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Spatial and Temporal Distribution of Microbial Pathogens in Poultry Litter and the Development of Microbial Inactivation Constants in Waste Application

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By

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The increase in production farming, also known as concentrated animal feeding operations (CAFOs), garners more investigations on the implications to public health regarding the disposal of the wastes of food production animals. In addition to the vast amount of animal manure produced, human biosolids is another waste residual that must be managed. The research focus was the sustainability of foodborne pathogens in waste products and the variables that manipulate these environments such as moisture, temperature, organic matter and time.

The first study was designed to analyze spatial differences in microbial populations in broiler litter by investigating the relationship of intra-house location, age of flock, bedding moisture, and seasonality. Antibiogram profiles of selected isolates were explored to determine if antibiotic resistant bacteria are common in these environments and if multiple class resistance is present. These findings provided insight

into new targets that may reduce zoonotic bacteria that are problematic from a food safety prospective as well as nuisance bacteria that threaten broiler health.

The second study was designed to establish current decay rates of viral and bacterial pathogens when seeded in various waste residuals and the effects soil type and application method have on those rates. Decay rates were established by standard culture and molecular methods, such as qPCR. A comparison of both derived inactivation rates were analyzed to determine if these methods were significantly different. Both cultural and molecular methods have limitation and advantages, and the argument that both are useful and needed is asserted. The decay rates associated with each method were used to simulate a one-time exposure to a land application site to assess the microbial risk of *Salmonella* using a Quantitative Microbial Risk Assessment model.

DEDICATION

This work is dedicated to my extraordinary family for all their love, encouragement, patience, and belief that all things are possible: to my husband, James Allen, who has always made this pursuit so much easier, and to my children, Jon Austin, Ethan and Jesse, all of this effort is for you. I appreciate the sacrifices you have made and the words of encouragement when it seemed overwhelming at times.

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CHAPTER I

LITERATURE REVIEW U.S. WASTE MANAGEMENT PRACTICES AND PROBLEMS IN THE 21ST CENTURY

Introduction

Food security has improved through the development of better agricultural practices in the U.S. Farming has become more efficient due to technological advances in farm equipment, farming practices, and genetic improvements in crop plants and food animals. A higher quantity and quality food product can now be produced with less input needed both in crop and animal production, which is called economy of scale. For example, in 1920, the average poultry farmer needed 16 weeks to produce a 2 pound broiler; today, it takes approximately 7 weeks to produce a 5 pound broiler (Lacy, 2000). The improvements to agriculture have reached all farming commodities including swine, poultry, dairy, and beef cattle. The dynamics of our farming system have changed since 1940 to comprise fewer but larger farming operations. From 1982 to 1997, the number of livestock farming operations declined by 24% in the United States (Sims and Maguire 2004). Concurrently, livestock farms trended toward raising larger numbers (200-1000+) of animals in more densely populated confinement operations, now known as concentrated animal feeding operations (CAFOs). As the efficiency of farming has improved, manure management practices have been adapted to the CAFO methods. There are approximately 238,000 operational animal feeding operations (AFOs)

(Burkholder et al., 2007; Dungan, 2010) and close to 20,000 CAFOs (USEPA, 2010) operating in the U.S. In the U.S. alone, more than 100 million dry tons of manure are produced per year (Burkholder et al., 2007). Manure comprises animal wastes (feces and urine), and waste-contaminated bedding material that is produced during animal farming operations. Traditionally manures were disposed of through on-farm land application as fertilizer. The large amounts of manure produced by CAFOs, limited land availability and high transportation costs have increased the difficulty of manure disposal.

To add to these previously stated problems, there are 16,000 municipal wastewater treatment plants in the U.S. producing 7 million dry tons of biosolids annually (USEPA, 1999). Biosolids are defined by the Environmental Protection Agency (EPA) as "the primarily organic solid product yielded by municipal wastewater treatment processes that can be beneficially recycled" (USEPA, 1995). The reuse of these wastes must be managed to provide sufficient nutrients for crop production without causing environmental harm. The purpose or use of the land application site dictates the restrictions and regulations which apply (USEPA, 1995). To maintain environmental and public health, animal and human waste must be properly managed.

The use of waste as fertilizer has been practiced for centuries. Both manures and biosolids can be beneficial soil amendments providing organic materials, arable composition and increased water capacity that in turn increases crop growth, which is economically and ecologically advantageous (Bhattarai et al., 2011; USEPA, 1999). Waste management requires not only the disposal of these byproducts but the attenuation or reduction of pathogens in the wastes. Manure and biosolid management practices have come under increased scrutiny in recent years. With new outbreaks of foodborne illness

(Berger et al., 2010; CDC, 2012), many people suspect land application of wastes to be the source of the problem. Fecal contamination is known to cause foodborne and waterborne illnesses but often the mode of contamination is unknown. To examine the research challenges associated with waste management, this literature review examines the pathogens associated with wastes, environmental health concerns, known illness outbreaks due to fecal contamination, and methods practiced to attenuate pathogens in waste materials. Disparities and gaps in current knowledge will be highlighted to identify areas for future research that may lend to a better understanding of waste management and challenges.

Pathogens in Wastes

Land application enables manure and biosolids to be utilized to provide nitrogen, phosphorus and organic matter for crops. The disadvantage of this practice is the potential to pollute groundwater, surface water, and soil if application rates are not appropriately managed. Specific EPA rules apply to biosolids according to the pathogen reduction processes employed (USEPA, 1995). Their objective is to protect the environment and public from pathogen exposures. If not managed correctly, nitrogen and phosphorus, in addition to pathogens, can contaminate water supplies. According to the World Health Organization (WHO), the primary source of contamination that poses the greatest problem in water systems is feces from animals or humans (WHO, 2004). The most recent United States Geological Survey (USGS) publication of impaired water systems lists about 40,000 as impaired, with approximately 25% of the impairments due to pathogen contamination (USGS, 2012).

Pathogens in waste include bacteria, viruses and parasites. In the U.S., six pathogens (*Salmonella*, *Listeria*, Toxoplasma, Norovirus, *Campylobacter* and *Escherichia coli* O157:H7) account for 90% of food-related deaths of known etiology (Mead et al., 1999). Scallan et al. (2011) agrees with these estimates but includes *Clostridium* spp. to this list of pathogens. In food related illnesses, these pathogens are transmitted by the fecal-oral route, but the source of food contamination is often difficult to trace. Although some pathogens are species specific, many pathogens, especially bacteria, can infect or cause disease in both animals and humans. Some wastes may have pathogens that are only attributable to a specific type of waste. For example, Hepatitis A is only attributable to biosolids, but *Salmonella* can be isolated from most wastes. Common pathogens and their associated wastes are listed in Table 1. Transmission of zoonotic pathogens is a public health concern, but little is known about the impact of CAFOs on this transmission. Several pathways can lead to transmission between animals and humans (Figure 1.1). Transmission of zoonotic pathogens can be facilitated by fomites, insect vectors, bioaerosols, and improper disposal of fecal matter leading to contaminated food crops or water-systems (USEPA, 1995). Understanding the risks associated with the pathogens that are harbored in these wastes could lead to more effective agricultural management practices. For example, more current information on pathogen decay rates in agricultural environments would improve our understanding of pathogen persistence in these environments and our assessment of risk from potential public exposure. Though numerous studies have documented the persistence of pathogens in manures and biosolids, a side-by-side comparison of bacterial and viral

pathogen survival in these wastes within shared agricultural matrices and environmental conditions has not been reported in the scientific literature.

Zoonotic Bacterial Pathogens in Biosolids and Manures:

Four of the six pathogens that account for the majority of annual food-related deaths in the U.S. are bacterial (Mead et al., 1999; Scallan et al., 2011). *Salmonella,* being one the most prevalent bacterial pathogens, has been isolated from 2 – 3% of fecal samples of cull sows analyzed (McKean et al., 2001). This incidence seems relatively low; however, positive isolation of *Salmonella* from sow carcasses was 41% of samples collected after transport to slaughter facility in this same study (McKean et al., 2001). *E. coli* O157:H7 was found at the similar levels (< 5%) in fecal samples of dairy cattle (Pell, 1997). Studies show that bacterial shedding in excrement is higher during animal stress (Freestone and Lyte, 2010; Volkova et al., 2011). In addition, bacteria are affected by seasonal variations and can be present at higher levels in animal feces during the specific times of the year (Hutchison et al., 2005). For example, *E. coli* O157 was frequently at higher levels during the summer, but *Campylobacter* peaked during winter months (Hutchison et al., 2005). Some zoonotic bacterial pathogens may elicit no adverse effects in their animal hosts, and may be part of the normal gut flora. *E. coli* is commonly found in the guts of all mammals and birds and is present in their excrement at levels of 6 $\log_{10}$ cfu g^{-1} of manure. *E. coli* O157:H7, however, is unique among strains of *E. coli* and is highly pathogenic and destructive when ingested by humans.

From 1993 to 1997, *Salmonella* alone accounted for 55% of foodborne illnesses caused by bacteria with known etiology (Olsen et al., 2000). Ten years later in 2008, this *Salmonella* statistic was approximately the same at 57% of the bacterial derived

foodborne illnesses (MMWR, 2011). Animal reservoirs allow these bacteria to survive and to cause zoonotic illness as humans consume fecal-contaminated crops, water, or other food products. These pathogens may also contaminate other surfaces (i.e. fomites) such as farmer's boots, animal stalls, and farm equipment, which may facilitate bacterial transfer (Vacheyrou et al., 2011; Volkova et al., 2011). Most bacterial pathogens survive less than 2 months in environments outside their hosts, but given adequate conditions some can survive almost 3 times longer (Gerba and Smith, 2005; Hutchison et al., 2005).

Campylobacter is a prominent bacterium that can be isolated from most livestock animals (Table 1). Hutchison et al. (2005) found that the levels of *Campylobacter* were consistent regardless of animal type. *Campylobacter* may be present in fecal material but difficult to enumerate. Due to reduced nutrients and stress responses, this pathogen, along with others, can become viable but not culturable (VBNC), making it difficult to get a representative level of bacterial populations. Direct molecular detection techniques, that do not rely on culturing, may overcome these limitations and allow better investigative analysis. Topp et al. (2009) estimated the risk of infection with *Campylobacter,* due to post-exposure of cattle manure applied to land, to be 1:100,000 assuming that a 3-log reduction was achieved by composting or other storage methods.

Listeria monocytogenes accounts for about 1600 illnesses each year in the U.S. and about 255 deaths (Scallan et al., 2011). Compared to other foodborne pathogens, *Listeria* has a relatively high mortality rate. The recent 2011 *Listeria* outbreak due to consumption of contaminated cantaloupe resulted in 20% mortality with 146 cases of infection and 30 deaths (MMWR, 2011). *Listeria* enumerated in various manures (swine, poultry, cattle, sheep) have been found at levels of 2 – 3 $\log_{10}$ cfu g^{-1} (Hutchison et al.,

2005) and around 2.3 $\log_{10}$ MPN g^{-1}(dry wt.)(Garrec et al., 2003). *Listeria* persist over a wide range of temperatures from 4 - 37 ^{o}C, making it a dangerous pathogen able to remain viable in a variety of environmental conditions. It proliferates at refrigeration temperatures and causes late term abortion in women.

Clostridium perfringens is considered an emerging pathogen (Moore and Gross, 2010) This spore-forming bacterium is commonly found in soil and in feces of many animal species. Sporulation allows this bacterium to endure stressful environments and proliferate when more advantageous conditions return. Brooks et al. (2009) determined *C. perfringens* levels to be 5 $\log_{10}$ cfu g^{-1} in poultry litter, and McLaughlin et al. (2009) reported similar levels in swine manure lagoon effluent. In the latter report, *C. perfringens* was the highest enumerated pathogen. *C. perfringens* accounts for 10% of the foodborne related illnesses in the U.S. (Scallan et al., 2011).

Viral Pathogens in Biosolids and Manure:

Viruses typically are host specific and can cause infections via fecal/oral routes of exposure. However, phylogenetic comparisons of Hepatitis E variants were similar in both humans and swine (Meng et al., 1997). Enteric viruses are commonly found in fecal waste, more often in biosolids than manure. Viruses common in biosolids include norovirus, adenovirus, enterovirus, hepatitis A and E, and rotavirus (Viau et al., 2011; Wong et al., 2010).

Norovirus are the most prominent gastrointestinal foodborne disease causing viruses, and account for more than 50% of all gastroenteritis across the globe (MMWR, 2011; Scallan et al., 2011) and 95 - 99% of all viral gastroenteritis cases (Karst, 2010; Mead et al., 1999; Scallan et al., 2011). Because low doses of viral particles induce

infection, fecal contamination with norovirus has the potential to infect many individuals. The replication of norovirus in the intestine of an infected individual is so efficient that 11 log10 viral particles g-1 can be isolated from feces (MMWR, 2011). It is estimated that 21 million norovirus infections occur per year in the U.S. (MMWR, 2011). The prominence of norovirus infections compared with other etiologies associated with foodborne outbreaks, is illustrated by the CDC (Figure 1.2) (MMWR, 2009; MMWR, 2010). In addition to the large number of viral particles shed in fecal matter another factor that assures transmissibility is viral stability in a range of environments. Fecal-oral exposures can occur via compromised water-systems and food sources, or by secondary transmission. Contaminated irrigation systems (Seymour and Appleton, 2001) and poor hygiene among food crop handlers (Berger et al., 2010) are two modes of transmission in agricultural environments. Secondary (person to person) spread is the most common mode of transmission. For instance, the initial fecal contamination of food or water can lead to a series of infections if not contained (MMWR, 2011). Studies conducted to determine groundwater quality across the U.S. revealed that 20% of the samples contained viruses from fecal contamination (ASM, 2000). Enteric viruses cannot replicate in water but remain viable and can cause disease if ingested (Li et al., 1998). Molecular detection of noroviruses allows investigators to track sources of contamination; however, it cannot distinguish viable and nonviable virus particles in environmental samples.

Using molecular methods, the concentration of norovirus in biosolids after mesophilic anaerobic digestion was found at levels of 4.5 log10 genomic units (GU) (Viau et al., 2011). This confirms that norovirus can be detected at relatively high levels,

although the quantitative measure of viable viral particles is unknown. The high prevalence of norovirus is evidence of its survival in the environment outside its host.

Hepatitis A virus (HAV) is an enteric virus that is commonly contracted by eating fecal-contaminated fruits, vegetables, or shellfish. It is difficult to trace HAV due to some infected individuals remaining asymptomatic yet infectious and shedding the virus (Pepper et al., 2000). Mead et al. (1999) estimated that 4,170 people in the U.S. are infected with foodborne HAV each year, but new estimates have determined that approximately 1600 foodborne illnesses are caused by HAV (Scallan et al., 2011). Approximately twice that estimate is laboratory confirmed each year, but most are associated with travel outside of the U.S. (Scallan et al., 2011). Outbreaks have been due to imported fruits or vegetables from countries that lack the same sanitary standards imposed in the U.S. Contaminated irrigation water can spread pathogens on ready-to-eat crops, as in the 2003 HAV outbreak associated with green onions from Mexico (Amon et al., 2005).

Members of the enterovirus group include enterovirus, coxsackievirus, poliovirus and echovirus. These viruses, along with adenoviruses and rotaviruses, have been isolated from biosolids, but have not been found in manure. The risks associated with these viruses and transmission via land application of biosolids are not clearly understood. Gerba et al. (2002) investigated the UV light inactivation rates of enteric viruses and found that adenoviruses were most resistant to UV light. The resistance to UV light enables this enteric pathogen to remain viable when other viruses are inactivated, which may pose human health risks if viruses are present in land-applied biosolids. Adenovirus can be found in biosolids at levels of 5.0 x 105 genomic units

(GU) g-1 (dry) (Viau and Peccia, 2009). Adenovirus was isolated from 100% of the raw sewage samples (Symonds et al., 2009) and 88% of Class B biosolids samples (Viau and Peccia, 2009). Adenovirus may be a good fecal indicator for enteric viruses (Symonds et al., 2009). Borchardt et al. (2003) were able to detect norovirus, HAV, enterovirus and rotavirus by qPCR in residential well water near land application sites in Wisconsin. Two concerns with these results are the question of viability of virus particles and the inability to isolate these viruses in subsequent water samples. Nevertheless, their finding that 8% of the samples were positive for viral contamination is a concern (Borchardt et al., 2003).

Parasites in Biosolids and Manure:

Parasites are another known threat that is often caused by fecal contamination of water. The most well-known parasites are *Giardia, Cryptosporidium*, *Toxoplasma gondii, and Cyclospora*. Some parasites can live in the soil for up to 24 months (Gerba and Smith, 2005). The parasite *Giardia* can be shed by infected persons via feces in concentrations as high as 10^{10} cysts $gram^{-1}$ (CDC, 2011). Due to low dose-response and high numbers of parasites shed in the feces of infected individuals, the risk of secondary infections is high. Incidence of *Giardia* exposure is twice as high in the summer months of June to October than in January to March (CDC, 2011). Rose et al. (1991) found that 26 to 43 % of surface waters tested were positive for *Giardia*. *Cryptosporidium* is a parasite which causes gastroenteritis in humans when very few oocysts are ingested. It is resistant to normal disinfectants such as chlorine and is stable in the environment, especially in water. *Cryptosporidium* is also resistant to lime stabilization, a common practice used to reduce pathogens in biosolids (Bean et al., 2007). Bartels et al. (2010)

found that *Cryptosporidium* was isolated from 43% of the 1-2 week old calves they tested that had diarrhea. In 1993, the largest waterborne outbreak in the United States occurred when approximately 403,000 people were infected with this parasite with 54 deaths (Curriero et al., 2001; Hoxie et al., 1997). Investigation of this outbreak determined that excessive rainfall caused pathogen infiltration from fecal contaminated surface waters to the local water supply. The affected water-system could not filter out the parasite and the chlorine treatments used had little or no effect on the parasite (Hoxie et al., 1997). From 1997-2006, approximately 13% of the gastroenteritis-associated waterborne outbreaks, and those which were due to untreated water sources such as lakes, creeks, and ponds, were caused by *Cryptosporidium* (Yoder et al., 2008). This parasite is more commonly contracted during warm summer months coinciding with increased exposure to recreational waters, including swimming pool water, despite chlorine treatment (Pepper et al., 2000).

Cyclospora is another parasite that can be contracted by ingestion of fecal contaminated food or water. Cyclospora infections from fecal contamination of food and water are not well documented in the United States, but in less developed countries poor sanitation practices have led to contamination of exported fruit (Manuel et al., 2000; Wright et al., 2011). This type of contamination on raspberries imported from Guatemala was linked to a 1996 U.S. and Canadian outbreak of *Cyclospora* (Manuel et al., 2000). The spread of new and potentially pathogenic microbes from contamination of water or food is a growing concern; moreover, increasing globalization of food markets intensifies this potential threat. It is now commonplace to purchase produce out of season. The United States routinely imports fresh foods directly from South and Central America and

Mexico, increasing the potential for introduction of zoonotic parasites and pathogens to U.S. fresh markets and also to U.S. agriculture (Manuel et al., 2000; Wright et al., 2011). Few countries impose U.S. quality standards, however, under FDA guidelines, *Good Manufacturing Practices* (GMP) are imposed on imported products to ensure that food is safe and sanitary (USDA, 1999). Adherence to FDA regulations on pesticides, fungicides, and herbicides is monitored by collecting samples and analyzing their presence on food products. However, similar restrictions on fertilizer residues, including organic residuals from biosoloids and manure, are not included (USDA, 1999).

Environmental Concerns

Emerging Pathogens

Fecal contamination of food crops have caused and continues to cause multiple outbreaks and many deaths (Calvin, 2007; MMWR, 2011; Pell, 1997; Scallan et al., 2011). Many of the foodborne pathogens require very few microorganisms (10 – 100 cells) to induce illness. Therefore, when these pathogens find their way onto our table, many individuals can become ill or die. Gaps in food safety may occur, for example, when previously unrecognized or new pathogens or stains emerge. A recent emerging bacteria, *E. coli* O104, affected European countries in 2011. This strain of *E. coli* had been isolated several years prior but subsequently expressed a new virulence factor (Kunne et al., 2012). Furthermore, expression of new virulence factors is not limited to bacteria; viruses are known to rapidly evolve in response to natural selection pressures. Avian influenza emerged at the end of the 20th century but concern about this zoonotic illness has peaked in the last 10 years. New cases of human avian influenza were identified in Viet Nam, Egypt, and Indonesia in 2012. Swine influenza has also been of

concern. Viral transmissibility and waste management concerns make these viruses a focus for emerging pathogens (Ziemer et al., 2010). Although many of these cases were due to close interaction with infected animals, the zoonotic transmission of these pathogens is poorly defined.

Bioaerosols

Exposure to bioaerosols generated by land application practices and large farming operations is a problem that regulatory agencies within the United States Department of Agriculture (USDA) and the EPA have addressed. CAFOs, especially swine farms are primary targets for odor emissions complaints and regulations (Miner, 1999; Schiffman et al., 1995). According to a recent American Society of Microbiology (ASM) publication, the community risk (chances of an individual becoming ill) due to land application of swine manure lagoon effluent is less than 1:1,000,000 (ASM, 2011). Residents living near biosolids land application sites have reported that their health was compromised due to contact with foul odors and bioaerosols from land application (Lewis et al., 2002). Although individuals living near CAFOs have complained of becoming ill and have attributed the illnesses to CAFO-generated air pollution, Brooks et al. (2005) quantified microbial risks of land application and reported little risk for persons near fields receiving recycled wastes.

Prevalence of Food and Water Borne Disease

All foodborne or waterborne diseases are not directly related to land application or waste disposal, but research to better understand these agronomic practices may reduce the risk of future outbreaks. There are several different scenarios associated with farming

practices that have been suspected to compromise water-systems and food crops and cause potential outbreaks (Heaton and Jones, 2008). Points of interest are CAFO waste management practices including manure removal and storage, feral animal interactions with livestock and nearby crops, land application of biosolids and manure, water drainage and irrigation systems, and the hygiene practices of workers (Heaton and Jones, 2008). The importance of preventing future outbreaks is evident in the frequency and severity of past events. Annually, approximately 9.4 million illnesses, 56,000 hospitalizations and 1,350 deaths can be attributed to foodborne related illnesses alone in the U.S. (Scallan et al., 2011). Figure 1.2 depicts the number of foodborne outbreaks reported in 2006 and 2007 and the respective pathogens, if known. According to the CDC (MMWR, 2009; MMWR, 2010), over 700 outbreaks of unknown etiology occurred in 2006 and 2007. The number of unknown etiologies is higher than all known pathogens except norovirus; this data emphasizes the need for more research on new techniques to rapidly identify pathogens.

Since 1971, the CDC and the EPA have collaborated to set up a surveillance system to help regulate and monitor waterborne illnesses (Brunkard et al., 2011). According to Reynolds et al. (2008), 575,457 people have become ill due to waterborne diseases and 79 have died in 764 recorded outbreaks that occurred over the period from 1971 to 2002. These numbers are significantly lower than current estimates due to lack of reporting or individuals not seeking medical attention. The CDC estimates that up to 900,000 cases of disease and 900 deaths occur per year due to waterborne outbreaks in the United States (ASM, 2000). Contaminated water systems and foodborne related

outbreaks have caused many diseases and brought more scrutiny to agriculture and water systems.

