PATHOGENS, PRECIPITATION, POOP AND PRODUCE: THE ECOLOGY AND CONTROL OF FOODBORNE PATHOGENS IN PRODUCE PREHARVEST ENVIRONMENTS

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PATHOGENS, PRECIPITATION, POOP AND PRODUCE: THE ECOLOGY AND CONTROL OF FOODBORNE PATHOGENS IN PRODUCE PREHARVEST ENVIRONMENTS.

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The complexity of the global food supply chain, and the demands of a growing population for safe, sustainable food requires novel, holistic, and adaptive approaches to produce safety. However, food is not produced in a vacuum; farms are closely linked to surrounding environments, which can function as pathogen reservoirs as well as pathways for pathogen dispersal into fields. Thus, a comprehensive understanding of the ecological processes that drive the presence, dispersal and persistence of bacterial pathogens in agricultural environments is essential for the development of adaptive approaches to fresh produce safety. In the studies presented here, we employed several approaches to investigate the ecological processes associated with pathogen dispersal in and contamination of produce production environments at multiple scales. Specifically, these studies investigated (i) spatial and temporal risk factors associated with *L. monocytogenes* isolation at the farm, field and sub-field levels, and (ii) factors associated with the transfer of generic *E. coli* from contaminated wildlife feces to and survival on individual, preharvest produce items. We observed that foodborne pathogens are not uniformly present in agricultural environments, and that specific spatial (e.g., proximity to pasture) and temporal (e.g., time between a rain event and harvest) factors were associated with an increased

likelihood of pathogen detection. Using this information, we validated geospatial models that predict when and where pathogen contamination of produce production environments is likely to occur. We were also able to identify specific management practices that were associated with pathogen contamination of preharvest produce. For example, irrigation water was found to be a key pathway for pathogen dispersal in agricultural environments. The transfer of bacteria from in-field contamination sources, such as wildlife feces, to preharvest produce during irrigation was significantly associated with the distance between the produce and the feces. Following contamination, bacteria were able to survive on in-field produce for >10 days. Die-off observed over these 10 days followed a biphasic pattern with more rapid die-off immediately following contamination (i.e., 0-106 hours post-contamination). Overall the findings of the studies reported here provide key data that can be used to develop targeted strategies for reducing the likelihood of preharvest produce contamination.

BIOGRAPHICAL SKETCH

Daniel Weller was born in Annapolis, MD to Donald and Deborah Weller. Daniel became involved in research at a young age. In grade school he participated in a citizen science program at the Arlington Echo Outdoor Education Center (AEC) to restore Atlantic White Cedar populations in southern Maryland; Daniel worked with AEC in various capacities through his sophomore year of college. Daniel earned a Bachelor of Arts degree in Anthropology with minors in Biology and Environmental Studies from Ithaca College. Under the mentorship of Dr. Paula Turkon, Daniel completed an Honors Thesis in Anthropology; his honors project focused on the development of novel methods for performing dendroarchaeological analyses on highly friable samples. During his collegiate career Daniel worked in several other labs including the terrestrial ecology and aquatic ecology labs at the Smithsonian Environmental Research Center. Daniel began his graduate studies in 2013 under the mentorship of Dr. Martin Wiedmann at Cornell University. As a graduate student Daniel has been fortunate to work and collaborate with industry, academic and government stakeholders within and outside New York State. After completing his degree Daniel will continue to work in Dr. Wiedmann's laboratory as a postdoctoral researcher.

Dedicated to my parents, Donald and Deborah, my sister, Sarah, and my grandparents. You have provided unwavering support, love and advice every day of my life and throughout my academic career, and without you I never would have made it this far.

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CHAPTER 1

INTRODUCTION

Foodborne illness is a major cause of morbidity and mortality worldwide. A study conducted by the Pan American Health Organization found that between 1993 and 2010 approx. 9,180 foodborne disease outbreaks were reported in 20 Latin American and Caribbean countries (1). However, this number accounts for only reported outbreaks; since many foodborne illness cases are not associated with outbreaks or do not result in hospitalization, this number underestimates the true burden of foodborne illness in these countries. Indeed, although 271,974 cases of foodborne illness were reported in the United States (USA) between 1998 and 2008 (2), experts estimate that 9.4 million cases of foodborne illness occur in the USA each year [i.e., an estimated 94 million cases between 1998 and 2008; (3)]. Preventing foodborne illness is clearly a public-health priority as evidenced by the passage of the Food Safety Modernization Act (4) and other food safety legislation (5, 6) as well as the proliferation of outreach materials focused on consumer food safety [e.g., (7, 8)]. Since pathogen contamination of food and the resulting illnesses also are associated with substantial economic costs, preventing microbial contamination of food products is also an economic priority. For example, the total estimated medical cost of salmonellosis in the USA is 312 million dollars annually; this number excludes the cost of premature death and productivity losses as well as costs associated with other etiological agents (9). In fact, the total estimated annual cost of foodborne illness in the USA is approx. 15 billion dollars; this value was estimated by summing the costs associated with the 15 pathogens that account for $>95\%$ of illnesses in the

Table 1.1: Estimated costs associated with foodborne illness due to specific etiological agents in the United States. [a]

Etiological Agent	Medical Costs [b]
Campylobacter spp.	$1,928,787,166
Clostridium perfringens	$342,668,498
Cryptosporidium parvum	$51,813,651.77
Cyclospora cayetanensis	$2,301,423
Escherichia coli O157	$271,418,690
non-O157 Shiga toxin-producing *E. coli*	$27,364,561
L. monocytogenes	$2,834,444,202
Norovirus	$2,255,827,318
Salmonella spp.	$3,666,600,031
Shigella spp.	$137,965,962
Toxoplasma gondii	$3,303,984,478
Vibrio parahaemolyticus	$40,682,312
Vibrio vulnificus	$319,850,293
Vibrio spp. [c]	$142,086,209
Yersinia enterocolitica	$278,111,168
Total	$15,603,905,963

[a] Data is from United States Department of Agriculture, Economic Research Service Reports (9).

[b] Includes cost of visits to the physicians' office, emergency room, and out-patient clinics as well as hospitalization, premature death, and productivity loss for non-fatal cases.

[c] Costs due to *Vibrio* spp. excludes costs due to *V. parahaemolyticus*, and *V. vulnifcus*.

USA [Table 1.1; (9)]. It is important to note that outbreak costs extend beyond the hospital. For example, following a 2008 *L. monocytogenes* outbreak linked to ready-to-eat-deli meat the Canadian government spent approx. $2.4 million on outbreak response, while the food facility implicated in the outbreak spent 77 million dollars [Table 1.2; (10)]. Even when pathogen contamination of food does not result in illness, it still necessitates food recalls. While recalls carry direct costs [e.g., lost product, shipping costs], consumer avoidance (11–13) and reductions in share price (14–16) following recalls negatively affect the bottom line of the company doing the recall as well as other companies in the same sector of the food industry. As such, the development of effective strategies for preventing or reducing foodborne pathogen contamination of food products is of substantial interest to public health, government, and industry stakeholders.

Foodborne Pathogens, Fresh Produce, and the Preharvest Environment

Fresh produce is increasingly recognized as a source of foodborne outbreaks worldwide (17–19). Between 1973 and 2015 the proportion of foodborne outbreaks attributable to fresh produce in the USA rose from 0.7% to 19% (18, 20). This trend is not limited to the USA; the number of outbreaks attributable to fresh produce in the European Union increased from 29 in 2006 to 34 in 2009 and 44 in 2010 (21). The globalized nature of the food supply chain, and the associated changes in the scale of and methods for harvesting, packing, and processing fresh produce means that produce contamination in one area has the potential to affect human health worldwide. This may explain, at least partially, the observed increase in produce-borne disease outbreaks (22, 23). Other potential causes include (i) increased

Table 1.2: Costs associated with select foodborne disease outbreaks and recalls [a].

Event	Food Vehicle	Country	Medical [b] Costs (USD)	Public Health and Government Costs (USD)	Industry Costs (USD)	Study
2009 *Salmonella* recall	Peanut products	USA	-	-	133 million	(24)
2008 *L. monocytogenes* outbreak	Deli meat	Canada	162 million	2.4 million	77 million	(10)
2008 *Salmonella* recall [d]	Tomatoes	USA	-	-	250 million	(24)
2008 *Salmonella* outbreak	Muskmelon	Canada; USA	-	-	50 million	(25)
2007 *Salmonella* recall	Peanut butter	USA	-	-	133 million	(24)
2006 *E. coli* O157:H7 outbreak	Spinach	USA	-	-	350 million	(24, 25)
1992 *E. coli* O157:H7 recall	Hamburgers	USA	-	-	160 million	(24)
1988 *Salmonella* outbreak	Eggs	UK	-	26.2 million [e]	-	(13)
1983 *Salmonella* outbreak	Milk	UK	114,800	31,000	-	(26)
1982 *Salmonella* outbreak	Chocolate	UK	42,000		206,000	(26)
1982 *Salmonella* outbreak	Diet drink	USA	-	-	2.4 million	(26)
1970-1 *Salmonella* outbreak	Chocolate	Sweden	-	-	83,000	(26)
1964 *Salmonella* outbreak	Canned corn beef	UK	2,577,798	727,470	160 million	(26)
1963 Botulinum intoxication outbreak	Canned tuna	USA	8,000	-	163 million	(26)

Table 1.2 Continued

[a] Where possible the costs for a given outbreak and the associated recall have been reported in separate rows.
[b] Costs were estimated by monetizing and summing the cost of medical care, premature death, and/or other costs to individuals.
[c] Data not reported.
[d] Reports costs of lost tomato sales due to incorrect identification of tomatoes as the food vehicle in a 2008 *Salmonella* outbreak traced back to contaminated peppers.
[e] Reports legal costs associated with a settlement that the United Kingdom Ministry of Agriculture, Fisheries and Food had to pay egg producers.

consumption of fresh produce, (ii) year-round availability of fresh produce, (iii) intensification of agricultural production, and (iv) improvements in outbreak detection and reporting (27–29). Regardless of why the number of foodborne illness cases attributable to contaminated produce has increased, fresh produce presents a unique food safety challenge because produce does not undergo a kill step prior to consumption. Since the health benefits of consuming fresh produce are well-recognized and substantial, reducing consumption of fresh produce is not a practical solution for reducing risk and improving public health. As a result, preventing contamination, as opposed to removing or killing pathogens present in the food product prior to consumption, is of particular importance for fresh produce commodities.

In 2012 a multistate *Salmonella* outbreak linked to cantaloupe sickened 261 people (30). As part of the environmental assessment performed following the outbreak *Salmonella* of the same pulsotype as the outbreak strain was isolated from multiple environmental samples collected from an implicated farm, including preharvest produce (30). While the 2012 outbreak illustrates the potential for preharvest produce contamination to directly cause illness, previous studies (31, 32) have also shown that once preharvest produce is contaminated, pathogens can be transferred to other produce items as well as the post-harvest environment during harvest and processing. Once the post-harvest environment is contaminated, pathogens can proliferate and contaminate large amounts of product. In fact, several studies have found that environmental pathogen levels and pathogen levels on produce increase the farther downstream the produce supply chain the samples are collected (33–35). Thus,

preventing preharvest produce contamination is instrumental for minimizing pathogen contamination throughout the produce supply chain.

Identifying and implementing effective strategies for reducing preharvest produce contamination is incredibly difficult due to the complexity of the preharvest environment. Indeed, preharvest environments are part of a larger landscape and are impacted by the surrounding natural and built environments. For example, agricultural water sources can act as a pathway for pathogen movement into produce fields. As a result, the use of contaminated water for produce production can serve as a direct route of produce contamination (20, 60). In fact, the probable contamination source for the aforementioned 2012 *Salmonella* outbreak was the use of contaminated water for irrigation (30). Similarly following a 2005 outbreak of *E. coli* O157:H7 linked to fresh lettuce, the outbreak strain was isolated from environmental samples collected at a dairy that was upstream of the implicated produce farm (55). The outbreak strain was also isolated from irrigation water samples collected at the implicated farm (55). As a result, contamination of the irrigation water source by cows at the upstream dairy, and the subsequent use of said water to irrigate crops on the implicated farm was identified as the probable contamination source for the 2005 outbreak (55). The 2005 outbreak is illustrative of how events that occur in farm-adjacent environments can effect on-farm produce safety.

Like agricultural water, wildlife can also act as a vector for the movement of pathogens within and between natural and agricultural environments; several foodborne outbreaks have been traced back to wildlife intrusion in produce fields

(Table 1.3). Indeed, multiple studies have shown that foodborne pathogens can be isolated from wildlife feces [e.g., (36, 37)], and that once defecation occurs pathogens present in the feces can transfer via splash to preharvest produce (38, 39). In addition to wildlife intrusion and the use of contaminated water for produce production, there are a multitude of other pathways for the dispersal of pathogens within and between farm and farm-adjacent environments. For example, agricultural inputs, such as biological soil amendments and contaminated seed, have been identified as potential sources for preharvest contamination of produce (40–43). While produce can be directly contaminated through wildlife defecation on or the application of contaminated water and manure onto the edible portions of the crop, wildlife intrusion and the use of contaminated water and manure can also contaminate the preharvest environment. Once the produce production environment is contaminated pathogens may survive and proliferate in produce field soils; the pathogens are then available to transfer to preharvest produce at a later time. A field study conducted in the United Kingdom examined the survival of *E. coli* O157:H7 and *Salmonella* in produce field soils, and found that following irrigation with contaminated water (inoculum = 10^8 CFU/mL) the inoculation strains persisted in the soil for more than 6 weeks (44). In a separate trial of the same study, the authors found that generic *E. coli* in field soils could transfer via splash to agar strips that were up to 45 cm (horizontal distance) and 20 cm (vertical distance) from the drop origin (44). A separate study conducted in

Table 1.3: Contamination sources for select foodborne disease outbreaks linked to preharvest bacterial contamination of produce.

Year	Organism	Food Vehicle	Country	Suspected Contamination Source(s)	Study
2014	*Salmonella*	Cucumbers	USA	Manure	(20, 45)
2014	STEC	Strawberries	USA	Wildlife Intrusion	(46)
2014	STEC	Sprouts	Germany	Contaminated Seeds	(47)
2008	*Camplyobacter jejuni*	Peas	USA	Wildlife Intrusion	(48, 49)
2008	*Salmonella*	Peppers	USA	Agricultural Water	(50, 51)
2006	*E. coli* O157:H7	Spinach	USA	Agricultural Water; Wildlife Intrusion	(52–54)
2005	*E. coli* O157:H7	Lettuce	Sweden	Agricultural Water	(55)
2005	*Salmonella*	Tomatoes	USA	Agricultural Water	(56)
2004	*Yersinia pseudotuberculosis*	Carrots	Finland	Wildlife Intrusion	(57)
2002	*Salmonella*	Tomatoes	USA	Agricultural Water	(56)

California found that, on average, during a 2.5 h irrigation event 0.006% of *E. coli* in wildlife feces (inoculum = $1.29*10^8$ CFU/5 g) transferred to preharvest lettuce that was < 1 m from the fecal pellet (39). However, the ability of pathogens to survive in the preharvest environment and transfer to preharvest produce appears to be mediated by weather (39, 44, 59). For example, in the aforementioned Californian study the *E. coli* concentration on lettuce upwind of the fecal pellet was significantly less than the concentration on lettuce that was downwind (39). Similarly, in an unpublished study conducted in Arizona the distance that *E. coli* in simulated wildlife feces (inoculum = 10^7-10^8 CFU/g) transferred via splash during irrigation was associated with wind speed (60). As these studies as well as the 2012 *Salmonella* outbreak and 2006 *E. coli* outbreak illustrate, events that occur in farm-adjacent environments as well as on-farm conditions can impact preharvest produce safety, which suggests that produce contamination risks are not uniform across space and time. Thus, a one-size fits all approach to preharvest produce safety may not be the most effective strategy for reducing produce safety risks. As such, targeted management practices that can be tailored to the risks for a specific farm or field are needed. To best develop these strategies a systems-based approach to preharvest produce safety is needed, which requires a comprehensive understanding of the ecological processes that underpin the distribution, survival and dispersal of foodborne pathogens in and between agricultural and natural environments.

The Food Safety Modernization Act's Produce Safety Rule

Recognizing the importance of preventing preharvest produce contamination, the produce industry, extension agents, and other stakeholders identified good agricultural

practices (GAPs) that growers can implement to reduce the likelihood of preharvest produce contamination. GAPs have been codified in the form of voluntary growers agreements (e.g., Ohio Produce Marketing Agreement, Leafy Greens Marketing Agreement), government guidelines (e.g., the USDA Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables), and the establishment of produce safety extension programs (e.g., National GAPs Program). As part of the Food Safety Modernization Act, the US federal government also recently established the first federal law to regulate fresh produce safety, the Produce Safety Rule [PSR; (4)]. Unfortunately, produce production environments are complex, and there was insufficient data on which to base many of the standards established by the PSR. For example, the PSR mandates that water used for preharvest applications must meet specific standards; if agricultural water sources do not meet these standards then corrective actions are required (4). Potential corrective actions listed in the PSR include water treatment, allowing time for pathogens to die-off between irrigation and harvest (a time-to-harvest interval) or between harvest and end of storage, or finding an alternative water source (4). However, limited data is available on pathogen die-off under field and storage conditions, which makes implementation of the suggested corrective actions impractical, and could result in the harvesting and consumption of contaminated produce. There are similar issues with PSR guidance and standards for surface water testing and treatment, wildlife intrusion, and the use of biological soil amendments of animal origin. For example, the PSR requires growers to take all measures reasonably necessary to identify and not harvest produce that may have been microbially contaminated by wildlife. However, relatively little information is

available to help growers determine which wildlife prevention measures are appropriate for a given operation. For example, while no-harvest buffers around in-field feces are often mentioned as a strategy that growers can use to reduce produce contamination risks associated with wildlife intrusion, there is limited data on the risk reductions associated with different buffer widths. These knowledge gaps mean that current standards are not only unclear, but are also likely to change. As a result, growers are unsure of how to best meet the standards established by the PSR, including which food safety measures they should implement on their farms and how these f measures should be implemented to best meet the needs of their operation. In fact, understanding and complying with ambiguous standards has been cited in industry magazines as one of the major obstacles facing growers (61). Clearly, additional research is needed to address these knowledge gaps, to facilitate the development of science-based, on-farm intervention and control strategies, and to support the implementation and revision of the PSR. Addressing these knowledge gaps was one aim of the studies included in this dissertation.

Specifically, the aim of the studies described here were to (i) increase our understanding of the ecological processes that drive the distribution, survival and dispersal of foodborne pathogens in produce production environments, (ii) generate experimental data that can be used in quantitative risk assessments to address knowledge gaps in the PSR, (iii) identify potential intervention and control strategies for reducing the likelihood of preharvest produce contamination, and (iv) create and validate tools that can be used in the development of said strategies. Thus, four studies were conducted to (i) validate and refine geospatial models that predict *L.*

monocytogenes prevalence in produce fields (at the field-level), (ii) identify factors that could be used to predict pathogen contamination patterns within a field (i.e., at the sub-field level), (iii) calculate coefficients for the transfer of *E. coli* in feces to preharvest lettuce, and (iv) quantify die-off rates for *E. coli* under field conditions and compare this rate to previously reported rates.

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CHAPTER 2

VALIDATION OF A PREVIOUSLY DEVELOPED GEOSPATIAL MODEL THAT PREDICTS THE PREVALENCE OF *L. MONOCYTOGENES* IN NEW YORK STATE PRODUCE FIELDS

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Abstract

Technological advancements, particularly in geographic information systems (GIS), have made it possible to predict the likelihood of foodborne pathogen contamination in produce production environments using geospatial models. Yet, few studies have examined the validity and robustness of such models. This study was performed to test and refine rules associated with a previously developed, geospatial model that predicts *L. monocytogenes* prevalence for produce farms in New York State (NYS). Produce fields for each of four enrolled produce farms were categorized into areas of high or low predicted *L. monocytogenes* prevalence using rules based on a field's available water storage (AWS), and its proximity to water, impervious cover, and pastures. Drag swabs (n=1,056) were collected from plots assigned to each risk category. Logistic regression, which tested the ability of each rule to accurately predict *L. monocytogenes* prevalence, validated the rules based on water and pasture. Samples collected near water (odds ratio (OR) = 3.0) and pasture (OR = 2.9) showed a significantly increased likelihood of *L. monocytogenes* isolation compared to samples collected far from water and pasture. Generalized linear mixed models identified additional land cover factors associated with an increased likelihood of *L. monocytogenes* isolation, such as

proximity to wetlands. These findings validated a subset of previously developed rules that predict *L. monocytogenes* prevalence for produce production environments. This suggests that GIS and geospatial models can be used to accurately predict *L. monocytogenes* prevalence on-farms, and prospectively used to minimize the risk of pre-harvest contamination of produce.

Introduction

Fresh produce presents a unique food safety challenge due to the absence of a kill-step between harvest and consumption. An increase in recalls and reported outbreaks linked to fresh produce over the past decade (1–3) has been associated with consumer avoidance of products linked to outbreaks (4, 5). This trend can negatively affect growers and the produce industry (4–6). For example, following a 2011 listeriosis outbreak in the United States (US) associated with fresh cantaloupe (7), cantaloupe consumption dropped 53% nationwide (6). Prevention of produce contamination in production environments is therefore a concern for growers, the produce industry and public health professionals. To develop effective prevention strategies, it is important to understand the ecological processes and environmental factors that affect foodborne pathogen prevalence in produce production environments. Technological advancements, such as geographic information systems (GIS), have the potential to drastically improve our ability to examine these processes, and to develop novel tools for ensuring fresh produce safety.

Numerous studies (8–21) have examined the ecology of foodborne pathogens in agricultural environments, and several (22–27) have used GIS and geospatial analysis. For example, Chapin et al. (26) used GIS to organize and extract remotely sensed data to show that different species of *Listeria* occupy distinct ecological niches in agricultural and natural environments. Despite a number of studies that have used GIS to extract or visualize remotely sensed data (22–27), only one study (25) has used GIS to predict the distribution and prevalence of a specific foodborne pathogen in produce production environments. This study by Strawn et al. (25) used classification

tree analysis (CART) to develop a geospatial model that predicts the prevalence of *L. monocytogenes* in New York State (NYS) produce fields. This model consisted of a set of hierarchical rules based on, in order, proximity of fields to surface water, temperature, proximity of fields to impervious cover, available water storage (AWS) and proximity of fields to pasture (25). Studies in other disease systems (e.g., Lyme Disease and West Nile Virus) have not only developed (28–34) but have also validated (35–40) geospatial, predictive risk models. These validation studies (35–40) demonstrate the utility of geospatial risk models, like the model developed by Strawn et al. (25), for accurately and prospectively predicting pathogen prevalence. Additionally, these studies (37, 39, 40) used the output of their models to prioritize and identify risk management strategies, suggesting that geospatial models can also be integrated with on-farm food safety plans to develop targeted approaches to disease prevention. Thus, the purpose of this study was to (i) validate the ability of the model developed by Strawn et al. (25) to predict on-farm areas with a significantly higher or lower prevalence of *L. monocytogenes* and to (ii) identify additional land cover factors that were associated with *L. monocytogenes* isolation from produce production environments. This research also aimed to increase our understanding of foodborne pathogen ecology, and to develop targeted mitigation strategies for risk management in produce production environments (e.g., tailored on-farm food safety approaches). While multiple pathogens can contaminate produce at the production level, we chose *L. monocytogenes* as a model organism to examine contamination at the production level due to its high prevalence in NYS produce production environments (11, 22, 23, 25). We recognize that the model developed by Strawn et al. (25) predicts *L.*

monocytogenes prevalence, however since *Listeria* spp. is an indicator for *L. monocytogenes* also tested the ability of the model to predict *Listeria* spp. prevalence.

Materials and Methods

Study design. A cross-sectional study was conducted over a six-week period in July and August of 2014 on four produce farms in NYS. Farms were located in three regions of NYS: Western New York (n=2), the Hudson Valley (n=1), and the Capitol District (n=1). Farms were not selected based on geographic location or management practices; farms were enrolled based on the willingness of the grower to participate.

All fields within a farm were classified into four high risk categories and one low risk category (see Figure 1) based on a set of hierarchical rules that were adapted from Strawn et al. (25). The rules were based on a field's proximity to water, impervious cover and pasture, and a field's AWS (Supplemental Figure 1; see header "Geographic Data and Predicting Field Risk" for more information). All field areas classified into a given category (e.g., areas within 37.5 m of water) were then divided into 5 x 5 m plots, and a subset of plots were randomly selected from each category for sampling. One area drag swab was collected per plot. Methods used in this study were similar to Strawn et al. (25) to avoid bias between studies. However, unlike Strawn et al. (25), whose unit of analysis was the field, and who collected drag swab, composite soil, water and fecal samples, the unit of analysis in the study reported here was the plot (i.e., sub-field) and only drag swabs were collected.

Geographic Data and Predicting Field Risk. All manipulations of geographic data were performed in ArcGIS (version 10.2.2, Environmental Systems

Research Institute, Redlands CA; 41). AWS data were obtained from the United States (US) Department of Agriculture (http://datagateway.nrcs.usda.gov/GDGOrder.aspx). Land cover data for NYS for 2006 were downloaded and extracted from the National Landcover Database (NLCD; http://www.mrlc.gov/nlcd06_data.php). Road data were downloaded from the Cornell University Geographic Information Repository (cugir.mannlib.cornell.edu). Hydrologic data were downloaded from US

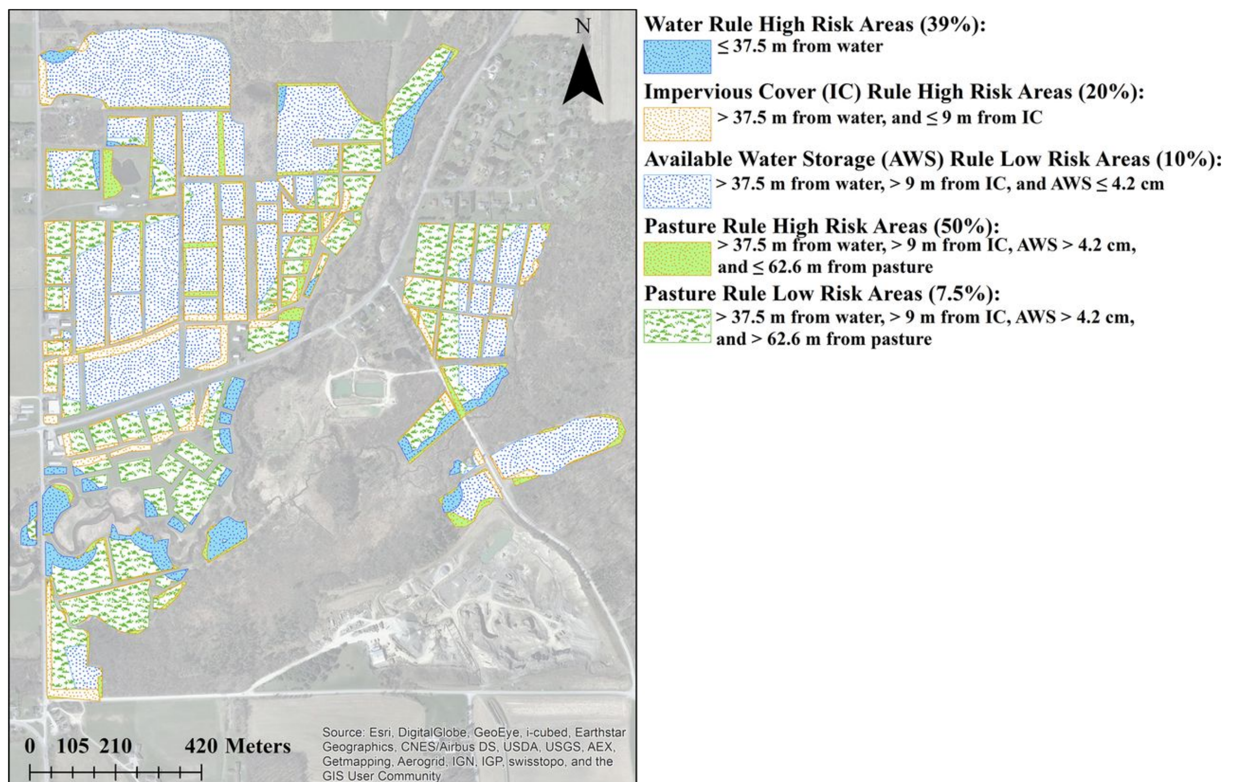


Figure 2.1: Map of predicted prevalence of L. monocytogenes on the Homer C. Thompson Vegetable Research Farm at Cornell University; the expected prevalence of L. monocytogenes is listed in parentheses in the key. Note that this map is not based on any of the farms included in this study for confidentiality reasons. Map created using ArcGIS software, and the base map is from ArcGIS (ESRI [all rights reserved]).

