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Thermal Reduction of Common Food-Borne Pathogens During Composting

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Abstract

Soil amended with manure has been implicated as a source of produce contamination leading to foodborne gastrointestinal-disease outbreaks. While current composting guidelines require temperatures ≥ 55°C for 3 days to destroy bacterial pathogens, these requirements have not been evaluated for all pathogens. Investigation of parasite survival in manure required development of a flow cytometry method integrating the cell-impermeant viability dye SytoX for simultaneous quantification and viability assessment of *Cryptosporidium* and *Giardia* oocysts/cysts. Further studies will be required to apply this method to investigate thermal reduction in parasites. Studies conducted with bacterial pathogens indicated that *E. coli* O157:H7 survived longer than other pathogens at 50°C to 55°C. *Listeria monocytogenes* survived significantly better in chicken manure compared to cow manure at 50°C to 55°C. Results suggest composting guidelines are adequate for bacterial pathogen reduction; however, testing for *E. coli* O157 along with *Salmonella* may increase confidence in compost safety.

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List of Abbreviations

a_w – water activity, the ratio of water pressure of the compound to the water pressure of pure water

C:N – carbon:nitrogen ratio, ratio of the mass of carbon to the mass of nitrogen in the compost material(s)

CCME – Canadian Council of Ministers of the Environment, the regulatory body responsible for compost quality

CJ 11168 – *Campylobacter jejuni* strain NCTC 11168

C. parvum – *Cryptosporidium parvum*

D_x-value – decimal reduction time, time required to reduce a population by 1-Log at temperature "x"

DMSO – dimethyl sulfoxide, solvent component in the SytoX brand dyes

DO – dissolved oxygen

E. coli – *Escherichia coli*

EC O157:H7 – *E. coli* strain O157:H7

EC O26:H11 – *E. coli* strain O26:H11

G. duodenalis – *Giardia duodenalis* also known as *Giardia lamblia*

GMO – genetically modified organism

ID – infectious dose

L. mono – *Listeria monocytogenes*

LM 5412 – *L. mono* strain 5412

MPN – most probable number

PI – Propidium iodide, a cell-impermeant viability stain

S. enterica – *Salmonella enterica*

SS 775W – *S. enterica* serovar Senftenberg, a documented heat-tolerant *Salmonella* strain

ST 611 – *S. enterica* serovar Typhimurium

T – temperature

RT – room temperature (25.0°C)

z-value – a thermal death time calculation, the temp. required for 1-Log reduction in D-value

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I. Introduction and Literature Review

1.1 Foodborne illness associated with Fresh Produce

Foodborne disease continues to have a large impact on public health causing approximately 4.0 million episodes of domestically acquired gastrointestinal illness, resulting in estimated costs of $3.7 billion related to healthcare and lost productivity in Canada annually (Thomas et al, 2008; Thomas et al, 2013). Recently the incidence of foodborne illness outbreaks linked to fresh produce has been rising, with infections resulting from multiple pathogens (Allen et al, 2013; Lynch et al, 2009). Pathogens such as *Salmonella enterica*, *Escherichia coli*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Cryptosporidium parvum*, and *Giardia duodenalis* have been of major concern due to their common environmental occurrence (Oliveira et al, 2011).

The awareness and desire for healthy active living has been increasing, this lifestyle includes not only physical activity but also a diet consisting of more fruit and vegetables. Consumption of fruits and vegetables by Canadians has increased 56% from 1963 to 2010 with a 19% increase occurring between 1990 and 2010, Canada being among the highest in the world for fruit and vegetable consumption per capita (Kozak et al, 2013; Olaimat and Holley, 2012; Statistics Canada, 2011). Significant proportions of recent foodborne outbreaks have been linked to consumption of fresh produce or produce-containing items in Canada (Ravel, 2009). Some more recent outbreaks include *Salmonella* in cucumber, tomatoes, mung bean sprouts, fruit salad, and onion sprouts; *E. coli* in spinach, lettuce, apple juice and spanish onions; *Campylobacter* in lettuce, sweet potatoes, cucumber, melon and

strawberries; and *Listeria* has been associated with contaminated coleslaw, and root crops (Brandl et al, 2004; Kozak et al, 2013; Ramaswamy et al, 2007; Warriner et al, 2009).

Risk of contracting food-borne illness is high when crops are unlikely to be cooked prior to consumption, such as salads, fruit, and vegetables. Organic, locally grown produce has also been growing in popularity due to a perceived reduction in pesticide residue and natural toxin risk associated with organic products (Campbell et al, 2013; Meerburg and Borgsteede, 2011). Although manure is used as fertilizer in both conventional (use of GMO's, chemical pesticides/herbicides and fertilizers permitted) and organic agriculture the use of manure as a source of plant nutrients is greater in organic production as use of synthetic fertilizers is not allowed (AIbthn, 2002; Meerburg and Borgsteede, 2011). Studies have detected *Salmonella* and *E. coli* in significantly higher percentages of organic sprouts and lettuce samples compared to those grown using conventional farming (Doyle, 2000; Mukherjee et al, 2004). These factors may play a role in the rate of outbreaks due to the consumption of fresh produce.

Fresh produce could become contaminated during production, processing, distribution, and/or preparation within the farm-to-fork continuum, and soil amended with manure has been implicated as a possible source of produce contamination (Guan and Holley, 2003; Hoar et al, 2001; Islam et al, 2004a/b/c; Natvig et al, 2002; Solomon et al, 2002). There are many knowledge gaps associated with microbiological safety of fresh produce including the difficulties in controlling the spread of human pathogens in the agricultural environment, post-harvest decontamination techniques being ineffective, and inability to trace contaminated items back to the source (Warriner et al 2009). Reports of food related

outbreaks are also limited for many pathogens as they are difficult to document and often under-reported.

Manures are an excellent source of plant nutrients and have often been used to improve soil quality and fertility. In 2010 almost half of all Canadian farms (approximately 48%) applied some form of manure to their soils (Dorff and Beaulieu, 2014). Recycling manure(s) on farm to agricultural land is practical and economic for farming. However there still remains much to be known about the interaction and survival of pathogens during the manure composting process. As many of these pathogens are ubiquitous in the environment, quite robust, and have a wide range of hosts it is unlikely that they will all be eradicated. Therefore, it is imperative we find interventions that prevent contamination before transfer of pathogenic organisms can occur during growth and harvest of fresh produce.

Though there are many benefits associated with use of composted manure, it has been demonstrated that pathogens are able to survive for long periods in manure (Oliveira et al, 2011). Mawdsley et al (1996a/b) confirmed the possibility of manure contaminating soils with *Cryptosporidium* oocysts through demonstrating oocyst(s) movement within the upper portion of manure-amended soil (containing oocysts) for several weeks to 70 days (Fayer et al, 2000). The possibility of soil cross-contaminating crops has also been confirmed through studies conducted by Oliveira et al (2011) who demonstrated that *Listeria innocua* (surrogate for *L. mono*) could survive for extended periods in soil, and that soil containing contaminated compost or irrigation water resulted in contamination of lettuce. Similar studies have been conducted using *E. coli* and *Salmonella*, resulting in contamination of lettuce, onions, radishes, and soil (Guan and Holley, 2003; Islam et al, 2004b/c; Natvig et al, 2002; Solomon et al, 2002).

Although the Canadian Council of Ministers of the Environment (CCME) has set time/temperature regulations for compost treatment, these requirements have not been extensively evaluated for their effectiveness in reducing all priority pathogens.

1.2 Characteristics of Pathogens Associated with Produce Outbreaks

Among the main causative agents of food-borne illness are the bacteria *Escherichia coli, Salmonella, Campylobacter,* and *Listeria.* This list is not limited to bacterial pathogens, and the protozoan parasites *Cryptosporidium* and *Giardia* are also often associated with severe gastrointestinal disease (Nicholson et al, 2005; Rimhanen-Finne et al, 2004). Common clinical characteristics of foodborne pathogens as well as the significant sources are summarized in Table 1.

Table 1. Characteristics of foodborne pathogens being studied.

Pathogen	Incubation Period	Infectious dose and symptoms	Significant sources	References
Campylobacter jejuni	2-5 days	Diarrhea.may contain blood. Guillain-Barre syndrome. Dose: >500 cells	Manure (especially from poultry) Raw poultry	Warriner et al 2009; Young et al, 2007
Cryptosporidium parvum	2-4 days	Severe watery diarrhea Dose: 9 – 1024 oocysts	Domestic animals, manure, sewage, infected food handlers	Okhuysen et al, 1999 Warriner et al 2009
Giardia duodenalis	1-2 weeks (varies from 1-45days)	Majority are asymptomatic, diarrhea, increased fat and mucus in feces, abdominal cramps and bloating, fever Dose: ≤ 10 cysts	Contaminated water, cattle, manure, domestic animals, infected food handlers	Ortega et al, 1997
Escherichia coli O157:H7	24-48h	Abdominal pain and diarrhea which becomes bloody Hemorrhagic colitis Dose: >100 cells	Manure from ruminants, sewage, raw beef	Warriner et al, 2009
Listeria monocytogenes	1-90 weeks	Flu-like symptoms, septicemia, meningitis, encephalitis. Still birth or abortion. Dose: $>10^4$ cells	Manure, sewage, soil, silage	Warriner et al, 2009
Salmonella	6 - 72 h	Nausea, vomiting, abdominal cramps, diarrhea, fever, headache. Dose: $>10^3$ cells	Manure, soil, wild and domestic animals, sewage, raw meat and poultry	Coburn et al, 2006;Warrin er et al, 2009

1.2.1. *Escherichia coli*

Escherichia coli (*E. coli*) typically colonizes the gastrointestinal (GI) tract of humans and animals and usually coexists with its host in a mutually beneficial relationship. *E. coli* colonizes the mucous layer of the mammalian colon and assists with breakdown of carbon compounds (van Eslas, 2011). However, some strains have acquired virulence factors and mutations (including haemolysin production, protein secretion, various O antigens and iron sequestration) enabling them to adapt to new niches and cause disease in the central nervous system, GI and urinary tracts of hosts. These pathogenic forms are divided into six separate categories: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) or shiga-like toxin producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (Kaper et al, 2004). EHEC and ETEC are also able to cause disease in animals.

Pathotypes of *E. coli* are characterized by the O (lipopolysaccharide, LPS) and H (flagellar) antigens. In North America EHEC strain O157:H7 has been the most studied pathogen but other serogroups, referred to as non-O157, are also prominent and are increasingly associated with disease (Bower 1999; CDC 2012; Fremaux et al, 2007; Gould et al, 2013; Warriner et al, 2009). The Center for Disease Control reported non-O157 STEC serotype O26 as being responsible for approximately 26% of all non-O157 *E. coli* infections in its most recent report, the highest incidence of non-O157 serotypes (CDC, 2012).

The bovine intestinal tract is the principal reservoir of EHEC, which is the category focused on in this study. EHEC is known to cause haemorrhagic colitis (bloody diarrhoea), non-bloody diarrhoea, and haemolytic uremic syndrome (Kaper et al, 2004). Though initial

outbreaks were associated with undercooked hamburgers and meat products, more recently outbreaks have also been linked to contaminated drinking water, lettuce, melon, radish sprouts, and spinach (Warriner et al, 2009).

It has been found that only half of the genetic factors required for virulence are present in *E. coli* serotypes associated with animals compared to those of human clinical isolates, and the most virulent STEC are often harbored by humans or introduced to animals that come into contact with sewage (Johnson et al, 2004; Warriner et al, 2009). A combination of genes encoding toxins, colonization factors and other functions are required to make *E. coli* pathogenic. Pathogenic *E. coli* strains possess specific adherence factors, adhesins, that allow them to colonize areas such as the small intestine which would normally not be colonized by commensals. These adhesins include fimbriae or fibrillae and outer membrane proteins such as intimin, which can induce signalling cascades resulting in cytoskeletal restructuring, cytokine activation, inflammation, and in some cases internalisation. (Kaper et al, 2004). Surface structures also allow adhesion to mucosal cells, and toxins are often secreted to interfere with host cellular processes.

Healthy cattle have been found to harbour *E. coli* O157:H7 asymptomatically, with prevalence of *E. coli* O157:H7 in cattle ranging from 0.3 to 11% (Fremaux et al, 2007; Kudva et al, 1998). Investigations in the United Kingdom and United States found O157:H7 positive cattle to shed from 100cfu/g to 10^6 cfu/g and 4×10^3 cfu/g to 1.3×10^7 cfu/g in faeces (respectively) (Rhoades et al, 2009). *E. coli* has demonstrated the ability to survive long periods of time in manure averaging culture-positive samples of at least 30 days and lasting over 217 days and even up to one year (Franz et al, 2011; Kudva et al, 1998; Oliveira

et al, 2011). Though fewer studies are conducted for non-O157 STEC strains, there is evidence for survival in manure from 42 to 90 days (Fremaux et al, 2007).

Franz et al (2011) found survival of human *E. coli* O157 isolates to be significantly higher in manure-amended soil (median 211 days) compared to those of animal isolates (median 70 days), reporting no relationship between survival and presence of virulence genes but suggesting oxidative capacity as a marker for enhanced survival of *E. coli* O157:H7 strains. In soils amended with contaminated compost, *E. coli* O157:H7 persisted for 154 to 217 days (Islam et al., 2004a) and were detected on lettuce and parsley planted in said soil for 77 to 177 days (Oliveira et al, 2012). Sinton et al (2007) also found final counts of *E. coli* to be higher than other indicator and pathogenic bacteria (i.e. *Salmonella enterica, Campylobacter jejuni,* enterococci, etc) in bovine faeces suggesting it may be a more practical indicator.

1.2.2 *Campylobacter jejuni*

Campylobacter jejuni is a species of gram-negative microaerophilic bacteria often commensally colonizing the gastrointestinal tract of domestic animals, poultry and cattle, and is the most common cause of bacterial enteritis in Canada (Inglis et al, 2010; Silva et al, 2011). After approximately 2-5 days incubation within a human host *C. jejuni* infection often presents as acute gastroenteritis with inflammation, abdominal pain, fever, and diarrhoea sometimes including blood and/or mucus. In order to establish infection *C. jejuni* utilizes motility along with its corkscrew morphology to pass through the mucus layer of the gastrointestinal epithelium and invade the underlying epithelial cells (Young et al, 2007).

This intracellular invasion is what elicits the host immune system to recruit inflammatory cells and induce the characteristic inflammation associated with infection.

Sporadic outbreaks of campylobacteriosis involving contaminated water and various food sources (chicken, raw milk, mud, vegetables) have been documented (Brandl et al, 2004; Warriner et al, 2009). Studies in the USA, Canada, Denmark, New Zealand, and elsewhere have reported isolating *Campylobacter jejuni* in approximately 10 – 50% of cattle samples tested (Bae et al, 2005; Gilpin et al, 2008; Inglis et al, 2003; Nielson, 2002). Natural occurring counts of *Campylobacter* spp. in cow faeces have been found to be highly variable, from <1 to 1.8 x 10^7 CFU/g (Sinton et al, 2007). One of the largest and most serious outbreaks in Canada occurred in 2000 in Walkerton, Ontario where over 2,300 people were infected with *Escherichia coli* O157:H7 and/or *C. jejuni* after ingestion of water that had been contaminated by cattle feces (Clark et al, 2003; Inglis et al, 2010).

Gilpin et al (2008) reported counts of *Campylobacter* spp. naturally present in cattle faeces to fall below detectable levels within 14 days in pasture, however found that naturally occurring species exhibited similar survival rates as laboratory strains. Although reports have indicated that *Campylobacter* does not persist well in solid manure after excretion (Gilpin et al, 2008; Hutchison et al, 2005a; Nicholson et al, 2005; Sinton et al, 2007), studies using molecular methods conducted by Inglis (et al, 2010) found *Campylobacter* spp. persisted in untreated manure pens for approximately 2 months and throughout composting (approx. 8 months).

1.2.3 *Listeria monocytogenes*

Listeria monocytogenes (*L. mono*) is a gram-positive opportunistic intracellular pathogen ubiquitous in a wide range of environments such as soil, water, food, humans and animals. Infection is acquired through consumption of contaminated food products, however disease (Listeriosis) mainly affects immunocompromised individuals. Listeriosis often presents as gastroenteritis but may also manifest as encephalitis, meningitis, and septicaemia resulting in a 25-30% mortality rate (Hamon et al, 2006; Ramaswany et al, 2007).

Following ingestion, *L. mono* induces uptake into non-phagocytic cells (epithelial cells, endothelial cells, etc) of the intestinal epithelium. The intracellular lifecycle allows it to escape from the cell vacuole, multiply in the cytoplasm, and spread directly to neighbouring cells. *L. mono* bacterium then cross the intestinal barrier after which they are able to access internal organs such as the liver, spleen, central nervous system, and uterus (Ramaswamy et al, 2007).

L. mono is able to survive and grow in a wide range of pHs and temperatures from 4.3 to 9.4 and below freezing (-7°C) to body temperature respectively (Grewal et al, 2007; Ramaswamy et al, 2007). It is a pathogen of major concern due to its ability to grow on vegetables and produce at refrigeration temperatures, as well as its demonstrated long survival times in soils (Oliveira et al, 2010; Oliveira et al, 2011). *L. mono* has been isolated from livestock, wildlife and human fecal sources in Ontario (Lyautey et al, 2007) and is found to have a prevalence of approximately 5-30% in cattle faeces (Rhoades et al, 2009).

1.2.4 *Salmonella enterica*

The genus *Salmonella* contains two species, *S. enterica* and *S. bongori*, which are further subdivided into serovars. *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteriditis are the two most frequently associated with human illness (Franz and van Bruggen 2008; Warriner et al, 2009). *Salmonella enterica* is a gram-negative facultatively anaerobic bacterium known to cause a variety of diseases in humans and animals with symptoms ranging from gastrointestinal to sepsis and typhoid fever. It is carried in the gastrointestinal tract of wild animals, poultry and humans.

Following consumption of contaminated food or water *Salmonella* cells colonize the intestine, trans-locating the intestinal epithelium via invasion of enterocytes, M cells and/or dendritic cells (Grassl and Finlay, 2008). Interaction with the intestinal epithelium then results in the production of cytokines and chemokines by the host immune system (Coburn et al, 2006). Symptoms are usually acute onset of cramps, abdominal pain, nausea, vomiting and diarrhea with or without blood. If untreated symptoms last 5-7 days and may resolve spontaneously (Coburn et al, 2006).

Salmonella has been transferred via manure application to vegetable crops such as radishes, arugula, carrots, lettuce and pasley (Natvig et al, 2002; Islam et al, 2004b/c). Multiple studies have isolated *Salmonella* in 1.4 – 8% of cattle manure and 18% of poultry manure (Hutchison et al, 2004; Warriner et al, 2009). Investigations of survivability in manure have found *Salmonella* to persist for long periods, from 16 up to 148 days, in manure as well as 3 months in biowaste composts (green waste, fruits and vegetables, cardboard, etc) (Lemunier et al, 2005; Nicholson et al, 2005; Sinton et al, 2007; You et al, 2006), *Salmonella*

survival being greatest in soils amended with poultry compost (Islam et al, 2004c). Ceustermans et al (2007) observed *Salmonella* Senftenberg 775W eradication within 7 days at temperatures between 50°C and 55°C, whereas Droffner et al (1995) found *Salmonella* Typhimurium survived at 60°C for 9 days in biowaste compost (Erickson et al, 2009). A survey of composting facilities in the United States found more than half (of 72) facilities' compost products still contained *Salmonella* sp. even though time-temperature criteria were met (Hay, 1996; Wichuk and McCartney, 2007).

1.2.5 *Cryptosporidium parvum*

Cryptosporidium is a genus of obligate intracellular protozoan parasite, species which are a major cause of gastroenteritis and diarrhoea worldwide. Although most species have some form of host specificity they are not strictly host specific and are known pathogens of domesticated livestock, poultry, house-pets and wildlife as well as humans. *Cryptosporidium parvum* in particular has been reported to infect over 150 mammalian hosts (Fayer, 2004; Fayer et al, 2000).

The infectious dose (ID) of *C. parvum* oocysts is quite low ranging from 9 to 1024 oocysts (Fayer et al, 2000; Fayer, 2004; Okhuysen et al, 1999). Infection occurs when spore-like oocysts, the environmental stage, are ingested via the oral-faecal route. In the small intestine of the host changes in temperature, pH, the presence of bile salts and enzymes results in the excystation of contents of the oocyst(s) to release four sporozoites which invade epithelial cells of the gastrointestinal tract (Carey et al, 2004). Sporozoites adhere to epithelial cells of the intestinal tract, where they continue an endogenous life cycle within a protective parasitophorous vacuole. Following merogony (asexual replication) to increase

numbers, merozoites infect other cells in the GI tract and type II meronts differentiate into macrogamonts and microgamonts to proceed to sexual reproduction. Sexual reproduction results in production of both thin- and thick- walled oocysts, which remain in the host for re-infection or are shed in feces (respectively) to continue the transmission cycle (Carey et al, 2004). While immunocompetent hosts may excrete oocysts for one to several weeks, immunocompromised hosts often experience chronic infections resulting in shedding for several months. Infection of healthy volunteers by Chappel et al (1996) found volunteers with diarrheal illness excrete more oocysts over the course of infection compared to those without diarrhea (asymptomatic), with 89% of infected volunteers excreting a greater number of oocysts throughout the study than their initial challenge dose.

Cryptosporidiosis is a health threat to humans and animals due to the lack of drugs to prevent or treat infection as well as the resiliency of *Cryptosporidium* oocysts in the environment (Carey et al, 2004; Jenkens et al, 2004). As *Cryptosporidium* spp. have been found to be prevalent in mammals such as cattle, sheep, pigs (etc), excretion and the process of manure spreading on farmland has the potential to contaminate the environment (Barwick et al, 2003a). Cattle have long been a suspected source of water and produce contamination of *Cryptosporidium* oocysts. Infected calves have been found to excrete 10^9 to 10^{10} oocysts over 7-10 days (Fayer, 2004). Studies conducted by Mawdsley et al (1996a/b) used a soil-tilting table to investigate movement of *C. parvum* oocysts in different soil types following application of contaminated livestock faeces. Oocysts were found in the upper portion of soils amended with contaminated manure where contact with crops would be possible and moved within the soil for several weeks to 70 days (Fayer et al, 2000). Barwick et al (2003 2) also found 17% of soil samples collected in New York State dairy farms were positive for

Cryptosporidium (Olson et al, 2004). Investigations of pathogen contaminated livestock faeces spread on pasture found *Cryptosporidium* oocysts significantly more hardy than the bacterial pathogens, requiring 8 to 31 days for a 1-log reduction compared to 1.94 days for the same reduction in bacteria (Hutchison et al, 2005a).

1.2.6 *Giardia duodenalis*

Giardia spp. is a unicellular microorganism that also commonly causes diarrheal disease throughout the world. There are few morphological differences between genetic variants of *Giardia*, so genotypes are categorized based on their genetics. To date two genotypes have been associated with cattle: *Giardia duodenalis* (synonyms *G. inetinalis, G. lamblia*) zoonotic genotype Assemblage A, and the livestock genotype Assemblage E (Olson et al, 2004). Known hosts of *G. duodenalis* include most mammals such as humans, livestock and pets. Like *Cryptosporidium, Giardia* is an obligate intracellular parasite that infects the intestines of host(s) and is spread via the faecal-oral route.

Giardia differs from *Cryptosporidium* in that it has a more simplistic life cycle consisting of two main stages: the trophozoites and the cyst (Ortega et al, 1997). Following ingestion, exposure of *Giardia* cysts to the acidic stomach results in excystation of cyst(s) to release two trophozoites in the small intestine of the host. These trophozoites replicate via asexual binary fission in the small intestine where they cause the symptoms of giardiasis, and have no sexual cycle compared to *Cryptosporidium*. Exposure of trophozoites to biliary fluids will result in trophozoites encysting into cysts, the environmental form of the parasite. Cysts are excreted from the host through the feces allowing completion of the transmission cycle.

As observed with *Crypto*sporidium, *Giardia* is prevalent in cattle populations (Barwick et al, 2003; Olson et al, 2004; Olson et al 1999), though the rates can vary depending on herd management and climate. Studies by Ralston et al (2003) in Calgary, Alberta have reported incidences of up to 100% prevalence of *Giardia* infection of ranch cattle. Barwick et al (2003) found an association between prevalence of *Giardia* infection in a cattle herd and detection of a cyst in the soil, where farms that had a prevalence rate of 18-39% were more likely to have soil positive for cysts. Cattle infections with *Giardia* are not only a public health concern for humans but could also influence the performance of animal species in areas of weight gain and feed intake, resulting in possible economic losses (Olson et al, 2004). Unlike *Cryptosporidium,* data on outbreaks of Giardiasis linked directly to manure are rare.

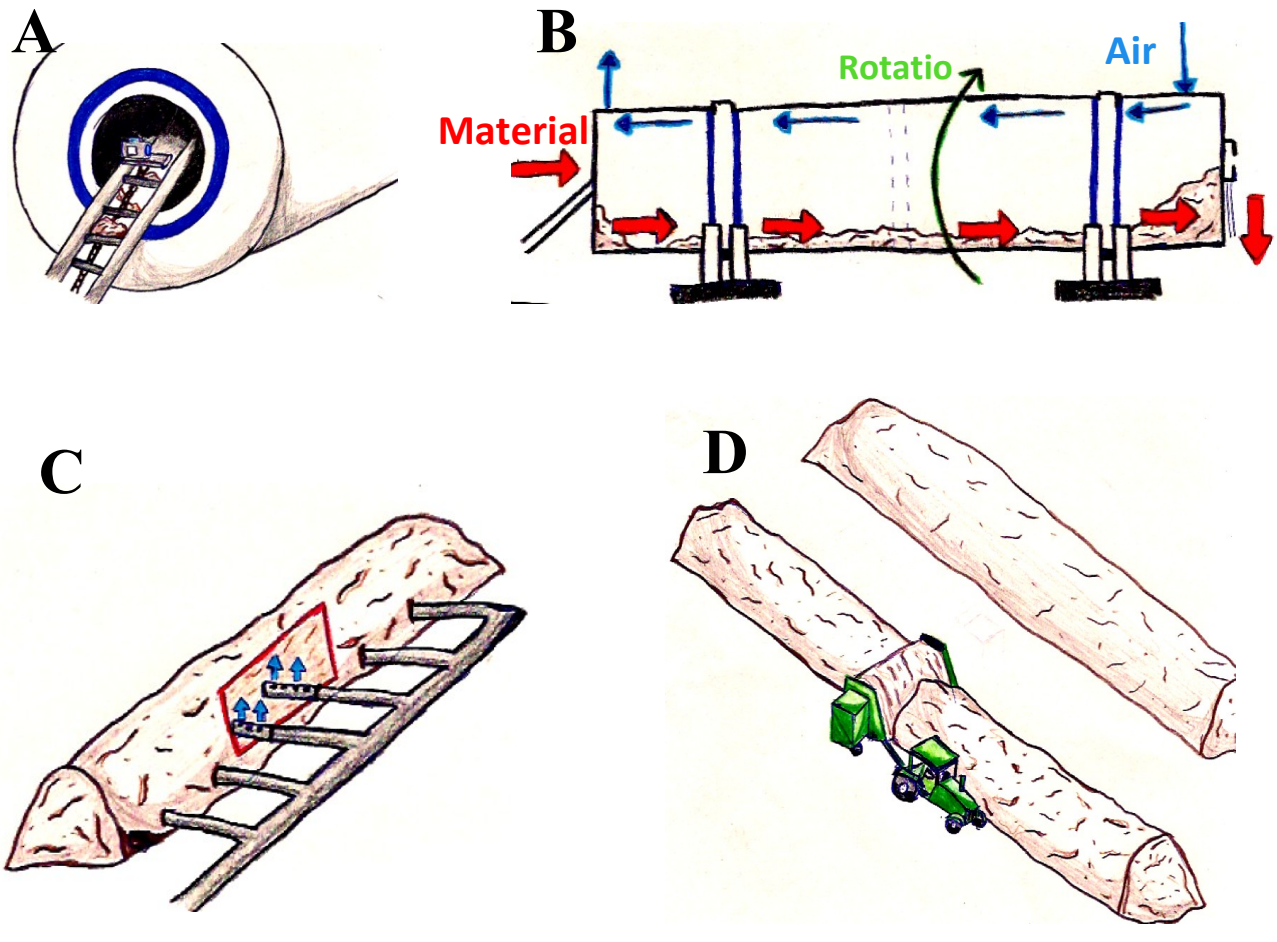
1.3 Composting

1.3.1 Composting Process

There are many benefits to the agricultural use of composted materials including reduction of pollution, improvement of agricultural soils, and control of soil erosion. Composting is a bio-oxidative process where organic matter is converted to humus through microorganism driven decomposition. Ideally, the final composted product is a reduced volume, free of weed seeds, contains nutrients to amend soil quality, and is pathogen free (Inglis et al, 2010).

Three methods of composting approved and regulated by the Canadian Council of Ministers of the Environment (CCME) are in-vessel, windrow, and aerated static pile composting. In-vessel composting is accomplished by containing the organic materials in a

reactor vessel under constant rotation or agitation, Figure 1A/B, thereby maintaining optimal conditions (temperature, air, etc). Windrow composting, Figure 1D, is a process where organic matter is piled in long rows (*windrows*). The rows are occasionally turned using a windrow turner, improving oxygen content, mixing in moisture, and redistributing portions of the pile that are at different temperatures. The aerated static pile method, Figure 1C, is conducted through passively or actively providing air circulation to windrows, open/covered piles, or in closed vessels. This method is commonly used by larger composting facilities due to the higher costs and complexities of the system. Regardless of the system used, effective composting is dependent on microbial communities, the generation of metabolic heat and enzymatic decomposition (Inglis et al, 2010). Guidelines set by the CCME require composting materials reach a minimum temperature of 55°C that is maintained for three days, however windrow composting requires temperatures greater than 55°C be maintained for 15 days with a minimum of 5 turnings to ensure all material is mixed into the high-temperature core at some point (Wichuk and McCartney, 2007).


A
B
Material
Rotatio
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C
D

Figure 1. Composting methods approved by the Canadian Council of Ministers of the Environment (CCME). (A) Front of in-vessel system where manure (or other materials to be composted) enter system. (B) In-vessel composting systems constantly turn or agitate (not shown) composted material through a system to provide constant air-flow and optimal temperature conditions to all composting materials. (C) Aerated static pile composting systems pile the organic material to be composted on perforated piping systems to provide constant air circulation and optimal conditions. (D) Windrow composting systems pile the organic matter or biodegradable waste to be composted in long rows (windrows), the piles are turned throughout the process using a windrow turner attached to a tractor. In-vessel and aerated pile systems must reach a temperature of 55C and maintain it for 3 consecutive days according to CCME guidelines. Windrow composting guidelines require piles to reach a minimum temperature of 55C for 15 consecutive days to ensure all material reaches temperature at some point during the composting period. Adapted from Rynk, 1992.

1.3.2 Composting Guidelines in Ontario

The North American regulatory bodies the United States Environmental Protection Agency and the CCME have similar composting guidelines and safety requirements. The CCME guidelines are based on four safety criteria: foreign matter, maturity, pathogens, and trace elements (CCME). Two compost categories are in place; Category A – compost that can be used in any application (unrestricted use), and Category B – compost that is restricted due to high trace elements or sharp foreign matter (restricted use). Those products that do not meet either criterion must be disposed of. For the purpose of this study we investigated lab-simulated compost materials that contained *other feedstock* (not yard waste which is vegetative matter from plant materials). The CCME guidelines require that these materials undergo the following:

1.
    a. In-vessel composting – maintained at 55°C or greater for three days
    b. Windrow composting – attain temperature of 55°C or greater for at least 15 days, turned at least five times during the high temperature period
    c. Aerated static pile – maintained at 55°C or greater for three days, it is preferable to cover the pile with an insulating material

AND

2. Organism content
    a. Fecal coliforms < 1000 MPN/g total solids (dry weight)
    b. No *Salmonella* sp., detection level <3 MPN/4g total solids (dry weight)

However, their guidelines "*…generally apply to the quality of compost rather than the composting process"*(CCME, 2005). As their quality of composting is based on MPN of *Salmonella*, it could theoretically be possible for untested pathogens, to still be present in composted materials. Note that enumeration of fecal coliforms is also conducted, with a

footnote indicating: *"...In cases where high levels of fecal coliforms are suspected to be due to environmental contamination, additional analysis for E.coli should be conducted*"(CCME, 2005).

The composting time-temperature regulations for the United States Environmental Protection Agency (EPA) are based on a study in 1979 on the inactivation of parasites, which are very heat resistant. This study found 51°C to be the threshold for helminth egg destruction, and that temperatures of 55°C with atmosphere near 100% relative humidity for 24 hours would destroy all pathogens (Hay, 1996). Though it is never directly stated in CCME guidelines it can be assumed that *Salmonella*, a human frank pathogen that can cause disease in both healthy and ill subjects, is important as an evaluation tool for the quality of compost in order to limit health risk to the population. *Salmonella*, as well as Enterococci and *E. coli*, have been found in final products indicating they are more resistant to the composting process and could be used to evaluate efficiency of composting (Briancesco et al, 2008). *Salmonella* sp. indicators are also often used due to their high numbers in biosolids and easier isolation and identification compared to other pathogens (Hay, 1996). A result of nonuniform temperature throughout the compost windrow requires that time-temperature monitoring also be supplemented with monitoring of indicator organisms to ensure pathogen destruction (Hay, 1996).

1.3.3 Fundamentals and Important Factors

The composting process is broken up into three general phases: (1) a rapid increase in temperature over a short period of one or two days; (2) a high temperature phase lasting months; (3) the curing phase, where there is a large drop in temperature. Survival of

pathogenic organisms in compost depend on a number of factors including temperature, moisture content, pH, treatment system, aeration, microbial competition, duration of incubation, and carbon and nitrogen content (C:N ratio more specifically) (Grewal et al, 2007; Nicholson et al, 2005; Pell 1997). Moisture and efficient aeration are essential to effective composting. Temperatures at or near 55°C are considered optimal, however temperature is temporally and spatially variable in the compost pile. A summary of important composting elements and their significance in the process is displayed in Table 2 below.

Table 2. Factors that significantly affect the composting process.