Waste Associated Outbreaks

Outbreaks potentially associated with breaches in waste management practices or processing in the field is a topic which has garnered interest. The unknown link that has enabled recent outbreaks in agriculture has many public health advocates seeking better understandings of the proliferation of bacterial and viral pathogens in the environment. For example, a new and emerging enterohemorrhagic *Escherichia coli* (EHEC) strain, *E. coli* O104:H4, caused concerns in Germany and several other European countries. The foodborne pathogen caused 4,321 people across Europe to become ill and 50 died (RKI, 2011). Globalization has changed the face of how these types of outbreaks have to be managed. Several countries were affected by this outbreak including Canada and the United States which traced the *E. coli* O104 transmission to people that had recently traveled to Germany. Approximately 852 patients with hemolytic uremic syndrome (HUS) were diagnosed due to the outbreak (RKI, 2011). HUS is a condition that develops as a progression of the infection in approximately 5-10% of those infected (Bower, 1999). Public officials originally warned that raw produce should not be eaten until further investigation had been completed. All bans on produce were eventually lifted, but the public had been advised that bean sprouts and seed sprouts should not be consumed. Sprouts were the most likely mode of transmission. This EHEC outbreak was the second largest in the world; though with the high number of fatalities, it is considered the deadliest outbreak thus far. The largest was the 1996 outbreak that infected approximately 10,000 people due to white radish sprouts (Michino et al., 1999).

The bacteria were never isolated from the sprouts, but the consumption of the sprouts was a commonality among those affected. Due to the low dose response of *E. coli* O104:H4, it may be difficult to isolate the organism from the sprout itself. Investigations into point source tracking could help decipher what changes are needed to eliminate pathogen transmission.

There have been several other outbreaks regarding food crops and water-related diseases such as the hepatitis A outbreak in 2003 from consumption of green onions and the 2006 *E. coli* O157:H7 outbreak that was traced to spinach. In the past 20 years, the number of individuals affected in an outbreak has geographically expanded. Many outbreaks involve multiple states and even multiple countries. The *E. coli* O104 outbreak is a great example of this. Table 2 highlights outbreaks that are associated with pathogens as a consequence of environmental contamination of food crops or water. Contributory factors pertaining to the environmental health may be the missing link that public health officials need to curtail these outbreaks. Table 2 summarizes selected outbreaks and the identified agents responsible. This list is a small snapshot to the thousands of cases of foodborne and waterborne outbreaks that occur across the globe each year.

History of Waste Management Regulations

Population increases require more vigilant concerns for environmental protection and conservation. In 1948, the Federal Water Pollution Control Act (FWPCA) was passed by Congress, 33 U.S.C. 1251 - 1376(1948). The FWPCA set in motion the current regulation established to protect and conserve the environment and waterways of the United States. There have been several amendments to this law and it has imparted

statutes to individual states with programs and funding to ensure that both recreational waters and groundwater are protected. The FWPCA also established guidelines for agriculture and industrial practices as well. The EPA was established in 1970. In 1972, the Federal Water Pollution Control Act was shortened to the Clean Water Act, and the EPA was designated with the authority to provide sanctions and permits to industries and farmers regarding waste disposal of any sort. In 1976, EPA established guidelines for waste management practices which corresponded to the 1977 amendment of the Clean Water Act that defined "Best Management Practices" (BMP) for industry and agriculture practices, 33 U.S.C. 1288(1948). BMP are set for these entities through permits that define the maximum amount of waste disposal based on the size of the operation and the means to which waste products can be eliminated. These provisions have been implemented so that the environment and public health will not be compromised. Topp et al. (2009) defines an "effective multi-barrier strategy" with 3 areas to reduce health risks to the environment and the public when implementing a waste management plan: 1) maintaining population health, 2) management of stored waste while attenuating, and 3) proper application rate during suitable environmental conditions. These three components can be applied to both animal and human wastes applications. One of the latest regulations imposed by EPA was the Part 503 ruling set in place in 1993. Regulations are limited for land application of manures; however, the EPA Part 503 (USEPA, 1995) governs the application of biosolids to land (NRC, 2002). This guide explains how biosolids should be handled and defines what constitutes Class A and Class B biosolids (Table 3). These terms and regulations are discussed later in land application section.

Methods for Attenuating Waste Products

Waste disposal protocols consist of multiple attenuation points to achieve pathogen reduction. For example, biosolids are a product of aerobic and anaerobic digestion combined with lime stabilization. The ultimate goals are to reduce pathogen load and to dispose of waste material with the least production cost necessary to implore this task. The cost of removal, attenuation, and transport from farms can be expensive (Adhikari et al., 2005; Melse and Timmerman, 2009). In conjunction to cost, the total solids (TS) associated with the end product of waste affects pathogen attenuation (Pell, 1997; USEPA, 1995). The state of the waste whether liquid manure (1-4% TS), slurry (4-15% TS) or semi-solid (15% or more TS) limits what constitutes an effective attenuation process (Pell, 1997; Ziemer et al., 2010). Solid and semi-solids are typically handled by mesophilic anaerobic digestion, liming, composting, air drying, incinerating or other alternatives such as pyrolysis. Little research discusses it but some wastes are fed to other animals for a supplemental feed additive. Liquid waste necessitates different methods of attenuation practices than solid and semi-solid wastes; these methods include aerobic digestion, chemical additives and lagoons. Attenuation methods are discussed along with effectiveness of pathogen reduction and disadvantages associated with each practice. Table 1.4 summarizes the effective log reduction based on waste and attenuation practice.

Solid and Semisolid Attenuation Methods

Mesophilic Anaerobic Digestion

Waste products are broken down in the absence of oxygen at temperatures between 35^{o}C to 55^{o}C for a minimum of 15 days or 60 days at 20^{o}C (USEPA, 1995).

Anaerobic digestion eliminates aerobic bacteria due to the lack of oxygen. Both coliforms and viruses are significantly reduced but parasites can remain unaffected by this attenuated process (Godfree and Farrell, 2005). To reduce parasites in wastes, other attenuation methods must be practiced such as liming and land application. Methane is produced by anaerobic digestion, and if captured, can be used as an energy source. Biogas production, endorsed by the U.S. as new methods of clean and renewable energy, is being explored. Companies have developed new technologies to dispose of waste products and produce methane by anaerobic digestion that can be converted to usable energy which reduces heating and energy costs to the farming operation.

Lime Addition

Lime stabilization is commonly practiced to increase the waste pH to 12 in order to inactivate bacteria and viruses. Contact for 2 hours with the waste is necessary (USEPA, 1999). Lime stabilization has the potential to reduce bacteria populations and virus concentrations by 7-logs and 4-logs, respectively (Bean et al., 2007). The longevity of the reduction in bacterial counts is questionable based on other studies. Hogan et al. (1999) determined the addition of lime initially caused a decrease in fecal coliform counts but by day 6 the number of bacteria had recovered to the same concentration as the control samples. Furthermore, lime stabilization is not effective on parasites (Bean et al., 2007; Godfree and Farrell, 2005). Garrec et al. (2003) found that liming was the only sufficient attenuating method for biosolids-borne *Listeria monocytogenes*.

Composting

Composting is a relatively cost-effective practice for pathogen reduction for all wastes produced; however, the process of composting is limited to only waste that consist thirty percent dry matter in order to achieve temperatures that are required to reduce pathogen load (Pell, 1997). The process of composting waste requires it to be stored for a minimum of 5 days at 40^{o}C and within that time the temperature must reach 55^{o}C for 4 hours (USEPA, 1995). Composting poultry litter, biosolids and cattle manure is a common practice prior to land application. Composted waste is promoted to reduce pathogens but some researchers have found that certain pathogens such as *Campylobacter jejuni* can be resistant to this attenuating process (Inglis et al., 2010). This evidence supports the idea that multiple attenuation processes should be used as a means to reduce the transmission of zoonotic pathogens.

Incineration / Combustion

Some waste products are disposed of by incineration. This method is beneficial because less material remains for disposal; however, cost effectiveness comes into question, since energy is required to incinerate large amounts of waste. Another disadvantage of incineration and combustion is the air filtration systems that are required to decrease air emissions. The ash produced does not provide the organic nutrient benefits of other attenuated waste products applied to land. On the other hand, pathogen attenuation is effective. One area of concern is the incineration of animal carcasses. The ash has the potential to contain prions if expired animals that were infected with this infectious protein are disposed during this process (Brown et al., 2000). Prions have the capability to survive extreme temperatures (Brown et al., 2000) and can remain in the

environment for many years (Woolhouse et al., 1998). However, the risk of this is not known and very few livestock have even been found to be infected in the United States.

Alternative Disposal Methods

Biocrude oils for energy production, by pyrolysis, is a new alternative to waste disposal. Finding new methods to produce energy is highly regarded. Pyrolysis is the process of heating the waste material at temperature between 400^{o}C and 600^{o}C for a determined amount of time in an anaerobic environment. The end product can be used as an energy source as a biofuel and the biochar can be applied to land as fertilizer (Agblevor et al., 2010). The biochar is rich in carbon and research suggests that the application to land can reduce carbon volatilization and create a slow release carbon sink to promote vegetation growth (Bell and Worrall, 2011). More research is required to investigate the effects of biochar application to land and the effects on the soil microbial community (Bell and Worrall, 2011).

Feeding livestock poultry litter is an alternative that cattle farmers practice. Mixing poultry litter in cattle feed is practiced by some farmers to increase protein levels in feeding regimen (Martin, 1998). However, the threat of bovine spongiform encephalopathy (BSE) caused by the infectious agent, prions, deter some farmers from this practice (Agblevor et al., 2010). At the turn of the century, the U.S. reported its first case of BSE, which caused large economic losses for the beef industry. To date, there has not been any evidence to support this mode of transmission. Furthermore, this feeding practice has also been speculated to cause infections in cattle due to *Clostridium spp.* (Payne et al., 2011).

Slurry and Liquid Attenuation Methods

Aerobic Digestion

Aerobic digestion is performed by a process of agitating waste products or in incorporating oxygen to activate the degradation of organic matter by microorganisms. Aerobic digestion is very efficient in the pathogen removal of both bacteria and viruses but falls short of eliminating parasites from waste. Secondary treatment of biosolids involves physiochemical separation of solids resulting in microbial degradation usually by a trickling filtration system, a form of aerobic digestion. Bacteria necessitate the process but are limited once the organic matter is expended and these organisms die off. Further attenuating methods may be needed and are generally practice before disposal of waste products.

Storage Pits – Lagoons

The construction of storage pits, also known as lagoons, is another method for pathogen attenuation for several waste management practices but especially in swine production farms. Almost all swine production facilities practice some method of lagoon attenuation (Ziemer et al., 2010). Generally more than one lagoon is constructed for waste storage so that one lagoon is able to remain static for a specific time for waste to age and new effluent flows into the receiving lagoon. Waste must remain lagooned for a minimal amount of time of 4 to 6 months depending on system temperature of $>5^{o}C$ or $\leq 5^{o}C$, respectively(USEPA, 1999). Because temperature affects pathogen attenuation the higher waste temperature requires a reduced amount of time for storage. Typically, once the designated time has elapsed, swine effluent is pumped from lagoons and applied to adjacent land.

Land Application of All Waste Products

Land application of waste is a process of attenuating pathogens by exposure to ultraviolet light, desiccation and predation by other microorganisms. The TS of the waste product determines the equipment needed and costs associated with the land application process and transport. Land application is the primary disposal method of biosolids. Sixty percent of biosolids are land applied (NRC, 2002). Class A biosolids are a result of treated solids which are the byproduct of wastewater treatment plants so that little to no pathogens are detected. Conversely, Class B biosolids are minimally treated and pathogens are present but must not exceed set concentration of fecal coliforms (Table 1.3). The application of Class B biosolids has more site application restrictions and public access restrictions than Class A biosolids. Research indicates if these recommendations are followed, it would allow for an environmentally safe application of biosolids (Brooks et al., 2005). CAFOs are required to develop nutrient management plans for waste disposal and maintain records of this on site. The nitrogen and phosphorus concentration of wastes intended for land application must be recorded annually; nitrogen and phosphorus concentrations of land receiving waste must be recorded every 5 years according to BMP (40CFR412.4)(USEPA, 2003). Monitoring of pathogens is not defined by federal regulations and state mandated requirements are poorly defined. Monitoring of adjacent watershed areas is required to ensure that waste contamination is not compromising these systems.

Agronomic Factors Potentially Influencing Waste Associated Outbreaks

Runoff potential

Runoff is defined as overflow of water or liquid that is applied to land or caused by heavy rainfall due to saturation of water capacity of soil. This can be due to one of many factors. Location to water systems can affect the likeliness of runoff. This factor can be compounded by the slope of the affected field. The conditions of the soil are important to take into account when considering runoff potential. A soil that is already saturated due to previous rainfall or liquid application of waste will cause a greater potential for runoff. Soil type plays an important role. Sandy soils have a low affinity to water and allow less absorption. Clay soils are more porous and hold on to water molecules with a greater affinity. (Brooks et al., 2009) found that bacterial counts were 3 to 6 logs higher for *Staphylococcus*, *Enterococcus* and *Clostridium perfringens* after application of poultry litter in a simulated study to determine the effects associated with runoff after several rainfall events. Many factors affect the transport of pathogens in waste, but this study highlights the potential of bacterial movement and the ability to contaminate water-systems and neighboring lands.

Heavy Rainfall

With more emphasis on safer foods and water, studies have been conducted to learn what elements play a role in outbreaks. Heavy rainfall has been linked to many waterborne outbreaks in the past. Heavy rainfall is responsible for the increased movement of microorganisms through the soil, which can contaminate ground water that normally would be free of pathogens (Curriero et al., 2001; Esseili et al., 2012). The 1993 Milwaukee outbreak of *Cryptosporidium* infecting 403,000 was associated with

heavy rainfall (Curriero et al., 2001). Figure 1.3 indicates all waterborne outbreaks across the United States and those associated with heavy rainfall that preceded the outbreak. Studies have shown that heavy rainfall is a common factor in many documented outbreaks (Curriero et al., 2001), (Kistemann et al., 2002). Likewise, LeChevallier et al. (1998) investigated the prevalence of *Giardia* and *Cryptosporidium* and found that the increase in rainfall was associated with the increase of these parasites in the Delaware River. Bacterial counts increased when studying the effects of heavy rainfall and runoff (Cooley et al., 2007; Kistemann et al., 2002). A positive correlation with heavy rainfall and an increase of pediatric cases of acute gastrointestinal illnesses were reported by Drayna et al. (2010); more research that addresses waterborne disease associated with heavy rainfall could help reduce acute gastrointestinal illnesses. Educating farmers that land application of manure is not recommended in times when heavy rainfall is eminent could potentially reduce the number of outbreaks due to waterborne diseases.

Climatic Variances

Manure application is generally practiced during March to September. It has been shown that seasonal factors affect water and foodborne outbreaks (Money et al., 2010). Many bacterial outbreaks peak in summer months due to increase in contact to untreated surface water and recreational waters. In addition, climatic variance can increase moisture due to rainfall leading to increased vertical and horizontal transport of microorganisms. Moist soils also promote survival of pathogens that may be introduced by waste residuals applied to soil (Gagliardi and Karns, 2000). Soil conditions due to climatic variance play a role in waste being able to contaminate water-systems.

Application of wastes is affected by these seasonal variances and determines the nitrogen availability factor. Extremely dry, cracked soil may lead to less absorption/filtration that could cause contaminated ground water. In contrast, climatic factors such as frozen soil will lead to runoff because the frozen soil will not absorb rainwater or waste water. If land application of waste is employed during these conditions, neighboring water systems could receive contaminated runoff. Hutchison et al. (2005) found that seasonal factors influenced the number of pathogens in animal manure; May and December months indicated significantly higher concentrations in animal manure.

Agronomic Practices

Land application of waste has been proven a beneficial practice that significantly increases crop yields. However, 1.37 billion wet tons of manure is produced by the farm industry each year (ASM, 2000). Application rates play a significant role in bacterial counts associated with runoff (Brooks et al., 2009). For manure application, BMPs have been set up more for environmental factors and most efficient practices for supplying the best nutrient demands for the crop (USDA-AMS, 2000). The nitrogen in the manure varies depending on the source of the animal and potentially the purpose as well. For example, in poultry manure, layers have about half the amount of nitrogen (37 lb/ton) than broilers (73 lb/ton); dairy cattle and beef cattle average about the same amount of nitrogen (Beegle, 1997). The waste management practice is necessary to consider due to nitrogen losses and availability factors; however, more attention needs to be given to the pathogen loads that could be harbored in these different waste products. Farm management of natural fertilizers has effects on not only provided nutrients but also the fate of pathogens that are associated. More nitrogen is available to the soil if immediate

incorporation occurs rather than delayed incorporation. Incorporation may increase nutrient uptake but at the same time may provide protective measures on pathogen survival by preventing UV irradiation and desiccation (Pepper et al., 2000).

Buffers

An agronomic management practice that ensures that land application of waste is most beneficial with less nutrient losses is the establishment of land buffers that prevent runoff from entering adjacent properties or water systems (Newton et al., 2003). The Code of Federal Regulation recommends the practice of establishing buffer, but gives no specifics for required practices (40CFR122.23)(USEPA, 2003). However, individual states implement these requirements for new CAFOs to protect neighboring residents. For example, Mississippi Department of Environmental Quality (MSDEQ) ACT4-L1 requires that land intended to receive waste products from established CAFOs not be any closer than 1000 feet from nearest resident or dwelling and 300 ft. from property line (MSDEQ, 1994). BMPs includes application of biosolids or manure to land with provisions that allow efficient absorption considering weather conditions promoting environmentally favorable conditions and reducing risks to conjoining ecosystems. Establishing vegetation buffers increases distance regarding public access; when studying bioaerosols during biosolid disposal, the isolation of indicator organisms were not able to be detected at distance >30 meters (Brooks et al., 2005). Pathogen concentrations associated with bioaerosols from land application of biosolids are affected by wind speed, temperature and distance traveled (Brooks et al., 2005). Buffers can include planted trees or grassland that establishes barriers around farmland. Trees can be great buffers not only for waste application surplus but also to eliminate odors for neighboring residents.

Anthropogenic Factors Influencing Waste Associated Outbreaks

Food Preference

Foodborne outbreaks have been a major focus in the media over the last decade. Many vegetable crops such as spinach, tomatoes and jalapenos have been linked to multiple state outbreaks due to bacterial contamination. From 1998 to 2008, there have been 11 outbreaks in the United States alone due to tomatoes contaminated with *Salmonella enterica*. (Barton Behravesh et al., 2011; CCDR, 2005; Cummings et al., 2001; Greene et al., 2008; MMWR, 2007; MMWR, 2008). Fresh produce is now the number one cause of *E. coli* O157:H7 outbreaks in the United States (Calvin, 2007); consumption of contaminated meat products was generally the mode of transmission. There are two contributing factors that have led to an increase of illness due to consumption of leafy green vegetables such as spinach and lettuce. One explanation is that consumers have increased consumption by 90% since 1992 (Sivapalasingam et al., 2004); and two, much of the leafy greens are processed by a single processing company (Calvin, 2007). Society as a whole has become more health conscious. Thus, eating more raw vegetables has increased over the past 15 years. Cooking these vegetables would kill associated pathogens that may be present. With many of the leafy greens going to the same processing plants, the possibility of cross contamination and likelihood affecting larger population is more prominent (Calvin, 2007). A prime example of this is the *E. coli* O157:H7 outbreak in 2006 due to consumption of spinach. This outbreak was attributed to feral pigs defecating on the spinach crops that had freely roamed between cattle farms and this food crop land (Jay et al., 2007). Cattle are an animal reservoir for *E. coli* O157:H7, and the feral pigs were the vector that bridged the gap for transmission

to humans. The contaminated spinach was disseminated to multiple state causing a wide spread outbreak that resulted in a large recall on fresh spinach sold in the United States. The interest of eating healthy may also be the goal of individuals that are more at risk to infection.

Vulnerable Populations

Although a progression of safer food and water systems has been implemented, some individuals are more vulnerable to illness. Immuno-compromised individuals, children, elderly, pregnant women and those that are living in unclean environments are more at risks for becoming ill due to foodborne and waterborne diseases (ASM, 2000; WHO, 2004). All of these individuals are more susceptible to infection due to weakened immunity. The number of individuals that are more susceptible has increased and represents approximately 20 to 25 percent of the United States' population as a whole (Gerba et al., 1996; Reynolds et al., 2008). Table 1.5 represents the populations in the United States that may be more prone to disease. It is often these individuals that are most affected by outbreaks in communities and cause exposure to many people at once. Daycares, hospitals and nursing homes are environments where a great number of vulnerable populations can be susceptible to secondary transmission. Adults 55 and older represent 78% of those that die due to gastroenteritis causing diseases (Figure 1.4) (Gerba et al., 1996).

Conclusions

Waste management is a growing concern as better technologies of pathogen detection and disease outbreak tracking has linked food crops and water resources as

means of contamination. Research asserts that regulations set in place provide evidence of minimal risks associated with waste disposal (Brooks et al., 2007; Brooks et al., 2005; Eisenberg et al., 2008). However, the disease outbreaks that have been discussed have been associated with fecal contamination. The route of transmission has not been fully understood and more research is needed to better determine the link of waste management, farming practices and contaminated foods and water resources. Pathogen fate is poorly understood in regard to waste management practices, and more research that defines which pathogens persist in the environment under and what variable alter the decay rates could potentially improve risk assessment models that are presently available.

Table 1.1 Pathogens of Concern Based on Specific Animal Manures or Human Biosolids

Pathogen	Biosolids	Cattle	Poultry	Swine	Other
Salmonella	✓	✓	✓	✓	✓
Pathogenic *E. coli*	✓	✓			
Campylobacter	✓	✓	✓	✓	
Listeria		✓	✓	✓	✓
Clostridium	✓		✓	✓	
Hepatitis A & E	✓				
Norovirus	✓				
Cryptosporidium	✓	✓		✓	✓
Giardia	✓	✓		✓	✓
Toxoplasma gondii				✓	✓

Table 1.2 Examples of Selected Foodborne and Waterborne Outbreaks Since 1990

Year	Mode	Location*	Pathogen	Cases	References
2012	Cantaloupe	U.S (26).	*Salmonella*	270	(CDC, 2012)
2011	Bean Sprouts	Europe	*E.coli* O104:H4	4,321	(RKI, 2011)
2011	Cantaloupe	U.S. (9)	*Listeria monocytogenes*	146	(MMWR, 2011)
2011	Lettuce	U.S. (10)	*E. coli* O157:H7	60	(CDC, 2011)
2010	Tomatoes	France	Hepatitis A	59	(Gallot et al., 2011)
2010	Oysters	North Carolina	Norovirus	280	(Alfano-Sobsey et al.,
2008	Peppers/Tomatoes	U.S./Canada	*Salmonella* Saintpaul	1,442	(Mody et al., 2011)
2008	Raw Peas	Alaska	*Campylobacter jejuni*	98	(Gardner et al., 2011)
2006	Spinach	U.S. (26)	*E. coli* O157:H7	205	(Jay et al., 2007)
2006	Lettuce	U.S.(5)	*E. coli* O157:H7	71	(Patel et al., 2010)
2003	Green Onions	U.S. (7)	Hepatitis A	555	(Amon et al., 2005)
2000	Drinking Water	Canada	*E. coli* O157/*Campylobacter*	2,300	(Hrudey et al., 2003)
1996	White Radish Sprouts	Japan	*E. coli* O157:H7	9,451	(Michino et al., 1999)
1996	Berries	U.S. /Canada	*Cyclospora*	1,465	(Manuel et al., 2000)
1993	Drinking Water	Wisconsin	*Cryptosporidium*	403,000	(Curriero et al., 2001)

*Numbers in parentheses after U.S locations are associated with the number of states that were affected.