Geological Survey National Hydrology Map

(http://viewer.nationalmap.gov/viewer/nhd.html?p=nhd). Maps of each farm were obtained from the grower, uploaded into ArcGIS, and georeferenced. If the image could not be accurately georeferenced, a farm map was drawn in ArcGIS by identifying field boundaries in satellite images using the original PDF of the farm fields as a reference.

Predicted field risk for *L. monocytogenes* was based on a hierarchical model developed by Strawn et al. (25) using classification tree analysis. Briefly, we adapted that model by removing the meteorological factors so the model only included spatial factors (i.e., proximity to water, proximity to impervious cover, AWS, and proximity to pastures; Supplemental Figure 1). This adapted model will be referred to as the CART model throughout this article. The CART model had four splits/rules, which in order, will be referred to as the Water Rule, the Impervious Cover Rule, the AWS Rule, and the Pasture Rule (Supplemental Figure 1).

Before division of each farm into areas of high or low predicted *L. monocytogenes* prevalence, the relevant shapefiles for each farm were generated using ArcGIS. Hydrology shapefiles were buffered to 39.5 m, road shapefiles were buffered to 19.5 m, and pasture shapefiles were buffered to 62.5 m. Roads and waterways were buffered by an additional 10 m and 2 m, respectively, to give these features realistic width. Additionally, the AWS data was converted from raster to shapefile format. The AWS shapefile was then split into (i) areas with AWS > 4.2 cm, and (ii) areas with AWS ≤ to 4.2 cm (i.e., high and low AWS areas, respectively). The NLCD raster was also converted to shapefile format and split, creating separate files for each land cover

class (e.g., pasture, grasslands, and woody wetlands). The NLCD shapefiles for developed areas were merged with the road map to create an impervious cover shapefile. Similarly, all NLCD shapefiles corresponding to wetland, and forest shapefile were merged to create a single wetlands shapefile and a single forest shapefile.

After creation of the relevant shapefiles, each farm was categorized into areas of high or low predicted *L. monocytogenes* prevalence by following the splits in the CART model (Supplemental Figure 1). For example, the buffered hydrology shapefile corresponded to all areas with a high predicted *L. monocytogenes* prevalence according to the Water Rule. Similarly, all areas that did not have a high predicted prevalence according to the Water Rule but were included in the impervious cover shapefile corresponded to areas with a high predicted prevalence according to the Impervious Cover Rule.

To assess additional risk factors, the distance was calculated from the center of each 5x5 m sampling plot to land covers of interest (i.e., barren land, grassland, forest, impervious cover, roads, scrubland, water, and wetlands). The split NLCD shapefiles were used to calculate distance to barren land, grassland, and scrubland. Similarly, the road and hydrology shapefiles were used to calculate distance to roads and water. Lastly, the merged forest, wetlands, and impervious cover shapefiles were used to calculate distance to those features.

Sample collection and preparation. Samples were collected and prepared as previously described by Strawn et al. (25). Briefly, latex gloves (Nasco, Fort Atkinson, WI) were worn and changed for each sample collected. For each plot, a pre-moistened

drag swab (30 mL of buffered *Listeria* enrichment broth (Becton Dickinson, Franklin Lakes, NJ) in a sterile Whirl-Pak bag) was dragged around the perimeter and diagonals of the plot for 3-5 minutes. All samples were transported on ice, stored at 4°C and processed within 24 h of collection.

Bacterial Enrichment and Isolation. *Listeria* spp. and *L. monocytogenes* enrichment and isolation were performed as previously described (25). Briefly, each sample was diluted 1:10 with buffered *Listeria* enrichment broth (Becton Dickinson), followed by incubation at 30°C. After 4 h, *Listeria* selective enrichment supplement (Oxoid, Cambridge, UK) was added to each enrichment. After incubating for 24 and 48 h, 50 µl of each enrichment was streaked onto *L. monocytogenes* plating medium (LMPM; Biosynth International, Itasca, IL) and Modified Oxford agar (MOX; Becton Dickinson); the plates were then incubated for 48 h at 35 and 30°C, respectively. Following incubation, up to four presumptive *Listeria* colonies were sub-streaked from MOX to LMPM and incubated at 35°C for 48 h. From all LMPM plates, up to four presumptive *Listeria* colonies were then sub-streaked onto brain-heart infusion plates (BHI; Becton Dickinson), followed by incubation at 37°C for 24 h. The species and *sigB* allelic type of one presumptive *Listeria* colony per sample was determined by PCR amplification and sequencing of the partial *sigB* gene as previously described (42–44).

Positive and negative controls were processed in parallel with field samples. *L. monocytogenes* FSL R3-0001 (45) and uninoculated enrichment media were used as the positive and negative controls, respectively. All isolates were preserved at -80°C and isolate information can be found at www.FoodMicrobeTracker.com.

Statistical analysis. All statistical analyses were performed in R (version 3.1, R Core Team, Vienna, Austria). The frequency and prevalence of *L. monocytogenes* was calculated for each predicted risk area for each rule. Although the outcome of the CART model was a predicted prevalence for *L. monocytogenes*, all statistical analyses were performed for both (i) *L. monocytogenes* and (ii) *Listeria* spp. (including *L. monocytogenes*) since *Listeria* spp. is more common than *L. monocytogenes* in NYS produce production environments and is often used as an index organism for *L. monocytogenes*.

In order to test the ability of each rule to accurately predict the prevalence of *Listeria* spp. and *L. monocytogenes* in produce fields, and to validate the CART model, multivariable logistic regression analyses were performed using the lme4 package (46). The multivariable model originally contained all four rules, but was reduced using backwards selection. The outcome for the multivariable model was the presence of *Listeria* spp. or *L. monocytogenes*. Farm was included as a random effect.

As the multivariable model used to validate the algorithm adapted from Strawn et al. (25) only contained four factors (i.e., proximity to surface water, impervious cover, and pasture, and AWS), univariable generalized linear mixed models (GLMM; 46) were developed to examine the effect of additional land covers (i.e., proximity to barren land, forests, grassland, roads, scrubland, and wetlands) on the likelihood of *Listeria* spp. and *L. monocytogenes* isolation. Since the CART model was based on a binary interpretation of AWS, and proximity to water, impervious cover, and pasture, univariable GLMMs were also developed to examine the relationship between these four factors, as continuous variables, and *Listeria* spp. and *L. monocytogenes*

prevalence. In this and all other GLMMs performed for this study, farm was included as a random effect and the outcome was the prevalence of *Listeria* spp. or *L. monocytogenes*. All factors that were significantly associated with the isolation of *Listeria* spp. or *L. monocytogenes* were tested for correlation with all other factors that were found to be significant by univariable analysis.

A multivariable GLMM was also developed de novo (i.e., not based on the rules reported by Strawn et al. (25)) to identify the most important land cover factors associated with *Listeria* spp. and *L. monocytogenes* isolation from drag swab samples. Factors that were not correlated and were significant by univariable analysis were considered candidate factors for inclusion in the multivariable model.

Predictive models, based on the GLMMs for *L. monocytogenes*, were then applied in a GIS platform to generate predictive maps of *L. monocytogenes* prevalence at the sub-field level to compare with the map that was developed using the CART model (Figure 1). Predictive risk maps were developed by inputting the univariable and multivariable GLMMs into ArcGIS. The Homer C. Thompson Vegetable Research Farm at Cornell University was used to develop these maps to ensure confidentiality of the commercial growers enrolled in our study.

Results

The overall prevalence of *Listeria* spp. and *L. monocytogenes* for field drag swabs collected from NYS produce farms was 20% and 12%, respectively. Overall, *Listeria* spp. (including *L. monocytogenes*) was isolated from 20% (208/1056) of samples. *L. monocytogenes* was isolated from 12% (128/1056) of samples, *L. innocua* was isolated from 4.0% (42/1056) of samples, *L. seeligeri* was isolated from

2.0% (21/1056) of samples, and *L. welshimeri* was isolated from 1.6% (17/1056) of samples.

Overall, the prevalence of *Listeria* spp. was greater for all field areas with a high predicted prevalence of *L. monocytogenes* isolation compared to field areas with a low predicted prevalence (Table 1; Figure 2). For example, *Listeria* spp. prevalence was 26% (51/195) in samples collected from areas with a high predicted prevalence according to the Water Rule, and 18% (157/861) in samples collected from areas with a low predicted prevalence according to the Water Rule (Table 1; Figure 2).

The prevalence of *L. monocytogenes* was greater for all field areas with a high predicted prevalence of *L. monocytogenes* isolation compared to the field areas with a low predicted prevalence according to the Water, Pasture and AWS Rules (Table 1; Figure 3). For example, *L. monocytogenes* prevalence was 22% (43/195) in samples collected from areas with a high predicted prevalence according to the Water Rule, and 10% (85/861) in samples collected from areas with a low predicted prevalence according to the Water Rule (Table 1; Figure 3).

Table 2.1: Frequency and prevalence of Listeria species-positive and L. monocytogenes-positive samples for farm fields that had either a high or a low predicted risk of L. monocytogenes isolation based on land cover factors

Rule	Description by predicted prevalence (No. of Samples = 1,056)		No. of samples positive for (prevalence [%]):			
	High	Low	*Listeria* spp. (No. of Positive Samples = 208)[a]		*L. monocytogenes* (No. of Positive Samples = 128)	
			High predicted risk	Low predicted risk	High predicted risk	Low predicted risk
Water	≤37.5 m from water (195)	>37.5 m from water (861)	51 (26)	157 (18)	43 (22)	85 (10)
Road	≤9 m from roads (168)	>9 m from roads (693)	36 (21)	121 (17)	11 (7)	74 (11)
AWS[b]	>4.2 cm^3 AWS (106)	≤4.2 cm^3 AWS (587)	23 (22)	98 (17)	20 (19)	54 (9)
Pasture	≤62.5 m from pasture (49)	>62.5 m from pasture (57)	12 (24)	11 (19)	11 (22)	9 (15)

[a] *Listeria* spp. include *L. monocytogenes.*
[b] AWS, available water storage.

Figure 2.2: Frequency and prevalence of positive Listeria species samples for farm fields that had either a high or a low predicted prevalence of L. monocytogenes isolation based on a hierarchical predictive risk model.

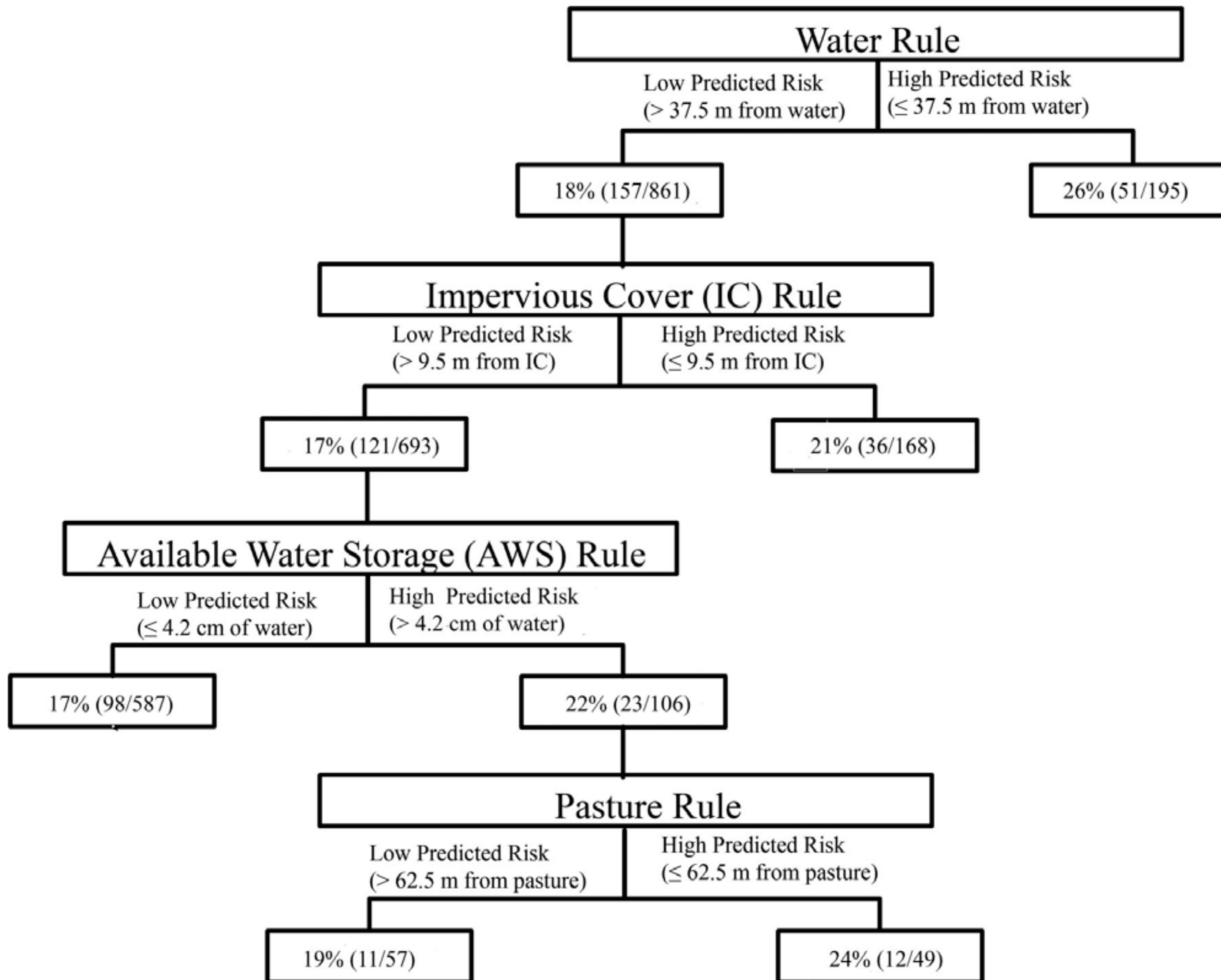
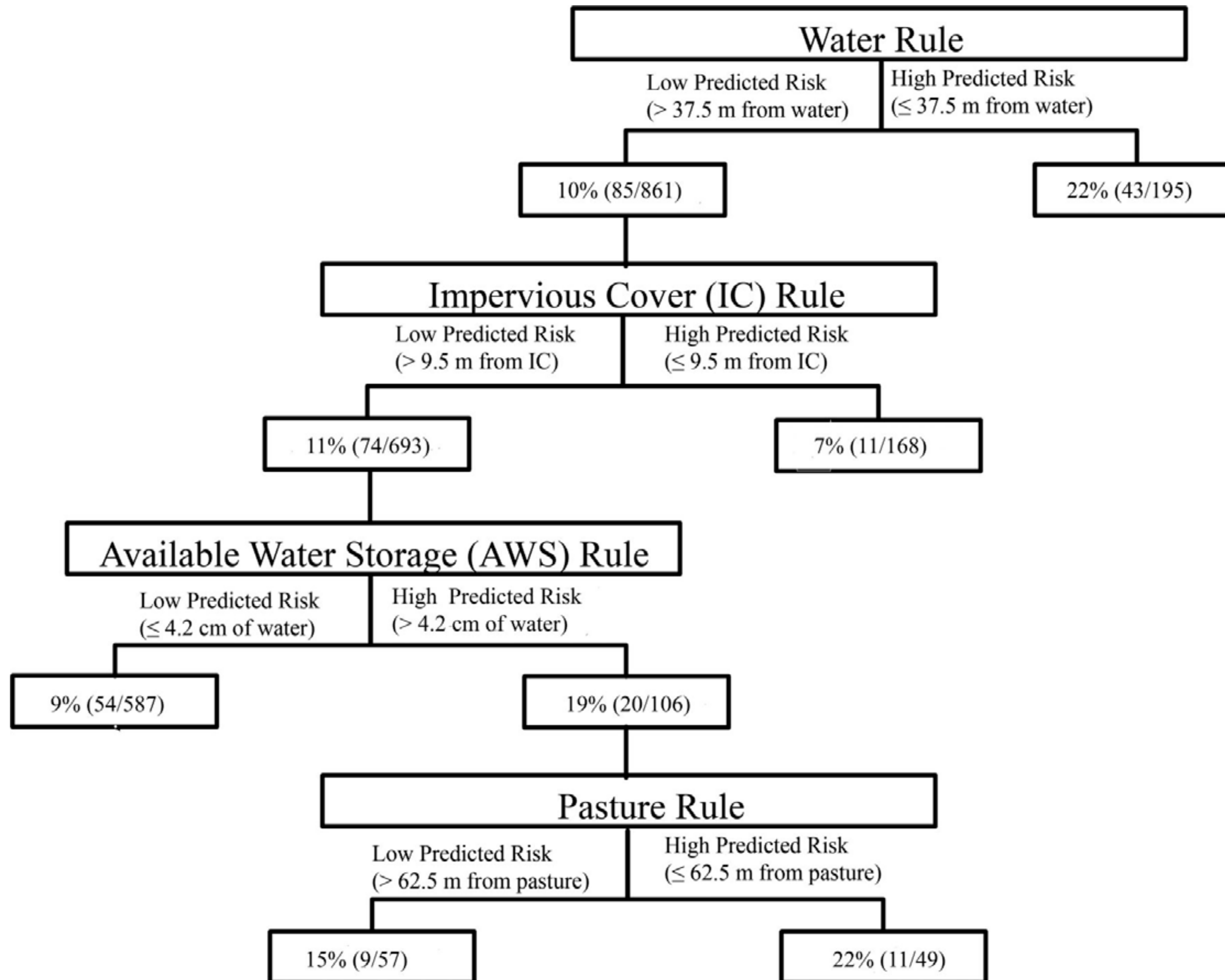

Water Rule
Low Predicted Risk
(> 37.5 m from water)
High Predicted Risk
(≤ 37.5 m from water)
18% (157/861)
26% (51/195)
Impervious Cover (IC) Rule
Low Predicted Risk
(> 9.5 m from IC)
High Predicted Risk
(≤ 9.5 m from IC)
17% (121/693)
21% (36/168)
Available Water Storage (AWS) Rule
Low Predicted Risk
(≤ 4.2 cm of water)
High Predicted Risk
(> 4.2 cm of water)
17% (98/587)
22% (23/106)
Pasture Rule
Low Predicted Risk
(> 62.5 m from pasture)
High Predicted Risk
(≤ 62.5 m from pasture)
19% (11/57)
24% (12/49)

Figure 2.3: Frequency and prevalence of positive L. monocytogenes samples for farm fields that had either a high or a low predicted prevalence of L. monocytogenes isolation based on a hierarchical predictive risk model.


Water Rule
Low Predicted Risk
(> 37.5 m from water)
High Predicted Risk
(≤ 37.5 m from water)
10% (85/861)
22% (43/195)
Impervious Cover (IC) Rule
Low Predicted Risk
(> 9.5 m from IC)
High Predicted Risk
(≤ 9.5 m from IC)
11% (74/693)
7% (11/168)
Available Water Storage (AWS) Rule
Low Predicted Risk
(≤ 4.2 cm of water)
High Predicted Risk
(> 4.2 cm of water)
9% (54/587)
19% (20/106)
Pasture Rule
Low Predicted Risk
(> 62.5 m from pasture)
High Predicted Risk
(≤ 62.5 m from pasture)
15% (9/57)
22% (11/49)

Rules based on surface water and pasture proximity accurately predict *L. monocytogenes* prevalence in environmental samples collected from NYS produce production environments. Logistic regression was performed to test the ability of each rule to accurately predict *L. monocytogenes* prevalence in NYS produce production environments. Logistic regression analysis showed that only the Water and Pasture Rules accurately predicted the prevalence of *L. monocytogenes* in NYS produce production environments (Table 2). Samples collected from field areas that had a high predicted prevalence of *L. monocytogenes* isolation by the Water Rule had an increased odds of *L. monocytogenes* isolation (OR = 3.0; 95% CI= 2.0, 4.6), compared to samples collected from field areas that had a low predicted prevalence. Samples collected from field areas that had a high predicted prevalence for *L. monocytogenes* by the Pasture Rule had an increased odds of *L. monocytogenes* isolation (OR = 2.9; 95% CI= 1.4, 6.0), compared to samples collected from field areas that had a low predicted prevalence.

While the outcome of the CART model was *L. monocytogenes* prevalence, the ability of the model to predict *Listeria* spp. prevalence was also validated because *Listeria* spp. is more common than *L. monocytogenes* and as a result, the findings based on *Listeria* spp. are more robust. Multivariable logistic regression showed that only the Water Rule was found to accurately predict the prevalence of *Listeria* spp. in NYS produce production environments (Table 2). Samples collected from field areas that had a high predicted *L. monocytogenes* prevalence by the Water Rule had an increased odds of *Listeria* spp. isolation (OR = 1.6; 95% CI= 1.1, 2.4), compared to samples collected from field areas that had a low predicted prevalence.

Table 2.2: Results of multivariable analyses built using backward regression (i.e., only factors with $P \leq 0.05$ were retained) that tested previously identified rules to accurately predict the effect of different binary land cover factors (e.g., either far away from or close to water) on the likelihood of Listeria species and L. monocytogenes isolation.

Species by rule	Odds ratio for *Listeria* species or *L. monocytogenes* detection	95% CI[a]	*P* value
Listeria spp.[b]			
Water[c]	1.6	1.1, 2.4	0.008
L. monocytogenes			
Pasture[d]	2.9	1.4, 6.0	0.005
Water[c]	3.0	2.0, 4.6	<0.001

[a] CI, confidence interval.
[b] *Listeria* spp. include *L. monocytogenes.*
[c] The water rule predicts a high prevalence of *L. monocytogenes* for areas within 37.5 m of surface water and a low prevalence for areas >37.5 m from surface water.
[d] The pasture rule predicts a high prevalence of *L. monocytogenes* for areas within 62.5 m of pasture and a low prevalence for areas >62.5 m from surface water.

Proximity to wetlands and scrublands were associated with an altered likelihood of *L. monocytogenes* isolation from produce production environments in NYS. As the multivariable model used to validate the CART model (25) only contained four factors, GLMMs were developed to identify additional land cover factors that were associated with the isolation of *L. monocytogenes* from NYS produce production environments. Of the nine land cover factors that were evaluated, six features (i.e., proximity to forest, grasslands, pasture, scrublands, water and wetlands) were significantly associated with *L. monocytogenes*-positive samples by univariable analysis (Table 3). For example, for a 100 m increase in the distance of a sampling site from forests, the likelihood of *L. monocytogenes* isolation decreased by 14% (OR =

0.86; 95% CI= 0.74, 1.0). Similarly, for a 100 m increase in the distance of a sampling site from surface water, the likelihood of *L. monocytogenes* isolation decreased by 23% (OR = 0.77; 95% CI= 0.66, 0.90; Figure 4).

Table 2.3: Results of univariable analyses that tested the effect of different land cover factors, treated as continuous variables, on the likelihood of Listeria species and L. monocytogenes isolation.

Proximity by land cover factor	Odds ratio[a]	95% CI[b]	*P* value
Listeria spp.[c]			
Forest	0.84	0.74, 0.95	0.009
Pasture	0.92	0.83, 1.0	0.117
Scrubland	0.93	0.88, 0.99	0.044
Water	0.85	0.76, 0.95	0.005
Wetlands	0.93	0.86, 1.0	0.058
L. monocytogenes			
Forest	0.86	0.74, 1.0	0.060
Grassland	1.04	0.99, 1.1	0.104
Pasture	0.92	0.81, 1.0	0.148
Scrubland	0.88	0.81, 0.95	0.002
Water	0.77	0.66, 0.90	0.001
Wetlands	0.92	0.84, 1.0	0.088

[a] For a 100-m increase in the distance of a given sampling point from the given land cover factors.
[b] CI, confidence interval.
[c] *Listeria* spp. include *L. monocytogenes.*

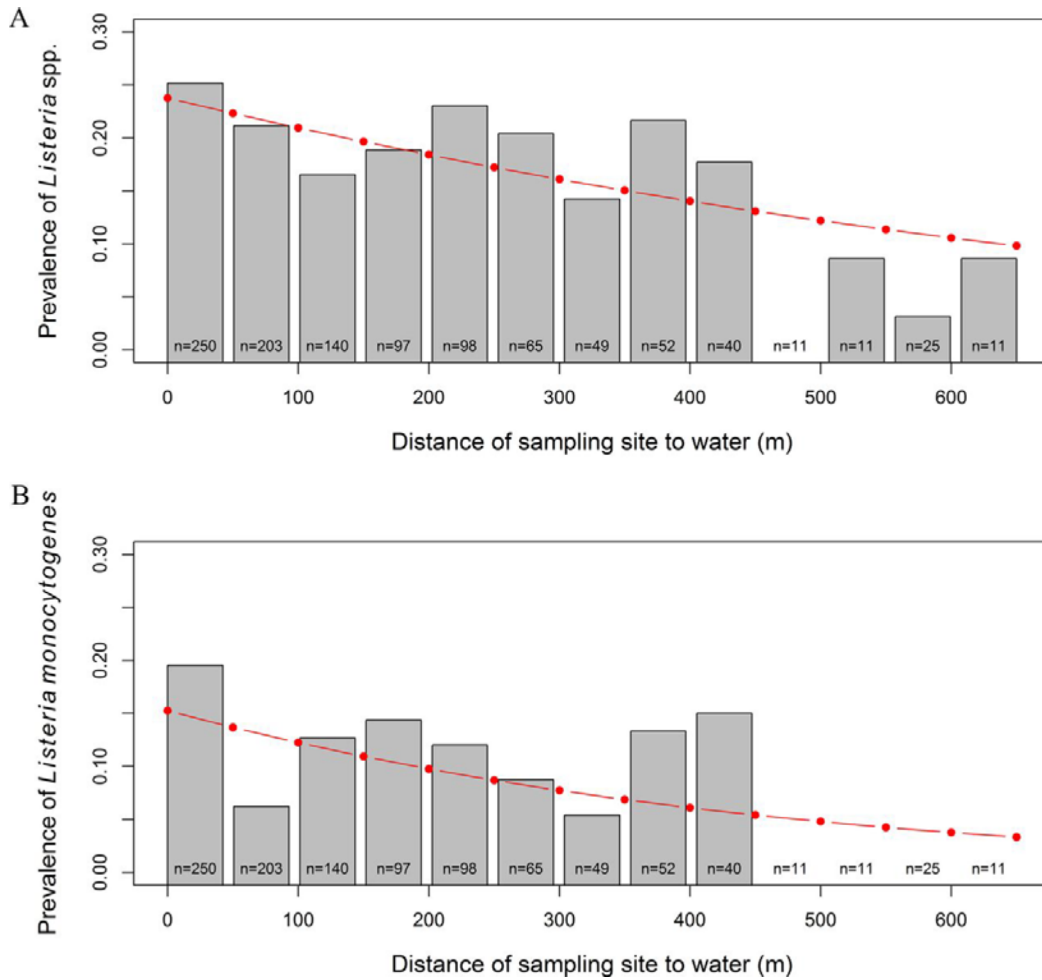


Figure 2.4: True prevalence (bars) and predicted prevalence of Listeria species-positive samples (A) and L. monocytogenes-positive samples (B) (line) based on mixed models that included proximity to water as a risk factor. True prevalence was calculated for 50-m bins (e.g., all samples that were between 0 and 50 m from water went into the first bin); the sample size for each bin is noted at the bottom of each column. Among five samples collected >650 m away from water, two were Listeria species positive and none were L. monocytogenes positive. Prevalence is reported as a decimal.

