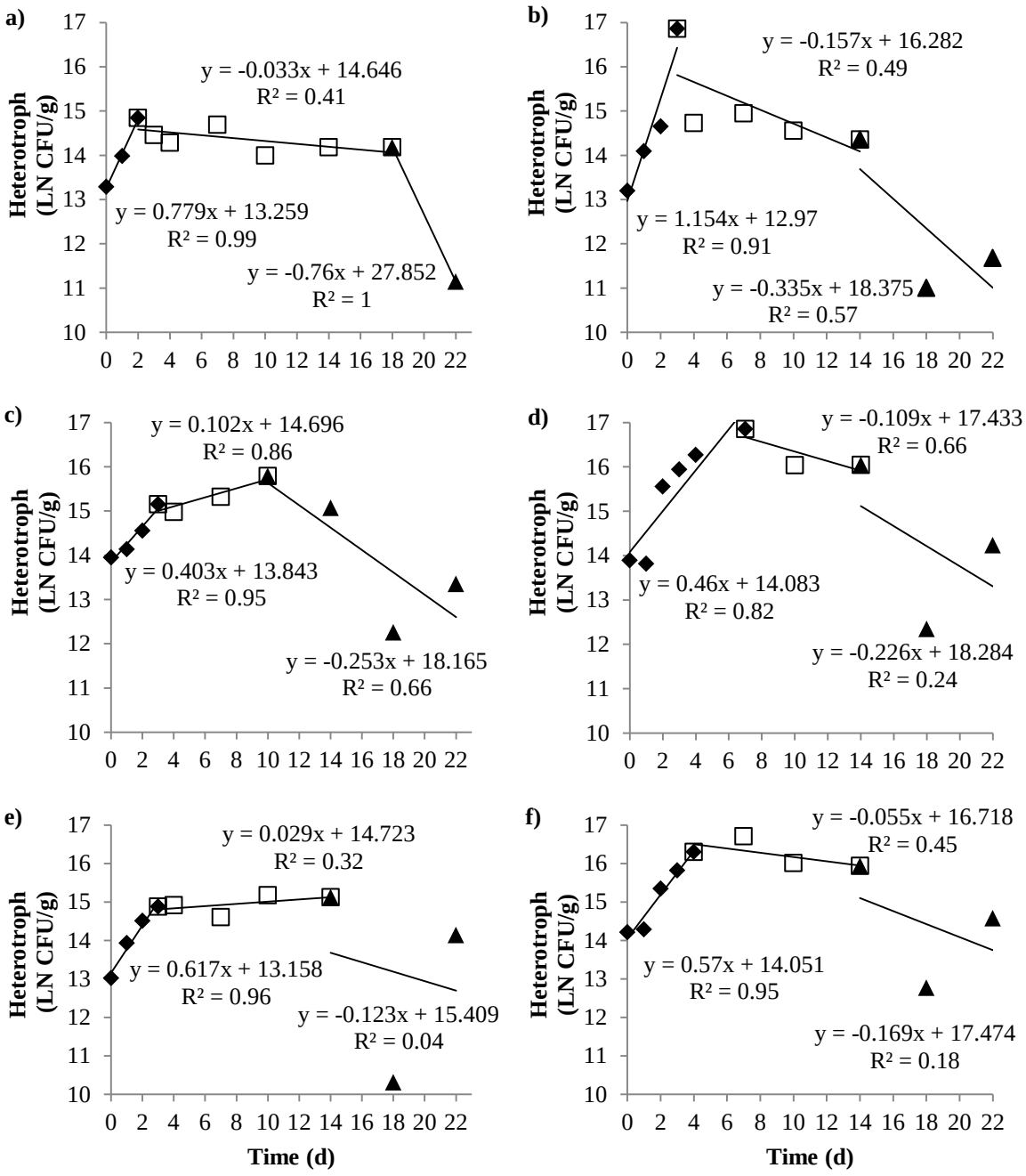

Figure B-5. LN of heterotroph concentrations in sediment recorded in trial 2 plotted over time to produce decay and growth constants. Graphics depict results for mesocosm: a) C1; b) C2; c) H1; d) H2; e) L1; f) L2

