ABSTRACT

Title of Document: INVESTIGATING METRICS PROPOSED TO PREVENT THE HARVEST OF LEAFY GREEN CROPS CONTAMINATED BY FLOODWATER.

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Pathogens can be transported by water through soil to contaminate distant crops. The California LGMA states that leafy green crops within 30ft of flooded soil should be destroyed due to potential contamination. Previously flooded areas should not be replanted for 60 days. This study investigated the transport of *Salmonella enterica* and *Citrobacter freundii* through soil in a model system with a positive slope (uphill). Field trials involving flooding one end of a spinach bed with a negative slope (downhill) with water containing *Escherichia coli* were also conducted. Soil type, soil moisture content, and slope affected bacterial movement. In field trials, *E. coli* was quickly transported to the 30ft boundary, and persisted significantly longer in the fall trial than the spring. These data suggest the LGMA metrics need to provide additional parameters to prevent the harvest of leafy green crops potentially contaminated by floodwater.

INVESTIGATING METRICS PROPOSED TO PREVENT THE HARVEST OF LEAFY GREEN CROPS CONTAMINATED BY FLOODWATER

By

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Chapter 1: Introduction

1.1 Background

Every year, an estimated 47.8 million people in the United States experience an illness caused by contaminated food (67). As high as 46% of those illnesses are linked back to fresh produce, including fruits and vegetables such as melons, tomatoes, spinach, lettuce, and sprouts (61). In recent years, the number of outbreaks of foodborne illness associated with fresh produce has increased dramatically as a result of many different factors. The promotion of healthy lifestyles where fruits and vegetables are a large portion of the daily diet has led to a huge increase in the consumption of produce per person (8). In North America alone, the daily sales of fresh-cut produce reached 6 million packages in 2005 (58). In response, the global production of fruits and vegetables increased by 94% between 1980 and 2004. By 2013, the average total consumption of fresh vegetables in the United States was approximately 140 pounds/person (80). Awareness that raw fruits and vegetables are potential vehicles for foodborne illness, and improved detection of pathogens on the fresh produce samples, may have also contributed to the increase in outbreaks linked back to produce (8, 9).

Leafy green vegetables have been identified by the Food and Agriculture Organization/World Health Organization as the fresh produce commodity group of highest concern from a microbiological safety perspective. Their cultivation is vulnerable to contamination from a variety of sources, including contaminated manure, soil, irrigation water, and contact with wildlife and/or their feces (26). In fact, a 2008 survey of 300 fresh produce samples found that leafy green vegetables, including arugula,

sprouts, and spinach, had the highest average microbial loads of all samples tested (1). Approximately 23% of all foodborne illnesses are caused by these leafy vegetables (61). Between 1998 and 2008, more illnesses were associated with leafy vegetables than any other food commodity category (see Figure 1-1) (61). Such vegetables can become contaminated with pathogenic microorganisms through contact with improperly composted manure, contaminated irrigation or post-harvest washing water, contaminated soil, wild animals, contaminated workers, or contaminated processing facilities.

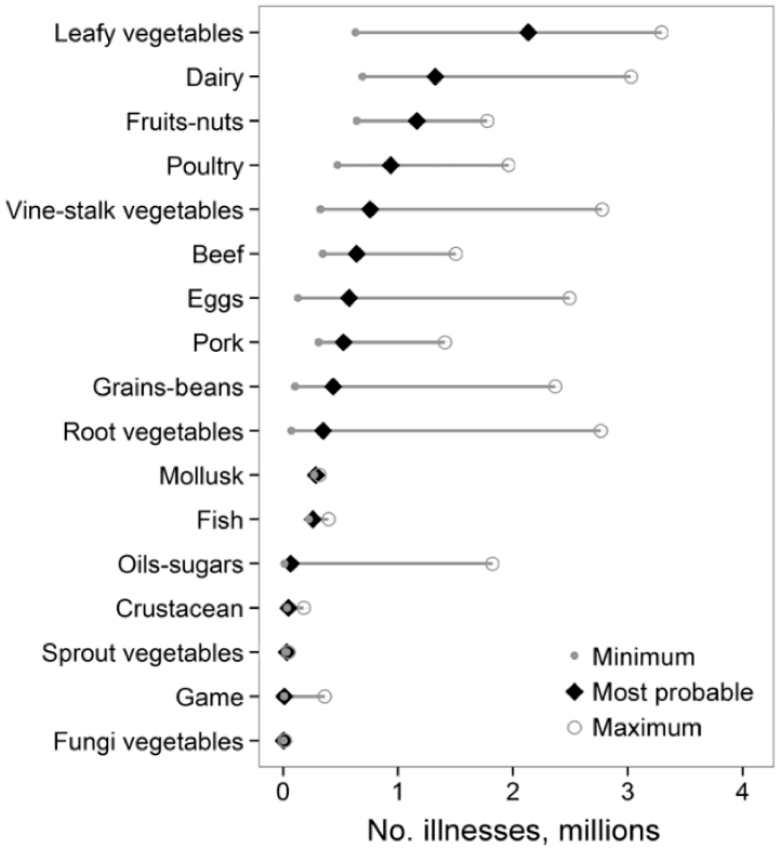

Figure 1-1: Estimates of the number of foodborne illnesses each year from all etiologies attributed to food commodities in the United States, 1998-2008 (61).

Livestock such as cows and chickens often harbor pathogenic bacteria such as *Escherichia coli* and *Salmonella enterica* in their gut, and shed these microbes in their

feces. While pathogenic bacteria are usually isolated in low numbers from feces, some studies have quantified high levels (>10^5 cfu/g) of *E. coli* O157:H7 in cattle manure (22, 50, 59). Farmers often use animal manure on produce crop fields as fertilizer, but if this manure has not been properly composted or stored for an adequate period of time, these pathogens may still be viable in the manure and potentially contaminate edible portions of the produce.

Rainfall and irrigation events may mobilize these cells out of the fecal matrix into the soil, where the bacteria can survive for extended periods of time, and move with infiltrating water to contaminate distant crops. Because of this, the United States Food and Drug Administration (FDA) has identified produce that has come in contact with flood water as "adulterated", and should be destroyed (11).

In 2012, the California Leafy Green Products Handlers Marketing Agreement (LGMA) elaborated on this recommendation to advise farmers to destroy all crops within 30ft of the edge of a flood, and to wait 60 days before replanting flooded fields, to allow sufficient time for previously flooded soils to dry out (16). The "30ft buffer zone", however, was based on the turning radius of a tractor, and not the potential movement of bacteria through soil. In fact, there is very little research that has investigated the movement of bacteria across a field, and virtually no research that uses a flood as the source of potential bacterial contamination transferred to leafy green crops. As such, there is a specific need for research that addresses this topic in order to provide more specific guidance to farmers to decrease the risk of harvesting leafy green vegetables that

have been contaminated by pathogenic bacteria while appropriately allowing otherwise safe produce to enter commerce.

1.1 Hypothesis of Research

The hypotheses of this study are that soil type and initial moisture content have a significant impact on the movement of bacteria across a soil core; as soil clay content increases and moisture content decreases, bacterial transport through soil will decrease. Additionally, *Citrobacter freundii* will be transported similarly through soil as *Salmonella enterica* serovar Newport, allowing *C. freundii* to be used as a surrogate for *Salmonella* in future field studies.

It is anticipated that the LGMA-recommended 30-ft "buffer zone" of crop destruction around a flood will be excessive, but the 60-day waiting period before replanting flooded areas will be appropriate. While bacteria can survive in soil for extended periods of time, movement across a field is limited because soil particles effectively retain bacteria and filter the cells out of percolating water.

1.2 Study Approach

This study was conducted in two phases: a laboratory-scale investigation and a field-scale experiment. In the laboratory-scale investigation, the effect of soil type and initial soil moisture content on the movement of bacteria through soil was determined using a model system. In the field-scale experiment, the survival of bacteria from flood water in soil, as well as the risk of contamination of spinach tissue by the bacteria, was investigated.

In the laboratory experiment, the movement of *Salmonella enterica* serovar Newport and *Citrobacter freundii* were compared through three soil types of varying clay content, at three different initial soil moisture contents. This experiment was designed to model a naturally occurring flood where heavy rainfall caused water to collect at the bottom of a field slope. The purpose of this experiment was to compare the influence of soil type and moisture content on the movement of bacteria through soil, as well as to elucidate if *C. freundii* may be a good candidate as a surrogate strain for pathogenic *Salmonella* strains for future field research.

In the field-scale experiment, the movement of *Escherichia coli* from floodwater through soil across a field of 4-week old spinach was evaluated. A flood was created on one end of rows of spinach with a negative slope to provide a worst case scenario where heavy rainfall caused the bank of a body of water to overflow and cause flooding of cropland. Soil and spinach samples out to 30-ft from the edge of the flood were evaluated for *E. coli* populations over a period of 63 days to address the LGMA metrics.

1.3 Potential Impact of Study

This research seeks to provide more quantitative information on the risk of contamination of leafy green produce by bacteria that have been mobilized by floodwater. Based on a literature review, this will be the first experiment to track the long-term movement and survival of bacteria from a flood through soil used to grow produce crops. The goal of this study is to directly address the validity of metrics the LGMA has outlined for leafy

green produce farmers to follow after a flooding event. Data generated from this research project will be vital to determining appropriate safety standards that protect both the general population from contaminated produce, as well as farmers from unnecessary crop and profit losses. Regulations that have been supported by scientific theory and validated research will protect farmers from the liability of harvesting crops contaminated by bacteria from distant floods, or having to unnecessarily destroy crops that have a low risk of contamination. Decreasing the risk of harvesting contaminated crops will subsequently decrease the risk of foodborne illnesses and outbreaks associated with leafy green crops.

Chapter 2: Literature Review

2.1 The Problem

From 1996 to 2005, 18 separate outbreaks of foodborne illness caused by *Escherichia coli* O157:H7 were linked to fresh or fresh-cut lettuce and spinach, for a total of over 400 illnesses and 2 deaths. Eight of the outbreaks were traced back to farms in the Salinas Valley, California, and sampling identified *E. coli* O157:H7 in creeks and rivers nearby (11)

As a result, in 2005, the United States Food and Drug Administration (FDA) published the "Letter to California Firms that Grow, Pack, Process, or Ship Fresh and Fresh-cut lettuce" where ready-to-eat produce crops that had been in contact with floodwater were characterized as "adulterated", due to the potential exposure to microorganisms or other contaminants in the water. The FDA advised these crops should be excluded from the food supply to protect human health (11). In response, the California Leafy Green Products Handler Marketing Agreement (LGMA) released guidelines in 2012 that suggested farmers destroy all leafy green crops within a minimum of 30ft from the edge of a flood, and to wait 60 days before replanting the soil that had been underwater (16). The 30ft buffer zone is based on the turning radius of farm production equipment, to prevent cross contamination between flooded crops and leafy greens that had not contacted floodwater.

The LGMA defines a flood as "the flowing or overflowing of a field with water outside a grower's control that is reasonably likely to contain microorganisms of significant public health concern and is reasonably likely to cause adulteration of edible portions of fresh produce in that field" (16). There are two main pathways that floodwater on cropland can become contaminated with human pathogens, both involving the leaching of contaminants from manure. Animal manure, especially ruminant feces, is a significant vehicle for human pathogens like *E. coli* O157:H7 and *Salmonella*. Often, manure is applied to crop fields as fertilizer, but research has shown that these pathogens may survive in manure for exceptional periods of time, over 200 days in some cases (44, 83). A heavy rainfall event on a field that has been fertilized by inadequately stored manure may cause a mobilization of the pathogens present in the manure into the water. Subsequently, the bacteria may move through the soil with infiltrating water to distant locations past the edge of the flood.

In a second scenario, cattle on a ranch may have access to streams or rivers. Defecation on the bank of, or directly into, the body of water leads to transport of the potentially pathogenic manure downstream, where cropland may be present. A heavy rainfall event either upstream near the pastureland or downstream at the cropland may cause overflowing of the contaminated water and transport of the bacteria with manure through the soil to potentially contaminate crops.

As will be discussed below, the vertical transport of bacteria through soil is well documented because of the risk of microbial contamination of ground water. Similarly,

the horizontal transport of bacteria down a hillslope has also been studied, in the context of the risk of contamination of water sources from bacteria leaching from a septic system (48, 62). However, little research has investigated the horizontal movement of bacteria through soil from contaminated floodwater and the risk of contamination of leafy greens.

2.2 The Pathogens

While a large number of bacterial genera are linked to foodborne illness, including *Listeria* and *Campylobacter* spp., this literature review will focus on *Salmonella enterica* and *Escherichia coli*.

Salmonella enterica is a gram-negative, rod-shaped bacterium commonly found in the intestines of a number of animals, including birds, lizards, poultry, and cattle. The species is further differentiated into serovars based on the antigens expressed, with a number of serotypes having been found on a variety of fresh produce, including various sprouts, cabbage, lettuce, parsley, salad greens, and spinach (42). For bacterial foodborne illness outbreaks in the United States between 2004 and 2012, *Salmonella* was the most commonly identified cause, accounting for 53% of outbreaks (see Figure 2-1). Further, a limited number of *Salmonella* serovars caused most multistate outbreaks of bacterial foodborne illness during this period (17). *Salmonella enterica* serovars Typhimurium and Newport were associated with the most produce-associated outbreaks.

Salmonella have been isolated from a variety of animal and environmental farm samples, including pigs, manure, water, and soil. A study of almost 8,000 animal and environmental samples from a U.S. dairy farm isolated *S. enterica* from as high as 72%

of cattle samples and over half of the environmental, insect, and bird fecal samples (62). Another study isolated *Salmonella* from 13% of chickens in a poultry farm (64). In addition to pigs and poultry, wild birds and reptiles are significant vehicles of *Salmonella* (27). Produce crops frequently become contaminated through contact with contaminated manure from these livestock, frequently if the manure has contaminated irrigation water or when the manure is deposited onto the soil.

Escherichia coli is another gram-negative rod shaped bacterial species found in the gut of animals. This species can also be further differentiated by antigen presentation. Ruminants, especially cattle, are a natural reservoir of *E. coli* strains that are pathogenic to humans. Omisakin *et al.* reported that *E. coli* O157:H7 was isolated from 7.5% of all cattle fecal samples tested (59). Another study found at least one fecal sample positive for *E. coli* O157:H7 from 21 out of 29 cattle lots sampled (24). This pathogen can be shed asymptomatically in the feces of the cattle and survive for extended periods of time in the environment.

Historically, enterohemorrhagic *Escherichia coli* outbreaks were linked to meat products, mainly ground beef. But with public health education, the number of meat-linked outbreaks has taken second place to produce-associated outbreaks, mainly by leafy greens and sprouts. Between 2004 and 2012, *E. coli* was the second most common cause of produce-linked foodborne illness, with O157:H7 being the most prevalent serotype (17). *Escherichia coli* strains were responsible for the largest number of outbreaks linked to salad vegetables like lettuce and spinach. Behind *Salmonella*, *E. coli* caused the second

largest number of multistate outbreaks during this period. Further, *E. coli* O157:H7 was the cause of the most widespread multistate outbreak in the United States when, in 2006, 238 individuals across 26 states became sick, and 5 died after consuming contaminated fresh spinach from California (17, 20).