Table 1.3 Tolerable Pathogen Concentration for Biosolids Published by EPA Regulations

	Class A Biosolids	Class B Biosolids
Fecal coliform density	1,000 MPN /g TS[a]	< 2,000,000 MPN /g TS[a]
Salmonella spp. density	3 MPN/4g TS[a]	NA
Enteric viruses	< 1 PFU / 4g	NA
Helminth ova	< 1 / 4g	NA

[a]TS- Total dry solids (USEPA, 1995)

Table 1.4 Reduction Potential for Waste Attenuation Processes

Attenuating Process	Waste Source	Indicator Organisms	Human Enteric Viruses	Parasites	Source Reference
Mesophilic anaerobic digestion	Biosolids	0.5 - 4.0	0.5 - 2.0	0	(Godfree and Farrell, 2005; Vanotti et al., 2005)
	Cattle	ND	ND	ND	
	Swine	4.1 – 4.5	ND	ND	
	Poultry	ND	†	ND	
Aerobic digestion	Biosolids	0.5 - 4.0	0.5 - 2.0	0	(Godfree and Farrell, 2005)
	Cattle	ND	ND	ND	
	Swine	ND	ND	ND	
	Poultry	ND	†	ND	
Composting	Biosolids	2.0 - >4.0	2.0 - >4.0	2.0 - >4.0	(Godfree and Farrell, 2005; Hutchison et al., 2005; Larney et al., 2003; Mohee et al., 2008)
	Cattle	4.0 – 5.9	4.0	ND	
	Swine	2.0	2.0	ND	
	Poultry	> 6.0	†	ND	
Air drying	Biosolids	0.5 - 4.0	0.5 - 4.0	0.5 - 4.0	(Godfree and Farrell, 2005)
	Cattle	ND	ND	ND	
	Swine	ND	ND	ND	
	Poultry	ND	†	ND	
Lime Stabilization	Biosolids	2.0 - 4.0	> 4.0	0	(Bean et al., 2007; Godfree and Farrell, 2005) (Maguire et al., 2006; Wong and Selvam, 2009)
	Cattle	ND	ND	ND	
	Swine	>4.0	ND	ND	
	Poultry	2.0 - 3.0	†	ND	
Lagoon	Biosolids	2.0 - 6.0	2.0 - 4.0	ND	(Gaasenbeek and Borgsteede, 1998; Godfree and Farrell, 2005; Hill and Sobsey, 2003; McGee et al., 2001; Venglovsky et al., 2009; Wong and Selvam, 2009)
	Cattle	3.5 – 5.5	ND	ND	
	Swine	2.0 – 3.0[a]	1.0 – 2.0	>4.0	
	Poultry	*	*	*	
Land Application	Biosolids	3.0 - 4.0	2.0 - 4.0	ND	(Brooks et al., 2009; Farrah et al., 1981; Gaasenbeek and Borgsteede, 1998; Nicholson et al., 2005; Zaleski et al., 2005)
	Cattle	2.0 - >4.0	ND	ND	
	Swine	1.0[a]	ND	<0.5	
	Poultry	3.0	†	ND	

* Denotes that data is not available because this attenuation practice is not common for this particular waste. † Denotes that these organisms are not generally isolated from this waste. ND - no data found to determine log reduction of organisms. All units are reported as Log_{10} PFU-MPN-CFU g^{-1} except those denoted as ([a]) which were reported as Log_{10} PFU-MPN 100 mL^{-1}.

Table 1.5 Vulnerable Populations in the United States

Vulnerable Populations	# of Individuals in U.S.	References
Children < 5	20,201,362	(Howden and Meyer, 2011)
Adults > 65	40,267,984	(Howden and Meyer, 2011)
HIV Infected Persons	1,178,350	(CDC, 2011)
Diabetics	25,800,000	(ADA, 2011)
Pregnant Women	6,000,000	(APA, 2012)
Cancer Patients	18,600,000	(CDC, 2011)
Organ Transplant	153,641	(OPTN, 2010)

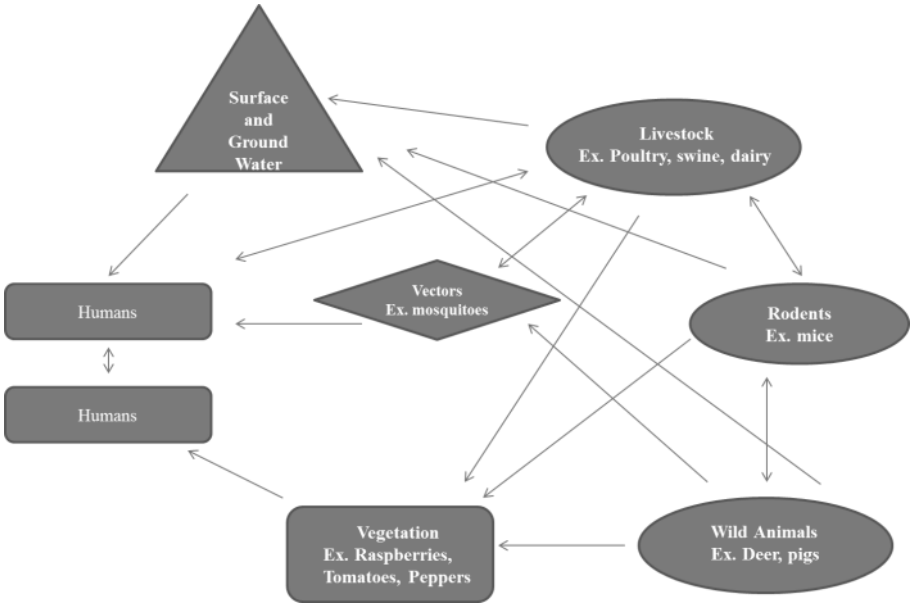

Figure 1.1 Schematic of possible zoonotic transmission pathways.

Double arrows imply multidirectional transmission and single arrow represents pathogen transfer one way.

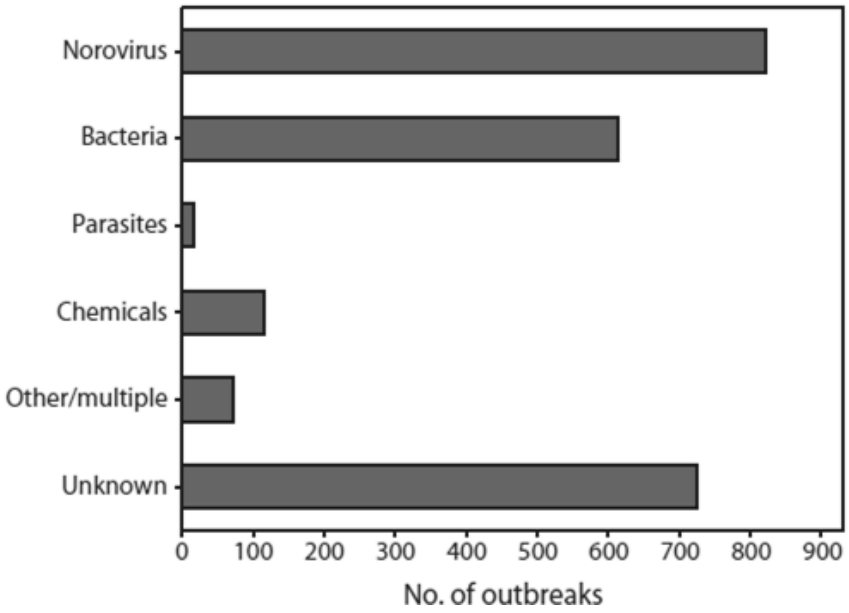

Figure 1.2 Number* of foodborne disease outbreaks reported to CDC, by etiology United States, 2006 - 2007.

Sources: CDC. Surveillance for foodborne disease outbreaks - United States, 2006 (MMWR, 2009); CDC. Surveillance for foodborne disease outbreaks - United States, 2007 (MMWR, 2010). * No. = 2,367.

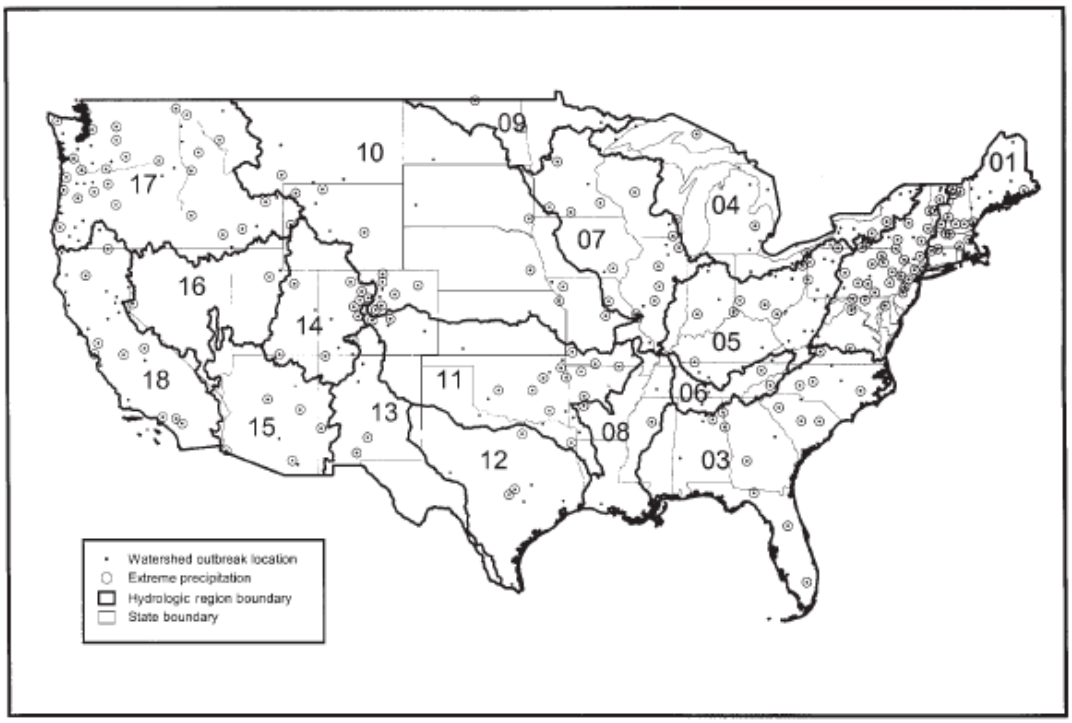

Figure 1.3 Locations of Waterborne Disease and High Levels of Precipitation

Source: Curriero, F.C., Patz, J.A., Rose, J.B., Lele, S., (2001). The association between precipitation and waterborne disease outbreaks in the United States, 1948-1994. *American Journal of Public Health*

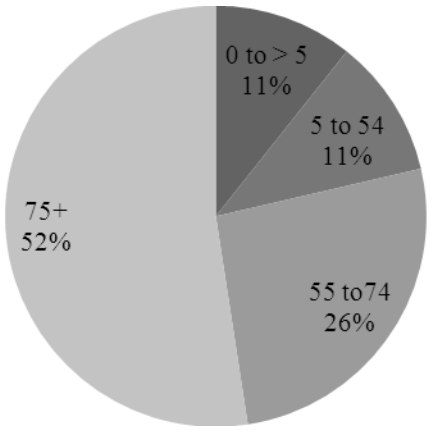

Figure 1.4 Percentage by Age of Deaths Due to Gastroenteritis

(Gerba et al., 1996)

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CHAPTER II

SPATIAL AND TEMPORAL ANALYSIS OF MICROBIAL POPULATIONS IN PRODUCTION BROILER HOUSE LITTER IN THE SOUTHEASTERN U.S.

Summary

Broiler production is one of the leading agricultural enterprises in the United States. In Mississippi the economic impact from broiler production and processing exceeds that of any other agricultural commodity. Reducing mortality rates is critical in broiler production; therefore, it is vital to reduce bacterial pathogen loads in broilers and broiler houses. The main objectives of this study were to discern intra-house spatial and temporal effects on foodborne and nuisance pathogen bacterial levels. A single broiler concentrated animal feeding operation house litter was monitored throughout 3 consecutive flocks; *Salmonella*, staphylococci, enterococci, *Clostridium perfringens*, *Campylobacter*, and *Listeria* levels were monitored throughout that time at the wall, feeder, water cup, and house end spatial positions. Nuisance pathogens *Clostridium perfringens*, staphylococci, and enterococci were consistently present at levels of 7 $\log_{10}$, 12 $\log_{10}$ and 8 $\log_{10}$ colony forming units (cfu) kilogram^{-1}, respectively; while *Salmonella*, *Campylobacter*, and *Listeria* were present at low levels. Among surveyed bacteria, *Salmonella* was more consistently detected at the ends of the house, while staphylococci levels were lower near feeder locations. Nearly all measured bacteria were

significantly associated with broiler age as *Salmonella* was found early in the flock, while *Clostridium perfringens*, staphylococci, and enterococci levels were greater late in the flock. The effect of season was noted only for staphylococci and *Listeria* which were positively associated with Flock 1 (summer). Overall, it appeared that pathogen levels were difficult to predict given house conditions, both spatially and temporally; however it was evident that high moisture supported *Salmonella* at the ends of the house and broiler age influenced the presence of most nuisance pathogens as broiler age increased. This suggests review of house management practices with particular attention to high moisture locations and precautions taken as the broilers age.

Description of Problem

The poultry industry is one of the leading agricultural enterprises in the United States. Poultry product consumption in the U.S. has increased over the last several decades (USEPA, 2009). Chickens grown for meat production are known as broilers and are produced over a 6 - 8 week period where they are continuously fed and watered to produce a 2.25 – 3.25 kg (5 - 7 lb.) bird. Approximately 8 billion broilers are produced per year in the U.S. (USEPA, 2009) with about 10% of those produced in Mississippi (USDA-NASS, 2011). The demands of the growing market are met by large broiler farms classified as concentrated animal feeding operations (CAFOs), capable of producing over 100,000 broilers per house per year (5 - 6 flocks). CAFOs are managed to house poultry under constant feeding regimens in order to efficiently produce a quality meat product in a short amount of time. Investigating pathogenic and nuisance microbial communities within the broiler house environment may lead to increased broiler productivity. The pathogen and nuisance microbial levels in these environments can be

detrimental to the food animal industries (Bailey, 1993). Food safety and animal health concerns are critical to the industry and to public health.

House design and broiler management practices impart natural spatial variability throughout the broiler house. This variability produces different microbial niches. Typically one end of the house, the 'fan end' (F) (Figure 2.1), is equipped with massive exhaust fans that draw fresh air through the house. The other end of the house, the 'brood end' (B) (Figure 2.1) is used to brood the baby chicks at the beginning of each new flock grow-out period. The brood end typically has a large door that is closed during broiler placement but opened for equipment entrance during harvesting of broilers due to the "all-in/all-out" method and subsequent management of litter between flocks. During brooding, the young chicks (0 - 2 weeks old) are confined to the brood end half of the house, which is partitioned off to reduce heating costs. As the birds increase in size the partitions are removed, and the full house is available to the broilers. Thus, the fan end has broilers from 2 weeks old until harvest (6 - 7 weeks old), while the brood end has broilers from day 1 through harvest. This two-week differential suggests inherent differences in the litter between the two ends of the house.

Other factors also influence litter. Some areas of the house are more subject to litter "caking", the compaction of bedding material and excreta in areas where broilers congregate. Litter is typically "decaked" between flocks. This process removes the top "cake" layer that is higher in excreta and moisture. Differential caking and decaking produce distinct niches that favor distinct microbial populations. The area immediately adjacent to the wall of the house is inimitable because equipment constraints preclude complete litter removal during decaking; often leaving 30 - 60 cm wide strips of

accumulated cake along the walls. Litter in high traffic areas near watering cups and around feeders also has more caking, but cake near water lines has higher moisture content.

Salmonella, Campylobacter and *Listeria* are foodborne pathogens that may be found in the broiler house environment (Bailey, 1993). These three zoonotic pathogens are responsible for the majority of bacterial foodborne diseases and fatalities in the U.S. (Mead et al., 1999). *Salmonella* is a major concern in the poultry industry and is responsible for several poultry-associated human disease outbreaks (Luber, 2009; 2011; 2010). Microbial ecology of the litter can affect broiler health during production and may affect public health by bacterial pathogen transfer during production and processing (Volkova et al., 2010). Marin et al. (2011) investigated common risk factors capable of introducing *Salmonella* into the house, including chick delivery box liners, farmers' boots, and broiler feed. Volkova et al. (2010) determined that the presence of *Salmonella* in litter prior to flock placement and throughout grow-out contributed to its presence on post-chill tank carcasses during processing. Reducing pathogen levels by targeting specific problem areas of the house may be a means to reduce broiler mortality and curtail the spread of zoonotic pathogens. Increased scrutiny and criticism of antibiotic uses in animal agriculture requires alternate strategies to reduce bacterial pathogens. Alternative methods to reduce pathogens in broiler litter have been investigated (Line and Bailey, 2006), but few have demonstrated effective long-term reduction. These alternative methods may, however, be more effective if data were available to guide decisions on site-specific treatments. The primary objective of this study was to determine bacterial profiles in broiler house litter, with emphasis on *Salmonella* spp. and

other pathogens, as affected by environment, management, and spatial and temporal variables. The goal of the research was to provide site- and pathogen-specific data that would allow better informed decisions and improve future control of microbial populations in broiler house litter.

Materials and Methods

House Litter Management

The single north central MS broiler house used in this study was selected due to previous instrumentation for emission analysis (Brooks et al., 2010; Miles et al., 2011). Litter was comprised of pine wood shavings and poultry manure. The broiler farm comprised 8 broiler houses approximately 12.8 x 152.4 m, housing approximately 26,000 - 28,000 broilers per house per flock. The flock cycle comprised 6.5 weeks, from placement to removal, with 2 weeks between flocks. The top 10 cm of litter was removed after each flock and the remaining litter dressed with fresh pine-shavings in preparation for the next flock.

Litter Sample Collection

Litter samples (100 g) were collected, during the flock cycle, throughout the house using a two dimensional grid corresponding to water (C) and feeder (F) lines, walls (W), and ends (E) of the house (Figure 2.1). Litter samples were collected from June to December of 2008 comprising three consecutive flocks. Flock 1 was sampled from June 16 – July 28, flock 2 from August 25 – October 6, and Flock 3 from October 27 – December 10 and are referred to as summer, fall, and winter flocks, respectively. Sixteen litter samples were collected bi-weekly (0, 2, 4, and 6 weeks) (Figure 2.1). Two replicate

samples were collected from each of eight locations, four sites on each half (brood end [B] and exhaust fan end [F]) of the house. Collection sites were identified by two letters; the first letter specified the specific sample site (C, F, W, or E), and second specified the end of the house (B or F) (Figure 2.1). Ambient air temperature, humidity, and litter temperature were monitored and recorded continuously throughout the study located at the F half only using a HOBO H21–002 microstation logger (Onset Computer Corp., Bourne, MA). Litter moisture content was determined for each litter sample by heating 10 g at 104^{o}C for 48 hours and measuring the dry weight.

Litter Processing

Litter was collected in whirl-pack bags and transported in a cooler to the laboratory. All samples were processed within 24 hours of collection. To assure sample homogeneity, litter was blended with an industrial stainless steel blender for a minimum of 30 seconds. Between each sample, blender was cleaned with 70% ethanol and rinsed with sterile deionized water. Prior to microbial analyses, 10 g of poultry litter was suspended in 95 mL of sterile physiological saline, stomached for 30 seconds, and serially diluted for analysis.

Microbial Assays

Staphylococcus (standard plating), *Enterococcus* and *Clostridium perfringens* (membrane filtrations), *Campylobacter* and *Listeria* (presence/absence), and *Salmonella* (MPN) were all assayed. Staphylococci were assayed in duplicate by spread plating 0.1 mL of a proper dilution on manitol salt agar (MSA) (Neogen-Accumedia, Lansing, MI) and incubated at 35°C for 24 to 48 hours. Enterococci were analyzed on mEnterococcus

agar (Neogen-Accumedia), incubated at 35°C for 24 h, transferred to bile-esculin agar (Neogen-Accumedia) and incubated for an additional hour at 35°C. *C. perfringens* samples were heated to 70°C for 10 minutes prior to membrane filtration and placed on mCP agar (Neogen-Accumedia). Samples were incubated at 44.5°C for 24 hours under anaerobic conditions created by an Anoxomat gas generation system (Mart Microbiology, Lichtenvoorde, the Netherlands). All mCP plates were exposed to ammonium hydroxide fumes for a minimum of 30 seconds for confirmation of presumed positive *C. perfringens* colonies. Only colonies that turned pink once exposed were considered *C. perfringens*. Randomly selected colonies were further confirmed by streaking each to 5% sheep (Ovisaries) blood (Hema Resources & Supply; Willamette Valley, OR) tryptic soy agar (BD-Difco, Sparks, MD), anaerobically incubating at 44.5°C, and noting the characteristic double zone of hemolysis.

For cultural analysis of *Campylobacter* and *Listeria* in broiler litter, pre-enrichments were performed by adding 10g broiler litter, respectively, to 95 mL *Campylobacter* enrichment broth (CEB) (Neogen-Accumedia), and to 95 mL UVM – *Listeria* enrichment broth (UVM) (Neogen-Accumedia). CEB was incubated microaerophillically at 35°C for 4 hours then moved to 42°C for 44 hours. *Campylobacter* was streaked for isolation onto 5% sheep blood tryptic soy agar and incubated microaerophillically at 42°C for 48 hours. A microaerophillic environment was achieved using the Anoxomat gas system as described above by placing inoculated media in chambers that reduce oxygen levels to a gas mixture of H:N:CO_2 at a ratio of 10:80:10 (Brazier and Smith, 1989). For *Listeria* isolation, UVM was incubated at 30°C for 48 hours. For each sample, triplicate 0.1 mL aliquots were transferred to 10 mL of Fraser's

broth tubes (Neogen) and incubated at 35°C for 24 hours. Positive tubes were streaked onto modified Oxford agar (Neogen) and incubated for 24 - 48 hours at 35 °C.

Salmonella were enumerated using a three-dilution, three-tube MPN (1998) in which 1.0, 0.1, and 0.01g of homogenized litter was suspended in tryptic soy broth (TSB) (BD-Difco) and incubated at 35°C overnight. An aliquot of 0.5 mL was transferred from each tube to Rappaport-Vassiliadis R10 broth (BD-Difco) and incubated at 42°C for 24 to 48 hours. Positive tubes were subsequently transferred (0.1 mL × 3) to six-well cell culture plates (Thermo Fisher Scientific-Nunc, Rochester, NY) containing modified semisolid Rappaport-Vassiliadis agar (BD-Difco). Presumed positive samples were streaked onto Hektoen Enteric agar (BD-Difco) and incubated overnight at 35°C. Dark blue and black colonies were considered positive and confirmed using PCR.

Representative bacterial isolates from each sample location were preserved in 15% glycerol TSB (BD-Difco) and stored at –80^{o}C for PCR confirmation. Twenty-five percent of all bacteria were confirmed by PCR using species specific primers (Table 1).

