ABSTRACT

Title of Thesis: Comparison of Pathogen Detection Methods in Compost and Compost Characteristics as Potential Predictors of Pathogen Regrowth

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Improperly thermally treated compost can allow human pathogens to survive. Pathogens can regrow in finished compost due to recontamination or incomplete pathogen-kill. U.S. Environmental Protection Agency's (EPA) and U.S. Composting Council's (USCC) methodologies were compared to recover populations of inoculated non-pathogenic *E. coli* and pathogenic *Salmonella spp.* from finished compost. Two immunomagnetic separation (IMS) techniques were additionally compared for the rapid recovery of inoculated pathogenic *E. coli* O157:H7. Twenty-nine point-of-sale composts were obtained from 19 U.S. states. EPA methods recovered more (generic *E. coli*, $p=0.0001$) or statistically equal (*Salmonella*, $p=0.27$) amounts of inocula compared to USCC methods. Both IMS techniques identified with 3-4% false negatives among replicates. Physicochemical parameters of compost were tested as predictors of *Salmonella* and O157:H7 regrowth in finished compost. *Salmonella* and O157:H7 populations increased over three days in 48% and 52% of compost samples, respectively. No physicochemical measurements could predict the regrowth of *Salmonella* or O157:H7.

COMPARISON OF PATHOGEN DETECTION METHODS IN COMPOST AND COMPOST CHARACTERISTICS AS POTENTIAL PREDICTORS OF PATHOGEN REGROWTH

By

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AFF.....Alliance for Food and Farming
ANCOVA.....Analysis of Covariance
ANOVA.....Analysis of Variance
ARS.....Agricultural Research Service

BARC.....Beltsville Agricultural Research Center
BPW.....Buffered Peptone Water

CDC.....United States Centers for Disease Control and Prevention
CFSAN.....Center for Food Safety and Applied Nutrition
CFU.....Colony Forming Units
CHROM.....CHROMagar O157 agar
CSLM.....Confocal Scanning Laser Microscopy

EC.....Electrical Conductivity
EC.....*Escherichia coli* medium
EHEC.....Enterohemorrhagic *Escherichia coli*
EMB.....Eosin-Methylene Blue agar
EMFSL.....Environmental Microbial and Food Safety Laboratory
EPA.....United States Environmental Protection Agency

FDA.....United States Food and Drug Administration
FDOSS.....Foodborne Disease Outbreak Surveillance System
FOOD.....Foodborne Outbreak online Database
FoodNet.....Foodborne Diseases Active Surveillance Network
FSIS.....Food Safety Inspection Service

HUS.....Hemolytic Uremic Syndrome

IMS.....Immunomagnetic Separation

LIA.....Lysine Iron agar
LTB.....Lauryl Trypose Broth

MAC.....MacConkey's agar
MIL.....Motility Indole Lysine agar
MPN.....Most Probable Number
MSRV.....Modified Semisolid Rappaport-Vassiliadis medium

NOP.....National Organic Program
NRC.....National Research Council

OPR.....Ongoing Precision and Recovery

PBS.....Peptone Buffered Saline
PCR.....Polymerase Chain Reaction
PFRP.....Processes to Further Reduce Pathogens
PSRP.....Processes to Significantly Reduce Pathogens

RT-PCR.....Real time PCR (Polymerase Chain Reaction)

STA.....Seal of Testing Assurance
STEC.....Shiga-toxigenic (Shiga-toxin producing) *Escherichia coli*

TMECC.....Test Methods for the Examination of Composting and Compost
TOC.....Total Organic Carbon
TSB.....Tryptic Soy Broth
TSI.....Triple Sugar Iron agar
TT.....Tetrathionate broth
TTP.....Thrombotic Thrombocytopenia Purpura

USCC.....United States Composting Council
USDA.....United States Department of Agriculture

XLD.....Xylose-lysine Desoxycholate agar
XLT4.....Xylose-lysine Tergitol 4 agar

Chapter 1: Literature Review

Foodborne Illness Reporting Background

A major difficulty in understanding the trends of foodborne illnesses is the variety of different reporting and estimation mechanisms used. When different reporting and estimation mechanisms are used they cannot necessarily be directly compared. This makes it difficult at times to get a clear picture of the issue of foodborne illnesses and may even lead to conflicting results. The United States Centers for Disease Control and Prevention (CDC) uses two different information gathering mechanisms for tracking the incidence of foodborne illnesses in the U.S.A. that are to be taken separately: the Foodborne Disease Outbreak Surveillance System (FDOSS) which is compiled for public access in the Foodborne Outbreak Online Database (FOOD); and the Foodborne Diseases Active Surveillance Network (FoodNet).

FDOSS is based on data gathered from outbreak reports submitted by each state and the District of Columbia. The information gathered by the CDC through this system is necessarily incomplete because reporting by the states and Washington D.C. are voluntary and the completeness of the investigations and reports are variable. Additionally, not all outbreaks reported to the CDC are caused by foodborne diseases. FOOD filters FDOSS and reports only food-related incidents. The reporting structure allows for comprehensive reporting. For "disease-causing agent," the report includes microorganisms (including viruses), chemicals, heavy metals, mycotoxins, pesticides, and several "other" categories to allow for more accurate reporting. Only biological sources (including viruses) will be used for this paper. Furthermore, when the source of the contamination is identified, it is categorized as at least one of 17 different

commodities (dairy, beef, leafy greens, fruits, etc.). This allows for more comprehensive analyses of the available data that, while incomplete, is very informative of the relative occurrence and source(s) of each disease causing agent.

FoodNet is a partnership that the CDC has with 10 state health departments to directly report 10 common bacterial infections often associated with food (*Campylobacter, Cryptosporidium, Cyclospora, Listeria, Salmonella,* Shiga toxin-producing *Escherichia coli* (STEC) O157 and non-O157, *Shigella, Vibrio*, and *Yersinia*). This allows the CDC to directly survey approximately 15% of the US population for occurrences of these infections. The reporting does not include if the infection actually did come from food, one of the drawbacks of this system, and so is merely a reporting mechanism for all infections by these agents within the reporting-catchment areas. However, this database reports the infections regardless of outbreak status, meaning FoodNet reports single-person infections. FDOSS requires an outbreak of at least two people to report. Both systems have their strengths and weaknesses

In light of the background for these two systems, both will be used here to analyze the current and long-term trends in foodborne illness. Painter et al. (2013) used the FOOD to analyze the overall trends from 1998 to 2008. Painter et al. (2013) did not use any data from FOOD where either the specific food vehicle or disease causing agent was not identified (see Painter et al., 2013, Technical Appendix 1, Table 1). Only FOOD reports where a single food vehicle or disease causing agent was either suspected or confirmed were used as they are the only useful data points for showing trends. Thus, when using data from FOOD, this study will be referenced unless otherwise noted. The FoodNet data will be used as analyzed and summarized by the CDC.