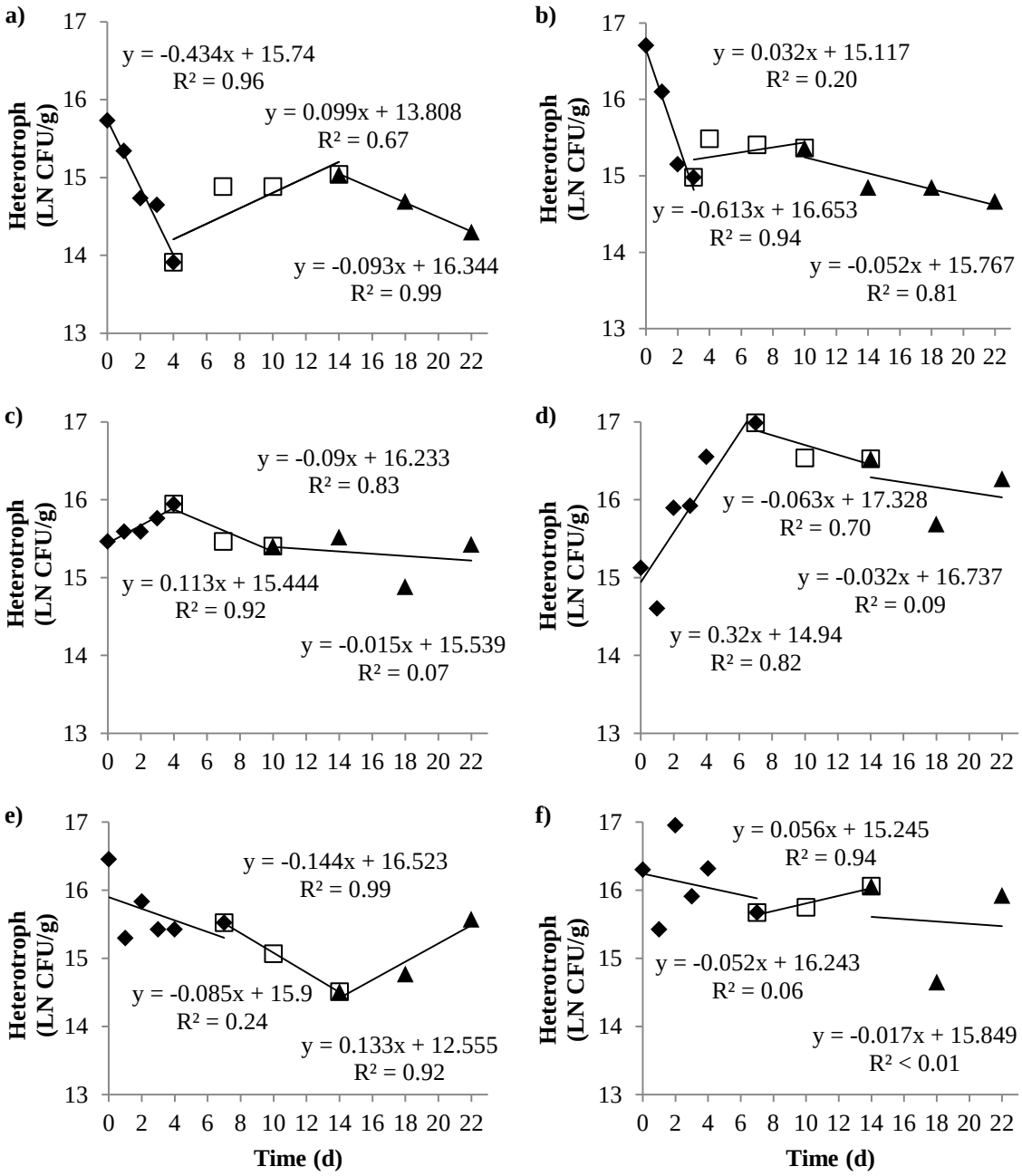

Figure B-6. LN of heterotroph concentrations in sediment recorded in trial 3 plotted over time to produce decay and growth constants. Graphics depict results for mesocosm: a) C1; b) C2; c) H1; d) H2; e) L1; f) L2

