Die off Rates of *Salmonella* and Shiga Toxin Producing *Escherichia coli* in Manure Amended Soil under Natural Climatic Conditions Using Novel Sentinel Chamber System

by

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ABSTRACT

DIE-OFF RATE OF SALMONELLA AND SHIGA TOXIN PRODUCING ESCHERICHIA COLI IN MANURE AMENDED SOIL UNDER NATURAL CLIMATATIC CONDITIONS USING NOVEL SENTINEL CHAMBER SYSTEM

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The die-off of Salmonella and Shiga Toxin producing Escherichia coli (STEC) in manure (swine or dairy) amended soil (loam or sandy loam) under field conditions was studied. Pathogen die-off occurred in three phases with an initial rapid decline that then slowed over a period followed by extended persistence at low levels. Survival of both pathogens was favored in the subsurface of loam soil. The decline of STEC was greater in swine manure amended soil compared to dairy manure amended soil, while Salmonella decline was greater in dairy manure amended soil. Fluctuations in moisture and temperature significantly affected the die-off while the season of application (spring vs fall) was less relevant. From the results it was apparent that in most cases pathogen die-off occurred within 120 days although low levels of persistent cells were recovered sometimes beyond this time. The food safety risks from persistent pathogens needs to be further studied.
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List of Abbreviations

AEM: Agricultural Environmental Management
ANOVA: Analysis of Variance
BCRC: Beef Cattle Research Council
BMPs: Beneficial management practices
CDC: Centers for Diseases Control and Prevention
CFIA: Canadian Food Inspection Agency
CFU: Colony Forming Units. The number of organisms (or clumps of organisms) able to grow to form colonies on a solid media.
DW: dry weight
FAO: Food and Agriculture Organization of the United Nations
Generic E.coli: E.coli endogenous to the manure
HUS: Hemolytic Uremic Syndrome
MPN: Most Probable Number. A unit of measure derived using a multiple tube and multiple dilution method of detection.
NESP: National Enteric Surveillance Program
PFRP: Process to further reduce pathogens
PSRP: Process to significantly reduce pathogens
PWGSC: Public Works and Government Services Canada
STEC: Shiga Toxin producing Escherichia coli
USDA: U.S. Department of Agriculture
U.S. EPA: Environmental Protection Agency
U.S. FDA: United State Food and Drug Administration
Introduction

Manure is an essential part of nutrient management of crops and over 150 million tonnes are applied on land in Canada each year (Statistic Canada, 2004, BRBC, 2015). On an annual basis, approximately 3.4 million hectares of land in Canada receives animal manure as an amendment to improve soil fertility and quality for crop growth (Statistics Canada, 2014). Despite being more environmentally sustainable, manure carries the risk of harboring virulent enteric pathogens such as *Escherichia coli* O157:H7 and *Salmonella*. Pathogens associated with soil can directly contaminate crops or be washed into water courses then indirectly introduced onto produce during irrigation. (Kudva et al., 1998)

Because manure represents a significant source of pathogens, guidelines have been published on handling and land application when used for crops produced for human consumption. The guidelines advise on application times (fall and spring), surface application versus incorporation or injection and pre-treatment options such as compositing. An additional recommendation is to set a time period from manure application to the time of harvest. Here, it is recommended to have a 90-120 day (120 days for crop that edible portion has soil contact and 90 days for all other food crops) period from manure application to harvest to enable pathogens to die off over time thereby negating food safety risks. However, the 90-120 day rule is largely derived from laboratory studies that did not take into account the intrinsic or extrinsic conditions that are encountered in the natural environment. Consequently, the 90-120 day guideline has yet to be verified under typical field conditions or factors impacting on pathogen survival clearly established. The following thesis was directed towards studying the pathogen die-off in different soils under fluctuating environmental conditions. The sentinel vials were introduced to the field experiment to enable direct measurement of pathogen die-off without the influence of washout via precipitation events (Huber, 2006). In the
way of background, the literature review outlines the characteristics of the main pathogens encountered, manure management practices and work performed to date to determine pathogen die-off in amended soil.
Chapter 1. Literature Review

1.1 Manure in agriculture productions

1.1.1 Manure usages in Canada

Manure has been used as a fertilizer since early civilization but is particularly important in the organic sector where synthetic fertilizers are not permitted in compliance with the Canadian Organic Standards (PWGSC, 2011). The majority of manure applied to land is in the animal production areas of Ontario, Quebec and Alberta (Statistics Canada, 2014) (Table 1-1).

Farms produce solid or liquid manure depending on the management practice during animal production. From Table 1-1, solid manure application areas are mainly located in Ontario (26.3%), Alberta (26.0%), followed by Saskatchewan (16.7%) and Quebec (15.8%). All these provinces are major cattle producing provinces which accounting for 83.8% of the total cattle counted in the 2011 census (Statistics Canada, 2014). Pig production and large dairy operations commonly use liquid manure systems due to the nature of the waste along with ease of handling (Statistic Canada, 2004, OMAFRA, 2015).

1.1.2 Manure types and handling

Solid manure frequently includes significant amounts of bedding materials such as straw or wood shavings resulting in a high solids content. In contrast, liquid manure has a solids content <10% and is relatively homogenous (OMAFRA, 2009). Liquid manure is more frequently encountered in pork production and large dairy operations with solid manure being common in beef and small dairy operations. Manure is stored until being applied to the surface or incorporated into land in the spring or fall as part of crop nutrient management (USDA, 2012b, USDA, 2006). Manure is typically used for low-risk crops as opposed to fresh produce
<table>
<thead>
<tr>
<th>Province</th>
<th>Solid manure incorporated (Hectares)</th>
<th>Solid manure not incorporated (Hectares)</th>
<th>Liquid manure injected into soil (Hectares)</th>
<th>Liquid manure left on surface (Hectares)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newfoundland and Labrador</td>
<td>718</td>
<td>835</td>
<td>633</td>
<td>1,892</td>
</tr>
<tr>
<td>Prince Edward Island</td>
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<td>6,582</td>
<td>3,438</td>
<td>3,588</td>
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<tr>
<td>Nova Scotia</td>
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<td>13,065</td>
<td>4,325</td>
<td>11,404</td>
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<tr>
<td>New Brunswick</td>
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<td>12,498</td>
<td>3,830</td>
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<td>61,519</td>
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<tr>
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<td>120,791</td>
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<td>British Columbia</td>
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<td>17,826</td>
</tr>
<tr>
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<td><strong>633,730</strong></td>
<td><strong>487,471</strong></td>
</tr>
</tbody>
</table>

Adapted from: (Statistics Canada, 2014)
(e.g. lettuce) due to the risks associated with pathogens (Figure 1-1). Nevertheless, fresh produce can still be indirectly contaminated by enteric pathogens when flooding events introduce contamination into irrigation water that is then applied to crops (Massey, 2006). Hence, pathogen die-off in manure amended soil is critical even for crops that are not to be consumed raw or sent for further processing.

![Figure 1-1 Crop Share of Manure Usage in U.S. in 2006. Adapted from: (USDA, 2006)](image)

Composting is an alternative to direct land application of stored manure although it has the disadvantages of increasing materials handling, equipment needs, labor and time (Urban-Garden-Center, 2014). The standards for commercial compost require that the temperature attains >55°C for at least 15 days during the composting process with frequent (daily) turning (CFIA, 2014). The major advantage of composting over direct field application is primarily increased microbiological safety through inactivation of enteric pathogens.
1.1.3 Manure application

Manure application primarily occurs in spring and fall, with some summer application on forage and wheat stubble with limited spreading during the winter (OMAFRA, 2005). The timing of applications can be affected by many on-site factors, such as weather, soil moisture, crop growth, labour availability and manure storage capacity.

Manure application management includes surface application and/or incorporation into the soil. Several application systems can be used, including solid spreader, liquid spreader, liquid injectors and irrigation systems (Alberta Agriculture and Forestry, 2015). Surface application of manure is the quickest and easiest approach although incorporation or injecting into the subsurface are recommended due to the perceived reduce risk of run-off or other environmental exposures (OMAFRA, 2005). From the FEMS survey in 2013, 52.4% of farms that produce livestock in Canada either left manure on the soil surface or incorporated it more than a week after application. Approximately, 32.6% of livestock farmers incorporated manure into soil in the 1 to 7 days following application (Statistic Canada, 2004).

Beneficial management practices (BMPs) were introduced to encourage farm production or management practices that reduce environmental risks or realize environmental benefits. Manure management BMPs in particular, are designed to prevent runoff, protect water sources, minimize manure nutrient losses and odour emissions (Statistic Canada, 2004). For example, from the Agricultural Environmental Management (AEM) program developed at Cornell University for dairy producers in New York watersheds, manure applications were suggested to follow such practices as: incorporation of manure into soil, implementing sound soil conservation and runoff management practices in crop land (e.g. reduced tillage systems, grassed filters), applying manure from higher risk animals (e.g. calves) to crop land with lowest risks of runoff, erosion, or
groundwater infiltration, or applying manure to tiled fields only when the soil is relatively dry to minimize run-off into water courses (New York State Soil and Water Conservation Committee, 2015).

1.2 Outbreaks linked to fresh produce and routes of contamination

The main pathogens of concern with fresh produce are of enteric origin and contaminate crops by a diverse range of routes (Figure 1-2).

![Pathogen contamination routes via the interacting animal, human, crop and environment domains. Adapted from: (FAO, 2012, Parker et al., 2012)](image)

Human pathogens associated with manure such as STEC and *Salmonella* are commonly implicated in foodborne illness outbreaks (PHAC, 2009) (Table 1-2; 1-3). Whilst not all can be traced to the directly application of manure on land, most the origins can be traced to animal production or contact with untreated sewage.
<table>
<thead>
<tr>
<th>Group</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
<th>2012</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli O157</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathogenicity Rate*</td>
<td>0.283‰</td>
<td>0.198‰</td>
<td>0.156‰</td>
<td>0.118‰</td>
<td>0.139‰</td>
<td>0.139‰</td>
</tr>
<tr>
<td><strong>Listeria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>132</td>
<td>124</td>
</tr>
<tr>
<td>Pathogenicity Rate</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.038‰</td>
<td>0.035‰</td>
</tr>
<tr>
<td><strong>Salmonella</strong></td>
<td>6419</td>
<td>6351</td>
<td>6084</td>
<td>7251</td>
<td>6809</td>
<td>6979</td>
</tr>
<tr>
<td>Pathogenicity Rate</td>
<td>1.942‰</td>
<td>1.899‰</td>
<td>1.797‰</td>
<td>2.117‰</td>
<td>1.968‰</td>
<td>1.994‰</td>
</tr>
<tr>
<td><strong>Shigella</strong></td>
<td>636</td>
<td>680</td>
<td>631</td>
<td>739</td>
<td>860</td>
<td>988</td>
</tr>
<tr>
<td>Pathogenicity Rate</td>
<td>0.192‰</td>
<td>0.203‰</td>
<td>0.186‰</td>
<td>0.216‰</td>
<td>0.249‰</td>
<td>0.282‰</td>
</tr>
</tbody>
</table>

* Pathogenicity Rates (in parts per ten thousand) calculated using the population estimates for Canada as of July 1 for years 2007 to 2012 as reported by Statistics Canada. Only cases of *E. coli* O157 are included in this table, as *E. coli* non-O157 is not consistently reported by provinces.

Reporting of *Listeria monocytogenes* to NESP began in July 2010 Adapted from (NESP, 2012)
Table 1-3 Outbreaks in recent five years in U.S. and the pathogen serotypes related to fresh product

<table>
<thead>
<tr>
<th>Year</th>
<th>Serotypes</th>
<th>Food Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>STEC</td>
<td></td>
</tr>
<tr>
<td>2013</td>
<td>O157:H7</td>
<td>Ready-to-Eat Salads</td>
</tr>
<tr>
<td>2012</td>
<td>O157:H7</td>
<td>Organic Spinach and Spring Mix Blend</td>
</tr>
<tr>
<td>2011</td>
<td>O157:H7</td>
<td>Romaine Lettuce,</td>
</tr>
<tr>
<td></td>
<td>Salmonella</td>
<td></td>
</tr>
<tr>
<td>2015</td>
<td>Poona</td>
<td>Cucumbers</td>
</tr>
<tr>
<td>2014</td>
<td>Newport</td>
<td>Cucumbers</td>
</tr>
<tr>
<td>2013</td>
<td>Saintpaul,</td>
<td>Cucumbers</td>
</tr>
<tr>
<td>2012</td>
<td>Typhimurium,</td>
<td>Mangoes, Cantaloupe</td>
</tr>
<tr>
<td></td>
<td>Braenderup,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Newport</td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>Agona, Panama</td>
<td>Fresh Imported Papayas(Whole), Cantaloupe</td>
</tr>
</tbody>
</table>

Compiled from (U.S. CDC, 2015)
Although previously considered to be restricted of meat products, *E. coli* O157:H7 has diversified into other foods including fresh produce (U.S.CDC, 2006). In addition, fresh produce outbreaks are more frequently implicating other STEC collectively known as non-O157 STEC. This has resulted in an increase in non-O157 STEC cases although this is likely attributed in part to better diagnostics being available (Figure 1-3; Table 1-3).

![Incidence rate of E.coli O157 and E.coli non-O157 serotypes reported to the Public Health Agency of Canada through National Enteric Surveillance Program (NESP). Compiled from (Public Health Agency of Canada, 2013b, NESP, 2012)](image)

Over the last decade, *E. coli* O157:H7 cases have decreased although illness associated with non-O157 STEC have increased (Lukacsovics et al., 2014) (U.S.CDC, 2015a).

Among the non-O157 *E.coli* serotypes there have been six singled out of most concern given they account for over 70% of non-O157 STEC infections. The serotypes O26, O45, O103, O111, O121, and O145 were designated adulterants in 2011, which essentially implies that if encountered
on foods then a mandatory recall should be initiated (Brooks et al., 2005). However, the lack of suitable diagnostics for the non-O157 STEC have made determining actual prevalence in beef problematic (CFIA, 2013).