Factor	Significance
Carbon**:**Nitrogen (C:N)	- Carbon = source of energy for microorganisms - Nitrogen = used for building cell structure - C:N too high = slows decomposition once N depleted - C:N >30 = slows process - C:N too low = microorganisms release excess N as ammonia (N is lost from the compost pile)
Temperature	- High temperature required for pathogen reduction - Decomposition is more rapid at greater temperatures - Too high = Nitrogen loss
Moisture	- Ideal = 40 - 60% - Too high = water displaces air resulting in anaerobic environment - Too low = hinders microorganism' metabolism
Aeration/Oxygen Content	- Necessary for rapid odor-free decomposition - Too low = favours anaerobes, is less efficient, produces odour
Additional factors:	- pH - electrical conductivity - Particle size - Mixing/proportioning - Climate - Microbial interactions

1.4 Thermal Reduction of Pathogens

As temperature is arguably the most important factor for the reduction of pathogens during composting, and used in many methods of sterilization, this research focused on

understanding the relationship between common foodborne pathogens and heat while in manure. Each species of pathogen exhibits a different level of heat tolerance. The heat resistance of an organism is commonly measured using the decimal reduction time or D-value. This value represents the time required to kill 90% or 1-log of the organism at a specific temperature. The z-value represents the temperature dependence of the reaction. It is the temperature change required to alter the D-value by a 1-log. If the z-value is low the reaction is highly temperature dependent, whereas a high z-value is attributed to reactions that require a larger temperature change to decrease the reaction time.

Figure 2 is a graphical representation of the D- and z-value graphs used to determine these values for each pathogen. Briefly, the surviving population is counted/determined over a period of time at a specific temperature after which surviving numbers are converted to Log_{10} values and plotted against time (Figure 2). The D-value can then be calculated as the slope of the declining population. The z-value is similar, however the Log_{10} value of multiple D-values are calculated and plotted against their respective temperatures. Though many studies have been conducted on the thermal reduction of various pathogens and other microorganisms in buffered systems, there is limited data of pathogens in different matrices.

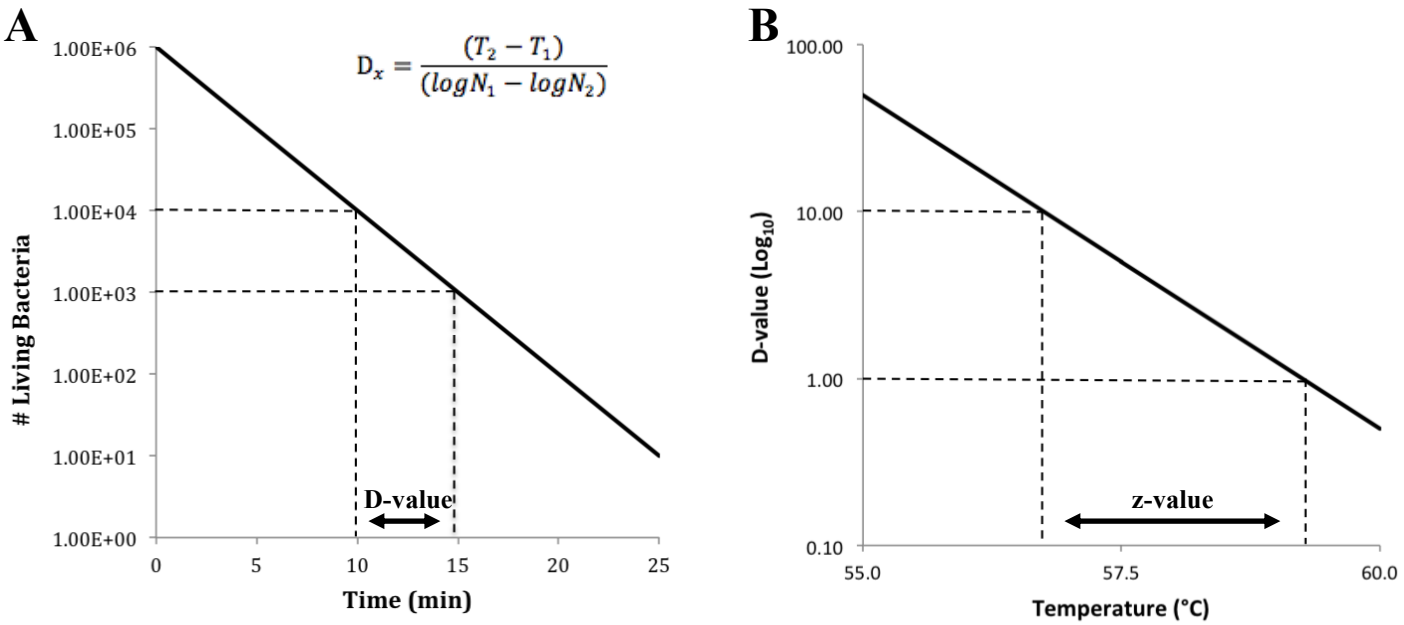

A
$D_x = \frac{(T_2 - T_1)}{(logN_1 - logN_2)}$
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Living Bacteria
D-value
0
5
10
15
20
25
Time (min)
B
100.00
10.00
1.00
0.10
D-value (Log10)
z-value
55.0
57.5
60.0
Temperature (°C)

Figure 2. Graphical representation of (A) decimal reduction, D-value, and (B) temperature dependence, z-value, calculations commonly used for microorganisms. (A) The D-value is measured by plotting the Log_{10} value(s) of the surviving population number of the microorganism vs the time that population count was calculated. The D-value is the negative reciprocal of the slope resulting in a 1-Log population reduction. The equation is displayed where D_x is the D-value for x temperature, T_2 is the number of sec/min/hrs/days elapsed at the final sample point since time zero, T_1 is the number of sec/min/hrs/days elapsed at the initial time point since time zero, N_1 is concentration/population at T_1 and N_2 is the concentration at T_2. (B) The z-value is calculated by plotting the Log_{10} value(s) of the calculated D-values against the temperature at which they were calculated. Z-value is the negative reciprocal of the slope where a reduction of 1-Log in D-value occurred.

1.5 Research Objectives

Bench-scale systems were utilized for this investigation to ensure control of environmental factors. This research aimed to test the hypothesis that the current CCME guidelines may not be adequate for the elimination of pathogens, as other pathogens may out-survive *Salmonella* spp. which is the current compost indicator for safety. The objectives of this study were:

1. Method development for fast detection and viability assessment of protists *Cryptosporidium parvum* and *Giardia duodenalis* in complex matrices such as manue (a brief introduction to this problem is section 3.1, pg 45).

2. To determine the D- and z-values of the common food-borne pathogens *Escherichia coli* O157:H7, *Campylobacter jejuni*, *Listeria monocytogenes*, *Salmonella enterica* Typhimurium, *Cryptosporidium parvum*, and *Giardia duodenalis*.

3. To evaluate variability in thermal reduction due to strain level differences such as heat resistance through experiments using *Escherichia coli* O26:H11, *Escherichia coli* AW1.7 and *Salmonella enterica* Senftenberg.

II. Methods

2.1 Strain Selection

Table 3. Pathogen strains being studied

Pathogen	Strain	Heat Resistant Strain	Isolation Source	Abbreviation Used
Campylobacter jejuni	NCTC 11168	N/A	Human clinical	CJ 11168
Cryptosporidium parvum	N/A	N/A	Calf faeces	*C. parvum*
Escherichia coli O157:H7 O26:H11	 C0283 02-6737	 AW1.7	 Cattle faeces Human clinical	 EC O157:H7 EC O26:H11
Giardia duodenalis	N/A	N/A	Gerbil faeces	*G. duodenalis*
Listeria monocytogenes	5412	N/A	Meat product(s) outbreak	LM 5412
Salmonella enterica Typhimurium Senftenberg	 611 (ATCC 13311) 775W (ATCC 43845)	 775W	 (611) Beef muscle (775) Unknown	 ST 611 SS 775W

The strains used in this investigation are listed in Table 3. Where possible, strains selected were isolated from produce related outbreaks or cattle sources. *Salmonella enterica* serovar Typhimurium 611 was isolated from beef muscle. The protists *Giardia* and *Cryptosporidium* were purchased from Waterborne^{TM}, Inc. (New Orleans, USA) where they are isolated from gerbil and calf sources respectively.

2.2 Growth and Maintenance of Cultures

Cultures of *Cryptosporidium parvum* and *Giardia duodenalis* were ordered at concentrations of 5 x 10^6 or 1 x 10^7 oocysts/cysts per 8mL from Waterborne Inc. (New Orleans, USA). Each organism is purified by sucrose and percoll gradients and stored in PBS with 0.01% Tween 20 and antibiotics. Parasite preparations were stored at 4°C until use

(*Giardia* cysts and *Cryptosporidium* oocysts remain viable at 4°C for 2 weeks and 2 months respectively)(Waterborne Inc.).

Cultures of *E. coli*, *Salmonella*, and *Listeria* were grown in two 500mL aliquots of sterile Brain Heart Infusion broth (BHI) (BD Bacto™, Mississauga, Canada). *Campylobacter* cultures were grown in two 500mL aliquots of Brucella broth containing an additional 0.25g sodium pyruvate (BD Bacto™, Mississauga, Canada).

To prepare bacterial cultures for inoculation, strains were incubated overnight at 37°C with agitation at 150 rpm in two 500mL flasks of BHI broth (*E.coli, Salmonella,* and *Listeria*) under aerobic conditions. *Campylobacter* strains were incubated overnight on biphasic plates (Mueller Hinton agar overlayed with 2mL of MH broth) at 37°C under microaerophilic conditions (5% oxygen, 10% carbon dioxide, 85% nitrogen).

2.3 Green Fluorescent Protein (GFP) Transformation of Heat Resistant *E. coli* AW1.7

E. coli AW1.7 is a generic strain that does not have chromogenic properties when plated on Rainbow agar (Biolog™, CA, USA) used in these experiments. To allow enumeration of heat tolerant AW1.7 and other *E. coli* strains on the same agar the AW1.7 strain was transformed with the *gfp*-containing plasmid pGFPuv (Clontech™, Mountain View, CA, USA) which encodes a variant of the *Aequorea victoria* GFP protein optimized for brighter fluorescence and confers resistance to ampicillin to allow isolation. The pGFPuv vector is depicted in Figure 3 (from the Clontech™ manual, 2012).

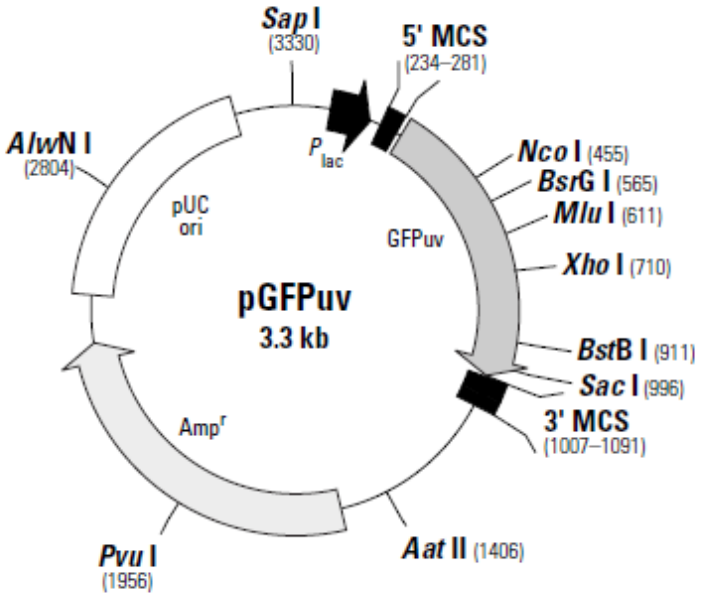

Sap I
(3330)
5' MCS
(234–281)
AlwN I
(2804)
P lac
Nco I (455)
BsrG I (565)
Mlu I (611)
pUC
ori
GFPuv
Xho I (710)
pGFPuv
3.3 kb
BstB I (911)
Sac I (996)
3' MCS
(1007–1091)
Amp r
Pvu I
(1956)
Aat II (1406)

Figure 3. Restriction map of pGFPuv Vector. A variant of GFP is located between the two MCSs of a pUC19 derivative. *lacZ* is the initiation codon for the GFPuv gene. This vector confers a resistance to ampicillin. Map and vector description is from Clontech™ (Mountain View, CA, USA) pGFPuv Vector Information document (2002). GenBank Accession #U62636.

As ampicillin resistance of the AW1.7 strain would interfere with selection of labeled transformants, the ampicillin resistance profile of this strain was determined by streaking fresh culture onto a Luria-Bertani (LB) plate supplemented with ampicillin (100µg/ml). After demonstrating that strains were not ampicillin resistant, fresh cultures of *E. coli* AW1.7 were grown overnight on LB agar at 37°C. Two hundred and fifty microliters of 15mM $CaCl_2$ (pH 6.1, Bio-Rad Laboratories Inc., California, USA) was pipetted into microcentrifuge tubes. Tubes were labelled in triplicate as +pGFPuv (containing plasmid), -pGFPuv (no plasmid, negative control), and +HT (plasmid with high temperature heat shock), and placed on crushed ice. Three colonies from LB plates were transferred to each of the nine tubes using a sterile loop, vortexed and placed back on ice. Ten microliters of pGFPuv was pipetted into the +pGFPuv and +HT tubes, 10µL of peptone water was pipetted into the –pGFPuv tubes. Tubes were incubated on ice for 10 min. The +pGFPuv and –pGFPuv tubes were transferred to the water bath set at 42°C, +HT tubes were transferred to the 52°C bath, for 50 s. Tubes were then incubated again on ice for 2 min.

Tubes were removed from the ice bath, and 250µL of LB broth was added to each tube and incubated for 10 min at room temperature. Tubes were vortexed, and 100µL from each tube was pipetted onto LB agar and LB agar + AMP (100µg/mL). Plates were incubated for 24 h at 37°C under aerobic conditions, then viewed for fluorescent colonies using ultraviolet light.

2.4. Parasite Detection in Manure and Berries

The lack of reliable detection/viability methods available for *Cryptosporidium* spp. and *Giardia duodenalis* required the development of an effective and high-throughput method.

The use of flow cytometry to detect *Cryptosporidium* and *Giardia* has been successfully documented for water samples (Barbosa et al, 2008; Hsu et al, 2005; Medema et al, 1998; Vesey et al, 1993).

Cattle manure was received from the Canadian Food Inspection Agency (CFIA) farm (Ottawa, ON). Fresh raspberries were purchased from local grocers.

2.4.1. Oocyst/Cyst Recovery from Manure

To recover oocysts/cysts from manure, 5g samples of manure were weighed in stomacher bags in duplicate. Samples were inoculated with 1mL each of *Cryptosporidium* and *Giardia* suspensions (approximately 625,000 oo(cysts)/mL), and manually massaged for 2 min to mix. Ten milliliters of 1% PBS Triton-X100 was added to each bag, which was then stomached at 230 rpm for 3 min. Two milliliters of retrieved liquid was layered over 15mL of sucrose (specific gravity of 1.25) in 50mL falcon tubes. Tubes were centrifuged at 25°C for 5 min at 800 x g. The top fraction of the sucrose gradient was transferred via pipette to a clean 50mL falcon tube and mixed with 10mL of PBS Triton-X100. The sample was concentrated by centrifugation at 1,000 x g for 10 min. The supernatant was removed and the pellet resuspended in 500µL of PBS.

For D- and z- value calculations 200g of both cow and chicken manure (1,250,000/mL) were inoculated with 14mL of each *Cryptosporidium parvum* and *Giardia duodenalis* oocysts/cysts in a large sterile container.

2.4.2. Oocyst/Cyst Recovery from Berries

Fifty grams of fresh berries were weighed and placed in stomacher bags in triplicate. Raspberries were purchased from local supermarkets for these experiments. One milliliter each of *Cryptosporidium* oocysts and *Giardia* cysts suspensions (625,000 oo(cysts)/mL) were added to each bag. One hundred and twenty milliliters of 0.01% Tween80 in PBS was added to each stomacher bag, and bags were then placed on an orbital shaker for 2 min at 100rpm. The rinse was retrieved from the filtered side of the stomacher bag and passed through one layer of gauze in a sterile funnel into 50mL centrifuge tubes. Tubes were centrifuged at 2,000 x g for 15 min at 20°C. Supernatant was aspirated and discarded. Fifteen milliliters of PBS-Tween80 was used to resuspend each pellet (via pipetting and vortexing) and the three pellets for each sample were pooled into one 50mL tube. One hundred microliters of Pectinex (pectinase) was added to each sample and vortexed to mix. Tubes were centrifuged at 5,000 x g for 30min at 25°C, and the supernatant was again discarded. Pellets were resuspended in 500µL of PBS. Fifty microliters of each sample was stained and analyzed via flow cytometry as described below.

Experiment was also conducted using strawberries, blueberries and blackberries and was effective for these berry types. Similar oocyst/cyst extraction protocol led us to continue with only one berry type.

2.4.3. Parasite Viability Staining

Monoclonal antibodies conjugated to Alexa Fluor 647 and fluorescein isothiocyanate (FITC) specific to *Cryptosporidium parvum* oocysts and *Giardia duodenalis* cysts, respectively, were purchased from Waterborne^{TM}, Inc. (New Orleans, USA) as 20X

concentrated solutions. One hundred microliter aliquots of each sample were transferred to 1.5mL microcentrifuge tubes. Forty microliters of each monoclonal antibody (1X concentration) was added to each tube, vortexed, and incubated at 37°C at 150 rpm for 20 min covered in aluminum foil. Antibody-stained samples were then divided into two 50µL samples; each was stained with varying amounts (1µL, 5µL, or 10µL) of either cell-impermeant viability dye SytoX or P.I and incubated at 37°C at 150 rpm for 20 min covered in aluminum foil.

2.4.4. Parasite Enumeration via Flow Cytometry

Stained parasites were analysed using the BD LSRFortessa^{TM} flow cytometer with FACS Diva software (Becton Dickinson Biosciences, San Jose, CA) equipped with three lasers. Fifty microliters of stained pure oocyst/cyst samples were mixed with 450µL of peptone water in a 5mL Falcon tube. For berry and manure samples, 50µL of sample was mixed with 1,450µL of peptone water.

The gating strategy for *Cryptosporidium* oocysts and *Giardia* cysts using flow cytometry was as follows: a dot plot was created with events fluorescing with FITC on the x-axis and events staining with AF647 on the y-axis. AF647-labelled events fluoresced brightly on the y-axis and represented AF647-bound *Cryptosporidium* oocysts. FITC-labelled events fluoresced brightly on the x-axis and represented FITC-bound *Giardia* cysts. Events were first gated based on fluorescence along these axes, which implied binding to that specific antibody. Gates were created around AF647 and FITC bound events. Additional dot plots with four quadrants were made whereby the x-axis represented staining with cell-impermeant viability dye SytoX Blue and the y-axis represented either AF647 or FITC staining.

Oocysts/cysts with no (or low) fluorescence, located in quadrant 1 (Q1) are viable. Those with fluorescence greater than 10^2 intensity staining with SytoX or PI and located in quadrant 2 (Q2) are labelled as nonviable.

2.5. D- and z- value Determination

2.5.1. Manure Characterization

The Evaluation Division Laboratory in the Food Directorate at Health Canada conducted manure characterization. The following 6 physio-chemical properties were tested: pH, water activity (a_w), percentage (%) moisture, dissolved oxygen (D.O.), oxidation-reduction potential (redox, E_h) and electrical conductivity (E.C.). All tests were conducted with sample(s) equilibrated to room temperature.

pH was measured using a pH meter. A 15g sample of manure was measured out, and as little water as possible to create a slurry was added. The pH meter probe was calibrated, placed in sample and used to gently mix the sample for 5 seconds.

Water activity of the slurry solution was measured using an AQUALAB meter.

Moisture content (% moisture): 10g samples were placed in a factory-set standard-drying program in a Mettler-Toledo HG63 Halogen Moisture Analyser (Toledo Inc., Greifensee, Switzerland). At the end of the cycle the machine records, calculates and displays the % moisture in the sample.

D.O., E_h , and E.C. were measured using an Orion 5 Star meter with a dissolved oxygen probe, Sure-flow comb, and DuraProbe Conductivity Cell, respectively (ThermoScientific Inc., Fort Collins, CO). Fifteen grams of manure was made into a slurry

with distilled water (1:2 manure/water ratio) and left to stand for 30 minutes at room temperature before tested with each probe.

2.5.2. Manure sampling

For each bacterium, two flasks containing 500mL of growth media (see section 2.2), were inoculated and incubated for 24 h prior to experimentation. (For *E. coli* 500mL of each EC O157:H7 and EC O26:H11 were grown). Cultures were retrieved from incubators and transferred to 50mL centrifuge tubes before being concentrated by centrifugation at 8,000 x g for 10 min. Supernatant was discarded and pellets were resuspended and combined to a total volume of 152mL of PBS (approximately 10^9 CFU/mL).

Control manure was "inoculated" with pure PBS. Sterile stomacher bags were labelled and a section of each bag was heat-sealed to create a pocket 2 inches wide. One hundred grams of cattle or chicken manure was weighed and transferred to a blending jar. One hundred milliliters of pure PBS was added before blending for 30 s on the lowest speed setting. Ten-milliliter portions were measured into a graduated cylinder before being funnelled into the pouch previously created in the stomacher bag. Each bag was then heat-sealed to dimensions of 2 inches x 3 inches. Before sealing the opening of each pouch, a data logger was placed in the center of the manure with the sensor against the inner mesh layer.

Experimental manure was inoculated in a similar fashion. One hundred and fifty grams of cow or chicken manure was weighed and transferred to a blending jar. Two milliliter of bacterial suspension was transferred to a sterile glass test tube (for inoculum calculations). The remaining 150mL was transferred to the blending jar, and mixed on the lowest speed for 30 s. Ten milliliter portions were measured into a graduated cylinder before

being funnelled into a stomacher bag which was heat-sealed to dimensions of 2 inches x 3 inches. Bags were heat-sealed in three adjacent lines to avoid spill, Figure 4.

One sample bag was set aside for time 0 calculations. Four bags were labelled for each temperature (6 temperatures x 4 bags) plus an additional control bag containing a data logger for each temperature. Metal test tube racks were used in water baths to ensure separation and water flow between sample bags as depicted in Figure 5. Bags were placed in water baths simultaneously and allowed to equilibrate to bath temperature for 2 min and 15 s before sampling time started.

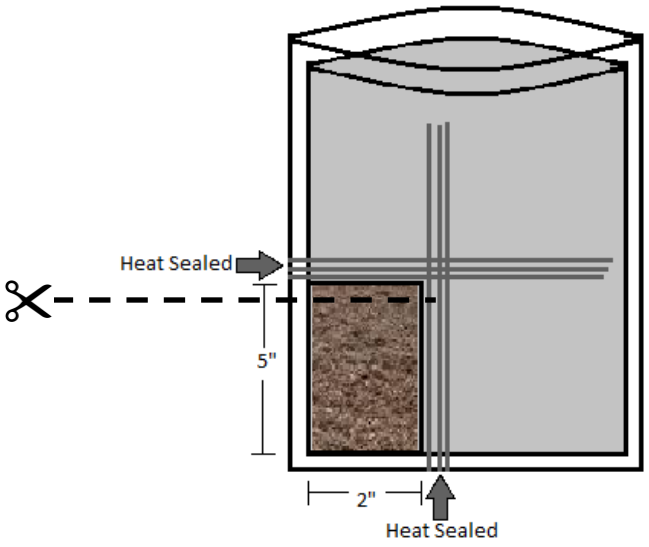

Heat Sealed
5"
2"
Heat Sealed

Figure 4. Experimental stomacher bag sample setup. Stomacher bag(s) with filtration layer (darker grey) in center were used to contain manure samples during heating. A 2-inch wide section of each bag was measured and heat-sealed 3 times vertically (dark grey arrow). 10g samples of manure-inoculum slurry were funnelled into pouch and the top was heat-sealed (3X) to create a pouch with 2"x 5" dimensions. Following experimentation, pouches were opened with a pair of sterilized scissors (ethanol-flamed) and 1mL of sample liquid was removed from the filtered side of the stomacher bag and transferred to a sterile test tube.

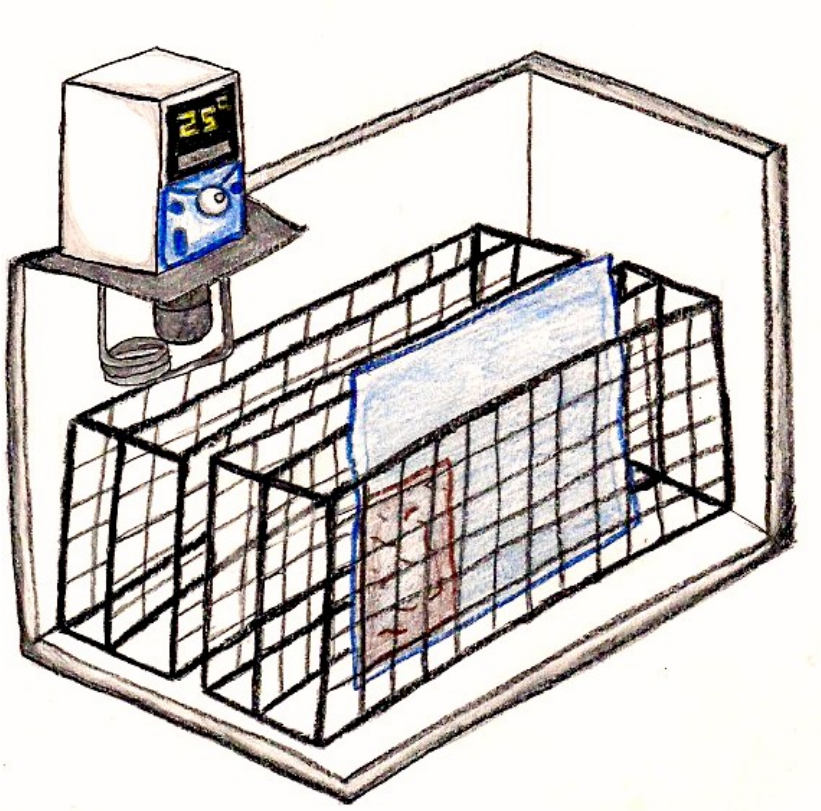

25°C

Figure 5. Waterbath setup and sample bag separation. Metal test tube racks were placed side-ways in waterbath, sample bags were then placed in separate slots (blue bag for example) to allow separation of water and temperature distribution between bags. A control bag containing a data-logger to monitor temperature, in case of error, was placed in bath during each run. If necessary an object was placed on top of racks to ensure bag(s) remained submerged.

Table 4. Temperatures, sampling intervals, and dilutions plated for each bacterium studied.

Bacteria	Temperature (°C)	Sampling Time Intervals	Dilutions Plated
Listeria monocytogenes 5415	25.0	25min	10^{-6}, 10^{-7}, 10^{-8}
	50.0	25min	25: 10^{-5}, 10^{-6}, 10^{-7} 50: 10^{-5}, 10^{-6}, 10^{-7} 75: 10^{-5}, 10^{-6}, 10^{-7} 100: 10^{-4}, 10^{-5}, 10^{-6}
	52.5	20min	20: 10^{-5}, 10^{-6}, 10^{-7} 40: 10^{-4}, 10^{-5}, 10^{-6} 60: 10^{-3}, 10^{-4}, 10^{-5} 80: 10^{-2}, 10^{-3}, 10^{-4}
	55.0	10min	10: 10^{-5}, 10^{-6}, 10^{-7} 20: 10^{-3}, 10^{-4}, 10^{-5} 30: 10^{-2}, 10^{-3}, 10^{-4} 40: 10^{-1}, 10^{-2}, 10^{-3}
	57.5	2min	2: 10^{-4}, 10^{-5}, 10^{-6} 4: 10^{-3}, 10^{-4}, 10^{-5} 6: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} 8: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}
	60.0	30s	30: 10^{-4}, 10^{-5}, 10^{-6} 60: 10^{-3}, 10^{-4}, 10^{-5} 90: 10^{-2}, 10^{-3}, 10^{-4} 120: 10^{-1},10^{-2},10^{-3},10^{-4}
Campylobacter jejuni NCTC 11168 (Study conducted in phosphate buffered peptone) Selective agar: mCCDA (Oxoid)	25.0	5min	10^{-5}, 10^{-6}, 10^{-7}
	52.5	2min	2: 10^{-4}, 10^{-5}, 10^{-6} 4: 10^{-3}, 10^{-4}, 10^{-5} 6: 10^{-3}, 10^{-4}, 10^{-5} 8: 10^{-2}, 10^{-3}, 10^{-4}
	55.0	30s	30: 10^{-4}, 10^{-5}, 10^{-6} 60: 10^{-2}, 10^{-3}, 10^{-4} 90: 10^{-2}, 10^{-3}, 10^{-4} 120: 10^{-1}, 10^{-2}, 10^{-3}

Table 4. (continued) Temperatures, sampling intervals, and dilutions plated for each bacterium studied.

Salmonella enterica 611 and 775W	25.0	45min	10^{-6}, 10^{-7}
	50.0	45min	45: 10^{-5}, 10^{-6}, 10^{-7} 90: 10^{-5}, 10^{-6}, 10^{-7} 135: 10^{-5}, 10^{-6}, 10^{-7} 180: 10^{-4}, 10^{-5}, 10^{-6}
	52.5	25min	25: 10^{-4}, 10^{-5}, 10^{-6} 50: 10^{-4}, 10^{-5}, 10^{-6} 75: 10^{-4}, 10^{-5}, 10^{-6} 100: 10^{-3}, 10^{-4}, 10^{-5}
	55.0	4min	4: 10^{-5}, 10^{-6}, 10^{-7} 8: 10^{-4}, 10^{-5}, 10^{-6} 12: 10^{-4}, 10^{-5}, 10^{-6} 16: 10^{-3}, 10^{-4}, 10^{-5}
	57.5	1min	4: 10^{-5}, 10^{-6}, 10^{-7} 8: 10^{-4}, 10^{-5}, 10^{-6} 12: 10^{-3}, 10^{-4}, 10^{-5} 16: 10^{-2}, 10^{-3}, 10^{-4}
	60.0	15s	15: 10^{-4}, 10^{-5}, 10^{-6} 30: 10^{-4}, 10^{-5}, 10^{-6} 45: 10^{-2}, 10^{-3}, 10^{-4} 60: 10^{-2}, 10^{-3}, 10^{-4}
Escherichia coli O26:H11 02-6737 O157:H7 C0283	25.0	45min	10^{-6}, 10^{-7}
	50.0	45min	45: 10^{-5}, 10^{-6}, 10^{-7} 90: 10^{-5}, 10^{-6}, 10^{-7} 135: 10^{-5}, 10^{-6} 180: 10^{-5}, 10^{-6}
	52.5	30min	30: 10^{-4}, 10^{-5}, 10^{-6} 60: 10^{-4}, 10^{-5}, 10^{-6} 90: 10^{-4}, 10^{-5}, 10^{-6} 120: 10^{-3}, 10^{-4}, 10^{-5}
	55.0	5min	5: 10^{-5}, 10^{-6}, 10^{-7} 10: 10^{-5}, 10^{-6}, 10^{-7} 15: 10^{-4}, 10^{-5}, 10^{-6} 20: 10^{-3},10^{-4},10^{-5},10^{-7}
	57.5	1min	1: 10^{-6}, 10^{-7} 2: 10^{-4}, 10^{-5}, 10^{-6} 3: 10^{-2},10^{-3},10^{-4},10^{-5} 4: 10^{-2}, 10^{-3}, 10^{-4}
	60.0	15s	15: 10^{-6}, 10^{-7}, 10^{-8} 30: 10^{-3}, 10^{-4}, 10^{-5} 45: 10^{-3}, 10^{-4}, 10^{-5} 60: 10^{-4},10^{-2}, 10^{-3},10^{-4}

Samples were removed from water baths at the specified time points, listed in Table 4, and placed in an ice water bath until plating.

To plate samples, scissors were immersed in ethanol and flamed using a bunsen burner, then the top seal of the sample bag was cut open. One milliliter of manure/inoculum slurry was then retrieved via pipette from the filtered side of the stomacher bag and mixed with 9mL of peptone water in a test tube. Serial dilutions were conducted separately for each sample, and 0.1mL of selected dilutions were plated on chromogenic agar specific to the bacterium being studied (see Table 5). Plates were incubated according to manufacturer specifications, and colonies were counted at 24 or 48 h.

CFU/g was calculated for each type of bacteria at each temperature (at each time point). Only plates containing 30 to 300 colonies were included. Three replicate experiments were conducted for *Listeria* and *E. coli*, two replicates were conducted for *Salmonella*.

Table 5. Selective media used for each bacterium.