Antibiotic Resistance Profiles

Representative isolates taken prior to flock placement (Week 0) and at final harvest (Week 6) of each flock were analyzed using the Kirby-Bauer technique for sensitivity to twelve antibiotics ranging from narrow to broad spectrum and encompassing eight classes of antibiotics (Table 2) (Bauer et al., 1966). Isolates were plated to Mueller Hinton (Neogen-Accumedia) (staphylococci), tryptic soy agar (enterococci*, Listeria*), or 5% sheep blood tryptic soy agar (*Clostridium perfringens*) in 150-mm petri dishes and were stamped with BBL Sensi-disc® antibiotics using a BBL antibiotic disc dispenser (BD-BBL; Franklin Lakes, NJ). Staphylococci, enterococci, and

Listeria isolates were aerobically incubated for 16 to 24 hours at 35 °C; *Clostridium perfringens* plates were placed in anaerobic Anoxomat chambers and incubated for 16 to 24 hours at 44.5 ^{o}C. Zones of inhibition (mm) were manually measured. *Staphylococcus aureus* ATCC 25923 (American Type Culture Collection; Manassas, VA), *E. coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were included as antibiotic effectiveness quality controls.

Mortality Data

As part of daily broiler house maintenance, the grower surveyed the house and removed dead birds. The grower kept a daily count of broiler mortalities removed from each house. Daily counts were totaled to determine the numbers of mortalities for each house and week sampled.

Statistical Analysis

SAS Enterprise Guide 4.3 (SAS Institute, Cary, NC) was used for all statistical analyses. All quantitative values (cfu or MPN 100 mL $^{-1}$) were adjusted by addition of 1 in order to convert zeros to positive numbers and $\log_{10}$ transformed. Chi square analysis was used for *Listeria* and *Salmonella* binomial data to determine effect on presence/absence ($\alpha = 0.05$). An ANOVA was performed for each bacteria species of interest to compare the effects of sample location, broiler age, and flock. Statistical differences between means were compared with Fisher's least significant difference at probability level of 0.05.

Results and Discussion

Effect of Location

Sample location had a minimal effect on bacterial levels and presence. Though there seemed to be unique management characteristics that could affect microbial constituents, no differences were noted for any of the surveyed bacteria when comparing brood versus fan house ends (data not shown). However, when house end was combined with specific site locations (i.e. cup, feeder, wall, end), the effect was more pronounced. When analyzing moisture content, the mean moisture content of (E) were consistently highest among locations, and the increased moisture may have favored bacterial populations in these areas (Table 2.3). Among surveyed bacteria, *Salmonella* and staphylococci levels were significantly associated with site location with a statistical p-value of 0.0207 and 0.0405, respectively (Figure 2.2 and 2.3). Specifically, EB and EF were found to harbor more *Salmonella*, while staphylococci levels were lower near FB and FF. Approximately 24% of samples collected throughout all 3 flocks were positive for *Listeria* with no association directly to location in the house. Though commonly associated with poultry, *Campylobacter* levels were below detection limits in all samples throughout the study. Environmental *Campylobacter* can be present in a reduced metabolic, viable but not culturable (VBNC) state which can prevent its isolation in harsh environments, yet still provide detection at processing plants where conditions improve (Lleo et al., 2005; Oliver, 2005; van Frankenhuyzen et al., 2011). Enterococci *were* found throughout the house at levels of 8 - 10 $\log_{10}$ cfu kg^{-1} with no effect of location (Figure 2.4). Similarly, levels of *Clostridium perfringens* were not affected by location and averaged 7.5 $\log_{10}$ cfu kg^{-1}(Figure 2.4).

Effect of Broiler Age

Broiler age had a significant effect among nearly all microbes collected ($p < 0.05$). *Salmonella*, *Listeria*, *Enterococcus*, *Staphylococcus*, and *Clostridium* levels were all associated with broiler age. Litter moisture content, temperature and mortalities were also associated with broiler age (data not shown). *Salmonella* was more commonly isolated prior to flock placement (36%). The increased *Salmonella* prior to flock placement may be attributed to lower competitive exclusion, as other bacterial populations increased each week. As the broilers age, their immunity improves which may reduce gut and fecal populations of these pathogens, thus also reducing their levels in litter. Competitive exclusion of *Salmonella* in the litter microenvironment is supported by concurrent decreased levels of *Salmonella* and increased levels of staphylococci, enterococci and *Clostridium perfringens* as flock age increased (Figure 2.4). Likewise, *Listeria* presence was also associated with broiler age, when broiler age by flock was considered. Chi square analysis per flock indicated *Listeria* presence was higher for Flock 1 and 2 during early and mid-weeks, and week 6 for Flock 3. This shift may be due to seasonal influences. The moisture content was found to be lower during the winter flock and litter moisture may have only provided viable conditions for *Listeria* to be isolated when accumulation of excrement and overflow of watering cups increased moisture and water activity at the final sampling time. Staphylococci were consistently present at higher levels than any other bacteria investigated in the study (Figure 2.4). A gradual per week increase in staphylococci was seen for all locations, with statistically significant increases occurring between weeks 2 and 4 (Figure 2.5). For *Clostridium perfringens* levels, Week 0 through 4 remained relatively constant; Week 6, for all flocks,

was significantly higher than other sampling times (Figure 2.4). *Clostridium* increased approximately 1 $\log_{10}$ cfu kg^{-1} from Week 4 to Week 6. Moisture content increased each week but was found to be statistically higher only for Flock 3 when comparing intra-flock changes. Statistical differences seen in Flock 3 were probably more pronounced due to the litter being drier initially. The moisture content was not statistically higher for each week for all flocks but an upward trend may have allowed for favorable anaerobic conditions to permit *Clostridium* to proliferate. *Enterococcus* was affected by broiler age as well; the difference was statistically significant between all sampling weeks except for Week 2 and Week 4 (Figure 2.4). Mortality rates peaked at Week 2 and Week 6 across all flocks. Mortality numbers from Week 2 are associated with initial broiler placement and may have little to do with litter or house environment. Young broilers are more susceptible to disease due to a less developed immune system and lack the necessary sustaining microflora in the gut that competitively excludes pathogens from overwhelming the gastrointestinal tract (Blankenship et al., 1993). *C. perfringens* causes necrotic enteritis and necrotizing fasciitis in poultry and is a major contributor to broiler mortality which may explain the increased mortality rates for Week 6 (Coursodon et al., 2012).

Seasonality (Flock)

Each flock was presented with different seasonal (environmental) characteristics. A part of broiler house maintenance is regulating house ambient temperature to reduce seasonal effects on the birds. The heating and cooling systems maintain approximate constant temperatures within the house; however, outside climatic factors may affect house environmental conditions. The moisture and temperature of the litter can be

altered due to outside ambient conditions. This was the case with litter temperature which was significantly lower for Flock 3 (winter) for all areas of the house (Figure 2.6). The moisture was also affected by seasonal differences. The moisture content of the litter during the winter flock was lower than that during the other 2 flocks (Table 2.4). The drier litter may be due to the heaters utilized during this time of grow out. Opara et al. (1992), when investigating the presence of pathogens in poultry litter, found a direct correlation to increased water activity and the ability to isolate these microbes. The drier litter during Flock 3 in the present study could explain why fewer bacteria were isolated.

Chi-square analysis among flocks indicated an association between seasonality and *Salmonella* isolation ($p = 0.0038$). When comparing the percentage of *Salmonella* isolates recovered, 54% of all positive samples were collected during Flock 1 (summer) followed by 39% and 7 % from Flock 2 (fall) and Flock 3 (winter), respectively. These findings were consistent with research which found that *Salmonella* is more likely to persist throughout the flock if the pathogen is detected prior to flock placement (Cardinale et al., 2004; Volkova et al., 2009; Volkova et al., 2011). For Flocks 1 and 2, *Salmonella* was detected more frequently at Week 0. A significant difference was associated with *Listeria* isolation and seasonality (Table 2.4). The distribution of all *Listeria* positive isolates across flocks 1, 2, and 3 was 57, 28, and 15%, respectively. *Staphylococcus* levels were highest during Flock 2, while *Clostridium* and *Enterococcus* were not affected by seasonal changes (Table 2.4).

Animal welfare is a major concern in the broiler production industry. Increasing feed conversion to broiler weight and decreasing mortality per flock are the ultimate goals for the broiler growers. In the present study, mortality varied seasonally as each

successive flock had a higher mortality rate. Total mortality rates were 2.3, 3.5, and 8.5%, respectively, for Flocks 1, 2, and 3. The cause(s) of increasing mortality in successive flocks was not identified in the present study and it is possible that the microbes responsible were not investigated. Future research involving molecular analysis of spatial microbial communities may give more information on broiler health and mortality.

Antibiograms

No apparent shifts in *Clostridium perfringens*' antibiograms can be seen when comparing antibiotic resistance profiles from isolates taken prior to flock placement until the final sampling week of flock grow-out. *Enterococcus* antibiograms had a greater number of resistant isolates for Flocks 1 and 2 than Flock 3 for cephalosporin (CF-cephalothin), glycopeptide (VA - vancomycin), tetracycline (TE - tetracycline), and quinolone (ciprofloxacin). *Enterococcus* was the only pathogen for which location may have influenced resistance. Twenty-five percent of the *Enterococcus* isolates taken from the ends of the broiler house were resistant to cephalosporin and 16% were resistant to vancomycin.

One quarter of staphylococci isolates were intermediately or completely resistant to erythromycin. Most staphylococci isolates were resistant to only one class of antibiotics, but one (EF - Time 0 Flock3) exhibited multi-class resistance to macrolide and aminoglycoside classes. There was no difference in antibiotic resistance for broiler age or seasonality. Most *Staphylococcus* isolates (29/48) were predominantly susceptible to all tested antibiotics.

Salmonella, *Enterococcus*, *Clostridium perfringens* and *Listeria* isolates possessed multiple antibiotic resistance (MAR) profiles (Table 2.2). Kelley et al. (1998) and Brooks et al. (2010) determined similar results of MAR in poultry house isolates. Brooks et al. (2010) concluded that these MAR profiles were contained within the house, since isolates from outside the poultry house did not share the same MAR properties. Future research should give more attention to antibiotic resistance profiles and the selective pressures which influence MAR bacterial persistence in the poultry house environment.

Conclusions and Applications

The goal was to identify microbial profiles specific to these areas through spatial analysis of targeted areas within the broiler house environment. Theoretically, environmental and house spatial characteristics should influence the litter to develop unique microcosm within the broiler house. However, after examining spatial differences, few associations could be determined based solely on location. One specific association is that *Salmonella* was found to be more commonly associated with the ends of the house. Isolating *Salmonella* in 15% of the 192 samples justifies the assumption that this pathogen is problematic and garners better methods of attenuation in broiler populations and litter. The EB/EF samples represented 32% of the positive samples collected.

When investigating antibiotic resistant profiles, staphylococci were not as alarming and most isolates were inhibited by all tested antibiotics. Antibiogram profiles of the bacteria collected from this poultry house confirmed that MAR *Salmonella, Clostridium perfringens*, *Listeria*, and *Enterococcus* are concerns. *Salmonella,*

Clostridium perfringens, *Listeria*, and *Enterococcus* microbial isolates were resistance to not only multiple antibiotics but multiple classes as well. These MAR are a concern not only for the poultry industry but from a public health view as well. Though antibiotic use has been limited by the poultry industry in recent years, the MAR profiles of pathogens studied provides evidence that it is still a concern.

The most significant factor that affected proliferation of bacteria was broiler age. Based on our investigation, spatial differences may provide limited effective methods of targeted treatments. Our findings determined that approximately one-third of *Salmonella* was isolated from the ends of the broiler house which may prove useful in targeted treatments. The increased presence may be due to limiting compaction of broiler litter, reduced competition prior to flock placement, or entrance of contamination sources from rodents or other outside vectors while flocks are removed and the end doors are open.

Based on our analysis, temporal differences appear to be the more relevant focus for effective treatment of pathogen reduction. To give more insight into broiler health and the potential to reduce pathogens in broiler litter, future studies investigating the overall microbial communities in these environments regarding temporal changes may provide useful data.

Table 2.1 Primers used for species specific confirmation

Bacteria	Primers	Primer Sequences (5' - 3')	Control Isolates
Salmonella	inv-f	CTGTTGAACAACCCATTTGT[1]	*S. enterica* Typhimurium
	inv-r	CGGATCTCATTAATCAACAAT	ATCC 14028
Staphylococcus	Staph756F	AACTCTGTTATTAGGGAAGAACA	*Staphylococcus aureus*
	Staph750R	CCACCTTCCTCCGGTTTGTCACC	ATCC 25923
Listeria	prs-F-Lys	GCTGAAGAGATTGCGAAAGAAG[3]	*L. monocytogenes*
	prs-R-Lys	CAAAGAAACCTTGGATTTGCGG	ATCC 51722
Enterococcus	tuf-ent1F	TACTGACAAACCATTCATGATG[4]	*Enterococcus fecalis*
	tuf-ent2R	AACTTCGTCACCAACGCGAAC	ATCC 19433
Campylobacter	ceu-E – f	CCTGCTACGGTGAAAGTTTTGC[5]	*C. jejuni*
	ceu-E – r	GATCTTTTTGTTTGTGCTGC	ATCC 33560

[1] (Lu et al., 2003), [2] (Zhang et al., 2004), [3] (Doumith et al., 2004), [4] (Ke et al., 1999), [5] (Gonzalez et al., 1997; Lu et al., 2003)

Table 2.2 Antibiotic class resistance profiles by bacterial group

Antibiotic Classes	*Staphylococcus* n = 48	*Enterococcus* n = 48	*Listeria* n = 22	*C. perfringens* n = 48	*Salmonella* n = 23
Penicillin[1]	0	1*	1*	3*	23*
Cephalosporin[2]	0	15*	7*	2*	13*
Glycopeptide[3]	0	8*	0	2*	22*
Peptide[4]	0	48*	9*	48*	21*
Macrolide[5]	12*	35*	9*	37*	23*
Aminoglycoside[6]	6*	48*	2*	48*	10*
Tetracycline[7]	1	11*	5*	40*	10*
Quinolone[8]	1	13*	4*	3*	1*
# of isolates with 2 class resistance	1	48	10	1	23
# of isolates with 3 or more class resistance	0	48	4	47	23

* Denotes multiple class antibiotic resistance included for at least one isolate.
[1]penicillin (penicillin, ampicillin).
[2]cephalosporin (cephalothin).
[3]glycopeptide (vancomycin).
[4]peptide (polymixin b).
[5]macrolide (erythromycin).
[6]aminoglycoside (amikacin, gentamicin, neomycin, kanamycin).
[7]tetracycline (tetracycline).
[8]quinolone (ciprofloxacin).

Table 2.3 Mean moisture content for each location.

Site	Moisture Content
Cups	42.6%[ab]
Walls	36.9%[b]
Ends	59.0%[a]
Feeders	26.8%[b]

Lettering denotes statistical differences. P-value <0.0001

Table 2.4 Seasonal differences among flocks.

Flock	Moisture % n=64	Staphylococci log_{10} cfu kg^{-1} n=64	*Salmonella*% Positives n=64	*Listeria* % Positives n=64
1	40.0%[ab]	12.4[b]	23%	42%
2	50.6%[a]	12.8[a]	17%	20%
3	33.4%[b]	12.6[a]	3%	11%

Flock 1, 2 and 3 represents summer, fall and winter seasons, respectively. Lettering denotes statistical differences among moisture content and staphylococci levels.

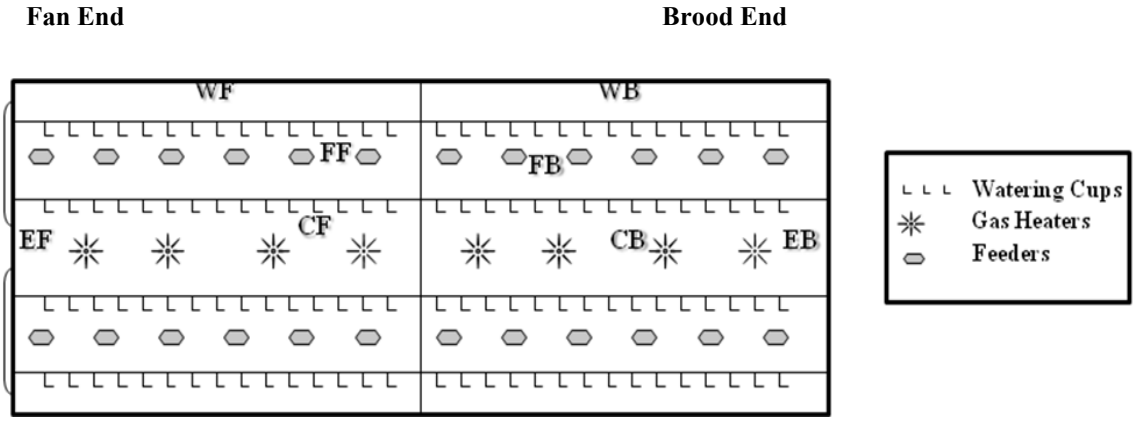

Figure 2.1 Broiler house litter floor layout.

Approximate locations of sampling areas defined as follows: CB, watering cups brood end; CF, watering cups fan end; EB, end sample brood end; EF, end sample fan end; FB, feeder samples brood end; FF, feeder samples fan end; WF, wall sample fan end; and WB, wall sample brood end.

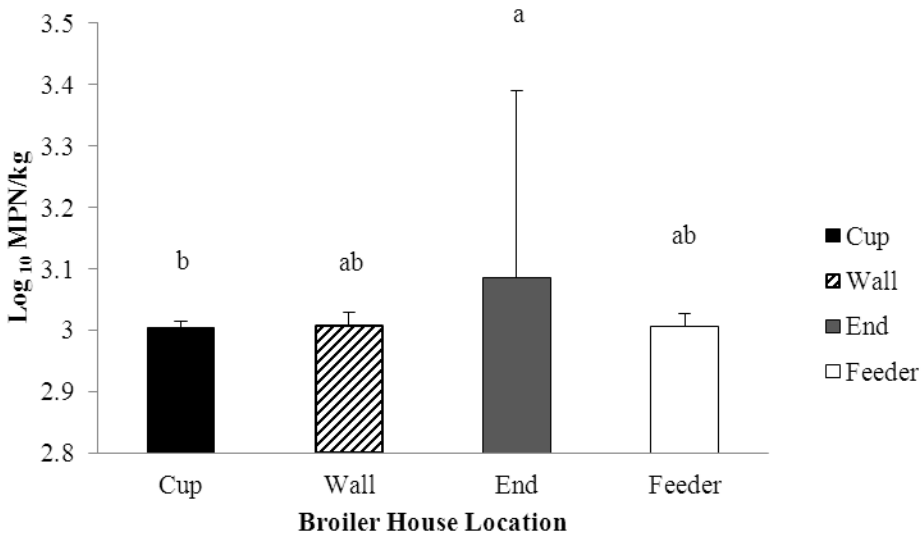

Figure 2.2 Mean *Salmonella* MPN kg^{-1} for all locations.

Bars represent standard deviation and lettering indicates statistical differences. P-value = 0.0207

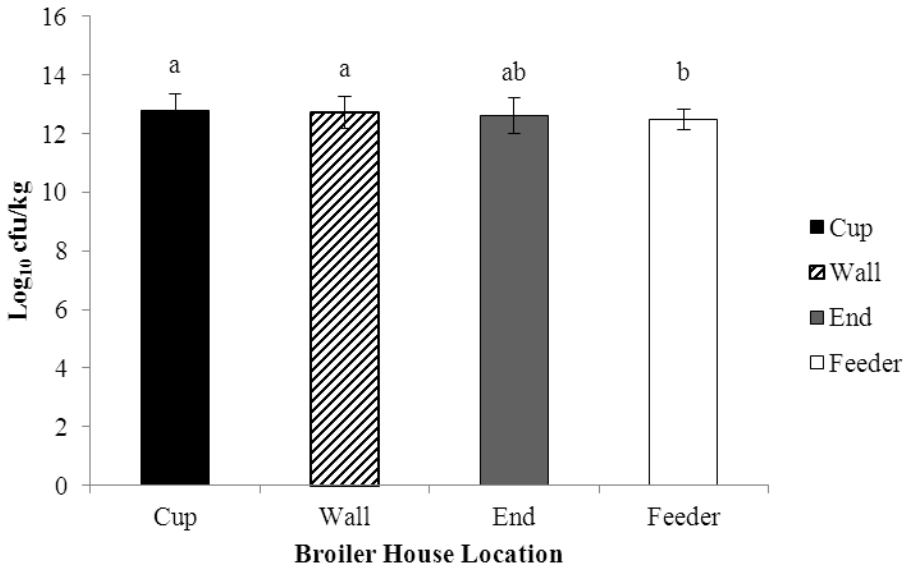

Figure 2.3 Mean staphylococci levels associated with each location.

Bars indicates standard deviation and lettering denotes statistical differences. (P-value = 0.0405)

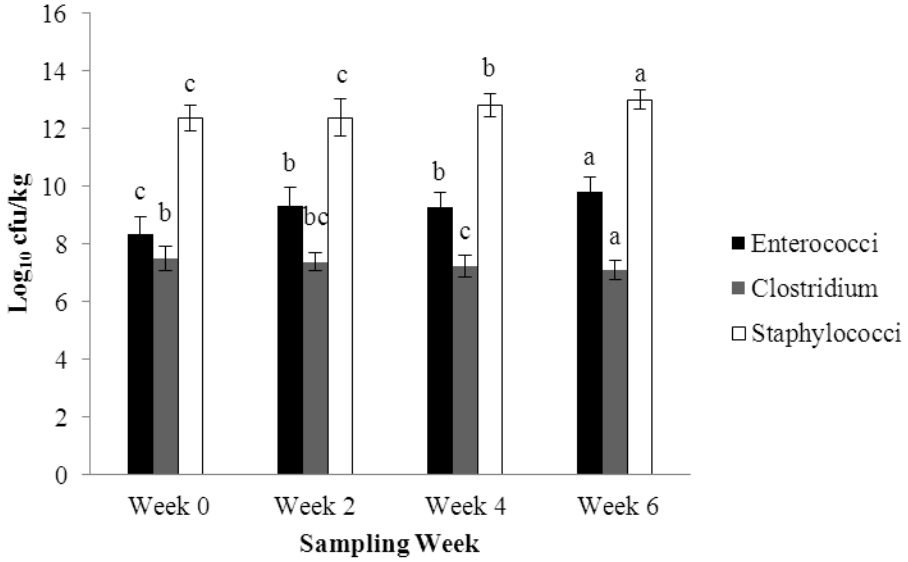

Figure 2.4 Enterococci, *Clostridium perfringens* and staphylococci levels according to broiler age.

Lettering denotes significant differences of sampling weeks only. Each bacteria was analyzed independently ($p < 0.0001$).