1.3 STEC

*Escherichia coli* exists as part of the normal intestinal flora of animals and humans (Adlerberth et al., 1998, Sunde et al., 1998). Most *E.coli* are harmless and play an important part of a healthy microbiome. The presence of non-pathogenic *E. coli* can benefit the hosts by producing vitamin K$_2$ (Bentley and Meganathan, 1982) and as a probiotic (Bicudo and Goyal, 2003). However, 4.5 million years ago a group of pathogenic *E. coli* diverged and evolved through acquisition of virulence factors to form 6 pathotypes (Reid et al., 2000). Of main concern was the emergence of *E. coli* that acquired the Shiga toxin prophage that subsequently formed Shiga Toxin producing *Escherichia coli* collectively referred to as STEC.

1.3.1 Mode of illness by STEC

The STEC group is comprised of 200 serotypes that have the common feature of harboring the *stx* gene encoding for Shiga-like toxin (U.S. CDC, 2014). However, the majority of serotypes do not express the toxin or lack all the virulence factors (i.e. *eae* gene for intimate attachment to epithelial cells in vitro). Consequently, the majority of STEC exhibit low to no pathogenicity. Yet a subgroup of STEC referred to as enterohemorrhagic *E. coli* (EHEC) do harbor the required virulence factors to induce the potentially fatal condition of hemolytic-uremic syndrome (HUS) (Sheng et al., 2006). Serotype O157:H7 is the most widely studied EHEC although non-O157 STEC are increasingly implicated in HUS cases (Paton and Paton, 2002). A common feature of
EHEC is the ability to produce stx2 although it can also harbor stx1 and attachment factors encoded on the LEE pathogenic island (Louie et al., 1993, Vallance and Finlay, 2000, Donnenberg et al., 1993). E. coli O157:H7 possess the pO157 plasmid that encodes for additional virulence factors that promote initial attachment and haemolytic activity. Non-O157 STEC may also harbor pO157 but not always, which may explain the lower virulence compared to O157:H7 (Kresse et al., 2000, Schmidt et al., 1996).

Upon ingestion of 10-100 cells, E. coli O157:H7 attaches to the gut mucosa and interacts with it, and produces histopathological changes in the epithelium. The EHEC introduce the Tir protein into the host cell via Type II secretion system that forms an attachment site for the E. coli via intimin. Actin polymerization then forms a pedestal that enables the STEC to adhere before producing Shiga toxin (Figure 1-3). Because stx is a prophage the producing bacterial cell is ultimately doomed as it undergoes lysis to release the new phages along with the toxin. The Shiga toxin is taken up by M cells and the toxinA component enters the host cell then cleaves the adenine unit of rRNA resulting in succession of protein synthesis. The host cell ultimately lyses thereby releasing iron (essential nutrient) to the remaining STEC cells. The symptoms of STEC infection vary from mild flu, similar to West Nile virus, through to the potentially fatal condition of HUS (Paton and Paton, 1998).

The main cause of HUS is O157:H7 although additional STEC have also been implicated, with the most common belonging to the Top 6 that express stx2 (Figure 1-5).
Figure 1-4 Mode of infection by Shiga Toxin producing Escherichia coli. Adapted from (Etienne-Mesmin et al., 2011).

Figure 1-5 Shiga toxin production in serotypes of the Top 6 STEC (incidence from 1999-2008; PulseNet Dataset, n=1422). Adapted from (U.S.CDC, 2011)
1.3.2 Prevalence in manure and carriage in animals

Ruminants are the primary carriers of STEC with cattle being the most significant source (Ferens and Hovde, 2011). Through surveillance studies the carriage of *E. coli* O157:H7 varies depending on season, geographical location and diagnostic methods applied (Table 1-4). Through control measures applied at the farm level the prevalence of *E. coli* O157:H7 has decreased although can transiently increase if super-shedders are within the herd (Ferens and Hovde, 2011, Ayscue et al., 2009).
Table 1-4 Prevalence studies of *E.coli* O157:H7

<table>
<thead>
<tr>
<th>Location</th>
<th>Source</th>
<th>Prevalence</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canada(AB)</td>
<td>Water from cattle farm regions</td>
<td>0.9% (n=1483)</td>
<td>Selective agar &amp; PCR</td>
<td>(Johnson et al., 2003)</td>
</tr>
<tr>
<td>Argentina</td>
<td>Downstream water from cattle pens</td>
<td>12.7% (n=251)</td>
<td>Multiplex PCR</td>
<td>(Tanaro et al., 2014)</td>
</tr>
<tr>
<td>U.S.</td>
<td>Cattle fecal samples from</td>
<td>1.14% (n=12664)</td>
<td>PCR</td>
<td>(Hancock et al., 1997)</td>
</tr>
<tr>
<td>France</td>
<td>Cattle manure, slurry, and sewage</td>
<td>24% (n=752)</td>
<td>PCR</td>
<td>(Vernozy-Rozand et al., 2002)</td>
</tr>
<tr>
<td>U.S.(MN)</td>
<td>Fresh product from organic farm</td>
<td>0.0% (n=467)</td>
<td>PCR</td>
<td>(Mukherjee et al., 2004)</td>
</tr>
<tr>
<td>U.S.(WA)</td>
<td>Cattle faecal</td>
<td>0.28% (n=3570)</td>
<td>PCR</td>
<td>(Hancock et al., 1994)</td>
</tr>
<tr>
<td>Argentina</td>
<td>Pre-slaughter cattle</td>
<td>4.1% (n=1622)</td>
<td>Enrichment</td>
<td>(Masana et al., 2010)</td>
</tr>
<tr>
<td>U.S.(OH)</td>
<td>Cattle</td>
<td>0.007% (n=750)</td>
<td>PCR</td>
<td>(LeJeune et al., 2006)</td>
</tr>
</tbody>
</table>

1.4 Salmonella

*Salmonella* are a group of bacteria that is commonly found in the intestines of animals and humans (PHAC, 2009). All serotypes of *Salmonella* can infect humans although virulence varies from weak to potentially lethal (NESP, 2011, Public Health Agency of Canada, 2013a).

*Salmonella enterica* subspecies *enterica* has 2610 different serotypes with Enteritidis, Heidelberg and Typhimurium being of most significant with respect to human health (NESP, 2012).

1.4.1 Mode of illness

*Salmonella* is an invasive pathogen that uses an array of virulence factors that gain entry into the host and then avoid detection by the immune system (Figure 1.6)(U.S. CDC, 2012). The infectious dose varies between 1000 – 100,000 cells depending on the strain and susceptibility of the host. Upon reaching the lower intestine, *Salmonella* expresses a Type III secretion system that introduces a protein (*InvA*) into the host epithelial cells. The protein causes the surface of the host cell to ruffle and form entry points for the *Salmonella* to invade. Once within the host cell the *Salmonella* undergoes phagocytosis and the pathogen starts growing. Eventually the *Salmonella* bursts out of the vacuole that triggers the cell immune system and localized host cell destruction by NK cells. Certain *Salmonella* can enter the blood stream and be taken up by macrophages that they subsequently utilize to become distributed in the lymph nodes, spleen, liver and gall bladder. This can result in long term colonization and persistent shedding of *Salmonella*. 
Most people infected with *Salmonella* develop diarrhea, fever, and abdominal cramps between 12 and 72 hours after infection. The illness usually lasts 4 to 7 days and most individuals recover without treatment. In some cases, diarrhea may be so severe that the patient needs to be hospitalized. In these patients, the *Salmonella* infection may spread from the intestines to the bloodstream, and then to other body sites. In these cases, *Salmonella* can cause death (U.S.CDC, 2015b).

### 1.4.2 Prevalence in animals and manure

*Salmonella* is inherently tolerant to a broad range of stresses and can persist for extended time periods in manure amended soil (Oliveira et al., 2011). The virulence factors used by *Salmonella* to avoid the immune-system are also considered to enhance survival in the environment through inducing dormancy in the enteric pathogen (Ibarra and Steele-Mortimer, 2009).
The major animal carriers of *Salmonella* include a range of farm animals. From US national enteric disease surveillance report of 2011, the top four sources of *Salmonella* come from porcine, chicken, bovine and turkey (U.S. CDC, 2011). The prevalence of *Salmonella* also varies among seasons. All prevalence studies indicate the *Salmonella* infections were highest in the summer (Brichta-Harhay et al., 2008). From U.S. national enteric disease surveillance report of 2011, the highest infection season was August (U.S. CDC, 2011).

1.5 Persistence of enteric pathogens in manure amended soil

1.5.1 The Methodology to Determine Prevalence of Environment Pathogens

Studying the persistence of pathogens in manure amended soil is complex due to multiple intrinsic and extrinsic factors that define the survival of the microbe. Specifically, the pre-history of the microbe can influence survival, in addition to soil characteristics and climatic conditions. Furthermore, the movement of pathogens from soil by physical forces of water flow is an additional factor to be considered. Beyond this point, there is also the restriction of releasing pathogens in the environment that can make generating data on persistence problematic.

There have been several approaches to assessing pathogen die-off in the soil environment. To avoid introducing pathogens into the environment, most studies are conducted in labs. The most common laboratory approach is to inoculate microcosms with the test pathogens then monitor die-off under static conditions. Although the approach allows the use of virulent pathogens the main drawback is that it is difficult to recreate the fluctuations in environmental conditions encountered under field conditions. In one example, Ma et al. (2012) determined the die-off of *E.coli* O157:H7 strains in manure amended into different soil types held within a plastic container with the moisture and temperature maintained at 50% and 22°C respectively. The researchers determine the time
taken for different strains to decrease from an initial inoculation level of $5 \times 10^6$ CFU per gram to the enumeration detection limit (1 log cfu/g). The results from the study were that *E. coli* O157:H7 decreased to the detection limit in 32 days in loamy sand compared to 80 days in sandy loam and 110 days for clay. Ma et al. (2012) went onto report that virulence factors required to cause infection did neither promote or decrease persistence in soil.

Erickson et al. (2013) studied the persistence of *E.coli* O157 and *Salmonella* in soil in the presence of growing lettuce plants. Here, lettuce was grown in an environmental growth chamber set at 70% humidity and a temperature cycle of 20°C during the day (12h) and 15°C at night (12h) with water sprayed to each tray at regular intervals to compensate for moisture loss. During the experiment, $10^3$ and $10^6$ log CFU/ml of *E.coli* O157:H7 and *Salmonella* were applied to the plant as low and high dose treatment, and pathogen levels were monitored with selective agar. The study found that both *E.coli* O157:H7 and *Salmonella* populations were lower for mid-age plants compared with seedlings, and pre-harvest internalization of *E.coli* O157:H7 and *Salmonella* was not observed at any time (Erickson et al., 2013).

Mubiru et al. (2000) compared the die-off of *E.coli* O157:H7 and avirulent *E.coli* in two types of soils. Inoculated soils at 20% water were placed in polyethylene bags which were sealed to maintain moisture and incubated at 25°C. *E.coli* O157:H7 exhibited similar die-off patterns to the avirulent *E.coli* in the same soil environment, and both E. coli strains had greater die-off rates in Pope silt loam (coarse-loamy) than Zanesville silt loam (fine-silty) (Mubiru et al., 2000). You et al. (2006) studied the survival of *Salmonella* Newport on manure amended soil. The inoculated soils were placed into polypropylene containers and incubated at 24.5°C. Experimental containers were covered with Parafilm to retard moisture loss while allowing air change. During the experiment, soil moisture was maintained by the periodic addition of water. In his study,
Salmonella increased by up to 4 log CFU/g in the first 1 to 3 days following inoculation then declined progressively. Time to reach two log reductions was 64 days with residual Salmonella being recovered up to 332 days into the trial (You et al., 2006).

Field trials are favored for determining die-off rates of pathogens because of the close representation of what occurs in the natural environment. However, given that variable conditions are encountered between trials attempting to generate consistent, reproducible, date is challenging. Vinten et al (2002) compared E.coli O157 die-off in manure amended soil in field and in laboratory conditions. Under the field environment, E.coli O157:H7 in all samples died off faster than under laboratory conditions. Under the temperature condition around 15°C in field and in lab temperature set as 15°C, the time for E.coli O157:H7 drop to 2 log reduction were 22 days for field test and >30 days for laboratory test. The results would suggest that lab based studies provide an over-estimation of survival but it should be noted that the result needs to be confirmed with data derived from multiple trials (Vinten et al., 2002).

Experimental introduction of pathogens into the field environment is normally accomplished by using naturally occurring pathogens associated with manure or using avirulent strains. The limitation of using endogenous pathogens is the variability in both levels (generally very low) and types present that results in highly variable data. The use of avirulent stains is problematic given that virulence factors can affect survival in the environment although studies to date have been contradicting (Law, 2000). The use of non-pathogenic indicators, such as generic E. coli, has also met with inconsistent results supporting the theory that by only using pathogens can accurate die-off rates be obtained (Bolton et al., 1999). Therefore, the general conclusions are that pathogens provide the most reliable data but containment should be exercised to prevent release into the wider environment. One approach is to conduct trials within secure greenhouse settings (Islam et al.,
2005). The disadvantage to the greenhouse approach is that the temperature and moisture fluctuations that occur in the natural environment cannot be recreated and hence similar to laboratory based studies.

Using a device that retains pathogens inside but yet exposes them to conditions similar to the surrounding environment is one of the best solutions to study pathogenic bacteria in field conditions. A small-volume sentinel chamber was developed by Jenkins (1999). Chambers were designed to equilibrate with external chemical and moisture conditions to assess the effects of environmental stresses on survival of pathogens in soil and animal wastes. The sentinel chamber consisted of a microfiltration system (2.5 cm long, internal diameter 0.7 cm) with a nylon 0.45 µm pore size filter on the end and 60 µm nylon mesh filter on the top. Pathogens were inoculated into animal wastes and mixed with soil. The spiked mixture were introduced into the vials and die-off rates of *Cryptosporidium parvum* oocysts determined to be months and can pose threat to the surface water (Jenkins et al., 2002, Jenkins et al., 1999).

1.5.2 Data reported on persistence

In general, pathogens survive the longest in water, followed by soil and manure (Guan and Holley, 2003). *E. coli* O157:H7 can survive more than 12 months when introduced into ovine manure compared to weeks in the water environment (Kudva et al., 1998).