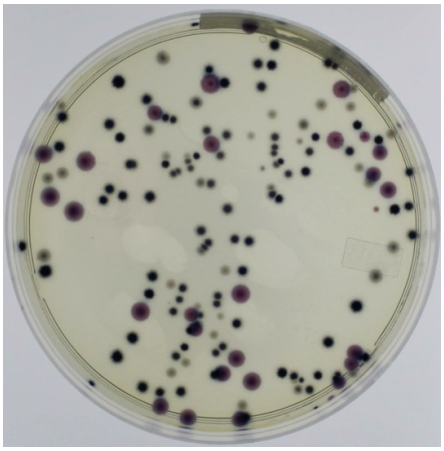
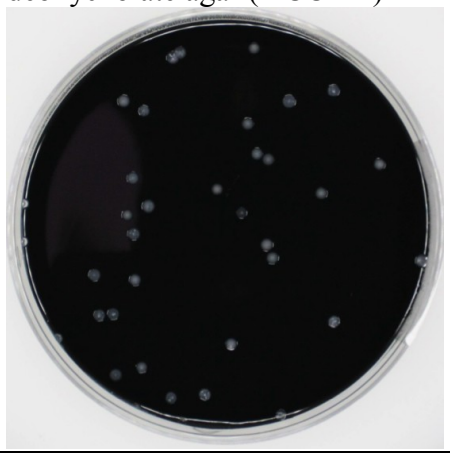
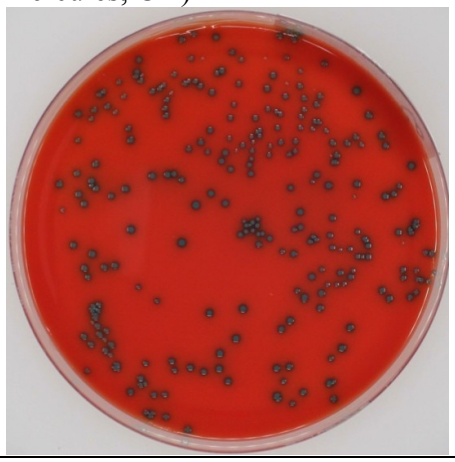
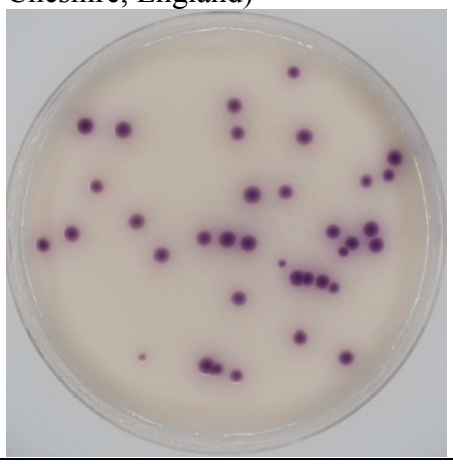
Bacterium	**Agar Selected**	**Characteristics**
Escherichia coli	Rainbow© Agar (Biolog, Hayward, CA) 	*incubated in aerobic conditions at 37°C *O157:H7 appear as small black or grey colonies *O26:H11 appear as purple-magenta colonies

Table 5. (continued)		
Campylobacter jejuni	Modified charcoal-cefoperazone-deoxycholate agar (mCCDA) 	* incubated in microaerophilic conditions *positive colonies appear milky-white on black background
Listeria monocytogenes	Rapid'L.mono Agar (RLM, Bio-Rad, Hercules, CA) 	*incubated in aerobic conditions at 37°C **L.mono* colonies appear purple on a red-orange background
Salmonella enterica	Brilliance Salmonella agar (Oxoid, Cheshire, England) 	*incubated in aerobic conditions at 37°C *colonies appear purple on white background *strains 611 and 723 were tested in separate experiments

2.5.3. D- and z value calculations

The total count for each bacterium was calculated by averaging the CFU/g counts from replicate experiments. D-values were calculated by plotting the Log_{10} of CFU/g against time (min) using Graphpad Prism software. The slope was found using linear regression to fit the curve of the data. The D-value was then determined as the negative reciprocal of the slope. The reported D-value for each bacterium and temperature was an average of replicate trials.

Z-values were calculated by plotting the Log_{10} of each of the five D-values against the temperature they were tested at for each bacterium. A trend line was calculated using linear regression in Graphpad Prism, and the *z*-value was calculated as the negative reciprocal of the slope.

2.6.Statistics

Statistical differences for comparisons of SytoX Blue and PI staining (of parasites) were conducted in Graphpad Prism using Two-Way Analysis of Variance (ANOVA) with multiple comparisons, and Tukey test for corrections with significant difference defined as $p<0.05$.

Statistical differences of D- and *z*-values were measured between manure types (cattle versus chicken) for each bacterial genus. Differences between bacterial genera were measured within the same manure type (cattle vs cattle, and chicken vs chicken). Graphpad Prism software was used to test for significant differences between linear regression lines with a significant difference defined as $p<0.05$.

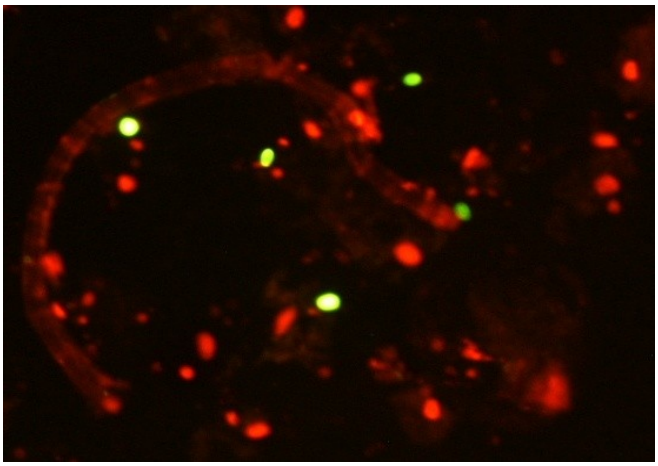
III. Results

3.1 Introduction to Current Parasite Detection Methods and use of Flow Cytometry

The most commonly used detection techniques for *Cryptosporidium* oocysts and *Giardia* cysts involve microscopy and/or polymerase chain reaction (PCR) technologies. Although these techniques are widely used they do not allow accurate viability determination on a large scale. Microscopy can be used for enumeration but requires expertise and is hampered by the presence of debris, other microorganisms, and auto-fluorescent particles (Figure 6). While qRT-PCR may correlate with viability, it would be difficult to quantify oocysts/cysts in a sample using gene expression values. Viability determination through measurement of mRNA using qRT-PCR requires time and a number of physical and chemical treatments. Therefore these methods are not only labour intensive but may be inaccurate and not suited for calculations of log reduction as would be required for D- and z-value determination. Flow cytometry is advantageous relative to microscopy and PCR as relatively large volumes can be analysed in a short time period, and multiple parameters can be analyzed, allowing viability determination as well as quantification simultaneously through measuring particle size, fluorescence (including multiple fluorochromes), and complexity. Flow cytometry has been used to detect *Cryptosporidium* and *Giardia* in water samples (Barbosa et al, 2008; Hsu et al, 2005; Medema et al, 1998), and some work has been conducted on the use of this technology for detection of parasites in more complex matrices such as soil and foods (Kato and Bowman, 2002).

The first objective of this study was to develop a method for a high-throughput method and protocol for the quantification and viability assessment of *Cryptosporidium parvum* and *Giardia duodenalis* oocysts/cysts in complex matrices, such as manure.

A



B

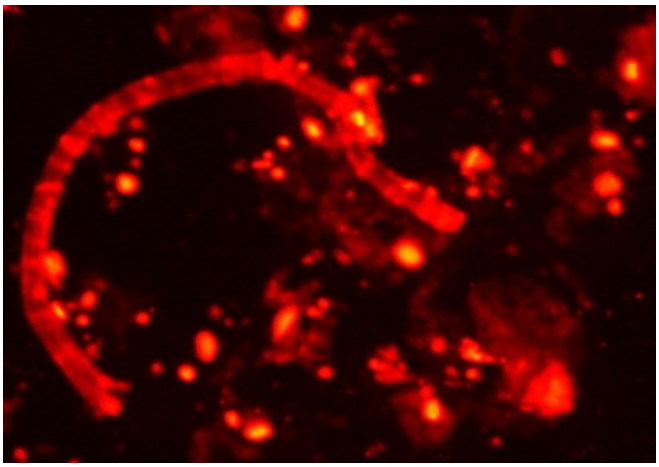


Figure 6. Microscopic images of Propidium Iodide (PI) stained *Giardia* cysts in cattle manure. Five grams of cattle manure was inoculated with oocysts/cysts. Cyst samples were recovered from manure via flotation in sucrose with a gradient of 1.5. Following flotation procedures to isolate oocysts/cysts, 50µL samples of isolated manure sample liquid were stained with *Giardia duodenalis* cyst specific monoclonal antibody conjugated to fluorescein isothiocyanate (FITC), then stained with cell-impermeant viability dye propidium iodide (PI). A: *Giardia* cysts stained with monoclonal antibody conjugated to FITC, visible in green, in a background of autofluorescent particles (red) in manure. B: (A) under a different fluorescent filter showing only red fluorescence, cyst particles are difficult to identify and dead cysts (stain bright red with PI) are also difficult to distinguish from background.

3.2 Enumeration of Viable Parasites

Pure suspensions of oocysts and cysts were analysed using the BD LSRFortessa (BD Biosciences) flow cytometer (equipped with three lasers) to ensure detection and adequate separation of *Cryptosporidium* and *Giardia* events, as well as to investigate possible viability dye – antibody fluorochromes combinations. There was separation of oocysts and cysts on different axes without event overlap using the AlexaFluor647 (AF647) antibody for *Cryptosporidium* and FITC labelled antibody for *Giardia*. Separation of *Cryptosporidium* oocysts and *Giardia* cysts on the y- and x-axes (respectively) is visible with small amounts of extra debris fluorescing dimly (black events, Figure 7A). The gating strategy used for parasite oocyst/cyst enumeration is also described in Figure 7. Briefly, oocysts/cysts were separated based on antibody-fluorochrome fluorescence. Those events fluorescing brightly were gated as either *Crytposporidium* (y-axis) or *Giardia* (x-axis) (Figure 7A), then were analysed separately for fluorescence with either SytoX Blue or PI viability dye (Figures 7B and 7C). Oocysts/cysts that fluoresced with either of the cell-impermeant dyes were counted as non-viable.

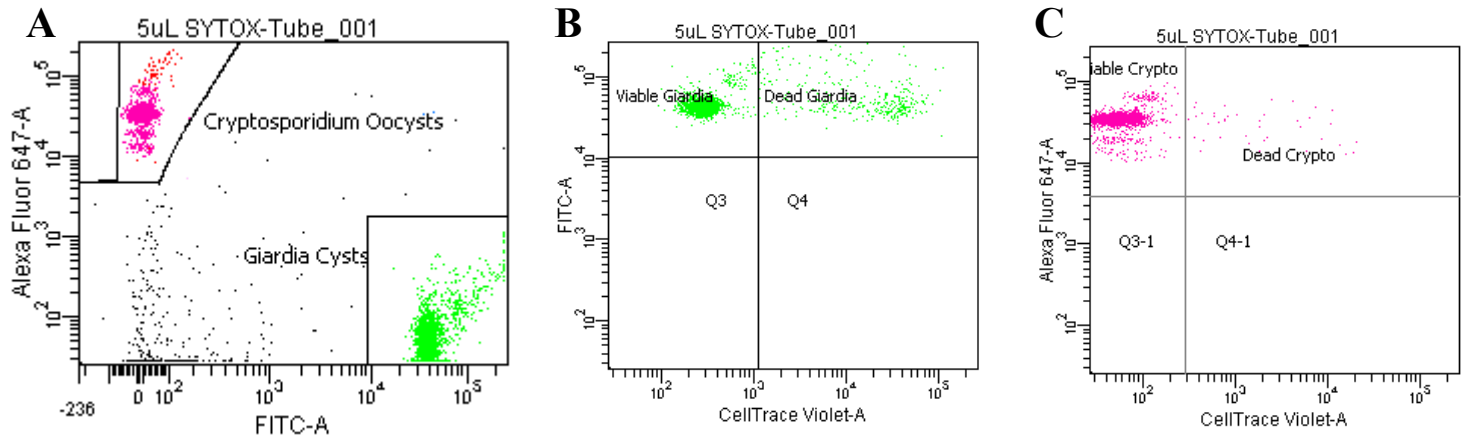

A
5uL SYTOX-Tube_001
Cryptosporidium Oocysts
Giardia Cysts
Alexa Fluor 647-A
FITC-A
-236
B
5uL SYTOX-Tube_001
Viable Giardia
Dead Giardia
Q3
Q4
FITC-A
CellTrace Violet-A
C
5uL SYTOX-Tube_001
Dead Crypto
Q3-1
Q4-1
Alexa Fluor 647-A
CellTrace Violet-A

Figure 7. Detection of *Cryptosporidium* oocysts and *Giardia* cysts using flow cytometry. Pure suspensions of oocysts/cysts were stained with monoclonal antibodies conjugated to Alexa Fluor 647 (AF647) (*Cryptosporidium*) and fluorescein isothiocyanate (FITC) (*Giardia*). (A) Dot plot with events (dots) fluorescing with FITC on x-axis and events staining with AF647 on y-axis. AF647-labelled events appear pink and represent AF647-bound *Cryptosporidium* oocysts. FITC-labelled events appear green and represent FITC-bound *Giardia* cysts. Events were first gated based on fluorescence on these axes, which implied binding to that specific antibody. Black events are background debris not bound to antibodies. (B and C) X-axis represents staining with cell-impermeant viability dye SytoX Blue. Oocysts/cysts with no (or low) fluorescence, located in quadrant 1 (Q1= Viable Giardia/Crypto) are viable. Those with SytoX or PI fluorescence greater than 10^2 (y-axis) and located in quadrant 2 (Q2 = Dead Giardia/Crypto) are labelled as nonviable

3.3 Confirmation of *Cryptosporidium* and *Giardia* oocysts/cyst detection

Flow cytometry has become a popular tool for sample analysis and data acquisition; however there is still some resistance to its use as data is represented by events ("dots"), and could potentially reflect particles in the sample matrix, rather than the microorganism of interest. In order to confirm that the events being counted were in fact *Cryptosporidium* and *Giardia* oocysts/cysts an experiment was conducted using the Amnis Image Stream Imaging Cytometer. This cytometer contains a high-resolution microscope, which allows the investigator to photograph all of the events being captured/recorded in a sample. Events predicted to be oocysts/cysts based on the gating strategies described above were visualized, and had typical organism morphology as is seen in non-imaging cytometers (Figure 8). Monoclonal antibodies conjugated to fluorochromes AlexaFluor 647 and FITC (*Cryptosporidium* and *Giardia* respectively) appear to be specifically binding the oocysts/cysts in tested pure oocyst/cyst samples in PBS, and are appropriate for detecting these organisms in complex samples such as manure.

Additional confirmation of flow cytometry analysis was conducted using a Cell Sorting Cytometer to sort selected *Giardia* cyst events onto a microscope slide. Using the cell sorter we were able to selectively mount cysts in specific areas on a microscope slide, as well as select the number of cysts mounted in each area. One cyst was mounted in 5 – 10 places on a slide (5 – 10 cysts/slide total), and then viewed via microscopy. Particles collected had typical size and morphology of *Giardia*, ovoid and 11-14μm in length, as well as fluorescing green due to the FITC-antibody (data not shown). *Cryptosporidium* oocysts were not visible, however drying of *Cryptosporidium* on microscope slides has been shown

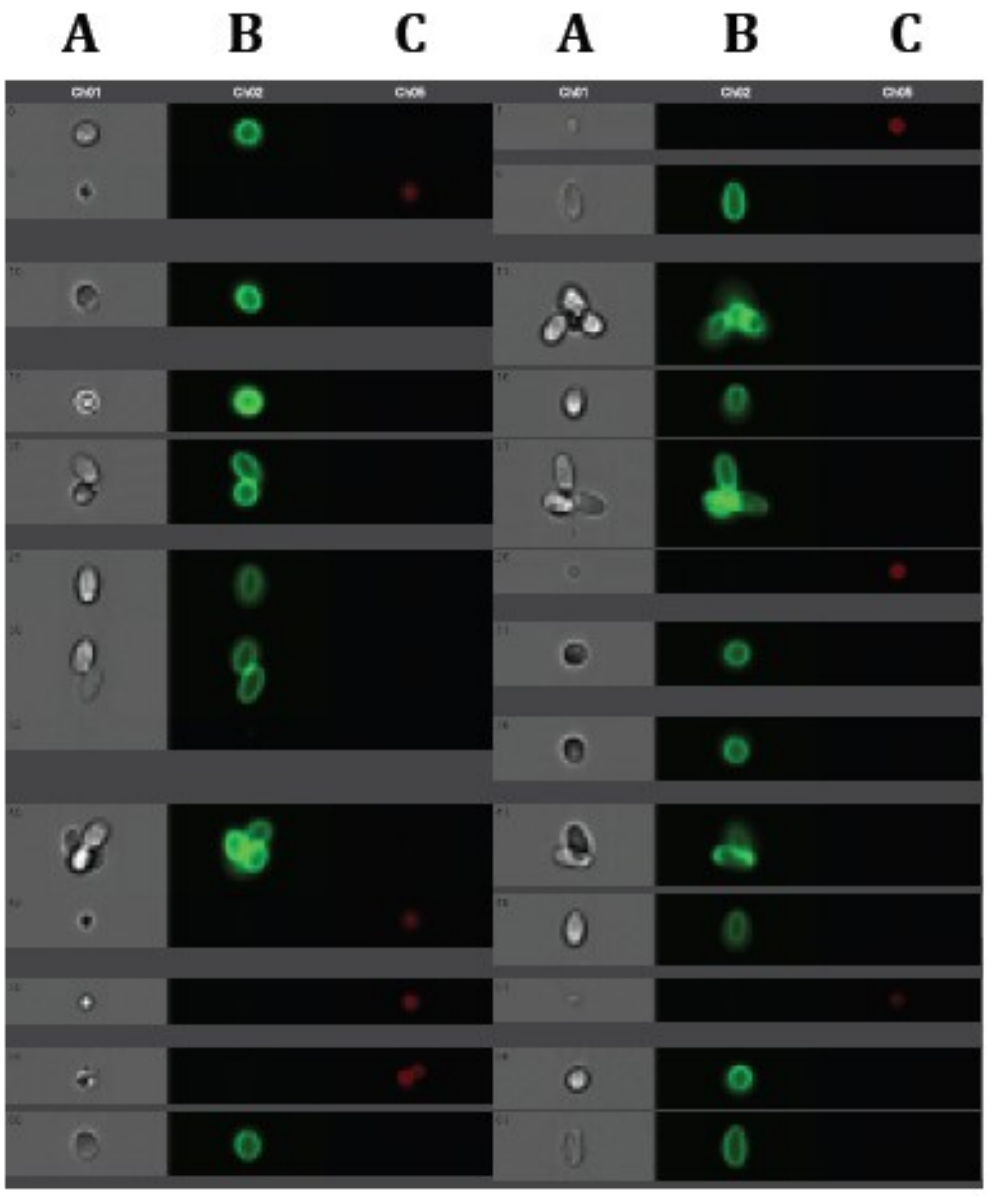

A
B
C
A
B
C
Ch01
Ch02
Ch05
Ch01
Ch02
Ch05

Figure 8. Imaging cytometry photograph of stained *Cryptosporidium* oocysts and *Giardia* cysts. Aliquots of pure samples of oocysts/cysts were stained with each of the monoclonal antibodies conjugated to AlexaFluor647 (AF647) and fluorescein isothiocyanate (FITC) (specific to *Cryptosporidium* oocysts and *Giardia* cysts respectively), fixed with 100uL of 10% buffered formalin and analyzed using an Amnis ImageStream Imaging Flow Cytometer. (A) Bright field image of events. (B) Channel displaying images of *Giardia* cyst events fluorescing green with FITC labeled bound antibody. (C) Channel displaying images of *Cryptosporidium* oocysts fluorescing red with AF647 labeled bound antibody.

previously as it is known that these oocysts are quite susceptible to desiccation (Fayer, 2004). Only one trial was conducted using the sorter due to equipment issues.

3.4 Dye Toxicity

Following successful detection of oocysts and cysts via flow cytometry a comparison of the two viability dyes SytoX Blue and PI was conducted. The SytoX brand of dyes is advantageous due to the availability in a variety of colours, a large (1000 X) increase in fluorescence on nucleic acid binding (compared to a 10X increase for PI), and a small excitation/emission spectrum that limits overlap into other fluorescent channels. It was observed in early trials that SytoX appeared to stain more oocysts/cysts as nonviable compared to PI which led to the investigation of whether SytoX was toxic to the oocysts/cysts or PI was overestimating viability.

Both dyes were evaluated using pure viable oocyst/cyst suspensions. Tubes were analysed on day one (time 0) after 20 minutes of staining with both monoclonal antibodies followed by 20 minutes staining with either SytoX Blue or PI at 37°C protected from light. Samples were analysed using the LSRFortessa Flow Cytometer with up to 5000 events collected. The remaining sample was stored at 4°C for 24 hrs protected from light and analysed again.

Neither SytoX nor PI had a toxic effect on *Cryptosporidium* oocysts after 24hrs of incubation (Figure 9). Variability in viability associated with staining methodology was observed for *Giardia* cysts, with more cysts staining as nonviable when a larger concentration of SytoX was added. Two-way ANOVA of dye concentration versus time data

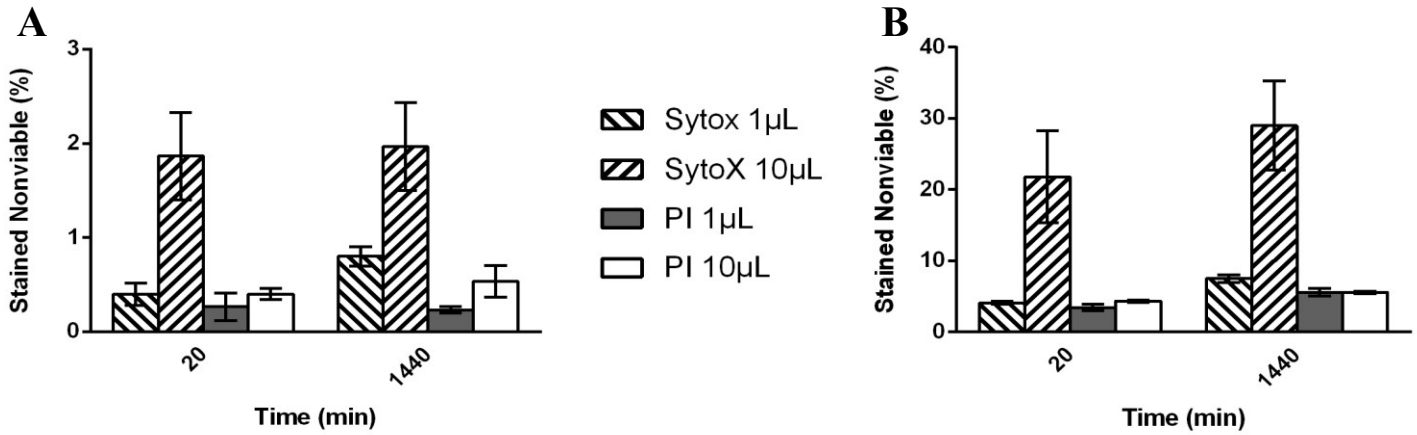

A
B
Stained Nonviable (%)
Time (min)
20
1440
Sytox 1μL
SytoX 10μL
PI 1μL
PI 10μL

Figure 9. Effect of SytoX Blue and PI stains on (A) *Cryptosporidium parvum* and (B) *Giardia duodenalis* viable oocysts/cysts over time. Viable suspensions of oocysts/cysts were stained with monoclonal antibodies conjugated to AlexaFluor647 (AF647) and fluorescein isothiocyanate (FITC) (specific to *Cryptosporidium* and *Giardia* respectively) for 20min, followed by staining with varying amounts of either propidium iodide (PI) or SytoX Blue cell-impermeant viability dyes for 20min at 37°C. Tubes were analyzed immediately after staining using a BD LSRFortessa flow cytometer and again after storage at 4°C for 24hrs. Means ± standard error are shown. n = 3. Two-way ANOVA was conducted using Graphpad Prism software. No significant difference was found between percentages of oocysts/cysts stained nonviable at 20min versus 24hrs for each dye concentration.

for each parasite showed no significant difference between the percentages of cysts staining nonviable at time 0 versus 24hrs.

Dyes were subsequently compared for the ability to accurately distinguish between viable and non-viable oocysts/cysts through the staining of different ratios of viable:heat-inactivated oocysts/cysts. A portion of pure suspensions of oocysts and cysts was heat inactivated at 60C for 20 min or 12 hours (*Cryptosporidium* oocysts and *Giardia* cysts respectively) to ensure they were non-viable. Based on previous experiments, viable suspensions of oocysts/cysts were mixed with heat-inactivated suspensions at ratios of 100:0, 50:50, and 0:100 (viable:heat-inactivated) and stained with10µL amounts of SytoX Blue or PI. These ratios were used as predictors to determine what percentage of the sample should stain as non-viable using flow cytometry assuming 100% of the heat-inactivated oocysts/cysts were non-viable, and 100% of the pure suspensions received from Waterborne Inc. (New Orleans, USA) were viable.

It is possible some oocysts/cysts may be damaged during the shipping and handling process. Each sample was tested after 20min staining and 24hrs, similar to the trial with pure viable oocysts/cysts, in order to monitor the possibility of toxicity (although the previous test suggested the dyes did not harm viable oocysts/cysts). Resultant ratios of viable:non-viable *Cryptosporidium* oocysts were closer to predicted values when oocysts were stained with SytoX Blue compared to PI (Figure 10). Results for *Giardia* found SytoX and PI staining not significantly different at 50:50 and 0:100 (viable:non-viable) predicted ratios, however SytoX Blue staining did result in slightly higher non-viable numbers than PI at 50:50 (though

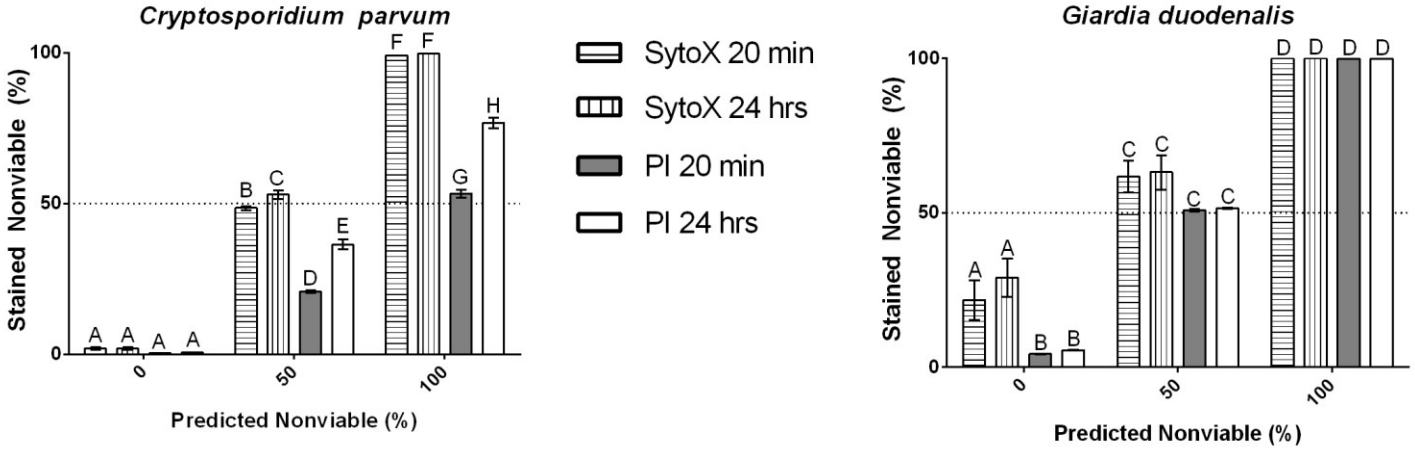

Cryptosporidium parvum
Stained Nonviable (%)
Predicted Nonviable (%)
SytoX 20 min
SytoX 24 hrs
PI 20 min
PI 24 hrs
Giardia duodenalis
Stained Nonviable (%)
Predicted Nonviable (%)

Figure 10. Predictive ability of SytoX Blue and PI for staining suspensions containing viable and heat-inactivated oocysts/cysts. Samples containing 100:0, 50:50, and 0:100 viable:heat-inactivated oocysts/cysts were stained with monoclonal antibodies conjugated to AlexaFluor647 (AF647) and fluorescein isothiocyanate (FITC) (specific to *Cryptosporidium* and *Giardia* respectively) for 20min, followed by staining with 10µL amounts of cell-impermeant viability dye SytoX Blue or PI for 20min at 37°C. Tubes were analyzed immediately after staining using a BD LSRFortessa flow cytometer and again after storage at 4°C for 24hrs. Means ± standard error are shown. n = 3.

not statistically significant), and there was a significant difference between SytoX and PI in viable samples with SytoX staining more cysts as non-viable (Figure 10).

Similar results were observed for *Giardia* cysts, with a higher initial nonviable count with the SytoX Blue dye compared to PI. Results using PI were very close to predicted ratios at both dye concentrations and time points compared to SytoX Blue, which had an increase in predicted nonviable cysts for the 100:0 and 50:50 viable:inactivated samples. Two-way ANOVA found no significant differences between SytoX and PI staining of *Giardia* oocysts at 50:50 and 0:100 viable:inactivated samples, however there was a significant difference between dyes in un-treated viable samples.

As there was a visible (and in some cases significant) increase in the percentage of *Cryptosporidium* oocysts staining dead when left at 4°C for 24 hours, a more extensive time analysis was conducted. Pure suspensions of 50:50 (viable:inactivated) oocysts/cysts were tested with 1µL or10µL of either SytoX or PI. Samples were then analysed immediately after staining (20 min), as well as after storage at 4°C for 24 hours (1440 min.) and 5 days (7200 min.) (Figure 11).

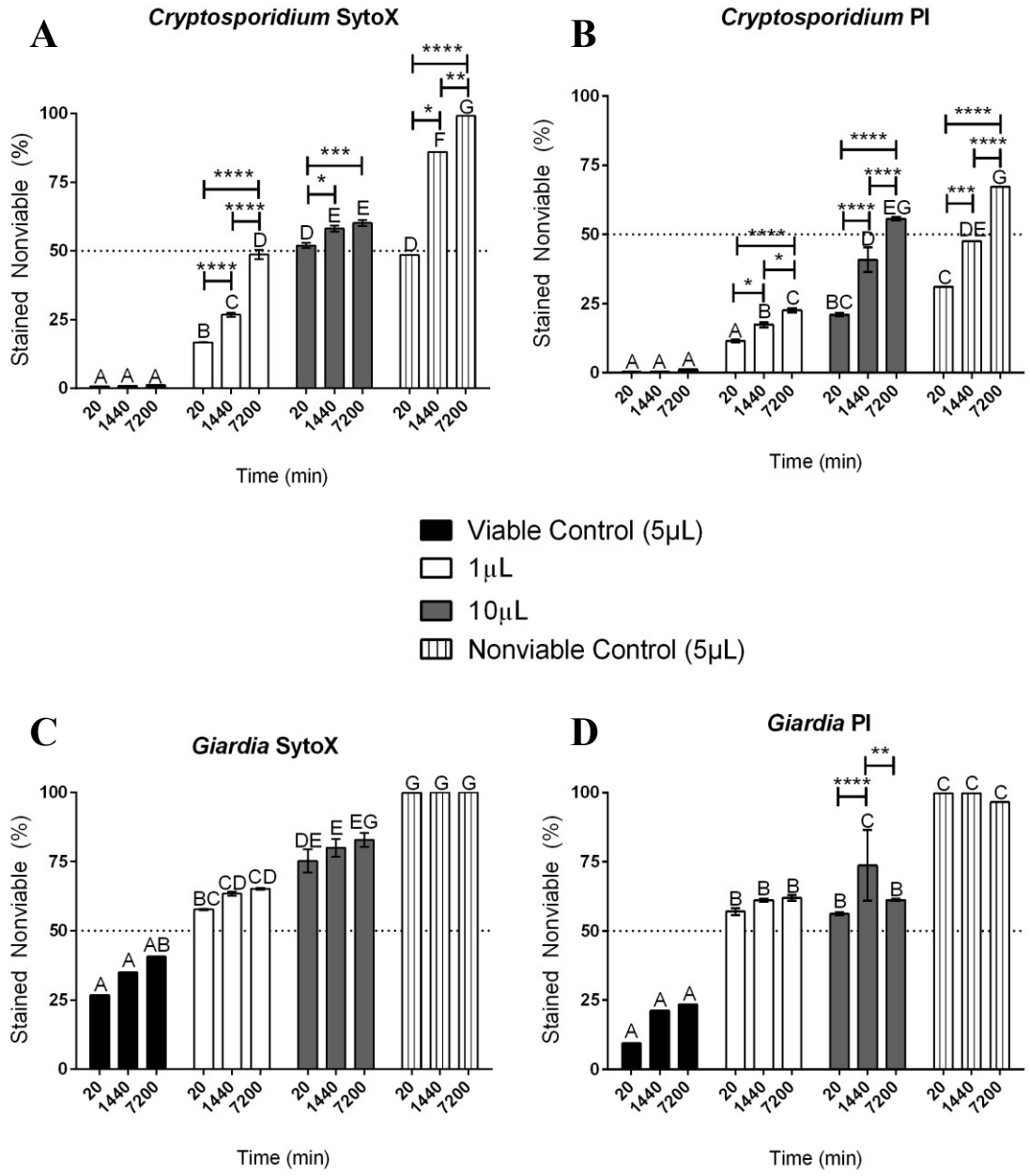

A
Cryptosporidium SytoX
Stained Nonviable (%)
Time (min)
B
Cryptosporidium PI
Stained Nonviable (%)
Time (min)
Viable Control (5µL)
1µL
10µL
Nonviable Control (5µL)
C
Giardia SytoX
Stained Nonviable (%)
Time (min)
D
Giardia PI
Stained Nonviable (%)
Time (min)

Figure 11. Comparison of viability dyes SytoX Blue and PI staining nonviable oocysts/cysts over time. Pure samples of *Cryptosporidium parvum* and *Giardia duodenalis* oocysts/cysts were mixed at a ratio of 50% viable : 50% heat-inactivated. oocysts/cysts were stained with monoclonal antibodies conjugated to AlexaFluor647 (AF647) and fluorescein isothiocyanate (FITC) (specific to *Cryptosporidium* and *Giardia* respectively) for 20min, followed by staining with 10µL amounts of cell-impermeant viability dye SytoX Blue or PI for 20min at 37°C. Samples were refrigerated until analysis via flow cytometry at 20 min., 24 hours (1440 min.), or 5 days (7200 min.) post staining. Mean percentages of oocysts/cysts stained nonviable from replicate experiments are shown with standard error. Means with different letters from A to D are significantly different. n =3. Asterisks (*)denote significant difference between time points at that concentration.
* significant at $0.05 > p < 0.01$, ** significant at $0.01 > p < 0.001$, *** significant at $0.001 > p < 0.0001$, **** significant at $p<0.0001$

Cryptosporidium samples required incubation for much longer time periods with even large amounts of PI (10uL) to achieve predicted results of 50% nonviable, compared to SytoX which only required 24 hrs for dye uptake at10µL concentrations (Figure 11 A and B, grey bars). No significant differences between incubation times were observed in viable controls of either dye (Figure 11 A and B, black bars). A significant increase in staining of heat-inactivated (nonviable) *Cryptosporidium* oocysts with PI was observed between 20min, 24hrs and 5 days at both concentrations (Figure 11B). There was no significant difference between incubation in dye for 24hrs versus 5 days for 10µL concentrations of SytoX, and only a slight significant difference ($0.5 > p < 0.01$) was found between 20min staining versus 24hrs staining at this concentration (Figure 11A). *Cryptosporidium* oocysts did require longer incubation periods to achieve predicted 50% staining using the lower 1µL SytoX concentration, exhibiting highly significant differences between all incubation times. There were no significant differences between nonviable staining with 10µL of SytoX at 24hrs or 5 days, suggesting larger amounts of dye or longer incubation periods may be required for dye uptake.