To identify the most important land cover factors associated with *L. monocytogenes* isolation from produce production environments, a multivariable GLMM was developed. The six factors that were found to be significant by univariable analysis were included as candidate factors. In the final GLMM only three land cover features were retained (Supplemental Table 1); and no significant interactions (i.e., $P < 0.05$) were observed between any variables in the model. For a 100 m increase in the distance of a sampling site from forests, the likelihood of *L. monocytogenes* isolation decreased by 13% (OR = 0.87; 95% CI= 0.76, 0.99). For a 100 m increase in the distance of a sampling site from scrubland, the likelihood of *L. monocytogenes* isolation decreased by 6% (OR = 0.94; 95% CI= 0.88, 1.0). Lastly, for a 100 m increase in the distance of a sampling site from water, the likelihood of *L. monocytogenes* isolation decreased by 15% (OR = 0.85; 95% CI= 0.76, 0.95).

Predictive risk maps (Figure 5) were then developed using the univariable and multivariable GLMMs for *L. monocytogenes* described above (Table 3; Supplemental Table 1). The maps were developed to allow for comparisons with the map based on the CART model (Figure 1), and as a proof of a concept to assess if the multivariable GLMM for *L. monocytogenes* could be used to predict *L. monocytogenes* prevalence at the sub-field level. This map shows that multivariable GLMM can be used to generate a map of *L. monocytogenes* prevalence, and that said map is at a finer scale compared to maps based on CART analyses.

Figure 2.5: Map of predicted prevalence of L. monocytogenes for the Homer C. Thompson Vegetable Research Farm at Cornell University based on the results of (i) univariable generalized linear mixed models in which proximities to scrubland (A), water (B), and wetlands (C) were included as risk factors and (ii) a multivariable generalized linear mixed model in which proximities to scrubland, water, and wetlands were included as risk factors (D). Note that this map is not based on any of the farms, included in this study for confidentiality reasons. Maps were created using ArcGIS software, and base maps are from ArcGIS (ESRI [all rights reserved]).

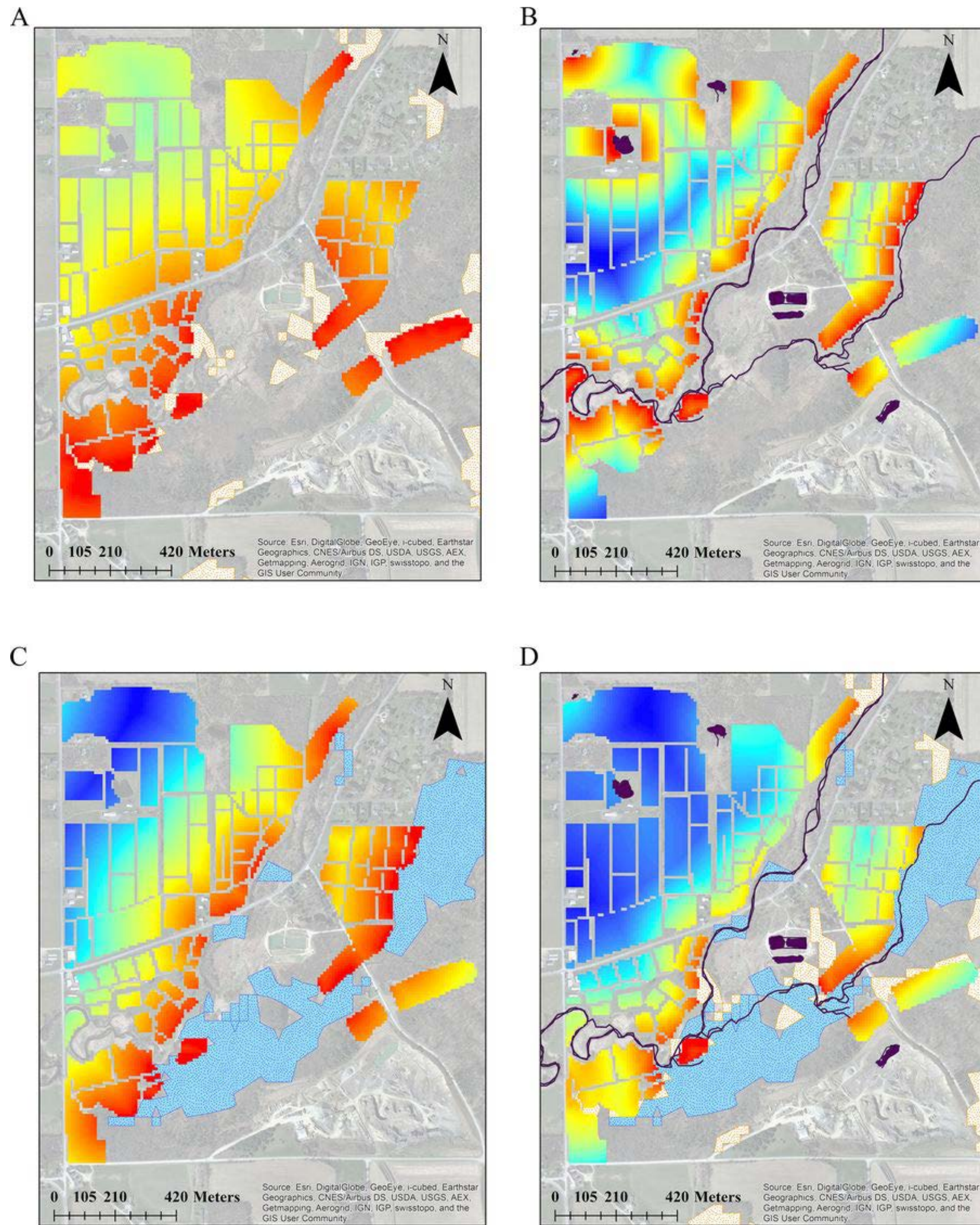
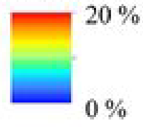


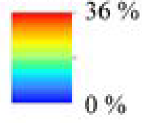
Figure 2.5: Continued

Predicted Prevalence of *Listeria monocytogenes*

Scale for Figures 4A-C



Scale for Figure 4D



Proximity to forests and scrublands were associated with an increased likelihood of *Listeria* spp. isolation from produce production environments in NYS. Similar to *L. monocytogenes*, GLMMs were also developed to identify additional land cover factors that were associated with the isolation of *Listeria* spp. from NYS produce production environments. Of the nine land cover factors that were evaluated, five features (i.e., proximity to forest, pasture, scrublands, water and wetlands) were significantly associated with *Listeria* spp.-positive samples by univariable analysis (Table 3). For example, for a 100 m increase in the distance of a sampling site from forests, the likelihood of *Listeria* spp. isolation decreased by 16% (OR = 0.84; 95% CI= 0.74, 0.95). Similarly, for a 100 m increase in the distance of a sampling site from surface water, the likelihood of *Listeria* spp. isolation decreased by 15% (OR = 0.85; 95% CI= 0.76, 0.95; Figure 4). No strong correlations (i.e., correlation coefficient less than 0.5) were observed between any of the significant factors by univariable analysis.

To identify the most important land cover factors associated with *Listeria* spp. isolation from produce production environments, a multivariable GLMM was developed. The five factors that were found to be significant by univariable analysis were included as candidate factors. In the final GLMM only three land cover factors were retained (Supplemental Table 1) and no significant interactions were observed between variables in the final model. For a 100 m increase in the distance of a sampling site from scrubland, the likelihood of *Listeria* spp. isolation decreased by 14% (OR = 0.86; 95% CI= 0.79, 0.93). For a 100 m increase in the distance of a sampling site from water, the likelihood of *Listeria* spp. isolation decreased by 24% (OR = 0.76; 95% CI= 0.65, 0.89). Lastly, for a 100 m increase in the distance of a

sampling site from wetlands, the likelihood of *Listeria* spp. isolation decreased by 9% (OR = 0.91; 95% CI= 0.83, 0.99).

Discussion

The primary objectives of this study were (i) to validate previously developed geospatial rules that predicted areas of significantly higher or lower prevalence of *L. monocytogenes*, and (ii) to identify additional land cover factors that may be associated with an increased or decreased likelihood of *L. monocytogenes* isolation in produce production environments. Our study validated two of the four rules (i.e., the Water and Pasture Rules) that comprised the CART model (25). Additionally, among land cover factors that were not included in the original CART model, but tested here, proximity to scrubland, and wetlands, were found to be significantly associated with an increased likelihood of *L. monocytogenes* isolation. These findings suggest that on-farm produce safety is complicated by the ecological context unique to each field, as well as the scale (e.g., farm, field, and sub-field levels) at which prevalence is assessed. Thus, it is essential to have tools that allow growers to account for both ecological context and scale when developing on-farm produce safety plans. The validation of the Water and Pasture Rules in this study demonstrates the application of geospatial models for prospective, and accurate prediction of pathogen prevalence on produce farms, suggesting that GIS is a promising tool for food safety.

Geospatial models have the ability to accurately predict the likelihood of *L. monocytogenes* isolation from produce production environments. In this study, proximity to surface water and pasture were significantly associated with *L. monocytogenes* isolation from produce production environments by logistic regression.

These findings validated two of the four rules from the CART model adapted from Strawn et al. (25). These findings were also consistent with other studies conducted on *L. monocytogenes* in NYS agricultural environments (22, 23, 26), as well as studies conducted on *L. monocytogenes* and other foodborne pathogens in agricultural and non-agricultural environments (19, 47–50). For example, in a Canadian study, Lyautey et al. (47) found that proximity to dairy operations was one of the most important predictors of *L. monocytogenes*-positive surface water samples. The repeated identification of an association between *L. monocytogenes* isolation, and proximity to water, pasture and other livestock associated areas, suggests that our findings are translatable to other farms in NYS. In our study reported here proximity to water and pasture were significantly associated with *L. monocytogenes* isolation by GLMM as well as logistic regression, further supporting the robustness of this association. By validating two of the rules adapted from the CART model, our study demonstrates that geospatial models can be used to accurately, and prospectively predict the prevalence of *L. monocytogenes* in produce production environments.

Interestingly while our findings were generally consistent with the previously reported CART model (25), neither the AWS nor the Impervious Cover Rules were validated by our findings. This may be the result of small differences in sampling protocols between Strawn et al. (25) and the study reported here. Strawn et al. (25) used drag swab, composite soil, fecal and water samples in their analyses, while in the study reported here only drag swab samples were collected. As each sample type likely represents a unique *L. monocytogenes* population from a distinct ecological niche (e.g., water versus soil), it seems plausible that different factors would be associated with the

isolation of *L. monocytogenes* in each study. Therefore, the fact that the AWS and Impervious Cover Rules were not validated may indicate that these rules are associated with *L. monocytogenes* isolation from one of the sample types that were collected by Strawn et al. (25) but not in the study here (e.g., water samples). Future studies that investigate geospatial factors associated with contamination risk for actual produce (i.e., not environmental samples) are thus needed to increase the accuracy of predictive models and allow growers to maximize surveillance efforts. However, these studies will require considerably larger samples sizes, as pathogen prevalence on produce tends to be significantly lower than in environmental samples (22). Also, in the study reported here more samples were collected from areas with low predicted risk as compared to areas with high predicted risk; this was due to the fact that samples were collected in commercial settings. Future studies should aim to collect comparable sample sizes from high and low risk areas as well.

Identification of additional factors (e.g., proximity to wetlands) that were not included in the original CART model, but were found to be associated with the prevalence of *L. monocytogenes* in produce production environments, may aid in the refinement of prediction models. Importantly, these same factors have also been identified as risk factors for *Listeria* and *L. monocytogenes* contamination in past studies of natural (26) and agricultural environments (23, 26). However, while the study reported here did not find any significant interactions between the different landscape factors studied, a previous report did find that interactions between landscape and meteorological factors significantly affected the probability of isolating *Listeria* spp. from soil, vegetation and water (24). Similarly, previous studies (9, 11,

19, 49, 51–53) found that management practices were significantly associated with the likelihood of isolating *L. monocytogenes* from on-farm environments. Management practices and meteorological factors, which were not considered in the study reported here, may thus affect the relationships between *L. monocytogenes* prevalence and landscape factors. Further improvement of geospatial models may therefore be achieved by integration of additional environmental (both landscape and meteorological) and management practice data. While development of such models would require larger datasets, these models could account for temporal (e.g., changes in management practices or meteorological factors over time), as well as spatial variation, and would thus facilitate identification of additional risk factors as well as additional control strategies.

Issues of scale need to be considered when developing and validating geospatial models for pre-harvest produce safety assessment. Despite the fact that the Pasture Rule was validated by logistic regression, proximity to pasture was not retained in the final multivariable GLMM. This difference may be a function of scale, which is defined by the resolution (i.e., grain) and extent of the available spatial data. Numerous studies (54–62) have found that changing study scale, changes the strength of associations and interactions. For example, in a study on habitat use by *Eleodes hispilabris*, McIntyre (62) found that *E. hispilabris* avoided shrubs at small scales, but selectively occupied shrubland at larger scales which may be due to different mechanisms influencing habitat selection at the different scales. Thus, studies that look at similar outcomes (e.g., *L. monocytogenes* prevalence) at different scales (e.g., field and sub-field level) may identify different predictor variables. The issue of scale is

complicated by the grain and accuracy of the remotely sensed data available, particularly if the scale of the input data differs from the model's scale (63). For example, while the 2006 NLCD has a national accuracy of 78% (64), the odds of misclassification increases as landscape heterogeneity increases (65). Therefore, in highly mosaic environments, such as produce farms, NLCD accuracy is lower. This may also explain why proximity to pasture was not retained in the final GLMM, particularly since misclassification of grass-dominated landscapes, such as pasture, accounted for 26% of all inaccuracies (64). It is therefore important that researchers are cognizant of the limitations associated with the use of remotely sensed data to develop geospatial predictive risk models. On the other hand, these limitations can be minimized by carefully designing studies, and using appropriate analyses that account for scale (54, 63, 66). In addition, improved data collection strategies (e.g., using drones) could be used to address these issues in the future. Despite differences in study scale, it is important to note that proximity to pasture was significantly associated with *L. monocytogenes* prevalence by univariable GLMM, which does support the validation of the Pasture Rule by logistic regression.

Ecological and food safety implications of edge interactions on farm landscapes. In the present work, edge interactions between produce farms and four other land cover types (i.e., forest, scrubland, water and wetland) were observed. The elevated prevalence of *L. monocytogenes* in ecotones (i.e., the transitional area where two ecological communities meet) is consistent with patterns observed in other disease systems (e.g. Lyme disease; 67–70). This is also consistent with our current understanding of infectious disease emergence; infectious diseases frequently arise at

the interface between human habitats and other ecosystems (67–71). Ecotones are most abundant in fragmented landscapes, and their presence intensifies ecological processes. For example, ecotones are often more diverse than surrounding communities (69, 72, 73), and provide ideal habitat for "edge species" (e.g., ticks and rodents; 69). Additionally, ecotones, and the associated habitat fragmentation, affects the nature and rate of species interaction (e.g., intensifying competition; 69, 74). In this context, our results suggest that food grown within short distances of ecotones, specifically the boundaries between farm fields, and forests, water, scrublands or wetlands are at an increased risk of *L. monocytogenes* contamination. Thus, risk management plans need to consider the potential for increased pre-harvest food safety risks associated with produce grown in or near ecotones. For example, growers could create buffer zones of unharvested product near the edges of fields, increase surveillance and/or decontamination of produce grown near field edges, or stage harvesting and processing so that higher risk material (i.e., produce grown near field edges) are harvested and processed last. These concerns are particularly pertinent for small farms who have a larger ratio of ecotone to field area; thus future studies should account for farm size when developing and validating on-farm intervention strategies

Predictive risk maps based on GIS-enabled models allow for the visualization of pre-harvest food safety risk at multiple scales. The CART model predicted prevalence at the field-level, while the GLMMs developed in the study reported here, predicted *L. monocytogenes* prevalence at every point within a field (i.e., at the sub-field level). Thus, the CART model generated a map of discrete areas of high and low predicted prevalence (Figure 1), while the GLMMs produced a risk

gradient map (Figure 5). As previously mentioned, different mechanisms drive ecological processes at different scales, so factors that are significantly associated with *L. monocytogenes* isolation at the field and sub-field levels may differ. Therefore, the model and predictive map that are most appropriate for use by the grower depend on the scale of their risk management plan (i.e., farm, field, or sub-field level). In general, maps based on the GLMM are more appropriate as those maps offer greater resolution, which allows for development of more targeted mitigation strategies, compared to maps based on CART models. However, the ability to develop both map types demonstrates the flexibility of geospatial tools, and the utility of GIS for visualizing the output of different model types. Overall, GIS offers a unique opportunity to look at variation across scales, and to account for cross-scale differences in predictive models by allowing for the integration and visualization of remotely sensed and field collected-data.

Conclusion

This study yielded quantitative data that showed that *L. monocytogenes* contamination on produce farms is dependent on the specific ecological context of a produce farm, and that geospatial, predictive risk maps can be used to accurately, and prospectively predict *L. monocytogenes* prevalence for NYS produce production environments. Additionally, other land cover factors were identified that should be examined in future studies to develop higher resolution models. The implementation of geospatial predictive models by the produce industry may increase the understanding of risk factors that promote foodborne pathogen prevalence and persistence in produce fields, and will assist growers in focusing their food safety efforts. Geospatial models

allow for the development of individualized preventive measures on produce farms, as they enable growers to proactively assess and address environmental factors that may increase the risk of contamination events on their specific farms. For example, predictive risk maps can identify areas of high predicted pathogen prevalence within farms, and enable growers to make more informed decisions about the management of crops in these areas including targeted pathogen surveillance programs and altered management practices. Thus, geospatial predictive risk models and maps have a promising future in pre-harvest food safety as they can be applied to any location and utilize a farms' unique combination of landscape characteristics (e.g., proximity to domestic animal operations), soil properties (e.g., available water storage), and climate (e.g., precipitation) in the prediction process.

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Supplemental Material

Supplemental material associated with this article can be found online at: http://aem.asm.org/content/82/3/797/suppl/DCSupplemental.

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CHAPTER 3

SPATIAL AND TEMPORAL FACTORS ASSOCIATED WITH AN INCREASED PREVALENCE OF *L. MONOCYTOGENES* IN SPINACH FIELDS IN NEW YORK STATE

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Abstract

While rain and irrigation events have been associated with an increased prevalence of foodborne pathogens in produce production environments, quantitative data are needed to determine the effects of various spatial and temporal factors on the risk of produce contamination following these events. This study was performed to quantify these effects and to determine the impact of rain and irrigation events on *Listeria* species (including *L. monocytogenes*) and *L. monocytogenes* detection frequency and diversity in produce fields. Two spinach fields, with a high and low predicted risk of *L. monocytogenes* isolation, were sampled 24, 48, 72 and 144-192 h following irrigation and rain events. Predicted risk was a function of a field's proximity to water and roads. Factors were evaluated for their association with *Listeria* spp. and *L. monocytogenes* isolation using generalized linear mixed models (GLMM). In total, 1,492 (1,092 soil, 334 leaf, 14 fecal and 52 water) samples were collected. According to GLMM, the likelihood of *Listeria* spp. and *L. monocytogenes* isolation from soil samples was highest during the 24 h immediately following an event (OR = 7.7 and OR = 25, respectively). Additionally, *Listeria* spp. and *L.*

monocytogenes isolates associated with irrigation events showed significantly lower *sigB* allelic type diversity (as determined by Shannon-Weiner Index) than isolates associated with precipitation events ($P = < 0.001$; T-Hutcheson test), suggesting that irrigation water may be a point source for *L. monocytogenes* contamination. Small changes in management practices (e.g., not irrigating before harvest) may therefore reduce the risk of *L. monocytogenes* contamination of fresh produce.

Introduction

Foodborne outbreaks have been increasingly linked to fresh produce in the United States (US; 1–6). In fact, the proportion of foodborne outbreaks that were attributed to produce between 1998 and 2008, 46%, was over twice that attributed to meat, 22% (6). Similarly, between 2002 and 2011 produce-associated outbreaks caused, on average, more illnesses per outbreak than any other food (1). As a result, the safety of fresh produce has come into question, negatively affecting produce growers, the food industry, and local economies (7, 8). For example, as a consequence of a 2011 listeriosis outbreak linked to fresh cantaloupes in the US (9), cantaloupe consumption dropped nationwide by 53% (10). The instability of the cantaloupe market following the 2011 outbreak is indicative of a larger trend of wide scale consumer avoidance of products associated with outbreaks, even when the outbreak is associated with point source events (7, 8). Thus, prevention of produce-associated outbreaks is of key concern for the produce industry. Although most listeriosis outbreaks associated with fresh produce are traced back to processing environments, the prevention of produce contamination in production environments is crucial. In fact, past studies have shown that low-level sporadic contamination of produce in production environments can result in pathogen proliferation and widespread contamination throughout the supply chain (11–13). In order to minimize pre-harvest produce contamination, it is necessary to understand how different spatial (e.g., proximity to water, roads) and temporal factors (e.g., time since irrigation) affect the likelihood of a contamination event in production environments.

Numerous studies have examined the relationship between environmental factors and the prevalence of *L. monocytogenes* (14–18) and *L. monocytogenes* surrogates (e.g., *Listeria* spp.; 19, 20) in produce production environments. Many of these studies (14–16) determined that water-related factors were significantly associated with the isolation of *L. monocytogenes* from environmental samples. Similar studies conducted in non-agricultural environments also found similar results (16, 21, 22). For example, Ivanek et al. (21) found that the isolation of *Listeria* spp. from samples collected in forested environments was positively associated with rainfall. Additionally, Strawn et al. (15) developed a geospatial algorithm, which included several water-related factors (e.g., available water storage (AWS) and proximity to water), to predict *L. monocytogenes* prevalence in NYS pre-harvest environments. Strawn et al.'s (15) findings also suggest that not all fields are at equal risk of pathogen contamination. Therefore, to identify and develop effective mitigation strategies to reduce the risk of on-farm produce contamination, it is essential to understand how contamination risk differs within and between fields due to variation in spatial (e.g., proximity to water) and temporal (e.g., time since a rain event) factors.

Past studies have also found that management practices affect the risk of contamination by *L. monocytogenes* (14, 23–28) and *L. monocytogenes'* surrogates (19, 20). For example, irrigation has repeatedly been associated with an increased risk of pre-harvest produce contamination by *L. monocytogenes* (14, 15, 26) and other foodborne pathogens (29–31). In fact, two studies (14, 25) have found that irrigation was one of the most important risk factors associated with *L. monocytogenes* isolation from samples collected in pre-harvest environments; both studies collected samples

from multiple farms growing a variety of crops. Moreover, contaminated irrigation water has been identified or suspected as the source of contamination for several produce-associated *Escherichia coli* and *Salmonella* outbreaks (32–36). Despite the repeated identification of irrigation as a risk factor for pre-harvest produce contamination, no study, to the authors' knowledge, has reported, quantitatively, the impact of irrigation over time (i.e., over subsequent 24 h periods following an irrigation event) on the risk of produce contamination in production environments.

Therefore, the purpose of this study was (i) to quantify the effects of various spatial (e.g., proximity to water) and temporal (e.g., time since an irrigation event) factors on the risk of produce contamination after rain and irrigation events, and (ii) to determine the impact of rain and irrigation events on *Listeria* spp. and *L. monocytogenes* diversity in spinach fields. The ultimate goal of this research was to identify potential mitigation strategies that can reduce the risk of produce contamination at the pre-harvest level.

Materials and Methods

Study design. A longitudinal study was conducted in two spinach (Unipack 15-F1, Harris Seeds) fields at the Homer C. Thompson Vegetable Research Farm over a seven-week period in May, June, and July 2014. Two 0.2 ha fields (Figure 3.1) were selected based on their respective predicted prevalence of *L. monocytogenes* (i.e., one high and one low risk field), which was a function of the fields' proximity to water and roads (see header "Landscape Data and Determining Predicted Field Risk"). Fields were prepared for planting by harrowing, and treatment with a 13-13-13 fertilizer at a rate of 789 kg per hectare. The herbicide metalolchlor (DuPont,

Figure 3.1: Locations of the low- and high-risk fields and the surface water sampling sites included in this study.

Wilmington, Delaware) was applied at a rate of 0.58 L per hectare immediately following seeding. Irrigation water was drawn from Fall Creek (Figure 3.1).

Each field was divided into 21 13x13 m plots. Soil sample sites were randomly selected from within each plot using the ‘Create Random Points’ function in ArcGIS (version 10.2.2, Environmental Systems Research Institute, Redlands CA) for each sampling trip (i.e., the same location within each plot was only sampled once during the course of the study). New sampling sites were selected for each sampling trip to ensure (i) that a representative sample of *Listeria* spp. and *L. monocytogenes* was collected from each plot during the course of the study, and (ii) that there was sufficient variation in sample location to statistically determine the effect of spatially-specific factors (e.g., distance of a sampling site to water) on the likelihood of *Listeria* spp. and *L monocytogenes* isolation. Soil samples were collected on the day of planting, and 24, 48, 72, and 144-192 h after an “irrigation” or “rain” event. An irrigation event was defined as any time irrigation water was applied to the field. A rain event was defined as > 6 mm of rain over a 24 h time period (i.e., 9 am to 9 am). If multiple subsequent 24 h periods each received > 6 mm of rain, then the first sample collection (i.e., t = 24 h) was performed 24 h after the last 24 h time period with > 6 mm of rain (i.e., if it rained more than > 6 mm on Tuesday and Wednesday then the 24 h samples were collected Thursday). Two multi-day rain events lasting 48 h occurred during the course of the study. To account for the effect of a multi-day rain event on our results, the amount of rainfall 0, 1, 2, 3, 0-1, 0-2 and 0-3 days preceding sample collection were included as risk factors in the statistical analyses (see header

"Statistical Analysis" for more information). If a rain or irrigation event did not occur between 144-192 h after a rain event, a "dry" event sampling was performed. Sampling at a later time point (e.g., 192 h versus 144 h) was given preference, if forecasts clearly indicated absence of rainfall for more than 144 h. Each set of samples (i.e., 24, 48, 72 h and 144-192 h, if collected,) was defined as representing either an irrigation or rain event depending on which "event type" initiated sample collection. Overall, seven sets of samples were collected: (i) five sets that represented rain events including three sets where samples were collected 144-192 h after the event, (ii) two sets that represented irrigation events including one set where samples were collected 144-192 h after the event. Additionally, a set of samples was collected prior to seeding on the day of planting. Overall, each plot was sampled 26 times.