APPENDIX C

REGRESSION MODELS FOR PREDICTING LOG_{10} CONCENTRATIONS OF *E. COLI* IN WATER

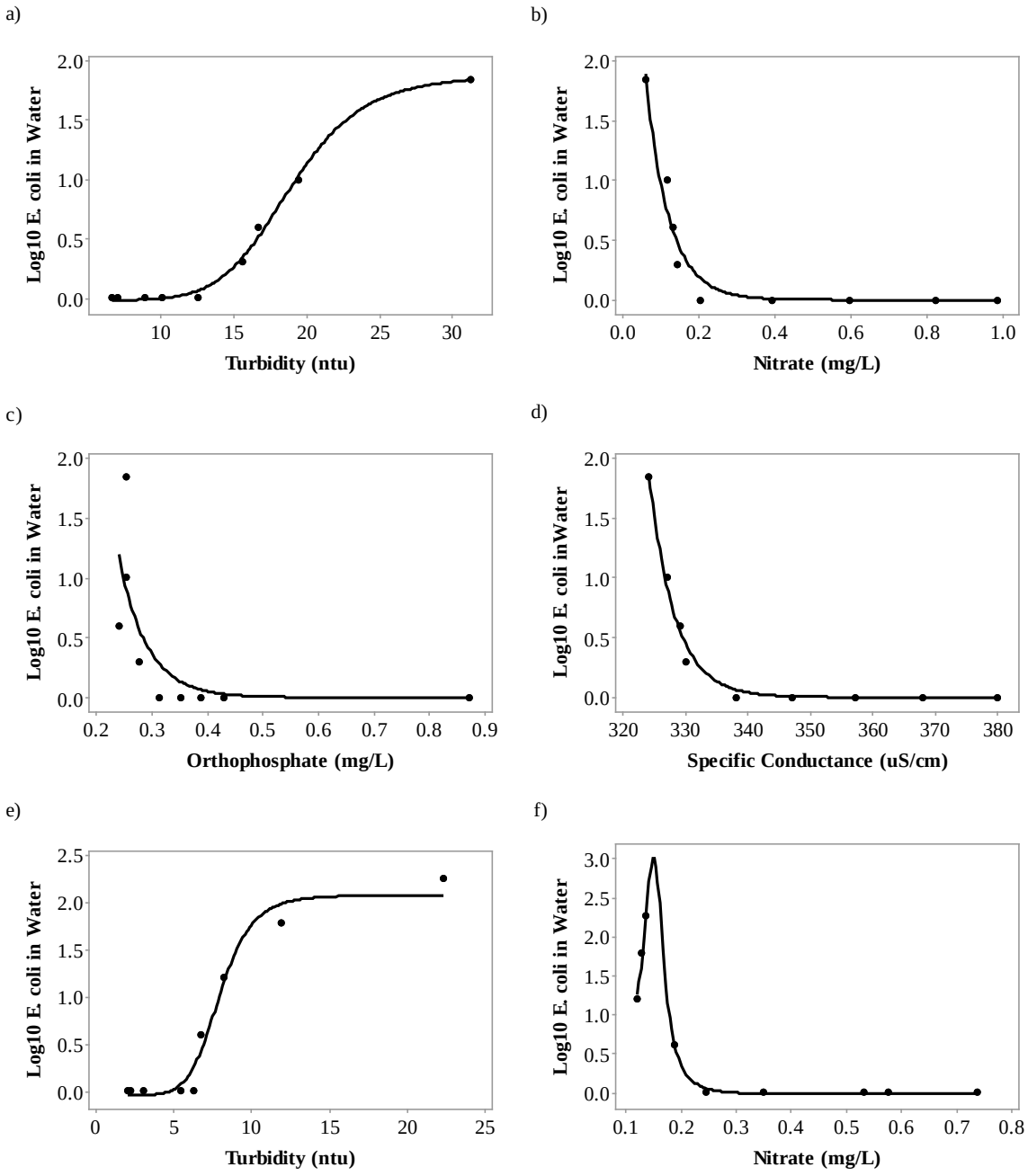

Figure C-1. Regression equations predicting $\log_{10}$ *E. coli* concentrations in water from respective predictor variables. a) C1-1 – turbidity; b) C1-1 – nitrate; c) C1-1 – orthophosphate; d) C1-1 – specific conductance; e) C1-2 – turbidity; f) C1-2 – nitrate

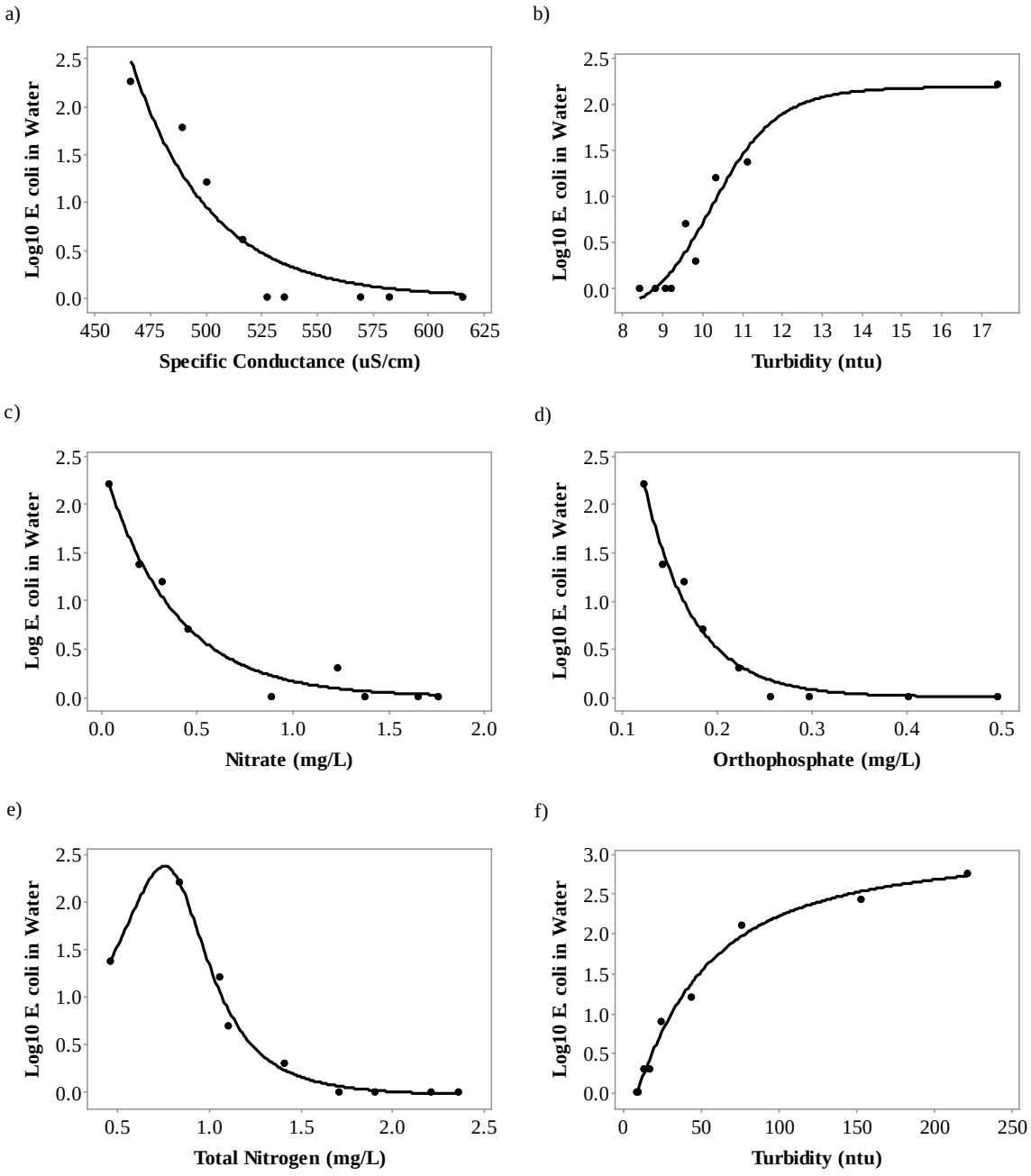

Figure C-2. Regression equations predicting $\log_{10}$ *E. coli* concentrations in water from respective predictor variables. a) C1-2 – specific conductance; b) C1-3 – turbidity; c) C1-3 – nitrate; d) C1-3 – orthophosphate; e) C1-3 – total nitrogen; f) C2-1 – turbidity