Organism Backgrounds

Of interest to this paper are the incidences of infection by *Salmonella spp.* and *E. coli* O157:H7. Scallan et al. (2011b) estimates *Salmonella spp.* causes 11% of the illnesses, 35% of the hospitalizations, and 28% of the deaths resulting from foodborne illnesses while *E. coli* O157:H7 causes 0.7% of the illnesses, 3.8% of the hospitalizations, and 1.5% of the deaths resulting from foodborne illnesses. Both of these organisms are associated with fecal matter of various animals, including humans. Many *Salmonella* infections are attributed to handling contaminated live animals (such as chickens, turtles, and snakes) rather than direct food contamination (FDA, 2012b). Regardless, the mode of infection is the fecal-oral route.

Salmonella only has three species, *S. bongori*, *S. enterica*, and *S. subterranean*. There are over 2400 serotypes of *Salmonella*, all of which are pathogenic to humans to some degree (Blaser and Newman, 1982; Pepper et al., 2006), with the majority of *Salmonella* serotypes occurring in the *S. enterica* species. Generally, *S.* Typhi is mentioned separately from the rest of *Salmonella* infections because this serotype causes Typhoid Fever, which is more serious than a typical *Salmonella spp.* infection. Depending on the *Salmonella* serotype and the health of the host, as few as one cell can cause infection (FDA, 2012b) but is quite often greater than 1,000 cells, at least in experimental settings (Blaser and Newman, 1982). Symptoms generally occur 6-72 hours after infection. Typical symptoms of salmonellosis include nausea, (bloody) vomiting, (bloody) diarrhea, fever, and headache and usually last four to seven days. Additionally, reactive arthritis, an autoimmune response to infection, is reported in about 2% of culture-identified cases (FDA, 2012b).

E. coli is a very common organism that is found in the digestive tract of most mammals and provides numerous benefits to the host organism. However, there is a relatively small subset of *E. coli* that is pathogenic to humans. The most common group associated with illness in humans is that which produces Shiga toxins, known as Shiga-toxigenic *E. coli* (STEC). Not all STEC have been associated with human illness. Of the STEC, the subset that is most associated with causing serious illness is called Enterohemorrhagic *E. coli* (EHEC). Among the EHEC, the most common group of serotypes associated with human illness is the O157 serotype group. This serotype group is commonly referred to as O157 EHEC or O157 STEC. Even among O157 EHEC, the *E. coli* O157:H7 serotype is the most common, accounting for approximately 75% of all EHEC infections worldwide (FDA, 2012a). Between 10 and 100 *E. coli* O157:H7 cells are typically needed to infect a host and cause symptoms that last two to nine days. Symptoms include severe abdominal cramps and (bloody) diarrhea. Approximately 3-7% of cases progress to potentially life-threatening conditions such as hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenia purpura (TTP). Some survivors of HUS have permanent kidney damage (FDA, 2012a).

Foodborne Illness Trends

Every year, approximately 1 in 6 people in the USA experience some form of gastroenteritis. Of these 48 million cases, about 80% of cases are attributed to unknown agents including unidentified microorganisms and non-living contaminants such as chemicals, toxins, etc. (Scallan et al. 2011a). In 2011 the remaining 20% were attributed to 31 known disease-causing organisms (including viruses), resulting in an estimated 9.4 million foodborne illnesses, 55,961 hospitalizations, and 1,351 deaths in 2011 (Scallan et al. 2011b)[1].

The total number of reported foodborne outbreaks seems to have declined from 2002 (Figure 1.1), though it is impossible to tell if this is a good predictor of future trends. The CDC defines an outbreak as two or more people acquiring the same illness from the same contaminated source (CDC, 2011). While some pathogens have fewer outbreaks per year, others have remained approximately the same (see *E. coli* O157 STEC in Figure 1.1). Additionally, a decrease in the number of outbreaks does not necessarily mean a decrease in the number of illnesses. Figure 1.2 shows, from the same datasets used for Figure 1.1, that the number of illnesses per year has been sporadic and no obvious downward trend can be attributed to the data. Indeed, it appears that

[1] It is important to note that the numbers used by Scallan et al. 2011a and 2011b are estimations and are not actual counts of reported cases. The study was very comprehensive and transparent about their assumptions and estimations, going so far as to include large online supplemental appendixes. Scallan et al. 2011 a and b used actual counts of reported cases (mostly from CDC sources including FOOD and FoodNet) when available as a basis for their estimations. Several pathogens required them to make estimations based solely on data from other countries or on vague assumptions (supported by the literature). Scallan et al. (2011a and 2011b) very clearly noted their own limitations and assumptions that were made in their modeling. These papers are very comprehensive with their treatment of statistical uncertainty and variability (Morris, 2011), making these papers the best-to-date estimations of foodborne illnesses in the United States despite questions of reliability simply due to lack of hard data. Therefore, these estimations, along with FOOD and FoodNet data, will be used when discussing the specific organisms involved in this study.

Salmonella spp. has even had an uptick in the number of illnesses associated with it despite the downward trend in number of outbreaks per year shown in Figure 1.1, indicating larger individual outbreaks.