A laboratory study by Wang et al. (1996) reported that *E. coli* O157:H7 can survival in bovine faeces for up to 49 d at 37 °C, 56 d at 22 °C and up to 70 d at 5 °C (Wang et al., 1996). In a field study conducted under tropical conditions, *Salmonella* Typhimurium persisted for 14 weeks at a high inoculum density at 7 log CFU/g for 6 weeks at a relatively low inoculum density of 4 log CFU/g (Ongeng et al., 2011a). *Salmonella* showed a slower decrease under lab conditions under
controlled temperatures and moistures compared to the field experiment. Multidrug-resistant *S.* Newport within microcosms maintained at 25% moisture and 25°C reduced by 5 log CFU/g over the course of 15 weeks (You et al., 2006). From addition persistence studies reported the general findings are that persistence was highly variable but enhanced at low temperatures (Table 1-5).

<table>
<thead>
<tr>
<th>Material</th>
<th>Duration of Survival</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>Salmonella</em></td>
<td><em>E.coli O157:H7</em></td>
</tr>
<tr>
<td>Water</td>
<td>-Frozen</td>
<td>&gt;6 months</td>
<td>&gt;300 days</td>
</tr>
<tr>
<td></td>
<td>-Cold (5°C)</td>
<td>&gt;6 months</td>
<td>&gt;300 days</td>
</tr>
<tr>
<td></td>
<td>-Warm (30°C)</td>
<td>&gt;6 months</td>
<td>84 days</td>
</tr>
<tr>
<td>Soil</td>
<td>-Frozen</td>
<td>&gt;12 weeks</td>
<td>&gt;300 days</td>
</tr>
<tr>
<td></td>
<td>-Cold (5°C)</td>
<td>12-28 weeks</td>
<td>100 days</td>
</tr>
<tr>
<td></td>
<td>-Warm (30°C)</td>
<td>4 weeks</td>
<td>2 days</td>
</tr>
<tr>
<td>Cattle manure</td>
<td>-Frozen</td>
<td>&gt;6 months</td>
<td>&gt;100 days</td>
</tr>
<tr>
<td></td>
<td>-Cold (5°C)</td>
<td>12-28 weeks</td>
<td>&gt;100 days</td>
</tr>
<tr>
<td></td>
<td>-Warm (30°C)</td>
<td>4 weeks</td>
<td>10 days</td>
</tr>
<tr>
<td>Liquid Manure</td>
<td></td>
<td>13-75 days</td>
<td>10-100 days</td>
</tr>
<tr>
<td>Composted Manure</td>
<td></td>
<td>7-14 days</td>
<td>7 days</td>
</tr>
<tr>
<td>Dry Surface</td>
<td></td>
<td>1-7 days</td>
<td>1 day</td>
</tr>
</tbody>
</table>

Adapted from: (Olson, 2001)

1.5.3 Factors affecting persistence

Weather conditions

The microenvironment of pathogens will greatly affect the survival of pathogens, including the temperature and moisture. Soil moisture is an important factor determining bacterial survival. In survival studies, precipitation and evapotranspiration are factors to monitor in the study of human pathogens such as STEC and *Salmonella* (Guan & Holley, 2003). Some studies have suggested that low soil moisture increase the *E. coli* die-off rate in the soil (Entry et al., 2000b, Sjogren, 1994), while other studies have found soil moisture have no significant effect of moisture on *E.*
coli die-off (Vinten et al., 2002, Ritchie et al., 2003a). A laboratory microcosm study compared two realistic extremes of soil moisture: saturated soil (50% water) and dry soil (25% waters as collected). *E.coli* declined faster in wet soil than dry soil when inoculated in slurry, but not when *E. coli* was inoculated in feces. The effect of soil moisture was not a critical effect for pathogen die-off (Oliver et al., 2006).

Studies have suggested that lower temperature (above freezing point) extend pathogen survival (Bolton et al., 1999). Survival of *E.coli* O157:H7 in bovine feces; when inoculated with same pathogen levels (about 7 log_{10} CFU/g), *E.coli* O157:H7 survived for 21 days at 37 °C, 56 days at 22°C, and 70 days at 5 °C (Wang et al., 1996). Another field study also revealed that warmer summer (daily average maximum temperature >20°C) were favored for the persistence of *S. Typhimurium* when compared a spring experiment (March) with a summer experiment (June) in Wisconsin, US (Natvig et al., 2002b). Meanwhile, Natvig conducted a freezing-thawing experiment in laboratory conditions and proved that freezing-thawing cycles were detrimental to both *S. Typhimurium* and *E.coli*. After 20 freezing-thawing cycles in 4 months, *S. Typhimurium* and *E.coli* declined from 4.8 log CFU/g to below detection limit (1.29 log_{10} CFU/g for *E.coli* and 1.68 log_{10} CFU/g for *S. Typhimurium*) for all soil samples (Natvig et al., 2002b).

**Characteristics of different soils**

All soils are made up of different percentages of sand, silt and clay (soil texture) and organic matter. Clay content and organic matter content can affect microbial survival times (Nicholson et al., 2005). Crop production soils are variable in physical and chemical properties. Higher organic content is good for crop growth, but organic matter can also provide rich nutrient favorable for pathogen(Hillel, 1982).
Texture of the soil refers to the size of the particles that make up the soil, and soil is characterized based on the proportion of sizes of the soil particles (sand, silt, and clay) as showed in Table 1-6. Furthermore, the soil texture triangle showed in Figure 1-7 gives names associated with various combinations of sand, silt and clay. For example, a soil with 30% clay, 50% sand, and 20% silt is called a sandy clay loam from the soil texture triangle (CSU Extension, 2014).

Table 1-6 The Size of Sand, Silt and Clay. Adapted from: (USDA, 2015)

<table>
<thead>
<tr>
<th>Name</th>
<th>Particle Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clay</td>
<td>below 0.002 millimeters</td>
</tr>
<tr>
<td>Silt</td>
<td>0.002 to 0.05 millimeters</td>
</tr>
<tr>
<td>Sand</td>
<td>0.05 to 0.10 millimeters</td>
</tr>
<tr>
<td>- Very fine sand</td>
<td>0.10 to 0.25 millimeters</td>
</tr>
<tr>
<td>- Fine sand</td>
<td>0.25 to 0.5 millimeters</td>
</tr>
<tr>
<td>- Medium sand</td>
<td>0.5 to 1.0 millimeters</td>
</tr>
<tr>
<td>- Coarse sand</td>
<td>1.0 to 2.0 millimeters</td>
</tr>
<tr>
<td>- Very coarse sand</td>
<td>greater than 75.0 millimeters (~2&quot;)</td>
</tr>
<tr>
<td>Gravel</td>
<td>2.0 to 75.0 millimeters</td>
</tr>
<tr>
<td>Rock</td>
<td>greater than 75.0 millimeters (~2&quot;)</td>
</tr>
</tbody>
</table>
The texture of soil affects the structure and therefore affects the transport and persistence of pathogens. Bech et al. (2010) conducted an experiment to study the survival of S. Typhimurium in loamy and sandy soils (Bech et al., 2010b). In the experiment that compared pathogen concentrations in different soil leachates, two types of intact soil monoliths were inoculated with 10.81 log CFU/ml. The loamy monoliths reach 5.11 log CFU/ml of Salmonella in leachates while sandy monoliths only have below 20 CFU/ml Salmonella over the 27 day trial. The results suggested that loamy monoliths had significantly higher pathogen concentrations than the sandy monoliths thereby representing an increased risk of contaminating crops. The structural differences of the loamy and sandy soils influence the transport of Salmonella, the sandy monolith’s lack of structure and macropores led to a higher degree of filtration. As a result, more Salmonella were
detected in loam soil, but more *Salmonella* were detected in draining water from sandy soil (Bech et al., 2010b).

### 1.6 Research hypothesis and objectives

According to the current organic production regulations, it is recommended to have a 90–120 day period (120 days for crop that edible portion has soil contact and 90 days for all other food crops) from manure application to harvest to enable pathogens to die off over time thereby negating food safety risks (USDA, 2012a, Government of Canada, 2015). Since the 90-120 day rule is largely derived from laboratory studies, it does not take into account the variation in survival based on environmental abiotic and biotic stresses. That is, the die-off rate could be slow or quick depending on the prevailing climatic conditions. Therefore, more accurate prediction of die-off rates are required to ensure sufficient time is provided to ensure inactivation of pathogens (Baird, 1994). To this end, the main objective of the current study was to generate data on the die-off of relevant pathogens under different conditions. Specifically, the experiment was arranged in a 4 factorial design with three replicates, including two pathogens, two manure types, two soil types, two depths of application, and two seasons of application. The specific objectives of the study were:

1) Determine the rates of *Salmonella* and STEC die-off in two different soil types amended with swine or cattle manure.

2) Assess the effect of spring versus fall manure application on the persistence of *Salmonella* and STEC.

3) Correlate the biotic and abiotic factors that affect pathogen persistence.

4) Verify the field trial data by using laboratory microcosms incubated under different moisture content and temperature.
Chapter 2 Material and Method

2.1 Experimental Design

The basic concept of the experimental design was to use confined pathogens to study the die-off patterns of Shiga toxin producing *E.coli* (STEC) and *Salmonella* under field conditions. The variables tested were those conditions encountered in typical agriculture production: manure type, soil type, application depth and season of application. The study matrix introduced in the this experiment include: two pathogen strains of STEC and *Salmonella*, two manure types of dairy manure and swine manure, two soil types of sandy soil and loam soil, two depths of application of surface and 15cm depth, and two seasons of application of spring and fall applications.

2.2 General Description

2.2.1 Description of the pathogens (STEC and *Salmonella*)

Four strains of *Salmonella* (S. Javiana 5913 (tomato), S. Enteritidis (poultry), S. Heidleberg (clinical), and S. new deli (environmental)) and two strains of *E.coli* derived from cattle manure were used as well as two strains of nalidixic acid resistant *E. coli* O157:H7, derived from cattle manure, donated by Dr. R. Johnson, Public Health Agency of Canada, Guelph.

As the *E.coli* O157:H7 strains came with nalidixic acid resistant markers, the other two strains of *E.coli* and four strains of *Salmonella* were trained to be nalidixic acid resistant. All six strains were streaked individually on tryptic soy agar (TSA) (CM0131, Thermo Fisher Scientific Inc., MA, USA) and incubated at 37°C for 24 h. Single colonies of STEC and *Salmonella* were transferred to 50 ml of tryptic soy broth (TSB), and incubated at 37°C for 24 h. When pathogen growth reach the desired density (OD >0.8), 0.1ml of STEC or *Salmonella* culture were transferred to 50 ml of tryptic soy broth containing 10 µg/ml nalidixic acid (TSB-NA), and incubated at 37°C.
for 24 h. After pathogens had grown to the desired density (OD >0.8), 0.1ml of STEC or 
*Salmonella* culture were transferred to 50ml of TSB-NA containing higher concentrations of 
nalidixic acid. Serial transfer from 10, 20, 30, and 40 to 50 µg/ml nalidixic acid were performed 
until the STEC and *Salmonella* were able to grow in TSB-NA containing 50 µg/ml nalidixic acid. 
Antibiotic resistant stability was verified through ten passages of the strains through TSB 
containing no nalidixic acid.

2.2.2 Description of media

*Salmonella* was cultivated on Xylose Lactose Tergitol 4 agar (XLT-4, CM1061, Thermo Fisher 
Scientific Inc., MA, USA). For STEC, sorbitol MacConkey agar (CM0813, Thermo Fisher 
Scientific Inc., MA, USA) with supplement of cefixime and potassium tellurite (CR0172, Thermo 
Fisher Scientific Inc., MA, USA) was used. Both agars were then supplemented with nalidixic acid 
sodium salt (N4382-5G Sigma-Aldrich Canada Co., Oakville, ON, CA) at a concentration of 50 
µg/ml.

2.2.3 Description of the sentinel chambers

The sentinel chambers used in the experiment were used in previous field experiments (Huber, 
2006). Two windows (approx. 4cm²) were cut on opposite sides of a 20ml HDPE Scintillation 
Vials (03-337-23C Fisher Scientific Company, Ottawa, ON, CA). 0.45µm pore size neutral Nylon 
membranes (N00HY00010, Fisher Scientific Company, Ottawa, ON, CA) was used to cover the 
windows and was sealed with clear marine silicone sealant (SE1134 General Electric Huntersville, 
NC,USA) (Figure 2-1). Four different coloured nylon strings were attached at the neck of the vial 
to categorize the vials into different manure and pathogen groups.
Figure 2-1 Sentinel vials (2.8cm diameter*6.5cm high), cut through with two windows (2cm*2cm), covered with nylon membrane (2.5cm*9cm)

2.2.4 Field Plots

The study took place on conventionally managed agriculture land (Centre Wellington, Ontario, Canada) located at altitude between 43.7°-43.8°, east of Arthur, Ontario, Canada. The agriculture land was operated in a four crop rotation of corn, soybean, wheat and canola. In the experiment years, this land was planted to soybeans for 2013 and corn for 2014. Two test plots were selected based on soil difference. Perth Loam (loam site) consisted of 27% sand, 54% silt and 19% clay. The Hillsburg Fine Sandy Loam soil (sandy site) was fine sandy loam which consists of 74% sand, 19% silt and 7% clay. The test plots were located approximately 1 km apart and hence were subject to the same climatic conditions.
2.2.5 Manure application rates

Recommended manure application rates are calculated based on the nutrient characteristics of
the specific manure, and incorporation of manure after application is recommended to optimize
nitrogen retention in the soil (OMAFRA, 2009). With the types of manure used in this study, a
typical application rate would be 45000 litres per hectare as part of the nutrient management. In
the current experiments, the incorporation depth of manure used was 15cm. In order to get same
concentration compared to typical agricultural production, the field trials were set up as follows:

\[
45000\text{L manure/hectare soil} = 4.5\text{L manure/m}^2\text{ soil for surface application} \\
= 4.5\text{L manure/ (m}^2\times15\text{cm depth)} = 30\text{ml manure/L soil for} \\
\text{manure addition to soils in sentinel vials}
\]

2.3 Preparation of inocula

Single colonies of STEC or Salmonella were transferred to 50 ml of tryptic soy broth containing
50 µg/ml nalidixic acid (TSB-NA) and incubated at 37°C for 24 h. All strains were streaked
individually on selective agar (XLT-4-NA or CT-SMAC-NA agar) and incubated at 37°C for 24 h. Single colonies formed on these plates were streaked onto a second plate of selective agar (XLT-
4-NA or CT-SMAC-NA agar) and incubated at 37°C for 24 h. Single colonies on the second set
of plates were transferred to tubes with 100 ml of tryptic soy broth containing 50 µg/ml nalidixic
acid (TSB-NA), and incubated at 37°C for 24 h. 0.5ml of each strain was transferred to 1.5 ml
centrifuge tube with 0.5 ml glycol for back up. Cells from each remaining culture were harvested
by centrifuging (Sorvall ST 16R, Thermo Fisher Scientific Inc., MA, USA) at 4500×g for 3 min,
washed three times in saline (8.5mg/ml) and re-suspended in saline (S642-500 Fisher Scientific
Company, Ottawa, ON, CA) to an OD 600 reading of 1.0 in the spectrophotometer (Smart Spec Plus, Bio-Rad, Mississauga, ON, CA) which indicated densities of approximating $10^8$ CFU/ml. All cultures were held at 4°C for two days to acclimatize cells to the low nutrient stress. Serial of dilutions were made from $10^{-1}$ to $10^{-7}$; $10^{-5}$, $10^{-6}$ and $10^{-7}$ were selected to determine the concentration of each strains. Aliquots (0.1ml) of each dilution were spread plated onto selective agar (XLT-4-NA or CT-SMAC-NA) and incubated at 37°C for 24 h. Based on the plate counts, equal calculated amounts of each strain’s culture were combined to get four-strain cocktail mixtures for both E. coli or Salmonella. The concentrations of the pathogen cocktails were verified by a second round of plating.