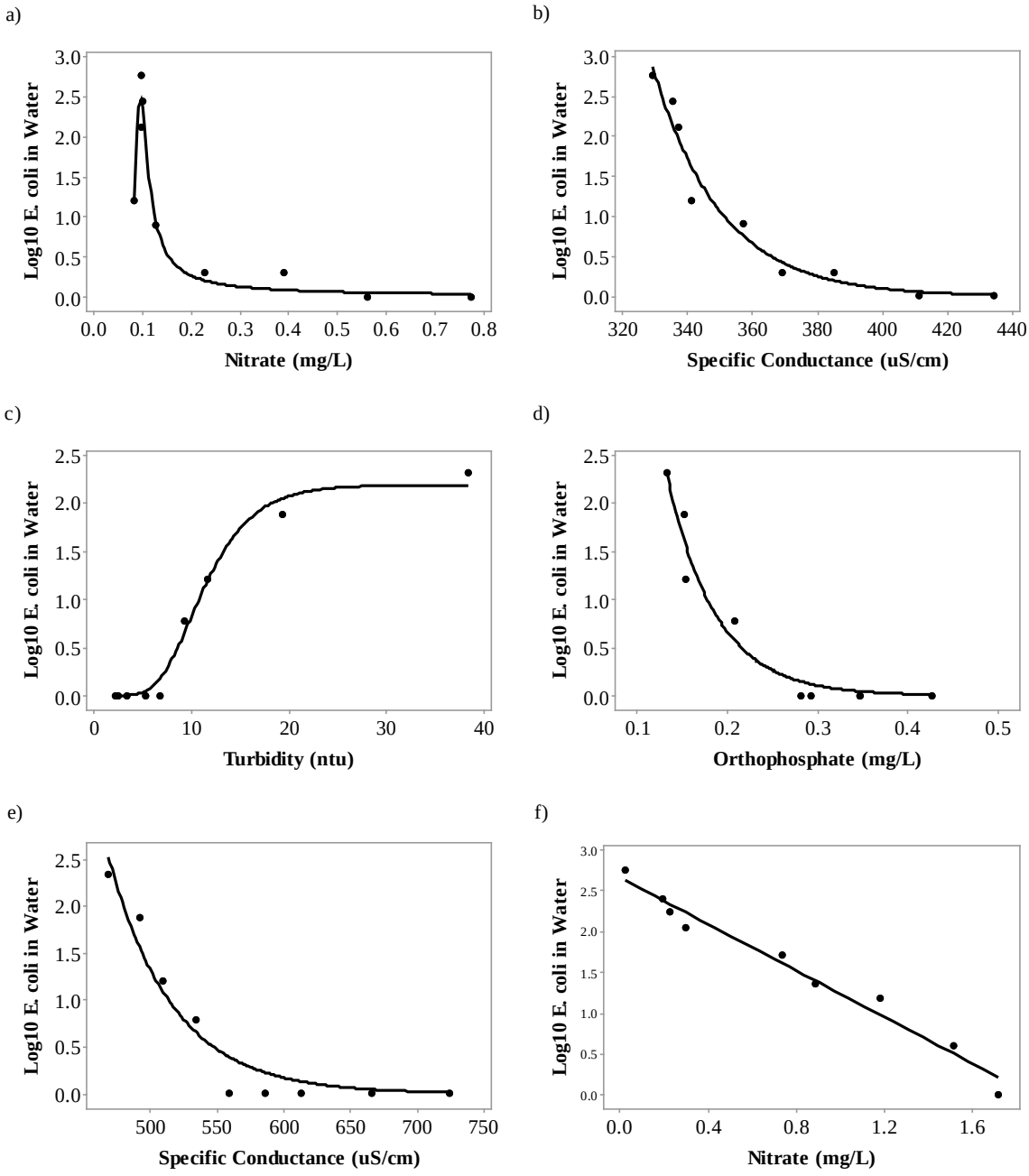

Figure C-3. Regression equations predicting $\log_{10}$ *E. coli* concentrations in water from respective predictor variables. a) C2-1 – nitrate; b) C2-1 – specific conductance; c) C2-2 – turbidity; d) C2-2 – orthophosphate; e) C2-2 – specific conductance; f) C2-3 – nitrate