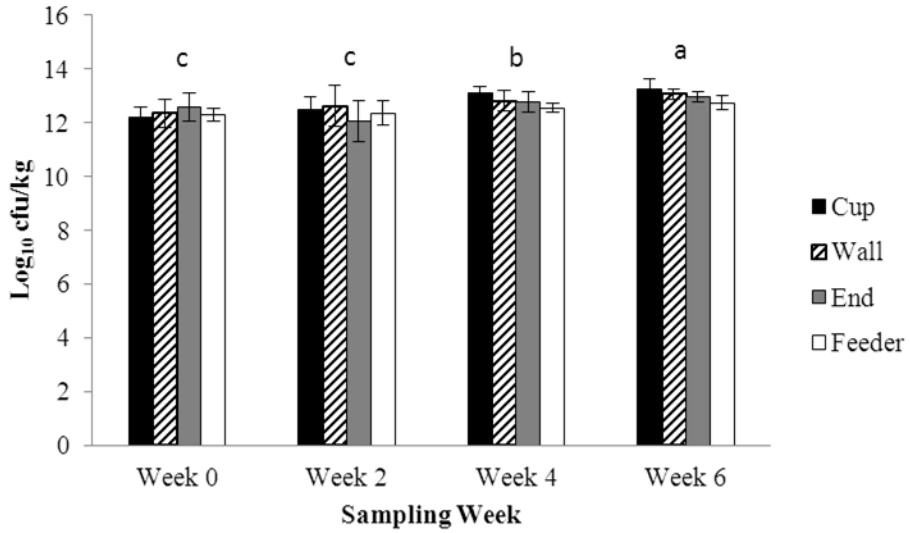

Figure 2.5 Staphylococci levels according to broiler age at specific locations.

Lettering denotes statistical difference among sampling weeks.

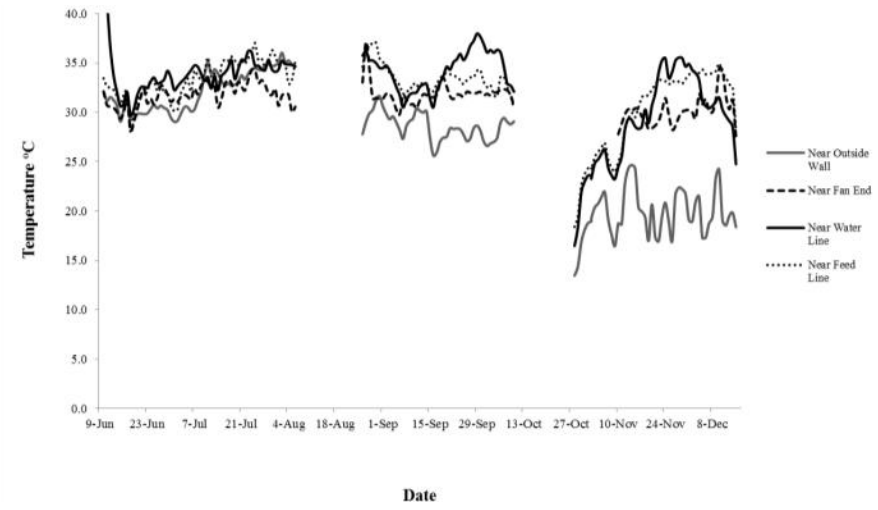

Figure 2.6 Temperature of litter grouped by flock for different areas in the broiler house.

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CHAPTER III

SURVIVAL OF BACTERIAL AND VIRAL PATHOGENS IN SWINE EFFLUENT, CATTLE MANURE AND BIOSOLIDS WHEN APPLIED TO SOUTHEASTERN U.S. SOILS

Abstract

Environmental and public health concerns associated with waste management involve pathogen survival and potential transport following land application. The question of whether there are pathogens that can survive longer periods of time in specific wastes and cause a public health risk needs to be investigated. The focus of this study was to determine the inactivation rates of common foodborne pathogens and coliphage. *Salmonella*, *Campylobacter*, *Listeria*, *Escherichia coli* O157:H7, *Clostridium perfringens*, MS2, and ØX174, were seeded in four waste matrices and applied to two soil types (sandy loam, clay loam) having two application plans (incorporated, non-incorporated). Waste matrices were comprised of Class B biosolids, swine effluent, cattle manure, and phosphate buffered saline (PBS) as a control. Microcosms were established as a factorial combination of the variables. All bacterial and viral pathogens were introduced into each waste to approximate soil levels of 10^6 dry g^{-1}. Temporal sampling and cultural analysis for pathogens and indicators was conducted from each microcosm held under constant ambient and moisture conditions. For comparative analysis of culture data, qPCR was performed on select samples for an understanding of

decay rates of each bacteria of interest and 16S analysis. *Salmonella* survived longer and at higher levels in the cattle manure than other wastes. *Salmonella* was generally present through day 30 for all wastes and 60 for cattle manure. Culturally, *Campylobacter* and *Listeria* were below detection limits (~200 cfu g^{-1}) by day 7 and 21, respectively, but molecularly, both were still detectable 30+ days. *Clostridium perfringens* was more prominent in biosolids and swine effluent, and temporally persisted regardless of waste, soil, or management. MS2 survived longer in biosolids while ØX174 had no statistical distinction between wastes. Both coliphages (phages) were below detection limits in all wastes by day 90. Class B biosolids and cattle manure seemed to sustain the pathogens of interest longer. The higher organic matter associated with these two waste matrixes potentially provided more substantial nutrients and protective measures from predation than the other wastes with reduced total solids. This study gives insight into the effect of waste residual on pathogen inactivation rates in soil and was used to determine quantitative microbial risk analysis of *Salmonella*. Because *Salmonella* can be found in cattle manure at higher levels than other waste residuals and has a slower inactivation rate, it poses the greatest risk to the public (2×10^{-4}) if exposure to land occurs post land application. Four months post application of cattle manure, the risks of *Salmonella* infection remained at 4×10^{-5}, a level that is within tolerable risks based on recommendations of 1:10,000 annual risk of infection set by the U. S. Environmental Protection Agency.

Introduction

Land application of animal manures and biosolids has been practiced for centuries. The practice of waste reuse is beneficial to the crops by adding nutrients and

organic matter and in turn improving crop yields. Not only is it advantageous towards improving farming efforts, as a nutrient amendment, it is cost effective and a seemingly sustainable solution to the vast amount of animal manure (~100 million dry tons) (Burkholder et al., 2007) and biosolids (~7 million dry tons) (USEPA, 1999) produced in the U.S. annually. Approximately 238,000 animal feeding operations (AFOs) and more than 20,000 concentrated feeding operations (CAFOs) are in the United States (Burkholder et al., 2007; Dungan, 2010); (USEPA, 2010). It is estimated that about 60% of biosolids are land applied as a means of disposal (NRC, 2002). Farm management has to be vigilant in controlling the robust amounts of waste that are produced in order to achieve the appropriate nitrogen and phosphorus ratios needed to improve crop yields yet not causing excess runoff that can have detrimental effects to nearby water-systems and property. Land application of biosolids and animal waste is a growing concern for environmental health risks, partially due to the pathogen loads found in these untreated waste products. Many neighboring residences have complained about compromised health status as a direct result from living in close proximity to the application sites (Harrison and Oakes, 2002; Lowman et al., 2011; Robinson et al., 2012).

All wastes harbor pathogens (Gerba and Smith, 2005; Hutchison et al., 2005); however, attenuation methods are employed prior to land application which reduces these levels. Waste management practices can be implemented through composting piles or lagoons, treatment facilities, and/ or land application. Land application is the predominant method of disposal even though other waste management practices may be employed prior to land application. Much of the concern surrounding the general practice of organic residual land application has been as a result of foodborne outbreaks

(Heaton and Jones, 2008; Pepper et al., 2008). Common foodborne bacterial pathogens which can be isolated from both human and animal wastes are *Campylobacter*, *Salmonella*, *Listeria*, *Escherichia coli* O157:H7 and *Clostridium perfringens*; all of which have been implicated in foodborne outbreaks (Moore and Gross, 2010; Scallan et al., 2011). These pathogens are known to survive in the environment for long time periods (Holley et al., 2006; Ingham et al., 2004; Inglis et al., 2010; Islam et al., 2004; Watkins and Sleath, 1981; You et al., 2006). However, site specific and waste specific survival rates for these pathogens are still not clearly understood.

Viral pathogen loads can be excessively high in feces of infected hosts. Because viruses are generally species specific, the main environmental source for human enteric viral transmission would be attributable to land application of biosolids (Gerba et al., 2011). Common viruses that are found in biosolids are norovirus, adenovirus, enterovirus, hepatitis A and E, and rotavirus (Pepper et al., 2010; Viau et al., 2011; Wong et al., 2010). Some animal derived viruses have similar genotypic properties to human acquired viruses such as Hepatitis E virus (HEV) (Kase et al., 2009), and evidence of zoonotic transmission has been provided (Meng, 2011). Kasorndorkbua et al. (2005) and McCreary et al. (2008) detected HEV in approximately 25 % of the swine lagoon samples tested. Though attenuation processes reduce these pathogen levels, viruses may still be present prior to land application at significant levels (Wong et al., 2010). The sustainability and transmission of viral pathogens during transport and land application is a concern to the public. Investigating phages as models may promote better understanding of viral pathogens' survival. MS2 can be used as an indicator of adenoviruses (Hansen et al., 2007), rotavirus (Hansen et al., 2007) norovirus (D'Souza

and Su, 2010) and enteric viruses as a whole in wastewater residuals (Katz and Margolin, 2007). The somatic DNA phage, øX174, has been correlated with adenovirus (Ballester et al., 2005) and an ideal contrast to MS2 which is an male specific, RNA coliphage. In this study, øX174 and MS2 phages were used to determine the viral decay rates in waste residuals applied to both sandy loam and clay loam soils.

When considering microbial survival, soil composition may be a variable that alters the inactivation of microbial pathogens. Soil composition can affects microbial transport by processes of adsorption and particle porosity. Adsorption can be affected by cations which may or may not be present in the soil matrix and affect the affinity of microorganisms to soil particles (Pepper et al., 2000). The porosity of the soil matrix may significantly affect microbial survival. For instance, small pores exclude some microorganisms from protection leaving them vulnerable to water motility and predation by other organisms. The number of pores also determines the soil water-holding capacity that is needed for microbial activity. Soils with high clay content typically have more pores and maintain moisture content while sandy soils have fewer pores and water travels through more rapidly. Clay loam soils have a higher affinity for water molecules, thereby, removing water otherwise available to the microbial population. These factors contribute to microbial transport and decay rates.

During low nutrient and stress induced environments, many pathogens can enter a reduced metabolic state called "viable but not culturable" (VBNC) (Besnard et al., 2002; Makino et al., 2000; Reissbrodt et al., 2002; Rollins and Colwell, 1986). Enrichment media can sometime recover these pathogens when standard methods are not sufficient to detect them. When VBNC bacteria cannot be isolated using standard culture methods or

enrichment processes, quantitative polymerase chain reaction (qPCR) can surpass this limitation and allows quantifiable detection of specific bacteria of interest.

The development of the risk assessment models have been used for decades to determine the risk imposed on exposed population concerning many different environmental contaminants dating back as early as the implementation of the Federal Water Pollution Control Act (1948). Haas et al. (1999) defined risk assessment as the "qualitative or quantitative characterization and estimation of potential adverse health effects associated with exposure to environmental hazards." The use of risk assessment models can be extremely useful and informative but are limited to the accuracy of the parameters used to determine such risk. The more information that simulates "real-world" events, the more improved the risk characterization should become. Quantitative microbial risk assessment was developed to predict microorganism's fate in the environment and the potential threat to populations exposed. Limited data is available that provides risk characterization of pathogens such as *Salmonella* when found in waste residual that is land applied to soils.

As part of environmental stewardship and the intent to understand our effect on public health, it is of great importance to determine what risks may be associated with waste management practices. The objectives of this study was to determine current decay rates for each bacterial pathogen (*Salmonella*, *E. coli* O157:H7, *Clostridium*, *Campylobacter* and *Listeria*) and bacteriophage (øX174 and MS2). In addition to determining inactivation rates for pathogens in wastes, the study aims to provide a comparative analysis of two methods of pathogen detection in multiple forms of wastes with varying composition and organic matter. A direct comparison of these established

decay rates established by both cultural and qPCR for select bacterial pathogens will validate the use of genetic markers and address the limitations of each method. Using the established decay rates associated with each waste applied to southeastern soils, risk simulations can provide useful data to understand what possible concerns are attributable to each waste management practice. Quantitative microbial risk assessment (QMRA) of *Salmonella* was simulated to determine the public risk characterization when exposed to land post application of waste residuals.

Materials and Methods

Study Design

This study was laid out in a factorial (4x2x2x2) design with four organic manures applied (Class B biosolids, cattle manure, swine effluent, and PBS control) to two types of southeastern soils (sandy loam and clay loam soils), using two farm management practices (incorporated and surface application), with two pathogen levels (concentrated cocktail of spiked microorganism and sterile PBS control) for a total of 32 treatments. Each treatment was replicated in triplicates and 11 time points assayed (0, 7, 14, 21, 28, 60, 90, 120, 150, 180, 210 days).

Soil Preparation

Stough (sandy loam) and Leeper (clay loam) soil types were collected from the Mississippi State North Research Farm. Stough soil is classified as coarse-loamy, siliceous, semi-active, thermic fragiaquic paleudults, while Leeper as fine, smectitic, nonacid, thermic vertic epiaquepts (NRC 2012). Each type of soil was homogenized via a #10 (2.54 cm) nominal sized sieve. Moisture content was assessed by weighing 10 g of

soil and heating at 104oC for 48 h. Table3.1 provides a comparative analysis of the two soils used in this study. The effect of soil composition was investigated to determine if contributing factors varied microbial decay for some pathogens.

Culture Preparation

E. coli O157:H7, *Salmonella* (ATCC 14028) and *Listeria* (ATCC 51722) cells were prepared by growing the bacteria to exponential phase (approximately 6 h) in tryptic soy broth (TSB) in 50-mL centrifuge tubes at 35°C shaking at 200 rpm. *Campylobacter jejuni* (ATCC 33560) was prepared by growing to exponential phase (approximately 24 h) in *Campylobacter* Enrichment Broth (CEB) in 50-mL centrifuge tubes at 42^{o}C. *Clostridium perfringens* was inoculated in TSB and grown overnight at 44.5 ^{o}C. C. perfringens inoculated TSB was then aseptically added to Duncan sporulation media (1:10) and grown at 35^{o}C for 2 weeks. Each culture was pelleted by centrifugation at 5000 × g for 30 min. Supernatants were decanted, and cells were re-suspended in an equal volume of sterile phosphate buffered saline, centrifuged and re-suspended for a total of 3 times to remove residual TSB, CEB or Duncan. Final washed cells were suspended in 25 mL phosphate buffered saline, then titered (10^{8} to 10^{9} ml^{-1}) and stored at 4^{o}C. All cells were used within 7 days of preparation.

Bacteriophage Preparation

MS2 and øX174 coliphages were propagated using host *E. coli* (ATCC 15597) and *E. coli* (ATCC 13706), respectively. The method used by Brooks et al. (2005) was repeated prior to seeding each coliphages in wastes. Once amplified, each was titered to

make sure that once seeded in waste the level of 10^6 plaque forming units (PFU) g^{-1} was achieved.

Microcosm Preparation

One hundred fifty grams (dry) of soil was placed in small Styrofoam cups with each of the four wastes applied at a rate of 10% (v/v) dry weight of soil (15 g or mL depending on waste matrix), mimicking the upper layer of soil, following a land application event. Microcosms were established in triplicate for each time-point to be analyzed for each land application scenario. Prior to waste application, each of the four wastes was seeded with each bacteria and phage of interest with the final concentration of each microorganism approximately 10^6 g^{-1} of soil. Each waste was spread evenly. For incorporated management practices, sterile wood sticks were used to mix waste into top layer of soil (~1 in.). A plastic lid was placed over each microcosm to reduce water evaporation. To maintain moisture content each week, moisture content of the microcosms was adjusted up to 25% by adding sterile distilled water.

Microcosms were randomly placed in 3 controlled growth chambers where temperatures were maintained at 30°C for 14 hours and 20°C for 10 hours each day. These parameters were established to mimic temperatures during the summer growing season in the Southeastern United States.

Cultural Enumeration

Prior to microbial analyses, 10 g of each sample was suspended in 95 mL of sterile physiological saline, stomached for 30 seconds, and serial diluted for analysis. Multiple dilutions were plated to respective media for analysis of microorganism

investigated. For isolation of *Salmonella* and *E. coli* O157:H7, samples were direct plated to Hektoen Enteric agar (BD-Difco) and Cefixime Tellurite Sorbitol MacConkey (CTSMAC) agar, respectively, and incubated overnight at 35°C. *Campylobacter jejuni* was enumerated by direct plating to Preston agar (Neogen-Accumedia) containing 5% horse blood (Hema-Resources; Aurora, OR) and incubated at 42°C under microaerophilic conditions for 48h. *Listeria monocytogenes* was direct plated to Oxford agar (Neogen) and incubated for 24 - 48 hours at 30°C. *Clostridium perfringens* was enumerated by membrane filtration on mCP media and incubating at 44.5 °C overnight. When pathogens of interest were no longer able to be isolated by direct plating, 1 g of representative microcosm sample was added to 10 mL of corresponding enrichment broth. After 24 – 48 h enrichment, each was plated to same corresponding media that was used for direct plating.

Coliphages, MS2 and øX174, were enumerated by the plaque assay using the previously stated *E. coli* hosts. Phages were enumerated by adding 0.1 ml of serial diluted sample into 0.1 ml of fresh exponential growth phase *E. coli* host culture specific to phage enumeration in TSB into 5.0 ml of melted soft TSA (0.75% agar) which was maintained in a water-bath at 50°C. Once combined, samples were vortexed and poured over the surface of TSA (1.5% agar) 96-mm-diameter plates. The melted soft agar was tilted back and forth to spread overlay evenly and allowed to harden at room temperature. Plates were incubated overnight at 35°C. Plaque forming units (pfu) were counted within 12 – 16 hours.

Quantitative PCR

DNA extraction was conducted on all samples from day 0, 7, 14, 30 and 60 using Qiagen QiAmp DNA stool mini kit (Cat No. 51504) following the manufacturer's recommended procedure. Reaction conditions consisted of the following: 2 µL DNA extract (diluted 1:10 or 1:100), 12.5 µL of the ABI syber green master mix (Applied Biosystems), 1.0 µL primer (10 µM), and 9.5 µL PCR H_2O for each real-time PCR reaction. For samples that contained clay loam soil 0.5 µL polyvinylpyrrolidone were added per reaction to reduced inhibition (Koonjul et al., 1999). Each reaction was set up in duplicate. *Salmonella*, *Campylobacter*, *E. coli* O157:H7, *Listeria* and 16S were all quantified using the ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA). Real-time PCR was performed by cycling conditions: 95°C (10 min); 40 repetitions: 95°C (15 s) and 60°C (1 min); and melt-curve analysis: 95°C (15 s), 60°C (30 s), and 95°C (15 s). Table 3.2 lists the genetic markers, primer sequence and size corresponding to each target analyzed.

Statistical Analysis

The SAS Enterprise Guide 4.3 (SAS Institute, Cary, NC) was used for all statistical analyses. Geometric mean was calculated prior to analysis. A one-way analysis of variance was performed for each bacteria and phage of interest to determine the effect of waste, soil type, farm management, and time. Statistical differences of means were compared with Fisher's least significant difference at probability level of 0.05.

Calculation of Decay Rates and QMRA

Decay rates (sr_t) (log d^{-1}) were calculated by modeling a first-order decay rate using the following equation:

$$\log_{10} \frac{N_t}{N_0} = sr_t * t + sr_0 \quad [3.1]$$

where N_0 is the initial microbial concentration, N_t is the observed microbial concentration at a subsequent time (t). Decay rates were determined using microbial levels from day 0, 7, 14, 21 and 30 for culture analysis and day 0, 7, 14, and 30 for molecular analysis. Log transformed counts, colony forming units (cfu) g^{-1} or plaque forming units (pfu) g^{-1} were used for bacteria and phage, respectively, to determine sr_t. Molecular data was reported in terms of log transformed genomic units (GU) g^{-1}.

Risk of *Salmonella* infection, associated with land application of waste residuals, was calculated using the approach outlined by NRC (1983) where four steps are defined: 1) Hazard identification, 2) Exposure Assessment, 3) Dose-Response, and 4) Risk Characterization. Soil-*Salmonella* contamination (sc), following land application of a given residual waste, was calculated by the following equation:

$$sc = rc * dr * \frac{1}{10^{sr}} * t * 1000 \quad [3.2]$$

where: *rc* is the level of *Salmonella* in each residual waste, and *dr* is the soil dilution ratio or application rate. To facilitate comparison of the newly developed inactivation rates with previously used rates, *Salmonella* levels (*rc)* were estimated from the literature, 5 - 105 cfu g^{-1} (biosolids) (Zaleski et al., 2005), 162 - 2500 cfu g^{-1} (cattle manure) (Hutchison et al., 2005), and 6.2 - 407 cfu g^{-1} (swine) (Hutchison et al., 2005; McLaughlin and Brooks, 2009; Vanotti et al., 2005). Herein, the PBS control was treated

as a liquid residual waste. For the current simulation, *dr* was assumed to be 1 x 10^{-1} (1:10) waste residual to soil. For comparison to a previously published QMRA, 1.75 x 10^{-3} (0.00175 g of residual per g of residual/soil mixture), an application rate equivalent to 6.75 Mg(dry) ha^{-1} (Gale, 2005) was also estimated. Dose exposure (*d*) was estimated using the following equation:

$$d = sc*v \quad [3.3]$$

where: *v* represents the average volume of soil ingested (4.8 x 10^{-4} kg^{-1}) by an individual during a one-time exposure as estimated by USEPA (1997). 3) The beta-Poisson dose-response model Haas et al. (1999) was used to determine probability of infection (P_I).

$$P_I = 1 - [1 + \frac{d}{N_{50}}(2^{1/\alpha} - 1)]^{-\alpha} \quad [3.4]$$

Alpha (α) is a constant (0.3126) that represents the host – pathogens interaction (Haas et al. (1999) in the dose-response model. The infectivity coefficient, (N_{50}) defined as 2.36 x 10^4 is based on the number of organisms required to induce infection.

Results and Discussion

Effects of Waste on Decay Rates

Figures 3.1 - 3.4 show decay rates as a function of waste residual type. Cattle manure sustained *Salmonella* longer than other wastes when analyzed by culture detection; swine effluent, biosolids and PBS had very similar log reductions over time. *Salmonella* survived twice as long in cattle manure in both soil types regardless of application method. Cattle manure was considered protective of *Salmonella*; it was isolated, through enrichment, from sandy loam soils, when surface applied, for up to four

months. The viability of *Salmonella* in cattle manure is justified because no chemical attenuation method was employed prior to application as was the case with biosolids. Biosolids are commonly lime stabilized to control pathogen levels and the vector attraction (i.e. insects). The increased pH (Bean et al., 2007) and the reduced moisture (Opara et al., 1992) associated with the treatment reduces bacterial colonization compared to cattle manure residuals. A major contributor to *Salmonella*'s survival in cattle manure may be due to increased nutrients when compared to all other wastes. The high percentage of total solids, including high organic content, provides more available nutrients than liquid residuals (swine effluent and PBS), which would explain the slower decay rates compared to these wastes.

According to USDA-AMS (2000), animal manure can be applied to food crops with a stipulated post-harvest delay of 90 to 120 days. This current study suggests that if high levels of *Salmonella* are present in animal manure applied to land, it may persist longer than the allotted waiting period for harvesting food crops. It is important to note, inactivation rates were established using high levels (10^6 cfu g^{-1}) of pathogens which is a caveat of laboratory studies. However, using data from Figure 3.1, if log reduction (~1 log) is similar to the observed levels of 2.5 x 10^3 cfu g^{-1} (Gale, 2005), then it would indicate that after one month cattle manure would still harbor *Salmonella* at levels of 2.5 x 10^2 cfu g^{-1}.