2.4 Set up for the field trial
2.4.1 Set up for the field trial (Lab part)

The study matrix introduced in the this experiment included: two pathogen strain cocktails of STEC and Salmonella, two manure types of dairy manure and swine manure, two soil types of fine sandy loam soil and loam soil, two depths of application of surface and 15 cm depth, and two seasons of application of spring and fall applications.

Fresh fine sandy loam soil and loam soil were collected from the field plots. Approximately 15 l fine sandy loam soil and 10 l of loam soil were sieved to 0.2 cm (200 mesh screen). After sieving, two types of soils were mixed separately in to two large bins, and covered with lid to retain moisture.

The manure samples were collected from pits at the dairy or swine farms on the day of inoculation. The manure was passed through a course sieve to remove large debris, inoculated with the appropriate pathogen cocktail, then incorporated into the soil (60 ml manure per 2000 g soil).
The mix was then dispensed into the sentinel vials and capped before transferring to refrigerated storage at 4°C until required (<24h). Negative controls with non-inoculated manure mix into soil and positive controls with both *Salmonella* and *E.coli* O157:H7 spiked into manure then mixed into soil were also included in the trials.

### 2.4.2 Set up for the field trial (Field part)

The packed sentinel vials were placed horizontally into 15 cm deep trenches at the test plots spaced at 40-45 cm apart (Figure 2-2). Care was taken to place the vials such that the windows were at the top and bottom of the placement to allow for free flow of moisture through the vials during precipitation events. The trenches were filled with the corresponding soil type, with a colored marker flag and attached string marking each vial position. Paired vials were placed on the surface of the plot then the area dosed with corresponding manure slurry to give coverage of 4.5 l/m². The final plot configuration was two pathogen strains of STEC and *Salmonella*, two manure types of dairy manure and swine manure, two soil types of sandy soil and loam soil, and two depths of application of surface and 15cm depth (Figures 2-3 A & B). A set of positive and negative control vials using dairy manure, as described above, were also placed at the sandy loam site. The sequence of plot installation for the field trial is shown in (Figure 2-4).

A weather station was established at the field plot to record the air and soil temperatures at 10 cm depth (measured using thermocouples probes; Modal Type: E). Soil moisture was monitored by volumetric water content reflectometer, and precipitation collected by automated tipping bucket (TE525). All devices were connected to a Campbell continuous automated data logger (CR10, Campbell Scientific, INC, Edmonton, AL, CA).
Figure 2-2 Flow Diagram of the vial inoculation and placement in the field plot.
Figure 2-3(A) The arrangement of all sentinel vials in the loam site with different treatment of soil, manure and depth

Figure 2-3(B) The arrangement of all sentinel vials in the Sandy site with different treatment of soil, manure and depth
Figure 2-4 Set up of field trials

A—Two trenches in the field plot, B—Paired sentinel vials on surface and in the bottom of the trench, C—Flagged vials, and trench filled with soil, and evenly packed, D—Seived dairy and swine manure collected freshly from barn, E—Application of dairy and swine manure at 4.5L/m$^2$, F—Completed field plots
Following field plot set up, bulk samples were taken from the field with a soil sampler (36 inch Model L, Oakfield Apparatus, Fond du Lac, WI, USA). Both surface (top 1 cm) and soil from the depth at 15 cm were taken for analysis of background *E. coli* and total coliforms. All bulk samples were sealed in plastic zip-lock bags to retain moisture. Bulk soil samples were analyzed to determine water content. Generic *E. coli* and total coliforms were enumerated on 3M™ Petrifilm™ *E. coli*/Coliform Count Plates.

### 2.5 Sampling

Sampling was performed on Days 0, 2, 5 and 8 with other times being adjusted depending on the measured die-off rates. At each sampling time, triplicate sets of vials were collected from each of the 10 treatment plots. Bulk samples (surface and deep) were taken from within each field plot with soil probe and were placed in sealed in bags to retain moisture. Samples were placed in a cooler and returned to the laboratory for microbiological analysis. The weather station data was downloaded periodically throughout the study.

The contents of each vial was weighed into a stomacher bag without filter (ST15X20 4M, Fisher Scientific Company, Ottawa, ON, CA) and suspended to 1:10 dilution through addition of 0.1% peptone water (CM1049, Thermo Fisher Scientific Inc., MA, USA). All samples were then stomached at 250 RMP for 1 min (STOMACHER 400 CIRCULATOR, Seward Laboratory Systems Inc. (USA), FL, USA). Serial dilutions were made based on the results of last set of samples. 0.1 ml of selected dilutions were spread plated onto selective agars (XLT-4-NA for *Salmonella* samples or CT-SMAC-NA for *E. coli* samples) and then incubated at 37°C for 24-48h. Dilutions (1 ml) were plated onto 3M™ Petrifilm™ *E. coli*/Coliform Count Plates (#6414, 3M Unitek, CA, USA) to enumerate the endogenous *E. coli* populations. The Petrifilms were incubated
at 37°C for 24 h with blue colonies with gas being counted as *E. coli*. The black color colonies on XLT-4-NA indicate *Salmonella*, the pink colonies on CT-SMAC-NA indicate non-O157 *E.coli* strains, and the straw color colonies on CT-SMAC-NA indicate O157 *E.coli* strains. The detection limitation for selective agar and Petrifilm were 1.52 log CFU/g soil and 0.52 log CFU/g soil, respectively. In the event that no colonies were recovered on plates the samples were enriched at 37°C in TSB containing 50 µg/ml nalidixic acid. The enriched sample was then streaked on selective agar plates (XLT-4-NA for *Salmonella* samples or CT-SMAC-NA for STEC samples) and incubated at 37°C for 24 h. Latex agglutination tests (DR120M, Thermo Fisher Scientific Inc., MA, USA) were conducted to identify STEC. Modified Semi-solid Rappaport Vassiliadis (MSRV) agar (CM1112, Thermo Fisher Scientific Inc., MA, USA) with novobiocin supplement (SR0181, Thermo Fisher Scientific Inc., MA, USA) were used to identify *Salmonella*.

Soil moistures were calculated as follows: 10-15 g of soil was weighed into an aluminum baking tray, placed in oven for 24 hours at 100°C and weighed for residual to determine the water content of each type of soil. The soil water content was calculated as followed:

\[
\text{Soil Water Content} = \left( \frac{W_{\text{wet with tray}} - W_{\text{dry with tray}}}{W_{\text{dry with tray}} - W_{\text{tray}}} \right) \times 100%
\]

### 2.6 Set up for Laboratory Microcosms

The preparation of inocula, soil and manure for the supplementary laboratory microcosm studies followed the same procedures as the field trials. Approximately 80 kg of soil were sieved to 0.2 cm (200 mesh screen). After sieving, all soils were mixed in a large bin, 13 bags of soil were weighed out at 5000g per bag, and stored in double sealed zipper bags (to keep moisture) at 4°C to minimize biological activity in the soil.
To produce low moisture soil, four bags of 5000g sieved soil were put in trays, spread evenly in lab with occasionally agitation to lower the moisture; after some water evaporated, all soils were stored at 4°C in double layered zipper bag and labeled as ”Low moisture”. The moisture of low moisture soil and regular soil were determined by oven drying as described above.

The microcosm containers were 27.9 cm (Length) × 16.8 cm (Wide) × 13.7 cm (High) with lid made from polycarbonate. To prevent rapid moisture loss, a layer of gravel stone (about 3cm) was put at the bottom of the experimental container with 100ml distilled water, covered with a mesh to prevent direct contact to soil. Calculated volumes of distilled water were added to soil to achieve the required moisture levels (5%, 15%, and 25%). Manure additions were prepared by adding the selected inocula into 50 ml manure, shaking well and storing at 4°C. Soil was mixed with inoculated manure and put on top of the mesh. The soil was filled to a level of 10 cm. Containers were covered with lid to prevent rapid evaporation. Three incubators were set at 4°C, 10°C, 20°C (room temperature) or 30°C. All experimental containers were filled with appropriate soil and stored in selected temperature.

Sampling of the experimental containers was designed to collect the samples with minimum disturbance of the soil structure. Soils were collected using wide straws to collect small soil cores; after tapping the soil into weighing tray, the straws were placed back to the in the holes made in the soils to hold the structure of the soil bed. Triplicate 10 g samples were collected from each container, and transferred into stomacher bags. Moisture of each type of sample was determined by oven drying and calculations as described above. Plating and counting of all samples were the same as described above for the field trials.
2.7 Statistics

Statistical analysis was conducted on the data collected from laboratory microcosms. One way ANOVA with Repeated Measures using SPSS was selected to analysis the data.

2.8 Biosafety permit

All analysis were conduct in biosafety level 2 lab in Centre for Public Health and Zoonoses. Biohazard permit is approved by University of Guelph biosafety committee for project: Die-off rates of human pathogens in manure amended soil under natural climate conditions. Permit number: 285-19-15-03. Permit effected from April 01 2013 to March 31, 2015. Permitted work location is University of Guelph, Bldg 49, Rms 1127 and 1140.
Chapter 3 Results:

3.1 Initial Analysis of the Soil and Manure

Soils samples were collected from the upper layer (0-15 cm) of the two field sites prior to initiating the trial. Perth Loam (Loam site) consisted of 27% sand, 54% silt and 19% clay. The Hillsburg Fine Sandy Loam soil (Sandy site) was fine sandy loam which consists of 74% sand, 19% silt and 7% clay. The dairy manure had a higher total solids and potassium concentration compared to that collected from swine (Table 3-1). Both manure types had comparable phosphorus and nitrogen content on a wet weight basis (Table 3-1). With respect to E. coli counts, the dairy manure contained 5.69 log CFU/ml and was not significantly different to swine manure that harbored 4.84 log CFU/ml.

Table 3-1 Analyses of liquid dairy and swine manures (Spring 2014 trials)

<table>
<thead>
<tr>
<th></th>
<th>Dry matter%</th>
<th>Nitrogen%</th>
<th>Potassium%</th>
<th>Phosphorus%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy manure</td>
<td>4.76</td>
<td>0.26</td>
<td>0.23</td>
<td>0.10</td>
</tr>
<tr>
<td>Swine manure</td>
<td>2.86</td>
<td>0.24</td>
<td>0.04</td>
<td>0.13</td>
</tr>
</tbody>
</table>

(Analysed by SGS Agri-food Laboratories)

3.2 Pathogen Die-off in field trials

Three field trials were set up in spring 2013 (May), fall 2013 (October), and spring 2014 (June) in the manner described above. For all field trials in different seasons, samples (n=3) of each
treatment were removed periodically for determining the residual levels of *E. coli* O157:H7 or *Salmonella*, in addition to endogenous *E. coli*.

### 3.2.1 Pathogens in bulk soil after surface applied manure

Inoculated vials were placed within the test plot that was overlaid with either dairy or swine manure. At each sampling time point, soil core samples of the surface manure amended soil (subsequently referred to bulk samples) were taken to enumerate *E. coli* and total coliforms at surface and depth (15 cm) (Figure 3-1). The levels of total coliforms in the bulk samples was low due to the dilution effect of soil. Correspondingly, the *E. coli* levels were also low and fell below the level of detection by Day 35 irrespective of soil or manure type (Figure 3-1A). The total coliform count fluctuated throughout the 140 day trial period although levels remained low (Figure 3-1B). The results would suggest that *E. coli* from enteric origins, has lower persistence compared to environmentally adapted coliforms.
Figure 3-1 Generic *E.coli* and total coliforms from bulk sample in spring trial 2014 (A—Generic *E.coli* in all bulk samples, B—Total coliforms from all bulk samples)
3.2.2 *E. coli* O157:H7 die-off in manure amended soil

From the result of two years field experiment, the extent of *E. coli* O157:H7 die-off was found to be dependent on the manure origins, soil type and location (surface vs sub-surface) but less so on the season of application. Generally, the decline in *E. coli* O157:H7 followed non-linear kinetics with three phases. The initial phase was a rapid decline whereby the population of the pathogens decreased by 99% (2 log CFU/g dry soil) within the first 10 days of the trial. Within the second phase, *E. coli* O157:H7 numbers declined at a slower rate and then entered a persistent state where low levels (lower than selective agar detect limitation) could be recovered by enrichment.