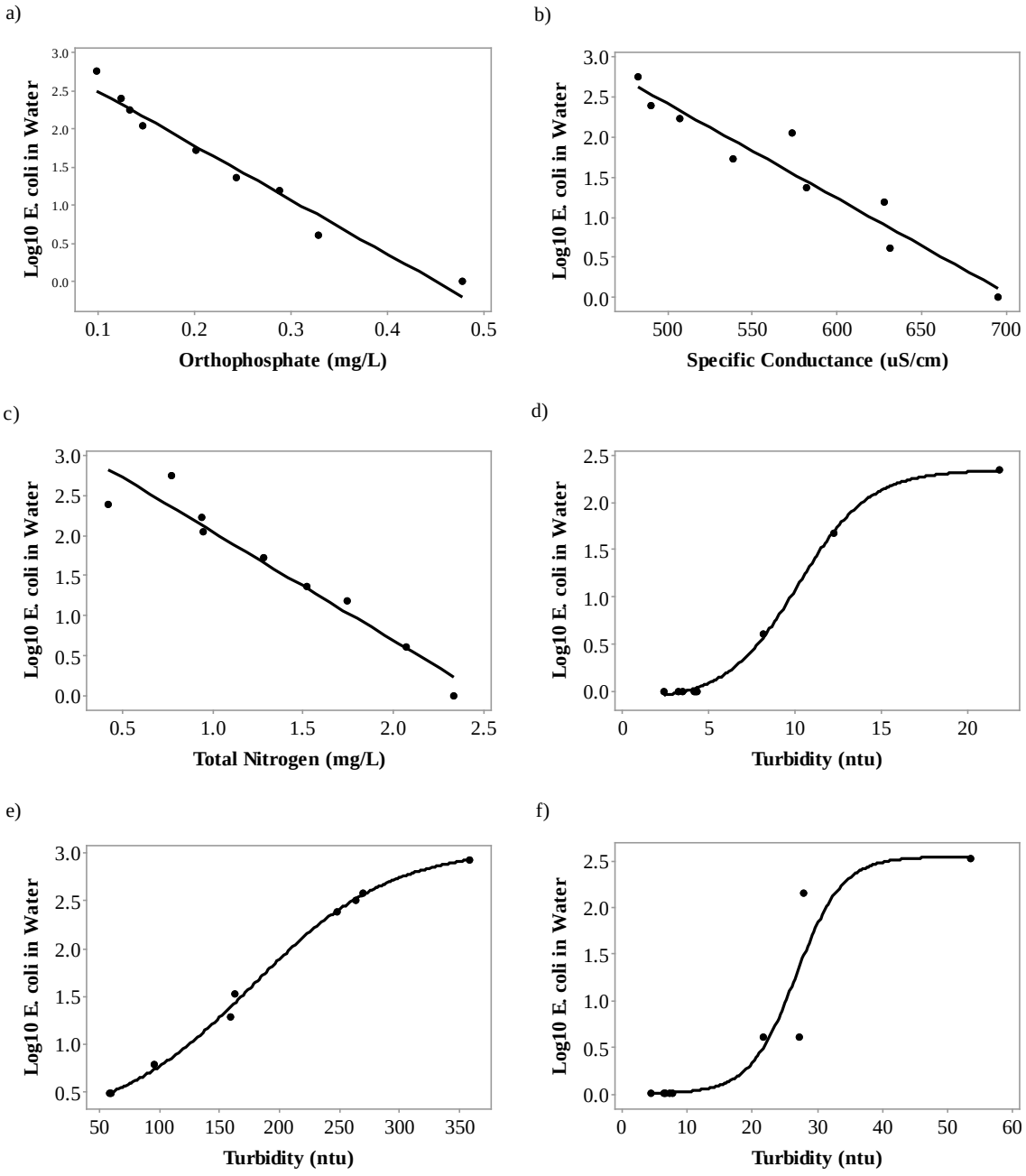

Figure C-4. Regression equations predicting $\log_{10}$ *E. coli* concentrations in water from respective predictor variables. a) C2-3 – orthophosphate; b) C2-3 – specific conductance; c) C2-3 – total nitrogen; d) H1-2 – turbidity; e) H2-1 – turbidity; f) H2-2 – turbidity