Determination of *Giardia* cyst viability varied according to dye used for staining. For all concentrations of both SytoX and PI (except 10µL PI at 24hrs) there were no significant differences between 20min, 24hrs, and 5days of analysis (Figure 11 C and D). Staining of *Giardia* cysts with 1µL SytoX for 24hrs and 5days was not significantly different from using 10µL SytoX staining for 20min (Figure 11C). A significant difference was observed in samples stained with 10µL PI staining for 20min compared to 5 days, as well as for 24hrs compared to 5 days, but not between 20min and 5 days (Figure 11D). The significant differences observed between other time points and 24hrs might be due to an outlier

observed in the 10µL 24hrs samples, which had 99.2% of cysts staining nonviable (Figure 11D, grey bars).

3.5 Effect of Dimethyl Sulfoxide (DMSO)

The SytoX brand dye is prepared in a solution containing dimethyl sulfoxide (DMSO), whereas PI is a 1mg/mL solution in water. DMSO is known to aid compounds in the penetration of tissues. To investigate whether DMSO was responsible for enhanced SytoX staining,samples of 50:50 viable:inactivated oocysts/cysts were stained with 1µL, 5µL, and 10µL of SytoX or PI and compared to samples stained with 1µL (SytoX or PI) + 9µL DMSO and 5µL (SytoX or PI) + 5µL DMSO (Figure 12).

Significantly higher numbers of *Cryptosporidium* oocysts stained as non viable with SytoX than with PI at all concentrations except viable controls, 1µL-20min, 5 µL+ 5 µL DMSO – 5 days, and 10µL-5 days staining (Figure 12, panel A). Samples containing 1µL SytoX were significantly lower from 1µL SytoX + 9µL DMSO at 20min and 24hrs analysis time points, but not at 5 days. No significant difference was observed in oocysts stained with 5µL SytoX and 5µL SytoX + 5µL DMSO at any time point. After 5 days, counts of viable oocysts stained with 1µL SytoX and 1µL SytoX + 9µL DMSO were no longer significantly different. Addition of DMSO to PI staining of *Cryptosporidium* oocysts did not appear to influence dye uptake, as no significant difference was observed between 1µL PI and 1µL PI + 9µL nor 5µL PI and 5µL PI + 5µL DMSO at any time point (Figure 12, panel A).

No significant difference in staining of viable and nonviable *Giardia* cysts was observed between SytoX and PI dyes (Figure 12, panel B). With the exception of the 10µL concentration(s) at 24hours, *Giardia* (Figure 12, panel B) exhibited significantly higher non-

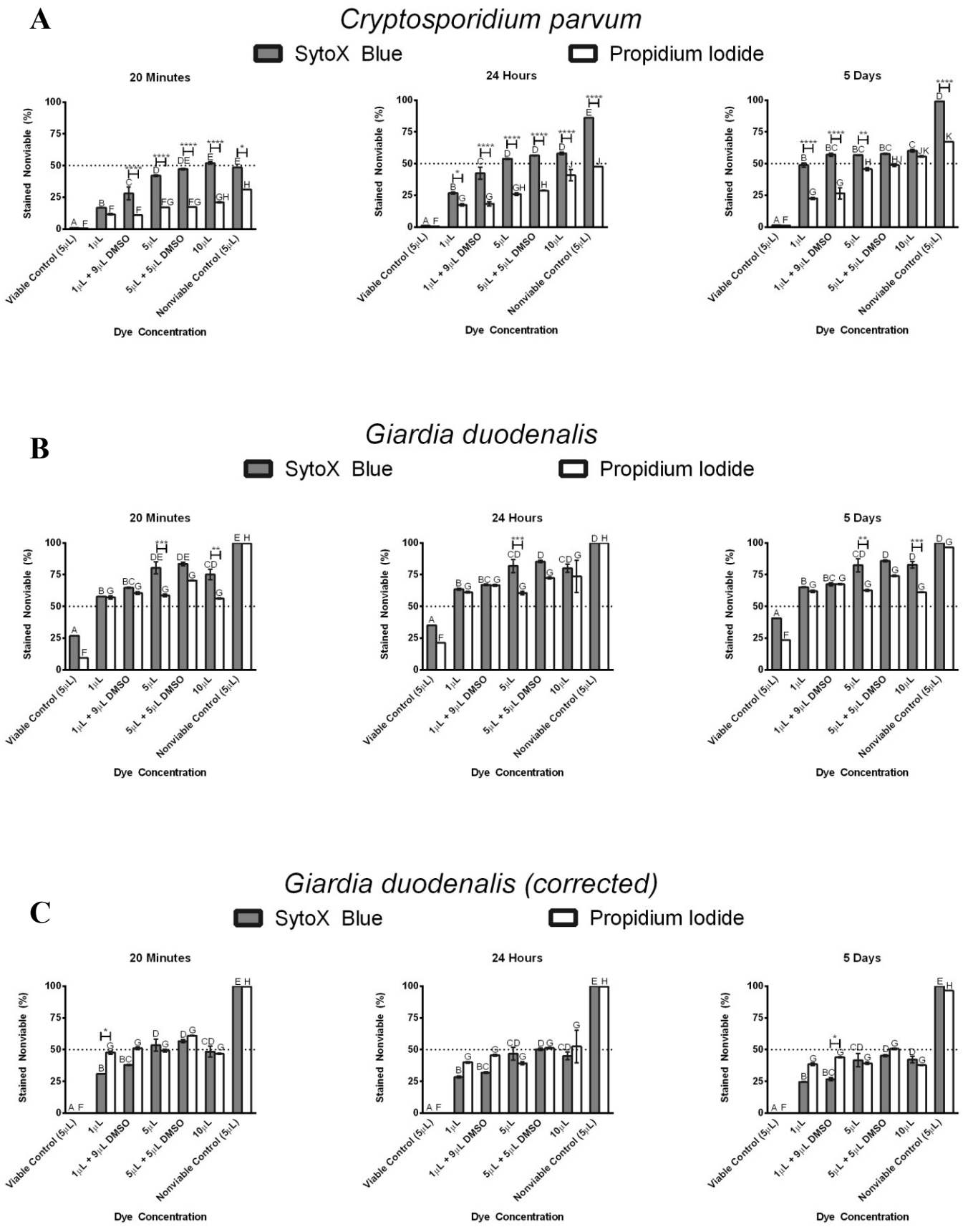

A
Cryptosporidium parvum
SytoX Blue
Propidium Iodide
20 Minutes
24 Hours
5 Days
Stained Nonviable (%)
Viable Control (5µL)
1µL
1µL + 9µL DMSO
5µL
5µL + 5µL DMSO
10µL
Nonviable Control (5µL)
Dye Concentration
B
Giardia duodenalis
SytoX Blue
Propidium Iodide
20 Minutes
24 Hours
5 Days
Stained Nonviable (%)
Viable Control (5µL)
1µL
1µL + 9µL DMSO
5µL
5µL + 5µL DMSO
10µL
Nonviable Control (5µL)
Dye Concentration
C
Giardia duodenalis (corrected)
SytoX Blue
Propidium Iodide
20 Minutes
24 Hours
5 Days
Stained Nonviable (%)
Viable Control (5µL)
1µL
1µL + 9µL DMSO
5µL
5µL + 5µL DMSO
10µL
Nonviable Control (5µL)
Dye Concentration

Figure 12. Effect of DMSO on vital dye staining of *Cryptosporidium parvum* (A) and *Giardia duodenalis* (B and C). Pure samples of *Cryptosporidium parvum* and *Giardia duodenalis* oocysts/cysts were mixed at a ratio of 50% viable : 50% heat-inactivated. oocysts/cysts were stained with monoclonal antibodies conjugated to AlexaFluor647 (AF647) and fluorescein isothiocyanate (FITC) (specific to *Cryptosporidium* and *Giardia* respectively) for 20min. Staining with cell-impermeant viability dyes SytoX Blue or PI in the following amounts; 1µL (SytoX or PI) + 9µL DMSO; 5µL (SytoX or PI) + 5µL DMSO; and 10µL (SytoX or PI); for 20min at 37°C. Mean percentages of oocysts/cysts stained nonviable from replicate experiments are shown with standard error. Means for SytoX Blue with different letters from A to D are significantly different. Means for PI with different letters from F to H are significantly different. n =3. Asterisks (*) denotes significant difference between SytoX and PI at that concentration.
* significant at $0.05 > p < 0.01$, ** significant at $0.01 > p < 0.001$, *** significant at $0.001 > p < 0.0001$,
**** significant at $p<0.0001$

viable counts when staining with SytoX compared to PI when staining with higher concentrations of 5µL and 10µL. Addition of DMSO to stained samples did not appear to affect dye uptake, as no significant difference was observed between 1µL SytoX and 1µL SytoX + 9µL DMSO nor with 5µL SytoX and 5µL SytoX + 5µL DMSO at any time point for *Giardia* cysts. Nor was there any significant difference between staining with higher concentrations of SytoX at any time point (5µL SytoX, 5µL SytoX + 5µL DMSO, and10µL SytoX) (Figure 12, panel B).

Although there were few significant differences between SytoX and PI staining of *Giardia* cysts, SytoX stained a higher percentage of cysts as nonviable (Figure 12, panel B). An approximately 25% higher nonviable count was recorded for all samples, including the viable controls which were predicted to contain no non-viable cysts. It has been found that *Giardia* cysts are less robust, and sometimes die during handling and experimentation (Medema et al, 1998). To control for the proportion of the purchased Giardia cysts were nonviable, initial viable control counts for SytoX and PI stained cysts were subtracted from the results of samples predicted to be 50% inactivated (Figure 12, panel C). Subtraction of viable control counts from the inactivated test samples resulted in relative proportions of viable:nonviable cysts that were closer to predicted values (Figure 12, panel C). Only two slightly significant differences ($0.5 > p < 0.01$) were found between SytoX and PI, both at low dye concentrations, one at 20 min incubation with 1µL dye and the other after 5 days incubation with 1µL dye + 9µL DMSO where SytoX stained samples were lower than PI. Correction resulted in no significant differences between numbers of cysts staining nonviable with concentrations of PI with/without DMSO at any incubation period. When cysts were stained with SytoX, 1µL SytoX and 1µL SytoX + 9µL DMSO were not significantly

different at any incubation time point, nor were higher concentrations; 5 µL, 5µL SytoX + 5µL DMSO, and 10 µL; significantly different from each other. However, the lower concentrations were significantly lower than the higher dye concentrations at each time point.

3.6 Parasite Detection in Complex Matrices

After completion of viability dye analysis, detection of oocysts/cysts was tested in complex matrices of manure and raspberries. Sample preparation and oocysts/cyst purification was slightly different for manure and berries, manure requiring a flotation method to extract oocysts/cysts from samples while berries employed a method that concentrated all of the smaller particles in samples into a smaller volume. A larger amount of debris was found in manure samples (Figure 13B), which required acquisition of 1,000,000 events compared to raspberries (Figure 13C) requiring acquisition of 100,000 events. The sensitivity of sampling parasites was also higher in raspberries compared to that of manure (Figures 14 and 15, respectively). A 10-fold higher weight of raspberries (50g) compared to manure (5g) per sample was inoculated with the same amount of oocysts/cysts. Therefore the concentration of oocysts/cysts was approximately 25,000 (oo)cysts/g and 250,000 (oo)cysts/g in raspberry and manure samples (respectively).

It should be noted that oocyst/cyst detection was also tested and possible in strawberry, blackberry and blueberry samples. Experiments were only continued with one berry type (raspberries) as concentration method and results were similar in all berries.

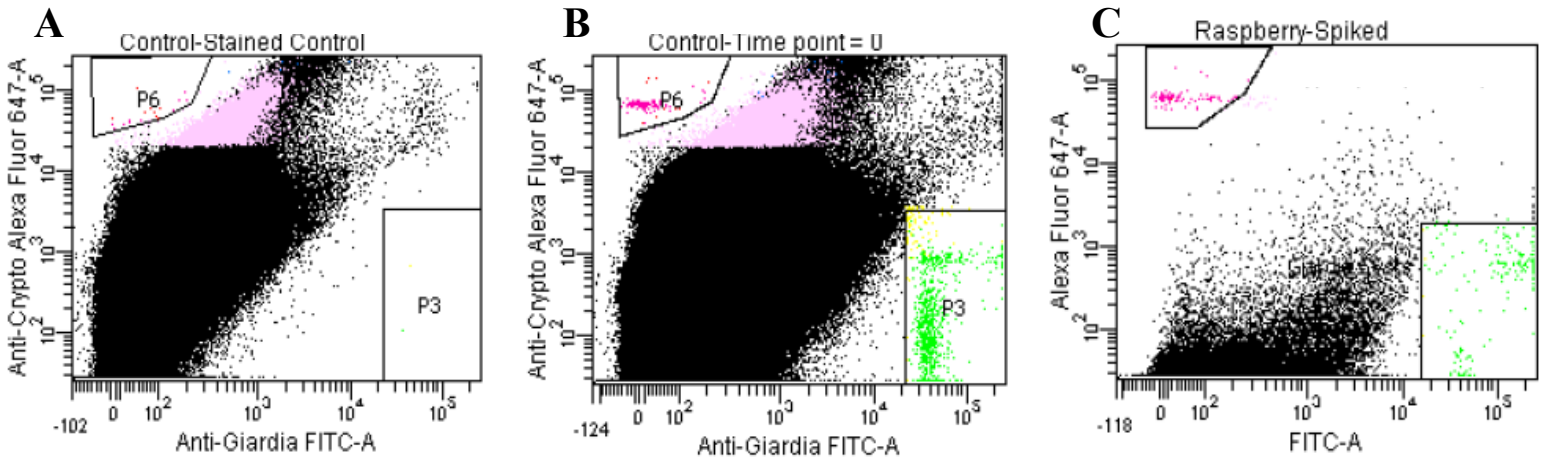

A
Control-Stained Control
P6
P3
Anti-Crypto Alexa Fluor 647-A
Anti-Giardia FITC-A
-102
B
Control-Time point = 0
P6
P3
Anti-Crypto Alexa Fluor 647-A
Anti-Giardia FITC-A
-124
C
Raspberry-Spiked
Alexa Fluor 647-A
FITC-A
-118

Figure 13. Flow cytometry dot plots of *Cryptosporidium* oocysts and *Giardia* cysts in manure and raspberries. Oocyst/cyst samples were recovered from manure via flotation in sucrose. Manure and raspberry samples were spiked with various amounts of pure oocysts/cyst suspensions. Oocysts/cysts were then extracted using sucrose flotation method (manure) or a concentration method with pectinase (berries). 50uL samples were stained with specific monoclonal antibodies conjugated AlexaFluor 647 (AF647) and to fluorescein isothiocyanate (FITC) (specific to *Cryptosporidium* and *Giardia* respectively). Samples were analysed using BD LSRFortessa Flow Cytometer. X-axis represents fluorescent intensity of event(s) emitting in the 519nm wavelength range (FITC stained events). Y-axis represents fluorescent intensity of event(s) emitting in the 668nm wavelength range (AF647). (A) Control unspiked manure sample. (B) and (C) Cow manure and raspberry samples (respectively) spiked with *Cryptosporidium* and *Giardia* oocysts/cysts. Black events represent background debris found in samples. Pink events are *Cryptosporidium* oocysts as determined by AlexaFluor 647 staining. Green events are *Giardia* as determined by FITC staining.

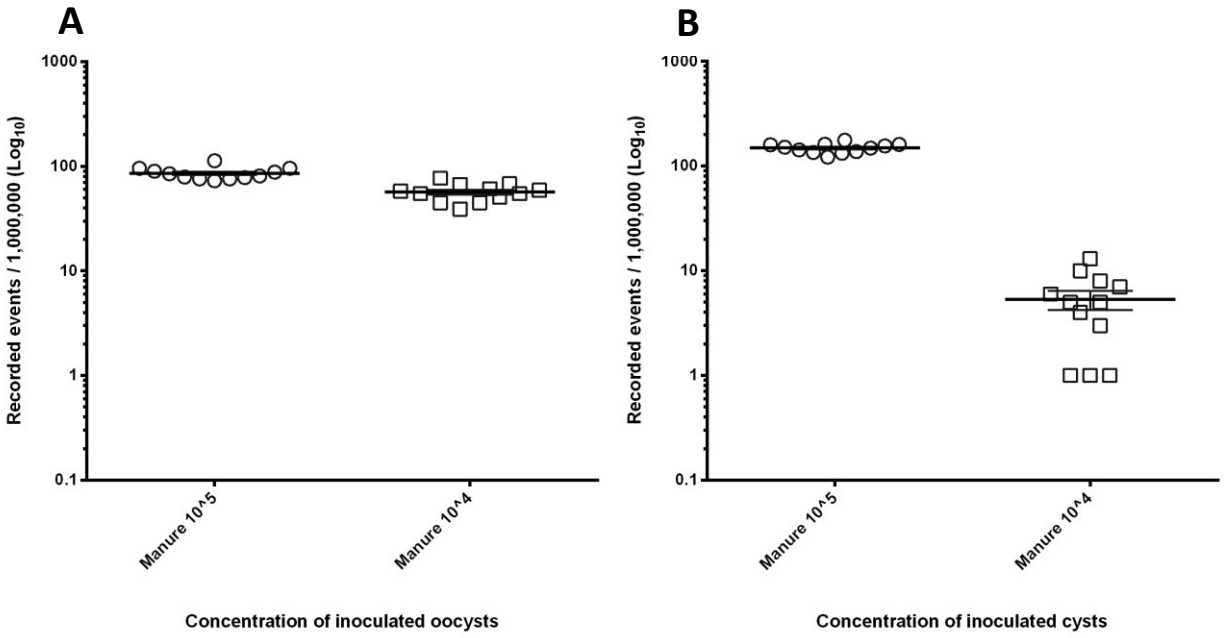

A
B
1000
100
10
1
0.1
Recorded events / 1,000,000 (Log10)
Manure 10^5
Manure 10^4
Concentration of inoculated oocysts
Concentration of inoculated cysts

Figure 14. Sensitivity of flow cytometry for detecting (A) *Cryptosporidium* oocysts and (B) *Giardia* cysts in cow manure. Manure samples were spiked with various concentrations of pure oocysts/cyst suspensions. Oocysts/cysts were then extracted using sucrose flotation method (manure). Fifty microliter samples were stained with specific monoclonal antibodies conjugated to AlexaFluor 647 (AF647) and fluorescein isothiocyanate (FITC) (specific to *Cryptosporidium* and *Giardia* respectively). Samples were analysed using BD LSRFortessa Flow Cytometer. X-axis represents inoculate oocyst/cyst concentration. Y-axis represents number of detected oocysts/cysts. Mean values of event counts from replicate experiments are shown with standard error. n = 12.

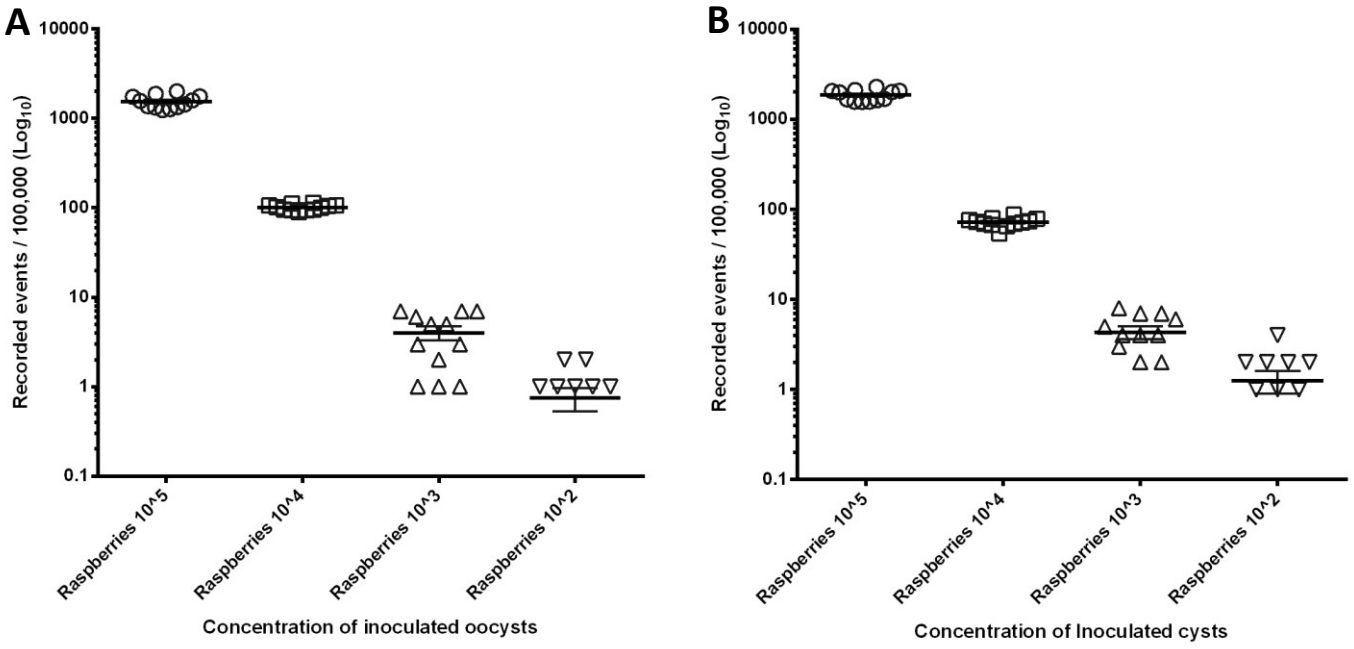

A
10000
1000
100
10
1
0.1
Recorded events / 100,000 (Log10)
Raspberries 10^5
Raspberries 10^4
Raspberries 10^3
Raspberries 10^2
Concentration of inoculated oocysts
B
10000
1000
100
10
1
0.1
Recorded events / 100,000 (Log10)
Raspberries 10^5
Raspberries 10^4
Raspberries 10^3
Raspberries 10^2
Concentration of Inoculated cysts

Figure 15. Sensitivity of flow cytometry for detecting (A) *Cryptosporidium* oocysts and (B) *Giardia* cysts in raspberries. Raspberry samples were spiked with various concentrations of pure oocysts/cyst suspensions. Oocysts/cysts were then extracted using a concentration method for berry samples. Fifty microliter samples were stained with specific monoclonal antibodies conjugated to AlexaFluor 647 (AF647) and fluorescein isothiocyanate (FITC) (specific to *Cryptosporidium* and *Giardia* respectively). Samples were analysed using BD LSRFortessa Flow Cytometer. X-axis represents inoculate oocyst/cyst concentration. Y-axis represents number of detected oocysts/cysts. Mean values of event counts from replicate experiments are shown with standard error. n = 12.

3.7 Characteristics of Manure

The chemical properties of both chicken and cow manure samples used in this study were determined before experimentation (Tables 7 and 8). According to these results the manure is in a reduced state with limited oxygen.

Table 6. Chemical characteristics of cow manure.

Physiochemical Property	Sample Replicate				Interpretation
	1	2	3	Average	
pH	7.47	7.52	7.63	7.54	- consistent with literature
Water Activity (T= 26.4°C) (availability of water, a_w)	1	0.998	1	0.999	- a_w pure water = 1.0 - a_w <0.85 = suppresses growth of most organisms - a_w 0.90 = minimum for most organisms
% Moisture	83.33	83.39	89.35	85.356	- ideal = 40 – 60%
Dissolved Oxygen (DO)	0.83	1.43	0.17	0.81	- depleted of oxygen - water saturated with DO has levels of 9-10
Redox Potential (E_h)	-45	-140.8	-69.2	-85	- E_h 100mV = poorly oxidized soil - E_h -200mV = much reduced soil - will support anaerobes
Electrical Conductivity (E.C.) (mS/cm)	2.119	2.036	2.26	2.138	- E.C. 1.8mS/cm to 23mS/cm = slightly salty - consistent with DO and E_h - not useful for crops
Average C:N*			26.8		

* samples were outsourced to EXOVA for C:N determination.

Table 7. Chemical characteristics of chicken manure.

Physiochemical Property	Sample Replicate				Interpretation
	1	2	3	Average	
pH	6.62	6.45	6.55	6.54	- consistent with literature
Water Activity (T= 26.4°C) (availability of water, a_w)	0.99	0.987	0.988	0.988	- a_w pure water = 1.0 - a_w <0.85 = suppresses growth of most organisms - a_w 0.90 = minimum for most organisms
% Moisture	70.59	71.37	70.11	70.69	- ideal = 40 – 60%
Dissolved Oxygen (DO)	0.14	0.1	0.11	0.116	- devoid of DO (more so than cow manure)
Redox Potential (E_h)	-381.5	-372.9	-389.2	-381.2	- E_h 200mV = much reduced soil - E_h -500mV = extremely reduced soil - will support anaerobes
Electrical Conductivity (E.C.) (mS/cm)	10.05	9.14	9.99	9.726	- E.C. 1.8mS/cm to 23mS/cm = slightly salty - consistent with DO and E_h - not useful for crops
Average C:N*			5.3		

* samples were outsourced to EXOVA for C:N determination.

3.8 D- and z- value results

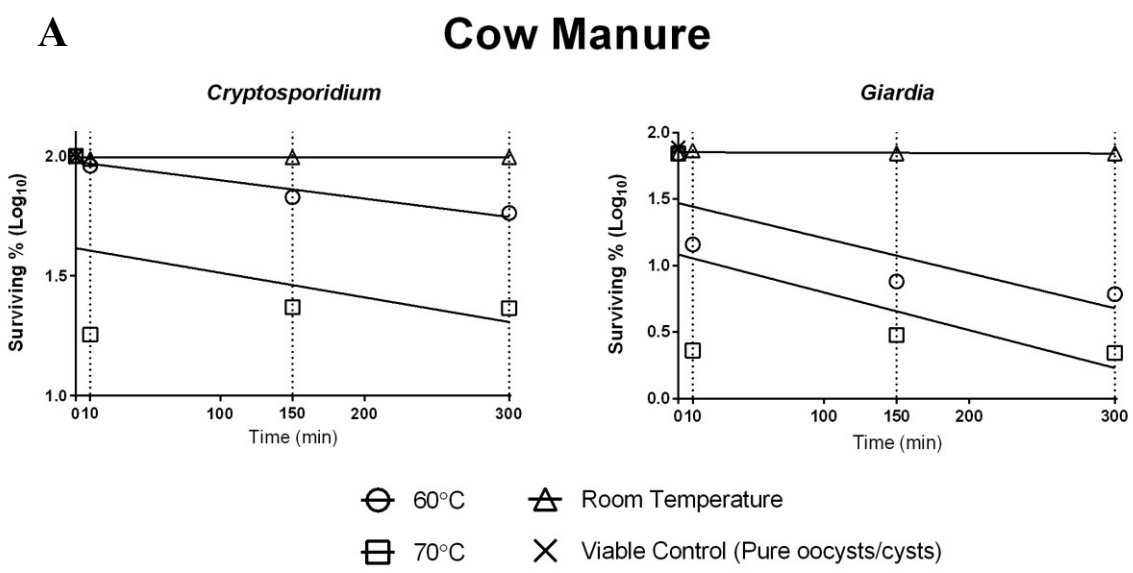
3.8.0 Thermal reduction of *Cryptosporidium* and *Giardia* oocysts/cysts in Cow and Chicken Manure

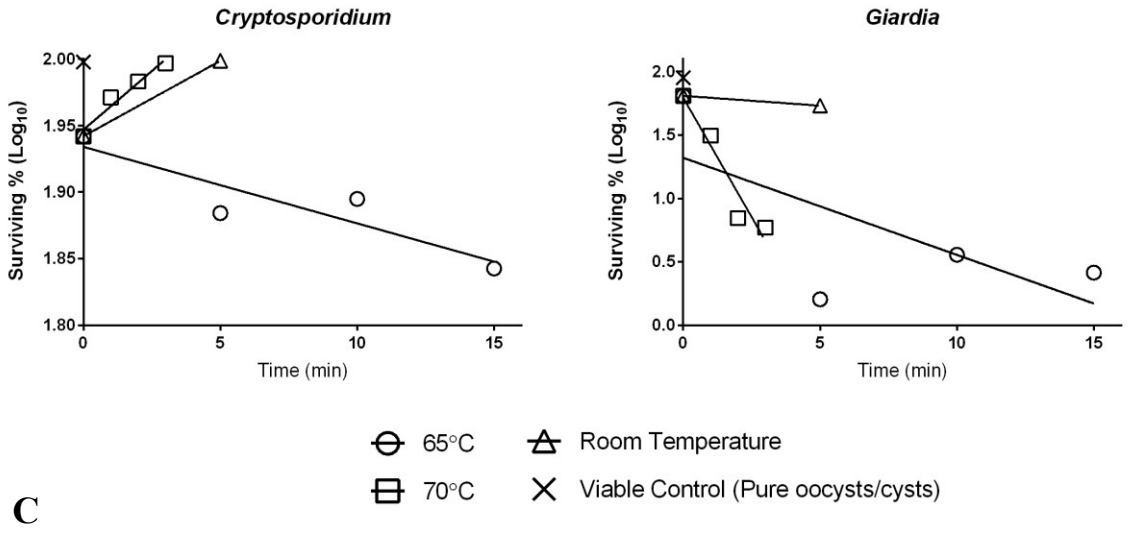
Following demonstration that viable *Cryptosporidium* and *Giardia* oocysts/cysts could be reliably detected in manure, experimentation was conducted to determine thermal reduction of these organisms. Due to time limitation, only a single trial to determine D-values of *Cryptosporidium* and *Giardia* in cow and chicken manure was attempted (Table 8). Both manure types were inoculated with oocysts and cysts, portioned into separate sterile sampling bags, and heated and sampled every 2.5hrs for each of six temperatures.

Thermal inactivation of *Cryptosporidium* in cow manure required longer time periods compared to *Giardia* as seen by the more gradual decline in the inactivation slope in Figure 16, Panel A. Results for *Cryptosporidium* inactivation in Chicken manure were uncertain as results appeared to show an increase in surviving percentage of *Cryptosporidium* over time at both 70°C and room temperature (Figure 16, Panel B). This error was still not corrected after attempts to correct the time 0 survival count (Figure 16, Panel C) as there was still an unexplained increase in survival percentage. *Giardia* cysts exhibited a sharp decline in survival at the higher temperature of 70°C in chicken manure, compared to a more steady decline at 65°C (Figure 16, Panel B).

Table 8. Thermal reduction of *Cryptosporidium parvum* and *Giardia duodenalis* in cow manure.

Manure Type	Species	Temperature (°C)	D_x value (x = temperature)	R2	Equation
Cow	*C. parvum*	60	1304.63 ± 188.56	0.946	y= -0.0007665x + 1.975
		70	974.65 ± 584.49	0.1822	y= -0.001026x + 1.616
	G. duodenalis	60	379.65 ± 137.63	0.6071	y= -0.002634x + 1.469
		70	351.86 ± 181.71	0.3048	y= -0.002842x + 1.083
Chicken	*C. parvum*	65	173.88 ± 42.58	0.8262	y= -0.005751x + 1.934
		70	-56.5 ± 10.67	0.9521	y= 0.01767x + 1.947
	G. duodenalis	65	13.04 ± 5.61	0.4675	y= -0.07667x + 1.321
		70	2.65 ± 0.44	0.9267	y= -0.3770x + 1.796


A
Cow Manure
Cryptosporidium
Giardia
Surviving % (Log10)
Time (min)
60°C
70°C
Room Temperature
Viable Control (Pure oocysts/cysts)


B
Chicken Manure
Cryptosporidium
Giardia
Surviving % (Log10)
Time (min)
65°C
70°C
Room Temperature
Viable Control (Pure oocysts/cysts)

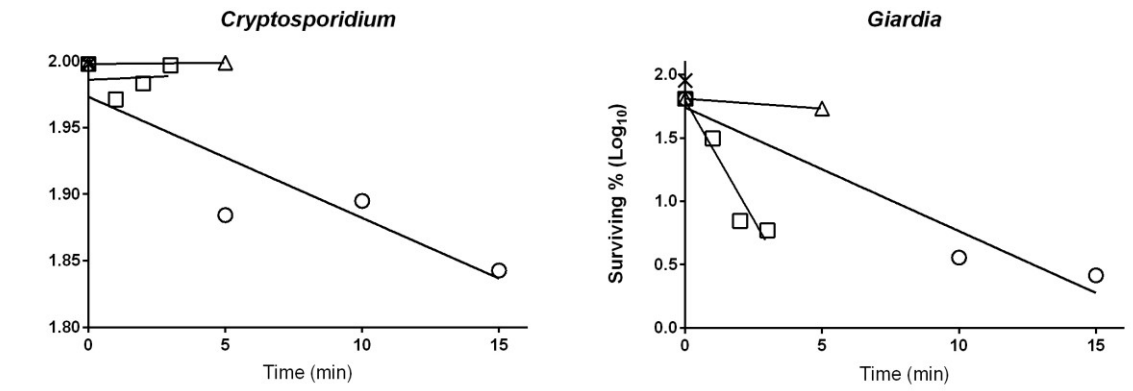

C
Cryptosporidium
Giardia
Surviving % (Log10)
Time (min)

Figure 16. Thermal reduction of *Cryptosporidium parvum* and *Giardia duodenalis* in sterile peptone water in cow manure (Panel A) and chicken manure (Panel B). Panel C shows results of inactivation in Chicken manure where starting viability counts were corrected. Manure samples were spiked with pure oocysts/cysts. Sample bags were placed in pre-heated water baths at 60°C, 65°C and 70°C. Oocysts/cysts were extracted using sucrose flotation method. Fifty microliter samples were stained with specific monoclonal antibodies conjugated to AlexaFluor 647 (AF647) and to fluorescein isothiocyanate (FITC) (specific to *Cryptosporidium* and *Giardia* respectively). Samples were analysed using BD LSRFortessa Flow Cytometer. X-axis represents time (min) samples were heated for. Log_{10} values of percentage viable oocysts/cysts are shown. n = 1. (Slopes and R^2 values are listed in Table 8).

The data obtained from the first trial for *Cryptosporidium* and *Giardia* D-values does not show a reliable decline in viability of oocysts/cysts over time for any temperature, displaying an increase in some cases as seen with *Cryptosporidium* in chicken manure (Figure 16, Panel B). After further literature review it was apparent that extensive experimentation using pure oocysts/cyst samples would be required for methodology development in order to find D-values in pure solution before investigating complex media. Due to time and resource limitations, further trials to determine thermal reduction of protists in manure were not conducted.