Water, leaf and fecal samples were also collected. Water samples were collected from Fall Creek (Figure 3.1), the water source used for irrigation. Fecal samples were collected when observed within 5 m of the sampled fields or Fall Creek. Fecal samples were not identified to species-level due to the high misclassification rate associated with visual identification of wildlife scat (37, 38). Composite leaf samples were collected for each plot once the spinach plants were large enough to survive harvesting (i.e., 36 d after planting). Composite leaf samples were hand collected by gathering leaves from 6-12 spinach plants growing along the perimeter and diagonals of each plot. Global positioning system (GPS) coordinates were recorded for each soil and water sample. In total, 1,092 soil, 52 water, 334 leaf and 14 fecal samples were collected (n=1,492 total).

Sample collection and preparation. Samples were collected and tested as previously described by Strawn et al. (15). Briefly, latex gloves (Nasco, Fort Atkinson, WI) were worn and changed for each sample collected. For each plot, a soil sample was collected from approximately 4 in (10.16 cm) below the soil surface by 5 mL sterile scoops (Fisher Scientific, Hampton, NH) and placed in a sterile Whirl-pak bag (Nasco, Fort Atkinson, WI). Twenty-five g of soil was then weighed into a separate sterile filter Whirl-pak bag. Water samples were collected directly into sterile jars using a sampling pole (Nasco) and processed according to the Environmental Protection Agency (EPA) standard methods (39). Briefly, a 250 mL water sample was passed through a 0.45 μm filter (Nalgene, Rochester, NY) and the filter was aseptically transferred to a sterile Whirl-Pak bag. Additionally, 10 g of each fecal sample and 25 g of each composite leaf sample were weighed out and aseptically transferred to separate sterile filter Whirl-pak bags. All samples were transported on ice and processed within 3 h of collection.

Bacterial enrichment and isolation. To enrich and isolate *Listeria* spp. and *L. monocytogenes,* samples were prepared as previously described by Strawn et al. (15). Briefly, each sample was diluted 1:10 with buffered *Listeria* enrichment broth (Becton Dickinson, Franklin Lakes, NJ) and incubated at 30°C for 24 h. After 4 h, *Listeria* selective enrichment supplement was added to each sample enrichment bag. At 24 and 48 h, 50 μl of each sample enrichment was plated onto *L. monocytogenes* plating medium agar (LMPM; Biosynth International, Itasca, IL) and Modified Oxford agar (MOX; Becton Dickinson). After incubation for 48 h at 35 (LMPM) and 30 (MOX) °C, up to four presumptive *Listeria* colonies were sub-streaked from LMPM and

MOX onto brain-heart infusion agar plates (BHI; Becton Dickinson). The BHI plates were then incubated at 37°C for 24 h. Presumptive *Listeria* colonies were confirmed by PCR amplification and sequencing of the partial *sigB* gene as previously described (40–42). Isolates were identified to allelic type (AT) by comparison of partial *sigB* sequences to an internal reference database (Food Safety Laboratory, Cornell University, Ithaca, NY; 40–42). The authors acknowledge that more discriminatory subtyping methods exist which are more translatable to other subtyping schemes commonly used (e.g., multi-locus sequence typing, and multi-locus genotyping); however, a previous study (43) showed that DNA-based subtyping methods, such as *sigB* AT, can efficiently differentiate between species of *Listeria.* More advanced subtyping schemes, such as whole genome sequencing, should be used in future studies to assess the relatedness of isolates across time and space.

Positive and negative controls were processed in parallel for each sample. *L. monocytogenes* FSL R3-001 (44) inoculated in BHI broth was used as the positive control and un-inoculated enrichment media was used as the negative control. All isolates were preserved at -80°C. Isolate information can be found at www.FoodMicrobeTracker.com and in Table S1.

Landscape data and determining predicted field risk. Landscape data (Table S2) were derived using ArcGIS as described by Weller et al. (14). Predicted risk was based on a geospatial algorithm previously described by Strawn et al. (15). Briefly, the GPS coordinates for each field and soil sampling site were imported into ArcMap using the Universal Transverse Mercator, North American Datum, 1983. Road and hydrologic data were downloaded from the Cornell University

Environmental Information Repository (cugir.mannlib.cornell.edu). Soil data were obtained from the Natural Resource Conservation Service Web Soil Survey (http://websoilsurvey.sc.egov.usda.gov/). Shapefiles for field edge and irrigation lines were created using the 'Create Features (Editor)' function. Data on the proximity of each sample collection point to field edge, irrigation lines, roads and surface water were derived using the 'Near (Analysis) ' function.

Based on the data and models described by Strawn et al. (15), a field was considered at high risk for *L. monocytogenes* if it was ≤ 37.5 m from water and ≤ 9.5 m from a road (15). A field was considered at low risk for *L. monocytogenes* if it was > 37.5 m from water and > 9.5 m from a road (15). The high risk field also had, on average, a higher AWS (i.e., an AWS for 0-100 cm below the soil surface of > 4.2 cm) compared to the low risk field. Soil in the high risk field was Eel silt loam, and for 0-100 cm below the soil surface was, on average, 60% sand, 30% silt, 11% clay and 2% organic matter; these values are based on representative values for several soil layers and a large area space (30 m^2) and therefore do not add up to 100%. Soil in the low risk field was Howard gravelly, loam, and for 0-100 cm below the soil surface was, on average, 47% silt, 39% sand, 13% clay, and 1% organic matter content. Both fields were level (i.e., slope < 5%). Lastly, spinach, and a clover-rye cover crop were planted in the high risk field in 2013 and 2012, respectively, while cucurbits and broccoli were planted in the low risk field in 2013 and 2012, respectively.

Meteorological data. Meteorological data (Table S2) were obtained from the Cornell University weather station located at the Homer C. Thompson Vegetable Research Farm (Rainwise Inc., Trenton, NJ). Data on leaf wetness were obtained from

the Cornell University Network for the Environment and Weather Applications (Network for Environment and Weather Applications, Cornell University, Ithaca, NY). Data were downloaded for each sample collection date and the three preceding 24 h periods (i.e., 9 am to 9 am). Average values for each factor for 0 to 1, 0 to 2, and 0 to 3 d before sample collection were also calculated. For a description of all meteorological factors included in this study see Table S2.

Statistical analysis. All statistical analyses were performed in R (version 3.1, R Core Team, Vienna, Austria). Prevalence was calculated for each field (high or low risk), time period (24, 48, 72 and 144-192 h), event type (rain versus irrigation event) and sample type (leaf, soil and water). The total number of ATs (i.e., allelic type richness) for *Listeria* spp. and *L. monocytogenes* was determined and the Shannon-Wiener index was calculated. A T-Hutcheson test (45) was performed to compare the Shannon-Wiener indices for the high risk and low risk fields, and for irrigation and rain events.

Univariable analyses were performed to determine the effect of spatial and meteorological factors, time since event, predicted field risk, and event type (i.e., irrigation versus rain event) on the odds of *Listeria* spp. and *L. monocytogenes* isolation. Correlation between significant factors (at $P \leq 0.20$) was assessed using the corrplot package (version 0.73, http://cran.r-project.org/web/packages/corrplot). Principal component analysis (PCA) was performed on each set of meteorological factors (e.g., all humidity factors), with the exception of rainfall. PCA was only performed if the factors were significant by univariable analysis and correlated, and if combination was biologically plausible. The first eigenvector from each PCA was

added to the dataset as a potential covariate for inclusion in the final model. Factors that were identified as significant by univariable analysis but not included in a PCA were included as potential covariates in the final model, as well.

Generalized linear mixed models (GLMM; Bates, Maechler, Bolker and Walker, submitted for publication) were developed using the logit link function. The outcome was the presence or absence of *Listeria* spp. or *L. monocytogenes*. Event type, hours and either predicted field risk, or proximity to water and road were included as fixed effects. Set and plot were included as random effects. The model was built using a backwards selection method (i.e., factors were removed from the model until only factors significant at $P \leq 0.05$ remained).

Spatial analysis. Model residuals were obtained for each GLMM, and a residual variogram was created to determine if there were spatial dependencies in the data that were not accounted for by the multivariable model (46).

Results

Prevalence and diversity of *L. monocytogenes,* and *Listeria* spp. in produce production environments. The overall prevalence of *Listeria* spp. was 14% (204/1492). The prevalence of *Listeria* spp. was higher in water samples, 90% (47/52) and fecal samples, 79% (11/14), compared to soil samples, 12% (126/1092) and leaf samples, 6% (19/334; Table 3.1). The prevalence of *Listeria* spp. was higher in soil samples collected from the high risk field, 15% (84/546), compared to the low risk field, 8% (42/546; Table 3.2; Figure 3.2). The prevalence of *Listeria spp.* was higher in soil samples collected 24 h after irrigation and rain events, 23% (68/294), compared to soil

Table 3.1: Effect of sample type on the frequency and prevalence of Listeria spp. and L. monocytogenes isolates from soil samples collected from spinach fields previously identified as being at high or low risk for L. monocytogenes isolation.

	No. (%) of samples positive for:	
Sampling site and sample type (no. of samples)	*Listeria* spp.[a]	*L. monocytogenes*
High-risk field (726)	109 (15)	73 (10)
Fecal (13)	11 (85)	9 (69)
Leaf (167)	14 (8)	2 (1)
Soil (546)	84 (15)	62 (11)
Low-risk field (714)	48 (7)	24 (3)
Fecal (1)	0 (0)	0 (0)
Leaf (167)	5 (3)	0 (0)
Soil (546)	43 (8)	24 (4)
Surface water[b] (52)	47 (90)	33 (63)

[a] *Listeria* spp. including *L. monocytogenes.*
[b] Surface water used for irrigation.

samples collected 48 h, 10% (28/294), 72 h, 5% (14/294), and 144-192 h, 3% (5/168) after irrigation and rain events (Table 3.2). The prevalence of *Listeria spp.* was higher in soil samples collected after irrigation events, 14% (40/294), compared to rain events, 10% (75/756; Table 3.2).

Twenty-seven different *Listeria* spp. allelic types were isolated from the *Listeria* spp. positive soil samples collected in this study (Table S3). While there was a greater diversity of AT in soil samples collected from the low risk field, compared to the high risk field, the difference was not statistically significant according to T-Hutcheson's test ($P = 0.08$; Table 3.3; Figure 3.3). The diversity of *Listeria* spp. AT types isolated from soil samples following rain events was significantly greater ($P < 0.001$) than the diversity of allelic types isolated from soil samples following irrigation events (Table 3.3). The diversity of *Listeria* spp. ATs isolated from water samples was

Table 3.2: Frequency and prevalence of Listeria spp. and L monocytogenes in soil samples collected 24, 48, 72, and 144 to 192 h after irrigation and rain events from two spinach fields previously identified as being at high or low risk for L. monocytogenes isolation.

		No. (%) of samples positive for:	
Event type[a]	Time (h)[b] (no. of samples)	*Listeria* spp.[c]	*L. monocytogenes*
Low-risk field			
Pre-sample	NA (21)	1 (5)	1 (5)
Irrigation	24 (42)	8 (19)	7 (17)
	48 (42)	3 (7)	2 (5)
	72 (42)	2 (5)	2 (5)
	144–192 (21)	0 (0)	0 (0)
Rain	24 (105)	16 (15)	7 (7)
	48 (105)	7 (7)	2 (3)
	72 (105)	4 (4)	2 (3)
	144–192 (63)	2 (3)	1 (1)
High-risk field			
Pre-sample	NA (21)	4 (19)	2 (10)
Irrigation	24 (42)	11 (26)	10 (24)
	48 (42)	11 (26)	11 (26)
	72 (42)	5 (12)	2 (5)
	144–192 (21)	0 (0)	0 (0)
Rain	24 (105)	33 (31)	28 (27)
	48 (105)	7 (7)	3 (3)
	72 (105)	3 (3)	5 (12)
	144–192 (63)	3 (5)	1 (16)

[a] Event type (i.e., irrigation or rain event) that initiated sample collection.
[b] Time in hours (i.e., 24, 48, 72, or 144 to 192 h) since the event. NA (not applicable) indicates that samples were collected before study initiation.
[c] *Listeria* spp. including *L. monocytogenes.*

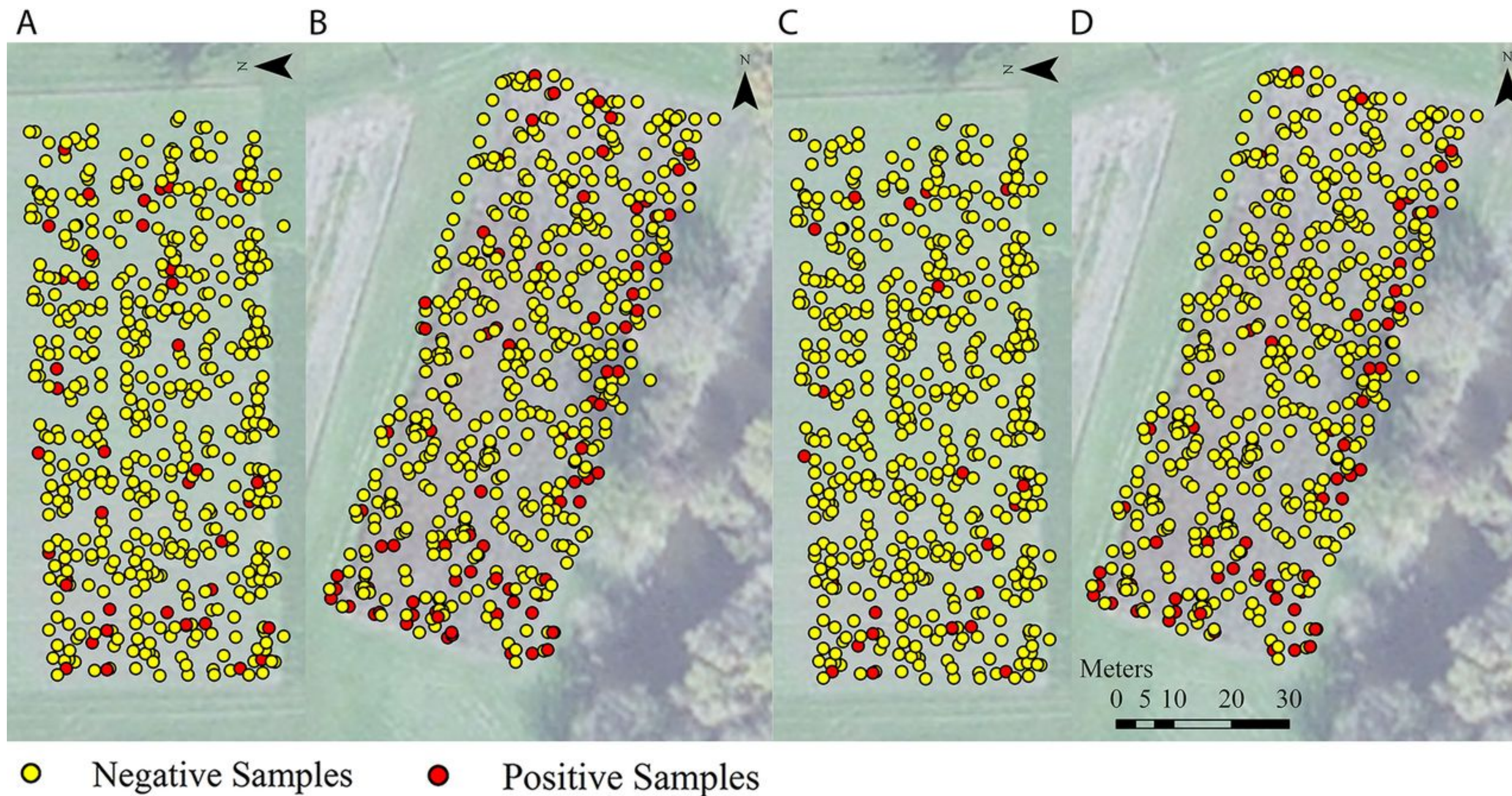

Figure 3.2: Distribution of Listeria species (including L. monocytogenes)-positive and -negative samples in the low-risk (A) and high-risk (B) fields and of L. monocytogenes-positive and -negative samples in the low-risk (C) and high-risk (D) fields. Fall Creek, the source of irrigation water in this study, is visible in the bottom right-hand corner of panels B and D.

Table 3.3: Diversity of Listeria species and L. monocytogenes allelic types isolated from soil and water samples collected from spinach fields previously identified as being at high or low risk for L. monocytogenes isolation.

		Listeria spp.[b]		*L. monocytogenes*	
Sampling site	Event type[a]	No. of allelic types	Shannon-Weiner index	No. of allelic types	Shannon-Weiner index
Low-risk field	—	18	2.4	7	1.2
	Irrigation	4	0.84	2	0.33
	Rain	16	2.5	6	1.4
High-risk field	—	21	2.0	7	1.1
	Irrigation	8	1.1	4	0.53
	Rain	18	2.2	6	1.2
Surface water	—	14	0.85	6	0.99
	Irrigation	4	0.67	3	0.39
	Rain	12	1.1	5	0.60

[a] Event type (i.e., irrigation or rain event) that initiated sample collection. — indicates information for all samples collected from the high-risk field, the low-risk field, or surface water regardless of the event type that initiated collection. The number of allelic types is not a simple summation of the numbers of ATs found following irrigation and rain events, as some ATs may have been found following events of both types.
[b] *Listeria* spp. including *L. monocytogenes.*

not significantly different from the diversity of *Listeria* spp. ATs isolated from soil samples following irrigation events (P=0.36). However, the diversity of *Listeria* spp. ATs isolated from water samples was significantly lower than the diversity of the *Listeria* spp. ATs isolated from soil samples following rain events (P<0.001).

The overall prevalence of *L. monocytogenes* was 9% (130/1,492). The prevalence of *L. monocytogenes* was higher in fecal samples, 64% (9/14) and water samples, 63% (33/52), compared to soil samples, 8% (86/1092) and leaf samples, 0.6% (2/334; Table 3.1). The prevalence of *L. monocytogenes* was higher in soil samples collected from the high risk field (11%; 62/546), compared to the low risk field (4%; 24/546; Table 3.1; Figure 3.2). The prevalence of *L. monocytogenes* was higher in soil samples collected 24 h after irrigation and rain events, 18% (52/294), compared to soil samples collected 48 h, 6% (18/294), 72 h, 4% (11/294) and 144-192 h, 1% (2/168), after irrigation and rain events (Table 3.2). Lastly, the prevalence of *L. monocytogenes* was higher in soil samples collected after irrigation events, 12% (34/294), compared to rain events, 6% (49/756; Table 3.2).

Nine different *L. monocytogenes* ATs allelic types were isolated from *L. monocytogenes* positive soil samples (Table S3); all isolates were from Lineage I or II. While there was a greater diversity of ATs in soil samples collected from the low risk field, compared to the high risk field (Figure 3.3), the difference was not statistically significant according to T-Hutcheson's test (P = 0.39; Table 3.3). The diversity of *L. monocytogenes* ATs isolated from soil samples following rain events was significantly

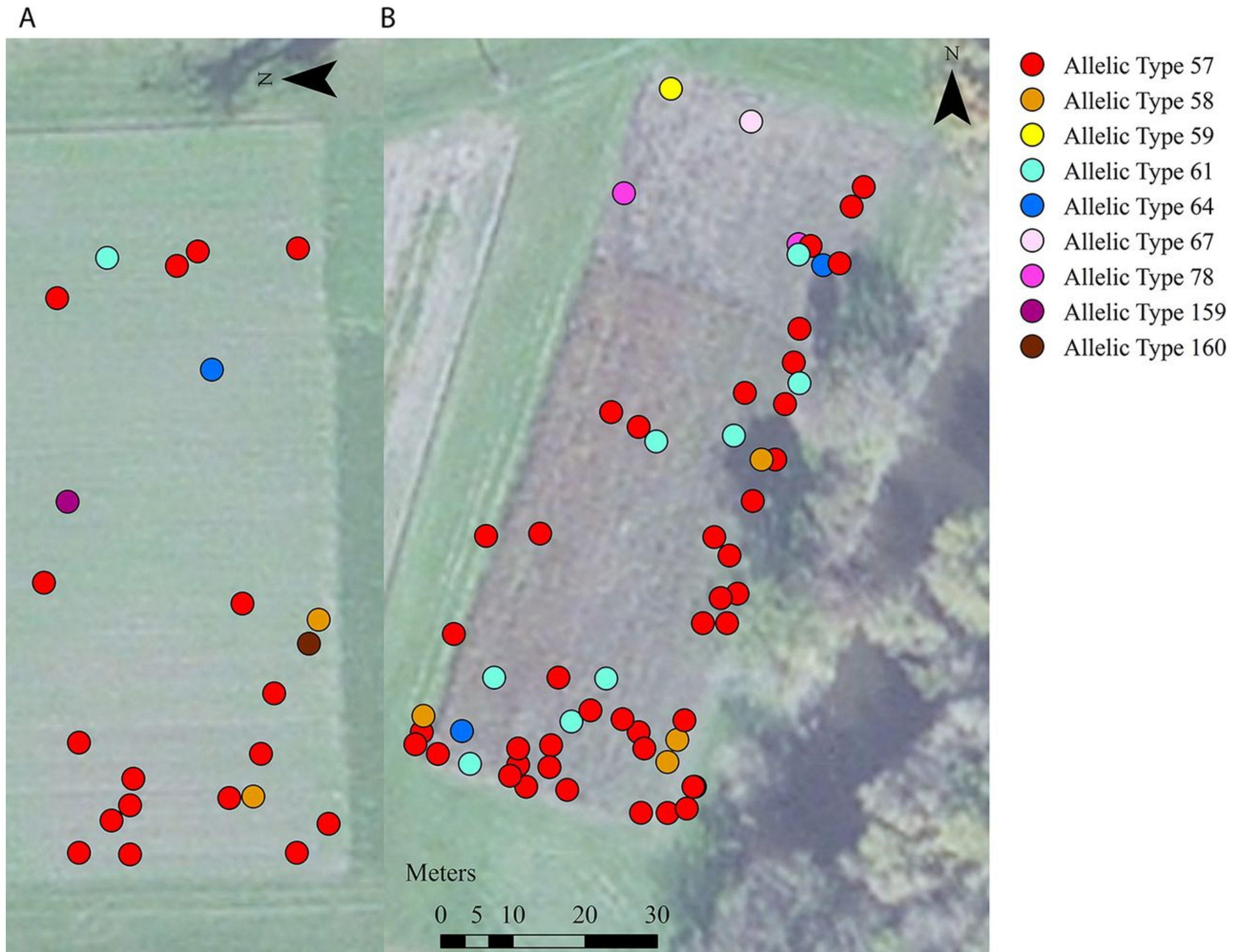

Figure 3.3: Distribution of L. monocytogenes allelic types in the low-risk (A) and high-risk (B) fields. Fall Creek, the source of irrigation water in this study, is visible in the bottom right-hand corner of panel B.

greater (*P* <0.001), than the diversity of *L. monocytogenes* ATs isolated from soil samples following irrigation events (Table 3.3). The diversity of *L. monocytogenes* ATs isolated from water samples was not significantly different from the diversity of *L. monocytogenes* ATs isolated from soil samples following irrigation events (*P*=0.45). However, the diversity of *L. monocytogenes* ATs isolated from water samples was significantly lower than the diversity of *L. monocytogenes* ATs isolated from soil samples following rain events (*P*=0.03).

Risk factors associated with *Listeria* spp. isolation from soil samples. Of the 107 factors that were evaluated, 39 factors were significantly associated with *Listeria* spp.-positive soil samples by univariable analysis including two study parameters, two spatial factors, one dew point factors, six humidity factors, three irrigation factors, three leaf wetness factors, 15 temperature factors, three precipitation factors, and four wind speed factors (Table S4). PCA was performed for the leaf wetness factors as a group, the temperature factors as a group, and the wind speed factors as a group (Table S5).

In the multivariable analysis, four factors (hours since event occurred, amount of irrigation water applied to the fields two days before sampling, amount of rain water that precipitated two days before sampling, predicted field risk) were retained (Table 3.4). Although event type was not found to be significant by multivariable analysis, it was retained in the final model so the effect of irrigation events compared to rain events could be quantified as this was of interest to the study. All factors retained in the final model were also retained when proximity to water and proximity to road were substituted for predicted field risk (Table S6). No significant interactions

between any factors were identified for either of the models. The model containing predicted field risk was selected as the final model because it had a lower Akaike information criterion (AIC), compared to the model containing proximity to water and road (AIC = 654.7 and 658.0, respectively). The odds of *Listeria* spp. isolation in soil samples were 8 times greater (OR = 7.7; 95% CI= 2.9, 20) for samples collected 24 h after an event, compared to soil samples collected 144-192 h after any event. The odds of *Listeria* spp. isolation in soil samples were 2 and 3 times greater for samples collected 48 and 72 h after an event (OR = 2.1; 95% CI= 0.74, 6.2 and OR = 2.5, 95% CI = 0.94, 6.9; respectively), compared to soil samples collected 144-192 h after an event. The odds of *Listeria* spp. isolation were 2 times greater (OR = 2.3; 95% CI= 1.5, 3.5) in soil samples collected from the high risk field, compared to soil samples collected from the low risk field. Lastly, for each 1 mm increase in the amount of irrigation water applied to a field, the odds of *Listeria* spp. isolation increased (OR = 1.1; 95% CI= 1.0, 1.2) and for each 1 mm increase in the amount of rain that fell on a field, the odds of *Listeria* spp. isolation also increased (OR = 1.4; 95% CI= 1.1, 1.8). The residual variogram for the *Listeria* spp. final model (Figure S1) suggests that the final model effectively accounts for all spatial dependencies within the data.

Risk Factors associated with *L. monocytogenes* isolation from soil samples. Of the 107 factors that were evaluated (Table S2), 46 were significantly associated with *L. monocytogenes*-positive soil samples by univariable analysis including two study parameters, two spatial factors, five dew point factors, ten humidity factors, three irrigation factors, two leaf wetness factors, 20 temperature factors, one precipitation factor, and one wind direction factor (Table S4). PCA was performed for

Table 3.4: Final multivariable model for the likelihood of isolating Listeria spp. and L. monocytogenes from spinach fields based on testing of soil samples and given a P value cutoff of 0.05.

Factor	OR[a]	95% CI[b]	*P*-value
Factors significant for *Listeria* spp.[c]			
Amount of irrigation water (mm) applied to fields 2 days before sample collection	1.1	1.0, 1.2	0.04
Event type that initiated sample collection			
Irrigation	0.71	0.40, 1.2	0.22
Rain	1.0		
Time since event occurred (h)			
24	7.7	2.9, 20	<0.01
48	2.1	0.74, 6.2	0.16
72	2.5	0.94, 6.9	0.07
144–192	1.0		
Predicted field risk			
Low	1.0		
High	2.3	1.5, 3.5	<0.01
Total amount of rain (mm) on day 2 before sample collection	1.4	1.1, 1.8	<0.01
Factors significant for *L. monocytogenes*			
Amount of irrigation water applied to fields 2 days before sample collection	1.2	1.1, 1.3	<0.01
Event type that initiated sample collection			
Irrigation	0.74	0.41, 1.3	0.33
Rain	1.0		
Time (h) since event occurred			
24	25	5.7, 99	<0.01
48	2.5	0.49, 12	0.27
72	3.4	0.74, 15	0.11
144–192	1.0		
Predicted field risk			
Low	1.0		
High	3.5	2.0, 6.0	<0.01

Table 4.4: Continued

[a] For continuous factors, OR refers to the change in the odds of isolating *Listeria* spp. or *L. monocytogenes* associated with a one-unit increase in the factor (e.g., a 1-mm increase in the amount of irrigation water applied).
[b] CI, confidence interval for the odds ratio.
[c] *Listeria* spp. including *L. monocytogenes.*

the dew point factors as a group, the humidity factors as a group, the leaf wetness factors as a group, and the temperature factors as a group (Table S5).