The *E. coli* O157:H7 decline on Spring trial 2013

In spring trial 2013, the climatic conditions were hot in the initial phase of the study that coincided with a 1 log increase in *E. coli* O157:H7 levels. Thereafter, a rapid decline of *E. coli* O157:H7 that reached the Level of Detection (LOD) of < 1.52 log CFU/g soil within 2 months (Figure 3-1). Although *E.coli* O157:H7 was detected by enrichment none were recovered in samples taken at the end of the 3 month trial (Figure 3-1 A). With dairy manure, the regrowth phase was more evident in vials placed in the subsurface and was observed in both soil types. After Day 3 the levels of *E. coli* O157:H7 declined and by Day 75 could only be sporadically detected thereafter although populations of the pathogen were recovered when the trial was completed by Day 124 (Day 3-1A).

When *E. coli* O157:H7 was introduced into swine manure a similar trend was observed to that of dairy manure amended soil. However, the first phase was more a reduction in rate of decline although numbers did increase in vials placed on the sub-surface of sandy soil (Figure 3-2 B).
Again, by Day 75 the numbers had declined to the limit of detection but were the sporadically detected through to the end of the 124 day trial period.

The endogenous *E. coli* counts recovered alongside *E. coli* O157:H7, followed the same trend in terms of an initial die-off rate followed by a plateau or regrowth, then a progressive decline to the limit of detection (Figure 3-2 C, D). The results suggested that the endogenous *E. coli* behaved the same as *E. coli* O157:H7 and hence would act as a suitable surrogate for the pathogen.
Figure 3-2 Persistence of *Escherichia coli* O157:H7 (A, B) and generic *E. coli* (C, D) in sandy or loam soil amended with dairy or swine manure in spring trial 2013. (A-- *E. coli* O157 Die-off in Dairy Manure  B-- *E. coli* O157 Die-off in Swine Manure  C--Generic *E. coli* Die-off in Dairy Manure  D--Generic *E. coli* Die-off in Swine Manure)
**E. coli O157:H7 decline on Fall trial 2013**

With dairy manure amended soil, *E. coli* O157:H7 decreased in the initial phase but then increased in numbers up to Day 5 (Figure 3-3 A). The regrowth phase was more evident in vials placed on the sub-surface of soil compared to the surface but was found in both soil types. After Day 5 the levels of *E. coli* O157:H7 declined and by Day 216 (over winter) could only be sporadically detected. Populations of the pathogen could not be recovered at the end of the 10 month trial (Figure 3-3A).

When *E. coli* O157:H7 was introduced into swine manure a similar trend was observed compared to that of the pathogen in the presence of dairy manure. The slight regrowth phase of *E. coli* O157:H7 occurred in the sub-surface of both types of soil (Figure 3-3 B). Again, by Day 216 (over winter) the numbers had declined to the limit of detection and could not be detected by the end of the 330 Day trial period (Figure 3-3B).

The endogenous *E. coli* counts recovered alongside *E. coli* O157:H7 and followed the same trend in terms of an initial die-off rate followed by a plateau or regrowth, then a progressive decline to the limit of detection (Figure 3-3 C, D).
Figure 3-3 Persistence of *Escherichia coli* O157:H7 (A, B) and generic *E.coli* (C, D) in sandy or loam soil amended with dairy or swine manure in Fall trial 2013. (A--*E.coli* O157 Die-off in Dairy Manure  B--*E.coli* O157 Die-off in Swine Manure  C--Generic *E.coli* Die-off in Dairy Manure  D--Generic *E.coli* Die-off in Swine Manure)
**E. coli O157:H7 decline on Spring trial 2014**

In spring trial 2014, *E coli* O157:H7 progressively decreased over the initial 20 days of the trial. *E coli* O157:H7 then transiently grew up to 1.5 log (CFU g\(^{-1}\) dry soil) although levels were significantly (*P* < 0.001) lower compared to the original pathogen concentration with 3-4 log (CFU g\(^{-1}\) dry soil) reduction (Figure 3-4).

With dairy amended soil, *E. coli* O157:H7 were inoculated at a higher levels as 6 log CFU/g soil. *E. coli* O157:H7 decreased relatively rapidly in surface samples and slower in deep samples, especially in the subsurface of loam soil (Figure 3-4 A). The regrowth phase was only observed in the surface vials placed in sandy soils. After Day 2 the levels of *E. coli* O157:H7 declined rapidly with a 99% decrease in numbers by Day 16. The greatest persistence was observed in samples in the subsurface of sandy loam soil. By Day 108, in all samples, *E. coli* O157:H7 could only be sporadically detected thereafter although populations of the pathogen were recovered at the end of the 141 Day trial (Day 3-4A).

When *E. coli* O157:H7 was introduced into swine manure, a similar trend to that observed compared to that when the pathogen in the presence of dairy manure. However, the regrowth phase of *E. coli* O157:H7 was observed in surface samples from both soil types (Figure 3-4B). Because the regrowth of the samples, the surface samples from sandy soil were still at 3 log CFU/g dry soil level at the end of the trial. Similar to the dairy manure treatment, sub surface samples from sandy loam soil appear to have slower die-off than other three treatments. Again, by the end of the 141 Day trial period, only sub surface sample from sandy soil and surface sample from sandy loam soil reach the detect limitations, but were still detectable with enrichment.

The endogenous *E. coli* counts recovered alongside *E. coli* O157:H7 followed the same trend in terms of an initial die-off rate followed by a plateau or regrowth. The regrowth in endogenous
*E. coli* correlated to the regrowth of *E. coli* O157:H7 in dairy manure and swine manure (Figure 3-4 C, D).
Figure 3-4 Persistence of *Escherichia coli* O157:H7 (A, B) and generic *E. coli* (C, D) in sandy or loam soil amended with dairy or swine manure in spring trial 2014. (A-- *E. coli* O157 Die-off in Dairy Manure  B-- *E. coli* O157 Die-off in Swine Manure  C--Generic *E. coli* Die-off in Dairy Manure  D--Generic *E. coli* Die-off in Swine Manure)
3.2.3 *Salmonella* Die-off in field trials

*Salmonella* decline on Spring trial 2013

The spring trial of 2013 was characterized by a period of hot dry weather that coincided with rapid die-off of *Salmonella*. *Salmonella* reached the limit of detection (<1.52 log CFU/g soil) within 2 months regardless of the location, soil or manure type.

In the trial initiated in Spring (May) of 2013, *Salmonella* decreased in the initial phase but then increased in numbers up to Day 35 when inoculated into soil amended with dairy manure (Figure 3-5A). The regrowth phase was more evident in vials placed in the sandy soil compared to the sandy loam soil. After Day 5 the levels of *E. coli* O157:H7 declined and by Day 69 could only be sporadically detected by enrichment. No recovery thereafter until the trial was completed on Day 140 (Day 3-5A).

When *Salmonella* was introduced into swine manure a similar trend to that observed in dairy manure was recorded. However, no regrowth of *Salmonella* was observed in the surface sample from sandy loam soil and sub surface sample from sandy soil (Figure 3-5B). By Day 69 the numbers had declined to the limit of detection and could not be detected through to the end of the 140 Day trial period.

The endogenous *E. coli* counts recovered alongside *Salmonella* followed the same trend in terms of an initial die-off rate followed by a plateau or regrowth, then a progressive decline to the limit of detection (Figure 3-5 C, D). The results suggested that the endogenous *E. coli* behaved the same as *Salmonella* and hence would act as a suitable surrogate for the pathogen.
Figure 3-5 Persistence of *Salmonella* (A, B) and generic *E. coli* (C, D) in sandy or loam soil amended with dairy or swine manure in spring trial 2013. (A--*Salmonella* Die-off in Dairy Manure  B--*Salmonella* Die-off in Swine Manure  C--Generic *E. coli* Die-off in Dairy Manure  D--Generic *E. coli* Die-off in Swine Manure)
**Salmonella decline on Fall trial 2013**

In fall trial 2013, *Salmonella* decreased then increased over the initial 20 days of the trial (before soil was frozen). The field plot was covered with a snow layer from December through to April and hence no sampling was possible during this period. After the winter, *Salmonella* persisted at low levels that could only be detected by enrichment (<1.52 log CFU/g soil), but was not detected 10 months into the trial period.

*Salmonella* decreased in the initial phase but then increased in numbers up to Day 5 when inoculated into soil amended with dairy manure (Figure 3-6 A). The regrowth phase was more evident in vials placed in the loam soil compared to the sandy soil. After Day 5 the levels of *Salmonella* declined and by Day 216 (over winter) could only be detected by enrichment. Populations of the pathogen could not be recovered when the trial was completed by Day 330 (Figure 3-6A).

When *Salmonella* was introduced into swine manure a similar trend observed to that of dairy amended manure. There was no regrowth phase of *Salmonella* was occurred on the sub-surface of sandy soil (Figure 3-6 B). Again, by Day 216 (over winter) the numbers had declined to the limit of detection, and cannot be detected by the end of the 330 Day trial period (Figure 3-6 B).

The endogenous *E. coli* counts recovered alongside *Salmonella* followed the same trend in terms of an initial die-off rate followed by a plateau or regrowth, then a progressive decline to the limit of detection (Figure 3-6 C, D).
Figure 3-6 Persistence of *Salmonella* (A, B) and generic *E. coli* (C, D) in sandy or loam soil amended with dairy or swine manure in fall trial 2013. (A-- *Salmonella* Die-off in Dairy Manure  B-- *Salmonella* Die-off in Swine Manure  C--Generic *E. coli* Die-off in Dairy Manure  D--Generic *E. coli* Die-off in Swine Manure)
Salmonella decline in Spring trial 2014

In spring trial 2014, Salmonella progressively decreased over the initial 20 days of the trial. Transiently growth of Salmonella appeared on the second phase of die-off. Salmonella persisted over the duration of the 5 month trials at low levels that could only be detected by enrichment (<1.52 log CFU/g soil) by the end of the spring trial.

In dairy amended soil Salmonella decreased rapidly in surface samples compared to samples at the subsurface. The rate of decline was slower in loam soil compared to sandy loam with higher persistence being observed at the subsurface (Figure 3-7 A). The regrowth phase was only observed in the surface vials placed in sandy soils. After Day 2 the levels of Salmonella declined fast, most samples were at 2 log CFU/g dry soil levels by Day 16. The decline in deep samples placed in sandy loam soil was slower than other three treatments. By Day 70, all samples could only be detected by enrichment, although populations of the pathogen were recovered by enrichment when the trial was completed by Day 141 (Day 3-7 A).

When Salmonella was introduced into swine manure a similar trend was observed compared to that when the pathogen in the presence of dairy manure. Similarly, the regrowth was only observed in the surface vials placed in sandy soils (Figure 3-7 B). Because the regrowth of the samples, the surface samples from sandy soil were still at 2 log CFU/g dry soil level by the end of the 141 Day trial period, while all other treatments reached detection limits by Day 69.

The endogenous E. coli counts recovered alongside Salmonella followed the same trend in terms of an initial die-off rate followed by a plateau or regrowth. The regrowth in endogenous E. coli correlated to the regrowth of Salmonella in dairy and swine manure (Figure 3-7 C, D).
Figure 3-7 Persistence of *Salmonella* (A, B) and generic *E. coli* (C, D) in sandy or loam soil amended with dairy or swine manure in spring trial 2014. (A--*Salmonella* Die-off in Dairy Manure  B--*Salmonella* Die-off in Swine Manure  C--Generic *E. coli* Die-off in Dairy Manure  D--Generic *E. coli* Die-off in Swine Manure)
3.2.4 *Salmonella* and *E.coli* O157:H7 die-off as positive control (mixed with both *E.coli* O157:H7 and *Salmonella* pathogens spike) and negative control (no pathogen spike) in Spring 2013, Fall 2013, and Spring 2014 field trials

The positive control samples were manure amended soil spiked with *E. coli* O157:H7 and *Salmonella*, and the negative control samples were un-spiked dairy manure amended soil. All control samples were put in sentinel vials and processed the same as other samples. The mix spiked *E. coli* O157:H7 and *Salmonella* had similar die-off patterns to *E. coli* O157:H7 and *Salmonella* on itself, all declined with three phases. The initial rapid decline phase, followed by pathogen decreased progressively over a period until cannot recovered by plate count method. The third phase was tailing with low levels of residual survivors persisting cells that could only be detected by enrichment (<1.52 log CFU/g soil).

In all three field trials, in positive control samples, *E. coli* O157:H7 show higher persistent than *Salmonella*, and deep samples survive better than surface samples (Compare Figure 3-8 (A) to (B)). In negative control samples, generic *E. coli* appears to have similar die-off with generic *E. coli* from positive control samples, which indicate the Petrifilm readings were not influenced by spiked pathogens.

Persistence of *E. coli* O157:H7, *Salmonella* and generic *E. coli* in sandy or loam soil amended with dairy or swine manure from positive and negative controls for all three field trials are presented in Figure 3-8 to 3-10.
Figure 3-8 Persistence of *Salmonella* and *E. coli* O157:H7(A, B) and generic *E. coli* (C, D) in sandy or loam soil amended with dairy or swine manure in spring trial 2013. (A-- *E. coli* O157:H7 Die-off in positive control, B-- *Salmonella* Die-off in positive control, C--Generic *E. coli* Die-off in positive control, D--Generic *E. coli* Die-off in negative control)
Figure 3-9 Persistence of *Salmonella* and *E. coli* O157:H7(A, B) and generic *E. coli* (C, D) in sandy or loam soil amended with dairy or swine manure in fall trial 2013. (A-- *E. coli* O157:H7 Die-off in positive control, B-- *Salmonella* Die-off in positive control, C--Generic *E. coli* Die-off in positive control, D--Generic *E. coli* Die-off in negative control)
Figure 3-10 Persistence of *E. coli* O157:H7 and *Salmonella* (A, B) and generic *E. coli* (C, D) in sandy or loam soil amended with dairy or swine manure in spring trial 2014. (A-- *E. coli* O157:H7 Die-off in positive control, B-- *Salmonella* Die-off in positive control, C--Generic *E. coli* Die-off in positive control D--Generic *E. coli* Die-off in negative control)
3.2.5 Comparative die-off of *E. coli*, *E. coli* O157:H7 and *Salmonella* in different soils amended with either dairy or swine manure

Because of the non-linear die-off curves it was not possible to determine rate constants to achieve a designated log (CFU/g dry soil) in pathogen levels. Therefore, the time for pathogens declined by 2 log (99%) or 3 log (99.9%) (CFU/g dry soil) under the different conditions was used as a metric to compare relative die-off rates (Figure3-11, 12).