3.9 Transformation of heat resistant *E. coli* AW1.7 with GFP plasmid

Transformation of the extremely heat resistant *E. coli* AW1.7 with the pGFPuv plasmid (Clontech^{TM}, Mountain View, CA, USA) was unsuccessful. Experiment was attempted in triplicate using heat-shock (in a solution of $CaCl_2$) at 42°C which is the standard temperature in heat-transformation protocols for *E.coli,* as well as 52°C as *E. coli* AW1.7 is heat tolerant and it was theorized that a higher temperature may be required for effective permeation. Control samples containing no pGFPuv were also tested in triplicate. Unfortunately neither the heat shock at 42°C nor 52°C (nor controls) resulted in growth on ampicillin containing Luria-Bertani plates (LB +amp) indicating unsuccessful uptake of the pGFPuv plasmid (data not shown).

3.10 Preliminary Thermal Trials of Bacterial Pathogens in Bovine and Chicken Manure

Preliminary studies were conducted using bacterial pathogens in order to select media that would allow quick and accurate enumeration of surviving colony forming units (CFU), as well as to estimate possible survival time(s) of the pathogens. For these experiments the

bacterial species being studied were tested two at a time (ex, *Campylobacter* and *E.coli* at one time, *Salmonella* and *Listeria* another) by inoculating concentrations of approximately 10^9 CFU/g of each species into100g of manure, and placed at 60°C in a water bath for 48hrs. Colonies were detected for each pathogen, even after incubation for 48 hrs, indicating that pathogens could survive for up to 48hrs at high temperatures in the manure matrix (Figure 17). *C. jejuni* and *E. coli* O157:H7 appeared to be the most susceptible to the heat, with *S.* Typhimurium surviving the longest (Figure 17).

The first trial to determine the thermal reduction times of these bacteria in manure was based on the preliminary experiment, where large quantities of manure were inoculated with all species simultaneously and portioned into 25g bags. Duplicate samples of both chicken and cow manure were then heated at each of five temperatures; 50°C, 55°C, 60°C, 65°C, and 70°C; as well as room temperature (25°C) as a control. Other than initial inoculation counts and room temperature controls (Figure 18) no growth was observed for any of the pathogens at 24, 48 or 72 hours for *Salmonella, E. coli,* and *Listeria,* nor the 8, 16 and 24 hour time points for *C. jejuni*.

The results from this initial trial revealed that while the manure matrix was not inhibitory to the survival of the bacterial pathogens at room temperature (Figure 18), pathogens were rapidly killed following heat exposure and did not survive past 48hrs as the preliminary trials had suggested. Note that a significant decline in viable *Campylobacter* and *Listeria* was observed at room temperature. The fact that the controls still exhibited growth allowed revision of temperatures and time points for sampling, to develop a sampling plan for measuring the thermal reduction of bacterial pathogens in compost.

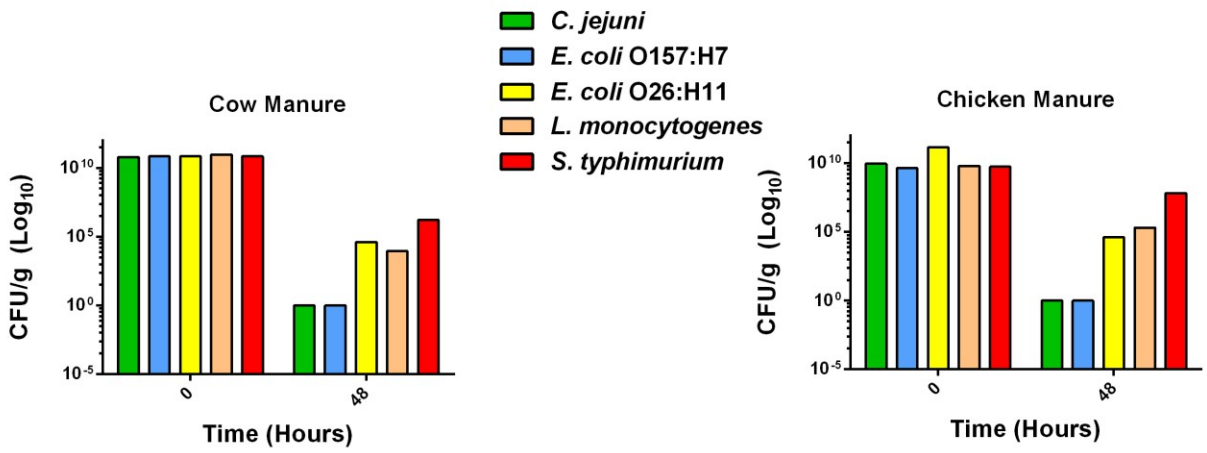

C. jejuni
E. coli O157:H7
E. coli O26:H11
L. monocytogenes
S. typhimurium
Cow Manure
Chicken Manure
CFU/g (Log10)
Time (Hours)
0
48

Figure 17. Preliminary study of pathogen survival in cow and chicken manure at 60°C. Approximately 10^9 CFU/g of each bacterial pathogen were inoculated into 100g of cow and chicken manure in a stomacher bag. Bag was closed and placed in water bath at 60°C for 48 hours. Following heating, buffered peptone water was added and bag(s) were manually massaged for 2 min, and stomached at 150 rpm for 3 min. Rinse was retrieved from the filtered side of the stomacher bag and plated on selective media. Mean $\log_{10}$ values of CFU counts are shown. n = 1.

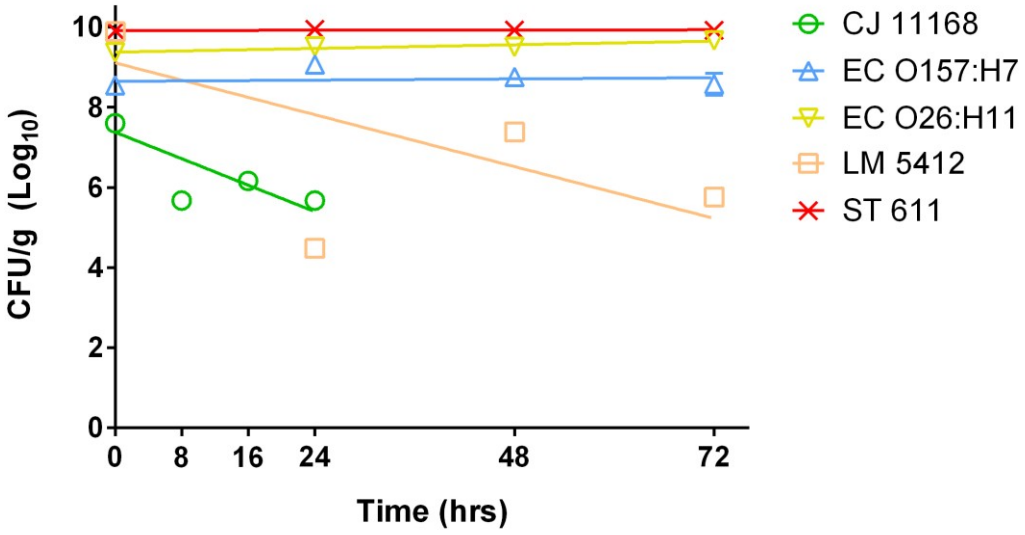

CFU/g (Log10)
10
8
6
4
2
0
0
8
16
24
48
72
Time (hrs)
CJ 11168
EC O157:H7
EC O26:H11
LM 5412
ST 611

Figure 18. Survival of pathogens in cow manure at RT. One litre of each *Campylobacter jejuni* (CJ 11168), *Escherichia coli* O157:H7 (ECO157:H7), *Escherichia coli* O26:H11 (EC O26:H11), *Listeria monocytogenes* (LM 5412) and *Salmonella enterica* serovar Typhimurium (ST 611) were grown in broth 24 hours prior to experimentation. Bacteria were pelleted and resuspended to a combined 15mL peptone water. Six hundred grams of cow manure was inoculated with combined bacterial cocktail. Manure was separated into 25g coupons, and three bags were placed in a water bath pre-heated to 25°C. Coupons were removed every 24 hours (every 8 hrs for CJ 11168), serially diluted and plated on selective media. Mean Log_{10} values of CFU counts from replicate experiments are shown. n = 6 (time 0) or n = 2 (subsequent time points).

3.11 D- value Determination of *Campylobacter jejuni* in sterile peptone water

Due to rapid decline of *Campylobacter jejuni* relative to the other pathogens, further analyses were conducted to compare resistance of CJ 11168 to heat. Using CJ 11168 as an initial reference strain, thermal inactivation studies were carried out at 52.5°C, 55°C, and room temperature in sterile Mueller Hinton (MH) broth. D-values for *Campylobacter* at these temperatures were low (Table 9), only requiring approximately 2 minutes at 52.5°C for a 10% population reduction, and less than 1 minute at 55°C (Figure 19). Due to the rapid decline of *C. jejuni* in pure solution at temperatures that were at the lower end of our sampling temperatures (52.5°C, 55°C), and preliminary results being low relative to other pathogens, it was unlikely that *Campylobacter jejuni* would outlive other bacterial pathogens through the composting process thus this pathogen was dropped in further experiments.

Table 9. D-values of *Campylobacter jejuni* 11168 in PBS.

Pathogen	Temperature (°C)	D value	R2	Equation
Campylobacter jejuni 11168	52.5	2.53 ± 0.059	0.9983	y= -0.3940x + 8.145
	55	0.78 ± 0.083	0.9464	y= -1.287x + 7.679

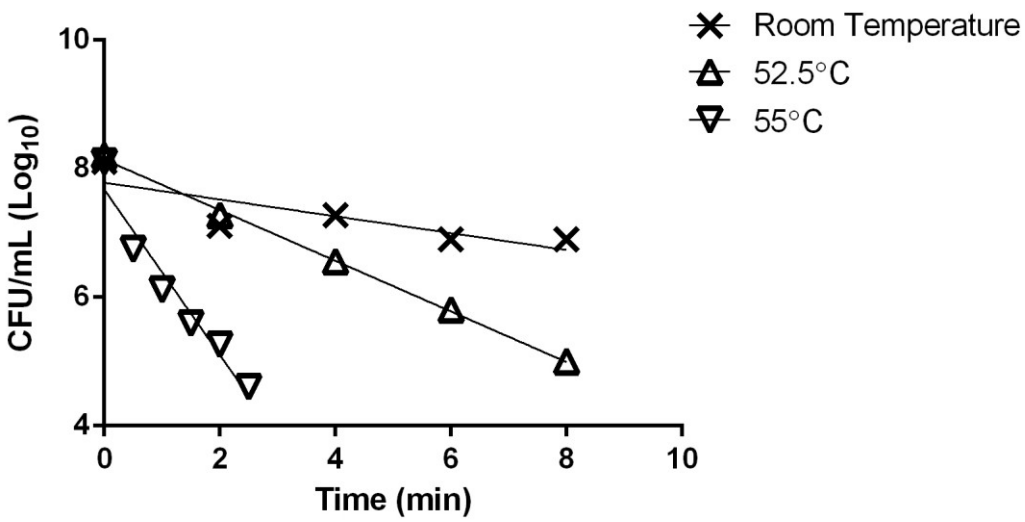

Room Temperature
52.5°C
55°C
CFU/mL (Log10)
10
8
6
4
0
2
4
6
8
10
Time (min)

Figure 19. Thermal reduction of *Campylobacter jejuni* 11168 in sterile Mueller Hinton broth. *C. jejuni* strains were grown on Mueller Hinton agar, retrieved, pelleted and re-suspended to 10^9 CFU/mL (optical density of 1.9) in sterile Mueller Hinton (MH) broth. Tubes of MH broth were pre-heated to desired temperatures; 25.0°C, 52.5°C, and 55°C. One milliliter of bacterial suspension was added to preheated tube(s) and left for a pre-determined time interval. Tubes were removed from bath, placed on ice, and 1mL was retrieved and serial dilutions were plated on selective agar. Log_{10} values of CFU are shown. n = 1. (Slopes and R^2 values are listed in Table).

3.12 Thermal reduction of Bacterial Pathogens in Bovine and Chicken Manure

The decimal reduction (D-) values of the bacterial pathogens *L. mono* 5412 (LM 5412), *S. enterica* serovar Typhimurium 611 (ST 611), *S. enterica* serovar Senftenberg 723/775W (SS 775W), *E. coli* O157:H7 C0283 (EC O157:H7), and *E. coli* O26:H11 (EC O26:H11) 02-6737 in bovine and chicken manure were investigated. Temperatures used in this study were 25°C, 50°C, 52.5°C, 55°C, 57.5°C, and 60°C. The temperatures were chosen in the range of 50°C to 60°C because current standards for composting require a minimum temperature of 55°C, thus this range allowed us to investigate results of temperatures in the range of this standard. The 25°C (room temperature) served as a control to ensure death was thermal and not an immediate reaction to the manure matrix.

Overnight cultures were centrifuged and resuspended in 0.1% peptone water. Concentrated cultures were blended with manure at a ratio of 1mL bacterial culture/ 1g of manure. Linear regression was established for each bacterial pathogen and temperature combination. The D-values are calculated as the inverse negative of the slope of the linear regression line of mean values of replicate experiments (Figures 21, 22, 23). D-values are reported in minutes (Table 10 and 12). D-values for the 25°C control temperature were not calculated, as experiments were not conducted for long enough time periods to make an accurate estimate of reduction time at this temperature.

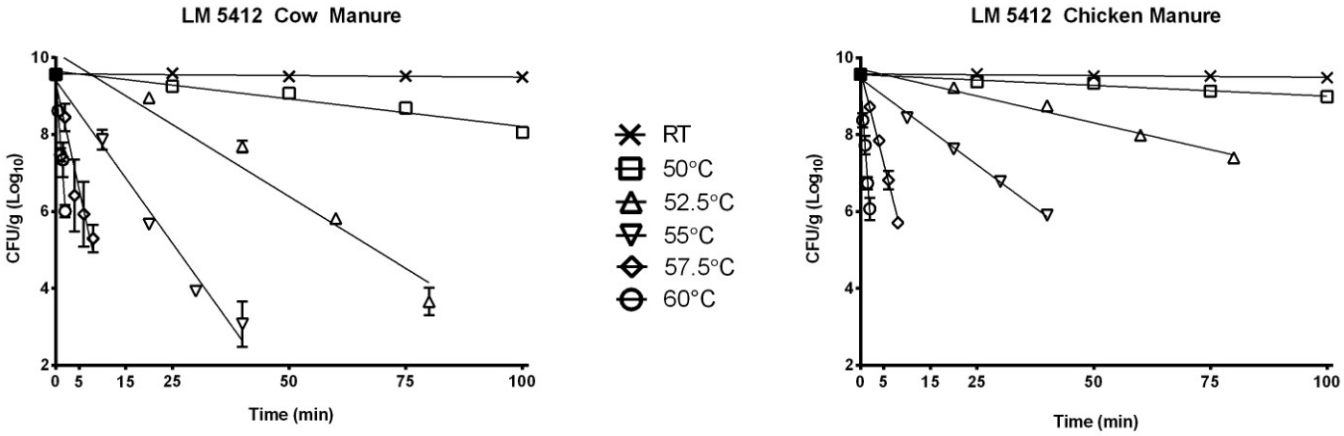

LM 5412 Cow Manure
CFU/g (Log10)
Time (min)
RT
50°C
52.5°C
55°C
57.5°C
60°C
LM 5412 Chicken Manure
CFU/g (Log10)
Time (min)

Figure 20. Thermal reduction of *Listeria monocytogenes* 5412 in cow and chicken manure matrices. *L. mono* was cultured in 1L of broth 24 hours before experimentation. Inoculum was pelleted and resuspended in 150mL of sterile peptone water. One hundred and fifty grams of cow or chicken manure was mixed with the resuspended *L. mono* using a blender. Sample was split into 10g coupons in stomacher bags, and bags were sealed. Coupons were placed in pre-heated water baths at 25.0°C, 55.0°C, 52.5°C, 55.0°C, 57.5°C, and 60.0°C. Samples were removed at different time intervals, serially diluted and plated on selective media. Mean Log_{10} values of CFU counts from replicate experiments are shown with standard error. n = 3. (Slopes and R^2 values are listed in Tables 10 and 11).

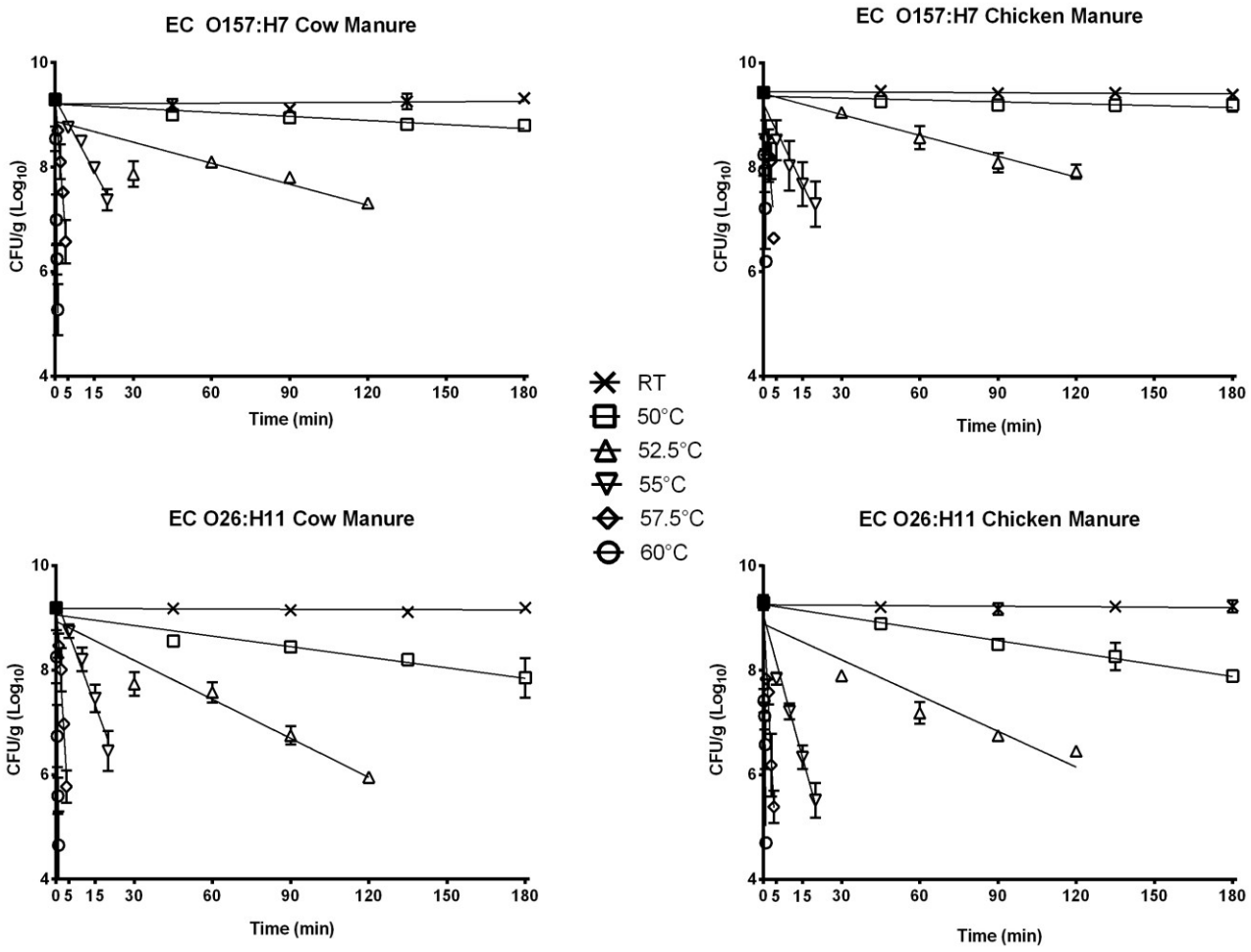

EC O157:H7 Cow Manure
EC O157:H7 Chicken Manure
EC O26:H11 Cow Manure
EC O26:H11 Chicken Manure
CFU/g (Log10)
Time (min)
RT
50°C
52.5°C
55°C
57.5°C
60°C

Figure 21. Thermal reduction of *Escherichia coli* O157:H7 and O26:H11 in cow and chicken manure matrices. EC O157:H7 and EC O26:H11 were each cultured in 500mL of broth 24 hours before experimentation. Inoculum were pelleted, and resuspended mixed together in 150mL of sterile peptone water. One hundred and fifty grams of cow or chicken manure was mixed with the resuspended *E. coli* using a blender. Sample was split into 10g coupons in stomacher bags, and bags were sealed. Coupons were placed in pre-heated water baths at 25.0°C, 55.0°C, 52.5°C, 55.0°C, 57.5°C, and 60.0°C. Samples were removed at different time intervals, serially diluted and plated on selective media. Graphs display each pathogen separately in each manure type. Mean Log_{10} values of CFU counts from replicate experiments are shown with standard error. n = 2 or 3. (Slopes and R^2 values are listed in Tables 10 and 11)

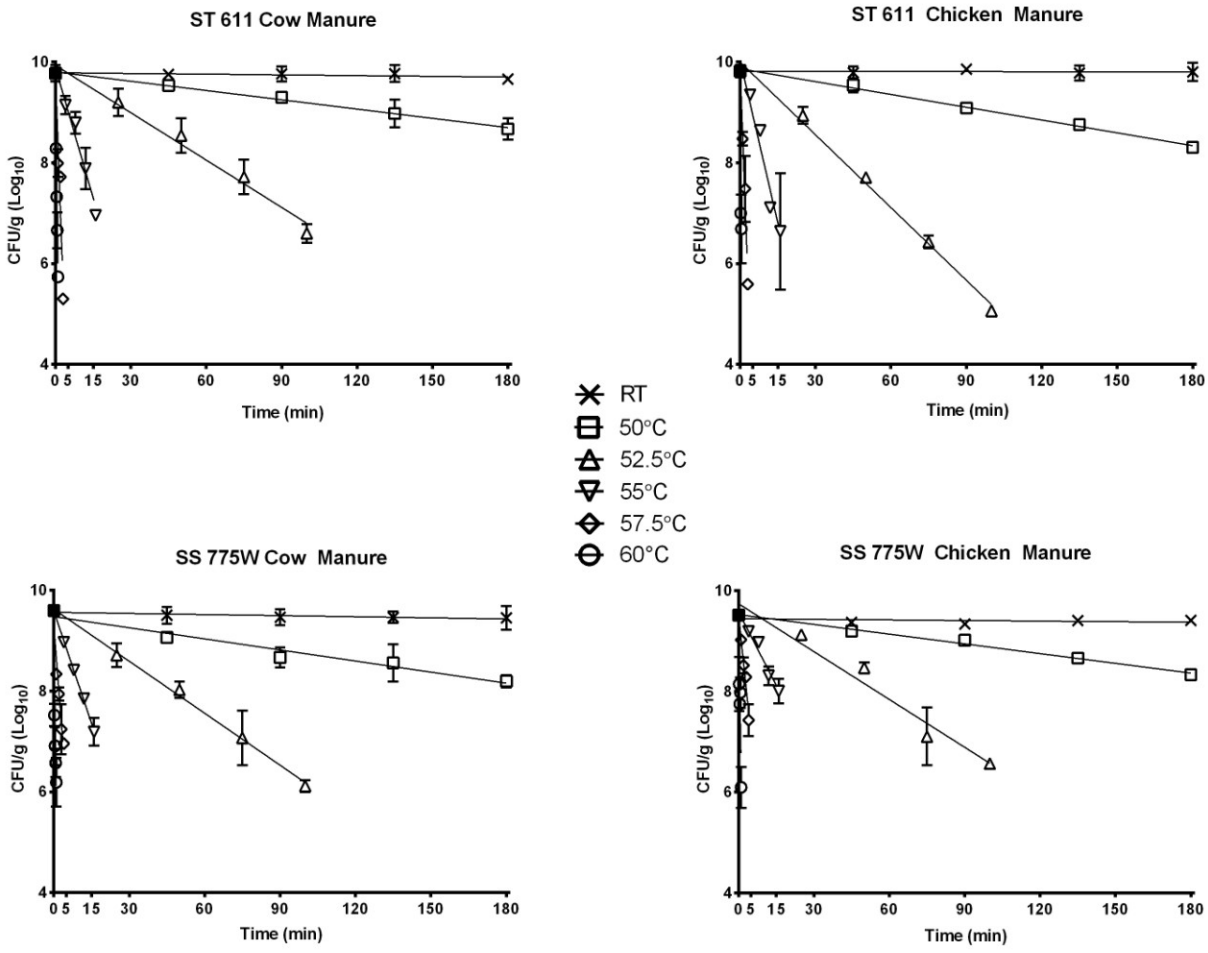

ST 611 Cow Manure
ST 611 Chicken Manure
SS 775W Cow Manure
SS 775W Chicken Manure
CFU/g (Log10)
Time (min)
RT
50°C
52.5°C
55°C
57.5°C
60°C

Figure 22. Thermal reduction of *Salmonella enterica* serovar Typhimurium 611 and *Salmonella enterica* serovar Senftenberg 775W in chicken and cow manure. *Salmonella* strains were tested on separate days. ST 611 or SS 775W was cultured in 1L of broth 24 hours before experimentation. Inoculum was pelleted and resuspended in 150mL of sterile peptone water. One hundred and fifty grams of cow or chicken manure was mixed with the resuspended *Salmonella* using a blender. Sample was split into 10g coupons in stomacher bags, and bags were sealed. Coupons were placed in pre-heated water baths at 25.0°C, 55.0°C, 52.5°C, 55.0°C, 57.5°C, and 60.0°C. Samples were removed at different time intervals, serially diluted and plated on selective media. Graphs display each pathogen separately in each manure type. Mean Log_{10} values of CFU counts from replicate experiments are shown with standard error. n = 3. (Slopes and R^2 values are listed in Tables 10 and 11)

Table 10. D-values of bacteria in cow manure.

Manure Type	Bacteria Strain	T (°C)	D-value (min) ± SE	R2	D-value Equation
Cow	ST 611	50.0	163.026 ± 7.231	0.9953	y= -0.006134x + 9.082
		52.5	31.918 ± 0.879	0.9815	y= -0.03133x + 9.933
		55.0	5.811 ± 0.117	0.9728	y= -0.1721x + 9.888
		57.5	0.973 ± 0.059	0.9243	y= -1.369x + 9.570
		60.0	0.258 ± 0.029	0.9783	y= -3.880x + 9.496
	SS 775W	50.0	136.370 ± 4.097	0.9558	y= -0.007333x + 9.476
		52.5	29.087 ± 1.995	0.9969	y= -0.03438x + 9.624
		55.0	6.739 ± 0.553	0.9991	y= -0.1484x + 9.590
		57.5	1.572 ± 0.196	0.9386	y= -0.6363x + 9.288
		60.0	0.322 ± 0.035	0.8354	y= -3.105x + 8.911
	EC O157:H7	50.0	384.763 ± 102.446	0.866	y= -0.002599x + 9.206
		52.5	74.516 ± 4.081	0.7415	y= -0.01342x + 8.880
		55.0	10.834 ± 1.511	0.9853	y= -0.09230 + 9.304
		57.5	1.510 ± 0.125	0.989	y= -0.6623x + 9.363
		60.0	0.247 ± 0.023	0.9868	y= -4.138x + 9.338
	EC O26:H11	50.0	148.280 ± 32.654	0.9375	y= -0.006744x + 9.058
		52.5	40.161 ± 0.404	0.9501	y= -0.02490x + 8.936
		55.0	7.386 ± 1.010	0.9726	y= -0.1354x + 9.366
		57.5	1.198 ± 0.194	0.971	y= -0.8348x + 9.353
		60.0	0.213 ± 0.018	0.9936	y= -4.701x + 9.238
	LM 5412	50.0	69.589 ± 2.34	0.9488	y= -0.01437x + 9.646
		52.5	13.373 ± 0.775	0.9574	y= -0.07478x + 10.13
		55.0	5.910 ± 0.571	0.9821	y= -0.1692x + 9.404
		57.5	1.809 ± 0.283	0.9417	y= -0.5527x + 9.342
		60.0	0.596 ± 0.0195	0.9623	y= -1.679x + 9.479

Table 11. D-values in chicken manure.

Manure Type	Bacteria Strain	T (°C)	D-value (min) ± SE	R2	D-value Equation
Chicken	ST 611	50.0	118.0498 ± 0.839	0.9944	y= -0.008471x + 9.863
		52.5	20.786 ± 1.664	0.9943	y= -0.04811x + 9.996
		55.0	4.647 ± 1.825	0.9609	y= -0.2152x + 10.03
		57.5	0.731 ± 0.188	0.984	y= -1.368x + 9.894
		60.0	0.1597 ± 0.029	0.8237	y= -6.260x + 9.401
	SS 775W	50.0	155.255 ± 10.973	0.9903	y= -0.006441x + 9.524
		52.5	31.596 ± 5.168	0.9619	y= -0.03165x + 9.740
		55.0	10.277 ± 1.189	0.9713	y= -0.09730x + 9.579
		57.5	2.038 ± 0.377	0.9707	y= -0.4906x + 9.536
		60.0	0.357 ± 0.042	0.8251	y= -2.8011x + 9.299
	EC O157:H7	50.0	832.639 ± 196.589	0.6709	y= -0.001201x + 9.360
		52.5	75.188 ± 5.882	0.9816	y= -0.01330x + 9.409
		55.0	9.766 ± 2.300	0.9545	y= -0.1024x + 9.212
		57.5	1.660 ± 0.603	0.8883	y= -0.6023x + 9.403
		60.0	0.334 ± 0.119	0.9702	y= -2.998x + 9.302
	EC O26:H11	50.0	130.668 ± 16.359	0.9923	y= -0.007653x + 9.259
		52.5	43.821 ± 0.765	0.9083	y= -0.02282x + 8.887
		55.0	5.501 ± 1.175	0.9807	y= -0.1818x + 9.058
		57.5	1.055 ± 0.140	0.9693	y= -0.9481x + 9.155
		60.0	0.249 ± 0.038	0.9209	y= -4.018x + 9.034
	LM 5412	50.0	178.603± 20.851	0.9686	y= -0.005599x + 9.563
		52.5	35.855 ± 1.474	0.9816	y= -0.02789x + 9.704
		55.0	11.1198 ± 0.212	0.9963	y= -0.08993x + 9.463
		57.5	2.078 ± 0.205	0.9964	y= -0.4813x + 9.660
		60.0	0.579 ± 0.053	0.99	y= -1.727x + 9.424

LM 5412 had significantly higher D-values in chicken manure compared to cow manure at 50°C, 52.5°C, and 55°C, but not at the two higher temperatures 57.5°C and 60°C where no significant differences (s.d.) were observed (Figure 23; Table 12). No other pathogen showed significant differences between D-values in the different manure types at 50°C, 57.5°C or 60°C. ST 611 had a significantly higher D-value in cow manure than chicken manure at 52.5°C (Figure 23; Table 12). EC O26:H11 had a significantly higher D-value in cow manure compared to chicken manure at 55°C, whereas SS 775W showed a significantly lower D-values in cow manure compared to chicken at that temperature (Figure 23; Table 12).

At 50°C the D-value for LM 5412 in cow manure was significantly lower than the other four bacteria and the D-value for EC O157:H7 was significantly higher than the other four (Table 13, Figure 23). EC O26:H11, ST 611, and SS 775W all had comparable D-values in cow manure at this temperature. In chicken manure EC O157:H7 again had a significantly higher D-value than the other four pathogens (Table 14, Figure 23). LM 5412, EC O26:H11, and SS 775W had similar D-values (not significantly different), whereas ST 611 was significantly different than all of the bacteria except for O26:H11.

At 52.5°C LM 5412 D-value in cow manure was significantly lower than all other pathogens, and EC O157:H7 D-value in cow manure was significantly higher than all other pathogens. EC O26:H11 and ST 611 D-values were comparable, as were ST 611 and SS 775W in cow manure. SS 775W and EC O26:H11 were significantly different (Table 13, Figure 23). In chicken manure D-value for EC O157:H7 was significantly higher than all other pathogens. LM 5412 and SS 775W were not significantly different in chicken manure,

Table 12. Statistical comparison of chicken versus cattle manure for each pathogen.

Bacteria Strain	T (°C)	D- values compared	p-value	Significant*
ST 611	50.0	cow vs chicken	0.0739	no
	52.5	cow vs chicken	0.0002	yes
	55.0	cow vs chicken	0.283	no
	57.5	cow vs chicken	0.3552	no
	60.0	cow vs chicken	0.0915	no
SS 775W	50.0	cow vs chicken	0.5014	no
	52.5	cow vs chicken	0.5856	no
	55.0	cow vs chicken	0.0019	yes
	57.5	cow vs chicken	0.1124	no
	60.0	cow vs chicken	0.7132	no
EC O157:H7	50.0	cow vs chicken	0.0709	no
	52.5	cow vs chicken	0.9686	no
	55.0	cow vs chicken	0.6704	no
	57.5	cow vs chicken	0.4675	no
	60.0	cow vs chicken	0.1101	no
EC O26:H11	50.0	cow vs chicken	0.514	no
	52.5	cow vs chicken	0.5226	no
	55.0	cow vs chicken	0.0249	yes
	57.5	cow vs chicken	0.3604	no
	60.0	cow vs chicken	0.2661	no
LM 5412	50.0	cow vs chicken	<0.0001	yes
	52.5	cow vs chicken	<0.0001	yes
	55.0	cow vs chicken	<0.0001	yes
	57.5	cow vs chicken	0.3872	no
	60.0	cow vs chicken	0.81	No

* $p<0.05$ = significant difference

nor were EC O26:H11 and SS 775W. ST 611 D-value was significantly lower than the other four pathogens in chicken manure (Table 14, Figure 23).

At 55°C the EC O157:H7 D-value in cow manure was significantly higher than all of the other four pathogens which had similar D-values (Table 13, Figure 23). In chicken manure D-values for LM 5412, EC O157:H7, and SS 775W were not s.d.. D-values for EC

O26:H11 and ST 611 were s.d. from each other and lower than the other three pathogens (Table 14, Figure 23).

At 57.5°C there were no significant differences between pathogen D-values in cow manure (Table 13, Figure 23). There were however differences in D-values in chicken manure, with EC O26:H11 and ST 611 being comparable (no s.d.) but lower than LM 5412, EC O157:H7, and SS 775W (the three of which were not significantly different) (Table 14, Figure 23).