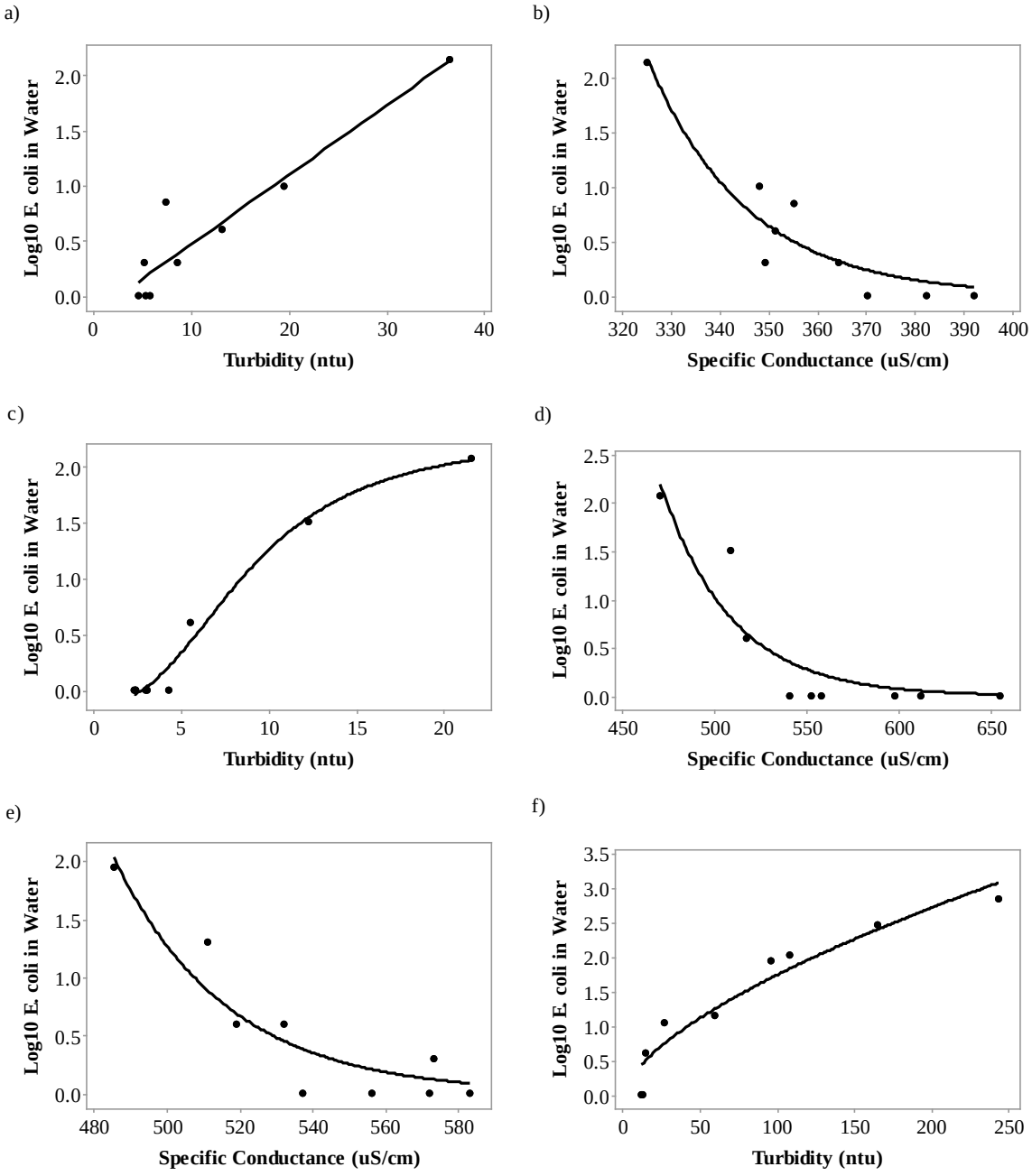

Figure C-5. Regression equations predicting $\log_{10}$ *E. coli* concentrations in water from respective predictor variables. a) L1-1 – turbidity; b) L1-1 – specific conductance; c) L1-2 – turbidity; d) L1-2 – specific conductance; e) L1-3 – specific conductance; f) L2-1 – turbidity

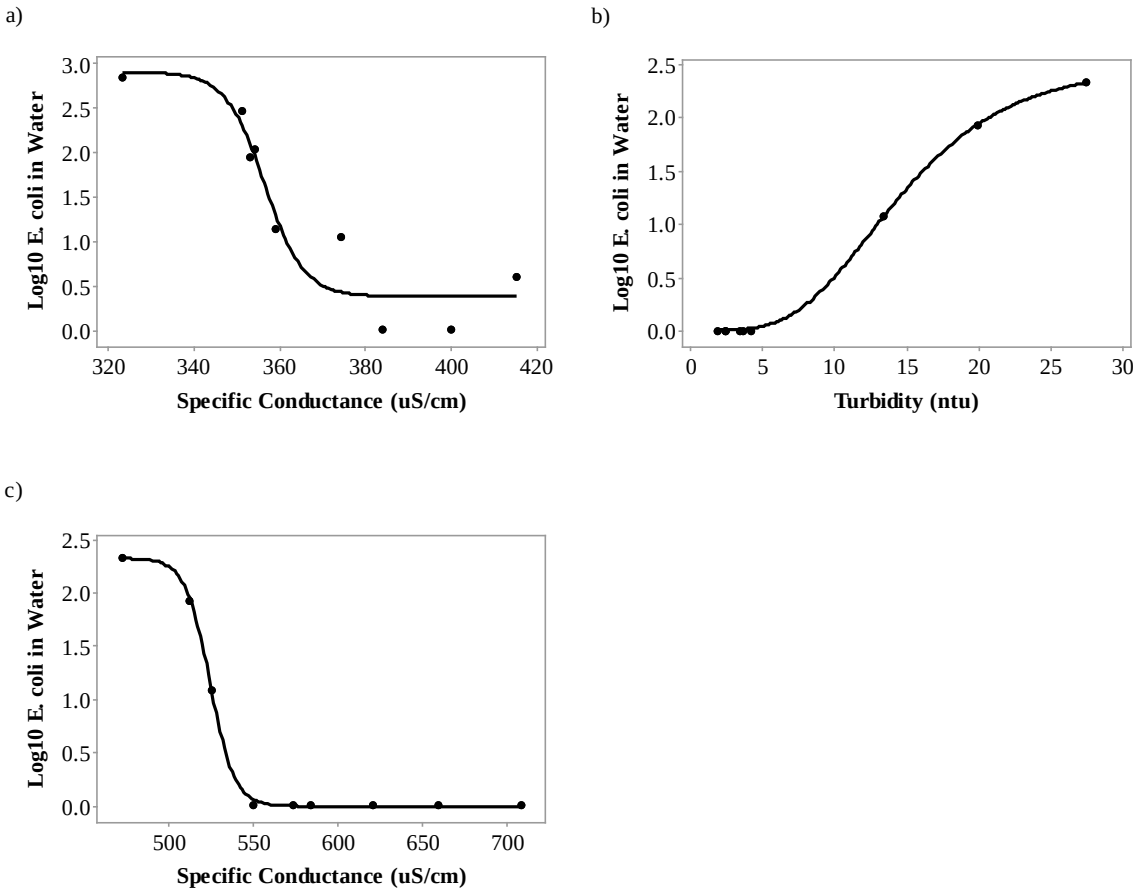

Figure C-6. Regression equations predicting $\log_{10}$ *E. coli* concentrations in water from respective predictor variables. a) L2-1 –specific conductance; b) L2-2 – turbidity; c) L2-2 – specific conductance