**Spring Trials**

In the Spring 2013 trial, STEC or *Salmonella* introduced into dairy manure decline by 2 log CFU/ dry soil within 2 days on vials placed on the surface of loam or sandy soil. However, differences were observed with vials containing dairy manure amended soil planted at depth. Here, STEC persisted longer in dairy manure compared to *Salmonella* independent of soil type. Yet, *Salmonella* persisted longer in loam soil at depth with time to reach the 2 log decline being 29 days compared to only 4 in sandy soil.

In swine manure amended soil there was a rapid decline in STEC with 99% reduction occurring within 4 days regardless of depth vs surface or soil type. The persistence of Salmonella was greater than that of STEC with vials in the subsurface persisting longer than those on the surface. Again, persistence of Salmonella was greater in loam soil compared to sandy soil (Figure 3-11, 12).

In the Spring 2014 trial for STEC the general trends identified in the 2013 trial were observed although time to decline by 2 log CFU/g did vary. Specifically, vials on the surface declined by 99% within 2 days. Those vials placed at the subsurface persisted for longer especially in loam soil compared to sandy soil. In a similar manner, *Salmonella* persisted longer in the sub-surface of loam soil but less so in sandy soil.
With swine manure amended soil the decline in STEC was more rapid compared to when dairy manure was applied. On the occasion of the Spring 2014 trial there were small differences in vials placed on the surface versus the subsurface. The same trend was observed for *Salmonella* in swine manure amended soil although vials in the subsurface of loam soil took 20 days to decrease by 99% compared to only 9 days in sandy soil.

**Fall Trial**

In the fall trial the time taken to decrease initial levels of STEC by 99% in dairy amended manure occurred within 10 days regardless of soil type or location (Surface vs deep). In contrast, STEC in swine manure amended soil persisted to a greater extent in the subsurface of loam soil and to a lesser extent in sandy soil. Yet, STEC decreased by 2 log cfu within 2 days when applied on the surface irrespective of soil type.

*Salmonella* die-off in dairy or swine manure amended soil was slowest in the subsurface of loam soil and took 28 days to decrease by 99%. This compares to 12 days in the subsurface of sandy soil that was longer for either of vials placed on the sub-surface of wither sandy or loam soil.

**Time for STEC or *Salmonella* to decrease by 3 log reduction**

The time to decrease pathogen populations by 99.9% represents the second phase of the die-off curve. That is, the phase that follows the initial rapid rate of decline but before the persistent state. The results essentially followed the same trends as observed for the time to achieve a 2 log reduction. Specifically, the most rapid decline of STEC was in vials on the surface compared to the sub-surface. Survival of STEC in the subsurface was greatest in loam soil when amended with
swine manure. However, in the subsurface of sandy soil longer persistence was obtained using dairy manure compared to that derived from swine.

The persistence of *Salmonella* was highest in the subsurface of loam soil irrespective if swine or dairy manure was applied. Survival of Salmonella in the subsurface of sandy soil was higher in dairy manure compared to swine manure. The time to decrease levels of Salmonella by 99.9% on surface was generally less than 10 days. Yet, there was extended survival of Salmonella in swine amended manure in loam soil on the surface.

Comparing between Spring and Fall applications was problematic given the limited number of trials performed. Yet, it is noteworthy that the highest persistence (>120 days) of Salmonella was obtained in both the Spring and Fall trails of 2013. In comparison, the longest persistence of Salmonella in the Spring 2014 trials was only 50 days. STEC was reduced by 99.9% before 120 days in all the different treatments with highest persistence (70 days) being in Fall application in swine manure in loam soil.
Figure 3-11 Time to reduce *Salmonella* or *E.coli* O157:H7 populations by 2 log (99%) CFU/g dry soil in different soils and manure type introduced in the Fall or Spring 2013 and 2014
3.2.6 Residual pathogen levels in manure amended soil samples

At the end of the three trials the final vials were collected and enriched for either *Salmonella* or *E. coli* O157:H7. There were three field trials. Spring 2013 trial started from May 17th 2013 and last for 140 days through the summer of 2013. Fall 2013 trial started from Oct 27th and last for 330 days through the winter 2013 until summer 2014. Spring 2014 trial started from Jun 4th 2014 and last for 141 days through the summer 2014.

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Figure 3-12 Time to reduce *Salmonella* or *E. coli* O157:H7 populations by 3 log (99.9%) CFU in different soils and manure type introduced in the Fall or Spring 2013 and 2014.
The only samples testing positive for the respective pathogen were from trials initiated in Spring 2014 (Table 3-2). Here, *E. coli* O157:H7 was recovered in all samples save for those in swine amended loam soil where 1 of 3 enrichments tested positive for the pathogen. In the same trial, Salmonella were recovered in the majority of samples of manure amended soil although sporadically samples containing dairy manure (Table 3-3). The persistence of residual populations was independent of soil type for both *Salmonella* and *E. coli* O157:H7.
Table 3-2 Enrichment result from last sample of each trial

<table>
<thead>
<tr>
<th>Experiment Period (Days)</th>
<th>E.coli</th>
<th>Salmonella</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spring 2013</td>
<td>Fall 2013</td>
</tr>
<tr>
<td>Dairy Sandy Surface</td>
<td>140</td>
<td>330</td>
</tr>
<tr>
<td>Dairy Sandy Deep</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Dairy Loam Surface</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Dairy Loam Deep</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Swine Sandy Surface</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Swine Sandy Deep</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Swine Loam Surface</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Swine Loam Deep</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Dairy Sandy Surface(Positive Control)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Dairy Sandy Deep(Positive Control)</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>
3.3 Environmental climatic conditions

Climatic conditions were monitored in experiment field plots, including air temperature, soil temperature, soil moisture and precipitation. Precipitation, air and soil temperatures, and soil moisture at the field plots were monitored by a Campbell continuous automated data logger through the experiment period; soil moistures for each plot were also measured on bulks soil samples at each sampling time.

Weather data during the experimental period including precipitation, air and soil temperatures are summarized in Table 3-4, and showed on Figure 3-13(A, B) and Figure 3-14(A, B).

Table 3-3 Summery of Soil, Air Temperature and Soil Moisture in Spring trial 2013 and 2014

<table>
<thead>
<tr>
<th></th>
<th>Soil Temperature 15cm(°C)</th>
<th>Soil Temperature 1cm(°C)</th>
<th>Max Air Temperature (°C)</th>
<th>Min Air Temperature (°C)</th>
<th>Precipitation (mm)</th>
<th>Moisture %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2013</td>
<td>High</td>
<td>24.19</td>
<td>27.62</td>
<td>37.72</td>
<td>22.30</td>
<td>53.40</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>1.39</td>
<td>-2.58</td>
<td>-1.84</td>
<td>-5.18</td>
<td>0.00</td>
</tr>
<tr>
<td>2014</td>
<td>High</td>
<td>22.99</td>
<td>28.68</td>
<td>29.91</td>
<td>20.62</td>
<td>23.00</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>11.24</td>
<td>9.54</td>
<td>9.50</td>
<td>-0.70</td>
<td>0.00</td>
</tr>
<tr>
<td>2013</td>
<td>Ave</td>
<td>15.16</td>
<td>15.70</td>
<td>21.91</td>
<td>10.36</td>
<td>3.49</td>
</tr>
<tr>
<td>2014</td>
<td>Ave</td>
<td>17.58</td>
<td>18.46</td>
<td>23.24</td>
<td>9.88</td>
<td>2.79</td>
</tr>
</tbody>
</table>
Figure 3-13(A) Max/Min Air temperatures and 1cm/15cm soil temperatures changes over the experimental period in 2013.
Figure 3-13(B) Max/Min Air temperatures and 1cm/15cm soil temperatures changes over the experimental period in 2014
Figure 3-14 (A) Precipitation and soil moisture changes over the experimental period in 2013

Figure 3-14 (B) Precipitation and soil moisture changes over the experimental period in 2014
Data from The Weather Network, 2014 for Arthur, Ontario, (The Weather Network, 2014), the closest available weather station to the plots indicates that both experimental years were slightly wetter years than average (Table 3-4).

Table 3-4 Comparison of rainfall in two experiment years

<table>
<thead>
<tr>
<th>Experiment Period</th>
<th>Spring Trial 2013</th>
<th>Spring trial 2014</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Rainfall</td>
<td>107mm</td>
<td>85mm</td>
</tr>
<tr>
<td>Average Rainfall annually over 30 years in experiment period</td>
<td>98.5mm</td>
<td>83mm</td>
</tr>
</tbody>
</table>

The soil moisture for the two field plots at each sampling time is shown in Figure 3-12 (A, B). In general, soil moisture increased with each precipitation event. High temperatures promote the evaporation and decrease the soil moisture. The periods of precipitation increased the moisture levels of the soil that fluctuated around 10% to 15% during June to July then increased to 25% in late summer. The fields encountered dry periods around July 2013 and June 2014.

The soil moisture content correlated to precipitation events in two spring trials. In Spring trial 2014, the average soil moisture dropped to the lowest at 10.1% on June 27th samples as there was no precipitation for six days in the experimental area, and the highest average soil moisture monitored was the Sep 20th samples at 22.0% as there was 17.1 mm precipitation in the area on that day. From each individual sample, loam surface sample applied with swine manure reach lowest moisture to 1% on June 27th sample, and loam surface sample applied with dairy manure reach highest moisture to 29.7% on Oct 23th.
Soil samples taken from the sub-surface had consistently higher moisture levels compared to those from the surface. With regards to soil type, loam samples had higher moisture content that sandy loam suggesting lower water binding and retention of the latter (Figure 3-15 D).

From July to October, the moisture content of loam soil was 5% to 10% higher compared to that of sandy soil (Figure 3-15 D).
Figure 3-15 Soil moisture changes in Spring Field Trial 2014 (A—Soil moisture changes in dairy manure, B—Soil moisture changes in swine manure, C—Soil moisture compare in surface and deep samples, D—Soil moisture compare in loam and sandy loam soil)
3.3.1 Correlations between weather conditions and pathogen die-off

Attempts were made to correlate events of pathogen regrowth and rapid die-off with climatic conditions especially in the early parts (40 days) of the trials. Figures 3-13 and 3-14 compared the die-off of *E.coli* O157:H7 and *Salmonella* with temperature, moisture and precipitation changes in the spring trials performed in 2013 or 2014.
Figure 3-16(A, B, C, D) Comparison of weather conditions in Spring 2013 and die-off of *Salmonella* and *E.coli O157* in loam soil and sandy loam soil amended with dairy and swine manure at surface and 15cm depth in spring trial 2013 (A--Max air temperature, soil temperature at 1cm and 15cm through the first 40 days of field trial, B-- *E.coli O157* Die-off in sandy and sandy loam soil amended with dairy and swine manure at surface and 15cm depth, C-- *Salmonella* Die-off in sandy and sandy loam soil amended with dairy and swine manure at surface and 15cm depth, D--Precipitation and soil moisture change 15cm through the first 40 days of Spring field trial 2013)
Figure 3-17(A, B, C, D) Comparison of weather conditions in Spring 2014 and die-off of Salmonella and E. coli O157 in loam soil and sandy loam soil amended with dairy and swine manure at surface and 15cm depth in spring trial 2014 (A--Max air temperature, soil temperature at 1cm and 15cm through the first 40 days of field trial, B--E. coli O157 Die-off in sandy and sandy loam soil amended with dairy and swine manure at surface and 15cm depth, C—Salmonella Die-off in sandy and sandy loam soil amended with dairy and swine manure at surface and 15cm depth, D—Precipitation and soil moisture change 15cm through the first 40 days of field trial 2014)
In the first 2 days of the 2013 trial there was an increase of 5°C as the weather went through a hot period without precipitation. The average soil moisture for the first two days were 12.4%, which were relatively low compare to the average of the whole trial of 40.0%. During the same period, both *E.coli* O157:H7 and *Salmonella* increased by 1 log before declining. The results suggest that high temperature correlates with the re-growth of both enteric pathogens in the soil environment.

In the spring 2013 trial from day 10 to 14, there are a sharp increase of air temperature from 17°C to 30°C. Accordingly, the soil temperature of the surface (1 cm) increased from 16°C to 23°C while soil deep (15 cm) increased from 16°C to 18°C. For *E.coli* O157 samples, all surface samples appear to increase in numbers in response to the temperature rise. For *Salmonella* samples, the growth of the pathogen was observed especially in vials on the surface of sandy soil amended with swine manure.

There were two major precipitation events that affected the soil moisture in the spring 2013 trial. Between days 6 to 9, there was precipitation in the field with a corresponding increase soil moisture levels. At the same time, *E.coli* O157 and *Salmonella* levels decreased progressively. The second precipitation event occurred from day 34 to 35, with over 20mm rainfall that increased the soil moisture content to 49%. The humidity also increased at the same time due to the rise in temperature from 20°C to 27°C. In the course of this period the die-off of *E. coli* O157:H7 and Salmonella was retarded although no pathogen growth was observed.

From Figure 3-16, 17, pathogen die-off was affected by the weather conditions showed in the second phase. In the first phase, the pathogen die-off was fast and cannot be correlated with the weather. Comparing Figure 3-16 (A) to (B, C), the increase of soil temperature correlated with the increase of the pathogens, especially for the surface samples. In addition, from the Figure 3-16
(C), re-growth of *Salmonella* occurred at day 5 to day 17 in sandy surface samples applied with swine manure when the soil temperature increased to 37°C in the presence of high moisture.

Comparing the first 40 days of the two seasons, the spring trial in 2014 had a sharp increase in temperature by 7°C by Day 2 followed by rapid temperature decrease to 10°C. The soil was saturated (35-40%) when the experiment was set up, followed by heavily rain (53mm) in the fourth day which increased the soil moisture to 50%. At the same time there was a rapid die-off of pathogens.