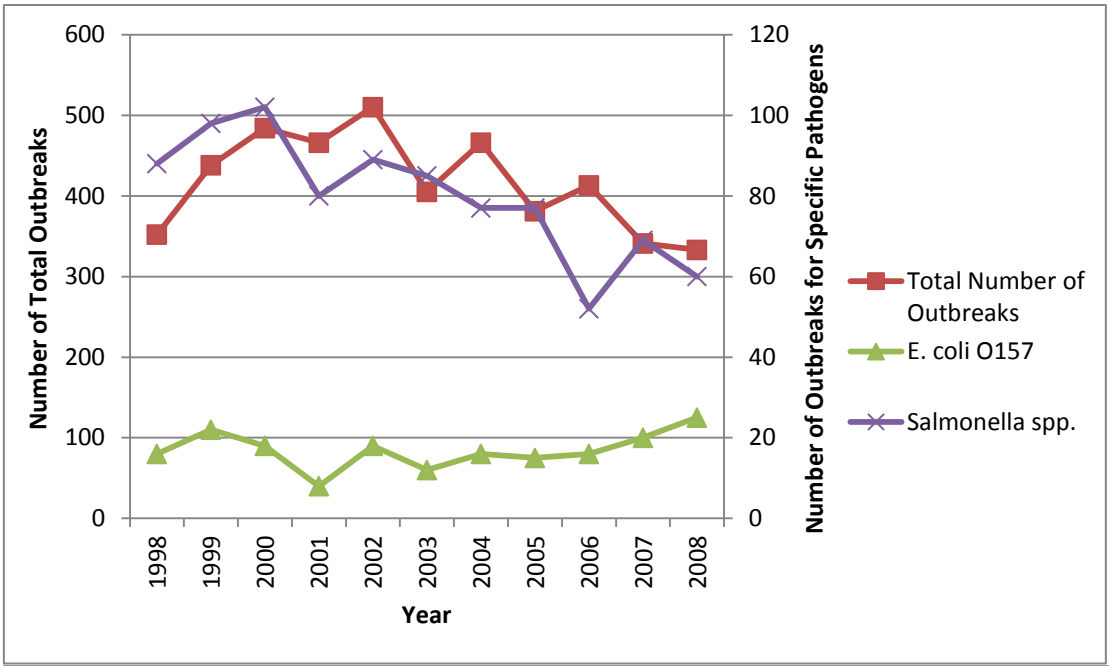

Figure 1.1: Number of reported foodborne illness outbreaks per year from 1998 – 2008. Note that *E. coli* O157 includes all O157 serotypes, not just *E. coli* O157:H7. Graph adapted from Painter et al. (2013), Technical Appendix 3.

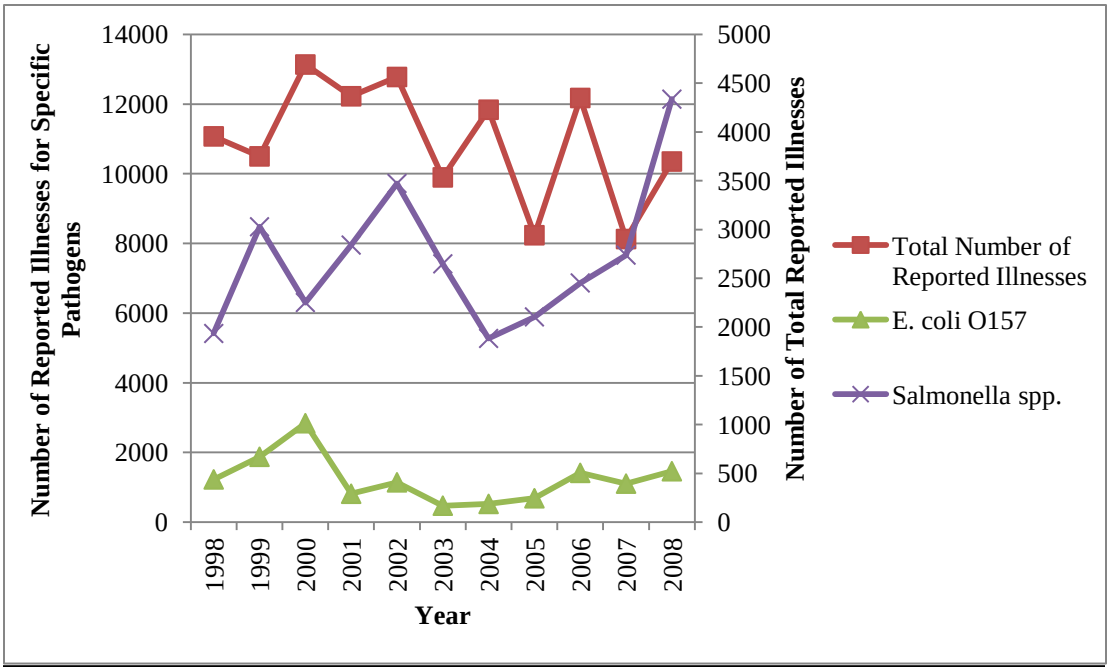

Figure 1.2: Number of reported foodborne illnesses per year from 1998 – 2008. Note that *E. coli* O157 includes all O157 serotypes, not just *E. coli* O157:H7. Graph adapted from Painter et al. (2013), Technical Appendix 3.

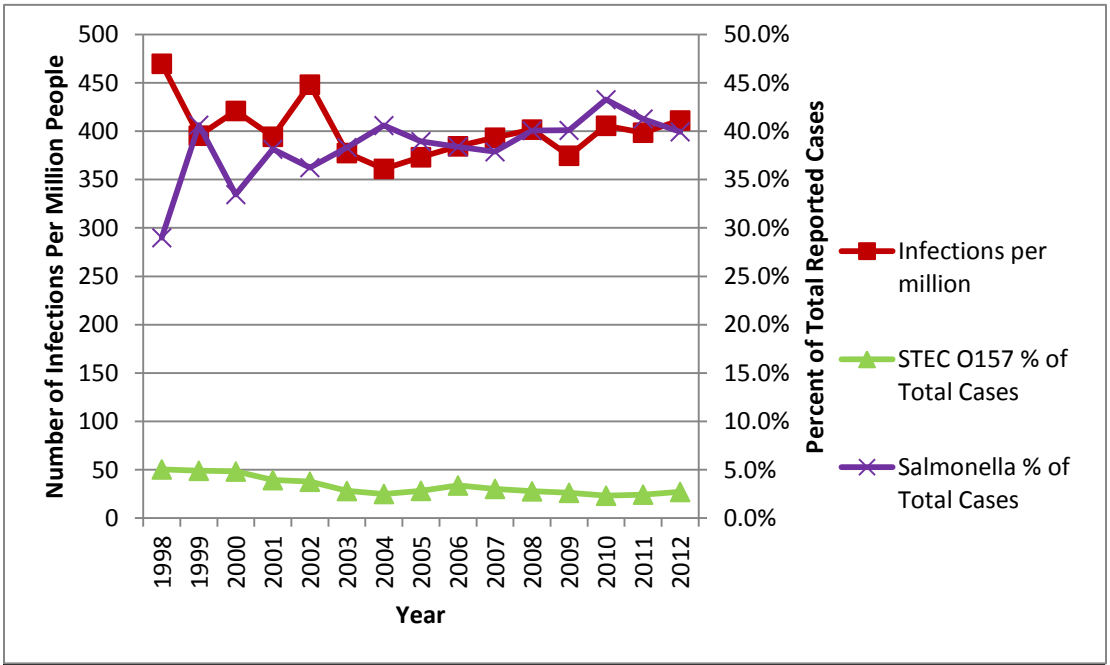

Figure 1.3: The rate of infection per million people by foodborne illnesses as reported by FoodNet from 1998 – 2012. Note that STEC O157 includes all O157 serotypes, not just *E. coli* O157:H7. Data adapted from CDC (2012).