APPENDIX D CHANGES IN TEMPORAL NUTRIENT CONCENTRATIONS IN MESOCOSM

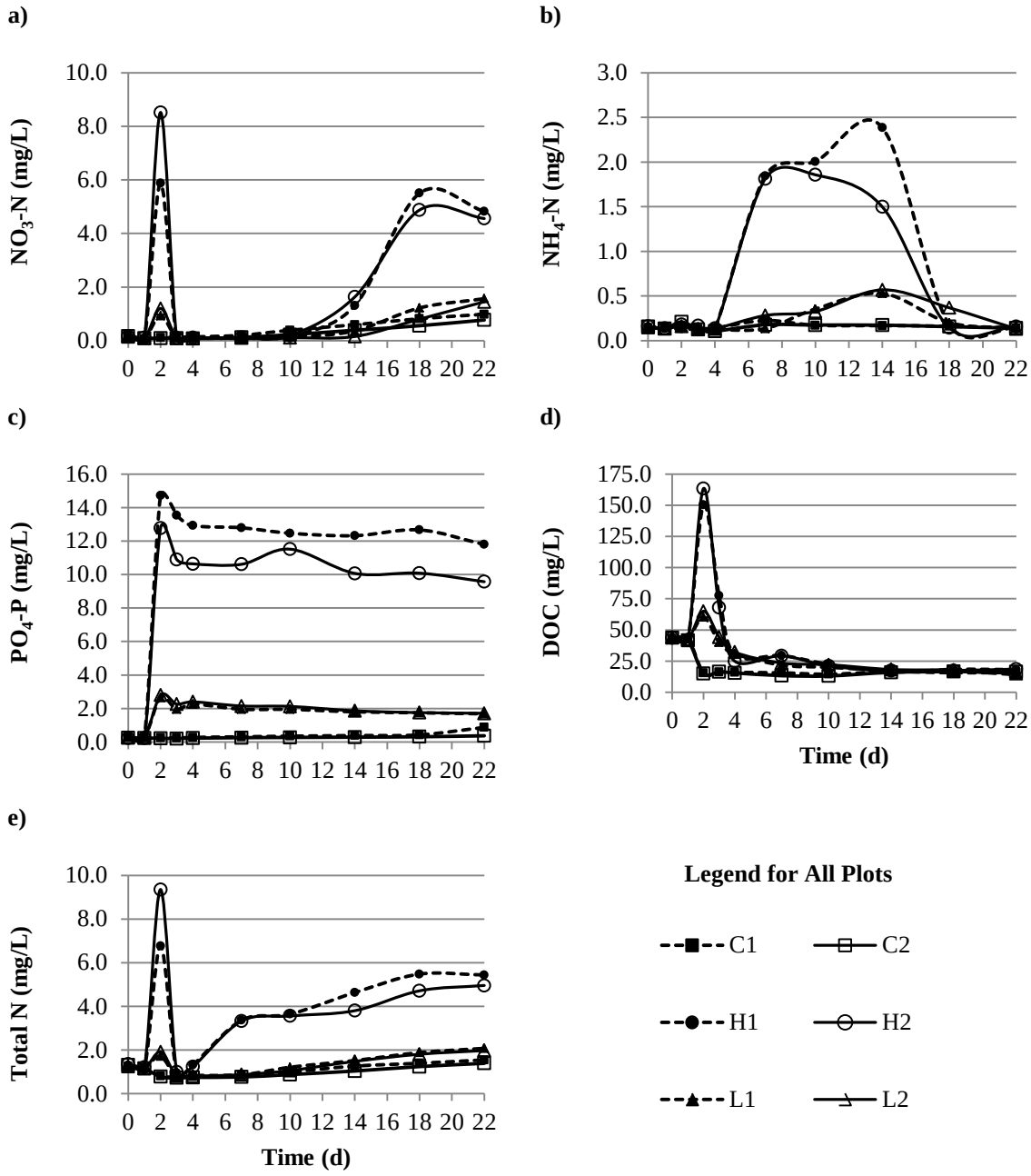

Figure D-1. Nutrient concentrations plotted over time in trial 1. Graphics depict: a) nitrate; b) ammonium; c) orthophosphate; d) dissolved organic carbon; e) total nitrogen