By culture methods, *Campylobacter* and *Listeria* were not isolated in any waste, regardless of soil or management practice, after 1 week and 3 weeks, respectively. Because these bacteria are known to enter a VBNC state, molecular analysis can provide more information about their environmental persistence. Inactivation of *E. coli* O157

was not significantly different when considering waste residuals alone. *Clostridium perfringens* survived in all treatments for most of the sampling times, except in cattle manure and PBS when surface applied to clay loam soil. The ability of *Clostridium perfringens* to survive is understandable, since it is known as a spore-forming bacterium that can endure environmental changes better than other microorganisms.

Both phages were below detection limits by day 90 for all treatments. A paired t-test was conducted to determine significant differences in phage isolation between waste, soil type and farming practice. Overall, we found biosolids yielded higher concentrations of MS2 phage. These findings are supported by Wei et al. (2010) who found that MS2 had a higher affinity for biosolids than swine or cattle manure potentially because of increased iron oxide (You et al., 2005). The possible explanation that MS2 host is more commonly found in biosolids could account for the increased survival in this residual. MS2 survived longer in biosolids than øX174 regardless of soil type or application method except for biosolids surface applied to clay loam soils which had very similar rates of inactivation (Table 3.3). Cattle manure maintained higher concentrations of øX174 phage in sandy loam soils longer when surface applied (60 days) while surface application of PBS was able to maintain øX174 phage when applied to clay loam soils for just as long. Liquid residuals (swine effluent and PBS) were more protective of øX174 in clay loam soils but a shift of øX174 persistence in solid residuals (biosolids and cattle manure) was observed in sandy loam. There was no significant difference in øX174 inactivation rates for any waste except PBS applied to clay loam soils ($p=0.0005$).

Amplification of 16S ribosomal RNA (rRNA) gives a quantitative snapshot of the microbial communities found in each waste / soil interface. No significant differences

were observed in regard to waste residuals. Application of biosolids provided the greatest decline, specifically when surface applied, of the microbial community as measured by 16SrRNA. Molecular analysis of the bacterial pathogens (*Salmonella*, *E. coli* O157, *Listeria* and *Campylobacter*) provided decay rates that were more conservative than culture data for most application scenarios, indicating that bacterial populations survive longer than culturing methods can capture. One of the most pronounced effects associated with wastes was identified with cattle manure. Cattle manure was most protective for all four bacteria when surface applied to sandy loam soil (Table 3.4).

Because inactivation constants associated with *Salmonella* were further analyzed to determine QMRA, each investigated land application scenario is given to provide waste residual effects on decay via qPCR (Figure 3.5 – 3.8). Figure 3.5 provides data of *Salmonella* log reduction for each waste residual surfaced applied to sandy loam soil. Cattle residuals sustained *Salmonella* the longest and PBS the least. *Salmonella* survival in waste residuals incorporated into sandy loam soil were similar for cattle manure, swine effluent and PBS (~2 log reduction), but biosolids did not sustain the bacteria (~5 log reduction) (Figure 3.6). *Salmonella* seeded in biosolid residuals and surface applied to clay loam soils had the least log reduction (~3.5) while cattle manure was least protective (~5.5 log reduction) (Figure 3.7). Swine effluent sustained *Salmonella* (~2.5 log reduction) when incorporated into clay loam soils but other residuals did not (~4.5 – 5 log reduction) (Figure 3.8).

Effects of Soil Type on Decay Rates

Salmonella, Campylobacter nor *Listeria* decay rates were affected by soil type. Considering both culture and molecular data, clay loam soil type significantly affected inactivation rate of *E. coli* O157 in swine effluent causing a slower decay rate than when applied to sandy loam soils ($p=0.0381$). Biosolids was able to harbor *E. coli* O157 longer when applied to sandy loam soils than clay loam soils according to molecular derived inactivation rates ($p<0.05$)(Table 3.5). Analysis of 16S rRNA via qPCR indicated that bacterial populations declined more when waste were applied to sandy loam soils than clay loam soils.

MS2 phage was significantly higher in all waste treated sandy loam soils except for cattle manure. According to Straub et al. (1992) comparison of viral decay in biosolids applied to clay and sandy soils, MS2 decay rates were not similar to this study. MS2 had a much slower inactivation rate than this previously published study. In addition, MS2 in all wastes applied to sandy loam had a much slower inactivation rate than clay soils, contrary to their findings (Straub et al., 1992). MS2 phage survival regardless of waste applied to sandy loam soils persisted longer than clay loam, indicating sandy loam was protective. It is possible that the adherence to clay particles made it more difficult to detect phage (Sobsey et al., 1980) but does not explain the distinct differences the Straub et al. (1992) study; this distinction may be due to differences in sampling and culturing methods.

Effects of Application Method on Decay Rates

Salmonella, *E. coli* O157 and *Campylobacter*, when viewing culture data, were not affected by application method alone. *Salmonella* was protected when incorporated,

but not significantly (p=0.0679). *Salmonella* may bind to soil particles and move into small pores when incorporated, thus being more protected due to binding and possibly increased moisture availability. *Salmonella* may have not survived as long when surface applied due to desiccation of waste residuals. *Listeria* was able to survive longer in waste residuals when surface applied to clay loam soils as opposed to being incorporated (p=0.0174). The reduction in survival when incorporated may be due to increased competition of other microorganisms in the soil. *Listeria* may become VBNC due to the increased environmental stresses when incorporated.

Molecular derived decay rates provided similar results compared to culture data, indicating that application method had no effect on *Salmonella* and *E. coli* O157. However, *Salmonella* was protected when incorporated, as seen with the culture data. When analyzed by qPCR, *Campylobacter* survived longer when surface applied to sandy loam soils (p=0.0191), but no significant difference was noted for clay loam soil (Table 3.4). Molecular analysis showed no effect of application method for *Listeria* survival.

Effects of Detection Method on Decay Rates

Molecular and culture enumeration are both useful tools for determining the presence of microorganisms in environmental samples. However, both have advantages and disadvantages. Culture analysis is cost-effective, simple, and can be used to analyze large samples aliquots, but the time to results is longer and some organisms cannot be cultured. Molecular detection via qPCR overcomes the challenges of culture analysis because result times are timelier and non-culturable organisms can be quantified. However, qPCR can be expensive, only small quantities can be analyzed, and genetic markers can persist longer than viability. These limitations must be recognized;

nevertheless, many labs are turning towards qPCR for pathogen detection, which ultimately will affect the way QMRA is interpreted.

Generally, qPCR produced slower inactivation rates for these bacteria except when bacteria were analyzed in cattle manure regardless of soil types or application methods (Table 3.6). Analysis of comparative decay rates for *Listeria* and *Campylobacter* resulted in slower inactivation derived by molecular detection than culture derived inactivation rates. In contrast, *Salmonella* and *E. coli* O157:H7 survival was significantly extended in culture analysis compared to molecular detection for cattle residuals ($p=0.0407$ and $p=0.0403$, respectively). Klein et al. (2011) noted that qPCR analysis of decay rates found that microorganisms yielded slower decay rates than culture data; however, *Salmonella* and *E. coli* O157 were not investigated. A possible explanation for the discrepancy may be due to *Salmonella* and *E. coli* O157 ease of culturing opposed to *Listeria* and *Campylobacter*. *Listeria* and *Campylobacter,* as an environmental response, may quickly enter the VBNC state. More importantly, the larger sample analyzed using culture (10g) opposed to qPCR (0.5g) may provide a more accurate indication of bacterial survival.

The most distinctive difference in analysis of decay rates was associated with *Campylobacter* detection. Viable, *Campylobacter* could not be detected after the first sampling time (day 1) but was detected for 30+ days via qPCR (Table 3.7). This difference was significant ($p < 0.0001$), and the inactivation rates reflect these differences (Table 3.6). *Listeria* inactivation in all waste residuals applied to sandy loam soils was significantly slower by molecular detection than culture detection ($p < 0.05$). qPCR can allow for quantitation of genomic units via the use of genetic markers when cells enter a

stress response state (VBNC) due to low nutrients or other harsh environments, which may explain these results A caveat associated with qPCR is distinguishing whether genomic units truly represent viable cell counts. Data showed no significant difference when comparing assay method for *Listeria* survival in all waste residuals applied to clay loam soils. Soils with a high percentage of clay have a higher cation exchange capacity (CEC) than soils that have a high sand content. CEC aids in the adherence of microorganisms to the soil particle. The adherence properties of the clay loam soil may have reduced the efficiency of pathogen detection associated with this soil.

***Salmonella* QMRA**

A critical component of QMRA modeling is the inactivation rates associated with a pathogen. Until recently, most inactivation rates were only investigated for a single soil type or waste residual. While this information is useful, the need to understand the effect of waste or soil type is crucial to fully implement QMRA. Information gained from the decay rates in this study provides critical data needed to calculate QMRA for analysis of land application and the risks of *Salmonella* infectivity to the public exposed to these fields after initial application (1 day) and at specified times (7, 30, 60, or 120 days) (Tables 3.8 & 3.9). Comparison of risks assessed using molecular and culture decay rates attested that bacteria in cattle manure had the highest P_I for all soil types and application methods for the initial day of application ($p<0.0001$), Day 7 ($p= 0.0004$) and Day 30 ($p=0.0277$); however, analysis of variance indicated no differences in risk across all wastes regardless of detection method after 30 days. *Salmonella* in biosolids indicated the least risk of infection post exposure to land application sites with application rates of 1:10 residual waste : soil dilution ratio (data not shown) and even less at application rates

of 1.75 x 10^{-3} residual per g (Table 3.8 & 3.9). Soil type had no significant difference in risk of infection, but a higher risk of *Salmonella* was noted across all wastes applied to sandy loam soil with a P_I 2 - 4 orders of magnitude higher than all waste residuals applied to clay loam soils.

QMRA: Comparison of Molecular and Culture Analysis

Method of detection is crucial to risk, and not all labs perform the same assay, thus adding to risk uncertainty. Recently, the advent of qPCR has enabled fast reliable detection of pathogens; however, current risk models were developed using live pathogens, so QMRA must adapt to these new technologies. Risk characterization from Day 1 to Day 30 are relatively similar, however, risks quickly diverge for one-time exposures, modeled post 30 days following land application. Cattle had the highest P_I for Day 1 exposures. The risks remained significantly higher than other waste residuals ($p < 0.05$) for the first 2 months regardless of detection method. Because culture data provided substantially slower decay rates for *Salmonella* in cattle manure for all soils and farming practices, risk of *Salmonella* infection was at least 4 orders of magnitude higher than molecular data (Table 3.8 & 3.9). Molecular derived decay rates provided the most conservative risks for the other waste residuals. Figures 3.9 – 3.12 provides a descriptive graphic of waste residuals produce high risks and the effects of application method (surface vs. incorporated) on each scenario derived by both culture and qPCR. Culture analysis of waste applied to sandy loam soil favored cattle manure regardless of application method (Figure 3.9), and clay loam soil highlighted that incorporated wastes produced highest risks, with cattle being the highest (Figure 3.10). Molecular analysis of waste applied to sandy loam indicated cattle manure as having the highest risk regardless

of application method and incorporated wastes exceeded surface application (Figure 3.11); risk associated with clay loam highlighted swine as the highest risk of *Salmonella* infection post 30 days but at significantly reduced levels (10^{-15}) (Figure 3.12).

A recent study using the same beta-Poisson model determined that risk of infection for one-time exposure of biosolids applied to soil was 1.42×10^{-9} (Brooks et al., 2012). This P_I is within the range of calculated risk in this study using both molecular (2.7×10^{-8}) and culture (7.46×10^{-11}) analysis. Figure 3.13 outlines the steps associated with land application of waste residuals and the subsequent reduction in probability of infection using the simulated model. Incidental exposure outlined by Brooks et al. (2012) for P_I of *Salmonella* via application of waste residuals of biosolids and swine effluent are very similar but much lower for cattle manure application. This comparison supports the assertion that both assay methods are useful to determine inactivation rates associated with pathogen fate and risk of infection. Using only the most conservative risk assessment calculations from both molecular and culture derived inactivation rates, P_I of *Salmonella* was still within acceptable risks (10^{-5}) if an individual is exposed to a land application site 4 months after application (Table 3.10). The most conservative risk is associated with culture derived decay rates when an assumption of *Salmonella* levels of 105 cfu g^{-1} in cattle manure are applied to soil regardless of soil type or application method on the initial day of application. No other application scenario indicated any higher risk for *Salmonella* infection. Molecular analysis was predominantly useful when waste residuals were surface applied but culture data offered more conservative risks when wastes were incorporated. As stated previously, risk associated with cattle manure regardless of soil type was more conservative when decay rates were analyzed by culture

detection. The regulations of delayed harvest of crops and restrictions imposed on the public to these sites are warranted and allow for substantially reduced risks.

Conclusion

The data attempted to look at the effect of waste residuals on pathogenic bacteria and viruses under different land application scenarios. This study was able to accomplish three objectives: 1) address which waste residual promotes or sustains pathogen levels under parallel events, 2) compare how farming scenarios (i.e. soil type or application method) affect establishing inactivation constants and 3) assess how detection methods affect determining inactivation constants and application of QMRA. Cattle manure was the most protective waste residual for *Salmonella*. For most bacterial inactivation constants, animal manures were more protective, but viral inactivation constants were associated with biosolids especially MS2 phage. Soil type and application method did prove to be significant variables that affected inactivation rates for certain bacteria and virus survival. For example, phage survived longer when surface applied than incorporated. The inactivation rates when compared via culture and molecular analysis did not always coincide, but quantitative analysis can be difficult to interpret as both assays have qualities and faults. Based on the differences associated with both assay methods, it is suggested that both be used to aid in the other's limitations. The differences that are associated with each detection method can aid in giving a more holistic and more conservative risk characterization of pathogens in the environment. Because the simulated risk models were established using culture detection methods, more investigation needs to be provided to determine how molecular detection techniques affect this paradigm.

Table 3.1 Soil Characteristics of Stough fine sandy loam and Leeper silty clay loam according to NCRS-USDA

Soil Characteristics	Stough fine sandy loam	Leeper silty clay loam
Sand	64%	20%
Silt	27%	49%
Clay	~10%	31%
pH	5	7

This data was obtained from the National Resources Conservation Service – USDA for the area of soil collected. (NRC, 2012)

Table 3.2 Quantitative PCR Primer Sequences Associated with Each Bacterial Targets

Target	Locus	Primer sequence (5' to 3')	Primer size	References
Salmonella[1]	spaQVicF	GCA ATT ACA GGA ACA GAC GCT	100 bp	(Kurowski et al., 2002)
	spaQvicR	CCT GAC GCC CGT AAG AGA		
Listeria monocytogenes[2]	hlyQF	CATGGCACCACCAGCATCT	64 bp	(Rodriguez-Lazaro et al., 2004)
	hlyQR	ATCCGCGTGTTTCTTTTCGA		
Campylobacter[3]	campF2	CACGTGCTACAATGGCATAT	109 bp	(Lund et al., 2004)
	campR2	GGCTTCATGCTCTCGAGTT		
E. coli[4,5]	vt2 (stx2) - f	TGT TGG CTG GGT TCG TTA ATA CGG	121 bp	(Lu et al., 2003)
	vt2 (stx2) - r	TCC GTT GTC ATG GAA ACC GTT GTC		
16S General[6]	16SFor	TCCTACGGGAGGCAGCAGT	460 bp	(Nadkarni et al., 2002)
	16SRev	GGACTACCAGGGTATCTAATCCTGTT		

Table 3.3 Culture Derived Decay Rates

Soil Type	Application Method	Residual Source	Decay Rates ($log_{10}(N_t/N_0)/t$)						
			Salmonella	*E. coli* O157	*Listeria*	*Campylobacter*	*Clostridium*	MS2 phage	ØX174 phage
Sandy loam	Surface	Biosolids	-0.1583	-0.1311	-0.2303	-0.5857	-0.0143	-0.0462	-0.0926
		Swine	-0.1997	-0.2266	-0.2098	-0.6033	-0.0236	-0.2342	-0.2047
		Cattle	-0.0060	-0.0228	-0.2255	-0.5667	-0.0042	-0.1839	-0.1252
		PBS	-0.1955	-0.2568	-0.2404	-0.5329	-0.0295	-0.2446	-0.2084
	Incorporated	Biosolids	-0.1041	-0.1160	-0.2440	-0.6525	-0.0010	-0.1248	-0.1886
		Swine	-0.1911	-0.2194	-0.2369	-0.7696	-0.0300	-0.1501	-0.1742
		Cattle	-0.0179	-0.0385	-0.2863	-0.6758	-0.0149	-0.1838	-0.0708
		PBS	-0.1431	-0.1910	-0.2371	-0.6365	-0.0105	-0.1531	-0.1729
Clay loam	Surface	Biosolids	-0.2018	-0.0792	-0.1351	-0.8020	-0.0631	-0.1806	-0.1738
		Swine	-0.1995	-0.0759	-0.0575	-0.7656	-0.0419	-0.2189	-0.1547
		Cattle	-0.1203	-0.0656	-0.0930	-0.8352	-0.1196	-0.2003	-0.1931
		PBS	-0.1789	-0.0504	-0.0863	-0.6967	-0.1001	-0.2058	-0.0612
	Incorporated	Biosolids	-0.0438	-0.1162	-0.1489	-0.6525	-0.0416	-0.1120	-0.1831
		Swine	-0.0378	-0.0498	-0.1635	-0.5856	-0.0167	-0.1094	-0.1529
		Cattle	-0.0145	-0.0385	-0.1514	-0.6772	-0.0286	-0.1338	-0.1732
		PBS	-0.1300	-0.1003	-0.1325	-0.4709	-0.0294	-0.1021	-0.0717

The underlined rates are the most sustaining residual per bacteria.

Table 3.4 Molecular Derived Decay Rates for Bacteria Analyzed

Soil Type	Application Method	Residual Source	Comparison of Molecular Derived Decay Rates ($log_{10}(N_t/N_0)/t$)			
			Salmonella	*E. coli* O157	*Listeria*	*Campylobacter*
Sandy loam	Surface	Biosolids	-0.0730	-0.1079	-0.1110	-0.0539
		Swine	-0.0762	-0.1568	-0.1041	-0.0276
		Cattle	-0.0593	-0.0460	-0.0909	-0.0419
		PBS	-0.2071	-0.1995	-0.1048	-0.0456
	Incorporated	Biosolids	-0.1607	-0.0831	-0.0871	-0.0787
		Swine	-0.0631	-0.0755	-0.0812	-0.0650
		Cattle	-0.0802	-0.0760	-0.1010	-0.0849
		PBS	-0.0560	-0.0460	-0.0333	-0.0536
Clay loam	Surface	Biosolids	-0.1313	-0.1655	-0.0683	-0.1199
		Swine	-0.1386	-0.0864	-0.0770	-0.0988
		Cattle	-0.1886	-0.1173	-0.0409	-0.0524
		PBS	-0.1549	-0.0226	-0.0683	-0.0674
	Incorporated	Biosolids	-0.1572	-0.1566	-0.0933	-0.1589
		Swine	-0.0972	-0.0843	-0.0548	-0.0538
		Cattle	-0.1668	-0.0956	-0.0760	-0.0676
		PBS	-0.1780	-0.1247	-0.0496	-0.0929

Underlined inactivation rates indicate the most protective waste residual for each scenario and bacteria analyzed.

Table 3.5 Comparison of inactivation rates when derived by culture and molecular detection.

Soil Type	Application Method	Residual Source	Comparison of Culture and Molecular Derived Decay Rates ($log_{10}(N_t/N_0)/t$)							
			Salmonella		*E. coli* O157		*Listeria*		*Campylobacter*	
Sandy loam	Surface	Biosolids	-0.1583	-0.0730	-0.1311	-0.1079	-0.2303	-0.1110	-0.5857	-0.0539
		Swine	-0.1997	-0.0762	-0.2266	-0.1568	-0.2098	-0.1041	-0.6033	-0.0276
		Cattle	-0.0060	-0.0593	-0.0228	-0.0460	-0.2255	-0.0909	-0.5667	-0.0419
		PBS	-0.1955	-0.2071	-0.2568	-0.1995	-0.2404	-0.1048	-0.5329	-0.0456
	Incorporated	Biosolids	-0.1041	-0.1607	-0.1160	-0.0831	-0.2440	-0.0871	-0.6525	-0.0787
		Swine	-0.1911	-0.0631	-0.2194	-0.0755	-0.2369	-0.0812	-0.7696	-0.0650
		Cattle	-0.0179	-0.0802	-0.0385	-0.0760	-0.2863	-0.1010	-0.6758	-0.0849
		PBS	-0.1431	-0.0560	-0.1910	-0.0460	-0.2371	-0.0333	-0.6365	-0.0536
Clay loam	Surface	Biosolids	-0.2018	-0.1313	-0.0792	-0.1655	-0.1351	-0.0683	-0.8020	-0.1199
		Swine	-0.1995	-0.1386	-0.0759	-0.0864	-0.0575	-0.0770	-0.7656	-0.0988
		Cattle	-0.1203	-0.1886	-0.0656	-0.1173	-0.0930	-0.0409	-0.8352	-0.0524
		PBS	-0.1789	-0.1549	-0.0504	-0.0226	-0.0863	-0.0683	-0.6967	-0.0674
	Incorporated	Biosolids	-0.0438	-0.1572	-0.1162	-0.1566	-0.1489	-0.0933	-0.6525	-0.1589
		Swine	-0.0378	-0.0972	-0.0498	-0.0843	-0.1635	-0.0548	-0.5856	-0.0538
		Cattle	-0.0145	-0.1668	-0.0385	-0.0956	-0.1514	-0.0760	-0.6772	-0.0676
		PBS	-0.1300	-0.1780	-0.1003	-0.1247	-0.1325	-0.0496	-0.4709	-0.0929

Inactivation rates derived by culture method on the left and qPCR on the right. The underlined rates are culture data that indicated bacterial inactivation rates that are slower than molecular analysis. The highlighted inactivation rates are the slowest per waste residual.

Table 3.6 Using qPCR, *Campylobacter* Log10 Reduction (GU g-1) at Day 30.

Waste Residual	Sandy loam†		Clay loam‡	
	Surface[a]	Incorporated[b]	Surface[a]	Incorporated[a]
Biosolids	1.5	2.1	3.7	4.3
Swine	0.5	1.9	2.8	1.4
Cattle	1.1	2.5	1.8	1.9
PBS	1.1	1.6	2.0	2.6

†[ab] denotes a significant difference among application method for inactivation rates when waste residuals were applied to Sandy loam soil. ‡[a] denotes that no significant differences were detected among application method for clay loam soils when analyzing inactivation rates of *Campylobacter*. Initial bacteria levels of 10^6 GU g^{-1} were seeded into each waste.

Table 3.7 QMRA associated with *Salmonella* and Culture Decay Rates using 1.75 x 10^{-3} kg manure (kg soil)$^{-1}$ application rate.