Similarly, using FoodNet data, the number of infections per million people from 1998 to 2012 has remained relatively constant (Figure 1.3). The percentage of reported cases that are attributed to *Salmonella spp.* has increased slightly while the incidence of *E. coli* O157:H7 has decreased slightly from 1998 to 2012 (Figure 1.3). However, due mainly to convenience and affordability, the participants in the FoodNet survey areas have increased the use of identification methods with higher false positive rates, resulting in the FoodNet possibly under-counting *E. coli* O157:H7 incidences by as much as 19% (CDC, 2013).

Regardless of reporting mechanisms, it appears obvious that foodborne illness is a continuing threat to the health of individuals in the USA, particularly *Salmonella spp.* and, to a lesser degree, *E. coli* O157:H7. There will likely always be a certain number of outbreaks and illnesses associated with these organisms. Negligence and accidents happen, so it is important to recognize that an incidence of 0 is unachievable. Increasing reporting, standardization of reporting and detection mechanisms, and identification of sources of contamination, as well as the education of the general populace in safe food preparation, storage, and handling techniques, are all needed to decrease the incidence of foodborne illnesses. Of particular interest to this paper is the incidence of foodborne illnesses in produce.

Sources of Contamination

The people of the USA have been gradually increasing their intake of fresh produce per capita (Huang and Huang, 2007; Clemens, 2004). American consumers are not only demanding more fresh produce per year, they are also demanding greater variety and year-round supply (Clemens, 2004). These demands have led to a huge increase in the importation of fresh produce from other countries, particularly Canada and Mexico as well as many Latin and South American countries. Greater demand plus greater importation, especially from countries that do not necessarily have the same quality standards and/or food safety oversight as the USA, can lead to a higher risk of contamination and transfer of foodborne illnesses (Beuchat, 1996; Sivapalasingam et al., 2004). While the United States Department of Agriculture (USDA) manages this risk through plant health certifications of trade partners, importation regulations, and inspections, most of these rules pertain to the accidental importation of invasive species and plant diseases rather than to the importation of produce contaminated with human pathogens (Huang and Huang, 2007). Though the USDA Food Safety Inspection Service (FSIS) does require equivalence in food safety standards to be met by imported food among other regulations, there is simply too much volume of imported food to ensure the safety of every batch, which is true for any imported good. The quantity of domestic supplies of fresh produce have also been increasing to meet the increased demand. Regardless of the source, whether from home or abroad, an increase in consumption will naturally increase the risk of foodborne illness simply due to increased exposure.

Salmonella spp. have historically been associated with poultry meat and eggs. *E. coli* O157:H7 has historically been associated with beef and other meats. However, both

have been increasingly recognized to have a variety of produce sources, including spinach, lettuce, cantaloupe, and sprouts (Beuchat, 1996; Berger et al., 2010). Fresh produce and produce products have increasingly been implicated in outbreaks over the past 20 years. Between 1998 and 2008, 46% of all foodborne outbreaks were associated with produce (Painter et al. 2013). Surprisingly, the single commodity type that accounted for the most total illnesses were leafy vegetables (22%), followed by dairy (14%), fruits/nuts (12%), and poultry (10%). The Alliance for Food and Farming, using FOOD data, found that only 22% of all foodborne illnesses from 1990 to 2007 were associated with produce (AFF, 2010), though it should be noted that several parameters were changed in the FOOD database in 1998 and so the combination of data before and after this point may not be appropriate (Sivapalasingam et al., 2004; Morris, 2011). Sivapalasingam et al. (2004), using FOOD data, found that the incidence of produce related outbreaks increased eightfold from 0.7% of all foodborne illnesses in the 1970's to 6% in the 1990's while the total number of foodborne incidents that could be attributed to a known food item remained relatively constant. This increase can be somewhat explained by improvements in local and national reporting mechanisms, the recognition of certain organisms as foodborne pathogens (including *E. coli* O157:H7 which was not recognized until the 1990's), and the aforementioned increase in fresh produce consumption (Sivapalasingam et al., 2004).

Despite these trends, *Salmonella spp.* and *E. coli* O157:H7 contamination of consumer-ready food is relatively rare (Mandrell, 2009). Numerous surveys and review articles have found that contamination by either one is generally between 0 and 10% of samples surveyed with rare occurrences of higher percentages (Beuchat,1996; Mandrell,

2009; Mukherjee et al., 2006; Gorski et al., 2011; Allen et al., 2013). Brandl (2006) noted that two large U. S. Food and Drug Administration (FDA) surveys found between 1.6% and 4.4 % of domestic and imported foods are contaminated with human pathogens. Regardless, due to very low infectious doses of many human pathogens, especially for *E. coli* O157:H7, even a very low rate of contamination can lead to infection and outbreaks.

Whether the main point of contamination is pre-harvest or post-harvest is unclear in the literature. Some sources say the majority of contamination occurs on the farm before harvest while others say that the majority of contamination occurs after the produce has left the farm, particularly in the food service industry (Mandrell, 2009; AFF, 2010). Regardless, contamination can, and does, occur at every step in production. Food processors are in a position that can help reduce the transference of pathogens from the field to the consumer by testing their products. By testing their products, food processors can more readily detect contamination issues and destroy or more quickly recall contaminated shipments before it is sent to customers. For example, the company that processed the spinach that caused an outbreak in 2006 with 204 illnesses (Natural Selection Foods) has since added extra monitoring to the processing procedures. Two "firewalls" are now in place. They test every batch that comes in before processing and every batch of processed product that leaves the facility for both EHEC (including *E. coli* O157:H7) and *Salmonella*. Between 2006 and 2007, only 0.1% of batches coming in have tested positive and none have tested positive going out (Benbrook, 2007). All contaminated batches are destroyed. This extra diligence in monitoring and testing can lead to a substantially safer product and fewer foodborne illnesses. Even if contamination occurs and makes it past the processing plants, proper handling and cooking in the

foodservice and consumer sectors can greatly reduce the risk of people actually becoming infected. However, many of the outbreaks are associated with fresh produce and salads which are purposely uncooked. Therefore, in these cases, understanding the mechanisms of contamination and reducing the initial rate of contamination are the best lines of defense for preventing foodborne illnesses.