In the multivariable analysis, three factors (hours since event occurred, amount of irrigation water applied to the fields two days before sampling, and predicted field risk) were retained (Table 3.4). Although event type was not significant, it was retained in the final model. All factors retained in the final model were also retained when proximity to water and proximity to road were substituted for predicted field risk (Table S6). No significant interactions between any factors were identified for either of the models. The model containing predicted field risk was selected as the final model because it had a lower AIC, compared to the model containing proximity to water and road (AIC = 461.5 and 465.9, respectively). The odds of *L. monocytogenes* isolation in soil samples were 25 times greater (OR = 25; 95% CI= 5.7,99) for samples collected 24 h after an event, compared to soil samples collected 144-192 h after any event. The odds of *L. monocytogenes* isolation in soil samples were about 3 times greater for samples collected 48 h (OR = 2.5; 95% CI= 0.49, 12) and 72 h (OR = 3.4; 95% CI= 0.74, 15) after an event, compared to soil samples collected 144-192 h after an event. While, statistically, the odds of isolating *L. monocytogenes* were greatest during the 24 h immediately following an irrigation or rain event, for the high risk field the observed prevalence of *L. monocytogenes* was higher 48 h compared to 24 h after irrigation (Table 3.2). The odds of *L. monocytogenes* isolation were 3.5 times greater (OR = 3.5; 95% CI= 2.0, 6.0) for soil samples collected from the high risk field, compared to soil samples collected from the low risk field. Lastly, for each 1 mm increase in the amount of irrigation water applied to a field, the odds of *L.*

monocytogenes isolation increased (OR = 1.2; 95% CI= 1.1, 1.3). The residual variogram (Figure S1) for the final model also suggests that the final model effectively accounted for all spatial dependencies within the data.

Discussion

The objectives of this study were (i) to quantify the effects of different spatial and temporal factors associated with the isolation of *Listeria* spp. and *L. monocytogenes* from produce fields following rain and irrigation events; and (ii) to determine how rain and irrigation events affect the detection frequency and diversity of *Listeria spp.* and *L. monocytogenes* in produce fields. Our study showed that the likelihood of isolating *Listeria* spp. and *L. monocytogenes* was greatest during the 24 h immediately following rain or irrigation events, and that the diversity of *Listeria* spp. and *L. monocytogenes* subtypes (ATs) was lower after irrigation events compared to rain events. Additionally, we show that proximity to water and roads were associated with an increased likelihood of isolating *L. monocytogenes* from soil samples collected in produce fields. These findings are consistent with previous research performed in NYS produce production environments (14–16, 25, 47), supporting a robust relationship between these factors, and *Listeria spp.* and *L. monocytogenes* isolation. Our study is unique as it quantified changes in *Listeria spp.* and *L. monocytogenes* prevalence over subsequent 24 h periods following rain and irrigation events. It is important to note that these findings are based on a single study conducted on two fields over the course of one growing season, and that additional studies are needed to determine if our findings are translatable to other farms. However, the results do

support previous studies'(14, 25, 48–50) conclusions that management practice-based interventions may reduce the risk of pre-harvest produce contamination.

Irrigation can be a point source of *Listeria* contamination, while rain appears to increase *Listeria* detection prevalence through non-point source mechanisms. Analysis of *sigB* AT diversity showed that, in our study here, the diversity of *Listeria* spp. and *L. monocytogenes* isolates was significantly lower in soil samples collected after irrigation events compared to rain events. The lower diversity following irrigation events suggests that irrigation water served as a homogenous, point source for *Listeria* spp. and *L. monocytogenes* contamination in the produce fields studied here. This is supported by the fact that the diversity of ATs in soil samples collected after irrigation events was not statistically different from the diversity of ATs in water samples collected from Fall Creek, the source of irrigation water used in this study. These findings are consistent with the findings of previous studies (14, 25, 26, 47, 51, 52) that identified irrigation water as a potential pre-harvest source of bacterial contamination of produce. Multiple studies have also reported significant associations between irrigation water and fresh produce contamination (12, 13, 19, 20). The relationship between irrigation and *L. monocytogenes* isolation in this study may be explained by the fact that surface water is a known reservoir for foodborne pathogens in produce production environments (22, 52–56). As our study and others (14, 21, 25, 47, 51, 52) have demonstrated, irrigation is an important risk factor for pre-harvest produce contamination, particularly if the irrigation water is drawn from a surface water source. Therefore, intervention at the irrigation-level may decrease the risk of *L. monocytogenes* contamination of produce. For example, in a

review of pre- and postharvest measures to reduce microbial contamination of fresh produce, Gil et al. (48) identified selection of proper irrigation methods, protection of surface water sources, and periodic testing of irrigation water as critical interventions for preventing microbial contamination.

The higher diversity of *Listeria* spp. and *L. monocytogenes* ATs associated with rain events suggests that rain increases the likelihood of *Listeria* spp. and *L. monocytogenes* detection. For example, rain may facilitate the movement of diverse *Listeria* into field environments, or facilitate the growth and detection of *Listeria* strains already present in the field. This is supported by past studies (15, 57), which found that pathogens transmitted by runoff and splash associated with rain events can bypass physical barriers to movement into and within fields. The findings of these earlier studies may also explain why all of the positive leaf samples in our study were associated with rain events. Additionally, rain events may create favorable conditions for foodborne pathogen growth (e.g., higher soil moisture; 55, 58–60), amplifying existing *Listeria* populations within the field and increasing the likelihood of detection during sampling. Similarly, higher nutrient loads associated with runoff (61–64) could facilitate microbial growth in fields (62, 65–67). As rain and irrigation events can affect the diversity of *Listeria* spp. and *L. monocytogenes* in produce production environments differently. Therefore, interventions to reduce the risk of pathogen contamination in fields may need to take into account the water source (i.e., surface water versus rain).

Meteorological factors were significantly associated with *L. monocytogenes* isolation from produce production environments. In previous studies, temperature-

related (e.g., heat index and maximum temperature; 15, 19–21, 60, 68, 69) and water-related (e.g., humidity and leaf wetness; 20, 21, 69) meteorological factors were significantly associated with pathogen isolation from produce production environments. For example, in a study conducted on Spanish lettuce fields, Oliveira et al. (20) found that humidity and temperature influence *L. innocua* survival following irrigation. Interestingly, in the study reported here, multiple meteorological factors (except rain) were significantly associated with *L. monocytogenes* isolation by univariable analysis but no meteorological factors were retained in the final multivariable model for *L. monocytogenes*; this is consistent with the findings of Weller et al. (14). Moreover, in the model developed by Strawn et al. (15) to predict risk of *L. monocytogenes* isolation from produce production environments, temperature was ranked below proximity to water, suggesting that spatial factors (e.g., proximity to water) have a greater influence on *L. monocytogenes* isolation than meteorological factors (e.g., temperature). Combined, the findings reported here and in other studies (14, 15) may indicate that, although meteorological factors are associated with *L. monocytogenes* isolation, they are not the most important risk factors for *L. monocytogenes* isolation. Thus, meteorological factors (other than rain) should not be the primary focus of risk management strategies for *L. monocytogenes* in produce production environments; rather risk management strategies that focus on landscape factors or management practices may be more beneficial long-term. However, due to the previously mentioned weaknesses of this study, further research is needed to determine if these conclusions are translatable to other farms both within and outside NYS.

Risk of produce contamination was highest within 24 h of irrigation and rain events. In our study, the odds of isolating *L. monocytogenes* in soil samples were greatest during the 24 h immediately following rain or irrigation events, compared to 48, 72 or 144-192 h following rain or irrigation events. Overall, our findings suggest that *L. monocytogenes* levels spike after an initial inoculation event, such as irrigation, and then decrease over subsequent 24 h periods. While no other study, to the authors' knowledge, has investigated *L. monocytogenes* survival in produce production environments over subsequent 24 h periods, previous studies (17, 19, 60, 68, 70) that investigated the persistence and survival of *L. monocytogenes* in non-produce production environments found similar patterns. For example, McLaughlin et al. (60) found that *L. monocytogenes* populations in soils collected from urban and forest environments declined after inoculation and were undetectable in 8-10 days. Similarly, Castro-Ibanez et al. (68) and Taylor et al. (70) both showed that fecal indicator bacteria levels peaked immediately following flooding events and then declined over subsequent sampling events. In the context of these studies, our findings suggest that waiting 24 h after irrigation and rain events to harvest crops may significantly reduce the risk of *L. monocytogenes* contamination. This time frame offers a tangible solution to growers that can be implemented with limited economic impact.

Landscape factors accurately predicted the risk of *L. monocytogenes* contamination. In our study the odds of isolating *L. monocytogenes* were significantly higher for samples collected from the high risk field compared to the low risk field, suggesting that landscape factors (e.g., proximity to road and water) may be useful for accurately predicting the likelihood of *L. monocytogenes* isolation from

produce production environments. This is not surprising since past studies have repeatedly associated landscape factors with foodborne pathogen isolation from produce production environments (14–16, 22, 71). However, it is important to note that the model that included predicted risk fit the data better than the model containing proximity to water and roads. This may suggest, that for the dataset discussed here, the model containing predicted risk accounted for additional differences between the two fields, such as soil type and field history. As previously mentioned, differences in soil properties are known to affect the likelihood of isolating *L. monocytogenes* from soil samples, and the high risk field had, on average, a higher AWS than the low risk field. Overall, the findings reported here and in other studies (14–16, 22, 60) support the conclusion that not all cropland is at equal risk of foodborne pathogen contamination. Clearly, preharvest contamination of fresh produce is the result of complex interactions between factors, including factors that were not included in this study (e.g., seasonal effects, and worker activity). However, our findings do suggest that the use of landscape factors to predict risk and to tailor cropping schemes to reduce risk (e.g., planting high risk crops in low risk areas) may be useful for developing targeted on-farm food safety risk management plans.

The association between *L. monocytogenes* prevalence, and proximity to water and road found in this study, is consistent with the existing literature (14–16, 71). For example, Strawn et al. (15) and Sauders et al. (71) found that the closer a field or location were to a road the greater the likelihood of *L. monocytogenes* isolation. Roadside ditches, like surface water, may act as a reservoir and transmission pathway for foodborne pathogens in produce productions environments (72). Heavy rain,

melting snow, wind, flooding, and human activity may also act as mechanisms for the spread of foodborne pathogens from ditches and waterways to produce fields. Additionally, roads, roadside ditches and riparian areas may act as corridors for animal movement. Therefore, fields that are closer to roads and water may be at greater risk for wildlife intrusion, which has previously been associated with produce contamination by foodborne pathogens (73–75). Since past studies have found that buffer zones (25, 76, 77) and wetlands (78) reduce the risk of microbial contamination in produce production and other environments, the construction of buffer zones and the conservation of wetlands around fields may reduce the risk of *L. monocytogenes* contamination of produce. However, more research is needed to quantify the impact of buffer zones and wetlands on the risk of produce contamination and to determine how buffer zones and wetlands can be most effectively used to reduce produce contamination risks.

Overall, our findings suggest that small changes in management practices may have a significant effect on the risk of *L. monocytogenes* contamination in produce production environments. For example, growers may reduce *L. monocytogenes* contamination risk by waiting 24 h to harvest crops following rain events, or by not irrigating within 24 h of harvest. Additionally, interventions at the irrigation-level, such as treatment of irrigation water (e.g., by chlorine tabs), may reduce the risk of pre-harvest contamination. Other potential intervention strategies may include constructing buffer zones or conserving wetlands around fields near water or roads, altering cropping schemes (e.g., planting high risk crops in low risk fields), and monitoring pathogen levels in irrigation water.

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Supplemental Material

Supplemental material associated with this article can be found online at: http://aem.asm.org/content/81/17/6059/suppl/DCSupplemental.

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CHAPTER 4

ESCHERICHIA COLI TRANSFER FROM SIMULATED WILDLIFE FECES TO LETTUCE DURING FOLIAR IRRIGATION: A FIELD STUDY IN THE NORTHEASTERN UNITED STATES

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Abstract

Wildlife intrusion has been associated with pathogen contamination of produce. However, few studies have examined pathogen transfer from wildlife feces to pre-harvest produce. This study was performed to calculate transfer coefficients for *Escherichia coli* from simulated wildlife feces to field-grown lettuce during irrigation. Rabbit feces inoculated with a 3-strain cocktail of non-pathogenic *E. coli* were placed in a lettuce field 2.5-72 hours before irrigation. Following irrigation, the *E. coli* concentration on the lettuce was determined. After exclusion of an outlier with high *E. coli* levels (Most Probable Number = $5.94*10^8$), the average percent of *E. coli* in the feces that transferred to intact lettuce heads was 0.0267% (Standard Error [SE] = 0.0172). Log- linear regression showed that significantly more *E. coli* transferred to outer leaves compared to inner leaves (Effect = 1.3; 95% Confidence Interval = 0.4, 2.1). Additionally, the percent of *E. coli* that transferred from the feces to the lettuce decreased significantly with time after fecal placement, and as the distance between the lettuce and the feces, and the lettuce and the sprinklers increased. These findings provide key data that may be used in future quantitative risk assessments to identify

potential intervention strategies for reducing food safety risks associated with fresh produce.

Introduction

Produce contaminated with pathogenic organisms is a significant source of foodborne illness in the United States [US; (1–3)] and internationally (2). In fact, researchers estimate that produce-related illnesses result in an annual loss of over 1,397 million dollars due to medical expenses in the US alone [calculated using data reported in Batz et al. (2012)]. Thus, there is interest in preventing or mitigating the food safety risks associated with fresh produce consumption, including through grower voluntary agreements [e.g., Leafy Greens Marketing Agreement (2012)] and government regulation [e.g. Food Safety Modernization Act (2015)]. While fresh produce contamination can occur throughout the post-harvest supply chain (6, 7), pre-harvest sources of contamination (e.g., wildlife) are also a food safety concern. Scientific studies that examine potential pre-harvest sources of contamination are needed to provide data to facilitate the development of effective, grower-level interventions that reduce the likelihood of produce contamination.

Past studies have identified wildlife as a potential source of bacterial pathogens, including pathogenic *Escherichia coli*, in pre-harvest environments (8–10). In fact, traceback analyses following produce-associated outbreaks have identified wildlife as a probable source of contamination in a number of instances (11–14). Understanding how pathogens transfer from wildlife to pre-harvest produce is critical for understanding and assessing the food safety risks associated with wildlife intrusion into produce fields. However, to the authors' knowledge only one peer-reviewed study has examined the transfer of bacterial pathogens from wildlife feces to field-grown produce via splash. Specifically, Atwill et al. (2015) conducted a field trial in Salinas,

California to investigate the transfer of *E. coli* O157:H7 from simulated wildlife feces (inoculum = $1.29*10^8$ CFU/5 g) to lettuce during a 2.5 h irrigation event. That study (15) reported that 0.006% of the *E. coli* present in the feces transferred to the lettuce, and that several spatial factors (e.g., distance between fecal pellets and lettuce) were associated with the transfer of *E. coli* from the fecal pellets to the lettuce. Such data has the potential to inform quantitative risk assessment, and facilitate the development of science-based food safety controls. However, additional studies are needed to verify that the Atwill et al.'s (2015) findings are reproducible in and generalizable to produce-growing regions outside California. Testing the robustness of a finding to minor variations in experimental procedures [e.g., conducting a field trial under different environmental conditions (e.g., weather, soil type), using slightly different management practices] is key for establishing that the finding is believable and informative (16). As such, this study was designed to repeat, in a different region of the United States and with slight modifications, the study conducted by Atwill et al. (2015) in order to obtain additional data on (i) transfer coefficients for *E. coli* in wildlife feces to field-grown lettuce during foliar irrigation, and (ii) the impact of spatial factors on these coefficients.

Materials and Methods

Field Setup. A field study was conducted in a romaine lettuce (*Lactuca sativa* L. var. longifolia cv. Green Towers; Harris Seeds, Rochester NY) field at the Homer C. Thompson Vegetable Research Farm in Freeville, NY during summer 2015. Throughout the growing season tine weeding, hand weeding and rototilling were used to thin the lettuce (at 4 weeks) and to weed the study field. The field consisted of (i) a

3.1 m bare ground buffer around the entire field, (ii) three 8.5 m x 59.5 m cells divided into seven 8.5 m x 8.5 m plots numbered 1-21, and (iii) two 8.5 m x 59.5 m bare ground buffers between each cell (Supplemental Material 1). Within each cell there were five longitudinal beds (each 1.2 m wide) separated by a 0.6 m furrow; each bed consisted of 4 rows of seed planted 0.4 m apart (Supplemental Material 1). Seeding was performed using a Monosem MS vegetable seeder (Monosem Inc., Edwardsville, KS) with a 1.5 in (3.81 cm) seeding rate. Overhead impact sprinklers (Nelson F33 sprinklers, Nelson Irrigation, Walla Walla, WA) were spaced around the field with approx. 15 m between sprinklers. Irrigation occurred, as needed, up to one week before harvest. On the day of harvest irrigation commenced at 7:30 am and ended at 10:00 am. During this time approx. 25 mm of water were applied to the field.

Twenty of the 21 plots were randomly assigned to one of four treatments (fecal placement at 72, 48, 24 or 2.5 h before irrigation; 5 plots per treatment); treatment will henceforth be referred to as fecal age. The remaining plot did not receive a fecal pellet. Fecal placement sites were randomly selected from within each plot so that only (i) one fecal pellet was placed in each plot, (ii) each fecal pellet was placed in a furrow between two lettuce rows, and (iii) all fecal pellets were at least 7 m from each other. The six lettuce heads closest to each fecal placement site were selected for sampling. If there were not six heads within 1 m of the fecal placement site, a different site within the given plot was selected. Each lettuce head that was selected for inclusion in this study received a unique identification number (the head ID).

Geographic positioning system (GPS) coordinates were obtained with +/- 10 cm accuracy for the lettuce heads, fecal pellets, sprinkler locations and field

boundaries using a Geo 7x series GPS unit (Trimble, Sunnyvale, CA). GPS coordinates for post-irrigation pools (i.e., pools of water that formed in the field during irrigation) were also recorded. The distance and degree angle between (i) the lettuce and other features (i.e., sprinklers and fecal pellets), and (ii) the fecal pellets and sprinklers were calculated using the Spatial Analyst extension in ArcGIS (17).

To determine the likelihood of false positives due to indigenous rifampicin resistant *E. coli*, environmental sampling was performed before the study began. Briefly, 3 composite soil samples, 3 vegetation samples, and 4 irrigation water samples were collected. Each 250-mL water sample was passed through a 0.45 μm filter (Nalgene, Rochester, NY). The filter was then transferred to a Whirl-Pak bag (Nasco, Fort Atkinson, WI), and enriched with 90 mL of tryptic soy broth (Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with rifampicin (EMD Chemicals, San Diego, CA) to a final concentration of 100 μg/mL (TSB+R). Following incubation at 37°C for 24 h, 50 μL of enrichment were streaked onto *E. coli* CHROMagar (DRG International, Inc., Springfield, NJ) supplemented with rifampicin to a final concentration of 100 ug/mL (ECC+R). The ECC+R plates were then incubated at 42°C for 18-24 h. Soil and vegetation samples were first diluted 1:2 in PBS, and then, in duplicate, serially diluted in 2 log steps to 10^{-11} in TSB+R. Following incubation at 37°C for 24 h, 3 μL of each dilution were streaked onto ECC+R. The ECC+R plates were then incubated at 42°C for 18-24 h. While one soil sample tested positive for rifampicin-resistant *E. coli*, *clpX* allelic typing (performed as described below) differentiated this isolate from the inoculation strains used in the study reported here.

Bacterial Strain and Inoculum Preparation. Three rifampicin-resistant, non-pathogenic *E. coli* strains [TVS 353, TVS 354 and TVS 355; (18)] were obtained from the University of California, Davis. Each strain was grown separately on tryptic soy agar plates (TSA; Becton, Dickinson and Company) at 37°C to stationary phase (18-24 h). Following incubation, each plate was flooded with 10 mL of phosphate buffered saline (PBS) and the cells were resuspended using a serological pipette. Bacterial suspensions were then separately transferred to a 15 mL Falcon tube, and centrifuged at 2500xG for 5 min. The culture pellet was washed twice with 10 mL PBS, and re-suspended in 5 mL of PBS. The bacterial suspension was diluted 1:32 in PBS, and the optical density (OD_{600}) was measured. Based on the optical density the culture was diluted in PBS to achieve a concentration of approx. 4×10^7 cfu/mL. The inoculum was then prepared by mixing the three strains in equal proportions (approx. 16.7 mL of each strain) in a 100 mL Erlenmeyer flask. While Atwill et al. (2015) used an attenuated *E. coli* O157:H7 strain (ATCC 700728) in their study, we used a cocktail of 3 non-pathogenic *E. coli* strains (TVS 353, TVS 354 and TVS 355). While the use of non-pathogenic, surrogate *E. coli* strains (as opposed to pathogenic strains) is a limitation of our study, pathogenic *E. coli* could not be used due to biosecurity concerns. The 3 strain cocktail used in this study was chosen for the reasons discussed in Weller et al. (19). Briefly, several studies (18, 20) have reported that the 3 strain cocktail used here demonstrated greater environmental fitness compared to individual *E. coli* strains, including ATCC 700728. For example, one study found that the non-pathogenic *E. coli* cocktail used in the study presented here persisted for a greater amount of time in hydroponic and soil environments compared to ATCC 700728 (20).

The authors attributed this to the fact that one of the three cocktail strains (TVS 355) was isolated from produce field soils, and therefore may be adapted to the stresses experienced during the course of their study (20). Gutiérrez-Rodríguez et al. (20) also reported that the survival and persistence of pathogenic and non-pathogenic *E. coli* was strain dependent. The use of strains isolated from relevant environments therefore may help to provide a conservative estimate of pathogen survival in feces and, pathogen transfer to lettuce.

Fecal Pellet Preparation and Placement. Fecal pellet preparation was performed in a similar manner to that described in Atwill et al. (2015). Briefly, laboratory rabbit (CoVance, Princeton, NJ; *Oryctolagus cuniculus*) feces were used as a proxy for wildlife feces. Fifty grams of feces and 50 mL of inoculum were placed in a Whirl-Pak bag and hand-massaged for five minutes. Five gram portions of the feces-inoculum mixture were weighed out and formed into 5 g pellets. The pellets were then placed in pre-labeled, sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI) and stored at 4°C for < 16 h. Pellets were placed on the soil surface 72, 48, 24 or 2.5 h before the start of irrigation on the day of harvest.

The concentration of *E. coli* in the fecal pellets was determined by separately mixing three pellets with PBS and spiral plating the 10^{-3}, 10^{-4}, and 10^{-5} dilutions on TSA+R. The TSA+R plates were then incubated at 37°C for 18-24 h. Colonies were then enumerated using a Q-Count (Advanced Instruments, Norwood, MA). On average the fecal pellets contained $3.65*10^{8}$ CFUs of *E. coli* per 5 g fecal pellet (Table 4.1). We inoculated the feces with a high concentration of *E. coli* to ensure comparability with Atwill et al. (2015).

Table 4.1 Concentration of non-pathogenic E. coli in the fecal pellets prior to fecal placement and at harvest.

Fecal age (h)	Geometric mean CFU per fecal pellet at placement (95% CI[a])	Geometric mean MPN per fecal pellet at harvest[b] (95% CI[a])	Change in the average log_{10} *E. coli* concentration between placement and harvest
2.5	$2.62*10^8$ ($1.99*10^8$, $3.45*10^8$)	$4.84*10^8$ ($1.07*10^5$, $2.18*10^{12}$)	0.27
24	$2.15*10^8$ ($1.20*10^8$, $3.87*10^8$)	$2.23*10^8$ ($6.21*10^5$, $7.99*10^{10}$)	0.02
48	$8.04*10^8$ ($9.66*10^7$, $6.69*10^9$)	$1.93*10^6$ ($1.52*10^2$, $2.44*10^{10}$)	-2.62
72	$1.77*10^8$ ($1.45*10^8$, $2.16*10^8$)	$2.82*10^6$ ($3.36*10^3$, $2.37*10^9$)	-1.80

[a] Confidence Interval (CI)
[b] Geometric mean was calculated by taking the average of the log10 MPN per fecal pellet across a time group and raising 10 to the power of this average.

Harvest. Lettuce heads were harvested immediately following 2.5 h of foliar irrigation. Briefly, harvesting was conducted by teams of two, consisting of a bagger and harvester. Heads were harvested by hand using a food-grade knife. Gloves and shoe covers were changed between collection of each sample, and the knife was decontaminated using a 10% bleach wipe followed by a 70% ethanol wipe. In total 120 lettuce heads were collected, including (i) 100 intact heads, and (ii) 20 heads where the inner 1/3 of the head, and the outer 2/3 were harvested separately. Inner and outer leaves were treated as separate samples for all analyses (hence the 120 lettuce heads harvested represent 140 samples that were tested). Following harvest, all samples were placed in pre-labeled, sterile Whirl-Pak bags, transported back to lab in insulated containers, stored at 4 °C, and processed within 6 h.

Following irrigation, the fecal pellets and approx. 1.27 cm of soil immediately underneath each fecal pellet were collected using 5 mL sterile scoops (Fisher Scientific, Hampton, NH) and placed in pre-labeled, sterile Whirl-Pak bags. Fecal pellets were transported back to lab in insulated containers, stored at 4 °C and processed within 6 h.

E. coli **Enumeration.** *E. coli* enumeration was performed on the lettuce samples and the post-harvest fecal pellets (i.e., the fecal pellets that were collected from the field following irrigation) using a tripartite scheme (i.e., a high concentration assay, a low concentration assay and an enrichment assay) that was adapted from Atwill et al. (2015). The tripartite scheme described here is sequential. The high concentration assay was performed on all lettuce heads. The low concentration assay was then performed only on those heads that were below the detection limit of the

high concentration assay (300 cells per lettuce head). Similarly, the enrichment assay was performed only on heads that were below the detection limits of both the high and low concentration assays (2 cells per lettuce head). A sample was only considered negative if it was negative according to all three assays. All samples were held at 4 C for the duration of the study.

Conceptually, the high concentration assay was a 2-tube, 6-dilution most probable number (MPN) determination, while the low concentration assay was a 3-tube, 4-dilution MPN determination. It is important to note that *E. coli* levels in the fecal pellets tested before field placement were enumerated by direct plating as described above (yielding CFU per fecal pellet). However, *E. coli* levels in the fecal pellets collected from the field following irrigation were enumerated using the MPN approach outlined here to allow for quantification of potentially low *E. coli* levels that would not be detected by direct plating.

For the high concentration assay 600 mL of PBS were added directly to each Whirl-Pak bag containing a lettuce sample; 90 mL of PBS was added to each Whirl-Pak bag containing a post-harvest fecal pellet. Samples were then hand-massaged for 1 min. In duplicate, 1 mL of washate was transferred to the first and seventh wells of a twelve-well deep well plate (VWR International, Radnor, PA), which contained 9 mL of TSB+R. Five serial 100-fold dilutions (0.1 mL into 9.9 mL of TSB+R) were performed starting from each of the two initial wells. Following incubation at 37°C for 24 h, 3 μL of each dilution were streaked onto ECC+R. The plates were then incubated at 42°C for 24 h. Blue colonies indicated the presumptive presence of one of the *E. coli* strains used in this study.

Since the detection limit for the high concentration assay was 300 cells per lettuce head, a low concentration assay was performed on all heads that were negative according to the high concentration assay. Briefly, 19 g of sterile tryptic soy broth powder and 0.75 mL of an 80 mg rifampicin/mL of dimethyl sulfoxide solution were aseptically added to the remaining washate to achieve a final concentration of 100 g/mL of rifampicin. The head was then shaken until the powder had dissolved. The washate was then divided into three tubes each of 100 mL, 10 mL, 1 mL and 0.1 mL. Following incubation at 37°C for 24 h, 3 µL from each tube were streaked onto ECC+R; the plates were then incubated at 42°C for 24 h.