### 3.4 Pathogen Die-off in Microcosm Study

Although field trials provide the only means to determine die-off rates under natural conditions the data analysis is complicated due to multiple contributing intrinsic and extrinsic factors. Therefore, supplementary studies were undertaken using pathogens inoculated into manure amended soil microcosms held under constant moisture or temperature (Figure 3-18 to 3-23).

#### 3.4.1 STEC die-off in microcosm study

A repeated measures ANOVA was used to conduct the analysis (lærd Statistics, 2015). *E.coli* O157, O145 or O26 levels at 12 sampling time points were analyzed in the form of recovered CFU/g dry soil. Temperature was set at 4 static levels (4°C, 10°C, 20°C or 30°C) with three moisture levels (5%, 10% or 15%).

The die-off rate of *E. coli* O26 or O145 was significantly higher than *E. coli* O157:H7 within the range of applied moisture and temperature conditions. The *E.coli* O26 or O145 at all moisture and temperature conditions tested reached a 3 log reduction within 5 days while it took >10 days for *E.coli* O157 to decrease to the same level (Figure 3-18, 19, 20). The persistence of STEC was
enhanced at 4°C and 10°C with regrowth being observed for *E. coli* O157:H7 held at 30°C but not with either O26 or O145 (Figure 3-18, 19, 20).

A Greenhouse-Geisser correction was used for *E.coli* O157, O26 or O145 because the Mauchly’s Sphericity test indicates the assumption of sphericity is not met (Table 3-5 A). Within subjects effects test compared the *E.coli* die-off over time, over time between different moisture, and over time between different temperatures in two *E.coli* group (*E.coli* O157 and *E.coli* O26 or O145).

<table>
<thead>
<tr>
<th>Within-Subjects Effects Test</th>
<th>df (E.coli O157)</th>
<th>df (E.coli O26 or O145)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>P&lt;0.0005</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Time * Moisture</td>
<td>0.232</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Time * Temperature</td>
<td>0.001</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Between Subjects Effects Tests</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moisture</td>
<td>0.015</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.001</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

*P < 0.05 indicate two compared value were significant different

*P > 0.05 indicate two compared value were not significant different

The Post Hoc Test for moisture temperature, with equal variances assumed as Tukey, the cooperation results are shown in Table 3-5 (B).
Table 3-5 (B) Statistic analysis for the *E.coli* O157, O26 and O145

<table>
<thead>
<tr>
<th>Moisture</th>
<th>df (E.coli O157)</th>
<th>df (E.coli O26 or O145)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>10% 0.031 P &lt; 0.05</td>
<td>0.057 P &gt; 0.05</td>
</tr>
<tr>
<td></td>
<td>15% 0.029 P &lt; 0.05</td>
<td>P&lt;0.0005 P &lt; 0.05</td>
</tr>
<tr>
<td>10%</td>
<td>15% 0.999 P &gt; 0.05</td>
<td>0.017 P &lt; 0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temperature</th>
<th>df (E.coli O157)</th>
<th>df (E.coli O26 or O145)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>10°C 1.000 P &gt; 0.05</td>
<td>0.962 P &gt; 0.05</td>
</tr>
<tr>
<td></td>
<td>20°C 0.008 P &lt; 0.05</td>
<td>P&lt;0.0005 P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>30°C 0.932 P &gt; 0.05</td>
<td>0.421 P &gt; 0.05</td>
</tr>
<tr>
<td>10°C</td>
<td>20°C 0.007 P &lt; 0.05</td>
<td>P&lt;0.0005 P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>30°C 0.956 P &gt; 0.05</td>
<td>0.709 P &gt; 0.05</td>
</tr>
<tr>
<td>20°C</td>
<td>30°C 0.002 P &lt; 0.05</td>
<td>P&lt;0.0005 P &lt; 0.05</td>
</tr>
</tbody>
</table>

*P < 0.05 indicate two compared value were significant different

*P > 0.05 indicate two compared value were not significant different

Endogenous *E. coli* associated with manure that was incorporated into soil was monitored under the same conditions as STEC. Comparison of generic *E.coli* with tested pathogens are showed in Table 3-5 (C).

Table 3-5 (C) Statistic analysis for generic *E.coli*

<table>
<thead>
<tr>
<th>df (Generic E.coli)</th>
</tr>
</thead>
</table>

**Between Subjects Effects Tests**
- Between different pathogen types P< 0.0005 P < 0.05

**Post Hoc Tests for three pathogens**
- Generic *E.coli* *E.coli* O157 P< 0.0005 P < 0.05
- *E.coli* O26 or O145 0.119 P > 0.05

*P < 0.05 indicate two compared value were significant different

*P > 0.05 indicate two compared value were not significant different

In summary, for *E.coli* O157, persistence at 5% moisture was significantly lower than persistence at 10% and 15%, and persistence at 10% was also lower than persistence at 15% but
not significantly. Persistence at 20°C was higher than persistence at 4°C followed by 10°C. All those three temperatures were significantly higher compared to 30°C.

For *E. coli* O26 or O145, persistence at 15% moisture was significantly higher compared to 10% and 5% moistures, and persistence at 10% was also lower than persistence at 5% but not significantly. An incubation temperature of 20°C was significantly higher than other three temperatures tested. Persistence at 4, 10 or 30°C was not significantly different. Generic *E.coli* had similar die-off trend with O26 but lower than serotype O157.
Figure 3-18 Die-off of *E.coli* O26/O145, *E.coli* O157, and generic *E.coli* in 5% moisture sandy soil amended with *E.coli* spiked dairy manure at 4°C, 10°C, 20°C and 30°C in lab trial 2014 (A-- *E.coli* O26/O145 die-off in 5% moisture sandy soil at 4°C, 10°C, 20°C and 30°C, B--*E.coli* O157 die-off in 5% moisture sandy soil at 4°C, 10°C, 20°C and 30°C, C-- Background of *E.coli* (Generic *E.coli*) die-off in 5% moisture sandy soil at 4°C, 10°C, 20°C and 30°C)
Figure 3-19 Die-off of *E.coli* O26/O145, *E.coli* O157, and generic *E.coli* in 10% moisture sandy soil amended with *E.coli* spiked dairy manure at 4°C, 10°C, 20°C and 30°C in lab trial 2014 (A-- *E.coli* O26/O145 die-off in 10% moisture sandy soil at 4°C, 10°C, 20°C and 30°C, B--*E.coli* O157 die-off in 10% moisture sandy soil at 4°C, 10°C, 20°C and 30°C, C-- Background of *E.coli* (Generic *E.coli*) die-off in 10% moisture sandy soil at 4°C, 10°C, 20°C and 30°C)
Figure 3-20 Die-off of *E. coli* O26/O145, *E. coli* O157, and generic *E. coli* in 15% moisture sandy soil amended with *E. coli* spiked dairy manure at 4°C, 10°C, 20°C and 30°C in lab trial 2014 (A-- *E. coli* O26/O145 die-off in 15% moisture sandy soil at 4°C, 10°C, 20°C and 30°C, B-- *E. coli* O157 die-off in 15% moisture sandy soil at 4°C, 10°C, 20°C and 30°C, C—Background (Generic *E. coli*) of *E. coli* die-off in 15% moisture sandy soil at 4°C, 10°C, 20°C and 30°C, D-- Generic *E. coli* of negative control (amended with unspiked manure) die-off in 10% moisture sandy soil at 20°C)
3.4.2 *Salmonella* die-off in microcosm study

Relative to STEC, *Salmonella* exhibited an extended persistence at all temperatures and moisture values. Persistence was extended at low incubation temperatures (4°C and 10°C) but transient growth at higher temperatures when moisture was high. Yet, growth was followed by rapid die-off thereafter (Figure 3-21 to 3-23).

A repeated measures ANOVA was used to conduct the analysis. The recovery CFU/g dry soil of *Salmonella* were analysed. A Greenhouse-Geisser correction was used for *Salmonella* because the Mauchly’s Sphericity test indicates the assumption of sphericity is not met (Table 3-7 A).

Table 3-6 (A) Statistic analysis for the *Salmonella*

<table>
<thead>
<tr>
<th>df (Salmonella)</th>
<th>Within-Subjects Effects Test</th>
<th>Between Subjects Effects Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>time</td>
<td>time * Moisture</td>
</tr>
<tr>
<td></td>
<td>P&lt;0.0005</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.05</td>
<td>P &gt; 0.05</td>
</tr>
</tbody>
</table>

*P < 0.05 indicate two compared value were significant different

*P > 0.05 indicate two compared value were not significant different
The Post Hoc Test for moisture temperature, with equal variances assumed as Tukey, the cooperation results are shown in Table 3-7 (B).

Table 3-6 (B) Statistic analysis for the *Salmonella*

<table>
<thead>
<tr>
<th>Moisture</th>
<th>df (Salmonella)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% 10%</td>
<td>0.015</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>15% 10%</td>
<td>0.518</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>10% 15%</td>
<td>0.111</td>
<td>P &gt; 0.05</td>
</tr>
</tbody>
</table>

Endogenous *E. coli* associated with manure that was incorporated into soil was monitored under the same conditions as *Salmonella*. Comparisons of generic *E.coli* with tested pathogens are shown in Table 3-7 (C).

Table 3-6 (C) Statistic analysis for the generic *E.coli*

<table>
<thead>
<tr>
<th>Between Subjects Effects Tests</th>
<th>df (Generic E.coli)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between different pathogen types</td>
<td>P&lt; 0.0005</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

*P < 0.05 indicate two compared value were significant different

*P > 0.05 indicate two compared value were not significant different*
In summary, for *Salmonella* die-off in the laboratory experiment, only 5% moisture was significantly higher than 10% moistures but not between other moistures. Persistence at 5% moisture was higher than persistence at 15% followed by persistence at 10%.

Four temperatures were also similar from adjacent temperature levels, but significantly different from parted temperature levels (P<0.05). Persistence at 4 °C was higher than persistence at 10 °C followed by 20 °C and persistence at 30 °C.

The die-off of *Salmonella* in microcosms held under constant moisture (5%, 10%, and 15%) and temperature (4°C, 10°C, 20°C and 30°C) are shown in Figure 3-18 to 3-20.
Figure 3-21 Die-off of *Salmonella* and generic *E.coli* in 5% moisture sandy soil amended with *Salmonella*-spiked dairy manure at 4°C, 10°C, 20°C and 30°C in lab trial 2014 (A-- *Salmonella* die-off in 5% moisture sandy soil at 4°C, 10°C, 20°C and 30°C, B-- Background (Generic *E.coli*) of *Salmonella* die-off in 5% moisture sandy soil at 4°C, 10°C, 20°C and 30°C)
Figure 3-22 Die-off of Salmonella and generic E.coli in 10% moisture sandy soil amended with Salmonella-spiked dairy manure at 4°C, 10°C, 20°C and 30°C in lab trial 2014 (A-- Salmonella die-off in 10% moisture sandy soil at 4°C, 10°C, 20°C and 30°C, B-- Background (Generic E.coli) of Salmonella die-off in 5% moisture sandy soil at 4°C, 10°C, 20°C and 30°C)
Figure 3-23 Die-off of *Salmonella* and generic *E.coli* in 15% moisture sandy soil amended with *Salmonella*-spiked dairy manure at 4°C, 10°C, 20°C and 30°C in lab trial 2014 (A-- *Salmonella* die-off in 15% moisture sandy soil at 4°C, 10°C, 20°C and 30°C, B--Background (Generic *E.coli*) of *Salmonella* die-off in 5% moisture sandy soil at 4°C, 10°C, 20°C and 30°C, C-- Generic *E.coli* of negative control(amended with unspiked manure) die-off in 10% moisture sandy soil at 20°C)
Chapter 4. Discussion

4.1 Discussion and comparison between trials

The primary objective of the study was to determine the die-off rates of STEC and Salmonella in manure amended soil to verify if current recommendations of 90-120 day wait period is sufficient to ensure pathogen inactivation.

A common feature in all of the field trials was the non-linear die-off kinetics that could be separated into three distinct phases. Specifically, there was an initial rapid decrease in pathogen numbers followed by a slower phase then an extended period of persistence. The non-linear decrease in pathogens in the soil environment is in agreement with previously reported studies (Brennan et al., 2014, Moynihan et al., 2013, Erickson et al., 2014, Erickson et al., 2015). However, other studies under laboratory settings more frequently observe linear kinetics (Liang et al., 2011). In the current study it was noted that laboratory based studies that applying constant moisture and temperature resulted in more linear die-off kinetics compared to those observed in field trials. The difference between field-based generated die-off kinetics with laboratory based studies can be attributed to the dynamic intrinsic and extrinsic factors that constantly vary under natural climatic conditions along with stress adaption of the pathogen.

Researchers have undertaken different approaches in presenting non-linear die off kinetics in the context of predicting modelling. For example, only the initial linear portion of the data is taken to describe die-off whilst others have used the time to reduce the population by 50%. Neither approach is ideal given that an underestimation of die-off time would be generated due to failing to account for persisted populations or pathogen regrowth (Oliver et al., 2010). A further approach is to apply Weibull modeling although valid models can only be generated by having a complete understanding of the influential factors of pathogen die-off which at the moment are lacking
(Bradford et al., 2014, Ongeng et al., 2015a). Although predictive modelling is the ultimate goal of studies determining die-off rates, at present the role of different factors from method of enumeration through to the effect of endogenous microflora and soil physical properties still require to be elucidated (Ongeng et al., 2015a). On this basis the current study undertook the relatively simplistic approach of determining the time taken for a given STEC or Salmonella population to decline by 99% or 99.9% to represent the first and second die-off phase respectively. There are limitations to the model as it does not directly correlate the intrinsic and extrinsic factors that contribute to the die-off rate. In addition, the approach does not account for the transient re-growth of pathogens that was observed in the current field trials with Salmonella and to a limited extent STEC.