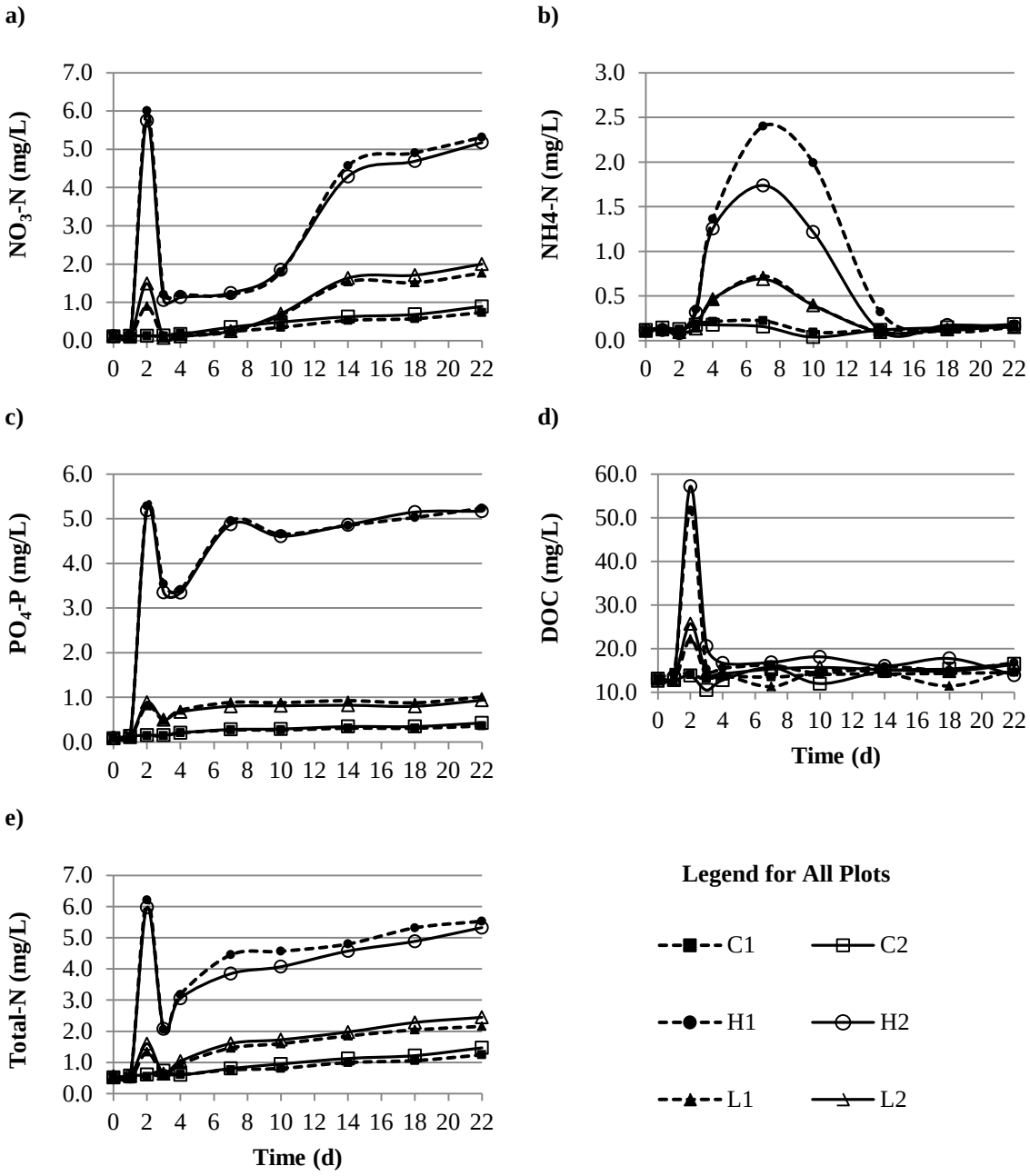

Figure D-2. Nutrient concentrations plotted over time in trial 2. Graphics depict: a) nitrate; b) ammonium; c) orthophosphate; d) dissolved organic carbon; e) total nitrogen

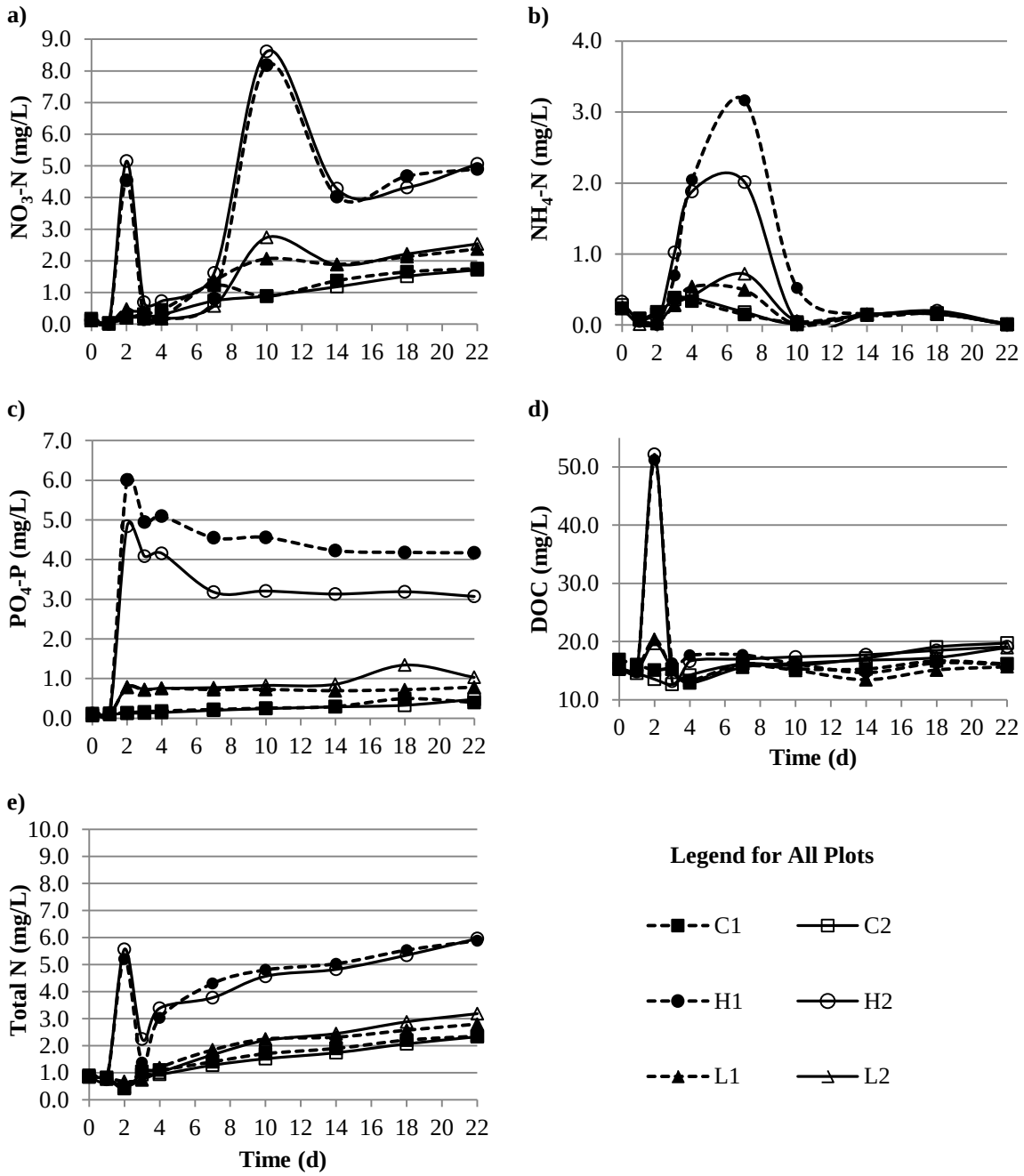

Figure D-3. Nutrient concentrations plotted over time in trial 3. Graphics depict: a) nitrate; b) ammonium; c) orthophosphate; d) dissolved organic carbon; e) total nitrogen 3