Whether the contamination occurs on the farm from wild animals or contaminated irrigation water, in the processing plant because a worker neglected to wash their hands after using the restroom , or other reasons, the starting point for all contamination of food products with *Salmonella spp.* and *E. coli* O157:H7 is fecal matter. To make the issue more complicated, both organisms are capable of surviving outside of a host organism for days to months, depending on the conditions. Both have been found in wild animals, livestock, and human waste streams (Doyle et al., 2006; Teplitski et al., 2012; Stephens et al., 2007), all of which can play a role in the contamination of produce on the farm.

Wild animals are of particular concern because there is very little that a farmer can do to prevent their incursion onto the fields. A large survey in California found that 20 out of 476 wildlife samples, or 4.2%, were positive for *Salmonella spp*. These 20 samples were from a variety of animals including various birds, deer, elk, opossum, coyote, feral pig, and skunk (Gorski et al., 2011). Interestingly, and somewhat troubling, one of the isolates from a skunk was a *Salmonella* Montevideo strain that was resistant to two antibiotics, streptomycin and gentamicin. This, and other studies, indicates that antibiotic resistant strains can occur in nature or be transferred to natural populations. Food contamination with these strains may be less treatable than others. *Salmonella spp.* has also been reported in deer, badgers, mice, turtles, and various birds (Mandrell, 2009).

E. coli O157:H7 has also been found in a variety of wild animals including deer, feral pigs, pigeons, seagulls, horses, dogs, and various birds (Doyle et al., 2006; Mandrell, 2009).

Unfortunately, the occurrence, distribution, and concentration of these pathogenic organisms within wild animals and their populations appear to be almost random. Some individuals will shed large amounts of a pathogen in their feces while another member of the species in the same geographic area will not shed any (Teplitski et al., 2012; Gorksi et al., 2011; Mandrell, 2009). There is very little that a farmer can do to prevent all wildlife from entering their fields. Fences will keep out some of the larger offenders such as deer and feral pigs, however these can be extremely expensive for large acreage farms. Additionally, smaller animals (such as fox, mice, and rabbits) and birds would be unhindered by many types of fences. Therefore, farmers should expect some contamination of their product from wildlife regardless of their deterrence mechanisms, and proceed accordingly.

Another large source of contamination is livestock. Cattle are natural carriers of the *E. coli* O157:H7 strain because they are not affected by the Shiga toxin once they are out of the neonatal stage (Priumboom-Brees et al., 2000). Once again, however, the prevalence of these pathogens among cattle is not well understood and seemingly sporadic. Between 0 and 61% of feedlot cattle carry *E. coli* O157:H7 (Jeon et al., 2013). Among feedlot cattle, Stephens et al. (2007) found that 31 of 73 cattle, or 42.5%, tested positive for *E. coli* O157:H7 while 50 out of 50 tested cattle were positive for *Salmonella spp.* The *Salmonella spp.* samples were not serotyped in this study. However, this study was unique in that it sampled more than just the fecal matter but instead also sampled,

through swabs, parts of the cows' bodies such as the back, neck, hock, and flank. Interestingly, this study found that just because the fecal matter was negative did not necessarily mean that the cow did not have a pathogen on it, and therefore capable of spreading the pathogen. Gorski et al. (2011) collected 795 cattle fecal samples in California and found only one positive for *Salmonella spp.* They suggested that the prevalence of contamination may be somewhat attributed to husbandry practices. The fecal samples obtained by Gorski et al. (2011) were from cattle on rangeland while much of the literature indicates feedlot cattle tend to have a higher rate of contamination. This suggests, as would be intuitively obvious, that a higher concentration of cattle can lead to a higher transference of fecal matter between cattle, which can lead to the inoculation of cattle with pathogens and a subsequently higher percentage of cattle that shed these pathogens as compared to cattle at low concentrations at pasture. Additionally, there are some individual cattle that seem to have an affinity to *E. coli* O157:H7 and release high concentrations in their feces. These cattle, and other animals that shed an extremely high concentration, are termed "super shedders". These are of particular concern both in transference between cattle but also between farms (Jeon et al., 2013). The use of livestock manure as fertilizer and runoff from livestock operations to adjacent produce farms have been implicated in the contamination of produce (Buck et al., 2003; Gerba and Smith, 2005).

Humans and human waste is the final major source of human pathogens. For various reasons, some people can be infected with *Salmonella spp.* or *E. coli* O157:H7 and be asymptomatic. One of the most famous examples of this is Typhoid Mary, an asymptomatic carrier of *S.* Typhi, who spread the disease to dozens of people through her

trade as a cook (Gopinath et al., 2012). A study in Mexico found that 11.4% of 1814 sampled children aged four months to seven years were asymptomatic carriers of several different *Salmonella spp.* serotypes (Zaidi et al., 2006). There appears to be a complex relationship between individual gut microflora, genotype, and immune system responses that explains why some people become asymptomatic carriers of *Salmonella spp.* (Gopinath et al., 2012; Ruby et al., 2012). Though there is not much literature on the subject, these factors likely hold true for *E. coli* O157:H7 as well. For *Salmonella spp.*, the introduction of antibiotics can increase fecal shedding or cause the induction of the carrier state of some humans. Increased shedding is likely due, in part, to the antibiotics reducing the competition in the gut by killing some of the normal gut microflora and allowing the *Salmonella spp.* to grow (Gopinathet al., 2012).