The growth of pathogens such as Salmonella and E. coli outside the primary environment of the gastrointestinal tract has been previously observed (Ishii et al., 2006). Growth is typically encountered under conditions of high organic content, moisture and temperatures >25°C. There is also a theory that strains of enteric bacteria (termed naturalized) have a genetic disposition of being adapted to environments outside the gastrointestinal tract to the extent of total adaption (Ishii et al., 2006). In the current study the re-growth of Salmonella and STEC was favored under high temperatures and moisture in both field trials and within laboratory microcosms. However, this could not be attributed to naturalization given growth was only transient and followed by a rapid decline. Nevertheless, the result does highlight that the potential for enteric pathogens to grow in the field environment exists and would ultimately impact on estimating die-off rates.

The study looked at the effect of climatic conditions, manure type (swine vs dairy), time of application, surface vs subsurface and two different soils (loam vs sandy) on the persistence of STEC and Salmonella. From the field trial data it was apparent that each of the above factors
contributed to pathogen survival although to different degrees. In the case of STEC it was found that enhanced persistence was promoted in the sub-surface of dairy amended loam soil. The same was found for Salmonella although dairy manure resulted in higher die-off rates compared to swine. Although not investigated to any great extent in the current study the effect of manure type is thought be due to pH, carbon composition and endogenous microflora. For example, the acidic pH of dairy manure is induces stress response in E. coli O157:H7 thereby enhancing persistence in soil (Franz et al., 2005). In the case of Salmonella, the ammonia and urea content of dairy manure has been demonstrated to compromise persistence in soil (Ongeng et al., 2015b). The composition of manure in terms of fatty acids, phenolics and other byproducts are also considered to influence pathogen survival in soil (Min et al., 2007, Wells et al., 2005). The endogenous microflora associated with both soil and manure has also been associated with affecting pathogen persistence in both positive and negative aspects (Fouladkhah and Avens, 2010, Moynihan et al., 2013, Liang et al., 2011). Yet, it should be noted that endogenous microflora affects cannot be studied in isolation and hence the area is still evolving. Yet, the fact that autoclaved soil has a tendency to increase the persistence of enteric pathogens in soil would underline the antagonistic activity of endogenous microflora that could be used for bioremediation in future (Jiang et al., 2002, You et al., 2006, Ongeng et al., 2011a). The resident microflora of manure amended soil in the current study was not investigated apart from the coliform levels. It has been proposed that the persistence of E. coli O157:H7 is negatively correlated to total coliform levels (Franz et al., 2007). This has raised the question if composting is a preferred approach to pathogen control given that coliforms are diluted during soil incorporation (Ongeng et al., 2011a).

The shorter persistence of both E. coli O157:H7 and Salmonella on the soil surface was likely due to rapid fluctuations in moisture and surface heating in the case of spring application. The
exposure to UV can also contribute to pathogen die-off (Meays et al., 2005) although in the current study this factor would be less significant given the vile system applied that would have provided shielding. The subsurface of soil was a more consistent environment that aided pathogen persistence. With respect to soil, sandy soil resulted in reduced persistent relative to loam. The significant differences are the structure and lack of clay and hence reduced capacity of the sandy soil to retain moisture and nutrients compared to loam soil. The effect of soil on the persistence of pathogens has primarily been studied in terms of mobility of bacteria cells during leaching. Here, it has been reported that pathogens such as *Salmonella* are more mobile in loam compared to sandy soil (Bech et al., 2010a, Stocker et al., 2015). In the current study the pathogens were within vials thereby negating the effect of leaching. Yet, it is possible that mobility of water (hence nutrients) within the vial resulted in localized areas with both moisture and nutrient depletion. In sandy soil the mobility of water would be less thereby promoting microcosms of depleted nutrients and dehydration.

The time of application (spring vs fall) was less significant to pathogen persistent when compared to the climatic conditions. In the current study the 2013 spring trial was noted for being hot with occasional rain events. This contrasts to the 2014 spring trial that was a lower average temperature with frequent rain compared to 2013. From the results obtained, the hot dry conditions of 2013 extended the time required to reach a 99.9% reduction. Yet, it was noted that the greater proportion of persisters were recovered during the 2014 spring trial. The results are in contrast to those reported by Semenov (Semenov et al., 2007) who suggested the wide temperature variations (as observed in spring 2013) results in rapid die-off rates of pathogens such as *Salmonella*. The discrepancy is likely due to additional factors since in field trials it is not possible to study temperature in isolation given the contribution of moisture. Indeed, fluctuations in moisture by
rain followed by dry periods negatively affects the persistence of *Salmonella* and STEC in manure amended soil (Ongeng et al., 2011b, Franz et al., 2014). There are conflicting reports on the effect of soil moisture content on the die-off of *E. coli*. Studies have reported that low moisture content promotes die-off (Entry et al., 2000a, England et al., 1993) whilst others report on no significant affect (Ritchie et al., 2003b, Oliver et al., 2005, Oliver et al., 2006).

The interplay between temperature and moisture cannot be separated by field trials given the fluctuations in each simultaneously. It is for this reason that complementary laboratory microcosm based studies were performed in the current study. The data generated from the laboratory based trials indicated that STEC persistence was favored under high temperature and moisture. This contrasts to *Salmonella* that exhibits extended survival at low moisture and temperature. The results from the laboratory trials partly explains the higher die-off rates of *E. coli* O157:H7 relative to *Salmonella* observed in the field trials given that the moisture content of soil was generally low.

The persistence of STEC and *Salmonella* in the Fall 2013 trial followed the trend of the Spring 2013 trials. This may have been unexpected given the Fall application was a time when the climatic temperatures were cooling then eventually freezing in contrast to the hot dry summer. The freeze-thaw cycles are noted to cause a decline in pathogen survival (Natvig et al., 2002a) and this was probably the case of pathogens held within surface vials. However, the vials on the subsurface were likely subjected to less variation in temperature thereby persisting for a longer period. Indeed, *Salmonella* persisted for over 120 days but eventually died off with no residual persisters being recovered.

The ultimate objective of the research was to assess if the 90-120 day period is sufficient to ensure adequate pathogen die-off. The recorded die-off rates observed in the current study were within the range of those published by others for field trials. Yet it should be noted that the
persistence of pathogens such as *Salmonella* and *E. coli* O157:H7 have varied between 16 days to 231 days so is very broad (Hutchison et al., 2004, Islam et al., 2004a, Islam et al., 2004b). The broad range of pathogen survival is due to multiple factors than includes geographical location, strain selection, manure and soil type, in addition to diagnostic methods. Irrespective of this fact is that comparisons between trials is problematic and only generalizations can be made with respect to factors that affect pathogen persistence.

For surface applied manure it was evident that pathogen die-off occurred well within the 120 day time period. However, it should be noted that surface application would lead to a greater risk of pathogen leaching into water courses thereby representing an indirect food safety risk if used for irrigation. For manure applied to the subsurface there was extended survival of *Salmonella* up to Day 120. Even in the case of STEC there was persistence of low levels observed during the spring 2014 study. Therefore, the adequacy of the 120 day period largely depends on the risk derived from low levels of pathogen survivors which at present remains unknown (Ceuppens et al., 2015, Chitarra et al., 2014). Of specific concern is the significant of persister cells that although exist in a dormant state, are considered to have enhanced virulence when ingested (Amato et al., 2013, Kussell et al., 2005, Putrins et al., 2015). Persister cells always have the potential to grow when reactivated although if this occurs during interaction with plants remains unclear. The persister state is considered to be controlled by environmental triggers and also dependent on the activity of rpoS (van Hoek et al., 2013, Kussell et al., 2005). This is likely the cause of the strain dependency on pathogen survival within the soil environment (van Hoek et al., 2013). In current study there was a clear higher persistence of *E. coli* O157:H7 compared to non-O157 STEC. The results are in agreement with others that reported on higher or the same persistence between STEC in the environment (Polifroni et al., 2014). Frenz et al (2011) compared the relative survival of 18
strains of *E. coli* O157:H7 and reported that those derived from human clinical cases exhibited greater persistence compared to those derived from animals (Franz et al., 2011). This was attributed to the ability of O157:H7 strains to utilize long chain fatty acids as opposed to composition of virulence factors (Franz et al., 2011). Yet, given that in the current study that persisters were only recovered in the Spring 2014 would indicate environmental factors also play a role in inducing dormancy beyond those related to strain dependency.

From the results generated it is clear that there are multiple factors that impact on pathogen die-off in soil, many of which remain unknown. Consequently, it maybe over optimistic at the present time to select a parameter or a combination of parameters, to predict the die-off rates of pathogens in manure amended soil. Yet, the monitoring of indicators naturally present in manure amended soil can be applied as an indirect metric of pathogen survival. The persistence of generic *E. coli* was comparable to that of O157:H7 or *Salmonella* in both laboratory and field trials. The results contradict those of others who have suggested lower persistence of generic *E. coli* compared to STEC and *Salmonella* (Astrom et al., 2006, Hurst et al., 1980). Yet, other studies have reported on comparable die-off rates (Erickson et al., 2014, Oladeinde et al., 2014). The differences are likely again due to the multiple factors and combinations that define persistence of bacterial cells in manure amended soil. Regardless of this fact, the results would suggest that studies using *E. coli* as indicators for pathogen die-off is not totally reliable without generating further verification studies. Yet, given the restrictions of releasing pathogens in the environment such studies will require to use laboratory microcosms. In this respect, the microcosms applied in the current study provided an over-estimation of the pathogen survival in the initial phases at least. In the literature there are contradictory reports on how representative laboratory based studies are in predicting die-off rates in the field. The conflicting reports are more associated with the lack of parallel trials.
that directly compare field vs laboratory trials. In general, laboratory based studies generate lower
die-off rates compared to field based studies (Soupir et al., 2008). This is likely due to the stable
as opposed to fluctuating conditions occurring in the field environment. Therefore, further studies
should be devoted to mimicking the fluctuations in temperature and moisture encountered in
natural environments to determine if better representative data can be generated.

4.2 Conclusions

The primary objective of the study was to determine the die-off rates of STEC and Salmonella
under different intrinsic (moisture, manure type, soil type) and extrinsic (temperature) conditions.

From the data generated the conclusions from the study are:-

1) Under-field conditions the pathogen die-off occurs in an initial rapid phase then a slower
rate followed by an extended persistent phase.

2) *Salmonella* persisted to a greater extent compared to *E. coli* O157:H7.

3) Extended persistence of both *Salmonella* and STEC were enhanced by being located in the
subsurface of loam soil with more rapid die off being observed in vials placed on the
surface.

4) Dairy manure promoted the persistence of *E. coli* O157:H7 compared to soil amended with
swine manure.

5) The season of manure application (Spring vs Fall) was less significant on pathogen die-off
with fluctuations in moisture and temperature being more critical.

6) The survival of *E. coli* O157:H7 was enhanced by high moisture and temperature. In
contrast, low moisture and temperature promoted the persistence of *Salmonella* in manure
amended soil.
7) In the majority of cases, both *Salmonella* and *E. coli* O157:H7 died-off before 120 days although on occasions viable cells were still recovered by enrichment. The food safety risk of persistent-low levels, of pathogens remains to be elucidated.

8) Generic *E. coli* exhibited similar die-off kinetics as *Salmonella* and STEC applied in the current study.

9) Laboratory based soil microcosms over-estimated the persistence of STEC and *Salmonella* compared to field trials.

The study has demonstrated that *Salmonella* and STEC can persist over 120 days in certain soil types. Consequently, it possible that pathogens could be present even after the 90 day wait period before planting crops. However, it should be noted that majority of the cells (>99%) within the bacterial population die-off within the initial 12 day period leaving a small residual population that persists over an extended time. Furthermore, the levels of pathogens spiked into the manure amended soil far exceeded those which might be expected under natural conditions. Laboratory based soil microcosm models underestimate pathogen die off in the real environment and hence are not suitable for risk assessment studies. On the other hand, the microcosm study was a good supplement study of the field experiment as it reflected the die-off without the environmental interference.

From an agricultural perspective, the following recommendations can be provided: Leaving the manure on soil surface for at least 12 days (average 2 log reduction time) is a simple and cost-effective treatment for lowering pathogen levels (including STEC and *Salmonella*) for liquid manure. Applying manure during a dry period can prevent contamination in run-off.
4.3 Further Study

4.3.1 Risk analysis models should be built to predict die-off

Algorithms should be developed based on predicting the die-off of pathogens under varying conditions of temperature and soil type. The models developed could then be used to predict the level of pathogen die-off under the measured climatic conditions. This would provide a more accurate estimate of the wait period from manure application to planting crops than using the arbitrary 90 or 120 day rule. Algorithms would be especially relevant to assessing the hazard associated with low residual levels of pathogens with respect to contaminating crops or water courses.

4.3.2 Detect the mechanism for sub-populations of pathogens

As mentioned in the result and discussion part, surviving sub populations of both STEC and Salmonella were observed in spring trial 2014 and lab trials. The re-growth of the pathogens can affect the evaluation of pathogen survivability. The physiology of sub-populations of pathogens (i.e. STEC and Salmonella) should be studied to determine if environmental fitness has been acquired at the cost of virulence.

4.3.3 More studies among different geographical areas

Further trials are required to verify that die-off models are applicable in different geographical areas. Pathogens die-off rates were largely affected by climate conditions. As climate difference among areas, multi-location study are needed to complete the pathogen die-off models. Further trials are also needed for other soil types, in particular clay and organic soils.
4.4 Conclusion and recommendation:

The study has demonstrated that *Salmonella* and STEC can persist at very low levels over 120 days in certain soil types. Consequently, it possible that pathogens could be present even after the 90 day wait period before planting crops. However, it should be noted that majority of the cells (>99%) within the bacterial population die-off within the initial 12 day period leaving a small residual population that persists over an extended time. Laboratory based soil microcosm models underestimate pathogen die off rates in the real environment and hence are not suitable on their own for risk assessment studies. On the other hand, the microcosm studies are good supplementary studies to field experiments as they can reflect the die-off without the variability of environmental interference.

From agriculture aspect, the following recommendations can be provided for reducing pathogen levels from land-applied manure: leaving the manure on soil surface for at least 12 days (average 2 log reduction time) is a simple and cost-effective treatment for lowering pathogen levels (including *E.coli* and *Salmonella*) from liquid manure. However, applying manure during a dry week is required to prevent contamination in run-off, and the losses on nitrogen need to be considered in nutrient management for crops.
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