Table 2-1: Summary of outbreaks associated with fresh vegetables and fruits in the United States, 2004-2012 (17)

	Food vehicle									
	Vegetables					*Fruits*				
Type of pathogen	*Salad*	*Leafy*	*Tomato*	*Other*	*Sprouts*	*Berries*	*Melon*	*Juices*	*Other*	*Total outbreaks*
Norovirus	97	62	5	9	0	5	9	3	33	223
Salmonella spp.	8	8	17	3	14	2	14	0	5	71
Escherichia coli	10	22	0	0	4	2	0	6	2	46
Campylobacter spp.	4	2	1	0	0	0	1	0	1	9
Shigella spp.	1	2	0	0	0	0	0	0	0	3
Clostridium spp.	0	0	0	0	0	0	0	0	0	0
Staphylococcus spp.	2	0	0	0	0	0	0	0	0	2
Yersinia spp.	0	0	0	0	0	0	0	0	0	0
Bacillus spp.	1	0	0	0	0	0	0	0	1	2
Giardia spp.	0	1	0	1	0	0	0	0	0	2
Cyclospora spp.	1	1	0	1	0	3	0	0	2	8
Cryptosporidium spp.	0	0	0	0	0	0	0	3	0	3
Other foodborne viruses (hepatitis A virus)	0	1	1	1	0	0	0	0	2	5
Other microorganism (*Listeria monocytogenes*)	0	0	0	0	2	0	1	0	0	3

2.3 Pre-Harvest Sources of Contamination

The demand for fresh produce has put stress on farmers to increase production in ways that may threaten the microbiological safety of the product. The largest factor may be the use of animal manure as fertilizer, rather than chemical fertilizers (9). Using animal manure provides the farmer numerous benefits, including saving the farmer the cost of the chemical fertilizer and providing a way to dispose of livestock waste. However, livestock may asymptomatically harbor microorganisms that are pathogenic to humans, and these zoonotic pathogens may be shed in feces. For example, a survey of fecal samples from 44 cattle found that while 61% of cattle harbored *E. coli* O157 at levels

below 10^2 cfu/g, 2 samples contained over 10^5 cfu/g *E. coli* O157 and 2 additional cattle contained between 10^4 and 10^5 cfu/g (59). This high rate of shedding has also been seen in other investigations of the levels of pathogens in cattle feces (22, 50).

Pathogens present in manure can persist to contaminate soils and plants, even if the manure has been stored for a time before land application. On organic farms, no manure may be applied to fields as fertilizer within 120 days of harvest, but *Salmonella* Newport has been shown to survive in manure for almost 200 days (83), and *E. coli* O157:H7, almost two years (44). Current guidelines advise a composting period before applying manure to a field as fertilizer, but pathogens may resist being killed by this process if the temperature of the pile does not reach the appropriate temperature, generally at least 55°C for several days (72).

The influence of cattle grazing on soils and water sources has been thoroughly studied. Howell *et al.* found a 51% increase in fecal coliform-positive well water samples after cattle were released to graze on various silt loam fields for the first time (39). Gagliardi and Karns (2000) found that the coliform concentrations in the surface layers of sandy loam soil were significantly higher, and detected to greater depths, during cattle grazing months than when fields were unused (30).

The proximity of produce fields to livestock may also contribute to crop contamination. Though the livestock manure may not be used as fertilizer, run-off from the animal pastures to crop fields after rain event may promote the transport of pathogens from

manure to the crops (8). In a three-year study of surface runoff from grazed and ungrazed pasture land in Nebraska, levels of fecal coliforms were found to be between 5 and 10 times greater in grazed pastures than ungrazed land (44). A 1999 study by Hagedorn *et al*. traced fecal contamination of stream water in Virginia to the presence of cattle nearby. The contamination could have been a result of the cattle having direct access to the stream or could have resulted from runoff from the feedlots, manure piles, or crop fields fertilized with manure. The installation of fencing to keep the cattle away from the stream correlated with a 94% reduction in fecal coliform levels in the water (36). In Colorado, the presence of cattle near creeks increased the levels of fecal coliforms detected in the waters by a factor of 1.6 to 12.5 (31).

Contamination of surface water is a concern both for the direct ingestion of contaminants, and the indirect exposure provided when this water is used to irrigate crops. As such, irrigation water has been implicated as a source of contamination of a variety of leafy greens in a number of epidemiological investigations and experimental studies. Contaminated irrigation water was traced as the source of a 1998 outbreak of *E. coli* O157:H7 infection from contaminated lettuce (3). Another study identified sewage-contaminated irrigation water as the source of *E. coli* detected on cabbage seedlings (78). Contaminated water used to irrigate lettuce was identified as the source of a multistate outbreak of *E. coli* O157:H7 in 1996 (37).

In addition, wild animals such as swine, deer, and rats may transport pathogens from distant sources via their fur or feces (3). In the aforementioned 2006 outbreak of

Escherichia coli O157:H7 from bagged spinach, trace-back investigations recovered the outbreak strains from both cattle feces and local feral swine (45). In 2004, Nielsen *et al* reported the isolation of Shiga toxin-producing *E. coli* isolates from a wild bird and a rat that were virtually identical to cattle isolates from nearby farms (56). Further, fecal contamination of surface waters in New York was traced back to wild bird feces (74).

2.4 Bacterial Survival in Soil

Research has shown that contaminated water does not need to come into direct contact with the plant for contamination to occur. In 2002, Solomon *et al.* demonstrated that *E. coli* O157:H7 in irrigation water added to soil could be detected on, and in, lettuce leaf tissue (72). This is an indication that, to some extent at least, pathogens are able to both survive in and move through soils to points distant from their source to contaminate edible portions of plants.

Just as *E. coli* and *Salmonella* are able to survive for long periods of time in manure, they can also survive an impressively long time in soil, if conditions are favorable. This is a significant accomplishment, as soil environments are highly dynamic, with constantly fluctuating temperature, nutrient levels, and pH, as well as a high density of competing native microorganisms. *Escherichia coli* have been isolated from soil in a huge variety of climates, from tropical soils to alpine soils (12). Recently, it was discovered that low levels of *E. coli* could be cultured from lysimeters of an Irish soil, over nine years after the last application of any fecal material, suggesting *E. coli* are able to become naturalized in soil and form distinct saprophytic populations (13).

While most pathogens survive for only a few months in soil, some studies have reported significantly longer survival rates. Research by Islam *et al*. showed *E. coli* O157:H7 persisted for 217 days in compost-amended soil where parsley was grown (41). A similar experiment looking at *S. enterica* serovar Typhimurium survival found the *Salmonella* survived for 161 and 231 days in soil where lettuce and parsley were grown, respectively (42). Other studies have found that *Salmonella* may persist in soils for up to two and a half years (43).

Bacterial survival in soil is dependent on a variety of factors, mainly temperature but also soil composition, nutrient content, water content, pH, UV exposure, and competition from indigenous microorganisms as summarized in Table 2-2. Many studies have indicated that human pathogens such as *E. coli* and *Salmonella* survive in soils significantly longer at lower temperatures (38, 77). Van Donsel *et al* showed a one-log reduction in the levels of coliform bacteria in soil within 3.3 days of application in the summer, while it took 13.4 days for the same reduction in the winter (77). Similarly, Stoddard *et al* (75) found the die off of fecal coliforms from manure was more rapid after fall application, due to the freezing temperatures and freeze-thaw cycles of the fall months, than after spring application. After compiling the data from a number of experiments, Reddy *et al* concluded that the bacterial die-off rate approximately doubles when the temperature increases by 10°C (65).

Moisture content is an important factor influencing bacterial survival in soil. Survival of both *E. coli* and *Salmonella* have been shown to be significantly enhanced at higher moisture contents compared to drier soils (25). Another experiment found *E. coli* survival to be greatest in soils that had been flooded (44). Multiple studies have shown the die-off rate increases when the soil moisture content decreases (65, 48). Further, soil type has an effect on bacteria survival but mainly as a function of the soil's ability to retain water. *E. coli* survival in sandy soils is generally significantly lower than other soil types, as a result of the sandy soil's relative inability to hold water (44).

Competition from indigenous microorganisms for resources is a significant factor influencing pathogen survival in soils, as evidenced by experiments showing prolonged survival in sterilized soil compared to field samples (44). One explanation is that established communities of soil bacteria are better adapted to the relatively harsh and transient environmental conditions in soil. Further, bacteriophages and other organisms have been shown to parasitize *E. coli* cells in soil (44). Finally, other microbes in the soil may produce antibiotics or other substances that are toxic to fecal bacteria like *E. coli* and *Salmonella* (65).

The effect of manure application on the survival of human pathogens in soil has not been conclusively determined. Some studies have reported that while it is often the source of the pathogens, manure significantly decreases the survival of *S.* Typhimurium in soil compared to non-amended soil (43). A possible explanation for this is that the manure supplies nutrients that promote the survival and (potentially antagonistic) activity of

native microbes. However, a separate study investigating the leaching of *E. coli* O157:H7 through intact soil cores found that the presence of manure in the soil cores promoted replication and survival of the bacteria (30). This effect may be source-dependent, as one study showed that the time for a one log reduction in *S.* Typhimurium levels took 15 days in poultry manure amended soil, but only 8.7 days in cattle manure-amended soil (57). Pathogens are also able to survive longer in manure slurry than farmyard manure, possibly because the lower temperature and anaerobic conditions in slurry favor pathogen growth (43, 68)

Table 2-2 Factors affecting pathogen survival in soil (2)

Factor	Comment
Humidity	Humid environments favor pathogen survival Dry environments facilitate pathogen die-off
Soil content	Clay soils and soils with high organic content favor pathogen survival
Temperature	The most important factor in pathogen die-off. High temperatures lead to rapid die-off; low temperatures lead to prolonged survival. Freezing temperatures can also cause pathogen die-off
pH	Some viruses survive longer in lower pH soils, while alkaline soils are associated with more rapid die-off of viruses; neutral to slightly alkaline soils favor bacterial survival
Sunlight (UV radiation)	Direct sunlight leads to rapid pathogen inactivation through desiccation and exposure to UV radiation
Foliage/plant type	Certain plants have sticky surfaces (e.g., zucchini) or can absorb pathogens from the environment (e.g., lettuce, sprouts), leading to prolonged survival of some pathogens; root crops such as carrots are more prone to contamination, and facilitate pathogen survival
Competition with native flora and fauna	Antagonistic effects from bacteria or algae may enhance die-off; bacteria may be preyed upon by protozoa

2.5 Bacterial Movement Through Soil

Very few studies have investigated the movement of bacteria across a field. Factors affecting such movement have been established through experiments investigating the downward movement of bacteria from the surface of soil to deeper distances, and even to groundwater sources. This section outlines the mechanism of

bacterial movement through soil, and the various factors affecting such movement, as determined by experiments that monitored the downward movement of bacteria through soil. Logically, these dynamics of bacterial movement should also play a similar role in the horizontal movement of bacteria across a field.

The major transport mode of bacteria through soil is with the gravitational flow of water, via preferential flow processes through macropores. This usually occurs when rainfall or irrigation promotes the transport of the bacteria with water through relatively large cracks or pores in the soil (4). Therefore, bacteria movement through soil is retarded in fine soils with many small pores as compared to dense soils that have large cracks running through them. These relatively larger pores and cracks may be formed by soil fauna such as earthworms and insects, cracks caused by the shrinkage of clay during freeze/thaw cycles, pores formed by plant roots, or pores caused by natural subsurface water flow (44).

An experiment comparing a sandy loam soil to a clay loam soil found that bacteria traveled farther in the clay loam soil, and were detected at significantly higher concentrations than in leachate from the sandy loam soil, where bacteria were rarely detected (4). This clay soil had large macropores of cracks and earthworm tunnels that promoted the movement of water through the column, as opposed to the sandy soil, which had a much finer structure where water moved through micropores that limited bacterial movement. However, even in the clay loam, less than 1% of the original inoculum was recovered from the leachate, indicating that the vast majority of bacteria

are retained in soil (4). Other studies have also found the presence of preferential flow paths to significantly increase the rate of bacterial transport through soil columns (7, 44, 54, 76).

Bacteria are mainly retained in soil via adsorption to the soil matrix, and simple physical filtration when the bacteria cannot fit through micropores. As bacterial cell size increases, the impact of straining by the soil increases as well. Research has indicated that when the cell size is greater than 5% of the average pore size in the soil, physical filtration becomes a significant factor in the rate of bacterial movement through the soil matrix (43).

Most filtration occurs at the soil surface, near the source of the contaminant via a combination of simple straining and adsorption (7). This implies a high risk of contamination of leafy green crops by these cells, as the plant leaves are well exposed to soil and may become contaminated themselves by rain splash of the contaminated soil onto the edible portions of the crop. As cell size increases, bacteria are more subjected to filtration by soil. Further, the bacteria themselves are rarely present as individual cells; they aggregate into larger clumps that are especially prone to filtration by soil micropores and other bacterial aggregates, further preventing transport through the soil matrix (43).

Soil filtration of bacterial cells out of water is highly efficient, and the concentrations of bacteria recovered from leachates are usually very low. Generally, the vast majority of bacteria cells are retained in the top layers of soil, though cells do still reach lower

depths. In experiments investigating the leaching of bacteria through soil columns, the amount of the inoculum recovered from the leachate is generally very low, usually less than 5%, even at the greatest concentrations (4, 6, 7, 65).

2.6.1 Factors Affecting Movement

Soil type, mainly the concentration of clay in the soil, has a significant effect on how far bacteria can travel through soil. As bacteria generally present a net negative charge, most bacteria cells will adhere to the positively charged clay particles in soil (65). Therefore as the clay concentration increases, microbial movement generally decreases. In an experiment comparing the movement of *E. coli* O157:H7 through three different soil types after a simulated heavy rainfall, a significantly lower concentration of bacteria was recovered from clay loam than from silt loam or sandy loam, with the most bacteria recovered from the sandy loam soil, the soil with the lowest clay content (30).

Particle size of the soil also plays a significant role in determining the extent of bacterial transport. Finer grained soils, like clays and silts, are more efficient at filtering bacteria because of their smaller pore sizes (44). Generally, the removal of bacteria from infiltrating water is inversely related to the particle size of the soil. However, as stated above, if a soil is subjected to significant weathering and underground movement of soil fauna such as worms, then cracks and preferential flow paths are generated that significantly promote movement of water and therefore bacteria.