E. coli O157:H7 and other STEC can also exist inside asymptomatic human carriers. STEC commonly produce one or both of two different forms of Shiga toxin which are commonly known by their gene names: *stx1* and *stx2*. There are many subtypes of both toxins. The *stx2* gene has been shown to be more commonly associated with the development of HUS in infected persons (Boerlin et al., 1999). However, Stephan and Untermann (1999) found that there was a surprising tendency for asymptomatic carriers of STEC to have *E. coli* with only the *stx2* gene as opposed to the *stx1* or both. Out of 14 asymptomatic carriers at a beef processing plant, only 3 were positive for only *stx1* and two more produced both toxins. There was also a noticeable lack of the *eae* gene in these isolates, which is a gene that is critical for the attachment of the *E. coli* to the host's intestinal tract. Upon further investigation using 37 isolates from asymptomatic carriers of STEC, Stephan and Hoelzle (2000) found that there may be a correlation between

which subtype of the *stx2* toxin the *E. coli* produces and its tendency towards producing symptoms. Interestingly, of the 37 samples, only three had the *eae* gene. Two of these were the only *E. coli* O157:H7 isolates in the study. These *E. coli* O157:H7 isolates, while positive for *eae*, were negative for *stx1*.

There is also evidence that host susceptibility to the *stx2* toxin changes with age of the host. Liptakova et al. (2004) describes an outbreak among an extended family in which three children under the age of 33 months progressed to HUS symptoms, two children ages 5 and 7 years progressed to bloody diarrhea and four adults were asymptomatic. All were infected with the same strain, an *E. coli* O157 that was s*tx1*$^{-}$, *stx2*$^{+}$, and *eae*$^{+}$ that was apparently contracted from cream made from unpasteurized milk. Whether this age disparity is a result of immune system functionality, a decrease in receptors for the toxin itself, maturity of gut microflora, or some other explanation or combination thereof is currently unknown.

The asymptomatic adults in the Liptakova et al. (2004) case shed *E. coli* O157:H7 for up to 21 days after infection, while the maximum reported duration of shedding for humans for *Salmonella* Typhi was set by Typhoid Mary at 40 years (Gopinath et al., 2012). Non-Typhi *Salmonella spp.* has a median duration of excretion of approximately five weeks (Buschwald and Blaser, 1984). All of this suggests that there are many complex mechanics associated with the production of symptoms and whether a person will become a carrier of these pathogens. This possibility is why food production facilities need to be mindful of employees washing their hands before handling products, especially after using the restroom, and the overall cleanliness of the facility.

In addition to asymptomatic carriers, the waste stream (i.e., sewage) is another potential source of pathogens. Since sewage is the combined waste streams of the population that the waste water treatment plant serves, all of the pathogens being shed by sick and healthy people alike are concentrated into one location. Thus, the content and concentration of pathogens in the waste stream is entirely dependent on the general health of the community being served (Jones and Martin, 2003). A variety of human pathogens are often found in sewage sludge, including viruses, bacteria (notably *Salmonella spp*, *Giardia*, and pathogenic *E. coli*), parasites, and helminthes (Pepper et al. 2006; Sidhu and Toze, 2009). The separated solids from a waste water treatment plant, called biosolids, are often used as fertilizer as a means of disposal (Zaleski et al., 2005b). The United States Environmental Protection Agency (EPA) has mandated that all biosolids must meet certain criteria to reduce the amount of pathogens before being land applied (see Pathogen Standards section), especially for human food crops. One mechanism for reducing pathogens is composting.

Composting

Biosolids, animal manure, and other feedstocks can be composted to reduce the risk of transferring human pathogens to food crops. Composting is an aerobic microbially-driven process of breaking down organic matter into a humus-like material. This final product is not physically recognizable as its feedstock predecessors but instead generally resembles a rich organic soil. During the composting process, the microorganisms metabolize most of the free and fast-release nutrients, either volatilizing or incorporating the nutrients. The incorporation of nutrients into biomass, both living and dead, yields more complex matrices that stabilizes the nutrients into slow-release

forms. Many different organic materials can be used as feedstocks including manure, plants (i.e., crop residues, yard trimmings, etc.), food wastes, and various industrial products and by-products (i.e., paper, wood chips, etc.). The metabolic activity of the microorganisms degrading the organic material produces heat which is instrumental in killing pathogens (human, animal, and plant) and weed seeds.

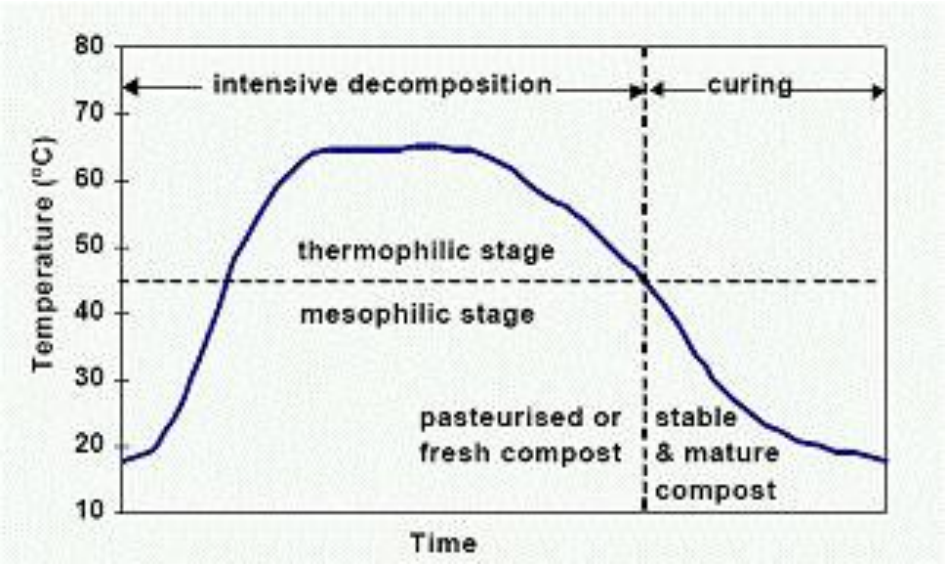

Figure 1.4: The idealized temperature progression of a typical compost pile. Source: State government of Victoria, Australia (Department of Environment and Primary Industries, 2013).