			Risk of Infection (One-time)									
			Decay Time (day)									
		Residual	1		7		30		60		90		120	
Soil Type	**Application**	**Source**	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
Sandy loam	Surface	Biosolids	3 x 10^{-7} - 7 x 10^{-6}		4 x 10^{-8} - 7 x 10^{-7}		8 x 10^{-12} - 2 x 10^{-10}		BL - 3 x 10^{-15}		BL - BL		BL – BL	
		Swine	4 x 10^{-7} - 2 x 10^{-5}		2 x 10^{-8} - 1 x 10^{-6}		6 x 10^{-13} - 4 x 10^{-11}		BL - BL		BL - BL		BL – BL	
		Cattle	1 x 10^{-5} - 2 x 10^{-4}		1 x 10^{-5} - 2 x 10^{-4}		1 x 10^{-5} - 2 x 10^{-4}		6 x 10^{-6} - 1 x 10^{-4}		4 x 10^{-6} - 7 x 10^{-5}		3 x 10^{-6} - 4 x 10^{-5}	
		PBS	4 x 10^{-7} - 2 x 10^{-5}		2 x 10^{-8} - 2 x 10^{-6}		8 x 10^{-13} - 5 x 10^{-11}		BL - BL		BL - BL		BL – BL	
	Incorporated	Biosolids	4 x 10^{-7} - 8 x 10^{-6}		9 x 10^{-8} - 2 x 10^{-6}		3 x 10^{-10} - 7 x 10^{-9}		3 x 10^{-13} - 5 x 10^{-12}		BL - 4 x 10^{-15}		BL – BL	
		Swine	4 x 10^{-7} - 2 x 10^{-5}		3 x 10^{-8} - 2 x 10^{-6}		1 x 10^{-12} - 7 x 10^{-11}		BL - BL		BL - BL		BL – BL	
		Cattle	1 x 10^{-5} - 2 x 10^{-4}		1 x 10^{-5} - 2 x 10^{-4}		6 x 10^{-5} - 4 x 10^{-4}		1 x 10^{-6} - 2 x 10^{-5}		4 x 10^{-7} - 6 x 10^{-6}		1 x 10^{-7} - 2 x 10^{-6}	
		PBS	4 x 10^{-7} - 3 x 10^{-5}		6 x 10^{-8} - 4 x 10^{-6}		3 x 10^{-11} - 2 x 10^{-9}		BL - 1 x 10^{-13}		BL - BL		BL – BL	
Clay loam	Surface	Biosolids	3 x 10^{-7} - 6 x 10^{-6}		2 x 10^{-8} - 4 x 10^{-7}		4 x 10^{-13} - 8 x 10^{-12}		BL - BL		BL - BL		BL – BL	
		Swine	4 x 10^{-7} - 2 x 10^{-5}		2 x 10^{-8} - 1 x 10^{-6}		6 x 10^{-13} - 4 x 10^{-11}		BL - BL		BL - BL		BL – BL	
		Cattle	1 x 10^{-5} - 2 x 10^{-4}		2 x 10^{-6} - 3 x 10^{-5}		4 x 10^{-9} - 7 x 10^{-8}		9 x 10^{-13} - 1 x 10^{-11}		BL - 3 x 10^{-15}		BL – BL	
		PBS	4 x 10^{-7} - 2 x 10^{-5}		3 x 10^{-8} - 2 x 10^{-6}		2 x 10^{-12} - 2 x 10^{-10}		BL - BL		BL - BL		BL – BL	
	Incorporated	Biosolids	4 x 10^{-7} - 9 x 10^{-6}		3 x 10^{-7} - 5 x 10^{-6}		2 x 10^{-8} - 5 x 10^{-7}		1 x 10^{-9} - 2 x 10^{-8}		5 x 10^{-11} - 1 x 10^{-9}		3 x 10^{-12} - 5 x 10^{-11}	
		Swine	5 x 10^{-7} - 3 x 10^{-5}		3 x 10^{-7} - 2 x 10^{-5}		4 x 10^{-8} - 3 x 10^{-6}		3 x 10^{-9} - 2 x 10^{-7}		2 x 10^{-10} - 1 x 10^{-8}		2 x 10^{-11} - 1 x 10^{-9}	
		Cattle	1 x 10^{-5} - 2 x 10^{-4}		1 x 10^{-5} - 2 x 10^{-4}		5 x 10^{-6} - 8 x 10^{-5}		2 x 10^{-6} - 3 x 10^{-5}		7 x 10^{-7} - 1 x 10^{-5}		3 x 10^{-7} - 4 x 10^{-6}	
		PBS	4 x 10^{-7} - 3 x 10^{-5}		7 x 10^{-8} - 5 x 10^{-6}		7 x 10^{-11} - 5 x 10^{-9}		9 x 10^{-15} - 6 x 10^{-13}		BL - BL		BL – BL	

BL – risks which were below reportable limits

Table 3.8 QMRA associated with *Salmonella* and Molecular Decay Rates using 1.75 x 10^{-3} kg manure (kg soil)$^{-1}$ application rate.

			Risk of Infection (One-time)											
			Decay Time (day)											
		Residual	1		7		30		60		90		120	
Soil Type	**Application**	**Source**	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
Sandy loam	Surface	Biosolids	4 x 10^{-7}	8 x 10^{-6}	1 x 10^{-7}	3 x 10^{-6}	3 x 10^{-9}	6 x 10^{-8}	2 x 10^{-11}	4 x 10^{-10}	1 x 10^{-13}	3 x 10^{-12}	BL	2 x 10^{-14}
		Swine	4 x 10^{-7}	3 x 10^{-5}	2 x 10^{-7}	1 x 10^{-5}	3 x 10^{-9}	2 x 10^{-7}	2 x 10^{-11}	1 x 10^{-9}	8 x 10^{-14}	5 x 10^{-12}	BL	3 x 10^{-14}
		Cattle	1 x 10^{-5}	2 x 10^{-4}	6 x 10^{-6}	9 x 10^{-5}	2 x 10^{-7}	4 x 10^{-6}	4 x 10^{-9}	6 x 10^{-8}	7 x 10^{-11}	1 x 10^{-9}	1 x 10^{-12}	2 x 10^{-11}
		PBS	4 x 10^{-7}	2 x 10^{-5}	2 x 10^{-8}	1 x 10^{-6}	3 x 10^{-13}	2 x 10^{-11}	BL	BL	BL	BL	BL	BL
	Incorporated	Biosolids	3 x 10^{-7}	7 x 10^{-6}	3 x 10^{-8}	7 x 10^{-7}	7 x 10^{-12}	1 x 10^{-10}	BL	2 x 10^{-15}	BL	BL	BL	BL
		Swine	5 x 10^{-7}	3 x 10^{-5}	2 x 10^{-7}	1 x 10^{-5}	7 x 10^{-9}	5 x 10^{-7}	9 x 10^{-11}	6 x 10^{-9}	1 x 10^{-12}	8 x 10^{-11}	2 x 10^{-14}	1 x 10^{-12}
		Cattle	1 x 10^{-5}	2 x 10^{-4}	4 x 10^{-6}	6 x 10^{-5}	6 x 10^{-8}	9 x 10^{-7}	2 x 10^{-10}	4 x 10^{-9}	9 x 10^{-13}	1 x 10^{-11}	4 x 10^{-15}	5 x 10^{-14}
		PBS	5 x 10^{-7}	3 x 10^{-5}	2 x 10^{-7}	2 x 10^{-5}	1 x 10^{-8}	8 x 10^{-7}	2 x 10^{-10}	2 x 10^{-8}	5 x 10^{-12}	3 x 10^{-10}	1 x 10^{-13}	7 x 10^{-12}
Clay loam	Surface	Biosolids	3 x 10^{-7}	9 x 10^{-6}	5 x 10^{-8}	1 x 10^{-6}	5 x 10^{-11}	1 x 10^{-9}	6 x 10^{-15}	1 x 10^{-13}	BL	BL	BL	BL
		Swine	4 x 10^{-7}	3 x 10^{-5}	6 x 10^{-8}	4 x 10^{-6}	4 x 10^{-11}	3 x 10^{-9}	3 x 10^{-15}	2 x 10^{-13}	BL	BL	BL	BL
		Cattle	1 x 10^{-5}	1 x 10^{-4}	7 x 10^{-7}	1 x 10^{-5}	3 x 10^{-11}	5 x 10^{-10}	BL	BL	BL	BL	BL	BL
		PBS	4 x 10^{-7}	3 x 10^{-5}	5 x 10^{-8}	3 x 10^{-6}	1 x 10^{-11}	8 x 10^{-10}	BL	2 x 10^{-14}	BL	BL	BL	BL
	Incorporated	Biosolids	3 x 10^{-7}	7 x 10^{-6}	4 x 10^{-8}	8 x 10^{-7}	9 x 10^{-12}	2 x 10^{-10}	BL	4 x 10^{-15}	BL	BL	BL	BL
		Swine	5 x 10^{-7}	3 x 10^{-5}	1 x 10^{-7}	8 x 10^{-6}	7 x 10^{-10}	5 x 10^{-8}	8 x 10^{-13}	5 x 10^{-11}	BL	7 x 10^{-14}	BL	BL
		Cattle	1 x 10^{-5}	2 x 10^{-4}	1 x 10^{-6}	2 x 10^{-5}	13 x 10^{-10}	2 x 10^{-9}	BL	2 x 10^{-14}	BL	BL	BL	BL
		PBS	4 x 10^{-7}	2 x 10^{-5}	3 x 10^{-8}	2 x 10^{-6}	3 x 10^{-12}	2 x 10^{-10}	BL	BL	BL	BL	BL	BL

BL – risks which were below reportable limits

Table 3.9 Merging the two QMRA of *Salmonella* Using Conservative Values

			Risk of Infection (One-time)											
			Decay Time (day)											
		Residual	1		7		30		60		90		120	
Soil Type	**Application**	**Source**	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
Sandy loam	Surface	Biosolids	4×10^{-7} - 8×10^{-6}		1×10^{-7} - 3×10^{-6}		3×10^{-9} - 6×10^{-8}		2×10^{-11} - 4×10^{-10}		1×10^{-13} - 3×10^{-12}		BL - 2×10^{-14}	
		Swine	4×10^{-7} - 2×10^{-5}		2×10^{-7} - 1×10^{-5}		3×10^{-9} - 2×10^{-7}		2×10^{-11} - 1×10^{-9}		8×10^{-14} - 5×10^{-12}		BL - 3×10^{-14}	
		Cattle	1×10^{-5} - 2×10^{-4}		1×10^{-5} - 2×10^{-4}		1×10^{-5} - 2×10^{-4}		6×10^{-6} - 1×10^{-4}		4×10^{-6} - 7×10^{-5}		3×10^{-6} - 4×10^{-5}	
		PBS	4×10^{-7} - 2×10^{-5}		2×10^{-8} - 2×10^{-6}		8×10^{-13} - 5×10^{-11}		BL - BL		BL - BL		BL – BL	
	Incorporated	Biosolids	4×10^{-7} - 8×10^{-6}		9×10^{-8} - 2×10^{-6}		3×10^{-10} - 7×10^{-9}		3×10^{-13} - 5×10^{-12}		BL - 4×10^{-15}		BL – BL	
		Swine	4×10^{-7} - 2×10^{-5}		2×10^{-7} - 1×10^{-5}		7×10^{-9} - 5×10^{-7}		9×10^{-11} - 6×10^{-9}		1×10^{-12} - 8×10^{-11}		2×10^{-14} - 1×10^{-12}	
		Cattle	1×10^{-5} - 2×10^{-4}		1×10^{-5} - 2×10^{-4}		6×10^{-5} - 4×10^{-4}		1×10^{-6} - 2×10^{-5}		4×10^{-7} - 6×10^{-6}		1×10^{-7} - 2×10^{-6}	
		PBS	5×10^{-7} - 3×10^{-5}		2×10^{-7} - 2×10^{-5}		1×10^{-8} - 8×10^{-7}		2×10^{-10} - 2×10^{-8}		5×10^{-12} - 3×10^{-10}		1×10^{-13} - 7×10^{-12}	
Clay loam	Surface	Biosolids	3×10^{-7} - 9×10^{-6}		5×10^{-8} - 1×10^{-6}		5×10^{-11} - 1×10^{-9}		6×10^{-15} - 1×10^{-13}		BL - BL		BL – BL	
		Swine	4×10^{-7} - 3×10^{-5}		6×10^{-8} - 4×10^{-6}		4×10^{-11} - 3×10^{-9}		3×10^{-15} - 2×10^{-13}		BL - BL		BL – BL	
		Cattle	1×10^{-5} - 2×10^{-4}		2×10^{-6} - 3×10^{-5}		4×10^{-9} - 7×10^{-8}		9×10^{-13} - 1×10^{-11}		BL - 3×10^{-15}		BL – BL	
		PBS	4×10^{-7} - 3×10^{-5}		5×10^{-8} - 3×10^{-6}		1×10^{-11} - 8×10^{-10}		BL - 2×10^{-14}		BL - BL		BL – BL	
	Incorporated	Biosolids	4×10^{-7} - 9×10^{-6}		3×10^{-7} - 5×10^{-6}		2×10^{-8} - 5×10^{-7}		1×10^{-9} - 2×10^{-8}		5×10^{-11} - 1×10^{-9}		3×10^{-12} - 5×10^{-11}	
		Swine	5×10^{-7} - 3×10^{-5}		3×10^{-7} - 2×10^{-5}		4×10^{-8} - 3×10^{-6}		3×10^{-9} - 2×10^{-7}		2×10^{-10} - 1×10^{-8}		2×10^{-11} - 1×10^{-9}	
		Cattle	1×10^{-5} - 2×10^{-4}		1×10^{-5} - 2×10^{-4}		5×10^{-6} - 8×10^{-5}		2×10^{-6} - 3×10^{-5}		7×10^{-7} - 1×10^{-5}		3×10^{-7} - 4×10^{-6}	
		PBS	4×10^{-7} - 3×10^{-5}		7×10^{-8} - 5×10^{-6}		7×10^{-11} - 5×10^{-9}		9×10^{-15} - 6×10^{-13}		BL - BL		BL – BL	

Molecular and culture derived risk characterization using the most conservative risks with application rates of 1.75×10^{-3} kg manure (kg soil)$^{-1}$. BL – risks which were below reportable limits. The underlined risk characterizations are derived from molecular decay rates. All other risks are associated with cultural decay rates.

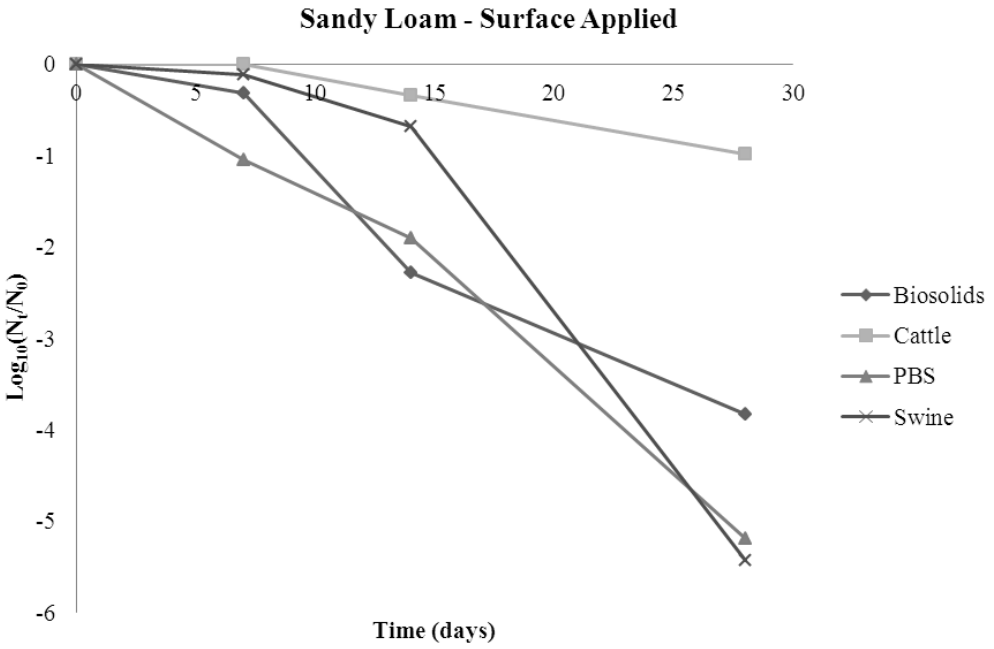

Figure 3.1 *Salmonella* survival in each waste residual when surface applied to sandy loam soils and enumerated by standard culture methods.

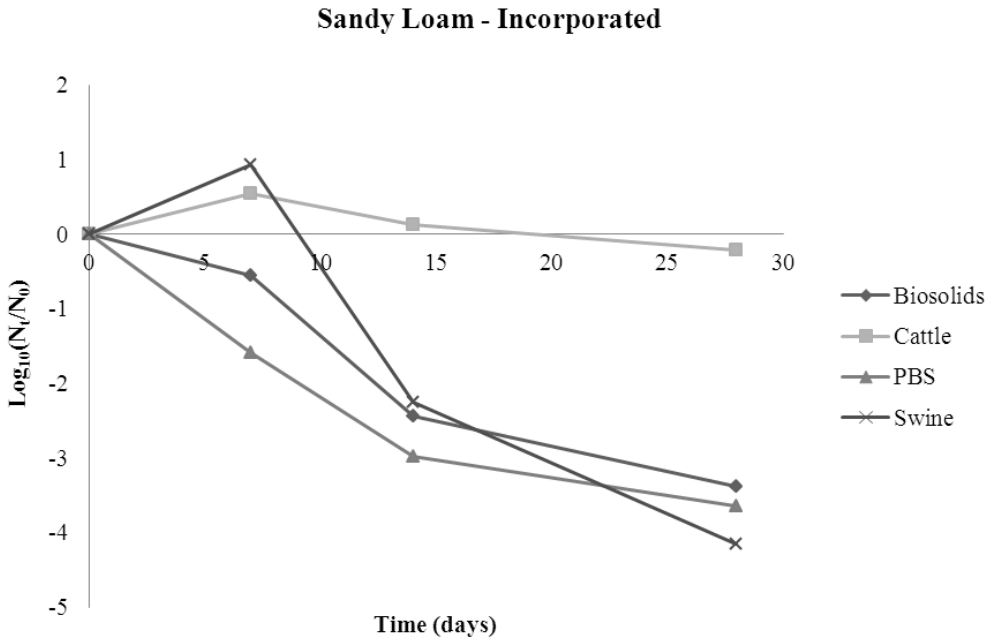

Figure 3.2 *Salmonella* survival in each waste residual when incorporated into sandy loam soils and enumerated by standard culture methods.

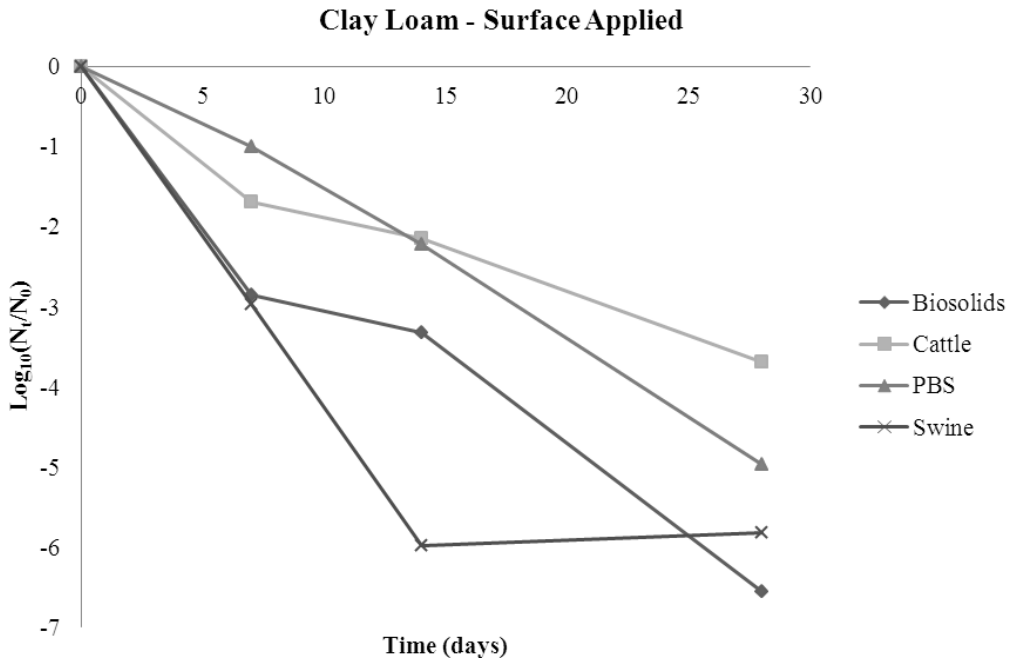

Figure 3.3 *Salmonella* survival in each waste residual when surface applied to clay loam soils and enumerated by standard culture methods.

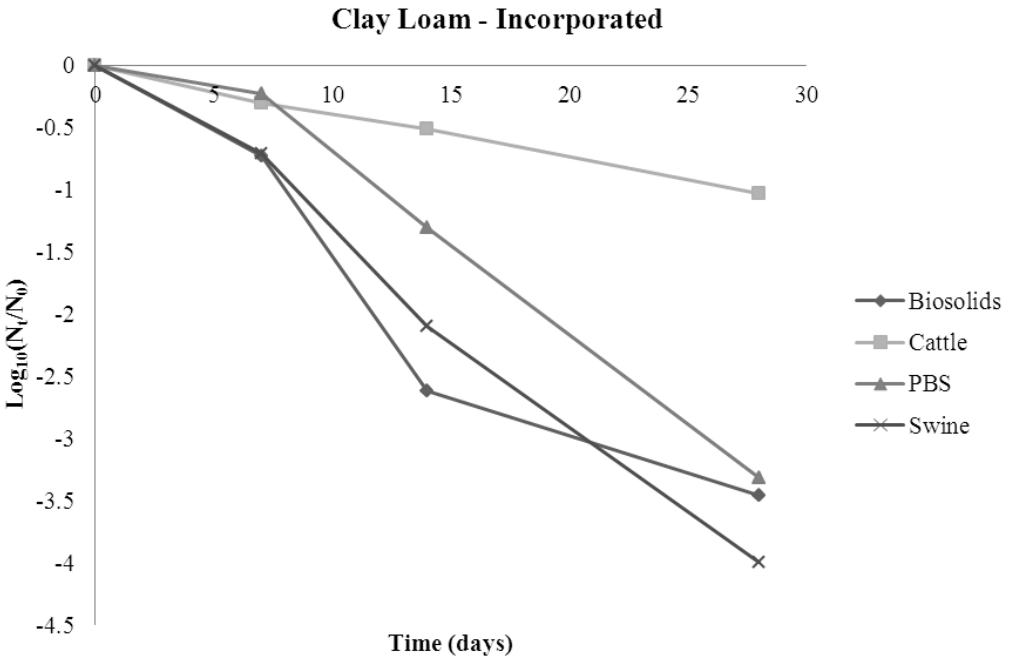

Figure 3.4 *Salmonella* survival in each waste residual when incorporated into clay loam soils and enumerated by standard culture methods.