At 60°C the cow and chicken manure D-values for LM 5412 were significantly higher than all of the other four pathogens. The D-values for EC O157:H7, EC O26:H11, ST 611, and SS 775W in cow manure were not significantly different (Table 13). In chicken manure the D-values for EC O157:H7, EC O26:H11 and SS 775W were comparable, and the D-values for EC O157:H7, EC O26:H11 and ST 611 were comparable (Table 14, Figure 23). ST 611 and SS 775W D-values in chicken manure were significantly different (Table 14, Figure 23).

Table 13. Statistical comparison of bacterial species survival at each temperature in cow manure.

Manure Type	T (°C)	Species	Species	p-value*	Significant
Cow	50.0°C	ST 611	SS 775W	0.4681	no
		ST 611	LM 5412	<0.0001	yes
		ST 611	EC O157:H7	0.0037	yes
		ST 611	EC O26:H11	0.6692	no
		SS 775W	LM 5412	0.0007	yes
		SS 775W	EC O157:H7	0.001	yes
		SS 775W	EC O26:H11	0.7199	no
		LM 5412	EC O157:H7	<0.0001	yes
		LM 5412	EC O26:H11	0.0001	yes
		EC O157:H7	EC O26:H11	0.0013	yes
	52.5°C	ST 611	SS 775W	0.4822	no
		ST 611	LM 5412	<0.0001	yes
		ST 611	EC O157:H7	<0.0001	yes
		ST 611	EC O26:H11	0.1061	no
		SS 775W	LM 5412	<0.0001	yes
		SS 775W	EC O157:H7	<0.0001	yes
		SS 775W	EC O26:H11	0.0182	yes
		LM 5412	EC O157:H7	<0.0001	yes
		LM 5412	EC O26:H11	<0.0001	yes
		EC O157:H7	EC O26:H11	0.0017	yes
	55.0°C	ST 611	SS 775W	0.4239	no
		ST 611	LM 5412	0.9555	no
		ST 611	EC O157:H7	0.0009	yes
		ST 611	EC O26:H11	0.2853	no
		SS 775W	LM 5412	0.4789	no
		SS 775W	EC O157:H7	0.0002	yes
		SS 775W	EC O26:H11	0.5639	no
		LM 5412	EC O157:H7	0.0002	yes
		LM 5412	EC O26:H11	0.1223	no
		EC O157:H7	EC O26:H11	0.0152	yes
	57.5°C	ST 611	SS 775W	0.1572	no
		ST 611	LM 5412	0.3428	no
		ST 611	EC O157:H7	0.0914	no
		ST 611	EC O26:H11	0.4061	no
		SS 775W	LM 5412	0.673	no
		SS 775W	EC O157:H7	0.9281	no

		SS 775W	EC O26:H11	0.277	no
		LM 5412	EC O157:H7	0.6184	no
		LM 5412	EC O26:H11	0.1789	no
		EC O157:H7	EC O26:H11	0.1605	no
	60.0°C	ST 611	SS 775W	0.2407	no
		ST 611	LM 5412	<0.0001	yes
		ST 611	EC O157:H7	0.7441	no
		ST 611	EC O26:H11	0.2755	no
		SS 775W	LM 5412	0.0105	yes
		SS 775W	EC O157:H7	0.1515	no
		SS 775W	EC O26:H11	0.0539	no
		LM 5412	EC O157:H7	<0.0001	yes
		LM 5412	EC O26:H11	<0.0001	yes
		EC O157:H7	EC O26:H11	0.3796	No

* p<0.05 = significant difference

Table 14. Statistical comparison of bacterial species survival at each temperature in chicken manure.

Manure Type	T (°C)	Species/strain	Species/strain	p-value*	Significant
Chicken	50.0°C	ST 611	SS 775W	0.0163	yes
		ST 611	LM 5412	0.116	yes
		ST 611	EC O157:H7	<0.0001	yes
		ST 611	EC O26:H11	0.5181	no
		SS 775W	LM 5412	0.3288	no
		SS 775W	EC O157:H7	<0.0001	yes
		SS 775W	EC O26:H11	0.3095	no
		LM 5412	EC O157:H7	0.0002	yes
		LM 5412	EC O26:H11	0.1717	no
		EC O157:H7	EC O26:H11	<0.0001	yes
	52.5°C	ST 611	SS 775W	0.0015	yes
		ST 611	LM 5412	<0.0001	yes
		ST 611	EC O157:H7	<0.0001	yes
		ST 611	EC O26:H11	<0.0001	yes
		SS 775W	LM 5412	0.353	no
		SS 775W	EC O157:H7	<0.0001	yes
	52.5°C	SS 775W	EC O26:H11	0.0768	no
		LM 5412	EC O157:H7	<0.0001	yes
		LM 5412	EC O26:H11	0.1429	no

	EC O157:H7	EC O26:H11	0.0022	yes
55.0°C	ST 611	SS 775W	0.0065	yes
	ST 611	LM 5412	<0.0001	yes
	ST 611	EC O157:H7	0.0131	yes
	ST 611	EC O26:H11	<0.0001	yes
	SS 775W	LM 5412	0.4644	no
	SS 775W	EC O157:H7	0.8781	no
	SS 775W	EC O26:H11	0.0003	yes
	LM 5412	EC O157:H7	0.4961	no
	LM 5412	EC O26:H11	<0.0001	yes
	EC O157:H7	EC O26:H11	0.0057	yes
57.5°C	ST 611	SS 775W	<0.0001	yes
	ST 611	LM 5412	<0.0001	yes
	ST 611	EC O157:H7	0.005	yes
	ST 611	EC O26:H11	0.1089	no
	SS 775W	LM 5412	0.812	no
	SS 775W	EC O157:H7	0.6745	no
	SS 775W	EC O26:H11	0.0026	yes
	LM 5412	EC O157:H7	0.4794	no
	LM 5412	EC O26:H11	<0.0001	yes
	EC O157:H7	EC O26:H11	0.0255	yes
60.0°C	ST 611	SS 775W	0.0456	yes
	ST 611	LM 5412	0.0003	yes
	ST 611	EC O157:H7	0.0648	no
	ST 611	EC O26:H11	0.1186	no
	SS 775W	LM 5412	0.0277	yes
	SS 775W	EC O157:H7	0.9091	no
	SS 775W	EC O26:H11	0.1857	no
	LM 5412	EC O157:H7	0.0366	yes
	LM 5412	EC O26:H11	<0.0001	yes
	EC O157:H7	EC O26:H11	0.2705	no

* $p<0.05$ = significant difference

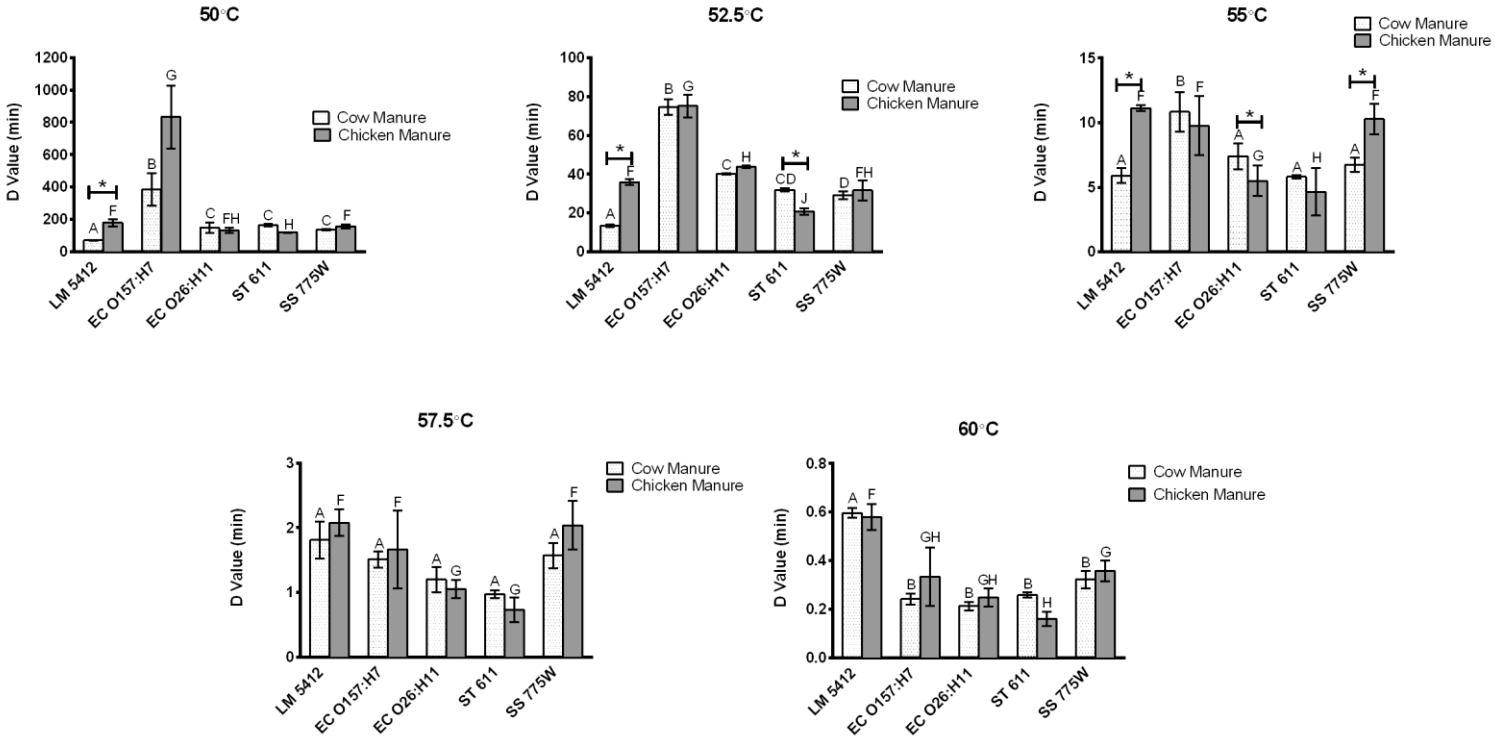

50°C
52.5°C
55°C
57.5°C
60°C
D Value (min)
Cow Manure
Chicken Manure
LM 5412
EC O157:H7
EC O26:H11
ST 611
SS 775W

Figure 23. Comparison of pathogen D values at different temperatures. Pathogens studied are LM 5412 (*Listeria monocytogenes* HPB 5412), EC O157:H7 (*Escherichia coli* O157:H7), EC O26:H11 (*Escherichia coli* O26:H11), ST 611 (*Salmonella* Typhimurium 611), SS 775W (*Salmonella* Senftenberg 775W). Strains were tested on separate days. Inoculum was pelleted and resuspended in sterile peptone water. Cow or chicken manure was mixed with the resuspended bacterium using a blender. Sample was split into 10g coupons in stomacher bags, and bags were sealed. Coupons were placed in pre-heated water baths at 25.0°C, 55.0°C, 52.5°C, 55.0°C, 57.5°C, and 60.0°C. Samples were removed at different time intervals, serially diluted and plated on selective media and incubated for 24hrs. CFU/g were calculated and the $\log_{10}$ graphed against time to calculate D-values for each temperature. Mean D-values from replicate experiments are shown with standard error. Means for cow manure with different letters from A to D are significantly different. Means for chicken manure with different letters from F to J are significantly different. * denotes significant difference between cow and chicken manure for that pathogen at that temperature. n = 2 or 3. $p<0.05$

3.13 *z*-Value Calculations for Pathogens in Cattle and Chicken Manure

Following D-value determination, *z*-Values for each pathogen were calculated by plotting the logarithmic of the D-values versus their respective temperature at 50°C, 52.5°C, 55°C, 57.5°C, and 60°C (Figure 24). The *z*-values for each were then calculated as the inverse negative of the slope of the linear regression line of the mean values of replicate experiments. *z*-values are reported in °C in Table 15.

Table 15. *z*-values of bacteria in manure.

Manure Type	Bacteria Strain	*z*-value (°C) ± SE	R^2	*z*-value Equation
Cow	ST 611	3.512 ± 0.087	0.9981	y= -0.2847x + 16.44
	SS 775W	3.834 ± 0.032	0.9998	y= -0.2608x + 15.17
	EC O157:H7	3.087 ± 0.052	0.9991	y= -0.3239x + 18.82
	EC O26:H11	3.466 ± 0.110	0.9967	y= -0.2885x + 16.68
	LM 5412	4.995 ± 0.272	0.9901	y= -0.2002x + 11.76
Chicken	ST 611	3.477 ± 0.059	0.9991	y= -0.2876x + 16.45
	SS 775W	3.865 ± 0.148	0.9953	y= -0.2587x + 15.14
	EC O157:H7	2.959 ± 0.134	0.9934	y= -0.3380x + 19.70
	EC O26:H11	3.541 ± 0.163	0.9931	y= -0.2824x + 16.31
	LM 5412	4.023 ± 0.113	0.9975	y= -0.2486x + 14.66

Only LM 5412 displayed a significant difference between *z*-values for cow and chicken manure, with cow manure being significantly higher and therefore more temperature dependent (Figure 25; Table 15).

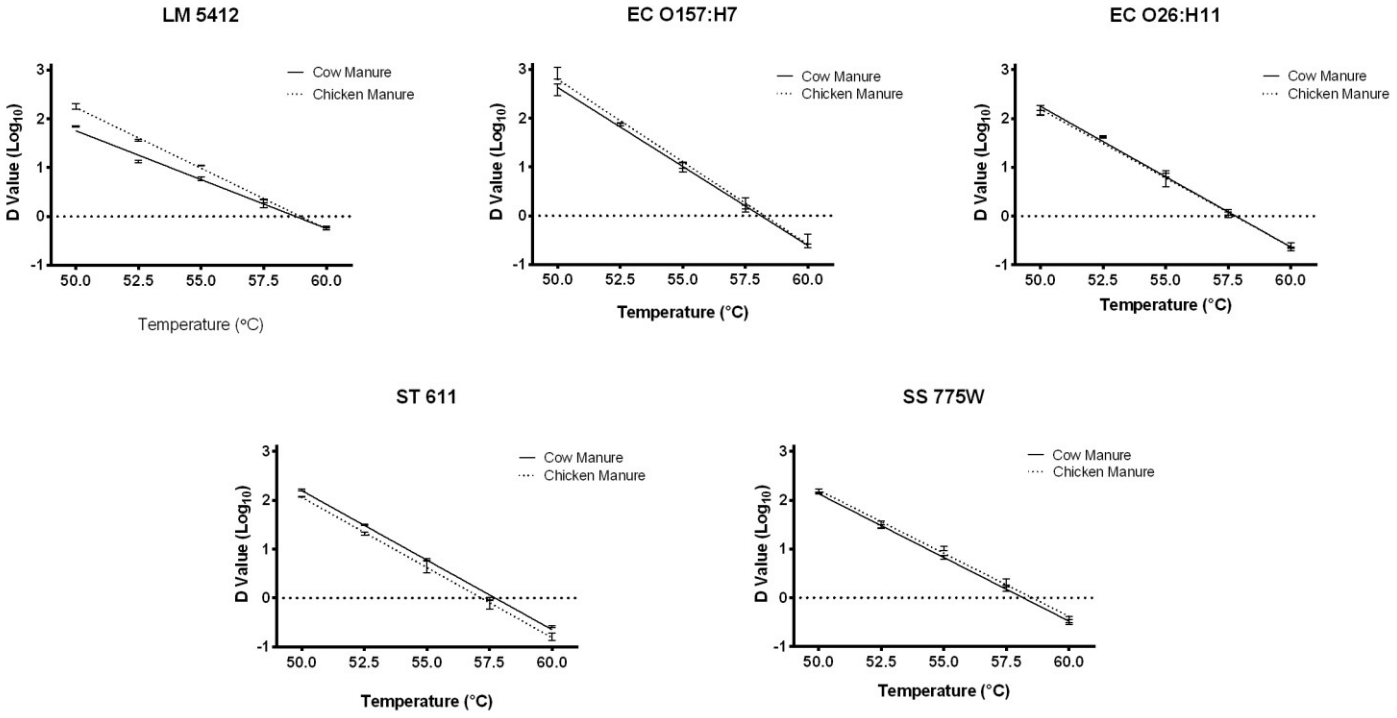

LM 5412
EC O157:H7
EC O26:H11
ST 611
SS 775W
Cow Manure
Chicken Manure
D Value (Log10)
Temperature (°C)
50.0
52.5
55.0
57.5
60.0
-1
0
1
2
3

Figure 24. Pathogen *z*-values. Pathogens studied are LM 5412 (*Listeria monocytogenes* HPB 5412), EC O157:H7 (*Escherichia coli* O157:H7), EC O26:H11 (*Escherichia coli* O26:H11), ST 611 (*Salmonella* Typhimurium 611), SS 775W (*Salmonella* Senftenberg 775W). To calculate D- values: Strains were tested on separate days. Inoculum was pelleted and resuspended in sterile peptone water. Cow or chicken manure was mixed with the resuspended bacterium using a blender. Sample was split into 10g coupons in stomacher bags, and bags were sealed. Coupons were placed in pre-heated water baths at 25.0°C, 55.0°C, 52.5°C, 55.0°C, 57.5°C, and 60.0°C. Samples were removed at different time intervals, serially diluted and plated on selective media and incubated for 24hrs. CFU/g were calculated and the $\log_{10}$ graphed against time to calculate D-values for each temperature. Graphs display each pathogen separately in each manure type. Mean Log_{10} values of D-values from replicate experiments in cow and chicken manure ± standard error are plotted against temperature. *z*-value is calculated as the inverse negative of the slope of the line with units in °C. n = 2 or 3.

EC O26:H11 and ST 611 were not significantly different in cow manure, whereas all other bacteria were (Table 16, Figure 25). In chicken manure EC O26:H11 and ST 611, SS 775W and LM 5412, SS775W and EC O157:H7, and LM 5412 and EC O26:H11 pairs did not have significantly different *z*-values. The *z*-value for EC O157:H11 in chicken manure was significantly lower than the other four pathogens; ST 611 and EC O26:H11 were not significantly different however *z*-value of ST 611 was significantly lower than LM 5412 and SS 775W.

Table 16. Statistical comparison of z-values between cow and chicken manure within a species and between species within manure type. $p < 0.05$ for significance

	Comparison 1	Comparison 2	p-value	Significant
ST 611	cow manure	chicken manure	0.7504	no
SS 775W	cow manure	chicken manure	0.8453	no
LM 5412	cow manure	chicken manure	0.0117	yes
EC O157:H7	cow manure	chicken manure	0.4353	no
EC O26:H11	cow manure	chicken manure	0.7254	no
Cow Manure	ST 611	SS 775W	0.0197	yes
	ST 611	LM 5412	0.008	yes
	ST 611	EC O157:H7	0.0052	yes
	ST 611	EC O26:H11	0.7644	no
	SS 775W	LM 5412	0.0021	yes
	SS 775W	EC O157:H7	<0.0001	yes
	SS 775W	EC O26:H11	0.03	yes
	LM 5412	EC O157:H7	<0.0001	yes
	LM 5412	EC O26:H11	0.001	yes
	EC O157:H7	EC O26:H11	0.0184	yes
Chicken Manure	ST 611	SS 775W	0.0446	yes
	ST 611	LM 5412	0.0041	yes
	ST 611	EC O157:H7	0.0236	yes
	ST 611	EC O26:H11	0.7278	no
	SS 775W	LM 5412	0.4525	no
	SS 775W	EC O157:H7	0.0058	yes
	SS 775W	EC O26:H11	0.2155	no
	LM 5412	EC O157:H7	0.0022	yes
	LM 5412	EC O26:H11	0.0708	no
	EC O157:H7	EC O26:H11	0.0379	yes

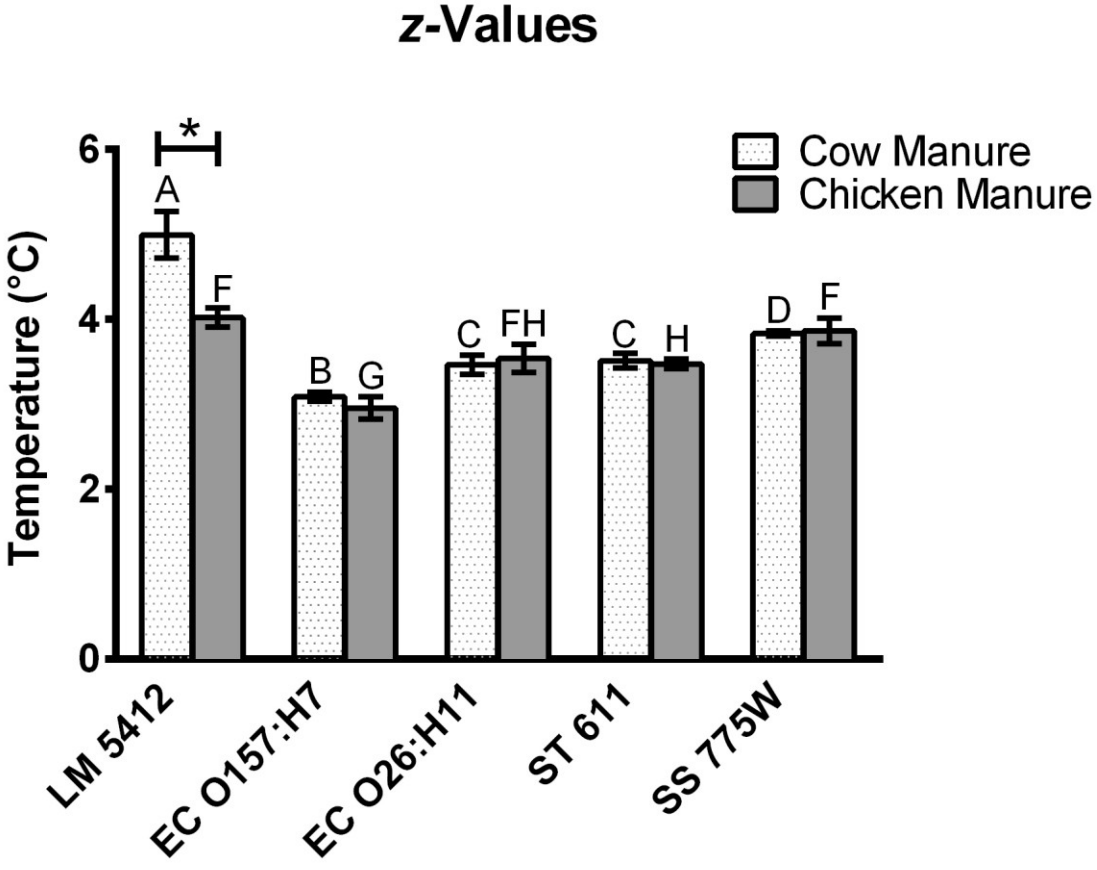

z-Values
Cow Manure
Chicken Manure
Temperature (°C)
0
2
4
6
*
A
F
B
G
C
FH
C
H
D
F
LM 5412
EC O157:H7
EC O26:H11
ST 611
SS 775W

Figure 25. Comparison of pathogen *z*-values. Pathogens studied are LM 5412 (*Listeria monocytogenes* HPB 5412), EC O157:H7 (*Escherichia coli* O157:H7), EC O26:H11 (*Escherichia coli* O26:H11), ST 611 (*Salmonella enterica* Typhimurium 611), SS 775W (*Salmonella enterica* Senftenberg 775W). To calculate D- values: Strains were tested on separate days. Inoculum was pelleted and resuspended in sterile peptone water. Cow or chicken manure was mixed with the resuspended bacterium using a blender. Sample was split into 10g coupons in stomacher bags, and bags were sealed. Coupons were placed in pre-heated water baths at 25.0°C, 55.0°C, 52.5°C, 55.0°C, 57.5°C, and 60.0°C. Samples were removed at different time intervals, serially diluted and plated on selective media and incubated for 24hrs. CFU/g were calculated and the $\log_{10}$ graphed against time to calculate D-values for each temperature. Graphs display each pathogen separately in each manure type. Mean Log_{10} of z-values ± standard error are shown. Means for cow manure with different letters from A to C are significantly different. Means for chicken manure with different letters from F to H are significantly different. * denotes significant difference between chicken and cow manure means for that pathogen at that temperature. n = 2 or 3. $p<0.05$

IV. Discussion

4.1 Parasite Method Development

The ability to determine viability in conjunction with detection and quantification is essential when an organism may impose a health threat. Use of dye permeability assays for determining oocyst/cyst viability are an indirect method of viability determination and may not reflect the infectivity of said oocyst/cyst. Though *in vitro* assays to determine the viability of oocysts/cysts are often conducted via viability dyes, an oocyst/cyst may appear as viable through rejection of cell-impermeant viability dye but not actually be infective where it is able to establish infection *in vivo* (in an animal model).

The use of fluorescent markers to determine viability has been well established for bacterial cultures (Schumann, 2003). In 1987, Schupp and Erlandsen demonstrated a correlation between the fluorescent dyes, fluorescein diacetate (FDA) and propidium iodide (PI)-positive *Giardia* cysts with infectivity of neonatal CF-1 mice. Cysts that stained positively with PI exhibiting red fluorescence were unable to infect mice, as PI is a cell-impermeant dye that is only able to cross compromised membranes (which is a characteristic of damaged or "dead" cells). PI has since been a standard for viability assessment of *Cryptosporidium* and *Giardia* oocysts/cysts. However, these experiments studied infectivity of mice inoculated with Fluorescence-Activated Cell Sorting (FACS) sorted PI positive cysts without comparing results to PI-negative cysts (Schupp 1987). The possibility that cysts negative for PI could be false-negatives, and non-viable was not tested in this study.

An additional test for viability determination of oocysts is excystation. Excystation is the process where infective sporozoites are released from the oocyst following ingestion by a

susceptible host. This process has been replicated *in vitro* under laboratory conditions as a qualitative assessment of viability of oocysts, being determined by their ability to excyst. Some studies have reported varying correlations between PI staining and excystation (Campbell et al, 1992; Medema et al, 1998; Smith and Smith, 1989), while others have concluded that PI staining and excystation methods overestimate oocyst/cyst infectivity in mice (Belosevic et al, 1997; Black, 1996; Smith and Smith 1989) and a failure to infect gerbils in spite of inoculation with PI-negative cysts (Wallis 1996). Therefore, there may be an underestimation of total viable oocysts/cysts using the PI method (Schumann, 2003).

Few studies have been conducted on the survivability of protists such as *Cryptosporidium parvum* and *Giardia duodenalis* during composting. Investigations are made more difficult due to the difficulty in culturing and detecting these protists in complex matrices such as manure and soil. In order to measure log reduction, large numbers of the organism in question must be quantified and viability must be determined. Traditional methods to enumerate oocysts/cysts in matrices such as manure employed microscopy, which is time consuming and unrealistic in a study measuring log reduction. Flow cytometry has been used previously in multiple studies to detect *Cryptosporidium parvum* and/or *Giardia duodenalis* oocysts/cysts in water samples (Hsu et al, 2005a/b; Medema et al, 1998; Vesey et al, 1994; Vesey et al, 1993).

The method developed to enumerate oocysts/cysts following extraction from manure samples was successful (Figure 13) as oocysts/cysts were identifiable even in the presence of a large amount of background debris found in manure. Similarly large amounts of debris were observed for both chicken and cow manure yet oocysts/cysts were separated with the use of monoclonal antibodies allowing gating on these specific populations and enumeration.

During cytometer analysis samples required occasional vortexing to prevent sedimentation. Chicken required more constant mixing to prevent clogging of the cytometer needle, suggesting an additional filtration step should be considered in future experimentation. Sensitivity of extraction and cytometer enumeration of oocysts/cysts was low, especially compared to extraction from fruit samples (Figures 14 and 15). Only a portion of the sample was analyzed via flow cytometry following extraction using a sucrose gradient. Analysis of just a portion would have resulted in lower numbers, however the recorded events per tube should have been greater still. In contrast, experimentation to enumerate oocysts/cysts inoculated into berries resulted in 10-fold higher recovery (Figures 14 and 15). The recovery method from berry a sample is much different from that used for manure and soil samples, being more of a concentration method, which removes and concentrates oocysts/cysts in the sample. Poor oocyst/cyst recovery from manure samples is likely hindered by particulate matter during sucrose flotation; a more efficient method of separation from manure should result in higher flow cytometry numbers.

Following successful detection using flow cytometry methods for viability assessment were investigated. The use of fluorogenic vital dyes has become a common method for determining not only protozoan viability but also stages of cell death (apoptosis and necrosis) of a variety of cell types. Membrane impermeant fluorescent stains are nucleic acid specific stains which are larger than cell permeant fluorescent markers, are often charged, and/or have groups that cause steric hindrance for membrane diffusion. Molecules of different size and charge may penetrate the bacterial cell wall depending on the species as well as the type and extent of membrane damage (Schumann et al 2003). PI has arguably become one of the most common fluorogenic compounds used in viability studies (King,

2000). PI binds nucleic acids by intercalating between bases as well as stoichiometrically allowing quantification of nucleic acid content. Once bound, its fluorescence enhances by 20-fold the ability to distinguish between viable and non-viable, as it is membrane impermeant and excluded from viable cells with intact membranes. However PI has been found to underestimate the number of non-viable cysts, only detecting a portion of non-viable cysts and, therefore, defining the lower limit of non-viable oocysts/cysts in samples (Belosevic et al, 1997; Black, 1996; Smith and Smith 1989; Wallis 1996).

More recently, investigations have focused on a variety of other fluorochromes that may better indicate viability and cell cycle stages. Neumann (et al, 2000) found *Cryptosporidium* oocyst staining with the cell-permeant dyes Syto-9 and Syto-59 correlated with infectivity of CD-1 mice (Belosevic et al, 1997), however the Syto dyes are cell-permeant and stain both viable and non-viable cell membranes. Studies on bacterial viability conducted by Roth et al (1997) found SytoX Green stain as a viability determinant with flow cytometry was superior to PI-based tests (King, 2000).

The SytoX brand of dyes are another, recently developed (Molecular Probes Inc.), type of nucleic acid stain that are cell-impermeant and only traverse compromised membranes. SytoX is advantageous due to its availability in a variety of colours, allowing one to conduct multi-parameter analyses with a variety of other fluorochromes in different cytometer channels. SytoX brand stains also result in a large (500-1000 X) increase in fluorescence on nucleic acid binding, much larger than that of PI and easier to detect. SytoX also comes with the advantage of small excitation/emission spectra which limits spectral overlap into other fluorescent channels, eliminating the need for cytometer compensation, whereas PI has a large excitation/emission spectrum that often leaks into other channels and

may interfere with fluorescence of additional parameters. In this study, we compared the ability of the SytoX brand nucleic acid dye (specifically SytoX Blue) to stain heat-inactivated *C. parvum* oocysts and *G. duodenalis* cysts compared to the more commonly used PI.

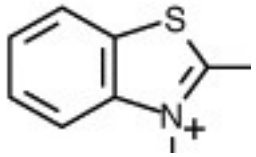
4.1.1 Cryptosporidium oocysts take longer to stain

We observed that viability staining of heat-inactivated *Cryptosporidium* oocysts required larger volumes or longer incubation periods (compared to *Giardia* cysts) to stain the oocyst population, especially in the case of PI. Schupp and Erlandsen (1987) reported that a proportion of *Giardia* cysts became FDA-positive over time although they did not initially stain with either FDA (stains viable cells) or PI (stains nonviable cells), whereas Smith and Smith (1989) found some cysts exhibited no fluorescence with FDA or PI after 4-6 hr of incubation (Belosevic et al, 1997). The different wall structures of *Giardia* and *Cryptosporidium* cysts/oocysts may explain the differences in staining (Chãvez-Munguãa et al, 2004). Oocysts are known to be quite robust and durable structures capable of withstanding a variety of environmental stressors, therefore it is possible that *Cryptosporidium* oocysts may undergo a gradual permeabilization after heat-inactivation.

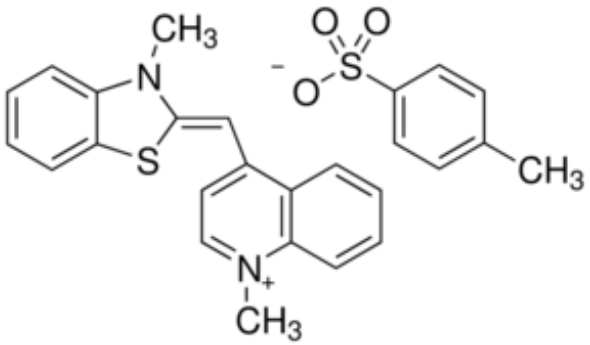
A characteristic of intact and potentially viable cells is the integrity of the cell membrane (Schumann et al 2003). Jenkins et al (2010) conducted studies on the wall structure of *C. parvum* oocysts and found it to have a complex chemistry of waxy hydrocarbons in the electron-translucent layer, proposing this as an explanation for the acid-fast staining properties of *C. parvum.* This wall chemistry may also explain a loss of impermeability when oocysts are exposed to large thermal changes, as is observed in

Mycobacterium which have similarities to *C. parvum* oocysts in the lipid composition of their walls (Jenkins et al, 2010).

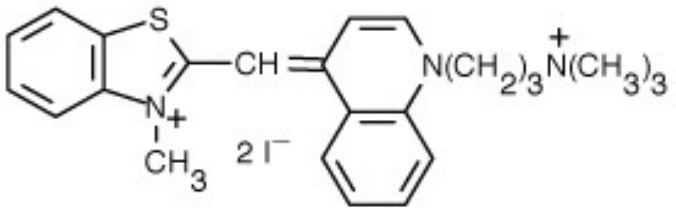
The cell membrane is hydrophobic, thereby allowing neutral molecules to traverse more easily than charged larger molecules. The size and charge of the fluorogenic compound may also be responsible for delayed oocyst uptake. Organic compounds that contain at least two positive charges, such as PI, DAPI and TO-PRO-1, are membrane-impermeant. They can only cross compromised cell membranes as membrane damage disrupts normal membrane potential allowing charged molecules to traverse. Positively charged dye molecules are attracted to negatively charged cell compounds such as nucleic acids (Shapiro 2003). Propidium iodide is a phenanthridium nucleic acid stain that carries two positive charges, whereas SytoX is an asymmetrical cyanine dye that likely has at least three positive charges, as it is highly impermeant (the structure is protected under patent). Though the molecular structure of SytoX is proprietary, it is one of the variants of fluorescent molecules developed by Molecular Probes that is related to thiazole orange (parent molecule). The fluorescent properties of these dyes are all based around a heterocyclic ring structure as outlined in Figure 26. The permeancy of the molecules depends on the ring substituents on this ring structure, and can influence whether the dye is permeant as well as how quickly it will enter and leave cells (Shapiro, 2003).