Soil moisture content is also a significant factor influencing the movement of bacteria through soil. Generally, infiltration rates are greater through wet soil, such as after a rainfall or irrigation event, than when the soil is dry (30). In unsaturated soil, bacteria adhere to the soil and accumulate at the air-water interface of soil pores. In saturated soil, few pores have air trapped in them, as they are full of water, and the bacteria are easily transmitted through the soil with the permeating water (55). An experiment investigating the movement of *E. coli* from manure to tile drains underneath a loam soil found that the highest concentrations of *E. coli* in the drainage water correlated with rain events (44). In a separate experiment, only 8.8mm of simulated rainfall were required to detect the *Salmonella* in leachate from soil columns, indicative of a high risk of bacterial movement away from a contaminant after just one average precipitation event (7). Mosaddeghi *et al*. investigated the transport of *E.* coli through saturated and unsaturated clay loam lysimeters, and found that the concentration of *E. coli* in effluent was always higher in the saturated columns than unsaturated (55). This was partially explained by the fact that the infiltrating water itself traveled farther in the saturated soil columns than the unsaturated columns. Thus, soil retention of bacteria increases when the soil water content decreases.

Plant roots may also impact the extent of bacteria movement through soil. While plant roots may promote the formation of cracks and pores in soils as stated above, the roots themselves might block bacterial movement through soil by acting as a physical barrier to the cells and/or providing a site for bacteria to adhere to. A study investigating the effect of plant roots on percolation of *S.* Typhimurium and *E. coli* O157:H7 through soil found

that in soils containing lettuce roots, neither species percolated to a depth beyond the roots. Further, concentrations of the two pathogens were significantly higher in rhizosphere soils than bulk soils at the same depths without plant roots (68).

In an experiment comparing the leaching of *E. coli* O157:H7 through different soils that had or had not received dairy manure solids, regression analysis showed that the bacteria was expected to continue to leach through the soil cores for a significantly longer period of time when manure was present (30). This finding, combined with the evidence that the presence of manure inhibited the *E. coli* replication within the core, suggests that the presence of manure promotes bacterial movement through soil, even though it may not promote growth. Similarly, a separate experiment found that the application of manure slurry led to higher survival and greater vertical transport of pathogens through soil columns than application of manure solids (68).

2.6 Contamination of Crops

Contamination of leafy green crops by human pathogens can happen through a number of different routes. Survival of both enterohemorrhagic *E. coli* and *S. enterica* serovars on the surface of such crops has been well documented. The bacteria can survive for extended periods of time in grooves of the leaves and potentially resist removal by post-harvest washing and sanitization steps (58). In the previously mentioned study that showed exceptionally long survival of *E. coli* O157:H7 in soil, the researchers also detected the bacterium for 77 and 177 days on lettuce and parsley (41). Further, *S.*

enterica serovar Typhimurium could be recovered for 63 and 161 days on lettuce and parsley, respectively (42).

Bacteria present on the surface of plant leaves may also enter the leaves through open stomata or damaged tissue, or contaminate leaves via uptake with water through the roots of the plant. These two routs of contamination provide significant protection against removal of the bacteria by washing, though the frequency of infiltration is widely debated for different pathogens.

In a 1999 experiment, Seo and Frank submerged lettuce leaves in a suspension of *E. coli* O157:H7 for 24 hours (69). The researchers found that the bacterial cells became entrapped 20-100μm below the surface of stomata and cut leaf edges, indicative of internalization. Further, these live cells were visualized after a 5-minute treatment with 20 mg/L chlorine solution, though it is worth noting this concentration is much lower than the common practice of rinsing in 100-200 mg/L chlorine (6, 69). This presumptive internalization may have been facilitated by the complete submersion of the leaves in the bacterial culture, as one investigation into *Salmonella* Typhimurium internalization into lettuce leaves found a significant increase in internalization when the *Salmonella*-contaminated soil was flooded with water than when a normal rainfall even was simulated (32).

In 2002, Solomon *et al.* reported experimental evidence that *E. coli* O157:H7 applied via inoculated manure or irrigation water to soil surrounding young lettuce plants was

recovered at a high frequency from surface sterilized leaves for as long as 5 days post inoculation (72). Experiments using *Salmonella* have yielded similar results (23, 29, 35). However Mitra *et al.* performed a similar study with spinach plants and found only a few plants to harbor *E. coli* O157:H7 at low concentrations after soil drench of contaminated water (53). Similarly, when *E. coli* O157:H7 was inoculated into manure and applied to soils, the bacteria was only detected on very few surface-sterilized spinach tissue samples (70).

Growing season may determine the extent of contamination of leafy green crops by pathogenic microorganisms. A recent survey of 32 organic and conventional farms in the mid-Atlantic region of the U.S. concluded that growing season was a significant factor for contamination of leafy green produce. Of the total leafy greens sampled, 2.2%, including spinach, were confirmed to be contaminated by *Salmonella*. All of the positive samples had been collected during the fall, from both organic and conventional farms in three states, but contamination was not observed in the spring sampling (51).

2.7 Knowledge Gap

While the factors affecting the movement of bacteria downward through soil is well understood, the movement of bacteria across a field has not been as thoroughly examined. One study tracked the movement of *Azospirillium brusilense* across a field, and found that when live plant roots were present, bacteria were detected as far as 1.6m from the original inoculation point, but did not travel beyond 30cm when there were no roots in the soil, or only dead roots (6). However, it should be noted that *A. brusilense* is a

rhizobial bacteria, and survival of the bacterium in the absence of live plant roots is minimal, so this lack of movement without the presence of plant roots is somewhat expected, and cannot be applied to bacteria such as *E. coli* and *Salmonella*, which can easily survive in the absence of plant roots.

Further, the effect of floodwater on the microbial safety of leafy green crops needs to be further investigated. Climate changes that affect the distribution and intensity of precipitation events may result in an increase of surface runoff and flooding events that may threaten the microbiological safety of leafy green produce (49). Floodwaters are frequently contaminated with pathogenic bacteria, viruses, helminthes, and protozoa. Microbiological analysis of floodwaters from the Category 4, 2005 Hurricane Katrina found fecal coliform concentrations in the water was significantly higher than water quality standards of 200 MPN/100mL (63). After severe flooding along the Mississippi River in 2001, an increase in gastrointestinal illness after contact with floodwater was observed, especially in children (79). However, very few studies look at the risk of contamination of produce crops after a flooding event. In 2008, Orozco *et al*. reported the isolation of *Salmonella* Newport from water samples and tomato fruits after flooding from a heavy rainfall event that had infiltrated several greenhouses and reached the top of the tomato pots (60). However, as this was a flooding event in a greenhouse with a concrete floor and individual tomato pots, it is difficult to apply the findings to a field setting.

To date, only one study has examined the potential for leafy green contamination in a field setting after a flooding event. After a natural flooding event in Spain in September 2012, Castro-Ibanez *et al.* sampled surface soil, irrigation water, and lettuce heads for seven weeks to determine the levels of indicator organisms, *Salmonella* spp., and verotoxigenic *E. coli* (VTEC). Levels of coliforms on lettuce decreased from about 10^6 CFU/g one week after flooding to about 10^3 CFU/g after seven weeks (19). *Salmonella* was only detected in soil and water samples, and on lettuce tissue, one week after the flooding event, and then remained undetectable for the duration of sampling. Non-O157 VTEC was identified by PCR in soil for 3 weeks after the flooding event, and for one week on lettuce, but was not detected in irrigation water (19). Unfortunately, it is difficult to apply these results to the threat of produce contamination from floodwater, as it is unclear where the soil and lettuce samples were collected in relation to the flood zone.

Currently, there are no specific regulations for the handling of leafy green crops after a flooding event. While the LGMA has outlined a 30ft zone of crop destruction and a waiting period of 60 days before replanting, these are just guidelines for farmers, there are no consequences for disregarding these metrics and harvesting crops close to a flood zone. Further, the 30ft buffer zone refers to the turning radius of farming equipment, not the underground movement of bacteria through soil. Because floodwaters are likely to contain zoonotic pathogens, likely from animal fecal matter, there is a significant need for a comprehensive study of the movement of bacteria from floodwater through soil to determine the specific risk of contamination of nearby crops. Such research can be used

to construct more specific guidelines and regulations for farmers after a flooding event to prevent the harvest of contaminated crops and protect public health.

Chapter 3: Project Objectives

The overall purpose of this study was to assess the suitability of the LGMA-proposed 30-ft 'buffer zone' and 60-day waiting period before harvest after a flooding event. The recommendations however, do not consider the potential for underground transport of bacteria through soil. To address this knowledge gap, the current study had two objectives, both with the goal of gaining a deeper knowledge of the movement of bacteria from floodwater through soil, and the potential for contamination of distant leafy green plants:

1. The first objective of this study was to compare the movement of pathogenic *Salmonella enterica* serovar Newport and the nonpathogenic *Citrobacter freundii* through three different soil types at three different initial soil moisture contents. The purpose of this experiment was to determine the effect of soil type and moisture content on bacteria transport through soil, and to establish if *C. freundii* may be a suitable nonpathogenic surrogate for pathogenic *Salmonella* in future field studies. These trials investigated the horizontal movement of the two microorganisms up a small positive slope to simulate the type of flooding that would occur when heavy rainfall led to the collection of contaminated water at the lowest points in a produce field.

2. The second objective was to directly assess if a 30-ft buffer zone around floodwaters and a 60-day waiting period to replant previously flooded soils is sufficient to prevent the harvest of contaminated leafy greens. Water containing dairy liquid manure and

nonpathogenic *E. coli* strains was applied to one end of a field of spinach to simulate a flood, and soil and spinach samples were collected at distances up to 9 meters (30ft) over 60 days to determine the prevalence of the inoculated strains. The movement and survival of bacteria during summer and fall months was compared. These trials examined the movement of *E. coli* under field conditions where the floodwater collected at the top of a small negative slope. This was designed to simulate a "worst case" scenario where floodwaters broke through a barrier and had the potential to travel greater distances, aided by gravity.

Chapter 4: Investigating the Influence of Soil Type and Soil Moisture Content on Movement of Bacteria from Floodwater through Soil

4.1 Introduction

While the downward movement of bacteria has been well studied, there have been fewer studies that characterize the horizontal movement of bacteria, or the movement of bacteria across a field. Coliforms have been recovered from soils as far as 456m from their septic tank source, depending on soil type (30). However, it is unclear how factors such as soil composition and moisture content may be involved in such a transport. Additionally, there is a need to investigate the spread of bacteria from a source such as a flood across a field.

Livestock and poultry manure are frequently used by vegetable producers as fertilizer, but zoonotic pathogens such as *Salmonella enterica* have been shown to be able to survive in manure for as long as 200 days (83). As such, even if the manure is stored for a time before being spread onto cropland, *Salmonella* may still be present to contaminate crops or nearby water sources. Rainfall, especially heavy rainfall that leads to flooding, may mobilize the *Salmonella* into the soil, where it can survive for significant periods of time (hundreds of days) and spread with infiltrating water to contaminate distant plants. Therefore, there is a need to assess the potential for *Salmonella*-contaminated floodwater movement through the soil to crops that were not directly exposed to the floodwater, and the specific factors influencing this transport.

The main transport mode for the downward movement of bacteria through soil is with infiltrating water. The main forces preventing movement are physical filtration by the soil matrix, and adhesion of bacterial cells to particles in the soil (4, 7). It is expected that these forces would play a similar role in the movement of bacteria across a horizontal plane of soil. Therefore, the distance traveled by bacteria across a field will likely depend on the composition and moisture content of the soil, and will correlate with the number of binding sites for bacterial cells in the soil. These binding sites are positive ions such as calcium and magnesium ions, among others, that are associated with clay particles in the soil (5, 73). A greater number of binding sites in the soil will hinder bacterial movement while fewer binding sites will lead to less adhesion to the soil matrix and therefore greater movement with infiltrating water. Because water travels greater distances through soils with higher moisture contents than drier soils (7, 30, 55), it is also likely that bacterial movement would be greater through wet soils than dry soils.

Because pathogenic *Salmonella* strains cannot be employed in field experiments, the horizontal movement of the pathogen was studied in a model system in a laboratory setting, using *Salmonella* Newport. The study investigated the effect of soil composition and initial moisture content on the movement of *S.* Newport. Additionally, *Citrobacter freundii* was co-inoculated with *Salmonella* Newport, to determine if *C. freundii* would be an appropriate surrogate for *Salmonella* Newport in future field trials.

4.2 Materials and Methods

Inoculum

This experiment used a rifampicin-resistant *Salmonella enterica* serovar Newport from a tomato-associated outbreak that was obtained from the University of Florida (34), and a *Citrobacter freundii* ATCC 8090 isolate that was made resistant to nalidixic acid by spontaneous mutation. Individual 10 mL cultures of *S.* Newport and *C. freundii* were grown for 24 hours at 37°C. *S.* Newport was grown in Tryptic Soy Broth (Difco, Franklin Lakes, NJ) supplemented with 80μg/mL rifampicin (Sigma-Aldrich, St. Louis, MO), and *C. freundii* was grown in Tryptic Soy Broth supplemented with 25μg/mL nalidixic acid (Sigma-Aldrich). 50μL of each culture was combined in 50mL sterile water to make an inoculum of 6 log CFU/mL.

Soil Preparation

Three soil types were used in this experiment. Hagerstown silt loam (HSL) was obtained from the Penn State Southeast Agricultural Research and Extension Center (Manheim, PA), and Keyport-Matawan sandy loam (KMSL) was acquired from the USDA Beltsville Agricultural Research Center (Beltsville, MD), through Dr. Manan Sharma. Fort Mott loamy sand (LS) soil was collected from the University of Maryland Lower Eastern Shore Research and Education Center (Salisbury, MD) through David Armentrout. Soil texture was approximated using methods described by Colorado State University Extension. Briefly, a jar was filled with ¼ soil and ¾ water, and a teaspoon of non-foaming dishwasher detergent (Cascade®) was added before the jar was shaken vigorously for 10 minutes. As the sand, silt, and clay layers settled out, the thickness of

the deposits were measured and expressed as a percentage of the total thickness (81). Soil pH was approximated using a SoilMaster™ Soil Testing Kit (Mosser Lee, Millston, WI). Texture and pH analyses were performed in triplicate. Further soil analyses were performed by A&L Eastern Laboratories (Richmond, VA) to determine soil organic matter, levels of phosphorus, potassium, magnesium, and calcium, and cation exchange capacity.

Soils were spread on metal trays and air dried for 24 hours prior to the experiment, then remoistened to a percentage of the maximum water holding capacity, as determined through procedures outlined in Franz *et al* (28). Briefly, approximately 50g of field-moist soil of each soil type was saturated with an excess of water, mixed, and allowed to sit undisturbed for 24 hours before being filtered to remove the excess water. 5g of each sample was dried in a drying oven at 105°C for 24 hours, then reweighed to determine the weight of water lost, or the maximum water holding capacity (WHC). For this experiment, soils were remoistened to 40, 60, or 80% of the WHC with sterile water, hand mixed thoroughly for several minutes, then passed through a #8 mesh screen with wire diameter of 0.028 inches and an opening of 0.097 inches (The United Company, Westminster, MD) to break up any clumps formed from mixing. At this point, a sample of each soil was saved in order to determine the exact moisture content of each experiment. As above, 5g of the soil was dried in a drying oven for 24 hours, and then reweighed to determine gravimetrically the loss of water. Maximum WHC, water added to equal each percentage of the WHC, and actual moisture content of each trial for each soil is summarized in Table 4-1.