Compost goes through a fairly predictable series of thermal changes. The initial feedstock is at ambient temperature. If proper C:N and moisture ratios are present, the pile will rapidly heat up to thermophilic temperatures (>40°C, though it can reach temperatures of 70°C if not properly maintained) due to the rapid increase in microbial activity (USDA, 2010). During this time, microorganisms rapidly respire the carbon in the pile as CO_2, causing the pile to lose mass and decrease the C:N ratio. Once much of the easily available nutrients have been metabolized into more complex compounds or volatilized, the temperature of the pile begins to decrease until mesophilic temperatures

are reached (10-40°C). This is termed the cooling or curing stage (Figure 1.4). During this time, spores germinate and mesophilic microbes reestablish throughout the pile, continuing the decomposition of the feedstocks. Once the pile no longer heats, even with mixing, the pile is considered mature or finished (USDA, 2010). In the composting industry sense, "finished" compost may refer to compost that is still curing, however, it must have already past the thermophilic stage and be ready for distribution.

The removal of pathogens, weeds, and most quick-release nutrients allow compost to be used in many applications including, but not limited to, soil amendments for poor soils, slow-release fertilizer, and as a soil alternative for landscape plants (Stoffella et al., 2003; USDA, 2010). Finished compost provides numerous benefits such as increasing water retention, fertility, soil structure and stability, decreased erosion, general suppression of various plant diseases and pests, and reduced synthetic fertilizer and pesticide use (Stofella et al., 2003). There are five interrelated aspects of composting that help shape its effectiveness in efficiently decomposing the organic material and killing pathogens: the C:N ratio, moisture, aeration, temperature, and composting techniques.

C:N Ratio

The ratio of carbon to nitrogen is one of the main drivers of microbial metabolic activity. All microorganisms need carbon and nitrogen to build biomass in the form of proteins, lipids, etc. Most of the microorganisms involved in the degradation of organic material are chemoheterotrophs, meaning that they use the organic material as their source of carbon for both energy and biomass. The metabolic processes that break down

the complex organic feedstocks produces heat and stabilizes nutrients through the assimilation of carbon and nitrogen into biomass.

In general terms, the ideal C:N ratio when starting the composting process is approximately 30:1 but anywhere from 25:1 to 35:1 will result in successful composting. This ratio allows microbes to efficiently utilize both the carbon and nitrogen without having a large excess of either. The actual ratio used is also determined by the availability of the carbon and nitrogen in the feedstocks. Using different feedstocks, the optimum initial C:N ratio may be as low as 15:1 or as high as 40-45:1. Carbon and nitrogen come in a variety of forms, both physically and chemically, that affect their relative availability to the microbes, carbon in particular. Very complex carbon molecules (e.g., lignins) or large pieces (e.g., woodchips) are less-readily broken down than less complex (e.g., glucose, amino acids) or smaller pieces (e.g., sawdust) (USDA, 2010). These differences in carbon availability can significantly change the amount and type of nitrogen that is needed to maintain high temperatures in the compost pile. In general, nitrogen sources are easily broken down, with the main exception being keratin (hair, horns, feathers, etc.).

Nitrogen can be in several forms. The first forms to be used are ammonia/um and nitrate because these are water soluble and easy to assimilate. If there is an excess of nitrogen, then these readily available forms will volatilize (ammonia), accumulate in the pile to toxic amounts, or leach out of the pile, which can cause nutrient runoff issues (USDA, 2010). Nitrogen is also found in more complex forms such as amino acids and proteins. These complex forms become the primary sources of nitrogen once the more readily available ammonium and nitrate are used or lost due to leaching or ammonia emissions. Unlike carbon, of which 30-50% volatilizes as carbon dioxide during

metabolism, most of the nitrogen in the pile is recycled through the complex forms being broken down and assimilated back again into new biomass (USDA, 2010). This means that the final compost will have a lower C:N ratio than the initial pile.

Since carbon is generally in forms that are harder, and therefore slower, to degrade and assimilate into biomass than nitrogen sources, and since the C:N ratio is one of the biggest drivers of microbial growth, excess carbon leads to a slower growth rate of the microorganisms. A slower growth rate leads to slower degradation rates of the compost and less heat production. Excess nitrogen leads to a large amount of nitrogen loss through emissions of ammonia and leaching. A rapid loss of nitrogen can lead to unfavorably high C:N ratio in the final compost material.

Moisture

Moisture control is a critical component of composting. The ideal initial moisture content for composting is 40-65% though preferably between 50 and 60% (Adhikari, 2005; University of Plymouth, 2005). A moisture content of less than 20% will greatly inhibit biological processes (Adhikari, 2005) due to a lack of available water and will therefore restrict the compost from decomposing any further. However, moisture cannot be consistently too high since this will cause a lower oxygen diffusion rate through the pile and cause anaerobic conditions (Kumar, 2010). Anaerobic conditions in the compost pile will induce anaerobic metabolisms which can reduce sulfur compounds into H_2S. This compound is extremely odorous and is a nuisance issue. Denitrification can also occur, which will produce nitrous oxides (potent greenhouse gases) and subsequently increase nitrogen losses from the pile. Maintaining optimal moisture conditions will

allow the microorganisms to operate at higher metabolic rates and in turn increase the temperature of the pile to an optimal range.

Aeration

Aeration of compost piles is critical to temperature and moisture control (Adhikari, 2005) and can vary greatly in cost. Several options are available for aeration. The simplest is the non-aerated static pile, which utilizes the natural diffusion of oxygen and requires there to be enough porosity to allow proper aeration to occur while maintaining a heated core. This is termed passive aeration. A second method is simply to place a perforated pipe under the pile to allow for increased passive aeration into the core of the pile (Adhikari, 2005; USDA, 2010). This helps to avoid anaerobic conditions in the core of a static pile.

Another method is turning. Turning the pile involves regularly mixing the pile so as to homogenize the compost and introduce oxygen into the middle of the pile while also mixing the material from the edge of the pile into the middle. This only temporarily aerates the pile and is mostly used to remix the feedstocks. Remixing the feedstocks can make more nutrients available in the center of the pile and cause reheating and can also be used to decrease the temperature when a pile is overheating simply by opening the pile up temporarily.