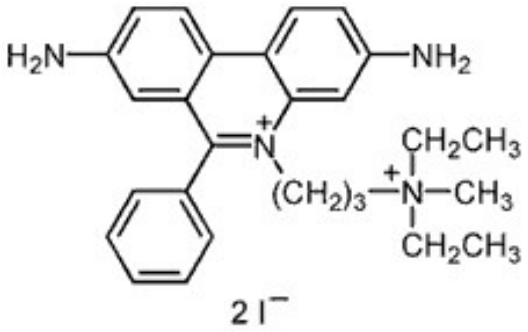
Heterocyclic Ring



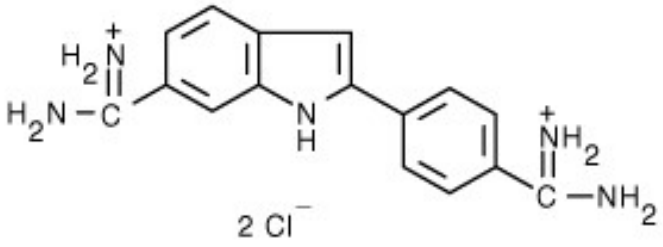
Thiazole Orange (TO)



TO-PRO-1



Propidium Iodide (PI)



4',6-diamidino-2-phenylindole (DAPI)

Figure 26. Nucleic acid dyes. The fluorescent properties of these dyes derive from the heterocyclic ring structure (top molecule). PI and DAPI have different heterocyclic ring structures than TO and TO-PRO-1. Thiazole Orange (TO) is the parent molecule to TO-PRO-1, as well as the cell-impermeant SytoX and cell-permeant Syto brand molecules (Not shown). Permeancy properties depend on the ring substituents. TO carries a delocalized positive charge and is permeant. Molecules containing at least two positive charges, such as TO-PRO-1, PI, and DAPI are membrane impermeant. Structures were provided by Molecular Probes Inc, except TO which was provided by Sigma-Aldrich Inc.

Mortimer et al (2000) reported that a smaller percentage of antibiotic treated *E. coli* cells fluoresced with PI compared to TO-PRO-1 and SytoX Green. Efflux pumps have also been found to remove ethidium bromide (EB), which is structurally similar to PI but cell-permeant, from bacterial cells, which could also explain poor staining with PI (Mortimer et al, 2000; Shapiro, 2003). Further studies comparing fluorogenic stains of known weight and charge may elucidate the differences between PI and SytoX, as well as the process of oocyst wall degradation. We suggest the possibility that it takes time for the melting of fatty acids and hydrocarbons present in the oocyst wall, thereby allowing wall deterioration and changes in potential allowing penetration of charged cell-impermeant viability dyes.

4.1.2 Giardia cysts exhibit a higher proportion of non-viability

Giardia cyst suspensions were found to contain a larger number of cysts staining non-viable compared to *Cryptosporidium* oocysts. Original studies on pure suspensions of viable cysts showed no significant increase in non-viable cysts over time (Figure 9), suggesting that the dyes SytoX and PI were non-toxic. Yet during experimentation with heat-inactivated cysts, results indicated a greater number of cysts staining as non-viable than were predicted to be contained in the sample. Correction of results through subtraction of initial non-viable values in viable controls resulted in values that were closer to predicted values (Figure 12, Panel C). This is not unexpected as there is the possibility of stress during handling and experimentation, as well as a gradual decrease in viability over time during storage (4˚C). Studies conducted by Medema et al (1998) found the percentage of *Giardia* cysts staining with PI, and therefore deemed non-viable, increased after sonication, flotation, and washing of the sample by approximately 20-35%. Other studies by Zhao et al (2010) also found that cell viability, proliferation, and responses to certain cytotoxic agents were not

affected by PI, SytoX Green, or SytoX Red, although these studies were conducted on A549 cell cultures and not protists. Similarly, Roth et al (1997) found SytoX Green not toxic to growing bacteria, while brightly staining cells with compromised plasma membranes.

The *Giardia* cyst wall is 0.3 to 0.5µm thick, composed of an outer filamentous layer and an inner membranous layer (Adam, 2001; Chãvez-Munguãa et al, 2004). The sugar component of the outer portion is predominantly *N*- acetylgalactosamine (Erlandsen et al, 1989). Deng and Cliver (1992) found *Giardia* cysts that stained non-viable with PI had lost their normal surface morphology and structures. PI-positive cysts exhibited a variety of damaged wall characteristics including increased thickness of cell wall filaments, ruptured, gapped and holed cyst surfaces, as well as collapsed cyst walls following heating at 65˚C in a water bath. This extreme degradation of the cyst wall in conjunction with the increased permeability properties of SytoX, and overestimation of viability with PI, may explain why in some cases SytoX stained more cysts as non-viable (although not significantly higher than PI) (Figure 12). This may also explain the differences seen between *Cryptosporidium* oocysts and *Giardia* cysts, as their walls are chemically and morphologically different. Further research should be done using a larger sample number to confirm possible losses of viability during experimentation, as well as including animal infectivity as the gold standard to determine viability of cysts.

4.1.3 Propidium Iodide results in lower proportions of non-viable oocysts/cysts than SytoX

Previous studies of apoptosis and cell cycle analysis using flow cytometry may also elucidate why PI stains a lower percentage of heat-inactivated oocysts/cysts as non-viable compared to SytoX. Apoptosis can be recognised by a pattern of morphological, biochemical

and molecular changes, which have been classified as early, intermediate and late apoptotic stages. Investigations of apoptotic stage and cell cycle analysis via alterations in membrane permeability are commonly done using flow cytometry (Haase, 2001; Mukhopadhyay et al, 2007). Studies on late stages of apoptosis indicate that PI may require more extensive membrane degradation in order to enter cells, thus are indicators of later stages of apoptosis (Zhao et al, 2010). Hasse and Reed (2002) found SytoX-stained cells allowed a more sensitive and accurate analysis of cell cycle phases of budding yeast compared to PI, and that SytoX may have a higher affinity for DNA than PI making it less sensitive to dilution or changes in cell number. Evidence suggests that SytoX is more cell-permeant than PI, and detects apoptotic cells at an earlier stage of membrane integrity loss. Although oocysts/cysts do not undergo apoptosis, as occurs in mammalian cells, one could theorize that SytoX may act in a similar manner when staining *Cryptosporidium* oocysts. Permeation of the probes may only occur after gradual permeabilisation of cells depending on the probes' molecular structure as was found by Schumann et al (2003) with bacteria. SytoX may be able to permeate the oocyst/cyst membrane at an earlier, less degraded, stage of wall decomposition compared to PI.

SytoX is prepared in a solution containing DMSO, which is known to aid in dye penetration. The addition of DMSO to PI samples as well as SytoX samples (already containing DMSO) did not significantly increase the proportion of non-viable oocysts/cysts when larger concentrations of dye were used, or after incubation for longer time periods. This suggests that the molecular structure of the SytoX dyes, and not the addition of DMSO, is responsible for the increased permeability and staining of oocysts/cysts compared to PI.

4.2 Thermal inactivation of different organisms at different temperatures

Heat processing of foods and other materials has long been an important sterilization and preservation tool to ensure microbiological safety. Moisture is also a component of composting materials; during wet heat exposure microorganisms are inactivated by protein denaturation and membrane damage (Smelt and Brul, 2014). The heat generated by the metabolic activity of microorganisms in the compost heap is essential for the inactivation of pathogens, which has resulted in the criteria for composting to be based on time-temperature conditions worldwide (Erickson et al, 2009). Ceustermans et al (2007) found temperature to be the most important factor in eradication of heat resistant *Salmonella* 775W during composting. Fremaux et al (2007) recorded thermal reduction values of 0.48 and 2.39 days at 65°C and 35°C, respectively, for non-O157 STEC *E. coli* during cow manure composting. The effect of temperature on *C. parvum* infectivity can differ by strain with higher temperatures resulting in more rapid loss of infectivity, for example oocysts held at 25°C and 30°C remained infective to 3 months whereas those heated to 59.7°C and 71.7°C for 5min and 5s, respectively, lost infectivity (Fayer et al, 2000). Van Herk et al (2004) found the inactivation of *Cryptosporidium* oocysts and *Giardia* cysts to be a result of temperature, and to some extent dessication, during windrow composting of beef feedlot manure.

EC O157:H7 had significantly higher D_x-values, and therefore survived longer than the other bacterial pathogens at 50°C, 52.5°C, and 55°C in cow manure. In cow manure at 57.5°C there were no significant differences between pathogens, compared to chicken manure where EC O26:H11 and ST 611 had significantly lower $D_{57.5}$-values than the other three pathogens. Fremaux et al (2007) found non-O157 STEC(s) to survive extended periods in cow manure heaps, although their larger scale studies allowed for temperature fluctuations

during compost-simulation. The lower survival of EC O26:H11 in this study may be attributed to the use of Rainbow agar to enumerate EC O157:H7 and O26:H11 simultaneously, competition between the two strains resulting in lower numbers of EC O26:H11.

LM 5412 had a significantly lower $D_{52.5}$-value compared to the other pathogens. However, LM 5412 survived significantly longer at 60°C in both cow and chicken manure. This is consistent with mean D-value(s) determined by Smelt and Brul (2014) where *Listeria monocytogenes* D_{60}-values were much higher than *Salmonella* spp., *E. coli*, and *Campylobacter*. Hutchison et al (2005a/b) also found *L. mono* decline in a variety of livestock manure types required longer compared to other enteric pathogens.

Few studies on pathogen decimal reduction (D_x-values) have been conducted in manure, making comparisons difficult. Those studies that are available are often larger on-farm studies measuring longitudinal reduction and relying on environmental conditions to determine manure/compost temperature. Spinks et al (2006) found *Salmonella* Typhimurium to be less resistant to heat during thermal inactivation analyses at sub-boiling temperatures in water compared to both *E. coli* O157:H7 and *E. coli* O3:H6. A temperature range from 55°C to 65°C was critical for elimination of enteric pathogenic bacteria, they concluded that a minimum temperature of 60°C should be used for hot water systems.

Laboratory scale studies have been found to show better survival for pathogens, as the organisms are not subjected to temperature changes, sunlight, or drying effects of air (Hutchison et al, 2005b). Sub-culturing of pathogens for years may also affect characteristics of laboratory strains (Fux et al, 2005). Further studies to compare more *Salmonella* and *E.*

coli strains should be conducted to compare possible differences in D_x-values.

4.3 Influence of composition and manure type on thermal inactivation

Composting by farming operations aims to not only convert animal waste into nutrient rich soil amendment material, but also reduce the microbial load and eliminate pathogens to allow use on fields for crop production. One of the fundamental factors for effective composting is the generation of high temperatures through anaerobic metabolism of microorganisms indigenous to the compost materials. High temperatures are not only used in composting, but also important in interventions of steam and hot water pasteurization. The effect of temperature on microorganisms, and their resistance to heat, depends on the nature of the matrix they are contained in.

Temperature is traditionally thought to be a controlling factor during composting, however other components of a compost pile can affect the structural and thermal properties of the material as well as influence the rate of biodegradation. The importance of other factors for pathogen inactivation was supported by Platz (1978) whereby a higher percentage of *Salmonella* Typhimurium and *E. coli* populations were inactivated when directly inoculated into a compost pile, compared to samples sealed in glass ampoules (to prevent direct contact with compost) and placed in the pile.

Though *Campylobacter* survival is mostly dependent on temperature, survival of *E.coli* and *Salmonella* has been found to be largely dependent on moisture content (Gilpin et al, 2008; Sinton et al, 2007). Reduced water content of composting materials resulted in a higher survival rate of *Salmonella* 775W (Ceustermans et al, 2007). Results found a

significant difference in *S. enterica* Senftenberg 775W chicken and cow manure D_{55}-values with chicken being significantly higher as well as exhibiting a lower moisture content and water activity compared to cow manure (Tables 7 and 8). However, *E. coli* O26:H11 and *S. enterica* Typhimurium 611 had significantly higher D_{55}-value and $D_{52.5}$-value (respectively) in cow manure compared to chicken manure suggesting a more drastic difference in moisture may be required, or this characteristic may vary between species and strains. *Cryptosporidium* survival is affected by moisture as well, desiccation being known to kill oocysts (Fayer, 2004; Fayer et al, 2000). Similarly Barwick et al (2003) found the likelihood of detecting *Giardia* cysts in soil increased significantly for each percentage of moisture content in soil. High moisture content can also limit oxygen diffusion creating anaerobic environments.

Changes in pH occur during composting due to decomposition and products of microorganism metabolism during the process. pH is important in determining the rate of inactivation next to the type of microorganism and temperature (Akterian et al, 1999; Smelt and Brul, 2014). The decline of *Salmonella* and *E.coli* in manure was found to be positively correlated with pH in manure by Franz et al (2005). Barwick et al (2003) found fewer *Cryptosporidium* oocysts in soils of neutral and basic pH than in soils with low acidic pH (Fayer, 2004). pH may also be affected by other factors during composting, such as the carbon:nitrogen ratio of a pile which may result in a decrease in pH at 20:1 and 30:1 ratios or increase in the case of 40:1 ratios (Erickson et al, 2009). This could be a result of bacterial production of acetic acid during metabolic processes.

Carbon and Nitrogen content of the material can affect the metabolism of microorganisms within the compost heap. Carbon (C) is essential for microorganism energy

and growth and nitrogen (N) is necessary for protein production and reproduction. C:N ratios of 25:1 to 30:1 are ideal, however ratios ranging from 20:1 to 40:1 have been deemed acceptable as well (Erickson et al, 2009; Rynk, 1992). Erickson et al (2009) investigated the effects of differing C:N ratios on *Salmonella* survival in cow manure. Higher C:N ratios (40:1) resulted in initial temperatures near maximum during the first day, whereas 30:1 exhibited a gradual increase from days 1 to 4, and 20:1 ratios required longer periods for temperature increases. 20:1 and 40:1 C:N ratios reduced *Salmonella* populations by 7-$\log_{10}$ compared to 30:1 which reduced populations 5-$\log_{10}$. However *Salmonella* was detected after 7 days of composting at a 40:1 ratio compared to the last day of detection being only 3 or 5 days after composting at 20:1 and 30:1 respectively (Erickson et al, 2009).

The chicken and cow manure used in this study were in a highly reduced state, with limited oxygen and high in salt content. The high percentage of moisture could have added to low oxygen levels through displacement of available oxygen. The chicken manure appears to be richer in salt-based minerals, which may be injurious to plants. The determined C:N values for the cow and chicken manure were consistent with literature (Tables 7, 8, and 19; Hartz et al, 2000; Sharpley and Moyer, 2000). It is not uncommon for manure to be in a reduced state prior to composting, as composting is a process to convert manure to an oxidized non-toxic material for agriculture. Although the cow manure samples would be adequate for composting, further studies in chicken manure would require addition of carbon sources such as sand or straw to increase the C:N ratio and ensure efficient microbial decomposition. Future studies should also control for each factor separately to compare effects on each organism.

Studies by van Asselt and Zwietering (2006) to provide a means for more precise studies of inactivation (D-values) collected and compared a large quantity of D-values and found few factors to have an effect on results. Exceptions were the presence of chocolate ingredients for *Salmonella* spp., the presence of 10% salt (or $a_w < 0.92$) for *L. mono*, significant differences for strains of *Bacillus cereus* in oily products, and significant differences between *Clostridium botulinum* strains.

Many different factors can play a role in composting of manure, and manure products from different animals often have different composition due to differences in diet, digestion, etc (Table 17). These different characteristics, such as moisture and nitrogen content, have been found to influence the composting process. Singh et al (2011) found *E. coli* O157:H7 survived for 72, 48 and 24h in optimal compost (C:N of 25:1) compared to 288, 72, and 48h in suboptimal compost (C:N 16:1) at 50°C, 55°C, and 60°C (respectively). They also found higher moisture content, 50% compared to 40%, resulted in faster decline. Inglis et al (2010) compared temperatures of cow manure compost between antibiotic treated cattle and cattle not administered antibiotics. Temperatures were more variable and the antibiotic treated cattle compost did not reach the high temperatures observed in untreated compost. C:N ratio and pH remained similar between antibiotic administered vs non-antibiotic administered manure compost, however there were significant differences in other characteristics such as water content, carbon content, nitrogen content, and electrical conductivity. Hutchison et al (2005a) found no significant differences in pathogen decline in liquid (slurry) and solid (farmyard manure) wastes, however levels of *Cryptosporidium* oocyst recovery were higher in slurries compared to manure with higher dry matter content.

Significant differences between D-values in cow manure versus chicken manure were observed for LM 5412 at the three lower temperatures, 50°C, 52.5°C, and 55°C, with chicken manure being significantly higher and therefore requiring longer periods for thermal inactivation. ST 611 exhibited a significantly higher D-value at 52.5°C, and EC O26:H11 an SS 775W had significantly higher cow and chicken (respectively) levels at 55°C (Table 12; Figure 23). LM 5412 was the only pathogen with significant differences in z-values between manure types (Table 14). The higher salt content of chicken manure, approximately 9.73mS cm^{-1} versus 2.138 mS cm^{-1} for cow manure, may explain the longer survival and therefore higher D-value(s) of *L. mono* in chicken manure as van Asselt and Zwietering (2006) found salt content to significantly affect D-value(s) for this pathogen. The differences between cow and chicken D_x-values for some pathogens suggests further comparisons of different manure types should be investigated to determine whether different composting guidelines should be required. No significant differences were observed at the higher temperatures of 57.5°C and 60°C which may indicate a more precise measurement of time, for e.g. in milliseconds, is required to further assess the D-value of the pathogens at higher temperatures. An alternative explanation is that when the temperature of a composting pile exceeds a certain threshold, say 60°C and above, temperature is the determining factor in pathogen reduction regardless of composition.

Table 17. Average composition of different manure types. Table adapted from Bernal et al (2009).

Manure Type (Dry)	Dry Matter	Organic Carbon (C)	Total Nitrogen (N)	pH
Cattle	140-300	65-126	4.2-8.1	8.6
Pig	150-330	42-132	3.5-11	8.1
Poultry	220-700	103-597	10-58	7.6

4.4 Thermal inactivation of Cryptosporidium and Giardia

This study achieved a method for simultaneous quantification and viability assessment of both *Cryptosporidium* and *Giardia* oocysts/cysts for chicken and cow manure samples as well as in berries and lettuce using flow cytometry. In addition to a high-throughput method for detection in these complex matrices, a possibly more efficient viability dye was also tested for effectiveness in evaluating oocysts/cysts. *Cryptosporidium* and *Giardia* are quite hardy and reported to survive extreme environmental conditions (Carey et al, 2004; Hutchison et al, 2005b ; Jenkens et al, 2004) and we hypothesized that the protozoan *Cryptosporidium* and *Giardia* oocysts/cysts may out-survive bacterial pathogens during the composting process.

Although D- and z-value trial results were inconclusive, a much more rapid reduction of *Giardia* cysts occurred in manure heating compared to *Cryptosporidium* oocysts suggesting *Giardia* is unlikely to pose a threat following composting. Similarly Olson et al (1999) also showed *Giardia* cysts to be more susceptible to environmental stress than *Cryptosporidium,* being more rapidly inactivated in water, faeces, and soil at -4°C, 4°C, and 25°C. Contrarily, Hutchison et al (2005b) found *Cryptosporidium* oocysts to survive significantly longer than bacteria, 1-log reduction requiring 8-31 days compared to 1.94 days for bacteria, in livestock waste spread onto pasture.

Trials in cow manure resulted in D_{70}-values of approximately 975 min and 350 min for *Cryptosporidium* oocysts and *Giardia* cysts respectively. The extremely lower D_{70}-value in chicken manure compared to cow manure may be a result of the composition of chicken manure. Chicken manure often resulted in clogging of the flow cytometer, suggesting an

additional filtration step may be necessary when analysing. D and z-value determination in manure required large amounts of time in order to inoculate, heat-treat, and extract oocysts/cysts from manure. As such, D- and z- values for *Giardia* and *Cryptosporidium* were not determined and further work should be done to determine D-values for *Cryptosporidium* oocysts.

Although Hutchison et al (2005b) state *Cryptosporidium* oocysts out-survived bacterial pathogens and were therefore more robust they admit poor recovery of oocysts from plots. D-values for their plot fescue study were calculated from the log decline of oocyst numbers recovered, not the log decline of viability as determined by cell-impermeant dye exclusion and microscopy. Mawdsley et al (1996) found similar declines in extraction, and that the use of a more rigorous extraction protocol using TRIS (tris(hydroxymethyl)aminomethane) and Tween (polysorbate surfactant, or detergent) gave significantly higher oocyst recoveries. They demonstrated the ability of oocysts to move within the soil profile, which may explain poor recovery when extracting oocysts from soil.

Future studies in pure solution (i.e. phosphate buffered saline, etc) should be conducted to determine D-values of *Cryptosporidium* oocysts and *Giardia* cysts to provide a reference for time-temperature sampling points in more complex matrices. Hutchison et al (2005a) found D- values for *Cryptosporidium* oocysts to range from 8 days in dirty water to 31 days in sheep manure in field experiments. However they reported difficulties in oocyst recovery (<0.005% of the inoculum) and measured viability using DAPI (4',6-diamido-2-phenylindole). In contrast Fujino et al (2002) reported 30s exposure of *Cryptosporidium parvum* at 55°C resulted in loss of infectivity (no oocyst production) in mice, as was 15s at

60°C and only 5s at 70°C. Although Fujino et al (2002) conducted experiments in distilled water and used infectivity as a measure of viability rather than dye exclusion.

4.5 Thermal inactivation of *Campylobacter jejuni*

Inglis et al (2010) reported persistence of *Campylobacter* spp. throughout windrow composting process for 8 months. Ethidium monoazide (EMA) added to DNA amplification techniques allowed them to amplify DNA from intact and therefore viable *Campylobacter* cells only. These results allowed for discussion of presence and amount of DNA, compared to other studies using direct plating and MPN techniques that require growth stating *C. jejuni* does not persist well in manure. DNA amplification with EMA is not practical for determining population reduction on a log scale (D-values) as presence of DNA does not prove existence of viable cells. D-value determination for CJ 11168 in manure was not completed in this study. Preliminary trials conducted for media selection and survival estimation suggested CJ 11168 could be recovered after 48 hrs at 60°C in cow manure (Figure 17). Experimentation at 50°C, 55°C, 60°C, 65°C, and 70°C resulted in no survival of CJ 11168 even after the shortest sampling period of 8 hrs. CJ 11168 was detected at all time points in manure at room temperature (25°C) suggesting heat was important for inactivation. It is possible that a higher inoculum would allow detection at later time points, as this would ensure levels would still be within our limit of detection.

Though there is limited data regarding the reduction of *Campylobacter* spp. during composting multiple studies have reported lower survival compared to other bacteria, similar to the results of this study. Nicholson et al (2005) described *C. jejuni* decline in stored

manure and during land spreading to be more rapid than that of *Listeria, E. coli,* and *Salmonella* under identical conditions. Sinton et al (2007) also found inoculated *C. jejuni* were rapidly killed in cow manure, although their experiments were conducted on pasture in an uncontrolled environment, and suggest manure that is freshly deposited may be a reservoir for this pathogen resulting in positive surveillance studies. In contrast, Hutchison et al (2005a/b) reported survival of 2.31 and 2.53 days in dairy cattle manure and poultry broiler litter (respectively) during simulated composting of livestock bedding wastes (Table 18). *Campylobacter* declines appeared similar to *Salmonella, E. coli,* and *Listeria*; however no reference to *Campylobacter* is mentioned in the statistical analyses.

The specific growth requirements as well as difficulty in isolation and enumeration of *C. jejuni* led to experimentation to determine thermal reduction in pure solution. Studies have found a range of D_x-values for *Campylobacter* from 50 min at 55°C in broths, to 0.2 min in scalding tap water at 55°C (Smelt and Brul, 2014; Yang et al, 2001). This study found D_x-values in the lower range, 2.53 min and 0.78 min at 52.5°C and 55°C respectively. These values were similar to Sörqvist (1989) and Waterman (1982) who reported D-values of 0.24-0.28 min at 60°C in saline solution and 1.1 min and 0.6 min at 55°C and 55.5°C (respectively) in milk. The faster inactivation of *C. jejuni* in various matrices compared to other pathogens (Table 18) suggests it will not out-survive other pathogens in composting and will not be a more accurate indicator for composting completion.

Table 18. D_x-values of bacterial pathogens in various matrices.

Source	D values				Temperature	Reference
	Salmonella	***E. coli***	***L. mono***	***C. jejuni***		
Liquids (i.e. milk products, broths, saline, etc)	222 min	266 min	643 min	50 min	55°C	Smelt and Brul, 2014; Sörqvist, 2003
	24 min	39 min	87 min	8.2 min	60°C	
	2.6 min	5.6 min	12 min	1.3 min	65°C	
	0.1 min	0.4 min	0.7 min	0.1 min	72°C	
Scalding Water	23.6 min			4.0 min	50°C	Yang et al, 2001
	1.1 min			0.2 min	55°C	
	0.2 min			<0.2 min	60°C	
Dairy cattle manure	1.79 days	1.47 days	2.97 days	2.31 days	Ambient air: 15-30°C Centre of heap: 20-65°C	Hutchison et al, 2005a/b
Dairy cattle slurry	1.61 days	1.63 days	2.01 days	2.65 days		
Beef cattle manure	1.62 days	1.49 days	1.72 days	3.05 days		
Beef cattle slurry	1.45 days	1.56 days	1.86 days	1.85 days		
Poultry broiler litter	1.77 days	1.63 days	2.13 days	2.53 days		
High fat ground beef	*S. senft.*: 0.92 min *S. typh.*: 0.16 min	0.18 min			63/64°C	Smith et al, 2001
Low fat ground beef	*S. typh.*: 0.15 min	0.16 min			63/64°C	
	S. typh.: 77 sec	223 sec			55°C	
Deionized water	*S. typh.*: 4 sec	67 sec			60°C	Spinks et al, 2006
	S. typh.: <2 sec	3 sec			65°C	

4.6 Escherichia coli AW1.7

Comparison of heat resistant *E. coli* AW1.7 to pathogenic organisms such as *E. coli* O157:H7 and *Salmonella* during thermal reduction in manure would have been valuable for understanding the effects of temperature during composting. Enumeration of AW1.7 with O157:H7 and O26:H11 simultaneously in manure samples required some distinctive feature due to difficulty distinguishing it from generic *E. coli* present in the sample. Attempts to

create a GFP-labelled AW1.7 were unsuccessful, as a simple heat shock transformation protocol was used, and time constraints did not allow for further investigation into electroporation and chemical methods. Ma et al (2011) were successful in transforming some isolates of *Salmonella* and *E. coli* with the pGFPuv vector with 100mM $CaCl_2$, suggesting future trials should also investigate use of different $CaCl_2$ concentrations.

E. coli AW1.7 is an exceptionally heat resistant strain of *Escherichia coli* with reported D_{60}-value of 71 min (Pleitner, 2011). Pleitner (2011) compared three strains of *E. coli*: *E. coli* GGG10 which was isolated more than 20 years ago before current intervention methods were practiced; extremely heat resistant *E. coli* AW1.7 isolated from a commercial beef processing facility; and *E. coli* AW1.7ΔpHR1, a heat sensitized strain of AW1.7 that has lost the largest plasmid ΔpHR1. Heat shock of these three strains resulted in increases of 1.5 and 3 logs for GGG10 and AW1.7ΔpHR1 (respectively) in D_{60}-values. Though AW1.7 was heat-shocked for 40min (compared to 6min for GGG10 and heat sensitive AW1.7ΔpHR1) no difference in heat resistance was observed following heat shock. However, increases of 2.5 to 4 logs in heat resistance were observed after all strains were exposed to osmotic stress before heating. The heat resistance of AW1.7 resulted from increased solute transport and mechanisms to protect ribosomal integrity. Studies by Franz et al (2011) found the oxidative capacity of *E. coli* O157 strains was significantly higher for longer-surviving strains in manure-amended soil. Further genetic studies of heat tolerant *Salmonella* and *Listeria* spp, comparing them to AW1.7, would be of value to investigate the possibility of increased solute transport improving heat resistance in these species as well.

4.7 Selection for Heat Resistant Organisms

During conditions such as heating, many organisms will activate stress response mechanisms that may lead to increased resistance to the stressor. Incubation at higher temperatures has been correlated to higher rigidity of the cell membrane thereby enhancing heat resistance of microorganisms (Smelt and Brul, 2014). Composting on a large scale involves piling raw materials (manure, vegetable compost, carbon source, etc) into large windrows or a vessel and allowing the metabolism of microorganism metabolism to generate heat. This process requires time and results in a gradual increase in temperature (depending on the C:N content of the mixture). This raises concerns as slow heating may allow cells to adapt to stressful conditions that result in lower inactivation of pathogens.

Studies conducted by Inglis et al (2010) found that temperatures of 55°C were not reached (or exceeded) until later in the active composting stage, and were not reached at all in composting manure from antibiotic treated cows (350mg of chlorotetracycline and 350mg of sulfamethazine, Aureo S-700 G; Alpharma Inc.). Álvarez-Ordóñez et al (2009) investigated the effects of growth temperature on the heat resistance of *Listeria* at temperatures from 56°C to 65°C. Time to reduce the bacterial population by 1-log at 62°C were 0.09 min when grown at 20°C compared to 0.49 min when grown at 45°C. Similar results were found for all other temperatures, population decline requiring longer time when inoculum was cultured at higher temperatures. Similarly Henry et al (1969) found *Salmonella* strains grown at 44°C were more resistant than those grown at 35°C or 15°C. Singh et al (2010) reported *E. coli* O157:H7, *Salmonella*, and *L. mono* heat shocked at

47.5°C for 1 hour survived longer than control cultures at 50°C, 55°C, and 60°C; heat-shocked *E. coli* O157:H7 and *Salmonella enterica* survived 7 and 2 days longer than non-shocked controls in dairy manure co-composted with vegetable waste (Shepherd et al, 2010).

Some evidence suggests the effectiveness of heat-shock may depend on the environmental conditions in the medium. Hassani et al (2007) reported an increase in heat tolerance of *Listeria monocytogenes* being dependant on duration and heat-shock temperature at pH 7.4, but heat-shock temperature had no effect at pH 5.5 and pH 4.0. Williams and Ingham (1997) also reported heat shock induced heat resistance of *E. coli* in tryptic soy broth but not beef slurry. It is a reasonable concern that a gradual increase in temperature during the beginning phase of composting could allow for heat-shock conditions and possibly produce more heat-tolerant and resistant bacterial strains that could survive longer than indicators.

4.8 Possibility of Regrowth

Composting consists of active and curing stages. During the active stage there is a rapid increase in temperature and biological decomposition. The curing stage is the maturation stage of the process when the temperature declines and mesophilic or less thermophilic organism populations are activated. Sterilization is not achieved during composting, it is not a sterilization process, therefore it is possible bacteria that were reduced to low levels may regrow following active stage completion when temperatures drop (Hay, 1996). *E. coli* and *Salmonella* concentrations have been shown to increase following compost sterilization suggesting indigenous microflora are important for pathogen suppression and destruction (Pietronave et al, 2002). Although conditions during the composting stage(s) are not suitable

for growth of enteric pathogens, growth of *E. coli* has been reported (Avery et al, 2012; Wichuk and McCartney, 2007). According to the results of this study, sustained temperatures over 55°C should result in the inactivation of most pathogenic bacteria found in manure compost. The distribution of heat throughout the compost pile is not uniform and could allow for survival of some organisms.

V. Conclusion

Efficacy of a cell-impermeant stain for distinguishing between viable and non-viable cells is dependent on the selectivity, brightness, excitation/emission spectrum, and toxicity of the compound. Use of the DNA binding dye PI has a number of drawbacks that confound accurate analysis by flow cytometry (Haase and Reed, 2002). Haase and Reed (2002) found PI fluorescence to be affected by stain and cell concentration, and suggested that PI may rapidly leak from cells as has been found by other investigators (Shapiro, 2003). They also found that SytoX green exhibited better coefficients of variation and decreased peak drift, increasing the resolution of cell cycle phases when compared to PI in analysis of yeast cell cycle.

This study highlighted a number of differences in viability staining between *Cryptosporidium* oocysts and *Giardia* cysts, as well as differences related to the cell-impermeant dye being used. SytoX Blue is advantageous compared to PI due to the small spectral overlap reducing the need for instrument compensation, increased permeability of compromised membranes, and availability in different colours to allow multiple colour combinations (Table 19). As the exact mechanisms of oocyst/cyst degradation are not fully known, we are only able to speculate as to the gradual permeabilisation of the oocyst wall

over time requiring longer incubation periods for dye uptake. However, SytoX Blue still performed better than PI at lower dye concentrations and shorter incubation periods. This study demonstrated the effectiveness of flow cytometry as a faster and less subjective means of quantification and viability determination of *Cryptosporidium* and *Giardia* oocysts/cysts compared to microscopy and can be easily adapted for use with dyes such as SytoX, which may be a more accurate estimate of oocyst/cyst viability than PI. Animal infectivity studies will need to be conducted to confirm the accuracy of SytoX staining with respect to viability determination of *Cryptosporidium* oocysts and *Giardia* cysts.

Table 19. Comparison of cell-impermeant nucleic acid binding dyes PI and SytoX.

	Propidium Iodide (PI)	**SytoX (Blue)**
Binding Site	Nucleic acids	Nucleic acids
Excitation/emission (ex/em)	536/617	431/480 (Note: is available in a variety of colours with different ex/em)
Molecular Weight	668.4 g/mol	~400 g/mol
Charge	2 positive charges	3 positive charges
Molar extinction coefficient (how strongly it absorbs light)	~6,000 cm^{-1} M^{-1}	~73,000 cm^{-1} M^{-1}
Quantum yield (emission efficiency, photons emitted:photons absorbed)	~0.2	~0.5

Composting of faeces, such as manure, is a frequent farming practice that allows recycling of organic wastes into a biologically stable, useful product. This process also results in reduction of pathogenic microorganisms found within the faecal matter through

thermal destruction, thereby permitting agricultural use as a soil amendment for crops. Most of the available data on thermal reduction of pathogens during composting focuses on faecal coliforms and traditional indicator organisms, leaving a gap in information on the reduction of other pathogens such as *Listeria, Campylobacter,* and protists *Cryptosporidium* and *Giardia.* Traditional indicators may not necessarily be accurate representations of all pathogens found in animal, on-farm, faecal material.