Table 4-1: Maximum water holding capacity (WHC) and water added to soil (mL/g) to reach 40, 60, and 80% of the maximum WHC

	Hagerstown Silt Loam	Keyport-Matawan Sandy Loam	Fort Mott Loamy Sand
Maximum water holding capacity (mL/g)	0.26	0.14	0.14
Water Added for 40% WHC (mL/g)	0.156	0.056	0.056
Water Added for 60% WHC (mL/g)	0.108	0.084	0.084
Water Added for 80% WHC (mL/g)	0.104	0.112	0.112

Tube Construction

A 33cm long piece of Tygon S3 E-3603 laboratory tubing (2.54cm inner diameter, VWR Philadelphia, PA) was connected to 1.27cm diameter rubber inlet tubing via a #6 rubber stopper and 1.27cm diameter glass tubing. A double layer of cheesecloth covered the rubber stopper to prevent soil from falling into the rubber tubing. Soil was added to the Tygon tubing 1cm at a time and tamped to promote settling until full. Tubes were taped to a metal tray to keep them straight, and the tray was tilted at a 10° angle, with the rubber stopper at the lower end, as shown in Figure 4-1. The overall experimental protocol is graphically summarized in Figure 4-2. Each combination of soil type and moisture content was performed in triplicate concurrently, with each combination being performed per day.

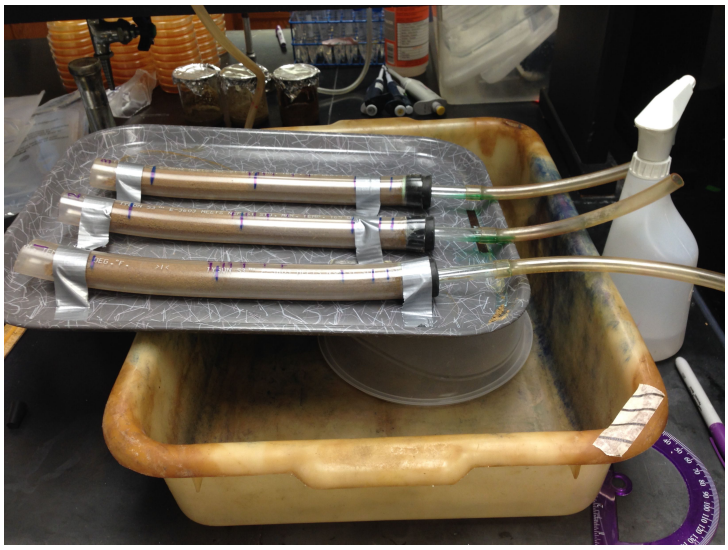

Figure 4-1: Experimental setup. Soil was added to Tygon tubing, which was connected to inlet tubing via a rubber stopper. Soil was prevented from falling into the inlet tubing by cheesecloth. Tubes were taped to a metal tray so they remained straight and placed at a 10° incline, with the inlet tubing at the lower end for inoculation

Inoculation

Five drops of green food dye was added to the inoculum to assist in visualizing the waterfront as it moved through the soil. After agitation to evenly disperse the dye, 15mL of the inoculum was added to the 1.27cm inlet tubing. The time it took the water/dye to move 25.5cm through the Tygon tubing was recorded. The experiment was stopped at 6 hours if the water had not traveled the full 25.5cm, and the distance traveled was recorded.

Sampling

Tygon tubing was cut into sections (0-1.25cm, 1.25-2.5cm, 2.5-5cm, 5-15.25cm, and 15.25-25.5cm) using a sterile knife (see Fig 4-2). The 0-1.25cm and 1.25-2.5cm samples were emptied directly into sample bags with a filter (Nasco, Fort Atkinson, WI) and weighed (each approximately 7g). The latter three sections were emptied into separate

sample bags (Nasco) and homogenized, before transferring 7g to separate filtered sample bags. All samples were then diluted 1:5 in Buffered Peptone Water (Neogen, Lansing, MI). Bags were mixed by hand for 2 minutes, and then serially diluted in 0.1% peptone (Neogen). 50μL of the appropriate dilutions were spread plated in duplicate onto Tryptic Soy Agar (Difco) supplemented with 80μg/mL rifampicin to enumerate *Salmonella* Newport, and MacConkey Agar (Difco) supplemented with 25μg/mL nalixidic acid to enumerate *C. freundii.* Plates were incubated at 37°C for 24 hours.

If plates were negative for growth, the samples in the Buffered Peptone Water were incubated at 37°C for an additional 24 hours, and then 50 μL of the enrichment was spread in duplicate on the above agars. Plates were incubated under the above conditions and presence/absence of growth was recorded. The lower limit of detection for plate counts was 2 log CFU/g soil, the lower limit of detection for the enrichment was -0.85 log CFU/g soil.

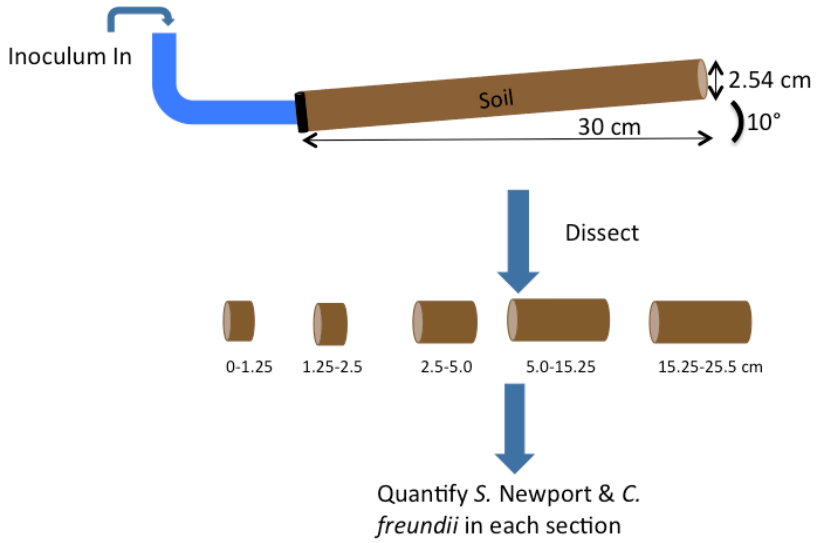

Figure 4-2: Summary of experimental procedures. After the water front traveled through 25.5cm of soil, or 6 hours, tubes were dissected into the sections shown, and the concentrations of *S.* Newport and *C. freundii* were quantified for each section using direct plating.

Statistical Analysis

The USDA Integrated Pathogen Modeling Program (iPMP) (40) was used to generate distance vs. population density distributions using a two-phase log-linear model to describe the movement of bacteria through the tubing. JMP® Pro 11.0.0 software was used to determine significant differences in *Salmonella* Newport and *C. freundii* movement in different soils and at different moisture contents. Because the distribution of values for regression parameters among replicates was not always normal, as determined by Normal Quantile plots, the nonparametric Wilcoxon test and Steel-Dwass (all pairs) comparison of means were used to determine significant differences. A p-value less than or equal to 0.05 was considered statistically significant.

4.3 Results

Table 4-2 summarizes the texture and pH of the three soils used in this experiment. The Hagerstown silt loam soil had the highest percentage of clay (37.8%) of the three soils, while the Fort Mott loamy sand soil had the highest percentage of sand (69.9%). The Hagerstown silt loam soil also had the highest percent organic matter (4%) and highest cation exchange capacity (10.6 meq/100g), while the Fort Mott loamy sand had the lowest values, (1% and 5.5 meq/100g, respectively).

Table 4-2: pH, texture, organic matter, and cation exchange capacity of soils used in the experiment*

	Hagerstown Silt Loam	Keyport-Matawan Sandy Loam	Fort Mott Loamy Sand
pH	6.7±0.1	7.8±0.3	7.3±0.3
% clay	37.8±3.3	12.0±2.7	9.5±0.7
% silt	31.0±2.0	33.3±4.4	20.7±4.9
% sand	31.2±1.3	54.7±1.7	69.9±5.6
% Organic Matter	4%	2%	1%
Phosphorus (ppm)	45	385	299
Potassium (ppm)	217	186	114
Magnesium (ppm)	162	117	131
Calcium (ppm)	1373	1052	621
Cation Exchange Capacity (meq/100g)	10.6	7.5	5.5

*Mean and standard deviation values are shown

Table 4-3 summarizes the maximum distances the waterfront traveled through each soil type and at each percent of the WHC. Experiments were stopped after six hours, regardless of how far the water had wicked. Generally, the distance traveled increased as the moisture content of the soil increased. The waterfront moved the least across tubes containing the Keyport-Matawan sandy loam soil at 40% WHC, only 13.4 centimeters in

the six hour experiment. Conversely, water traveled the farthest through the Hagerstown sandy loam soil at 80% WHC, traveling the entire 25.5cm in just 1.75 hours. The only other soil to travel the maximum distance was the Keyport-Matawan sandy loam at 80% WHC.

Table 4-3: Average maximum distances traveled (cm) through tubing for each soil type at each percent of the maximum water holding capacity (WHC)

Soil	% WHC	Distance (range) (cm)	Time (hr)
HSL	**40**	15.90 (15.50 - 16.25)	6.00
	60	20.40 (19.70 - 21.10)	6.00
	80	25.53 (25.50 - 25.70)	1.75
KMSL	**40**	13.40 (13.20 - 13.60)	6.00
	60	15.40 (15.25 - 15.75)	6.00
	80	25.50 (25.40 - 25.70)	3.50
LS	**40**	15.25 (15.00 - 15.50)	6.00
	60	15.30 (15.25 - 15.50)	6.00
	80	20.40 (19.70 - 21.00)	6.00

Figure 4-3 summarizes the concentration of bacteria in each section of tubing. Because values were determined by homogenizing sections of soil, each data point is plotted at the midpoint from each section, except the last data point indicates the farthest distance traveled by the waterfront.

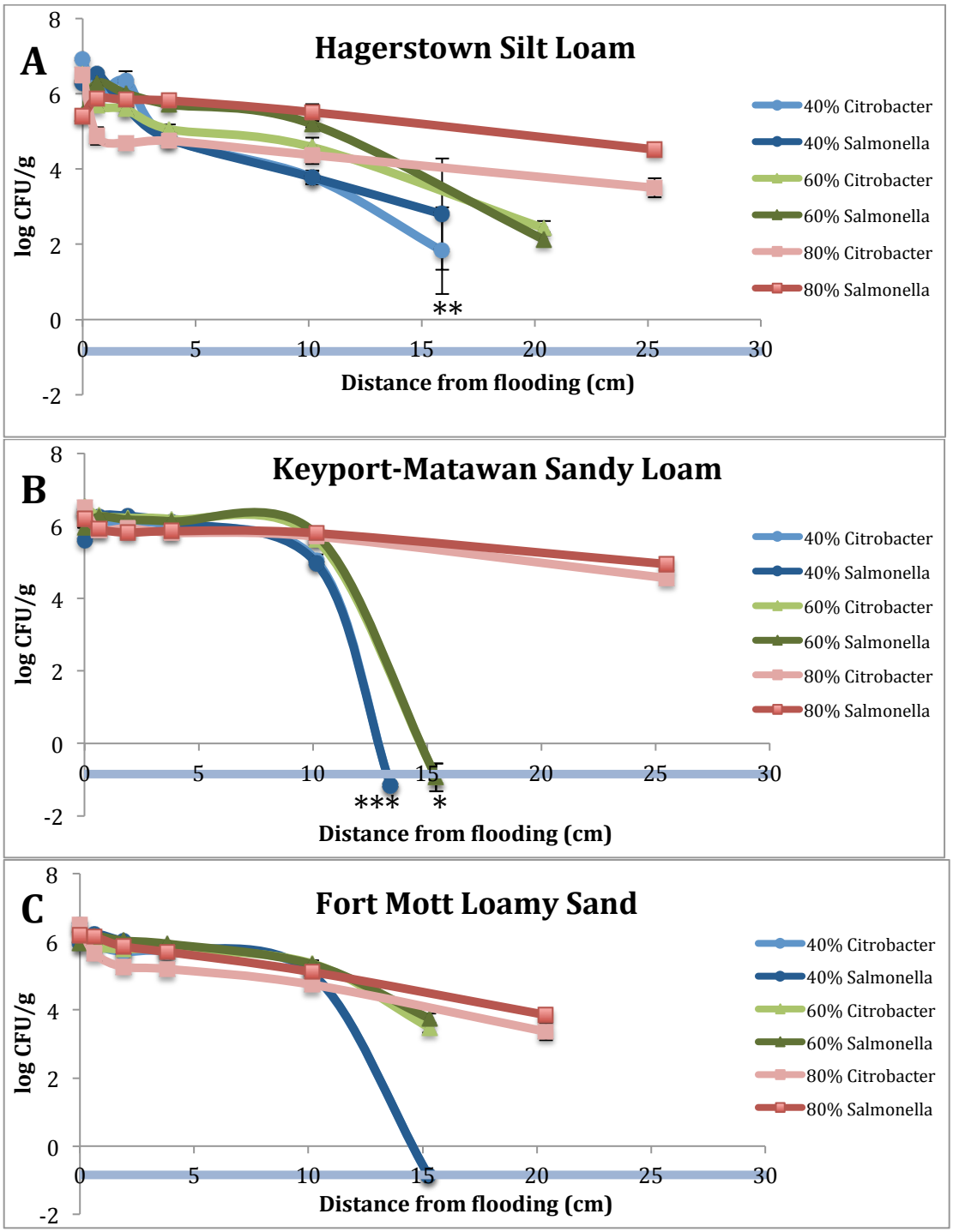

Figure 4-3: log CFU/g S. Newport and *C. freundii* at each distance and soil moisture content, where 40% = 40% WHC, 60% = 60% WHC and 80% = 80% WHC, in a) Hagerstown silt loam, b) Keyport-Matawan sandy loam, and c) Fort Mott loamy sand soils. Each data point is an average of three replicates, and plotted as the midpoint of each cut section of tubing, except for the last data point, which represents the farthest distance traveled by the waterfront. Error bars indicate the standard error of the mean. The limit of detection (gray line) for enrichment was -0.85 log cfu/g. Asterisks below a data point indicate the total number of negative

enrichments for that data point (Maximum of 3 for 3 replicates, each enrichment negative for *S.* Newport was also negative for *C. freundii*).