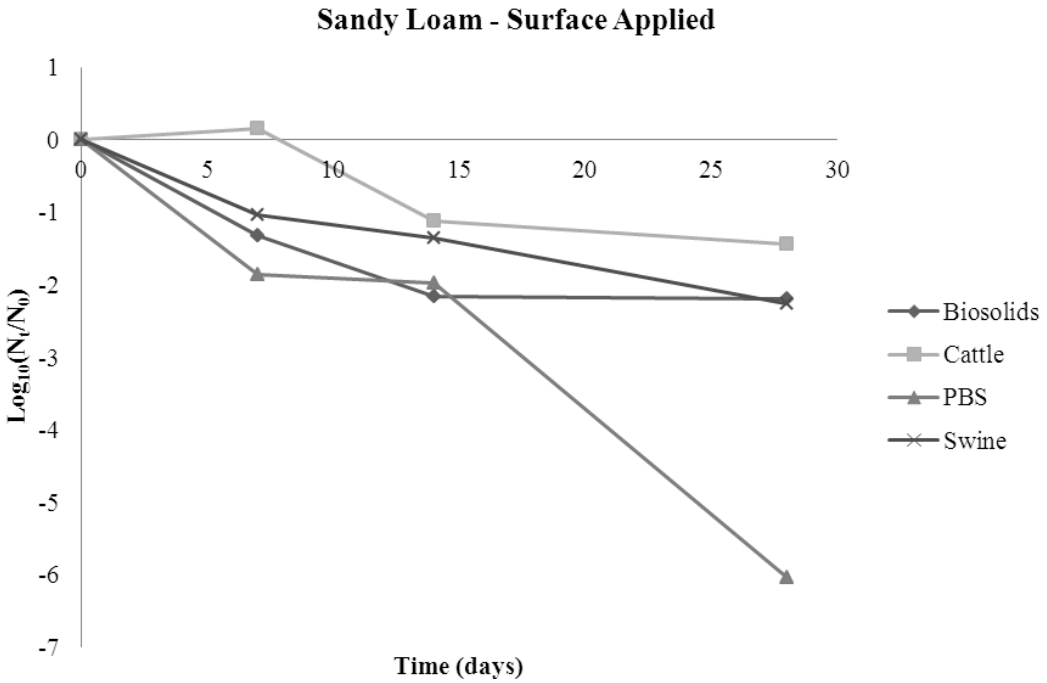

Figure 3.5 *Salmonella* survival in each waste residual when surface applied to sandy loam soils and determined by qPCR.

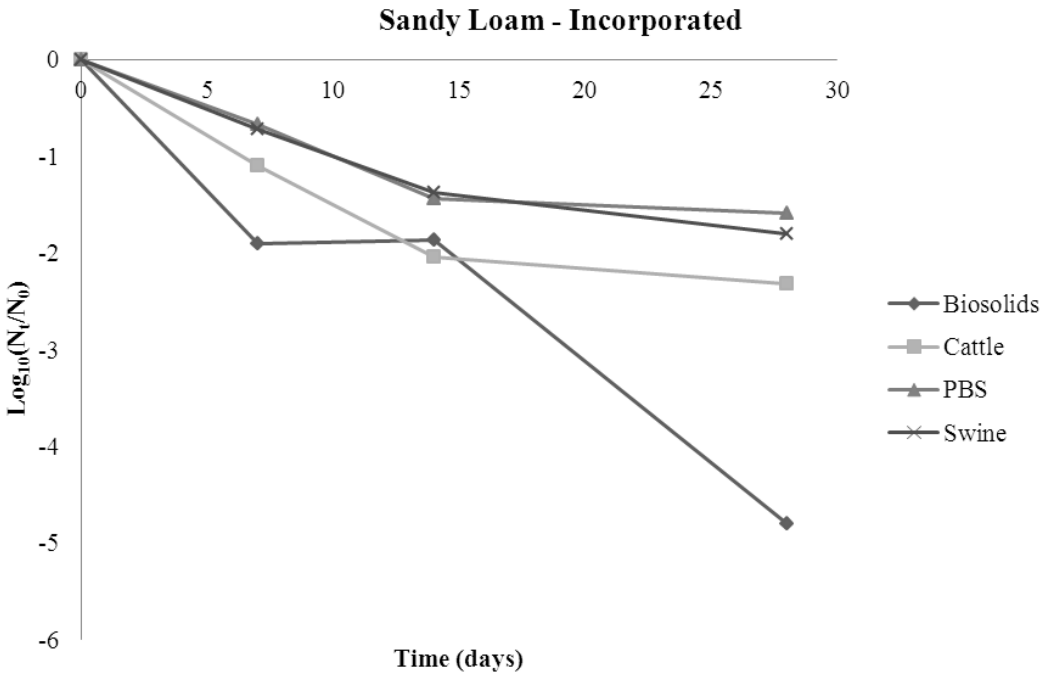

Figure 3.6 *Salmonella* survival in each waste residual when incorporated into sandy loam soils and determined by qPCR.

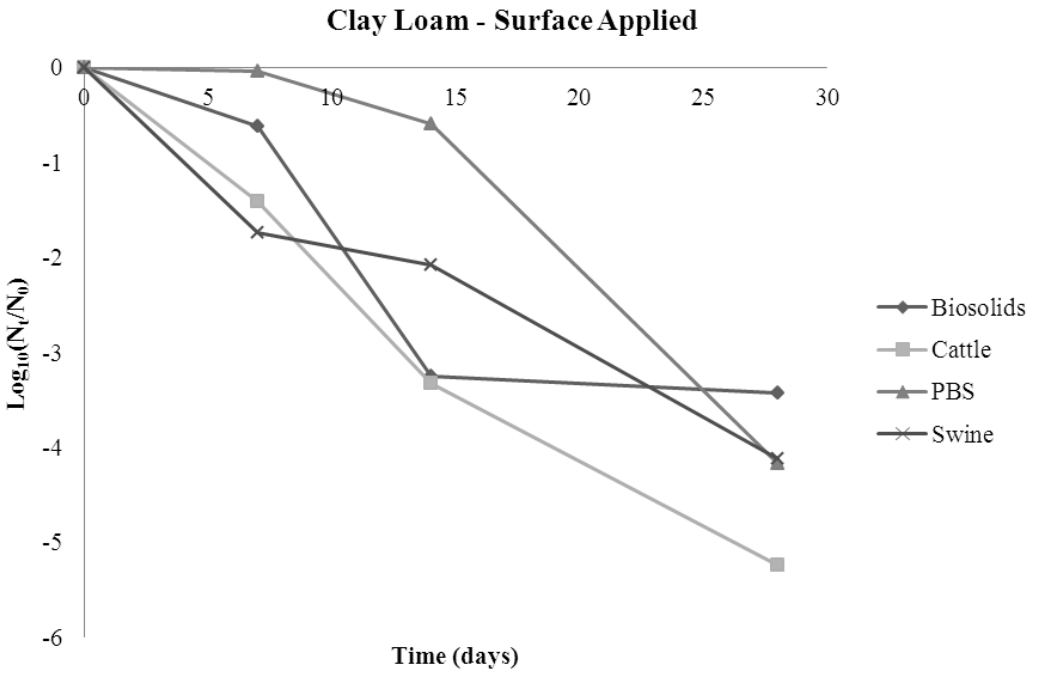

Figure 3.7 *Salmonella* survival in each waste residual when surface applied to clay loam soils and determined by qPCR.

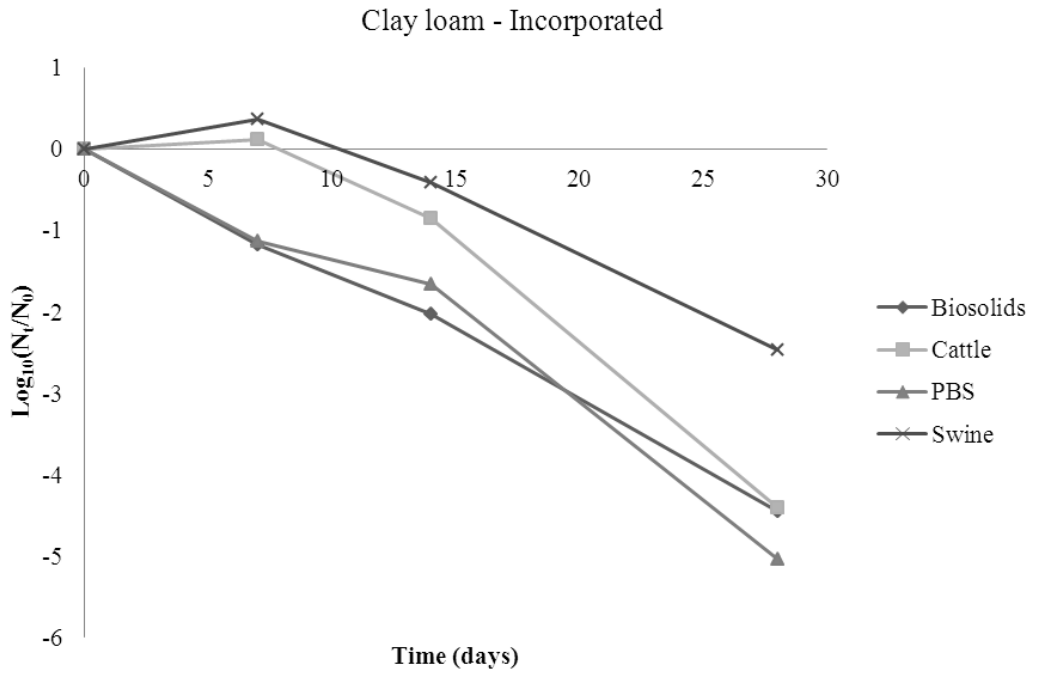

Figure 3.8 *Salmonella* survival in each waste residual when incorporated into clay loam soils and determined by qPCR.

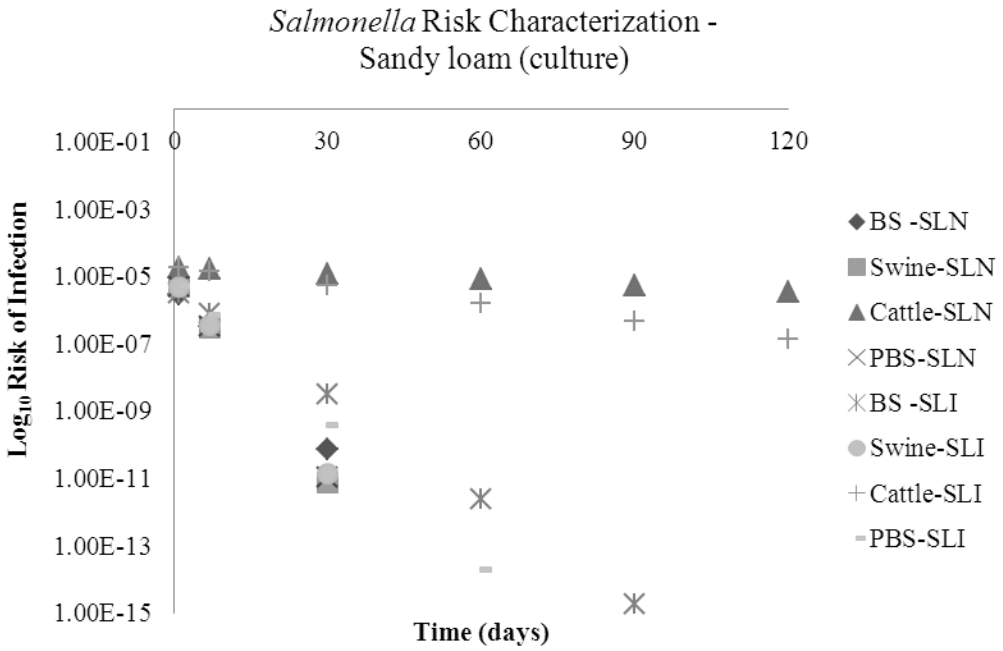

Figure 3.9 *Salmonella* risk characterization associated with each waste residual when applied to sandy loam soils using decay rates derived by standard culture methods.

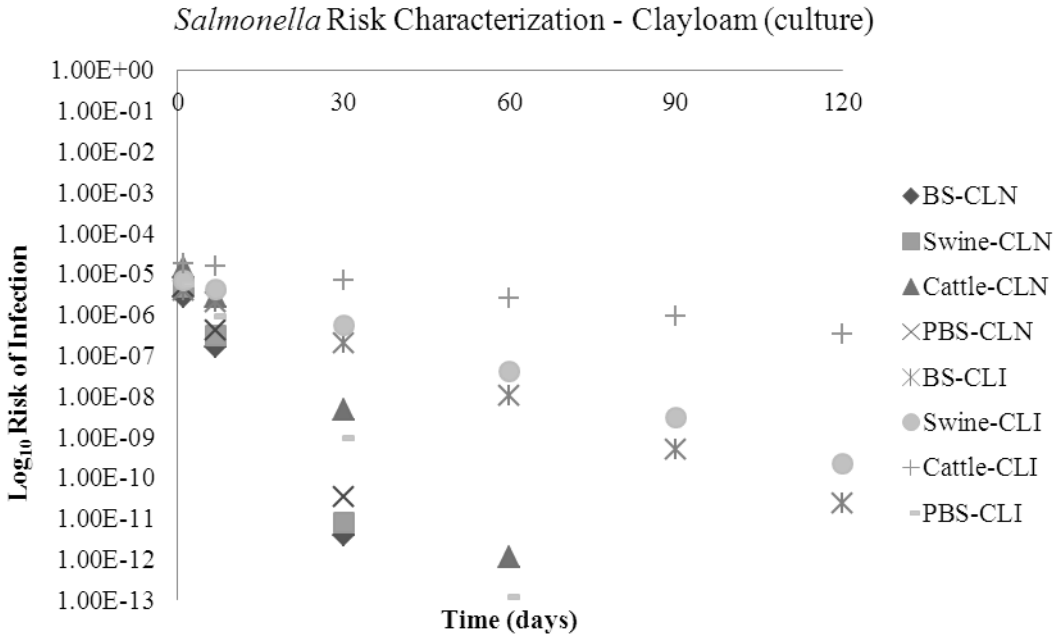

Figure 3.10 *Salmonella* risk characterization associated with each waste residual when applied to clay loam soils using decay rates derived by standard culture methods.

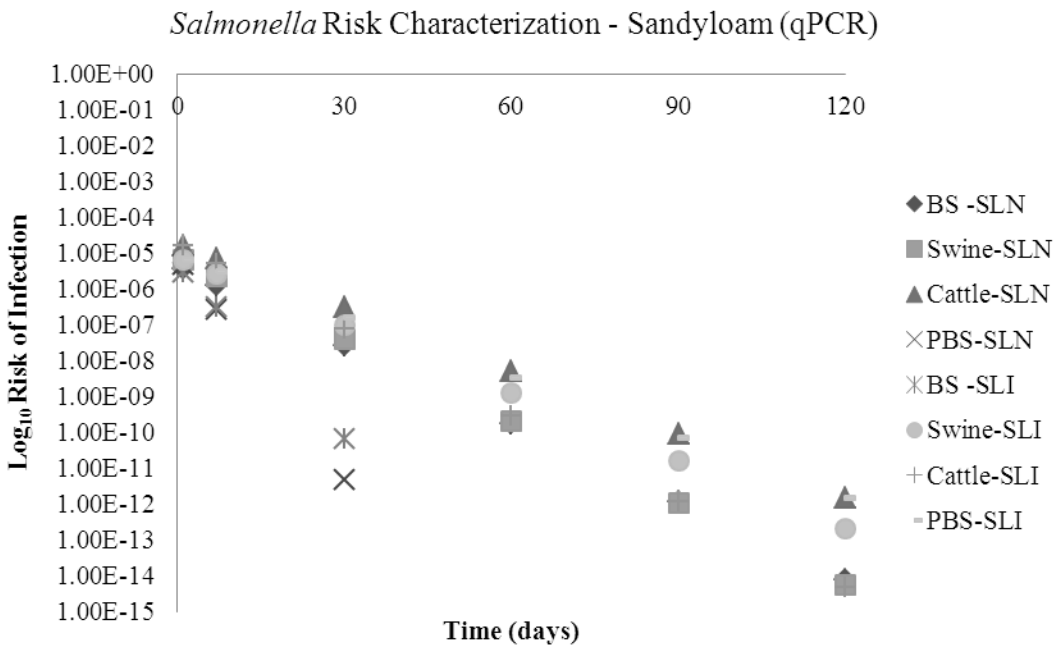

Figure 3.11 *Salmonella* risk characterization associated with each waste residual when applied to sandy loam soils using decay rates derived by molecular (qPCR) methods.

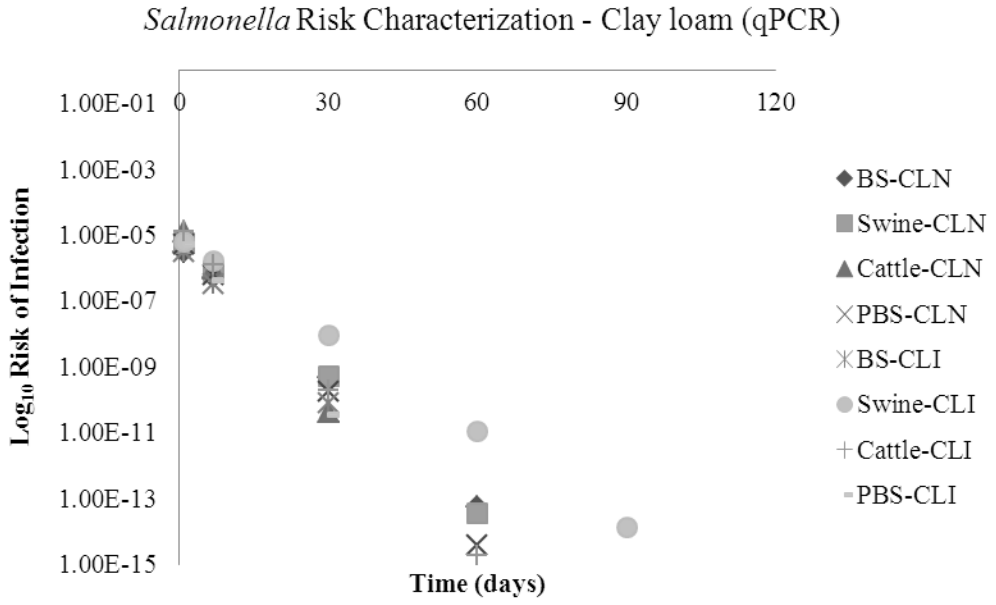

Figure 3.12 *Salmonella* risk characterization associated with each waste residual when applied to clay loam soils using decay rates derived by molecular (qPCR) methods.

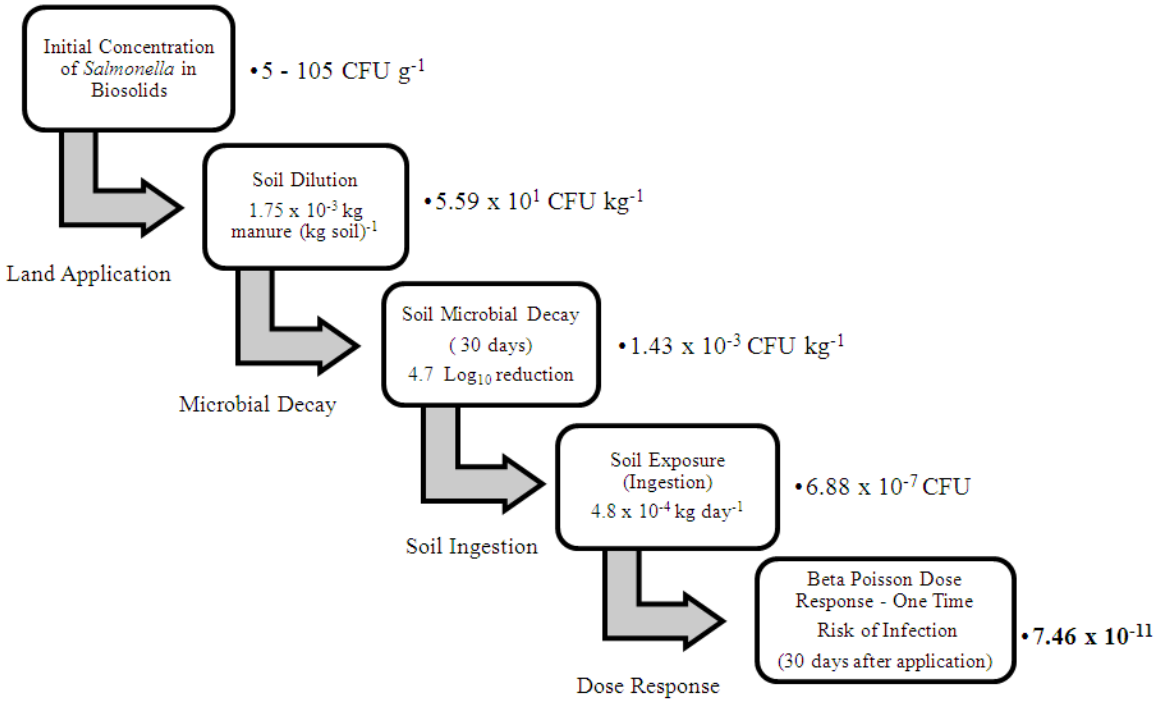

Figure 3.13 Schematic of risk of infection associated with land application of surface applied biosolids on sandy loam soils using culture derived decay rates.

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We would like to acknowledge Agriculture and Food Research Initiative Grant 3556 from the USDA National Institute of Food and Agriculture for funding this research. We appreciate our cooperators for allowing us to collect manure and municipal biosolids from their facilities. In addition, a special thanks to Cindy Smith and Renotta Smith, who have been a tremendous help with the research.

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CHAPTER IV

CONCLUSIONS

Escalading human populations have driven the need for more efficient food-animal production. In turn, the evolution of more confined animal production facilities has necessitated the need for innovative methods of waste disposals. The ever increasing amount of waste residuals produced by humans (biosolids) and animal production farming (animal manure) has the potential to be a source of pathogen proliferation and transport, if vigilance of our environmental stewardship is not employed when disposing of these. This dissertation was focused on two different areas in regard to the animal production and the waste management continuum. The progression of these findings begin with an on-farm study of pathogen levels associated with the broiler litter and culminates with the final study investigating inactivation of bacterial and viral pathogens via land application scenarios, which were applied to a beta-Poisson model to predict probability of *Salmonella* infection.

First, an observational study of pathogens pervasively found in a broiler production houses was investigated to identify spatial differences of distinct litter characteristics within production broiler houses and the effects of broiler age, moisture content and seasonality. Antibiogram profiles were also investigated to determine if multiple antibiotic resistance (MAR) of pathogens isolated from broiler litter is of concern. *Salmonella* isolation was discovered in 15% of litter sample and one-third of the

15% was associated with house ends. Broiler age was the most pronounced affect associated with the presence of bacterial pathogens in broiler litter. MAR was common in many bacteria isolates and warrants concern of being a possible source of antibiotic resistance genes that may transfer among bacteria. These findings may be instrumental in new strategies to reduce pathogens that induce human infections (i.e. *Salmonella, Campylobacter* and *Listeria*) and influence broiler health (i.e. *Clostridium perfringens* and staphylococci).

Second, a laboratory experiment investigated pathogen sustainability in waste residuals with varying soil composition and farming application methods. By surveying multiple farming scenarios, inactivation constants were established with much more decisive semblance of bacterial and viral decay given so many dynamics that potentially alter the survival of microorganisms. These decay rates were established using both standard plating methods and quantitative PCR allowing a direct comparison of these assays. Cattle manure was the most protective for *Salmonella* while biosolids was most protective for MS2 phage. Decay rates of all other bacteria were not significantly associated with waste residual alone. Using the established inactivation constants of *Salmonella*, quantitative microbial risk assessment (QMRA) provided evidence that application practices of biosolids and animal manure pose little threat to the public in the event of a one-time exposure post land application of residuals investigated. QMRA data is extremely limited for land application events, and this study is the first to establish inactivation constants for both bacteria and viruses under parallel events.

APPENDIX A

SUPPLEMENTAL FIGURES

Microcosms for Land Application Analysis