Since the detection limit for the low concentration assay was 2 cells per lettuce head, an enrichment assay was performed on all heads that were negative according to both the high and low concentration assays. The enrichment assay was designed to allow for detection of one *E. coli* cell per sample (i.e., detection limit = 1 MPN of *E. coli* per lettuce head). Briefly, heads were enriched by adding 500 mL of TSB+R, and then hand-massaged for 1 min. Following incubation at 37°C for 24 h, 50 µL from each enrichment were streaked onto ECC+R; the plates were then incubated at 42°C for 24 h.

Since one soil sample collected before the study began tested positive for rifampicin resistant *E. coli*, detection of the inoculation strains (TVS 353, TVS 354 and TVS 355) was confirmed on 10% of presumptive positive lettuce heads using PCR amplification and Sanger sequencing of the *clpX* gene as described in Walk et al. (2009).

The MPN of *E. coli* per lettuce head or fecal pellet (for fecal pellets collected after irrigation) was calculated using an unpublished R package (D. Kent, dk657@cornell.edu, 316 Stocking Hall, Cornell University, Ithaca, NY 14853; see Supplemental Materials S1 in Weller et al. (22)).

Statistical Analysis. All statistical analyses were performed in R (version 3.1, R Core Team, Vienna, Austria). For each fecal age (2.5, 24, 48, and 72 h) and head type (inner, outer, and intact) we calculated (i) the number and percent of lettuce heads that had detectable levels of *E. coli*, (ii) the percent of *E. coli* that transferred from the closest fecal pellet to each lettuce head (percent of *E. coli* transferred), and (iii) the geometric mean and 95% confidence interval for the MPN of *E. coli* on positive lettuce heads at harvest. The percent of *E. coli* transferred was calculated by dividing the MPN of *E. coli* per lettuce head at harvest by the CFU of *E. coli* per fecal pellet at fecal placement, and multiplying the product by 100. Variability in the *E. coli* concentration on the lettuce was visualized by plotting the mean and standard deviation of the $\log_{10}$ MPN of *E. coli* per lettuce head for each fecal age and head type. The proportion of positive and negative inner, outer and intact heads observed in this study were compared to the proportion reported by Atwill et al. (2015) using chi square and Fisher's exact tests.

To characterize the change in *E. coli* levels in the fecal pellets over the course of the study the geometric mean and 95% confidence interval for the CFU of *E. coli* per fecal pellet at fecal placement, and for the MPN of *E. coli* per fecal pellet at harvest were calculated for each fecal age (2.5, 24, 48 and 72 h). It is important to note that the MPN of *E. coli* per fecal pellet at harvest was likely affected by bacterial die-off in

the feces as well as loss due to splash and run-off during irrigation. The daily change in the $\log_{10}$ *E. coli* concentration per fecal pellet was determined by subtracting the average $\log_{10}$ MPN per fecal pellet at harvest from the average $\log_{10}$ CFU per fecal pellet at placement.

Using the lme4 package (23) two generalized linear mixed models (GLMM) were developed to describe (i) the percent of *E. coli* transferred from the fecal pellets to the lettuce (the transfer model), and (ii) the concentration of *E. coli* on the lettuce at harvest (the concentration model). The outcome of the transfer and concentration models were the $\log_{w0}$ percent of *E. coli* transferred, and the $\log_{w0}$ MPN of *E. coli* on the lettuce, respectively. The data used in the regression analyses was transformed using a $\log_{w0}$ rather than a $\log_{10}$ transformation because the $\log_{w0}$ transformation preserves relationships within the data when the data includes zeros [(24, 25); see Supplemental Materials 2 for the R code for the $\log_{w0}$ function as well as the function to back transform $\log_{w0}$ values]. Head ID nested within plot, and plot nested within fecal age were included as random effects in both models. The candidate explanatory variables for both models were (i) fecal age (fecal placement 2.5, 24, 48, and 72 h before irrigation), (ii) head type (intact, inner, and outer), (iii) the proximity of the lettuce to other features (i.e., lettuce-feces distance, lettuce-sprinklers distance, lettuce-field edge distance, and lettuce-post-irrigation pool distance), (iv) the sine of the wind direction relative to the lettuce-feces axis, and (v) the sine of the wind direction relative to the lettuce-sprinkler axis. Fecal age and head type were both coded as categorical variables. Fecal age was treated as a categorical variable due to the relatively small number of fecal ages (i.e., 4), the variability in the data, and the small

number of lettuce heads associated with each fecal age (i.e., 25 intact heads, 5 inner heads and 5 outer heads for each fecal age). All candidate variables were included in a full model. To test for multicollinearity the variance inflation factor (VIF) for each variable in the full model was determined; any variable with a $VIF^{1/2} > 2$ was removed from consideration as a candidate factor. Notably, feces-sprinkler distance was removed from consideration as a candidate factor due to multicollinearity with feces-lettuce distance. The models were reduced by backwards stepwise regression based on the Akaike's Information Criteria (AIC). Briefly, each variable was removed from the full model. The AIC was then determined and the variable whose removal resulted in the largest decrease in AIC was removed from the model. This process was repeated until the removal of additional variables failed to reduce the AIC.

To statistically describe the concentration of *E. coli* in the fecal pellets at harvest a linear model was developed. The outcome of the model was the change in the $\log_{10}$ MPN of *E. coli* per fecal pellet between fecal placement and harvest. The candidate explanatory variables included in the full model were (i) fecal age (2.5, 24, 48 or 72 h), (ii) the distance between the fecal pellets and other features (i.e., feces-sprinkler distance, feces-field edge distance, feces-post-irrigation pool distance), and (iii) the sine of the wind direction relative to the feces-sprinkler axis. All candidate variables were included in a full model, which was reduced by backwards stepwise regression based on AIC.

Wind direction relative to the lettuce-feces axis is defined as the angle between the average wind direction between 7:30 am and 10:00 am on the day of harvest and the the line connecting the lettuce and the closest fecal pellet. This is calcuated using

the formula, |Y-W|, where W is the compass bearing of the average wind direction between 7:30 and 10:00 on the day of harvest (when the field was irrigated), and where Y is the compass bearing of the line connecting the lettuce and the closest fecal pellet (Supplemental Material 3). Similarly, wind direction relative to the feces-sprinkler axis can be similarly described by the formula, |X-W|, where W is as above and where X is the compass bearing of the line connecting the fecal pellet and the closest sprinkler.

Weather conditions for the entire course of the study are reported to enable readers to better assess our findings (Supplemental Material 4). Weather data was obtained from a weather station (Rainwise Inc., Trenton, NJ) located at the Homer C. Thompson Vegetable Research Farm as described in Weller et al. (2015).

Results

***E. coli* transfer to intact heads, and inner and outer lettuce leaves.** Eighty-nine percent (88/99) of intact heads had detectable levels of *E. coli* (≥1 *E. coli* per lettuce head; Table 4.2). One intact lettuce head near a fecal pellet that was placed 24 h before irrigation had a substantially higher MPN of *E. coli* ($5.94*10^8$) compared to other intact heads near 24 h fecal pellets, which had *E. coli* levels that ranged from $1.00*10^0$-$4.08*10^4$ MPN per lettuce head (Table 4.2). Due to the presence of this outlier all analyses using the lettuce data were conducted with the outlier excluded (results reported in the tables included in the main text) and with the outlier included (results reported in Supplemental Materials 5-7). The average percent of *E. coli* that transferred to a given intact lettuce head from the closest fecal pellet was 0.0267% when the outlier was excluded from the dataset (Table 4.2). When the outlier was

included in the dataset, the average concentration of *E. coli* that transferred to a given intact lettuce head was 0.5976% (Supplemental Material 5). Similarly, the geometric mean MPN of *E. coli* per lettuce head for positive intact heads was 872 when the outlier was excluded from the dataset (Table 4.2), and 1,014 when the outlier was included in the dataset (Supplemental Material 5).

Among the inner and outer leaf samples, 75% (15/20) and 80% (16/20), respectively, had detectable levels of *E. coli*. The geometric mean MPN of *E. coli* per lettuce head was 35 for positive inner leaves, and 1,106 for positive outer leaves (Table 4.2). The average percent of *E. coli* that transferred from the closest fecal pellet to the outer leaves (0.0030%) was 1.5 log greater than the percent that transferred to the inner leaves (0.0001%; Table 4.2).

On average the $\log_{w0}$ MPN of *E. coli* per lettuce head decreased with the increasing age of the associated fecal pellet (Figure 4.1). For example, the geometric mean $\log_{10}$ MPN of *E. coli* per positive, intact lettuce heads associated with the 2.5, 24 and 48 h fecal pellets was 5,256, 1,042 (without the outlier), and 111, respectively (Table 4.2).

1 *Table 4.2 Summary of E. coli test results for each lettuce head type and different times between fecal placement and harvest. Note*
2 *that information in bold summarizes all data for lettuce heads of a given head type (i.e., inner, outer, and intact heads).*

3

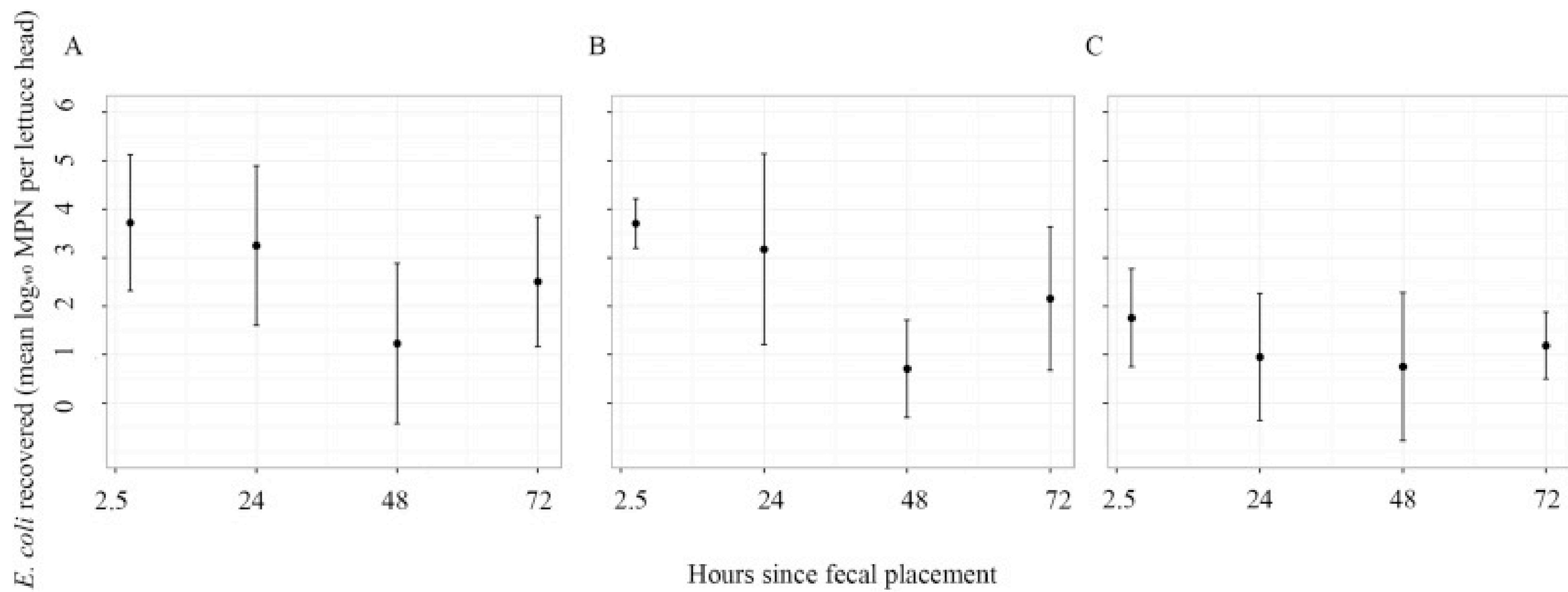
Head Type	Fecal Age (i.e., time between fecal placement and harvest)	No. of Negative Heads (% Negative Heads)	No. of Positive Heads (% Positive Heads)	Minimum and Maximum MPN per positive head [a]	Average MPN per positive head (95% CI [b]) [c]	Average percent of *E. coli* that transferred from the closest fecal pellet to the lettuce [d]
Inner	**-**	**5 (25%)**	**15 (75%)**	**$1.00*10^0$-$3.00*10^3$**	**35 ($2.53*10^{-1}$, $4.90*10^3$)**	**0.0001 %**
	2.5 h	0 (0%)	5 (100%)	$2.16*10^0$-$4.08*10^2$	57 ($5.41*10^{-1}$, $5.98*10^3$)	0.0001 %
	24 h	3 (60%)	2 (40%)	$1.38*10^2$-$4.08*10^2$	237 ($5.12*10^1$, $1.10*10^3$)	< 0.0001 %
	48 h	2 (40%)	3 (60%)	$1.00*10^0$-$3.00*10^3$	18 ($2.40*10^{-3}$, $1.31*10^5$)	0.0003 %
	72 h	0 (0%)	5 (100%)	$2.16*10^0$-$1.38*10^2$	15 ($6.44*10^{-1}$, $3.65*10^2$)	< 0.0001 %
Outer	**-**	**4 (20%)**	**16 (80%)**	**$1.00*10^0$-$2.76*10^5$**	**1,106 ($2.64*10^0$, $4.62*10^5$)**	**0.0030 %**
	2.5 h	0 (0%)	5 (100%)	$3.00*10^3$-$4.08*10^4$	5,056 ($4.90*10^2$, $5.22*10^4$)	0.0059 %
	24 h	1 (20%)	4 (80%)	$3.00*10^3$-$2.76*10^5$	9,291 ($1.01*10^2$, $8.55*10^5$)	0.0055 %
	48 h	2 (40%)	3 (60%)	$1.00*10^0$-$1.38*10^2$	15 ($1.02*10^{-1}$, $2.26*10^3$)	< 0.0001 %
	72 h	1 (20%)	4 (80%)	$2.52*10^1$-$3.00*10^3$	492 ($5.01*10^0$, $4.83*10^4$)	0.0005 %
Intact [e]	**-**	**11 (11%)**	**88 (89%)**	**$1.00*10^0$-$3.00*10^6$**	**872 ($1.00*10^0$, $7.58*10^5$)**	**0.0267 %**
	2.5 h	0 (0%)	25 (100%)	$2.16*10^0$-$3.00*10^6$	5,256 ($8.21*10^0$, $3.36*10^6$)	0.0968 %
	24 h [f]	0 (0%)	24 (100%)	$1.00*10^0$-$4.08*10^4$	1,042 ($4.08*10^0$, $2.67*10^5$)	0.0009 %
	48 h	10 (40%)	15 (60%)	$1.00*10^0$-$2.76*10^5$	111 ($4.28*10^{-2}$, $2.87*10^5$)	0.0056 %
	72 h	1 (4%)	24 (96%)	$1.00*10^0$-$8.40*10^4$	408 ($1.23*10^0$, $1.35*10^5$)	0.0023 %

4

Table 4.2 Continued.

[a] The detection limit of the tripartite enumeration scheme used in the study reported here was 1 MPN per lettuce head.
[b] Confidence Interval
[c] Geometric mean was calculated by taking the average of the $\log_{10}$ MPN per positive head across a time group and raising 10 to the power of this average.
[d] The percent of *E. coli* transferred to the lettuce head was calculated by dividing the MPN per lettuce head by the average CFU of *E. coli* per fecal pellet at the time of placement for the given fecal age and multiplying the product by 100. The average percent transfer was calculated by summing percent transfer for a given head type and fecal age (e.g., inner leaves 2.5 h) and dividing by the number of heads; both positive and negative heads were included in this calculation
[e] One intact head collected from a plot where the fecal pellet was placed 24 h before irrigation had extremely high levels of *E. coli* (i.e., $5.94*10^8$) and could be considered an outlier; this head was excluded from the calculations reported here. When this head was included in the calculations, (i) the maximum MPN per head changed to $5.94*10^8$ (from of $3.00*10^6$), (ii) the average MPN per head changed to 1014 (from 872), and (iii) average percent of *E. coli* that transferred to lettuce heads changed to 0.5976% (from 0.0267%).
[f] When the outlier is included in the calculations for the intact, 24 h row, (i) the maximum MPN per head changed to $5.94*10^8$ (from $4.08*10^4$), (ii) average MPN per head changed to 1,771 (from 1,042), and (iii) average percent of *E. coli* in the fecal pellets that transferred to lettuce heads changed to 2.2855% (from 0.0009%).

Figure 4.1. Mean logw0 MPN of E. coli per lettuce head on (A) intact heads, (B) outer leaves, and (C) inner leaves for each fecal age (i.e., fecal pellet placement at 2.5, 24, 48 and 72 h before irrigation); specific data points are shown in Table 4.2. The bars represent the standard deviation for each fecal age.



Factors associated with the percent of *E. coli* transferred from simulated wildlife feces to lettuce during foliar irrigation. Of the 8 factors that were included in the full model, only 5 factors (fecal age, head type, lettuce-feces distance, lettuce-sprinkler distance, and lettuce-post-irrigation pool distance) were retained in the final model for the $\log_{w0}$ percent of *E. coli* that transferred from the fecal pellets to the lettuce (transfer model; Table 4.3). The same 5 factors were also retained in the final model for the $\log_{w0}$ MPN of *E. coli* on the lettuce heads at harvest (concentration model; Table 4.4). The full transfer and concentration models were pared down using backwards selection based on the AIC as described in section 2.6. According to the transfer model the $\log_{w0}$ percent of *E. coli* that transferred from the fecal pellets to the lettuce was significantly greater for lettuce heads near 2.5 h fecal pellets compared to heads near 24 h (effect= -1.5), 48 h (effect= -2.6) and 72 h (effect= -1.0) fecal pellets (Table 4.3); this translates to a 97%, 100% and 90% decrease in the percent of *E. coli* that transferred to heads near 24 h, 48 h, and 72 h feces, respectively. For each 10 cm increase in the distance between the lettuce and the closest fecal pellet the $\log_{w0}$ percent of *E. coli* that transferred from the fecal pellets to the lettuce heads decreased, on average, by 0.3, representing a 54% decrease in the percent of *E. coli* transferred for each 10 cm increase in the distance between the lettuce and closest fecal pellet (Table 4.3). Moreover, the $\log_{w0}$ percent of *E. coli* that transferred from the fecal pellets to the lettuce heads was also significantly greater for outer leaves (effect = 1.3) and intact heads (effect = 1.9) compared to inner leaves (Table 4.3); this translates to an 1,884%, and 7,120% increase in the percent of *E. coli* that transferred to outer leaves and intact heads, respectively (relative to inner leaves).

Table 4.3 Final multivariable model for the log_{w0} *percent of E. coli transferred from the fecal pellets to the lettuce heads.*[a]

Factors	Effect	95% CI[b]	*P-value*
Fecal age	-	-	<0.001
2.5 h	0.00 [c]	-	-
24 h	-1.49	(-2.14, -0.84)	<0.001
48 h	-2.56	(-3.24, -1.89)	<0.001
72 h	-1.02	(-1.70, -0.33)	0.004
Head Type	-	-	<0.001
Inner Leaves	0.00 [d]	-	-
Outer Leaves	1.29	(0.45, 2.14)	0.003
Intact Head	1.86	(1.19, 2.52)	<0.001
Distance between Lettuce and Feces (10 cm) [e]	-0.34 [f]	(-0.44, -0.24)	<0.001
Distance between Lettuce and Post-irrigation pools (m) [g]	-0.08 [h]	(-0.15, -0.01)	0.022
Distance between Lettuce and Sprinkler (m) [i]	-0.08 [j]	(-0.15, -0.02)	0.008

[a] Percent *E. coli* transferred is the MPN for a given positive lettuce head divided by the average CFU of *E. coli* per fecal pellet at the time of placement for the given fecal age. For use in the model these values were transformed using the log_{w0}transformation. The effect estimates and 95% confidence intervals for the effect estimates are reported for the transformed data.
[b] Confidence Interval
[c] Compared to lettuce heads that were near fecal pellets that were placed 2.5 before irrigation there was a 97%, 100% and 90% decrease in the percent of *E. coli* transferred to lettuce heads near fecal pellets that were placed 24 h, 48 h and 72 h before irrigation, respectively.

Table 4.3 Continued.

[d] Compared to inner heads there was a 1,884%, and 7,120% increase in the percent of *E. coli* transferred to outer heads and intact heads, respectively.
[e] The minimum, maximum and mean distance between the lettuce heads and the closest fecal pellet were 3 cm, 99 cm and 49 cm, respectively.
[f] There was a 54% decrease in the percent of *E. coli* transferred for each 10 cm increase in the distance between the lettuce heads and the closest fecal pellet (e.g., from 0 to 10 cm, from 50 cm to 60 cm).
[g] The minimum, maximum and mean distance between the lettuce heads and the nearest post-irrigation pool were 0.0 m, 11.9 m and 3.4 m, respectively.
[h] There was a 17% decrease in the percent of *E. coli* transferred for each 1 m increase in the distance between the lettuce heads and the closest pool (e.g., from 0 to 1 m, from 5 to 6 m).
[i] The minimum, maximum and mean distance between the lettuce heads and the nearest sprinkler upwind of the lettuce head were 3.0 m, 17.9 m and 11.2 m, respectively.
[j] There was a 18% decrease in the percent of *E. coli* transferred for each 1 m increase in the distance between the lettuce heads and the closest sprinkler head (e.g., from 0 to 1 m, from 5 to 6 m).

Table 4.4 Final multivariable model for the log_{w0}MPN of E. coli on lettuce heads.[a]

Factors		Effect	95% C[b]	*P-value*
Fecal age		-	-	< 0.001
	2.5 h	0.00[c]	-	-
	24 h	-0.62	(-1.15, -0.09)	0.024
	48 h	-1.84	(-2.38, -1.29)	< 0.001
	72 h	-0.78	(-1.34, -0.23)	0.007
Head Type		-	-	< 0.001
	Inner Leaves	0.00[d]	-	-
	Outer Leaves	1.24	(0.56, 1.93)	0.001
	Intact Head	1.65	(1.11, 2.19)	< 0.001
Distance between Lettuce and Feces (10 cm)[e]		-0.28[f]	(-0.36, -0. 20)	< 0.001
Distance between Lettuce and Post-irrigation pools (m)[g]		-0.06[h]	(-0.12, -0.01)	0.029
Distance between Lettuce and Sprinklers (m)[i]		-0.08[j]	(-0.13, -0.03)	0.003

[a] For use in the model the MPN of *E. coli* on each lettuce head was transformed using the log_{w0}transformation; the effect estimates and 95% confidence intervals for the effect estimates are reported for the transformed data.
[b] Confidence Interval
[c] Compared to lettuce heads that were near fecal pellets that were placed 2.5 before irrigation there was a 76%, 99% and 84% decrease in the MPN of *E. coli* on the lettuce heads near fecal pellets that were placed 24 h, 48 h and 72 h before irrigation, respectively.

Table 4.4 Continued.

[d] Compared to inner leaves there was a 1,815%, and 4,821% increase in the MPN of *E. coli* on outer leaves and intact heads, respectively.
[e] The minimum, maximum and mean distance between the lettuce heads and the closest fecal pellet were 3 cm, 99 cm and 49 cm, respectively.
[f] There was a 48% decrease in the MPN of *E. coli* on the lettuce heads for each 10 cm increase in the distance between the lettuce heads and the closest fecal pellet (e.g., from 0 to 10 cm, from 50 cm to 60 cm).
[g] The minimum, maximum and mean distance between the lettuce heads and the nearest post-irrigation pool were 0.0 m, 11.9 m and 3.4 m, respectively.
[h] There was a 14% decrease in the MPN of *E. coli* on the lettuce heads for each 1 m increase in the distance between the lettuce heads and the closest pool (e.g., from 0 to 1 m, from 5 to 6 m).
[i] The minimum, maximum and mean distance between the lettuce heads and the nearest sprinkler upwind of the lettuce head were 3.0 m, 17.9 m and 11.2 m, respectively.
[j] There was a 16% decrease in the MPN of *E. coli* on the lettuce heads for each 1 m increase in the distance between the lettuce heads and the closest sprinkler head (e.g., from 0 to 1 m, from 5 to 6 m).

Survival of *E. coli* in simulated wildlife feces under field conditions. All fecal pellets had detectable levels of *E. coli* at the time of harvest (detection limit = 45 MPN of *E. coli* per fecal pellet; Table 4.1). *E. coli* concentrations increased between fecal placement and harvest for fecal pellets placed 2.5 h and 24 h before irrigation, but decreased for the fecal pellets placed 48 h and 72 h before irrigation (Table 4.1). A regression model for the change in the *E. coli* concentration per fecal pellet between fecal placement and harvest showed, that among the 5 factors included in the full model, only fecal age ($P=0.04$) was retained in the final model (Table 4.5). While fecal pellets placed 48 and 72 h before irrigation had approx. 2-3 log lower *E. coli* concentrations at harvest (relative to the average concentration of *E. coli* in the 48 and 72 h fecal pellets at placement, respectively; Table 4.1), only the die-off in the fecal pellets placed 48h before irrigation was significantly different from the 2.5 h baseline (Table 4.5).

Table 4.5. Final multivariable model for the change in the log_{10} MPN of E. coli per fecal pellet between fecal placement and harvest.[a]

Factors		Effect	95% C[b]	*P-value*
Fecal Age		-	-	0.041
	2.5 h	0.00	-	-
	24 h	-0.25	(-2.51, 2.00)	0.816
	48 h	-2.89	(-5.14, -0.63)	0.015
	72 h	-2.06	(-4.32, 0.19)	0.070

[a] For use in the model the MPN of *E. coli* in each fecal pellet at harvest (C_h) was divided by the average CFU of *E. coli* in the fecal pellets at the time of fecal placement (C_0). The quotient (C_h/ C_0) was then transformed using the log10 transformation; the effect estimates and 95% confidence intervals for the effect estimates are reported for the transformed data.
[b] Confidence Interval

Discussion

Comparison of transfer coefficient estimates for 2 studies conducted in different produce-growing regions of the United States. The objective of this study was to estimate *E. coli* transfer coefficients from simulated wildlife feces to field-grown lettuce during foliar irrigation. Our study adapted the methods used by Atwill et al. (2015), who examined the transfer of an attenuated *E. coli* O157:H7 strain from simulated wildlife feces (inoculum = $1.29*10^8$ CFU per 5 g fecal pellet) to field-grown lettuce during a 2.5 h irrigation event in Salinas, CA. While our findings were generally consistent with Atwill et al. (2015), the number of positive heads that we observed and the transfer coefficients that we calculated were substantially greater than those reported by Atwill et al. (2015). For example, the proportion of intact heads (89%), and outer (80%) and inner (75%) leaves with detectable levels of *E. coli* in our study were significantly greater ($P<0.001$ for all head types) than the corresponding proportions (38%, 25% and 0%, respectively) reported by Atwill et al. (2015). The maximum MPN per lettuce head observed in our study ($3.00*10^6$) was also an order of magnitude greater than the maximum MPN per lettuce head observed by Atwill et al. [$2.30*10^5$; (2015)] when the outlier was excluded from the dataset; the difference was approx. 3 orders of magnitude when the outlier (i.e., a lettuce head with $5.94*10^8$ MPN of *E. coli*) was included in our dataset. Additionally, the percent of *E. coli* in the fecal pellets that transferred to intact heads was approx. 5 times greater in our study (0.0267%) compared to Atwill et al. [0.006%; (2015)]. Since we used a 3 strain-cocktail of non-pathogenic *E. coli* and Atwill et al. (2015) used an attenuated strain of *E. coli* O157:H7, strain differences (e.g., with regard to stress resistance, expression of

adhesins) could contribute to the higher transfer coefficients observed here as compared to Atwill et al. (2015). However, differences in management practices may be more likely to account for the observed differences in transfer coefficients; specifically, the amount of irrigation water applied in our study (approx. 25 mm) was at least 6 times the amount applied by Atwill et al. [1.25-3.85 mm; (2015)] during the same time period (~2.5 h). The larger volume of water used in our study may have (i) altered fecal pellet consistency (e.g., softening the pellets) facilitating the release of *E. coli* from the feces (e.g., transfer via splash, erosion of the fecal pellets), which will be discussed in more detail later, and (ii) facilitated transfer by moving *E. coli* within the field (e.g., in runoff, flooded furrows, and in-field pools). In fact, we found a significant, positive association between the percent of *E. coli* transferred, and the distance between the lettuce head and pools that formed during irrigation. Environmental conditions, including weather, also may have impacted the findings of both studies. Further studies, with a standardized strain cocktail and study design, in different regions, and under different weather conditions will thus be needed to assess the specific effect of management practices, weather, and other factors on the transfer of *E. coli* from wildlife feces to field-grown produce via splash.