To account for differences in inoculum levels, data was log-normalized for statistical analysis. Each replicate was individually fit to a two-phase survivor curve adapted from Buchanan *et al.* 1997 (14). In the two-phase model, there is first a lag distance where no significant decrease in bacterial concentration is observed, before the concentration of cells decreases in a linear fashion. Such a curve has the following equation:

$$y = y_o, \; x \leq x_{shoulder}$$

$$y = y_o - \frac{(x - x_{shoulder})}{D}, \; x > x_{shoulder}$$

where y is the concentration of bacteria at distance x, y_o is the log-normalized average concentration of cells in each section of soil before a significant decrease in bacterial concentration is observed, $x_{shoulder}$ is the distance at which the significant decrease in bacteria concentration begins and D is the negative reciprocal of the slope of the linear portion of the model. A shoulder value of zero is indicative of immediate filtration at 0 inches and an entirely linear regression model. Parameters for each soil type and initial % WHC are summarized in Table 4-4. There was no significant difference ($p >> 0.05$) between parameters for *S.* Newport and *C. freundii* regressions, so results were averaged together.

Table 4.4 Average two-phase linear regression parameters by soil type and initial percent water holding capacity*

Soil	% WHC	$x_{shoulder}$ (cm)	y_o (log (N/N_o)/g)	Slope (log (N/No)CFU/g / cm)
Hagerstown Silt Loam	**40**	5.397±3.968	-0.812±0.484	-0.673±0.350
	60	4.210±3.286	-0.125±0.379	-0.280±0.114
	80	5.042±3.797	-0.714±0.149	-0.100±0.048
Keyport-Matawan Sandy Loam	**40**	9.218±0.166	0.166±0.407	-1.197±0.040
	60	9.712±0.138	-0.142±0.178	-1.172±0.105
	80	9.055±0.925	-0.544±0.118	-0.102±0.024
Fort Mott Loamy Sand	**40**	9.294±0.163	-0.339±0.191	-0.987±0.025
	60	8.334±0.721	-0.310±0.195	-0.345±0.049
	80	6.199±1.163	-0.770±0.180	-0.180±0.021

*Mean and standard deviation values are shown

Because the concentration recovered from each section can be considered the average concentration throughout that soil section, to determine the model parameters for each replicate, each normalized bacterial concentration was plotted at the median distance for each soil section (i.e. the concentration of cells in the first soil section was plotted at 0.625cm, the midpoint of 0 – 1.27cm.). For the last soil section, because the waterfront often did not travel the full 25.5cm, the concentration recovered was plotted at the midpoint of the final section's starting distance, 15.25cm, and the average distance traveled by the water front. For example, the waterfront in the HSL/60% replicates traveled approximately 20.4cm during the experiment, so the concentration of bacteria recovered in the last soil section was plotted at 17.83cm, the midpoint of 15.25 – 20.40cm. It must be noted that has likely led to a slight underestimation of the slope parameter, because if the average concentration was plotted at the distance where that number of cells was present in the soil, that distance would be somewhere shorter than the midpoint, and result in a more negative slope value. However, this exact distance

cannot be determined, so the midpoint between the start of the final section of soil and the distance traveled by the waterfront has been used as the best estimate.

Wilcoxon Test and Steel-Dwass comparison of means were performed to determine the effect of soil type and initial percent water holding capacity on the extent of bacterial movement. Nonparametric tests were performed because the values for the parameters from each replicate were not normally distributed. While the filtration shoulder was only significantly affected by soil type ($p<0.0001$), the slope of the linear decline was significantly affected by both soil type ($p<0.0001$) and initial soil moisture content ($p<0.0001$) as seen in Tables 4.5 and 4.6.

Steel-Dwass test showed the average $x_{shoulder}$ parameters for each soil type were significantly different; the KMSL soil had the longest average shoulder (9.328cm), followed by the LS soil (7.942cm), and the HSL soil had the shortest (4.883cm). The slope of the HSL soil was the smallest, while the slope of the KMSL soil was the largest, indicating the rate of bacterial filtration was greatest in the KMSL soil and smallest in the HSL soil.

When Steel-Dwass test was used to determine the impact of initial soil moisture content on the slope parameter, it was found that the slopes for the three moisture contents were all significantly different, with the greatest slope at 40% WHC and the smallest slope at 80% WHC.

The y_o parameter was also found to also be significantly affected by both soil type (p=0.0035) and initial moisture content (p=0.0001). A significantly greater fraction of the inoculum was retained in the lag distance in KMSL soil than the HSL soil, and in the 40% and 60% WHC soils than 80% WHC.

Table 4.5: Wilcoxon and Steel-Dwass test results for effect of soil type on two-phase linear regression parameters of bacterial movement through soil*

Soil	$x_{shoulder}$ (cm)	y_o (log (N/N_o) CFU/g)	Slope (log (N/No) CFU/g / cm)
Hagerstown Silt Loam	4.883^{A}	-0.550^{A}	-0.351^{A}
Keyport-Matawan Sandy Loam	9.328^{C}	-0.190^{B}	-0.842^{B}
Fort Mott Loamy Sand	7.942^{B}	-0.473^{AB}	-0.504^{AB}

*Values in each column not connected by same letter are significantly different.

Table 4.6: Wilcoxon and Steel-Dwass test results for effect of initial moisture content on two-phase regression parameters of bacterial movement through soil*

% WHC	$x_{shoulder}$ (cm)	y_o (log (N/N_o) CFU/g)	Slope (log (N/No) CFU/g / cm)
40	7.97	-0.345^{A}	-0.953^{A}
60	7.419	-0.192^{A}	-0.599^{B}
80	6.765	-0.676^{B}	-0.127^{C}

* Values in each column not connected by same letter are significantly different. No significant difference among initial moisture contents for $x_{shoulder}$

4.4 Discussion

In this experiment, soil cores were placed at a shallow incline, and the lower end was inoculated in such a way as to simulate a flood that may occur when a heavy rain event causes water to collect in a depression in a crop field. Results showed that both soil type and soil moisture content have a significant effect on bacterial movement through soil.

After data collection, it was determined that the data best fit a two-phase linear model based on the previously mentioned equation. In this model, there is a "lag distance" before a quantifiable, linear decrease in the concentration of bacteria occurs. In other words, the population density of the inoculum exceeds the ability of the soil to retain bacterial cells sufficiently to observe an observable filtration of bacteria from the soil. This is similar to results obtained by Mosaddeghi *et al*. in 2010, who found that more filtration of bacteria from infiltrating water occurred at 20-40cm deep in a saturated soil core than at the surface 0-20cm (55).

A potential interpretation of this lag distance is that there are a limited number of binding sites in the soil and these are overwhelmed close to the point of flooding. When all binding sites were occupied by infiltrating bacteria, there was no quantifiable decrease in the concentration of bacteria, and a lag distance was observed. Logically, as the bacterial concentration fell with increasing distance traveled, fewer biding sites were occupied, and fewer cells were quantified at increasing distances from the flood.

As water travels through the soil, random Brownian motion of the negatively charged bacterial cells will cause the cells to adhere to the clay particles in the soil, aluminosillicates that are stacked into sheet-like structures and generally carry a net positive charge because they attract and bind positive ions in the soil (5, 73). When these positive charges are on functional groups present at the soil:water interface, bacterial cells in the water can adhere to the clay particles, generally through electrostatic interactions. This binding removes the bacteria from the infiltrating water, resulting in a lower concentration of cells traveling through the soil at greater distances from the flood.

Therefore, it would be expected that as the concentration of clay, and consequently bacterial binding sites, increases, then the lag distance would decrease, and there would be a faster decrease in the concentration of cells recovered as the distance from the flood increased. In the present experiment, the lag distance, represented by the x_{shoulder} parameter, was significantly different among all three soil types, with the longest lag phase in the Keyport-Matawan sandy loam soil (KMSL), and the shortest in the Hagerstown silty loam soil (HSL). However, the KMSL has a higher clay content than the Fort Mott loamy sand (LS), the soil with the median length lag distance, though the difference did not reach statistical significance. Therefore, it must be concluded that clay content did not fully determine the length of the lag distance in this experiment, as was expected.

Another factor of soil composition that may affect the length of the lag distance is soil organic matter. Soil organic matter is any soil material originally produced by living

organisms that decomposes in the soil. This organic matter serves to both store nutrients in a plant-available form, and to bind soil particles into aggregates in order to support soil aeration and water infiltration, and prevent soil erosion (10). This increase in aggregate formation promotes the movement of water through soil, and theoretically would also promote the movement of bacteria through soil. Soil organic matter tends to increase as clay content increases because binding between clay particles and organic matter slows the decomposition process, while increasing soil aggregate formation.

When it was concluded that clay content could not predict the length of the lag distance, further soil analyses were performed. Soil analysis showed the HSL soil had the highest percent organic matter content, 4%, while the LS soil had the lowest at 1%. This was expected, as these results correlate with the percentage of clay particles in each soil, but indicates that, like clay content, the concentration of organic matter in soil cannot predict the extent of movement of bacteria across a field.

As bacteria cells in water almost always have a net negative surface charge (5), the number of positively charged ions in soil could be the factor influencing the length of the lag distance observed in different soil types. The cation exchange capacity (CEC) is the ability of soil to hold on to such positively charged ions as calcium, magnesium, potassium, sodium, and many others (46). As the CEC of the soil increases, more positive ions would be present to bind to bacterial cells and prevent their further infiltration through soil, increasing the number of binding sites and therefore decreasing the length of the lag distance as described above. However, soil analysis showed similar results for

clay content and organic matter, the HSL soil had the highest CEC, 10.6 meq/100g, and the LS soil had the lowest, 5.5 meq/100g. This result is not entirely surprising, as CEC tends to increase with clay and organic matter contents (46).

Soil pH could be a determinant of the number of binding sites available in a soil for bacteria cells to adhere to. The ionization of the clay as the pH drops allows positively charged ions such as calcium, potassium, and magnesium, to bind to the clay (47). Therefore, as soil pH decreases, the number of available binding sites for bacteria on clay increases, and the lag distance should decrease, as described above. However, in this experiment, the pH's of each soil type were not significantly different, though there was a trend of increasing pH with increasing lag distance. Therefore, pH cannot fully explain the differences in lag distance found in this experiment.

At this point, it must be concluded that the length of the lag distance observed in this experiment cannot be predicted by any one soil characteristic measured in this experiment. Because the concentrations of clay in the KMSL and LS soils were not significantly different, it is possible that the method of determining soil texture used in this experiment was not sensitive enough, and the LS soil could actually have a higher clay concentration than the KMSL soil. However this is highly unlikely, as other measured soil characteristics, organic matter and CEC, correlate with the KMSL soil having a higher concentration of clay than the LS soil. While individual considerations of soil components may provide the basis for the length of the lag distance, it is very likely that the length of the lag distance is a function of a combination of factors, such as the

concentration of clay in the soil and the soil pH, rather than just clay content or pH. Further analyses are therefore necessary to determine the factor(s) controlling the length of the lag distance in bacterial movement across soil.

The second main parameter of the two-phase linear model, the slope, was significantly affected by both the initial soil moisture content, and the soil type. As the percent WHC increased, the slope of the decline in cells recovered decreased, and greater concentrations of bacteria were recovered in the section of soil farthest from the flood. In other words, the rate of bacterial removal from infiltrating water decreased as the initial moisture content increased. This may be a combination of three factors: greater movement of water through more saturated soils, greater movement of bacteria with water through more saturated soils, and decreased probability of bacterial cell attachment with increasing moisture content. Research has shown that infiltration rates of water are greater through more saturated soil than dry soil (30, 44). This is reflected by the observation that as soil moisture content increased, the distance traveled by the waterfront during this experiment also increased. If the rate of infiltration of water across soil is assumed to be constant, then in this experiment, the rate of the waterfront increased as initial soil moisture content increased for all three soil types. Additionally, the concentration of bacteria recovered from the final section of soil generally increased as soil moisture content increased for each soil type. Bacterial movement has been shown to be greater through more saturated soils, as there are fewer air-water interfaces in soil pores for bacteria to adhere and become trapped in the soil (55).

Soil type also had a significant effect on the slope parameter for this experiment, with a similar trend as the length of the lag distance, though the trend did not reach statistical significance. As the length of the lag distance increased, the rate of the linear decline in bacterial concentration increased, becoming more negative. Logically, this was expected, as soils with more binding sites for bacteria will have, in addition to a shorter lag distance, a slower decrease in cells recovered as distance from the flooding increases. When fewer binding sites are present, the probability a cell will bind to one of the binding sites will also be smaller, resulting in a longer distance required to decrease the concentration of cells recovered.

The final parameter of the two-phase linear model is the y_o value, or the average fraction of the inoculum recovered within the lag distance, before a significant decrease in bacterial concentration was observed. This value also correlated with the lag distance, as expected, when the effect of soil type was examined. As the lag distance increased, and the number of binding sites for the bacteria decreased, a smaller fraction of the initial inoculum was retained within the lag distance. The y_o value was also significantly lower in soils at 80% WHC than in soils at 40% or 60% WHC. This is likely because the greater infiltration rate of water and bacterial cells through wet soils, as described above, resulted in a smaller fraction of the cells in the flood water being retained within the lag distance of the soil.

In conclusion, both soil texture and initial soil moisture content significantly affect bacterial movement through soil. Different soil textures have different bacterial filtration efficiencies – the distance at first quantifiable decrease in concentration of bacteria. However, the difference could not conclusively be connected to any of soil properties measured: texture, organic matter, cation exchange capacity, or pH. However, it can be concluded that as moisture content increases, the rate of filtration decreases, and bacteria are able to travel farther distances through soil. Finally, as expected, no significant difference in movement of *S.* Newport and *C. freundii* was observed in any soil type or at any initial moisture content, indicating *C. freundii* may be a suitable surrogate for *S.* Newport in field studies investigating bacterial transport through soil.

Chapter 5: Investigating Metrics Proposed to Prevent the Harvest of Leafy Green Crops Contaminated by Floodwater

5.1 Introduction

Leafy green vegetables, such as spinach and lettuce, are the vehicles for approximately one quarter of all foodborne illness outbreaks related to plant products (61). Such produce is especially vulnerable to bacterial contamination from soil or water because edible portions grow low to the ground and have no protective layers such as peels, shells, or skins (49).

There are many pathways by which these crops may become contaminated, most of which trace back to animal feces. Two such pathways are through manure contamination of irrigation water, and through the spread of inadequately composted manure onto fields. The direct contamination of leafy green vegetables in contact with such sources is well researched. However, there is a need to further investigate the movement of pathogens through soil from these sources and the risk of contamination of distant crops.

Flooding, or the uncontrolled overflowing of a field with water, may significantly promote the translocation of bacteria, as the major transport mode of bacteria through soil is with the gravitational flow of infiltrating water (4, 16). Floodwater may become contaminated with zoonotic pathogens if intense rainfall causes flooding where manure has been deposited directly onto the crop field, or if such a rain event causes the overflow of a river or creek adjacent to the cropland that has been contaminated by manure.