Studies of the protozoa *C. parvum* and *G. duodenalis* in samples such as soil and manure have been scarce due to the limited methods currently available. This study was able to produce a new method for rapid quantification and viability assessment of these protists in complex matrices such as manure and berries. The possibility of a more accurate cell-impermeant viability dye, SytoX, was also evaluated next to the current standard PI. It is theorized that this method may correlate more closely with actual infectivity of oocysts/cysts. The use of imaging cytometry to investigate dye-permeability of *C. parvum* and *G. duodenalis* as well as other parasites will also be advantageous for understanding the complexities of dye uptake and oocyst/cyst inactivation. Future work should focus on infectivity studies.

The current standard for compost safety (microbiologically) is to test for *Salmonella* spp. The results of this study found *Listeria monocytogenes* to survive longer than all other bacterial species in both manure types at 57.5°C and 60°C, although it did not survive the longest at temperatures below this. The significantly higher D_x-value of EC O157:H7 at 50°C, 52.5°C, 55°C suggests testing for *E. coli* levels should be conducted in conjunction with *Salmonella.* Extrapolation of D_x-values for the bacterial pathogens studied show these pathogens will be eliminated within the current 3-day(s) minimum at 55°C requirement.

Minimal differences were found between cow and chicken manure types, and in these cases D_x-values would still result in reductions acceptable for safety. Therefore different standards for these two manures do not need to be set. Studies should be conducted in other manure types including swine and slurries.

VI. Bibliography

Adam, R.D. (2001). Biology of *Giardia lamblia*. Clin. Microbiol. Rev. *14,* 447-475.

AIbthn, A. (2002). Recycling Biowaste - Human and Animal Health Problems. Acta Veterinaria Scandinavica *43,* S69-S75.

Akterian, S.G., Fernandez, P.S., Hendrickx, M.E., Tobback, P.P., Periago, P.M., and Martinez, A. (1999). Risk analysis of the thermal sterilization process.: Analysis of factors affecting the thermal resistance of microorganisms. Int. J. Food Microbiol. *47,* 51-57.

Allen, K.J., Kovacevic, J., Cancarevic, A., Wood, J., Xu, J., Gill, B., Allen, J.K., and Mesak, L.R. (2013). Microbiological survey of imported produce available at retail across Canada. Int. J. Food Microbiol. *162,* 135-142.

Álvarez-Ordóñez, A., Fernãndez, A., Bernardo, A., and Lã“Pez, M. (2009). Heat-Resistance Prediction Of Listeria Innocua Grown At Different Temperatures. J. Food Saf. *29,* 474-483.

Avery, L.M., Booth, P., Campbell, C., Tompkins, D., and Hough, R.L. (2012). Prevalence and survival of potential pathogens in source-segregated green waste compost. Science of the Total Environment *431,* 128-138.

Bae, W., Kaya, K.N., Hancock, D.D., Call, D.R., Park, Y.H., and Besser, T.E. (2005). Prevalence and Antimicrobial Resistance of Thermophilic Campylobacter spp. from Cattle Farms in Washington State. Appl. Environ. Microbiol. *71,* 169-174.

Barbosa, J., Costa-de-Oliveira, S., Rodrigues, A.G., and Pina-Vaz, C. (2008). Optimization of a flow cytometry protocol for detection and viability assessment of Giardia lamblia. 2nd International Conference of the Journal of Travel Medicine and Infectious Disease. *6,* 234-239.

Barwick, R.S., Mohammed, H.O., White, M.E., and Bryant, R.B. (2003a). Factors Associated with the Likelihood of Giardia spp. and Cryptosporidium spp. in Soil from Dairy Farms 1. J. Dairy Sci. *86,* 784-791.

Barwick, R.S., Mohammed, H.O., White, M.E., and Bryant, R.B. (2003b). Prevalence of Giardia spp. and Cryptosporidium spp. on dairy farms in southeastern New York state. Prev. Vet. Med. *59,* 1-11.

Belosevic, M., Guy, R.A., Taghi-Kilani, R., Neumann, N.F., Gyürék, L.L., Liyanage, L.R.J., Millard, P.J., and Finch, G.R. (1997). Nucleic acid stains as indicators of Cryptosporidium parvum oocyst viability. Int. J. Parasitol. *27,* 787-798.

Bernal, M.P., Alburquerque, J.A., and Moral, R. (2009). Composting of animal manures and chemical criteria for compost maturity assessment. A review. Bioresour. Technol. *100,* 5444-5453.

Black, E.K., Finch, G.R., Taghi-Kilani, R., and Belosevic, M. (1996). Comparison of assays for Cryptosporidium parvum oocysts viability after chemical disinfection. FEMS Microbiol. Lett. *135,* 187-189.

Bower, J.R. (1999). Foodborne Diseases: Shiga Toxin Producing E. coli (STEC). Pediatr. Infect. Dis. J. *18,* 909-910.

Brandl, M.T., Haxo, A.F., Bates, A.H., and Mandrell, R.E. (2004). Comparison of Survival of Campylobacter jejuni in the Phyllosphere with That in the Rhizosphere of Spinach and Radish Plants. Appl. Environ. Microbiol. *70,* 1182-1189.

Briancesco, R., Coccia, A.M., Chiaretti, G., Della Libera, S., Semproni, M., and Bonadonna, L. (2008). Assessment of microbiological and parasitological quality of composted wastes: health implications and hygienic measures. Waste Manage. Res. *26,* 196-202.

Campbell, B.L., Mhlanga, S., and Lesschaeve, I. (2013). Perception versus Reality: Canadian Consumer Views of Local and Organic. Canadian Journal of Agricultural Economics *61,* 531-558.

Campbell, A.T., Robertson, L.J., and Smith, H.V. (1992). Viability of Cryptosporidium parvum oocysts: correlation of in vitro excystation with inclusion or exclusion of fluorogenic vital dyes. Appl. Environ. Microbiol. *58,* 3488-3493.

Carey, C.M., Lee, H., and Trevors, J.T. (2004). Biology, persistence and detection of Cryptosporidium parvum and Cryptosporidium hominis oocyst. Water Res. *38,* 818-862.

Carreno, R.A., Matrin, D.S., and Barta, J.R. (1999). Cryptosporidium is more closely related to the gregarines than to coccidia as shown by phylogenetic analysis of apicomplexan parasites inferred using small-subunit ribosomal RNA gene sequences. Parasitol. Res. *85,* 899-904.

CCME (Canadian Council of Ministers of the Environment). (2005). Guidelines for Compost Quality. 1-26.

CDC. (2012). Foodborne Diseases Active Surveillance Network (FoodNet): FoodNet Surveillance Report 2011 (Final Report).

Ceustermans, A., De Clercq, D., Aersten, A., Michiels, C., Coosemans, J., and Ryckeboer, J. (2007). Inactivation of *Salmonella* Senftenberg strain W 775 during composting of biowastes and garden wastes. Journal of Applied Microbiology *103,* 53-64.

Chappell, C.L., Okhuysen, P.C., Sterling, C.R., and DuPont, H.L. (1996). Cryptosporidium parvum: Intensity of Infection and Oocyst Excretion Patterns in Healthy Volunteers. J. Infect. Dis. *173,* 232-236.

Chãvez-Munguãa, B., Cedillo-Rivera, R., and Martãnez-Palomo, A. (2004). The Ultrastructure of the Cyst Wall of Giardia lamblia. J. Eukaryot. Microbiol. *51,* 220-226.

Clark, C.G., Price, L., Ahmed, R., Woodward, D.L., Melito, P.L., Rodgers, F.G., Jamieson, F., Ciebin, B., Li, A., and Ellis, A. (2003). Characterization of Waterborne Outbreak-associated *Campylobacter jejuni*, Walkerton, Ontario. Emerging Infectious Disease *9,* 1231-1241.

Coburn, B., Grassl, G.A., and Finlay, B.B. (2006). *Salmonella*, the host and disease: a brief review. Immunology and Cell Biology *12,* 112-118.

Deng, M.Y., and Cliver, D.O. (1992). Degradation of *Giardia lamblia* Cysts in Mixed Human and Swine Wastes. Applied and Environmental Microbiology *58,* 2368-2374.

Dorff, E., and Beaulieu, M.S. (2014). Analytical Paper Canadian Agriculture at a Glance: Feeding the soil puts food on your plate.

Doyle, M.P. (2000). Reducing Foodborne Disease: What Are the Priorities? Nutrition *16,* 647-649.

Droffner, M.L., and Brinton, W.F. (1995). Survival of *E. coli* and *Salmonella* populations in aerobic thermophilic composts as measured with DNA gene probes. Zentralblatt Fur Hygiene and Umweltmedizin = International Journal of Hygiene and Environmental Medicine *197,* 387-397.

Erickson, M.C., Liao, J., Ma, L., Jiang, X., and Doyle, M.P. (2009). Inactivation of Salmonella spp. in cow manure composts formulated to different initial C:N ratios. Bioresour. Technol. *100,* 5898-5903.

Erlandsen, S.L., Bemrick, W.J., and Pawley, J. (1989). High-Resolution Electron Microscopic Evidence for the Filamentous Structure of the Cyst Wall in Giardia muris and Giardia duodenalis. J. Parasitol. *75,* 787-797.

Fayer, R. (2004). Cryptosporidium: a water-borne zoonotic parasite. Vet. Parasitol. *126,* 37-56.

Fayer, R., Morgan, U., and Upton, S.J. (2000). Epidemiology of Cryptosporidium: transmission, detection and identification. Int. J. Parasitol. *30,* 1305-1322.

Fischer, J.A., Muller-Weeks, S., and Caradonna, S. (2007). A Pitfall of Propidium Iodide Staining in Fluorescence-Activated Cell Sorting Cell Cycle Analysis? Cancer Res. *67,* 5577-5577.

Franz, E., and van Bruggen, Ariena H. C. (2008). Ecology of E. coli O157:H7 and Salmonella enterica in the Primary Vegetable Production Chain. Crit. Rev. Microbiol. *34,* 143-161.

Franz, E., van Diepeningen, A.D., de Vos, O.J., and van Bruggen, A.H.C. (2005). Effects of Cattle Feeding Regimen and Soil Management Type on the Fate of Escherichia coli O157:H7 and Salmonella enterica Serovar Typhimurium in Manure, Manure-Amended Soil, and Lettuce. Appl. Environ. Microbiol. *71,* 6165-6174.

Franz, E., van Hoek, A.H.A.M., Bouw, E., and Aarts, H.J.M. (2011). Variability of Escherichia coli O157 Strain Survival in Manure-Amended Soil in Relation to Strain Origin, Virulence Profile, and Carbon Nutrition Profile. Appl. Environ. Microbiol. *77,* 8088-8096.

Fremaux, B., Delignette-Muller, M., Prigent-Combaret, C., Gleizal, A., and Vernozy-Rozand, C. (2007). Growth and survival of non-O157:H7 Shiga-toxin-producing Escherichia coli in cow manure. J. Appl. Microbiol. *102,* 89-99.

Fujino, T., Matsui, T., Kobayashi, F., Haruki, K., Yoshino, Y., Kajima, J., and Tsuji, M. (2002). The Effect of Heating against *Cryptosporidium* Oocysts. Journal of Veterinary Medical Science *64,* 199-200.

Fux, C.A., Shirtliff, M., Stoodley, P., and Costerton, J.W. (2005). Can laboratory reference strains mirror 'real-world' pathogenesis? Trends in Microbiology *13,* 58-63.

Gilpin, B.J., Robson, B., Scholes, P., Nourozi, F., and Sinton, L.W. (2009). Survival of *Campylobacter* spp. in bovine faeces on pasture. Letters in Applied Microbiology *48,* 162-166.

Gould, L.H., Mody, R.K., Ong, K.L., Clogher, P., Cronquist, A.B., Garman, K.N., Lathrop, S., Medus, C., Spina, N.L., Webb, T.H.*, et al.* (2013). Increased recognition of non-O157 Shiga toxin-producing Escherichia coli infections in the United States during 2000-2010: epidemiologic features and comparison with E. coli O157 infections. Foodborne Pathogens and Disease *10,* 453+.

Grassl, G.A., and Finlay, B.B. (2008). Pathogenesis of enteric Salmonella infections. Current Opinion in Gastroenterology *24,* 22-26.

Grewal, S., Sreevatsan, S., and Michel, F.C. (2007). Persistence of *Listeria* and *Salmonella* During Swine Manure Treatment. Compost Science & Utilization *15,* 53-62.

Guan, T.Y., and Holley, R.A. (2003). Pathogen Survival in Swine Manure Environments and Transmission of Human Enteric Illness—A Review Sponsoring organizations: Manitoba Livestock Manure Management Initiative and Manitoba Rural Adaptation Council. Journal of Environmental Equality *32,* 383-392.

Haase, S.B. (2001). Cell Cycle Analysis of Budding Yeast Using SYTOX Green. In Current Protocols in Cytometry, John Wiley & Sons, Inc)

Haase, S.B., and Reed, S.I. (2002). Improved Flow Cytometric Analysis of the Budding Yeast Cell Cycle. Cell Cycle *1,* 117-121.

Hamon, M., Bierne, H., and Cossart, P. (2006). *Listeria monocytogenes*: a multifaceted model. Nature Reviews Microbiology *4,* 423-435.

Hartz, T.K., Mitchell, J.P., and Giannini, C. (2000). Nitrogen and Carbon Mineralization Dynamics of Manures and Composts. Hortscience *35,* 209-212.

Hassani, M., Mañas, P., Pagán, R., and Condón, S. (2007). Effect of a previous heat shock on the thermal resistance of Listeria monocytogenes and Pseudomonas aeruginosa at different pHs. Int. J. Food Microbiol. *116,* 228-238.

Hay, J.C. (1996). Pathogen Destruction and Biosolids Composting. Biocycle *37,* 67.

Hoar, B.R., Atwill, E.R., Elmi, C., and Farver, T.B. (2001). An examination of risk factors associated with beef cattle shedding pathogens of potential zoonotic concern. Epidemiol. Infect. *127,* 147-155.

Hsu, B., Wu, N., Jang, H., Shih, F., Wan, M., and Kung, C. (2005). Using the Flow Cytometry to Quantify the Giardia Cysts and Cryptosporidium Oocysts in Water Samples. Environ. Monit. Assess. *104,* 155-162.

Hutchison, M.L., Walters, L.D., Avery, S.M., and Moore, A. (2005a). Decline of zoonotic agents in livestock waste and bedding heaps. J. Appl. Microbiol. *99,* 354-362.

Hutchison, M.L., Walters, L.D., Avery, S.M., Synge, B.A., and Moore, A. (2004). Levels of zoonotic agents in British livestock manures. Lett. Appl. Microbiol. *39,* 207-214.

Hutchison, M.L., Walters, L.D., Moore, T., Thomas, D.J.I., and Avery, S.M. (2005b). Fate of Pathogens Present in Livestock Wastes Spread onto Fescue Plots. Appl. Environ. Microbiol. *71,* 691-696.

Inglis, G.D., and Kalischuk, L.D. (2003). Use of PCR for Direct Detection of Campylobacter Species in Bovine Feces. Appl. Environ. Microbiol. *69,* 3435-3447.

Inglis, G.D., McAllister, T.A., Larney, F.J., and Topp, E. (2010). Prolonged Survival of Campylobacter Species in Bovine Manure Compost. Applied and Environmental Microbiology *76,* 1110-1119.

Islam, M., Doyle, M.P., Phatak, S.C., Millner, P., and Jiang, X. (2004a). Persistence of Enterohemorrhagic *Escherichia coli* O157:H7 in Soil and on Leaf Lettuce and Parsley Grown in Field Treated with Contaminated Manure Composts or Irrigation Water. Journal of Food Protection *67,* 1365-1370.

Islam, M., Morgan, J., Doyle, M.P., Phatak, S.C., Millner, P., and Jiang, X. (2004b). Persistence of *Salmonella enterica* serovar Typhimurium on Lettuce and Parsley and in Soils on Which They Were Grown in Fields Treated with Contaminated Manure Composts or Irrigation Water. Foodborne Pathogens and Disease *1,* 27-35.

Islam, M., Morgan, J., Doyle, M.P., Phatak, S.C., Millner, P., and Jiang, X. (2004c). Fate of Salmonella enterica Serovar Typhimurium on Carrots and Radishes Grown in Fields Treated with Contaminated Manure Composts or Irrigation Water. Appl. Environ. Microbiol. *70,* 2497-2502.

Jenkins, M., Higgins, J., Kniel, K., Trout, J., and Fayer, R. (2004). Protection of Calves against Cryptosporiosis by Oral Inoculation with Gamma-Irradiated Cryptosporidium parvum Oocysts. J. Parasitol. *90,* 1178-1180.

Jenkins, M.B., Eaglesham, B.S., Anthony, L.C., Kachlany, S.C., Bowman, D.D., and Ghiorse, W.C. (2010). Significance of Wall Structure, Macromolecular Composition, and Surface Polymers to the Survival and Transport of Cryptosporidium parvum Oocysts. Applied and Environmental Microbiology *76,* 1926-1934.

Johnson, L.K., Brown, M.B., Carruthers, E.A., Ferguson, J.A., Dombek, P.E., and Sadowsky, M.J. (2004). Sample Size, Library Composition, and Genotypic Diversity among Natural Populations of Escherichia coli from Different Animals Influence Accuracy of Determining Sources of Fecal Pollution. Appl. Environ. Microbiol. *70,* 4478-4485.

Kaper, J.B., Nataro, J.P., and Mobley, H.L.T. (2004). Pathogenic *Escherichia coli*. Nature Reviews Microbiology *2,* 123-140.

Kato, S., and Bowman, D.D. (2002). Using flow cytometry to determine the viability of Cryptosporidium parvum oocysts extracted from spiked environmental samples in chambers. Parasitol. Res. *88,* 326-331.

King, A.M. (2000). Detection of dead cells and measurement of cell killing by flow cytometry. Journal of Immunological Methods *243,* 155-166.

Kozak, G.K., MacDonald, D., Landry, L., and Farber, J.M. (2013). Foodborne Outbreaks in Canada Linked to Produce: 2001 through 2009. J. Food Prot. *76,* 173-83.

Kudva, I.T., Blanch, K., and Hovde, C.J. (1998). Analysis of Escherichia coli O157:H7 Survival in Ovine or Bovine Manure and Manure Slurry. Appl. Environ. Microbiol. *64,* 3166-3174.

Lemunier, M., Francou, C., Rousseaux, S., Houot, S., Dantigny, P., Piveteau, P., and Guzzo, J. (2005). Long-Term Survival of Pathogenic and Sanitation Indicator Bacteria in Experimental Biowaste Composts. Appl. Environ. Microbiol. *71,* 5779-5786.

Lyautey, E., Hartmann, A., Pagotto, F., Tyler, K., Lapen, D.R., Wilkes, G., Piveteau, P., Rieu, A., Robertson, W.J., Medeiros, D.T.*, et al.* (2007). Characteristics and frequency of detection of fecal Listeria monocytogenes shed by livestock, wildlife, and humans. Can. J. Microbiol. *53,* 1158-1167.

Lynch, M.F., Tauxe, R.V., and Hedberg, C.W. (2009). The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. Epidemiology & Infection *137,* 307-315.

Ma, L., Zhang, G., and Doyle, M.P. (2011). Green Fluorescent Protein Labeling of *Listeria*, *Salmonella*, and *Escherichia coli* O157:H7 for Safety-Related Studies. Plos One *6,* e18083. 1-7

Mawdsley, J.L., Brooks, A.E., and Merry, R.J. (1996a). Movement of the protozoan pathogen Cryptosporidium parvum through three contrasting soil types. Biol. Fertility Soils *21,* 30-36.

Mawdsley, J.L., Brooks, A.E., Merry, R.J., and Pain, B.F. (1996b). Use of a novel soil tilting table apparatus to demonstrate the horizontal and vertical movement of the protozoan pathogen Cryptosporidium parvum in soil. Biol. Fertility Soils *23,* 215-220.

Medema, G.J., Schets, F.M., Ketelaars, H.A.M., and Boschman, G. (1998). Improved detection and vital staining of *Cryptosporidium* and *Giardia* with flow cytometry. Water Science and Technology *38,* 61-66.

Meerburg, B.G., and Borgsteede, F.H.M. (2011). Organic agriculture and its contribution to zoonotic pathogens. In Zoonotic Pathogens in the Food Chain, Krause, D. O., and Hendrick, S. eds., (Cambridge, Massachusetts: CAB International) pp. 167.

Mortimer, F.C., Mason, D.J., and Gant, V.A. (2000). Flow Cytometric Monitoring of Antibiotic-Induced Injury in Escherichia coli Using Cell-Impermeant Fluorescent Probes. Antimicrobial Agents Chemother. *44,* 676-681.

Mukherjee, A., Speh, D., Dyck, E., and Diez-Gonzalez, F. (2004). Preharvest Evaluation of Coliforms, *Escherichia coli, Salmonella,* and *Escherichia coli* O157:H7 in Organic and Conventional Produce Grown by Minnesota Farmers. Journal of Food Protection *67,* 894-900.

Mukhopadhyay, P., Rajesh, M., Hasko, G., Hawkins, B.J., Madesh, M., and Pacher, P. (2007). Simultaneous detection of apoptosis and mitochondrial superoxide production in live cells by flow cytometry and confocal microscopy. Nat. Protocols *2,* 2295-2301.

Natvig, E.E., Ingham, S.C., Ingham, B.H., Cooperband, L.R., and Roper, T.R. (2002). Salmonella enterica Serovar Typhimurium and Escherichia coli Contamination of Root and Leaf Vegetables Grown in Soils with Incorporated Bovine Manure. Appl. Environ. Microbiol. *68,* 2737-2744.

Neumann, N.F., Gyürek, L.L., Gammie, L., Finch, G.R., and Belosevic, M. (2000). Comparison of Animal Infectivity and Nucleic Acid Staining for Assessment of Cryptosporidium parvum Viability in Water. Appl. Environ. Microbiol. *66,* 406-412.

Nicholson, F.A., Groves, S.J., and Chambers, B.J. (2005). Pathogen survival during livestock manure storage and following land application. Bioresour. Technol. *96,* 135-143.

Nielsen, E.M. (2002). Occurrence and strain diversity of thermophilic campylobacters in cattle of different age groups in dairy herds. Lett. Appl. Microbiol. *35,* 85-89.

Okhuysen, P.C., Chappell, C.L., Crabb, J.H., Sterling, C.R., and DuPont, H.L. (1999). Virulence of Three Distinct Cryptospovidium parvum Isolates for Healthy Adults. J. Infect. Dis. *180,* 1275-1281.

Olaimat, A.N., and Holley, R.A. (2012). Factors influencing the microbial safety of fresh produce: A review. Food Microbiol. *32,* 1-19.

Oliveira, M., Usall, J., Solsona, C., Alegre, I., Viñas, I., and Abadias, M. (2010). Effects of packaging type and storage temperature on the growth of foodborne pathogens on shredded Romaine lettuce. Food Microbiol. *27,* 375-380.

Oliveira, M., Usall, J., Viñas, I., Solsona, C., and Abadias, M. (2011). Transfer of Listeria innocua from contaminated compost and irrigation water to lettuce leaves. Food Microbiol. *28,* 590-596.

Oliveira, M., Viñas, I., Usall, J., Anguera, M., and Abadias, M. (2012). Presence and survival of Escherichia coli O157:H7 on lettuce leaves and in soil treated with contaminated compost and irrigation water. Int. J. Food Microbiol. *156,* 133-140.

Olson, M.E., Goh, J., Phillips, M., Guselle, N., and McAllister, T.A. (1999). Giardia Cyst and Cryptosporidium Oocyst Survival in Water, Soil, and Cattle Feces. Journal of Environmental Quality *28,* 1991-1996.

Olson, M.E., O'Handley, R.,M., Ralston, B.J., McAllister, T.A., and Andrew Thompson, R.C. (2004). Update on Cryptosporidium and Giardia infections in cattle. Trends Parasitol. *20,* 185-191.

Ortega, Y.R., and Adam, R.D. (1997). Giardia: Overview and Update. Clinical Infectious Diseases *25,* 545-550.

Pell, A.N. (1997). Manure and Microbes: Public and Animal Health Problem? J. Dairy Sci. *80,* 2673-2681.

Pietronave, S., Fracchia, L., and Martinotti, M.G. (2002). Researchers Analyze How Microorganisms Suppress Pathogen Regrowth. Biocycle *43,* 57.

Platz, S. (1978). Survival of pathogenic bacteria and protozoa after short-time composting of poultry manure. In Animal and human health hazards associated with the utilization of animal effluents, Kelly, W. R. ed., (United Kingdom: Commission of the Eurpoean Communities) pp. 209-215.

Pleitner, A.M. (2011). Heat Tolerance Mechanisms of an Exceptional Strain of *Escherichia coli*.1-96.

Ralston, B.J., McAllister, T.A., and Olson, M.E. (2003). Prevalence and infection pattern of naturally acquired giardiasis and cryptosporidiosis in range beef calves and their dams. Vet. Parasitol. *114,* 113-122.

Ramaswamy, V., Cresence, V.M., Rejitha, J.S., Lekshmi, M.U., Dharsana, K.S., Prasad, S.P., and Vijila, H.M. (2007). *Listeria* - review of epidemiology and pathogenesis. Journal of Microbiology Immunology and Infection *40,* 4-13.

Ravel, A., Greig, J., Tinga, C., Todd, E., Campbell, G., Cassidy, M., Marshall, B., and Pollari, F. (2009). Exploring Historical Canadian Foodborne Outbreak Data Sets for Human Illness Attribution. J. Food Prot. *72,* 1963-1976.

Rhoades, J.R., Duffy, G., and Koutsoumanis, K. (2009). Prevalence and concentration of verocytotoxigenic Escherichia coli, Salmonella enterica and Listeria monocytogenes in the beef production chain: A review. Food Microbiol. *26,* 357-376.

Rimhanen-Finne, R., Vuorinen, A., Marmo, S., Malmberg, S., and HÃ¤nninen, M.-. (2004). Comparative analysis of Cryptosporidium, Giardia and indicator bacteria during sewage sludge hygienization in various composting processes. Lett. Appl. Microbiol. *38,* 301-305.

Roth, B.L., Poot, M., Yue, S.T., and Millard, P.J. (1997). Bacterial Viability and Antibiotic Susceptibility Testing with SYTOX Green Nucleic Acid Stain. Applied and Environmental Microbiology *63,* 2421-2431.

Rynk, R., van de Kamp, M., Willson, G.G., Singley, M.E., Richard, T.L., Kolega, J.J., Gouin, L., Laliberty, J., Kay, D., Murphy, D., Hoitink, H.A.J., and Brinton, W.F. (1992). *On-Farm Composting Handbook* (Ithaca, NY: NRAES-54. Natural Resource, Agriculture, and Engineering Service).

Schumann, R., Schiewer, U., Karsten, U., and Rieling, T. (2003). Viability of bacteria from different aquatic habitats. II: Cellular fluorescent markers for membrane integrity and metabolic activity.*32,* 14.

Schupp, D.G., and Erlandsen, S.L. (1987). A new method to determine Giardia cyst viability: correlation of fluorescein diacetate and propidium iodide staining with animal infectivity. Appl. Environ. Microbiol. *53,* 704-707.

Shapiro, H.M. (2003). Parameters and probes. In Practical Flow Cytometry, Wiley, New York) pp. 273.

Sharpley, A., and Moyer, B. (2000). Phosphorus Forms in Manure and Compost and Their Release during Simulated Rainfall. J. Environ. Qual. *29,* 1462-1469.

Shepherd Jr., M.W., Singh, R., Kim, J., and Jiang, X. (2010). Effect of heat-shock treatment on the survival of Escherichia coli O157:H7 and Salmonella enterica Typhimurium in dairy manure co-composted with vegetable wastes under field conditions. Bioresour. Technol. *101,* 5407-5413.

Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P.A., and Teixera, P. (2011). *Campylobacter* spp. as a foodborne pathogen: a review. Frontiers in Microbiology *2,* 1-12.

Singh, R., Jiang, X., and Luo, F. (2010). Thermal Inactivation of Heat-Shocked *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in Dairy Compost. J. Food Prot. *73,* 1633-1640.

Singh, R., Kim, J., Shepherd, M.W., Luo, F., and Jiang, X. (2011). Determining Thermal Inactivation of Escherichia coli O157:H7 in Fresh Compost by Simulating Early Phases of the Composting Process. Applied Environmental Microbiology *77,* 4126-4135.

Sinton, L.W., Braithwaite, R.R., Hall, C.H., and Mackenzie, M.L. (2007). Survival of Indicator and Pathogenic Bacteria in Bovine Feces on Pasture. Appl. Environ. Microbiol. *73,* 7917-7925.

Smelt, J.P.P.M., and Brul, S. (2014). Thermal Inactivation of Microorganisms. Crit. Rev. Food Sci. Nutr. *54,* 1371-1385.

Smith, S.E., Maurier, J.L., Orta-Ramirez, A., Ryser, E.T., and Smith, D.M. (2001). Thermal Inactivation of *Salmonella* spp., *Salmonella typhimurium* DT104, and *Escherichia coli* O157:H7 in Ground Beef. Journal of Food Science *66,* 1164-1168.

Smith, A.L., and Smith, H.V. (1989). A comparison of fluorescein diacetate and propidium iodide staining and in vitro excystation for determining Giardia intestinalis cyst viability. Parasitology *99,* 329-331.

Solomon, E.B., Yaron, S., and Matthews, K.R. (2002). Transmission of Escherichia coli O157:H7 from Contaminated Manure and Irrigation Water to Lettuce Plant Tissue and Its Subsequent Internalization. Appl. Environ. Microbiol. *68,* 397-400.

Sörqvist, S. (2003). Heat Resistance in Liquids of *Enterococcus* spp., *Listeria* spp., *Escherichia coli*, *Yersinia entercolitica*, *Salmonella* spp., and *Campylobacter* spp. Acta Veterinaria Scandinavica *44,* 1-19.

Sörqvist, S. (1989). Heat resistance of *Campylobacter* and *Yersinia* strains by three methods. Journal of Applied Bacteriology *67,* 543-549.

Spinks, A.T., Dunstan, R.H., Harrison, T., Coombes, P., and Kuczera, G. (2006). Thermal inactivation of water-borne pathogenic and indicator bacteria at sub-boiling temperatures. Water Res. *40,* 1326-1332.

Statistics Canada. (2011). Tables by subject: food and nutrition, food available, by major food groups. Available at: http://www5.statcan.gc.ca/cansim/a47.*2014,* 1.

Thomas, K.M., Murray, R., Flockhart, L., Pintar, K., Pollari, F., Fazil, A., Nesbitt, A., and Marshall, B. (2013). Estimates of the Burden of Foodborne Illness in Canada for 30 Specified Pathogens and Unspecified Agents, Circa 2006. Foodborne Pathogens and Disease *10,* 639-648.

Thomas, M.K., Majowicz, S.E., Pollari, F., and Sockett, P.N. (2008). Burden of Acute Gastrointestinal illness in Canada, 1999-2007: Interim Summary of NSAGI Activities.*2014,* 2.

van Asselt, E.D., and Zwietering, M.H. (2006). A systematic approach to determine global thermal inactivation parameters for various food pathogens. Int. J. Food Microbiol. *107,* 73-82.

van Elsas, J.D., Semenov, A.V., Costa, R., and Trevors, J.T. (2011). Survival of Escherichia coli in the environment: fundamental and public health aspects. Isme j *5,* 173-183.

Van Herk, F.H., McAllister, T.A., Cockwill, C.L., Guselle, N., Larney, F.J., Miller, J.J., and Olson, M.E. (2004). Inactivation of Giardia Cysts and Cryptosporidium Oocysts in Beef Feedlot Manure By Thermophilic Windrow Composting. Compost Sci. Util. *12,* 235-241.

Vesey, G., Slade, J.S., Byrne, M., Shepherd, K., Dennis, P.J., and Fricker, C.R. (1993). Routine monitoring of *Cryptosporidium* oocysts in water using flow cytometry. Journal of Applied Bacteriology *75,* 87-90.

Vesey, G., Hutton, P., Champion, A., Ashbolt, N., Williams, K.L., Warton, A., and Veal, D. (1994). Application of flow cytometric methods for the routine detection of Cryptosporidium and Giardia in water. Cytometry *16,* 1-6.

Wallis, P.M., Erlandsen, S.L., Isaac-Renton, J., Olson, M.E., Robertson, W.J., and van Keulen, H. (1996). Prevalence of Giardia cysts and Cryptosporidium oocysts and characterization of Giardia spp. isolated from drinking water in Canada. Appl. Environ. Microbiol. *62,* 2789-2797.

Warriner, K., Huber, A., Namvar, A., Fan, W., and Dunfield, K. (2009). Chapter 4 Recent Advances in the Microbial Safety of Fresh Fruits and Vegetables. In Advances in Food and Nutrition Research, Steve L. Taylor ed., Academic Press) pp. 155-208.

Waterman, S.C. (1982). The heat-sensitivity of *Campylobacter jejuni* in milk. Epidemiology & Infection *88,* 529-533.

Wichuk, K.M., and McCartney, D. (2007). A review of the effectiveness of current time-temperature regulations on pathogen inactivation during composting. Journal of Environmental Engineering and Science *6,* 573-586.

Williams, N.C., and Ingham, S.C. (1997). Changes in Heat Resistance of *Escherichia coli* O157:H7 Following Heat Shock. Journal of Food Protection *60,* 1128-1131.

Yang, H., Li, Y., and Johnson, M.G. (2001). Survival and Death of *Salmonella* Typhimurium and *Campylobacter jejuni* in Processing Water and on Chicken Skin during Poultry Scalding and Chilling. J. Food Prot. *64,* 770-776.

You, Y., Rankin, S.C., Aceto, H.W., Benson, C.E., Toth, J.D., and Dou, Z. (2006). Survival of Salmonella enterica Serovar Newport in Manure and Manure-Amended Soils. Appl. Environ. Microbiol. *72,* 5777-5783.

Young, K.T., Davis, L.M., and DiRita, V.J. (2007). Campylobacter jejuni: molecular biology and pathogenesis. Nature Reviews Microbiology *5,* 665-679.

Zhao, H., Oczos, J., Janowski, P., Trembecka, D., Dobrucki, J., Darzynkiewicz, Z., and Wlodkowic, D. (2010). Rationale for the real-time and dynamic cell death assays using propidium iodide. Cytometry *77A,* 399-405.

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