Removal of outer leaves may reduce pre-harvest food safety risks associated with fresh lettuce. Both Atwill et al. (2015) and the study reported here found that outer leaves were significantly more likely than inner leaves to become contaminated via splash. While no other peer-reviewed studies, with the exception of Atwill et al. (2015), have examined the splash of *E. coli* from feces to lettuce, one study (Oliveira et al., 2012) investigated the transfer of *E. coli* O157:H7 from (i)

contaminated compost (inoculum = 10^6 CFU/g; calculated using data reported by Oliveira et al., 2012), and (ii) soil irrigated with contaminated water (inoculum = 10^7 CFU/mL) to lettuce in two separate trials. In both trials *E. coli* O157:H7 was more frequently detected on outer leaves compared to inner leaves (Oliveira et al., 2012). Interestingly, several studies (Brandl and Amundson, 2008; Mootian et al., 2009; Van der Linden et al., 2013) that irrigated lettuce with or immersed lettuce in contaminated water found that *E. coli* concentrations on inner leaves were, on average, higher than the concentration on middle and outer leaves. This is logical since past studies (Brandl and Amundson, 2008; Peleg, 2006; Van der Linden et al., 2013) also found that bacteria are more likely to survive in protected sites, and sites with conditions conducive to bacterial growth, such as the inner leaves. For example, Brandl and Amundson (2008) found that bacteria, including *E. coli,* colonized younger, inner leaves at higher densities and more locations compared to older, outer leaves due to greater nutrient availability on the inner leaves. Based on the findings of this and other studies (Atwill et al., 2015; Oliveira et al., 2012) we can conclude that *E. coli* is more likely to splash to outer leaves than inner leaves. Thus, while outer leaves may act as a source of inner leaf contamination during harvest and post-harvest, once contamination occurs *E. coli* may be more likely to survive and proliferate on inner leaves. Therefore, removing the outer leaves at harvest, which is current industry practice, eliminates the part of the lettuce head that is most likely to become contaminated with a high bacterial load by splash from in-field wildlife feces.

Effect of time on the transfer of *E. coli* from simulated feces to lettuce under field conditions. In our study we observed limited evidence for significant

changes in the concentration of *E. coli* in the fecal pellets between placement and harvest, which occurred at 2.5, 24, 48, and 72 h after placement. The change in the *E. coli* concentration in the fecal pellets between placement and harvest ranged from an increase of 0.27 $\log_{10}$ MPN (2.5 h) to a decrease of 2.62 $\log_{10}$ MPN (48 h). However, the only change that was significantly different from the 2.5 h baseline was the change in the *E. coli* concentration in the fecal pellets that were placed 48 h before irrigation. This is not unexpected as past studies that examined *E. coli* survival in feces did not observe die-off until several days after fecal deposition [3-13 days; (Guber et al., 2015; Oladeinde et al., 2014; Soupir et al., 2008; Van Kessel et al., 2007)]. Although *E. coli* die-off in the fecal pellets placed 24 h and 72 h before irrigation did not differ significantly from the 2.5 h baseline, the $\log_{w0}$ percent of *E. coli* in the fecal pellets that transferred to the lettuce was significantly greater for lettuce near 2.5 h fecal pellets compared to 24, 48 and 72 h pellets. This may be attributable to the larger sample size per fecal age for the lettuce (N=35) compared to the fecal pellets (N=5), which provided for greater power to detect differences in *E. coli* concentration on the lettuce head. This difference may also suggest that, in addition to bacterial die-off and growth, other factors also affected the transfer of *E. coli* from the fecal pellets to the lettuce heads. For example, changes in fecal pellet consistency or moisture content may have altered the structure of the pellet surface, increasing the amount of energy needed to detach fecal particles and *E. coli*. While no study, to the authors' knowledge, has examined the impact of fecal structure on the splash kinetics of *E. coli*, several studies have examined the effect of fecal structure on the transfer of other organisms. For example, a study that examined the dispersal of *Cooperia* larva from

cow pats via splash found that immediately following a rain event no *Cooperia* was transferred from the pats due to the formation of a dry crust on the surface of the pats (Gronvold, 1984). Studies (Kress and Gifford, 1984; Thelin and Gifford, 1983) on the release of fecal coliforms from cow pats during rain events also noted that a dry crust formed on older pats, and that pat moisture content was positively associated with the release of fecal coliforms. Fecal pellet consistency may also be affected by the volume of water applied during irrigation (e.g., by softening the pellet, causing puddles to form on the pellet surface). Therefore, the larger volume of irrigation water applied in this study compared to Atwill et al. (2015) may have facilitated transfer, and may explain the larger transfer coefficients reported here compared to Atwill et al. (2015). To effectively model *E. coli* survival in feces, and its impact on the transfer of *E. coli* from feces to pre-harvest produce, future studies need to (i) increase the number of fecal pellets collected per time point, and (ii) collect data on changes in fecal structure, moisture content and consistency over time.

The effect of geospatial factors on the transfer of *E. coli* from feces to lettuce under field conditions. In the study reported here the same geospatial factors were retained in the final model for the $\log_{w0}$ percent of *E. coli* transferred, and the final model for the $\log_{w0}$ concentration of *E. coli* on the lettuce at harvest. One of these geospatial factors (lettuce-feces distance) was also retained in one the models for the concentration of *E. coli* on the lettuce at harvest reported by Atwill et al. (2015). This suggests that the relationship between *E. coli* transfer and lettuce-feces distance is reproducible. This is consistent with previous studies (Butterworth and McCartney, 1991; Girardin et al., 2005; Gronvold, 1984; Monaghan and Hutchison, 2012; Penet et

al., 2014) that found a strong negative correlation between the number of splash droplets and the distance from the splash origin, which was the fecal pellets in the study reported here. For example, under laboratory conditions, the number of *Colletotrichum gloeosporiodes* spores transported by splash decreased exponentially as the distance from the origin increased (Penet et al., 2014). Similarly, a study that examined the transfer of *E. coli* from contaminated soil to agar strips found that bacterial growth covered 16-18% of the strips 0-10 cm from the splash origin but covered less than 2% of the strips 25-45 cm from the origin (Monaghan and Hutchison, 2012). Since more energy is required to transport drops farther, one would expect fewer splash droplets and less bacteria per droplet to reach lettuce heads that were farther from the fecal pellets compared to heads that were closer to the fecal pellets, which was observed. Thus, the likelihood of *E. coli* transfer from feces to produce should be minimal past a given distance. Establishing a no-harvest buffer at this distance around in-field feces may therefore reduce the risk of harvesting microbially contaminated produce. Since all lettuce heads in our study were within 1 m of the fecal pellets we cannot make any recommendations on the use of buffers > 1 m in size. However, our findings suggest that a no-harvest buffer of 0.5 m around in-field wildlife feces would reduce the proportion of *E. coli* transferred by approximately 1.5 $\log_{10}$ (i.e., a 98% decrease in the percent of *E. coli* transferred to lettuce heads 0 cm from a fecal pellet compared to lettuce heads 50 cm from a fecal pellet). Additional research is needed (i) to test the robustness of our findings, and (ii) to quantify the transfer reductions expected for produce that is more than 1 m from in-field feces.

Similar to lettuce-feces distance, lettuce-sprinkler distance, which was another of the geospatial factors retained in our lettuce models, may also affect splash kinetics. While Atwill et al. (2015) did not include lettuce-sprinkler distance as a candidate factor in their models for the concentration of *E. coli* on the lettuce, they did include and retain feces-sprinkler distance. The identification of lettuce-sprinkler and feces-sprinkler distance makes logical sense because the horizontal and vertical distance that a drop travels from the sprinkler to the fecal pellets will affect the force of the drop at impact. Interestingly, studies on splash kinetics have also found that drop size is correlated with the kinetic energy of the drop, and therefore the distance that splash droplets will travel (Kincaid, 1996; Ntahimpera et al., 2007; Perryman et al., 2014). Past studies have also related rain intensity to drop kinetic energy, and splash mediated dispersal (Madden, 1997; Quansah, 1981; Yang et al., 1990). In fact, a study (Quansah, 1981) that examined the detachment and splash of soil particles during a simulated rain event found that rain intensity was one of the most important factors associated with splash mediated transport of soil particles. Since the amount of irrigation water applied to the field in the study reported here (approx. 25 mm over 2.5 h) was at least 6 times greater than that applied by Atwill et al. [1.25-3.85 mm over 2.5 h; (2015)], one can conclude that the intensity of water application was greater in this study compared to Atwill et al. (2015). This difference in irrigation intensity may explain the discrepancy between the transfer coefficients reported here and by Atwill et al. (2015). Irrigation intensity, and similar factors also may affect the subsequent wash-off of *E. coli* from the lettuce following splash; splash and wash-off are both captured in the transfer coefficients presented here. Overall, this suggests that

irrigation system set-up (e.g., water pressure, sprinkler setting, sprinkler height) may affect the transfer of *E. coli* from feces to lettuce. Therefore, altering the irrigation set-up (e.g., reducing water pressure, changing sprinkler head to reduce drop size) or type (e.g., from overhead sprinkler to drip) may be a risk management strategy that growers can use to reduce the likelihood of pathogen transfer from in-field feces to pre-harvest produce.

Conclusion

Our study was designed to simulate a study conducted by Atwill et al. (2015) in Salinas, CA, in another region of the country and with slight modifications. The study presented here and Atwill et al. (2015) are, to the authors' knowledge, the only peer-reviewed studies that have examined the transfer of *E. coli* from wildlife feces to field-grown lettuce via splash. Many of our findings (e.g., that fecal age is significantly associated with *E. coli* transfer from the fecal pellets to the lettuce heads) are consistent with those of Atwill et al. (2015). However, the average transfer coefficient associated with *E. coli* splash from wildlife feces to intact lettuce was approx. 5 times greater in the study reported here compared to Atwill et al. (2015). This difference could be due to a number of factors, including management practices (e.g., amount of irrigation water applied), and environmental conditions (e.g., New York versus California). Additional research is therefore needed to determine which of these factors are most important in determining the efficiency of transfer, and to assess the validity of generalizing field data on pathogen transfer to growing regions other than those in which the data were originally collected. Despite the need for additional

research, we can conclude that bacteria are readily transferred from in-field wildlife feces to field-grown lettuce via splash during foliar irrigation.

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Supplemental Material

Supplemental material associated with this article can be found online at: www.sciencedirect.com.proxy.library.cornell.edu/science/article/pii/S0740002016310310#appsec1.

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CHAPTER 5

SURVIVAL OF *ESCHERICHIA COLI* ON LETTUCE UNDER FIELD CONDITIONS ENCOUNTERED IN THE NORTHEASTERN UNITED STATES

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Abstract

While wildlife intrusion and untreated manure have been associated with microbial contamination of produce, relatively few studies have examined the survival of *Escherichia coli* on produce under field conditions following contamination (e.g., via splash from wildlife feces). This experimental study was performed to estimate the die-off rate of *E. coli* on pre-harvest lettuce following contamination with a fecal slurry. During August 2015, field-grown lettuce was inoculated, via pipette, with a fecal slurry that was spiked with a 3-strain cocktail of rifampicin resistant, non-pathogenic *E. coli*. Ten lettuce heads were harvested at each of 13 different time points following inoculation (0, 2.5, 5, and 24 h after inoculation, and every 24 h thereafter until day 10). The most probable number (MPN) of *E. coli* on each lettuce head was determined and die-off rates were estimated. The relationship between time and the $\log_{10}$ MPN of *E. coli* per head was modeled using a segmented linear model. The segmented linear model had a breakpoint at 106 h (Confidence Interval = 69, 142 h) following inoculation, with a daily decrease of 0.70 and 0.19 $\log_{10}$ MPN, for 0-106 h and 106-240 h following inoculation, respectively. The findings reported here are consistent with die-off rates observed in similar studies that assessed *E. coli* survival

on produce following irrigation. Overall, the findings reported here provide die-off rates for *E. coli* on lettuce that can be used in future quantitative risk assessments.

Introduction

Between 2003 and 2012 *Escherichia coli* O157 outbreaks reported in the United States (US) sickened 4,928, hospitalized 1,272, and killed 33 people (1). A number of recent *E. coli* O157 outbreaks have been associated with leafy greens [e.g., (2–4)], including a 2006 outbreak linked to fresh spinach that sickened 199, hospitalized 102, and killed 3 people throughout the United States (5). Microbial contamination of fresh produce, including leafy greens, can occur in the field (6–8), in processing environments [e.g., in packing houses or fresh-cut operations; (7, 8)], and immediately prior to consumption [e.g., in the home (8)]. Multiple foodborne disease outbreaks associated with leafy greens also have been traced back to probable pre-harvest contamination events (9–12). Thus, understanding the survival and transmission of foodborne pathogens in the pre-harvest environment is essential for developing effective and feasible strategies for reducing the foodborne disease risks associated with the consumption of produce.

Foodborne pathogens, including *E. coli* O157 and other Shiga-toxin producing *E. coli*, have been isolated from a variety of wild and domestic animals, indicating their potential to serve as a source of microbial contamination (13–17). Past studies have shown that pathogens present in wildlife scat and untreated manure can be transferred to produce following defecation in or application to produce fields (6, 18–20). For example, Atwill et al. (6) showed that *E. coli* O157:H7 in simulated wildlife feces could be transferred to field-grown lettuce via splash during foliar irrigation. The use of fecally-contaminated water for irrigation or frost protection can also serve as a direct route of produce contamination (21, 22). In fact, cases of foodborne disease

have been associated with wildlife intrusion into produce fields (23–26), and the use of contaminated surface water for produce production (12, 27, 28). However, pathogen populations that transfer to produce die off under field conditions over time (22, 29–31). Thus, die-off rates can be used in quantitative risk assessments to identify potential intervention and control strategies for reducing food safety risks associated with fresh produce consumption. For example, die-off rates can be used in risk models to estimate levels of contamination on produce at specific times following potential contamination events (32).

A number of studies have investigated bacterial die-off rates on field-grown produce, and reported mean die-off rates for *E. coli* (22, 29, 31, 33) ranging from 0.4 to 1.64 log_{10} CFU d^{-1}. For example, Wood et al. (22) observed die-off rates ranging between 0.54 to 1.64 log_{10} CFU of *E. coli* O157:H7 d^{-1} on field-grown spinach in Nova Scotia, Canada. By comparison, daily die-off rates for *Salmonella* on field-grown spinach and lettuce in the United Kingdom ranged from 0.43 to 0.76 log_{10} CFU d^{-1} (31). Variation in bacterial die-off rates on produce has been associated with multiple factors, including plant health and leaf age (34, 35), environmental conditions (22, 36), and pathogen transfer matrix. For example, Wood et al. (22) found that the time to reach the detection limit for *E. coli* O157:H7 on spinach grown in full sun and in partial shade was 72 to 100 hours, and > 150 hours, respectively. Due to the variability in previously reported die-off rates for *E. coli* on pre-harvest produce, more research is needed to evaluate existing data and generate new data that can be used to determine appropriate risk management strategies for reducing risks associated with the consumption of produce. The study reported here was thus conducted to generate

experimental data on the die-off rate of *E. coli* on fresh produce under field conditions in the Northeastern US, and to subsequently compare the observed die-off rate to previously reported rates for *E. coli* on pre-harvest produce.

Materials and Methods

Field Setup. This field study was conducted in a romaine lettuce (*Lactuca sativa* L. var. longifolia cv. Green Towers; Harris Seeds, Rochester NY) field at the Homer C. Thompson Vegetable Research Farm in Freeville, New York. Throughout the growing season a combination of tine weeding, hand weeding and rototilling was used to thin the lettuce heads (at ~ 4 weeks) and weed the study field. The field consisted of an 8.5 m x 59.5 m planted area with 5 longitudinal beds (each bed was 1.2 m wide) separated by 0.6 m furrows (see map provided in Supplemental Materials 1; https://foodsafety.foodscience.cornell.edu/research-and-publications/supplementary-materials-manuscripts/2017). Each bed consisted of 4 rows of seed planted 0.4 m apart (20 rows in total). Seeding was performed using a mechanical seeder (Monosem Inc., Edwardsville, KS) and a 1.5 in (3.81 cm) seeding rate was achieved. The field was surrounded by a bare ground buffer of at least 3.1 m on each side. Overhead impact sprinklers were spaced around the field (with approx. 15 m between sprinklers); irrigation occurred as needed up to one week before inoculation of the lettuce with *E. coli*.

One hundred and thirty lettuce heads growing in the study field were randomly selected for inclusion in the study. The 130 heads were randomly divided into one of 13 treatment groups. Each treatment group of 10 lettuce heads was harvested and quantitatively tested for *E. coli* at a given time point after inoculation of the lettuce

heads; the time points were 0 h, 2.5 h, 5 h, and 24 h after inoculation, and every 24 h thereafter until day 10.

Bacterial Strains. Three rifampicin-resistant non-pathogenic *E. coli* strains [TVS 353, TVS 354 and TVS 355 (37)] were obtained from the University of California, Davis; these strains were used to prepare a 3-strain cocktail that was used for lettuce inoculation. Briefly, each strain was grown in duplicate on tryptic soy agar plates (TSA; Becton, Dickinson and Company, Franklin Lakes, NJ) at 37°C to stationary phase (18-24 h). Each plate was then flooded with 10 mL of phosphate buffered saline (PBS) and the cells were resuspended using a 10 uL loop and 10 mL stripette. Bacterial suspensions were separately transferred into 15 mL Falcon tubes. Following centrifugation at 2,500xG (for 5 min) the culture supernatant was removed. The pellet was washed twice with 10 mL PBS, and re-suspended in 5 mL of PBS. The bacterial suspension was diluted 1:32 in PBS, and the optical density (OD_{600}) was measured. Based on the optical density the culture was diluted in PBS to achieve a concentration of ~ 10^{10} CFU mL^{-1}.

To assess the potential of false positives due to naturally occurring rifampicin resistant *E. coli*, sampling was performed prior to the start of the study. Briefly, 3 composite soil samples, 3 vegetation samples, and 4 water samples were collected from nearby environments, including Fall Creek, the source of irrigation water used in this study. Soil and vegetation samples were diluted 1:2 in PBS, and then, in duplicate, serially diluted to 10^{-11} in tryptic soy broth (Becton, Dickinson and Company) supplemented with 100 mg L^{-1} rifampicin (TSB+R; EMD Chemicals, San Diego, CA). After incubation for 18-24 h at 37°C, 3 µL of each dilution were cross-streaked on *E.*

coli ChromAgar (DRG International, Inc., Springfield, NJ) supplemented with 100 mg L^{-1} rifampicin (ECC+R). The ECC+R plates were then incubated at 42°C for 18-24 h. Water samples were processed as described in Weller et al. (38). Briefly, a 250-mL sample of water was passed through a 0.45 μm filter unit (Nalgene, Rochester, NY). The filter was then transferred to a Whirl-Pak bag (Nasco, Fort Atkinson, WI), and enriched with 90 mL of TSB+R. Following incubation at 37°C for 18-24 h, 50 μL of enrichment were streaked onto ECC+R agar plates, which were incubated at 42°C for 18-24 h. While one soil sample was culture-positive for rifampicin-resistant *E. coli* following plating on ECC+R, we were not able to confirm this culture as *E. coli* using *clpX* PCR.

It is important to note that in this study we used a cocktail of 3 non-pathogenic *E. coli* strains. While the use of non-pathogenic surrogate strains (rather than pathogenic wild type strains) may be considered a drawback, pathogenic *E. coli* could not be used in our study due to biosecurity concerns. The 3-strain cocktail used here was selected since several studies (37, 39) have found that the *E. coli* cocktail used in our study demonstrated greater environmental fitness compared to individual attenuated *E. coli* O157:H7 strains. As a result, the die-off rates for non-pathogenic *E. coli* that are reported in our study provide conservative estimates for pathogenic *E. coli* die-off on pre-harvest, field grown lettuce. Moreover, Gutiérrez-Rodríguez et al. (39) reported that the survival and persistence of pathogenic and non-pathogenic *E. coli* was strain dependent. The use of non-pathogenic surrogates can therefore provide valuable information to further define *E. coli* strain variability with regard to survival on produce.

Fecal Slurry Preparation. Laboratory rabbit (*Oryctolagus cuniculus*; CoVance, Princeton, NJ) feces were used as a proxy for wildlife feces in the study reported here. Past studies (13–15) have identified wild and domestic rabbits as reservoirs for pathogenic *E. coli*. Fifty grams of feces, 200 mL of PBS and 2.5 mL of the 3-strain *E. coli* cocktail (consisting of 0.833 mL culture of each strain) were combined in a sterile filter Whirl-Pak bag (Nasco, Fort Atkinson, WI) and hand massaged for 5 min. The solid matter was then separated from the liquid portion of the fecal-culture mixture by pipetting the liquid portion (designated as the "fecal slurry") into a 50 mL Falcon tube. The fecal slurry was then stored overnight at 4°C. The final concentration of *E. coli* in the fecal slurry ($3.5x10^8$ CFU mL^{-1}) was confirmed on the morning of inoculation by, in triplicate, diluting a 1 mL aliquot of the fecal slurry with PBS, and spiral plating 50 μL of the 10^{-3}, 10^{-4}, and 10^{-5} dilutions on tryptic soy agar plates supplemented with 100 mg L^{-1} rifampicin. Plates were incubated at 37°C for 18-24 h and colonies were enumerated using a Q-Count (Advanced Instruments, Norwood, MA).

Inoculation. Inoculation of each lettuce head was performed by pipetting 1 mL of fecal slurry onto the northernmost lettuce leaf from a height of ~ 7 cm. Due to the volume of inoculum, and lettuce growth structure and leaf shape, the slurry tended to spread across the inoculated leaf toward and onto the stem, and to drip onto lower leaves. It is important to note that the inoculation method used in this study is not representative of all feces-related contamination events in terms of bacteria (rifampicin resistant non-pathogenic *E. coli*), source (rabbit feces) or deposition (in a single spot on the lettuce in the form of a slurry with a high bacterial count). However,

our experimental design allowed us to control for confounders (e.g., feces sources, location of inoculum on the lettuce leaf), and to track *E. coli* die-off in the pre-harvest environment. For example, although *E. coli* contamination of fresh produce is likely to occur at much lower levels than that used in this study, a higher concentration was used here to ensure comparability with previous studies (29, 33, 40). A high initial inoculum was also used to allow for accurate quantification of die-off (33), which was expected to be > 4 log over the 10 day time frame (22, 29, 30). In fact, in their review of studies that examined pathogen die-off on produce Snellman et al. (33) only included studies that used a high initial inoculum because of the difficulty in determining cell densities accurately at low inoculum densities.

Harvest. Harvest occurred at pre-determined time points following inoculation (0, 2.5, 5, and 24 h after inoculation, and every 24 h thereafter until day 10); inoculation occurred 84 days after seeding. Lettuce heads were harvested by teams of two, consisting of a bagger and a harvester each of whom wore gloves. Heads were harvested with gloved hands using a food grade knife. Gloves were changed between collection of each lettuce head, and the knife was decontaminated with a 10% bleach wipe followed by a 70% ethanol wipe. In total 130 lettuce heads were collected (10 heads per time points). All heads were placed in pre-labeled Whirl-Pak bags (Nasco), stored at 4°C, and processed within 3 h of harvest.

Enumeration of *E. coli* on Lettuce. The enumeration methods used in the study described here were adapted from Atwill et al. (6). Briefly, 600 mL of PBS were added directly to each of the Whirl-Pak bags (Nasco) containing a lettuce head. Samples were then hand-massaged for 1 min. Rifampicin resistant *E. coli* were then

enumerated using a most probable number (MPN) determination with 6 dilutions tested in duplicate. In a twelve-well deep well plate (VWR International, Radnor, PA), 1 mL of sample suspension was transferred to each of two wells, which contained 9 mL of TSB+R. Five serial 100 fold dilutions (0.1 mL into 9.9 mL of TSB+R) were subsequently performed starting from each of the two initial wells. Following incubation for 24 h at 37°C, 3 μL from each well were streaked onto ECC+R. The ECC+R plates were then incubated at 42°C for 18-24 h. Blue colonies indicated the presumptive presence of one of the inoculation strains (TVS 353, TVS 354 and TVS 355) used in this study. Detection of the inoculation strains was confirmed on 10% of presumptive positive lettuce heads using PCR amplification and Sanger sequencing of *clpX* as described in Walk et al. (41). Only a subset of isolates from positive lettuce heads was tested by *clpX* PCR and sequencing since all *E. coli* isolates that were tested were confirmed as a *clpX* allelic type that matched one of the inoculation strains. The MPN of cells per head was calculated as described by Cochran (42); the R script used to implement the method outlined by Cochran (42) is reported in Supplemental Materials 2 (https://foodsafety.foodscience.cornell.edu/research-and-publications/supplementary-materials-manuscripts/2017).

Statistical Analysis. All statistical analyses were performed in R (version 3.1, R Core Team, Vienna, Austria). The p-value cut-off for significant results was 0.05 for all analyses. Die-off was visualized by plotting the average $\log_{10}$ MPN per head against time. Die-off per unit time between each time point (e.g., 0 and 2.5 h, 2.5 and 5 h) and each day (e.g., 0 and 24 h, 24 and 48 h) was calculated using the formula:

$\frac{\Delta log_{10}MPN}{\Delta t} = average\ log_{10}MPN_{t-1} - average\ log_{10}MPN_t$, where t-1 and t are the two sampling points of interest, and Δt is the length of time between these two sampling points. To statistically describe the change in log_{10} MPN per head over time a linear regression model was developed. However, past studies have shown that microbial die-off can be biphasic (43, 44), and may be better modeled using a segmented linear model or a Weibull model. Using the segmented package in R (45, 46) we conducted a Davies test to determine if the linear model included a non-constant regression parameter (this is the breakpoint), and developed a segmented linear model. We then retested the segmented linear model to determine if there was a second breakpoint. Using the nlsMicrobio package in R (47) we developed a Weibull model as parameterized by Mafart et al. (48). The formula for the Weibull model is $n_t = n_0 - (t/\delta)^p$, where n_t = log_{10} MPN of *E. coli* at time t, n_0 = log_{10} MPN of *E. coli* at time 0, δ = time to the first decimal reduction, and p = a parameter that describes the concavity of the curve described by the model. Akaike's information criteria (AIC) was used to determine whether the linear, segmented linear or Weibull model best fit the data.

Die-off was calculated using a log_{10} transformation because this is the transformation traditionally used by microbiologists, as well as by industry and government stakeholders. To provide decay rates for modeling purposes (i.e., k in $C_t = C_i + e^{kt}$, where C_t is concentration at time *t,* and C_i is initial concentration), the slopes of the linear and segmented linear models are also reported using a natural logarithm transformation.