In the recent years, there have been a number of foodborne illness outbreaks associated with the contamination of leafy green vegetables with *E. coli* O157:H7 where the source of the contamination could be traced back to livestock on farms near the contaminated produce. In 2005, an outbreak in Sweden that caused 135 illnesses was associated with the consumption of lettuce contaminated by *E. coli* O157 (71). Investigations into the outbreak recovered isolates identical to the outbreak strain in stream water used to irrigate the lettuce. Contamination of the stream was traced back to a cattle farm upstream from the lettuce field (71). In 2006, an outbreak involving 80 *E. coli* O157:H7 illnesses in Minnesota and Iowa was attributed to lettuce that had been irrigated with water from the same pipes used to transport wastewater from a nearby dairy farm (15). As with the Sweden outbreak, *E. coli* O157:H7 isolates identical to the outbreak strain were recovered from the dairy farm. In the previously mentioned 2006 spinach outbreak that affected 26 states in the U.S., the outbreak strain was isolated from streams near the suspect fields, as well as from cattle and feral swine that had access to the stream (45, 33). While researchers were unable to determine if heavy rainfall may have caused flooding that transferred the contamination to the spinach, they did conclude that the bacteria likely percolated through the soil to contaminate the groundwater used to irrigate the spinach (33).

The purpose of this experiment was to evaluate the transport of bacteria from floodwater through soil in a field setting, and the potential for contamination of spinach leaf tissue. This experiment was designed to assess the LGMA metrics that advise destruction of crops within 30ft of the edge of a flood and a waiting period of 60 days before replanting.

One end of a spinach field was flooded with water containing dairy liquid manure and *Escherichia coli* to simulate a natural flood. Soil and spinach samples were collected at distances up to 30-ft from the edge of the flood for 60 days to assess the movement of *E. coli* through soil and the potential for contamination of leafy green crops.

5.2 Materials and Methods

Field Site and Plot Design

The field used in this experiment was a lysimeter plot (-5% grade) at Beltsville Agricultural Research Center (BARC), United States Department of Agriculture (USDA). This slope was selected to assess the impact of gravity on the potential spread of the bacterium horizontally through the soil. Nine 33ft long by 18 inches wide rows of spinach cultivar Racoon were planted in groups of three, with 18 inches between rows and 8ft separating the three groups (see Figure 4-1). Spinach seed was applied via broadcasting at the standard rate of 37.5lb/acre. Over time, seedlings were thinned to a density of one plant every 2 inches. Prior to flooding, a 3ftx3ft soil berm was hand-built at the upper end of each center row of spinach to 4-5 inches high to contain the floodwater (see Figure 4-2). Additionally, immediately before flooding, the entire plot was saturated with irrigation water to promote standing water formation.

The experiment was performed for an April planting of spinach (henceforth known as the spring trial) and a September planting (fall trial). Immediately following the end of the spring trial, the rows were covered with clear plastic tarp (1 mil, Home Depot, College Park, MD) to heat the soil and inactivate any residual *E. coli*. Tarps were removed 16 days later, and Round Up was sprayed to kill the weeds in the plot. Six

weeks before planting for the fall trial, compost was applied to the plot, and tilled into the top six inches of soil.

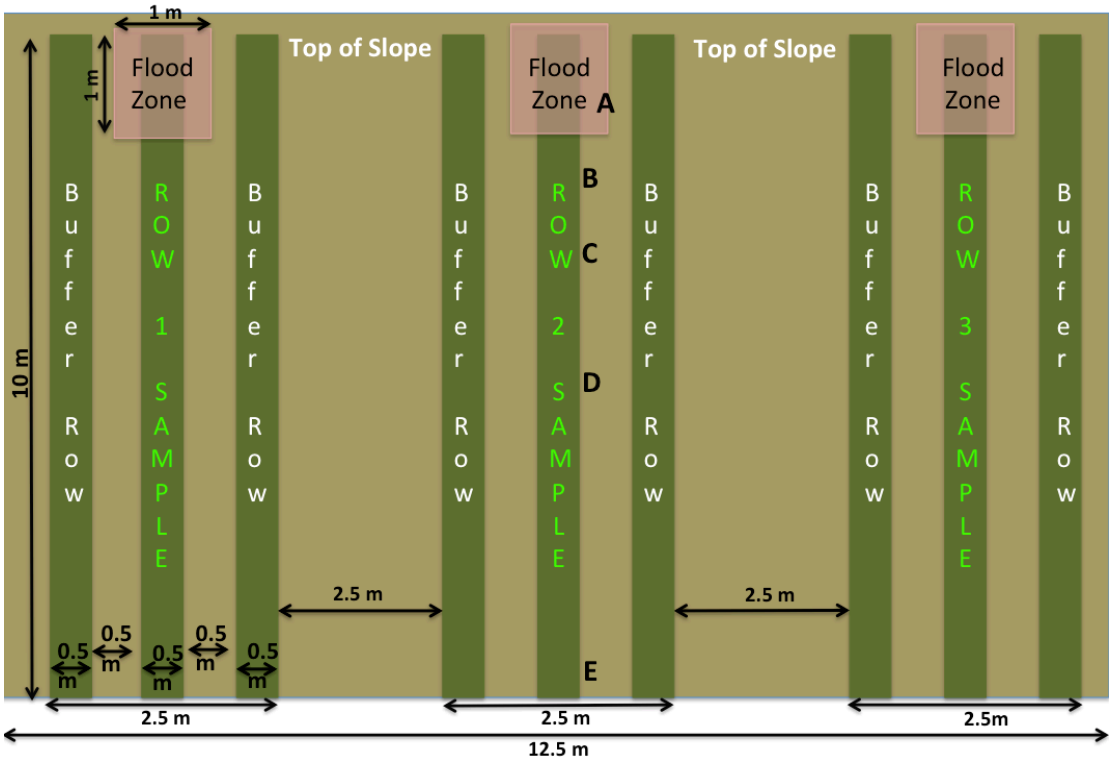

Figure 5-1: Layout of lysimeter plot (-5% grade) used for flooding experiment, where A-E indicate sampling distances. Individual floods were created at the top of the slope for each sampling row.

Figure 5-2: Example of soil berm built to contain floodwater

Soil Analysis

Soil texture was approximated from a composite field sample using methods described by Colorado State University Extension. Briefly, a jar was filled with ¼ soil and ¾ water, and a teaspoon of non-foaming dishwasher detergent (Cascade®) was added before the jar was shaken vigorously for 10 minutes. As the sand, silt, and clay layers settled out, the thickness of the deposits were measured and expressed as a percentage of the total thickness (81). Soil pH was approximated using a SoilMaster™ Soil Testing Kit (Mosser Lee). Texture and pH analyses were performed in triplicate.

Inoculum Preparation and Field Flooding

Nalidixic-acid resistant strains of *Escherichia coli* (MW416, 423, 425) that have been previously shown to be suitable surrogates for *E. coli* O157:H7 attachment to leafy greens (78) were grown separately overnight at 37°C in 150mL Tryptic Soy Broth (Neogen) supplemented with 50μg/mL nalixidic acid (Sigma-Aldrich) (TSBN). Cultures were combined with liquid dairy manure diluted 1:10 in sterile water to serve as the inoculated floodwater. Liquid dairy manure was obtained from a solid/liquid extractor for the USDA Northeast Area BARC dairy herd the day of inoculation. Specifically, 33mL of each *E. coli* culture was combined with 1.8L liquid dairy manure in 16.1L sterile water for a final *E. coli* concentration of ~6 log CFU/mL. One inoculum was created for each individual flood. Using a spigot to control flow, approximately 11L of the inoculum was applied to the area inside the soil berm to create a flood of standing water to the top of the 4-week old spinach plants (see Fig. 5-2). A small amount of floodwater was allowed to overflow the berm; the edge of this water was marked as the edge of the flood.

Sampling

Five individual samples were taken from each flooded spinach row: surface soil in the spinach row (0-5cm), rhizosphere soil 5-10 cm deep, bulk soil at the surface (0-5cm), bulk soil 5-10cm deep, and spinach leaf tissue. The soil samples were taken using soil probes, with three cores taken at each distance for both bulk and rhizosphere soil. The soil probes were rinsed and sanitized using 70% ethanol between each individual core. Cores were divided into surface (0-5cm) and subsurface (5-10cm) halves using sterile plastic knives (Figure 5-3). Spinach leaf tissue was collected by harvesting all the leaves of 3 to 5 spinach plants (depending on size) with scissors (Figure 5-3). Scissors were sanitized with 70% ethanol between each sampling distance.

Sampling distances were as follows: inside the flood zone, 0.5m, 1.5m, 4.5m, and 9m (30ft) from the edge of the flood. Sampling was performed on days 0, 1, 3, 7, 14, 21, 28, 35, 42, 49, 56, and 63. Soil and spinach tissue samples were collected on day -1 (the day before the start of the study) to confirm the absence of nalidixic acid resistant bacteria in the plot. Samples were stored in coolers on ice packs for transport to the laboratory, and processing began immediately upon arrival.

Figure 5-3 Sample collection. Top left: leaf tissue from 3-5 spinach plants was cut using sterile scissors and placed into sample bags. Top right: soil samples were collected using soil corers, 3 cores at each sampling distance for bulk and rhizosphere soil, and divided in to surface (bottom left) and subsurface (bottom right) samples.

Sample Analysis

E. coli in soil samples was quantified using MPN analysis. 30g of each soil sample were diluted 1:5 in Buffered Peptone Water (Neogen), then hand massaged for 2 minutes. Samples were then serially diluted 1:10 in quadruplicate in TSBN and incubated at 37°C for 24 hours. 1µL of each dilution was then streaked onto ¼ of a MacConkey agar (Neogen) plate supplemented with 50µg/mL nalidixic acid (MACN) using a 1µL inoculating loop (VWR). Spinach leaf tissue was weighed and diluted 1:10 in TSBN,

stomached for 1 minute, incubated at 37°C for 24 hours. 50μL of each enrichment were spread onto MACN plates in duplicate.

All plates were incubated at 37°C for 24 hours and examined for presence/absence of *E. coli*. MPN/g was calculated for soil samples using the MPN Calculator Build 23 VB6 version (http://i2workout.com/mcuriale/mpn/index.html). The limit of detection for MPN analysis was 1.1 MPN/g.

Dry Weights

The *E. coli* per gram of dry weight soil was determined for each sample by drying 10g of the soil at 105°C for 24 hours and weighing the residual. MPN/g values were then adjusted to reflect MPN/g of dry weight soil. All values were reported as MPN/g of dry weight soil.

Confirmation of Isolates

To confirm the *E. coli* recovered from the soil were the same nalidixic acid-resistant *E. coli* strains inoculated into the flood, DNA was extracted from *E. coli* isolates recovered from soil MACN plates from MPN analysis on sampling days 35, 42, 49, 56, and 63, using Insta-Gene™ matrix (Bio-Rad, Hercules, CA). DNA was also extracted from pure cultures of each of the three inoculated *E. coli* strains. BOX-A1R-based repetitive extragenic palindromic-PCR (BOX-PCR) was performed using primer sequences described previously (18) using a Mastercycler ProS (Eppendorf, Hauppauge, NY). The reaction mixtures (20μL) consisted of 2μL template DNA, 50pM of the BOX-A1R primer, 4μL of a 5X MyTaq™ reaction buffer containing 3mM $MgCl_2$ and 1 mM dNTPs,

and 0.5 units of 2 units of MyTaq™ HS DNA polymerase (Bioline, Taunton,MA) The amplification conditions consisted of an initial denaturation at 95°C for 10 minutes, 30 amplification cycles (94°C for 1 min, 53°C for 1 min, 72°C for 4 min) and a final extension at 65°C for 10 minutes. The amplification products were separated by electrophoresis on 2% agarose gels containing 0.5μL GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA), and 1X Lithium Borate buffer (Faster Better Media LLC, Hunt Valley, MD) at 250V. Gels were visualized using a Gel Doc™ EZ Imager (BioRad).

Statistics

The USDA Integrated Pathogen Modeling Program (40) was used to generate a linear regression, using the survival curve program, of the concentration of *E. coli* at each measured distance over time. JMP® Pro 11.0.0 was used to perform ANOVA tests to determine significant differences between sample type (bulk, rhizosphere, surface, or subsurface) and between peak concentrations and rate of decline at different distances and for the two seasons. A p-value less than 0.05 was considered statistically significant.

5.3 Results

Three rows of spinach were individually flooded with ~6 log CFU/mL *Escherichia coli* on June 3, 2014 for the spring trial and October 7, 2014 for the fall trial. Surface and sub-surface samples of bulk and rhizosphere soil were collected from inside the flood zone, 0.5, 1.5, 4.5, and 9m (30ft) from the edge of the flood zone over a 63-day period. In the spring trial, spinach foliar samples were collected over a 14-day period before the plants bolted and were no longer fit for sampling. In the fall trial, spinach samples were collected through day 42 before being seemingly consumed by wildlife that accessed the

plot. In addition to the sampling days stated above, spinach samples were collected 9 days post-inoculation in the spring when it was determined the spinach would not be suitable to sample for much longer. For consistency, a day 9 sample of spinach was also collected during the fall trial.

The soil in the lysimeter plot was determined to be a silty clay soil of approximately 50±2.3% clay, 24±3.6% silt, and 26±2.6% sand, and a pH of 6.83±0.29.

Soil

No nalidixic acid resistant *E. coli* was detected in soil or on spinach one day prior to inoculation for either trial. MANOVA with repeated measures analysis showed no significant difference between the MPN/g values for surface and subsurface bulk and rhizosphere samples taken at the same distance on the same day ($p=0.9987$ in spring; $p=0.4586$ in fall). As such, these samples were averaged together for further analysis, to yield one value for each sampling distance on each day.

The average log MPN/g *E. coli* at each sampling distance during the spring and fall trials are shown in Figure 5-4. In the spring trial, *E. coli* was recovered in soil from the 4.5m sampling distance in one row at approximately 0.82 log MPN/g, and from one soil sample (~1.82 log MPN/g) at 9m(30ft) from the edge of the flood immediately following inoculation. A sharp increase in concentrations outside the flood zone was seen on day 1, followed by a generally linear decline at all distances. By day 42, an enrichment step was

required to detect any *E. coli* outside the flood zone, and by day 63, *E. coli* could not be recovered from outside the flood at all.

Similar to the spring trial, in the fall trial *E. coli* was detected by enrichment from soil samples at 1.5m, 4.5m, and 9m (30ft) immediately after flooding. On day 1, there was a significant increase in recovery of *E. coli* from 0.5m from the edge of the flood compared to day 0, but such an increase was not seen at 1.5m until day 3. Throughout the experiment, *E. coli* was generally only detected by enrichment at 4.5m from the flood edge, though populations could be detected by MPN analysis from some 9m(30ft) surface soil samples through day 49. On day 63, *E. coli* populations in the flood zone soil had only declined approximately 2 log MPN/g. Outside the flood zone, *E. coli* was only detected on day 63 by enrichment beyond 0.5m from the edge of the flood. However, on day 63, *E. coli* populations in the flood zone soil were significantly higher in the fall (2.77 log MPN/g) than in the spring (0.4 log MPN/g).

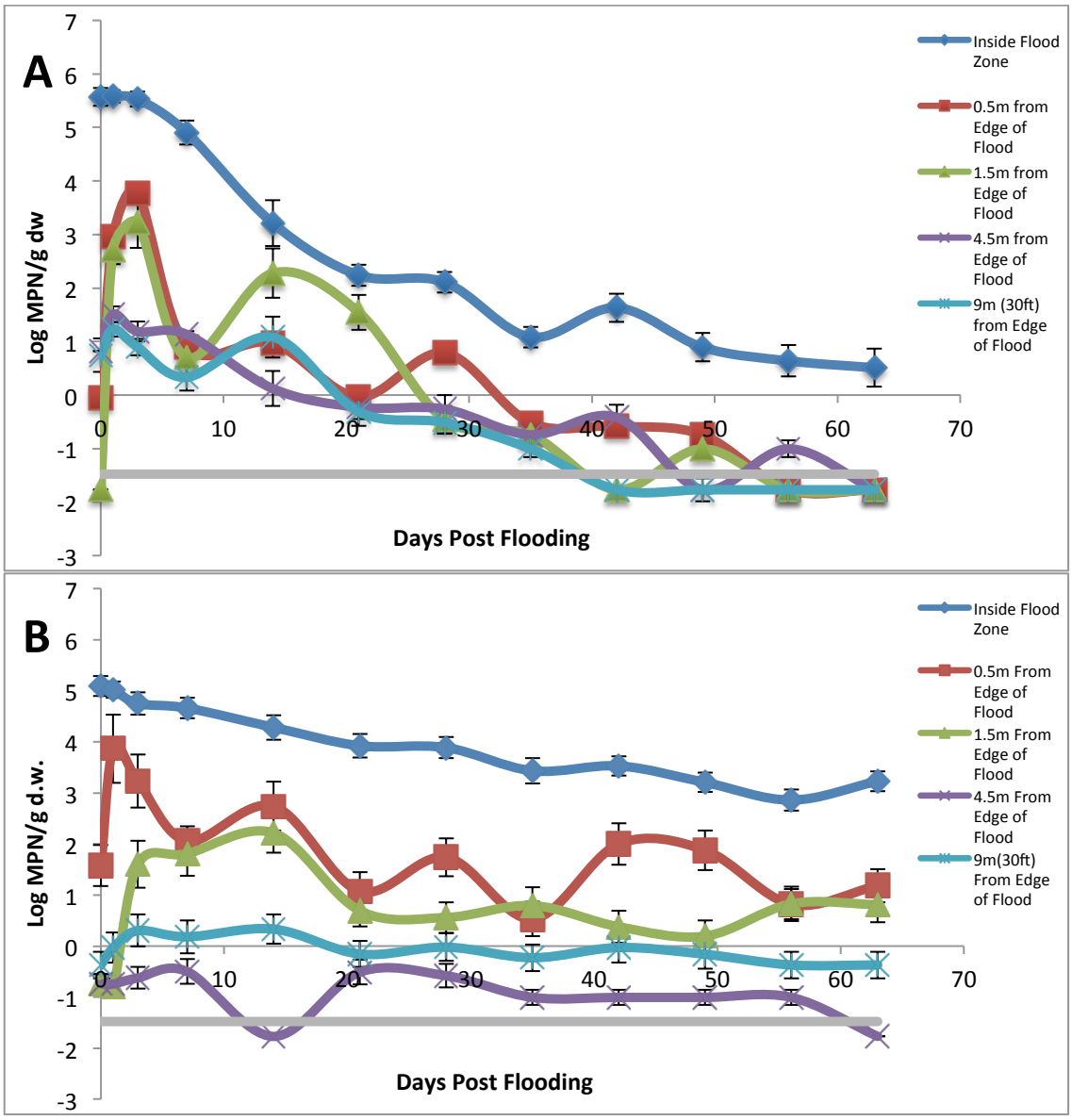

Figure 5-4: log MPN/g d.w. of *E. coli* present in soil at varying distances from the edge of the flood zone in A) the spring trial, June – August 2014 and B) the fall trial, October – December 2014. Each data point represents the average of 12 samples, i.e., the four soil samples at each distance from the three rows of spinach. The limit of detection for enrichment was -1.48 log CFU/g (gray line). Error bars indicate the standard error of the mean.

The concentrations of *E. coli* recovered from each distance over time were individually fit to linear regressions using the IPMP software. A regression from the peak concentration at each distance to when populations reached the limit of detection was

calculated. One-way ANOVA revealed a significant effect of distance ($p<0.0001$) and season ($p=0.0460$) on the peak concentration of bacteria recovered. The rate of decline was significantly affected by season ($p<0.0001$) but not sampling distance ($p=0.5557$). Table 5-1 summarizes the average peak concentration and rate of decline of *E. coli* at each sampling distance over time for each season. Further analysis showed the peak concentration to be significantly greater in the spring trial than the fall trial at all distances except 0.5m from the flood edge. The rate of decline was significantly faster in the spring than the fall.

The time required for a three-log decrease in *E. coli* concentration was calculated for each distance using the linear regression equation, as shown in Table 5-1. Oneway ANOVA analysis showed the time required for the three-log decrease was overall significantly affected by season ($p<0.0001$) but not distance (0.4349). Further analysis showed the time required was significantly longer in the fall at all sampling distances except 4.5m from the edge of the flood.

Table 5-1: Average peak concentration and rate of decline of *E. coli* in soil at each sampling distance over time*

Distance from Edge of Flood	Peak Concentration (log MPN/g)			Rate of Decline ((log MPN/g)/day)			Time to Three Log Decrease in Recovery (Days)		
	Spring	Fall	P-value**	Spring	Fall	P-value	Spring	Fall	P-value
Inside Flood Zone	5.76^{A}	4.83^{A}	**0.0082**	-0.153^{AB}	-0.034	**<0.0001**	20^{B}	90	**0.0001**
0.5m	3.59^{B}	1.99^{B}	0.2431	-0.179^{B}	-0.037	**0.0154**	18^{B}	105	**0.0226**
1.5m	3.14^{BC}	1.13^{BC}	**0.0247**	-0.178^{B}	-0.020	**0.0121**	18^{B}	172	**0.0238**
4.5m	1.42^{CD}	-0.43^{C}	**0.0108**	-0.066^{A}	-0.034	**0.0415**	47^{A}	112	0.1817
9m(30ft)	1.23^{D}	0.32^{BC}	**0.0051**	-0.111^{A}	-0.013	**0.0071**	28^{B}	250	**0.0038**

*Different letters down each column indicate significant differences as calculated using one-way ANOVA and Tukey's Test. No significant difference in rate of decline of *E.* coli at the different sampling distances in the fall season or in the time to a three-log decrease in the fall.

**p-value for the difference between values for spring and fall trials at each distance using one-way ANOVA. Bold indicates significant difference.

Spinach

The number of positive spinach enrichments (out of three replicates) during the spring planting is shown in Table 5-2. No spinach samples were positive by enrichment outside the flood zone on day 0. After just one day post-flooding, one of three spinach samples was positive by enrichment 30ft from the edge of the flood zone and two were positive on day 9. Inside the flood zone, all spinach samples were positive for *E. coli* throughout the 14 days of sampling. However, outside the flood zone, *E. coli* could only be detected from one spinach tissue enrichment on day 14.

Table 5-2: Number of spinach tissue samples positive by enrichment for *E. coli* out of three replicates, June – August 2014

Distance From Edge of Flood	Day 0	Day 1	Day 3	Day 7	Day 9	Day 14
0m	3	3	3	3	3	3
0.5m	0	3	3	3	2	0
1.5m	0	2	1	1	1	0
4.5m	0	3	2	0	0	1
9m (30ft)	0	1	1	0	2	0

The number of positive spinach enrichments (out of three replicates) for the fall trial is shown in Table 5-3. Inside the flood zone, all spinach samples were positive by enrichment for the duration of sampling. The number of positive samples peaked on day 3 for all distances outside the flood zone. After day 14, few enrichments were positive for *E. coli* outside the flood zone, and by day 42, only one spinach enrichment was positive.

Table 5-3: Number of spinach tissue samples positive by enrichment for *E. coli* out of three replicates, October – December 2014

Distance From Edge of Flood	Day 0	Day 1	Day 3	Day 7	Day 9	Day 14	Day 21	Day 28	Day 35	Day 42
0m	3	3	3	3	3	3	3	3	3	3
0.5m	2	2	3	0	3	1	1	1	0	0
1.5m	0	0	3	1	2	0	0	1	0	0
4.5m	0	0	1	1	1	0	0	1	1	1
9m (30ft)	0	0	2	1	1	0	0	0	0	0

Temperature and Rainfall

Figure 5-5 show the maximum temperature and total rainfall each day for the spring and fall trials. Student's t-test showed the average maximum daily temperature was significantly lower ($p<0.0001$) in the fall (~13°C) than the spring (~29°C). There was no significant difference in the average rainfall during each season. Correlation analysis was

used to attempt to correlate peaks in bacterial recovery over time to heavy rainfall events or to peaks in the daily maximum temperature. However, no such correlations were found.

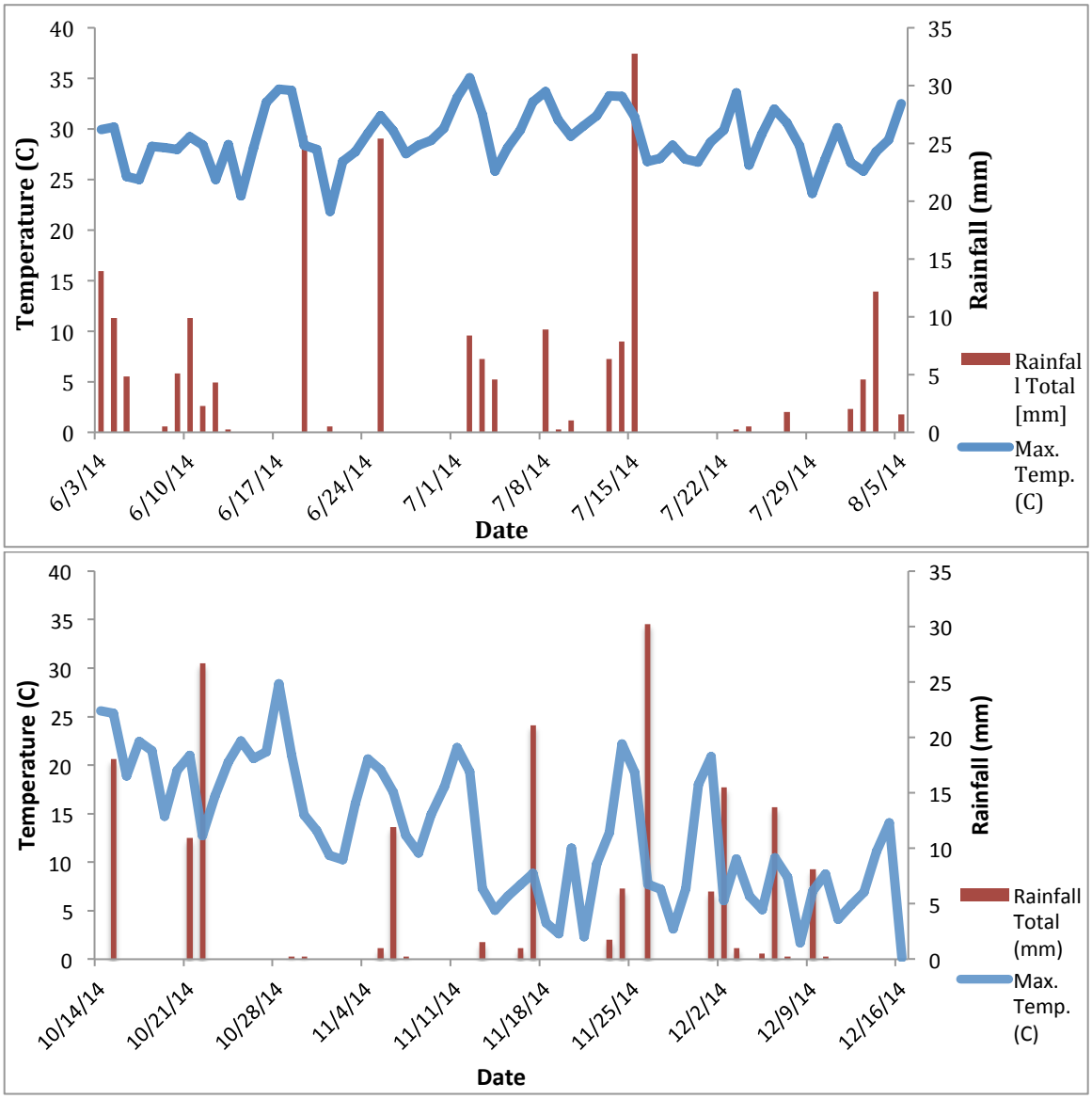

Figure 5-5: Temperature and rainfall data for spring (A) and fall (B) trials. Bars indicate total rainfall (mm) each day, and line indicates maximum daily temperature (°C)

Isolate Confirmation

All isolates collected during the second month of sampling (day 35 and beyond) were confirmed by BOX-PCR to one of the three nalidixic acid-resistant *E. coli* strains inoculated into the floodwater. An example gel is shown in Figure 5-6, where columns 2, 3, and 4, are the gel banding patterns for pure cultures of *E. coli* MW416, 423, and 425, respectively, and columns 5-17 are isolates recovered on MACN from soil samples on day 63 in the fall trial.