Weather conditions for the day of lettuce head inoculation, and 1-9 d after lettuce head inoculation are reported in Supplemental Materials 3 (https://foodsafety.foodscience.cornell.edu/research-and-publications/supplementary-materials-manuscripts/2017). Weather data were obtained from the Cornell University weather station (Rainwise Inc., Trenton, NJ) located at the Homer C. Thompson Vegetable Research Farm as described in Weller et al. (38). Linear regression was used to statistically describe the relationship between the log_{10} MPN of *E. coli* per head and weather (this will be referred to as the weather model). The dependent variable of the model was the log_{10} MPN of *E. coli* per head. The explanatory variables were (i) the period of time between inoculation and harvest (hours), (ii) average temperature, relative humidity, and wind speed for the 24 h preceding harvest, (iii) total leaf wetness for the 24 h preceding harvest, and (iv) whether the lettuce head was harvested before or after the rain event (~7.1 mm) that occurred between 64 and 69 h after inoculation (post-rain). The interaction between hours and the post-rain variable was also included in the model. The full model was reduced by backwards stepwise regression based on AIC. Briefly, each variable was removed from the full model and the AIC determined. The variable whose removal resulted in the largest decrease in AIC was removed from the model. This process was repeated until the removal of additional variables failed to reduce the AIC.

Results and Discussion

The observed die-off rate for *E. coli* was 0.52 log_{10} MPN per day. During the 240 h between inoculation and harvest on day 10 the average log_{10} MPN per head decreased from 8.86 to 3.64 (Figure 5.1), a log reduction of 5.22. On average, we

observed a die-off rate of 0.52 log_{10} MPN d^{-1} [95% Confidence Interval (CI) = 0.17, 0.87; Table 5.1]; this falls within the range of previously reported daily die-off rates for *E. coli* on produce, which ranged from 0.4 to 1.64 log_{10} MPN d^{-1} (22, 29, 31, 33). The observed die-off rate was also similar to die-off rates that can be calculated using the findings of Barker-Reid et al. [0.44 log_{10} d^{-1} for non-pathogenic *E. coli* on uninjured lettuce (49)], and Bezanson et al. [0.56 log_{10} CFU d^{-1} for *E. coli* O157:H7 on lettuce (30)]. Further comparisons of the die-off rate observed in this study and previous studies will be presented in subsequent sections.

Figure 5.1. E. coli *levels (log MPN per lettuce head) for each time point (e.g., 0, 2.5, and 5 h) reported as mean (gray points) and standard deviation (gray bars) and minimum and maximum (blue shading). The linear regression (A), segmented linear (B), and Weibull (C) models describe* E. coli *die-off over time.*

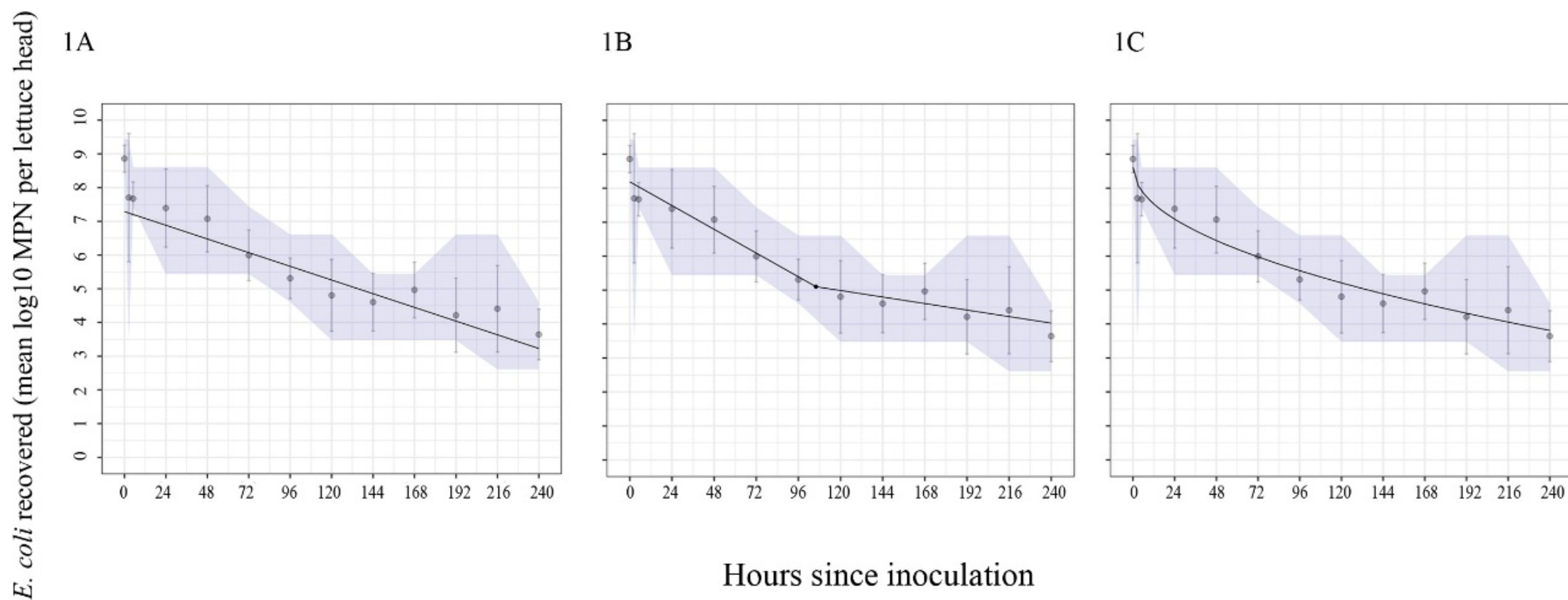

Table 5.1. Average die-off of inoculated E. coli *on lettuce heads grown under field conditions*

First time point (h)[a]	End time point (h)	Mean daily die-off rate (log MPN day^{-1})[b]
0	24	1.47
24	48	0.32
48	72	1.08
72	96	0.68
96	120	0.51
120	144	0.20
144	168	−0.36
168	192	0.75
192	216	−0.19
216	240	0.76
Mean for each 24-h period		0.52[c]

[a] Total die-offs within the first 24 h were 1.16, 0.03, 0.28 log MPN for 0 to 2.5, 2.5 to 5, and 5 to 24 h, respectively.
[b] Difference in log MPN per lettuce between first and end time points.
[c] 95% confidence interval = 0.169, 0.871 log MPN per day

***E. coli* is still detectable on the lettuce 10 d after inoculation.** It is important to note that 10 days after inoculation, *E. coli* was still detectable on the lettuce heads [Figure 5.1; mean $\log_{10}$ MPN of *E. coli* 10 days post inoculation (dpi) = 3.64; standard deviation = 0.75]. By comparison past studies that used high inoculum levels for *E. coli* (10^5 to 10^9 CFU mL^{-1}) found a range of times until *E. coli* numbers dropped below detection limits for the respective methods used (29, 50–52). For example, a study conducted in Georgia, US (40) found that *E. coli* O157:H7 was still detectable on lettuce 77 days after fertilization with contaminated manure (inoculum = 10^7 CFU g^{-1}); similarly *E. coli* O157:H7 was still detectable on lettuce 77 days after irrigation with contaminated water (inoculum = 10^5 CFU mL^{-1}). However, another study conducted in Georgia, US, found that *E. coli* was not detectable by enrichment 7 days after irrigation with contaminated water [inoculum = 10^6 CFU mL^{-1}; (51)]. In comparison, 82% of lettuce samples in a multi-year study in California had less than 10 *E. coli* cells per head 7 dpi [inoculum = 10^7 CFU of *E. coli* O157:H7 mL^{-1}; (29)]. Past studies have also found that environmental conditions, including weather and season, (22, 36, 37, 53) appear to be associated with *E. coli* survival on pre-harvest produce. For example, Xu et al. (36) found that *E. coli* populations on field-grown spinach increased by up to 1 log following < 20 mm of rain, while *E. coli* populations decreased following > 35 mm of rain. Future studies with multiple replicates over time (e.g., multiple growing seasons, multi-year studies, staggered planting of fields) and/or space (e.g., trials on different farms, regions) will be needed to further assess and quantify the effect of environmental conditions on *E. coli* die-off on field-grown produce.

Segmented linear model indicates that *E. coli* die-off follows a biphasic pattern with rapid initial die-off over the first approximately 100 h and more gradual die-off thereafter. A linear regression model built with the data generated here predicted a mean daily decrease of 0.46 $\log_{10}$ MPN of *E. coli* per lettuce head (95% CI = 0.38, 0.50; Table 5.2; Figure 5.1A), which is similar to the daily die-off rate reported by previous studies (22, 29, 31, 33). The linear model accounted for 66% of all variation in the $\log_{10}$ MPN decrease per head observed here ($R^2 = 0.66$). However, the raw data suggested a biphasic decrease (Figure 5.1B), which may be better represented by a segmented linear model. Using a Davies test, we determined that there was a non-constant regression parameter in the linear predictor ($P < 0.001$). Therefore, we developed a segmented linear model (Table 5.2; Figure 5.1B) with a breakpoint at 106 hours (95% CI= 69 h, 142 h). After visually examining the data (Figure 5.1B), we thought there might be a second breakpoint in the first 48 h immediately following inoculation. We therefore ran a second Davies test using the segmented linear model, and identified a second breakpoint at ~5 h. However, this breakpoint was not statistically significant ($P = 0.38$), and was not included in the final segmented model (Table 5.2). The segmented linear model predicts a mean daily decrease of 0.70 (95% CI= 0.55, 0.86) $\log_{10}$ MPN, and 0.19 (95% CI= 0.05, 0.36) $\log_{10}$ MPN, for 0-106 h and 106-240 h, respectively (Table 5.2). The segmented linear model accounted for 71% of all variation in the decrease in $\log_{10}$ MPN per head observed in this study ($R^2 = 0.71$). Since past studies have found that Weibull models accurately describe bacterial die-off (44, 48), we also developed a Weibull model. The formula for the Weibull model is $n_t = n_0 - (t/\delta)^p$, where $n_t = \log_{10}$ MPN of *E. coli* at

time t, $n_0 = \log_{10}$ MPN of *E. coli* at time 0, δ = time to the first decimal reduction, and *p* describes the concavity of the curve described by the model. The Weibull model for the study reported here is $n_t = 8.62 - (t/10.21)^{0.50}$ (Table 5.3). The AIC for the segmented linear (380) and Weibull (380) models were the same, and were lower than the AIC for the linear model (396; Tables 2 and 3). This suggests that the segmented model and Weibull model are comparable, and better fit the data than the linear model. However, since the parameters of the segmented model have a more intuitive interpretation than the parameters of the Weibull model, we will focus on the segmented model in our discussion.

As part of our analyses we also examined the relationship between the $\log_{10}$ MPN of *E. coli* per head and weather using a linear regression model. Of the 7 factors that were included in the full model, 5 factors were retained in the final model (Table 5.4). The model accounted for 70% of all variation in the decrease in $\log_{10}$ MPN per head observed in this study ($R^2 = 0.70$). The AIC for the model is 381 and the BIC for the model is 401.

Table 5.2. Average die-off of inoculated E. coli *on lettuce heads grown under field conditions*

Model	Slope (95% CI)[b]	Intercept	P	R^2	AIC[c]	BIC[d]
Linear	−0.019 (−0.021, −0.016)	7.79	<0.001	0.66	396	404
Segmented linear			<0.001	0.71	380	395
0–106 h	−0.029 (−0.036, −0.023)	8.19				
106–240 h	−0.008 (−0.015, −0.002)	5.97				

[a] Confidence Interval
[b] The reported parameters are for $\log_{10}$ transformed data. For natural log transformed data, the slope for the linear model is -0.043 (-0.048, -0.037). The slopes for the segmented linear model are -0.068 (-0.084,-0.052), and -0.020 (-0.034, -0.005), for 0-106h and 106-240 h, respectively.
[c] Akaike's information criteria
[d] Bayesian information criterion

Table 5.3. Parameters for a Weibull model that statistically characterizes the relationship between hours from inoculation to harvest and the E. coli level per lettuce head

Model	n_0	Δ	p	AIC[a]	BIC[b]
Weibull Model[c]	8.62	10.21	0.50	380	391

[a] Akaike's information criteria
[b] Bayesian information criterion
[c] The formula for the Weibull model is $n_t = n_0 - (t/\delta)^p$, where $n_t = \log_{10}$ MPN of *E. coli* at time t, $n_0 = \log_{10}$ MPN of *E. coli* at time 0, δ = time to the first decimal reduction, and p describes the concavity of the curve described by the model.

Table 5.4. Parameters for a linear regression model that characterizes the relationship between hours from inoculation to harvest, weather, and the E. coli *level per lettuce head.* [a]

Factor	Effect estimate	95% confidence interval	*P*
Avg relative humidity (%)	−0.115	−0.237, 0.007	0.064
Rain[b]			
Before	0.000		
After	−4.258	−6.880, −1.635	0.002
Hours[c]	−0.040	−0.058, −0.023	<0.001
Interaction between hours and rain	0.039	0.014, 0.064	0.003
Total leaf wetness (min)	0.004	0.000, 0.008	0.083

[a] Lettuce heads that were harvested before or during the rain event were coded 0; heads that were harvested after the rain event are coded 1.
[b] Hours between inoculation and harvest.

Our findings suggest that during the first ~100 hours following inoculation there is a period of rapid *E. coli* die-off, which has also been observed in previous studies (22, 29, 34, 37). Our findings that die-off was biphasic, and was best represented by the segmented linear and Weibull models are also consistent with past studies on *E. coli* die-off conducted in agricultural (30, 44, 54) and non-agricultural environments (43, 55). For example, McKellar et al. (44) evaluated different approaches for modeling *E. coli* die-off on field-grown lettuce using previously published datasets, and found that *E. coli* die-off followed a biphasic pattern with a rapid initial decline. As the study reported here only collected data over 10 days, and only included 4 data points for the first 48 h immediately following inoculation, we were not able to model die-off after 10 days or determine whether additional break-

points occurred during the first 48 h immediately following inoculation. Future studies should therefore (i) collect samples for more than 10 d, and (ii) collect additional data points during the first 48 h following inoculation. However, our data does indicate that the time immediately following inoculation (the first ~100 h) is the most important for *E. coli* reduction due to rapid die-off during this time.

Various mechanisms may explain the biphasic die-off pattern observed here and in other studies (29, 44, 56, 57). One possible explanation could be heterogeneity within the microbial population of the inoculum (e.g., use of multiple strains, heterogeneous bacterial populations in stationary phase), or adaptation of the surviving microbial population to field conditions. Variation in environmental conditions (e.g., inner versus outer leaves) could also cause the biphasic pattern observed in this study. For example, Peleg et al. (58) postulated that microbial die-off is driven by environmental conditions, and as a result, exposed populations (e.g., on outer leaves) decline more rapidly than protected populations (e.g., on inner leaves). In fact, past research has shown that the contamination of inner, younger leaves and other protected areas [e.g., shaded leaves; (22, 35, 51, 59)] facilitates survival. Moreover, studies have associated environmental conditions, such as UV radiation (22, 60, 61) and moisture levels (49, 59) with microbial die-off rates. While analysis of weather patterns showed no evidence of a significant association between temperature and die-off, there appeared to be a significant association between precipitation and die-off (Table 5.4). Specifically the breakpoint identified in the segmented model (at ~106 h, 95% CI = 69, 142 h) occurred shortly after a moderate rain event (~7.1 mm; at 64-69 h). In fact, according to linear regression analysis (i.e., the weather model) the die-off

rate was significantly lower on heads harvested after the rain event that occurred between 64 and 69 h after inoculation compared to lettuce heads that were harvested before the rain event (Table 5.4). While the moderate rain event may have washed bacteria off of the leaves, other factors including relative humidity and leaf wetness also were found to be associated with bacterial die-off (Table 5.4). Since our study was conducted over the course of a single growing season (and we thus lacked a comparison group), the impact of weather is difficult to separate from the impact of time since inoculation. Since the weather model reported in Table 5.4 accounts for slightly less variation in the data (R^2=70%) compared to the segmented model (R^2=71%), the observed biphasic pattern in microbial die-off could be explained almost equally well with or without the explicit consideration of weather. Interestingly, a biphasic die-off pattern for *E. coli* on produce has been reported previously (44) based on experiments conducted under presumably different environmental, including weather, conditions. As such, further studies with larger data sets collected over multiple growing seasons are needed to confirm our findings, and build upon the data presented here.

The die-off rates reported in this and other studies appear to be comparable. The die-off rates that were observed (0.52 $\log_{10}$ MPN d^{-1}) and calculated (0.70 and 0.19 $\log_{10}$ MPN d^{-1}, for 0-106 h and 106-240 h, respectively) as part of this study are at the lower end of the range reported by previous studies [0.4 to 1.64 $\log_{10}$ MPN d^{-1}; (22, 29, 31, 33)]. However, past studies (22, 30, 31, 49) have shown that die-off rates of 0.70 $\log_{10}$ MPN d^{-1} and lower do occur. Moreover, McKellar et al. (44) found that die-off rates were positively associated with inoculum concentration.

Since the inoculum levels for natural contamination events are likely lower than the inoculum levels used in this and other studies (22, 29, 31, 49), die-off rates following actual contamination events may be lower than those reported by past studies. Thus, while die-off rates similar to the rates reported in this study provide conservative estimates for calculating time-to-harvest intervals, their use may overestimate die-off following contamination with low levels of *E. coli* or other, similar bacteria. However, the daily die-off rates reported in this and previous studies (22, 29, 31, 33) were all within an approximately 1 log range, even though the studies used different study designs [e.g., *E. coli* strains (including the use of pathogenic and non-pathogenic strains), produce type, inoculation procedures] and were performed under different conditions (e.g., weather, soil type). This suggests that the die-off rates reported to date are reasonable and comparable, and can be used in quantitative risk assessments to evaluate the public health impact of pre-harvest risk management strategies.

Overall, the findings reported here are consistent with the die-off rates observed in past studies. As such, the die-off rates reported by this and similar studies can be used in quantitative risk assessments, and may therefore contribute to the development of effective risk management strategies, including the development of time-to-harvest recommendations following potential contamination events. The study reported here is also the first to calculate die-off rates for field-grown, pre-harvest produce in New York State, and as such, provides a foundational dataset on which future studies can build.

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Supplemental Material

Supplemental material associated with this article can be found online at: https://doi-org.proxy.library.cornell.edu/10.4315/0362.028X.JFP-16-419.s1.

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CHAPTER 6

CONCLUSION

Due to the increasing number of foodborne outbreaks attributed to pathogen contamination of fresh produce, growers face increased pressure to minimize the likelihood of preharvest produce contamination. However, to develop these approaches a comprehensive understanding of the ecological processes that drive the presence, dispersal and persistence of bacterial pathogens in produce production environments is essential. The studies presented here investigated these processes, and identified targeted risk management strategies that growers can use to reduce the likelihood of preharvest produce contamination. Specifically, the first two studies presented here focused on factors associated with the detection and diversity of *L. monocytogenes* with the aim of developing and validating decision-support tools (e.g., predictive models and risk maps) that growers can use to identify on-farm areas with a higher or lower predicted pathogen prevalence. While the first two studies investigated processes at the field and subfield-levels, the third and fourth studies focused on processes that occur at the level of the individual plant, specifically the transfer of bacteria in wildlife feces to and die-off on preharvest produce.

Factors associated with the detection and diversity of *L. monocytogenes* at the field and subfield levels. In the first two studies we investigated factors that could be used to predict pathogen contamination patterns within a farm (i.e., at the field-level), and within a field (i.e., at the subfield level), and used this information to validate and refine geospatial models (*5*) that predicted the likelihood of isolating *L.*

monocytogenes from produce field soils based on a field's soil properties, and its proximity to certain land covers (water, impervious cover and pasture).

In the first study (the validation study), fields located on four commercial farms were categorized into areas of high or low predicted *L. monocytogenes* prevalence using existing geospatial models (*5*). Drag swabs were collected from a subset of fields within each category and tested for *L. monocytogenes* presence. Logistic regression, which tested the ability of each rule to accurately predict *L. monocytogenes* prevalence for each field, validated the rules based on water and pasture. Since the geospatial model predicted *L. monocytogenes* prevalence at the field-level, factors associated with *L. monocytogenes* isolation at the sub-field level were also identified. Although, only one factor (proximity to water) was found to be significantly associated with the odds of *L. monocytogenes* isolation at the sub-field and field levels, we consistently found that the likelihood of *L. monocytogenes* isolation was higher in edge areas. This suggests that, regardless of scale, produce grown within a short distance of ecotones is at an increased risk of *L. monocytogenes* contamination.

In the second study (the irrigation study), soil, leaf, water, and fecal samples were collected from spinach fields 1, 2, 3, and 6-9 d following irrigation and rain events. Samples were then tested to determine *L. monocytogenes* presence; all isolates were identified to allelic type (AT) by comparison of partial *sigB* sequences to an internal reference database. Environmental factors (e.g., time since irrigation or rain event) were then evaluated for their association with *L. monocytogenes* isolation using regression analysis. Similar to the validation study, we found that the likelihood of *L.*

monocytogenes isolation was higher in edge areas. We also found that the odds of *L. monocytogenes* isolation from soil samples was greatest during the 24 h immediately following irrigation and rain events. In fact, we found that the odds of *L. monocytogenes* isolation were approx. 25 times higher during the 24 h immediately following rain or irrigation events compared to 144-192 h following rain or irrigation events. Thus, waiting 24 h after rain events to harvest crops may significantly reduce the risk of *L. monocytogenes* contamination of preharvest produce. Since waiting 24 h should have a limited economic impact on growers, it offers a tangible solution that growers can use to reduce produce contamination risks.

Overall, the findings from the validation and irrigation studies suggest that landscape structure (e.g., proximity to certain land-cover types) drives the spatial distribution of *L. monocytogenes* within a field, while meteorological factors (i.e., precipitation) and management practices (i.e., irrigation) drive the temporal distribution of *L. monocytogenes* within a field. As such, on-farm produce safety is complicated by the ecological context unique to each field as well as the conditions during the time that risk is being assessed. Thus, it is essential to have tools that allow growers to account for ecological context when developing on-farm produce safety plans. Since geographic information systems (GIS) platforms offer users the unique opportunity to look at spatial variation and to account for cross-scale differences by allowing for the integration and visualization of remotely sensed and field-collected data, GIS-enabled tools and geospatial models may allow growers to account for ecological context when developing on-farm produce safety plans. The validation of the geospatial model in the validation study demonstrates the utility of geospatial

models for predicting pathogen prevalence on produce farms, further suggesting that GIS-enabled tools may be promising for food safety. Indeed, by knowing where and when *L. monocytogenes* is likely to be found within a field growers will be able make small changes in their management practices that can greatly reduce the risk of produce contamination, such as planting high risk crops (e.g., cantaloupe) in low risk areas (away from ecotones). Additional research is needed to determine if the validated models can accurately predict the prevalence of *L. monocytogenes* for farms outside New York State. Studies are also needed to determine if the findings of the irrigation study are replicable in other produce-growing areas, and for other pathogens (e.g., *Salmonella*).

***Escherichia coli* transfer from simulated wildlife feces to and die-off on individual, preharvest produce items.** The third and fourth studies included in this dissertation focused on the transfer of bacteria in simulated wildlife feces to preharvest produce items (the transfer study), and the die-off of bacteria on produce following fecal contamination (the die-off study). In the transfer study rabbit feces inoculated with a 3-strain cocktail of non-pathogenic *E. coli* were placed in a lettuce field 2.5–72 h before irrigation. Following irrigation, the *E. coli* concentration on the lettuce was determined. Regression analysis showed that significantly more *E. coli* transferred to outer leaves compared to inner leaves. Therefore, removing the outer leaves at harvest, which is current industry practice, eliminates the part of the lettuce head that is most likely to become contaminated with a high bacterial load by splash from in-field feces. This may reduce the likelihood of harvesting contaminated produce reducing the potential for pathogen transfer to equipment and other produce items during harvest

and post-harvest processing. Additionally, we found that the percent of *E. coli* that transferred from the feces to the lettuce decreased significantly as the distance between the lettuce and the feces increased. It is therefore logical that the likelihood of *E. coli* transfer from feces to produce via splash during irrigation should be minimal past a given distance. Establishing a no-harvest buffer at this distance around in-field feces may reduce the risk of harvesting microbially contaminated produce. However, a dissertation published since completion and publication of the transfer study (*2*) showed that bacteria in feces can transfer to lettuce heads that are up to 1.63 m from the feces via splash during irrigation. Since all lettuce heads sampled in the transfer study were within 1 m of the nearest fecal pellet additional research is needed to examine bacterial transfer from feces to produce that is > 1 m from the feces. Despite this limitation, the transfer study generated key data that can be used, in conjunction with the results of future studies, to calculate transfer reductions associated with no-harvest buffers of various sizes.

In the survival study, lettuce was inoculated with *E. coli*, and harvested 0-10 days following inoculation. The *E. coli* concentration on the lettuce was determined and die-off rates were calculated. We found that die-off followed a biphasic pattern, and that the relationship between sample time and the log MPN of *E. coli* per head was best modeled using a segmented linear model. This model had a breakpoint at 106 h (95% confidence interval = 69, 142 h) after inoculation, with a daily decrease of 0.70 and 0.19 log MPN for 0 to 106 h and 106 to 240 h following inoculation, respectively. Overall, our findings were consistent with die-off rates observed in past studies; in fact the die-rates calculated as part of our study were within an

approximately 1 log range of those reported by previous studies (*1*, *3*, *4*, *6*). This suggests that the die-off rates reported to-date are reasonable and comparable, and can be used in quantitative risk assessments.

The findings of the transfer and die-off studies provide data that can be used in quantitative risk assessments to identify potential intervention and control strategies for reducing food safety risks associated with fresh produce consumption. The studies reported here are also the first, to the author's knowledge, to investigate the transfer of bacteria in wildlife feces to pre-harvest produce and to quantify die-off rates for field-grown, pre-harvest produce in the Northeastern United States. As such these studies provide foundational datasets on which future studies can build. The next generation of research should utilize similar methods to the studies reported here to ensure comparability between these and future studies, and allow for meta-analysis. However, these future projects need to address some of the limitations of the studies reported here. For example, because the transfer and die-off studies were both conducted in one field over one growing season the impact of weather is impossible to distinguish from the impact of time in both studies. As such future projects should include multiple replicates over time (e.g., multiple growing seasons, multi-year studies, staggered planting of fields) and/or space (e.g., trials on different farms, regions) to allow for more comprehensive analysis of the impact of environmental conditions on the transfer of bacteria in feces to and die-off on pre-harvest produce

Conclusion. Since it is highly improbable that a technology will be developed in the near future that can remove 100% of pathogens on fresh produce prior to consumption preventing produce contamination is critical for reducing food safety

risks associated with fresh produce consumption. The four studies included here further our understanding of the ecological processes that underpin the distribution, dispersal and persistence of foodborne pathogens in produce production environments. The information generated by these studies provides key data that can be used in the development of science-based strategies for preventing preharvest produce contamination. In fact, as part of the discussion in each study we identified potential intervention and control strategies that our data suggested may reduce the likelihood of preharvest produce contamination. Specifically, we identified strategies that growers can use to target produce safety risks for individual fields, individual parts of fields and individual plants. The types of targeted and adaptive strategies discussed in the four studies included here are key to ensuring fresh produce safety as these strategies will provide growers with the flexibility needed to deal with complex interactions that underpin pathogen dispersal, distribution, and persistence in produce production environments.

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