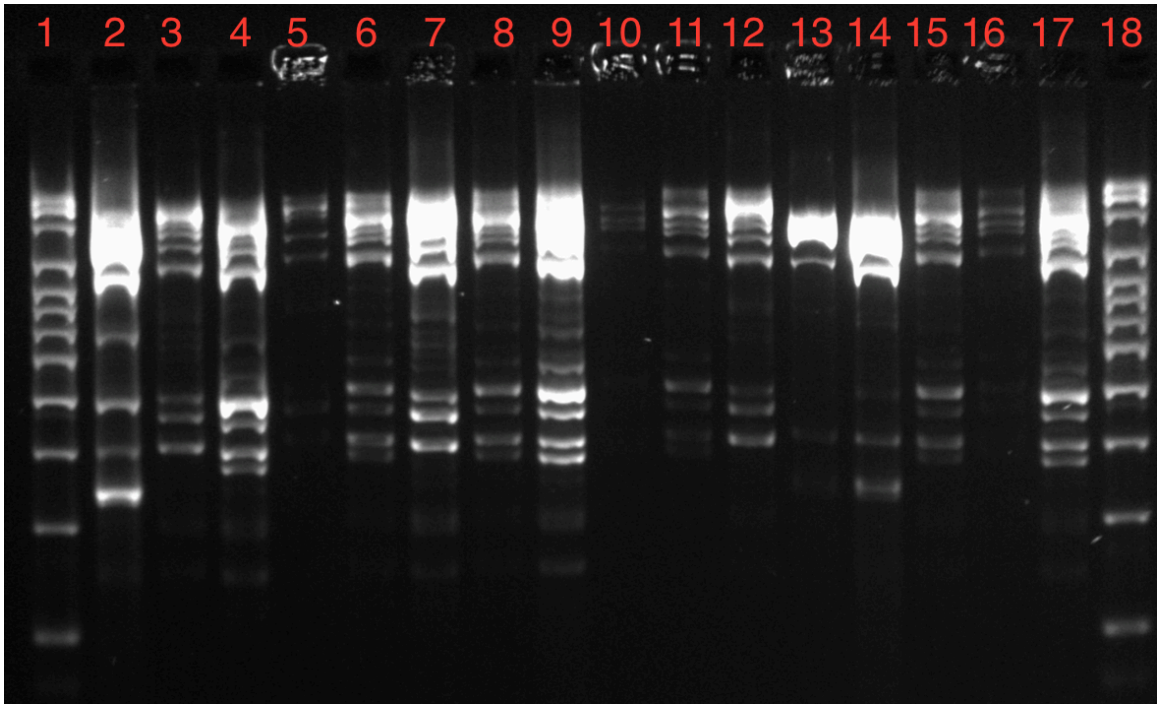

Figure 5-6: Gel electrophoresis of BOX-PCR products. Lanes 2-4 are the products from pure cultures of *E. coli* MW416, 423, and 425, respectively. Lanes 5-17 are products of isolates recovered from soil by MPN analysis. All isolates matched the banding profile of one of the inoculated strains.

5.4 Discussion

In this study, the movement of *E. coli* through soil and the subsequent contamination of spinach leaf tissue were examined using a lysimeter plot with a -5% grade. The *E. coli* was inoculated into water containing fresh dairy liquid manure at the higher end of rows of spinach in order to simulate a flood that may occur when a heavy rainfall event causes river or creek water contaminated with animal manure to overflow its banks and cause flooding on cropland at the higher end of a field slope. This introduces a potential enhancement of the horizontal movement of an enteric bacterium through the soil by gravity.

After flooding, *E. coli* was quickly detected in soil and on leaf tissue beyond the flood zone. In the spring trial, approximately 1.5 log MPN/g *E. coli* was recovered from soil at the farthest sampling distance, 9m(30ft) from the edge of the flood zone on day 1. On the same day, one spinach tissue enrichment was positive for *E. coli* presence, out of 3 enrichment samples. Potentially, this could have been the chance detection of an *E. coli* that was not from the inoculum. However, this possibility is reduced when its nalidixic acid resistant phenotype is considered. Composite soil and leaf tissue samples taken the day prior to flooding showed no presence of nalidixic acid resistant *E. coli* in the plot. The identity of the inoculum was confirmed by BOX-PCR (Fig. 5-6). Similarly in the fall trial, but on day 3, *E. coli* was detected at ~1 log MPN/g in soil at 9m(30ft), and on two of the three spinach tissue samples at the same distance. However, heavy rainfall after inoculation made it difficult to determine if this fast detection was a result of quick transportation of the bacteria through soil, or simply overland runoff down the slope to

the farthest sampling distances. Rain splash of contaminated soil onto the spinach leaves may be the cause of the positive tissue samples, as rain splash has been shown to be a significant method of dispersal of bacteria in soil to plant tissue (19).

The concentration of *E. coli* in soil was quantified separately for bulk and rhizosphere soils at surface and subsurface depths. Previous research has shown that the transport of pathogens through soil is significantly retarded in the presence of plant roots (68). Recovery of *E. coli* O157:H7 and *S.* Typhimurium was higher in the rhizosphere of lettuce plants than in bulk soil after contaminated slurry was applied to soil and a rainfall event was simulated (68). Further, a number of experiments investigating the downward movement of bacteria have concluded that the concentration of bacteria in soil is greatest at the soil surface, where filtration of bacteria is most efficient (7, 65). Contrary to these results, the current study found no significant differences between the concentrations of *E. coli* recovered from the bulk and rhizosphere soil, or between surface and subsurface soil, in either season.

The concentration of *E. coli* recovered from soil over time at each sampling distance was fit to a linear regression from the highest recovery to when the populations reached the limit of detection for MPN analysis. The peak concentration in soil dropped significantly as the distance from the flood zone increased for both trials but there was no definitive pattern for the rate of decline in recovery for either season. However, the peak concentration of *E. coli* recovered was significantly higher in the spring than the fall at all sampling distances except for 0.5m. At 0.5m from the edge of the flood, the peak

concentration was higher in the spring, but the difference did not reach significance, possibly because of high variation in the parameters for the linear regressions between replicates in the fall. These results suggest greater numbers of *E. coli* traveled out of the flood zone, and to farther distances, in the spring than the fall. This could be because more rain fell in the first few days post-flooding in the spring than the fall, mobilizing more bacteria out of the flood zone in the spring than the fall.

Inside the flood zone, *E. coli* populations were significantly higher on day 63 in the fall (2.77 log MPN/g) than in the spring (0.4 log MPN/g). While the recovery of *E. coli* was significantly higher on day 0 in the spring compared to the fall, despite fall inoculums being about 0.5 log cfu/mL higher, the rate of decline of recovery was significantly greater in the spring than the fall. Additionally, the significantly greater rate of decline in the spring compared to the fall was observed at all sampling distances. This is likely due to the higher spring trial temperatures, as bacterial survival in soil is significantly greater at cooler temperatures (2, 38, 65, 75, 77).

In this experiment, floodwater was contaminated with ~6 log cfu/mL *E. coli.* This level of contamination is generally higher than levels of bacteria in manure that may leach out to contaminate floodwater. A survey of cow feces at time of slaughter found 75% of samples to have equal to or less than 10^3 log CFU/g *E. coli* O157 (59). As such, the time for a 3-log decrease in population levels was calculated for each sampling distance in each season. The number of days required for a three-log decrease in the population of *E. coli* was significantly longer in the fall than the spring at all sampling distances, except

for 4.5m from the flood edge. At this distance, the time requirement was noticeably longer in the fall, but variation between the rates of decline for each row, due to the very low level of recovery of *E. coli*, is likely the reason the difference did not reach significance.

The purpose of this study was to investigate the movement of bacteria from floodwater through a spinach field, to determine if LGMA-proposed metrics were sufficient to prevent the harvest of contaminated spinach crops. The LGMA suggests destroying all crops within 30ft of the edge of a flood zone. In both seasons, *E. coli* was detected from spinach leaf tissue from at least one sample at the 9m(30ft) sampling distance, indicating that there is the potential for bacteria mobilized by floodwater to contaminate leafy green crops throughout the 30-ft "buffer zone" of crop destruction suggested by LGMA. However, in both trials, heavy rainfall occurred within 24 hours of flooding, potentially confounding results. The rainfall may have promoted the rapid transport of bacteria underground through the soil, but could have also washed contaminated soil across the field with surface runoff. In both cases, rain splash may have led to the contamination of the spinach leaves. Therefore, it can be concluded that a buffer zone of at least 30ft is necessary to prevent the harvest of potentially contaminated spinach after a flooding event involving heavy rainfall during or immediately after the flood accumulated when flooding occurs at the top of a field slope.

These results from the field trials are substantially different from those of the model system where relatively little movement of bacteria was observed. The comparative

findings clearly identify an additional factor in assessing the effectiveness of the LGMA guidance, i.e., the slope of the field being evaluated. In the model system, the soil was inoculated in such a way as to simulate a flood that would occur at the bottom of a field slope (movement up a slope), whereas the field trial simulated a flood at the top of a field slope. In the former, the force of gravity slowed the spread of the floodwater through the soil, and thus impaired bacterial movement. In the latter, the force of gravity worked to promote the flow of water down the slope, and in turn, promoted the movement of the bacteria with the infiltrating water. Further, the soils used in the model system were sieved to a somewhat uniform particle size, and soil cores were constructed in such a way as to prevent the presence of channels and cracks that would artificially promote the movement of water and bacteria around the soil, instead of through it. In a field setting, soils are subjected to a number of factors, including soil fauna such as worms, and the natural freeze-thaw cycle that generate cracks and macropores in the soil that may facilitate water and bacterial movement (4, 7, 76). While the model system was adequate for determining the effect of soil type and soil moisture content on the movement of bacteria, the homogeneity of the soil in the tubing may have led to an underestimation of the extent of bacterial movement through a field with a more heterogeneous soil distribution with naturally-formed cracks and channels.

The LGMA-proposed metrics suggests waiting 60 days before replanting previously flooded fields, provided the soil sufficiently dries during this period. In this experiment, *E. coli* in the flood zone could only be recovered by enrichment after 42 days whereas in the fall, there was only a ~2 log decline in the flood zone population over the entire 63

days of sampling. In fact, linear regression analysis predicted that the time required for a 3-log decrease in the concentration of *E. coli* in the flood zone was 90 days in the fall trial, but only 20 days in the spring. Therefore, it can be concluded that the 60-day waiting period may not be a sufficient length of time to allow for bacterial contamination of floodwater to die off before replanting previously flooded fields if the average daily maximum temperature is relatively low. Further research would be needed to investigate the potential for the contamination of the next crop of spinach if it is planted in the flood zone before the level of pathogens in the soil has dropped below the detection limit.

Chapter 6: Summary, Conclusions, and Future Directions

6.1 General Findings

In this experiment, the movement of bacteria through soil was investigated under both laboratory and field conditions. The movements of *Salmonella enterica* serovar Newport and *Citrobacter freundii* were compared through three soil textures at three initial moisture concentrations. There was no significant difference between the recoveries of the two species for any experimental conditions. In this experiment, the movement of bacteria through soil cores was found to best fit a two-phase linear model, where there is a length of soil with no observable decrease in the concentration of cells recovered from the soil, the "lag distance", before the recovery of cells decreases in a linear fashion. Soil type significantly affected both phases of the model, though no correlation to known soil parameters, including soil texture, organic matter, and cation exchange capacity, could be established. While the soil with the highest clay concentration had the shortest lag distance, as expected, the soil with the median clay content had the longest lag distance. As expected, as the initial soil moisture content increased, the rate of decline in bacterial concentration also decreased, indicating greater movement of bacteria through saturated soils than unsaturated soils.

The second phase of this experiment investigated the validity of metrics proposed by the California Leafy Green Products Handler Marketing Agreement (LGMA) that recommend specific practices to prevent the harvest of leafy greens contaminated by human pathogens from floodwater. According to the LGMA, after a flood, all leafy green

crops within 30 feet of the edge of a flood should be destroyed, and previously flooded areas should not be replanted for 60 days. The results of the current research suggest that additional factors, such as slope or seasons, may need to be considered to reach scientifically valid decisions regarding the disposition of leafy greens that have been exposed to flood conditions. The results of this experiment showed that under a scenario where the flood occurs in a field with a negative slope, low levels of *Escherichia coli* from floodwater could be recovered in soil and on spinach tissue 30-ft from the edge of the flood zone, especially during the spring trial. In general, *E. coli* persisted longer in the soil in the fall trial than the spring. This is likely due to the fact that the significantly lower temperature in the fall (~13°C) was better suited for bacterial survival than in the spring (~29°C). The time required for a three-log decrease in *E. coli* recovery was significantly longer in the fall than the spring at all distances. In the fall trial, the time required for a three-log decrease in recovery inside the flood zone was predicted to be 90 days. This finding is concerning, as the LGMA metric advises farmers to only wait only 60 days before replanting previously flooded soils.

6.2 Conclusions

- The movement of bacteria from floodwater can be best modeled using a two-phase linear model; where there is first a lag distance without significant removal of cells from infiltrating water before a linear decline in recovery as distance from the flood increases.

- The length of the lag distance is significantly affected by soil type, while the rate of filtration is significantly affected by both soil type and initial soil moisture content.
- *Citrobacter freundii* is an appropriate surrogate for *Salmonella* Newport in field experiments, as the movement of *C. freundii* through soil in a model system was not significantly different from that of *Salmonella* Newport.
- The LGMA metrics may not be sufficient to prevent the harvest of leafy green produce that has been contaminated by bacteria from floodwater. *E. coli* was recovered from soil and on spinach leaf tissue 30-ft from the edge of a flood in both the spring and fall growing seasons. In the fall season, the time required for a 3-log decrease in the concentration of *E. coli* in the flood zone was predicted to be 90 days, significantly longer than the 60-day waiting period proposed by LGMA.
- The LGMA metrics must be revised to include considerations of the effects of additional rain events after the initial flooding event on the movement of bacteria through soil, and of temperature on the survival of bacteria in soil.

6.3 Future Directions

Additional research is needed to more conclusively determine the extent of bacterial movement through soil after a flooding event, and the potential for contamination of leafy green crops.

Further soil analysis is needed to determine the specific effect of soil type on the movement of bacteria through soil in the model system. Similarly, it would be beneficial

to repeat the field-scale experiment in additional soil textures. Such knowledge would be used to determine the extent of bacterial movement through soil in a field setting: does the radius of the zone of crop destruction need to be greater in soils with a higher clay concentration than sandier soils?

Secondly, it could not be determined if *E. coli* recovered from spinach leaf tissue was a result of rain splash propelling contaminated soil onto the leaf surface, or internalization of the bacteria from the soil via the spinach root system. As stated in the literature review, the rate of internalization of bacteria into leafy green plant tissue is widely debated. In future studies, the spinach tissue harvested could be first subjected to a common washing method, or surface sterilized then homogenized, before quantification of bacteria. Additionally, the contamination of other leafy greens, such as lettuce and kale, by bacteria from floodwater should be assessed to determine if there are differences in the risk of contamination between different leafy green crops.

In this study, the movement of bacteria through soil was much greater through soils with a negative slope (downhill) than soils with a positive slope (uphill). This contrast is likely due to the effect of gravity on the movement of water through each system, but further field trials investigating the specific effect of the slope of the field on the extent of the movement of bacteria away from a flood zone would provide useful information regarding the movement of bacteria through soil.

Finally, further investigation into the validity of the LGMA metrics would provide more support for the results of this research. Because of space limitations, spinach and soil samples could not be collected beyond 30 feet from the edge of the flood. This research shows that bacteria in floodwater are rapidly detectable at the 30ft distance in both soil and spinach, but it is not known how far past the proposed buffer zone that bacteria are able to contaminate leafy green crops. Moreover, while it is clear that *E. coli* may persist in soil for significantly longer than the 60-day waiting period, the microbiological safety of produce planted in previously flooded soils is not known. Planting leafy greens 60 days after flooding a field with water containing *E. coli*, then determining the contamination of leaf tissue as the plant matures, would provide further information that may be used to establish more specific metrics to farmers in order to prevent the harvest and consumption of contaminated leafy green produce.

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