Alternatively, two different forced aeration systems can be utilized: positive and negative pressure aeration. Both systems require a perforated pipe on the bottom of the pile. Positive pressure pushes air through the pile from the bottom and out to the atmosphere. Lin (2008) reports that this may cause excessive drying in the core of the pile and can cause odorous gases to escape from the pile more readily. However, positive

pressure also pushes the high core temperatures to the outer layers of the pile. This can aid in the killing of pathogens on the outer surface of the pile which can often harbor of pathogens in unturned piles (Pereira-Neto et al.,1986). Negative pressure aeration causes a vacuum and brings air through the pile from the outside towards the pipe in the center. If properly watered, this will allow for a more even moisture distribution in the pile and will help prevent odorous gases from escaping, making this method of temperature control more suitable for urban environments (Lin, 2008). To prevent odorous gases from escaping, negative pressure systems pump the air that has been drawn through the compost pile through a biofilter, oftentimes simply a large pile of woodchips. The biofilter absorbs the gases and prevents their escape. Forced aeration systems also have the possibility of over-cooling the piles and extending the time necessary for the compost to cure and possibly reducing pathogen-kill (University of Plymouth, 2005; Kutsanedzie et al., 2012). Forced aeration systems thus require proper regulation of airflow to ensure optimal composting conditions. Airflow control is a major component of compost management and cannot be overlooked.

A bulking agent provides carbon as well as structural support to allow oxygen into the piles and reduces moisture through adsorption, reducing the chances of anaerobic conditions (Iqbal et al., 2010; USDA, 2010). Bulking agents are essential to composting techniques that utilize passive aeration. Many different materials can be used as a bulking agent including, but not limited to, wood chips, wood shavings, paper, leaves, straw, peanut shells, and bagasse (Iqbal et al., 2010; Adhikari et al., 2008). Larger bulking agents provide more diffusion of oxygen into the pile while smaller bulking agents provide more readily available carbon. Small bulking agents or those that tend to become

matted, such as leaves, will need to be turned more often to allow for sufficient oxygen to come into the pile. Larger bulking agents will not break down as quickly and so should not be expected to contribute a large amount of the carbon in the beginning of the composting process. Each bulking agent will affect the overall C:N ratio of the pile differently due to its own C:N ratio and the availability of its carbon. Even non-degradable items, such as shredded tires, can be used as bulking agents. These non-degradable items, and quite often large bulking agents such as woodchips, will be sieved from the finished compost and reused. Every bulking agent has advantages and disadvantages.

Temperature

The temperature of the pile is a direct result of the metabolic activity of the microorganisms decomposing the organic material. Thus, anything that affects the metabolic activity of the microbes will affect the temperature as well. The main factors that affect temperature have already been discussed: the C:N ratio, moisture, and aeration. Temperature has a substantial effect on the decomposition rate (Eklind et al., 2007) and the ammonia emission rates (Pagans et al., 2006). Temperatures that are too low (<45°C) will not sufficiently kill pathogens (plant, animal, and human) or weed seeds and leads to slower overall decomposition rates. Temperatures that are too high (>60°C) can lead to high ammonia emissions, a final product with a high C:N ratio(which limits its use as a fertilizer or soil amendment), and can kill beneficial microbes (Adhikari, 2005; Eklind et al., 2007). Though many studies have looked at the optimum temperature for the most efficient decomposition of materials, results have varied. The difference likely has to do with different feedstock and ratios used between different studies.

However, the optimum temperature for decomposition seems to be around 52° – 60° C (Eklind et al., 2007). Unfortunately, temperature can be difficult to control in such a small range, and therefore composts will often either exceed 60°C or fail to reach 52°C. Coincidentally, most animal and human pathogens are not able to survive at 55°C or above (Jones and Martin, 2003). Therefore, composts are required to reach at least 55°C and maintain that for at least three consecutive days in order to be considered essentially pathogen-free and safe for human handling. Aeration techniques are typically the means for controlling temperature (University of Plymouth, 2005; Lin, 2008).

The temperature of the pile is additionally influenced by the ambient temperature. Composting in the winter is likely to produce composts with lower overall temperatures than those produced by composting in the summer (USDA, 2010; Shepherd et al., 2010). The main way that is used to alleviate the cold ambient temperature effect on unturned piles is to add an insulation layer. This can be several inches of finished compost or specialized membrane coverings (Shepherd et al., 2011; USDA, 2010). Shepherd et al. (2011) reports that utilizing finished compost as a covering, they achieved 7 – 15°C of temperature elevation compared to uncovered controls. Maintaining high temperatures, and especially high temperatures at the outer edges of the pile, is imperative for ensuring pathogen-kill.

Composting Techniques

Composting can be a complicated process even when only considering the types of materials, the proper ratios, temperature control, etc. There are also multiple composting techniques. The most common composting mechanisms are static piles, windrows, and in-vessel containers.

Static piles are simply piles of feedstock. These are low-maintenance by design and often do not require specialized machinery. In general, static piles are used for relatively small amounts of material though they can be used for very large volumes when turning would be impractical or expensive. If the pile is small enough and no insulating layer is used, these piles can be turned using a front end loader or similar machinery. However, if the pile is large or utilizes an insulating layer, then the pile will remain unturned. Static piles have little temperature control and will generally heat up past the optimal composting temperature (University of Plymouth, 2005) unless an aeration technique or turning is used.

Windrows are long stretches of compost that can be up to 10 feet tall, 20 feet wide, and as long as is necessary (USDA, 2010). Windrows are more energy intensive than static piles because they are turned on a regular basis to control the temperature. Turning is used to prevent excess heating of the pile at the beginning of the composting process by dissipating the heat and to encourage reheating of the pile at the end of the composting process by remixing the material. This requires the windrows to be turned more frequently at the start of the composting process and less at the end. Windrows require a large turning machine as well as a front end loader to efficiently create, turn, and organize the large piles.

In-vessel composting systems range greatly in size, complexity, technology, and expense. The basic principle behind in-vessel composting is to containerize the feedstocks and allow it to compost under more controlled conditions rather than to have it exposed to the weather, as is the case for most windrow and static pile systems. In-vessel systems offer more control over the parameters of the composting process, often

including temperature, aeration, odor, and leachate control. However, the compost in in-vessel systems may be harder to maneuver due to loading and unloading requirements. Efficient methods of loading and unloading are currently also expensive.

Survival and Regrowth of Pathogens in Compost

Human pathogens can survive during the composting process for a variety of reasons. Detection of human pathogens in compost is relatively rare but does happen. In an evaluation of 15 different composting facilities from across the USA, Ingram (2009) only found 1 *E. coli* sample out of 105 (1%) positive for the *stx2* toxin gene and only 6 out 105 (6%) that contained *Salmonella spp.* All samples that were positive for *Salmonella spp.* came from facilities that composted biosolids. In an evaluation of 94 non-sludge composting facilities, Brinton et al. (2009) found only one sample containing *Salmonella spp.* (1%) but three that were contaminated with *E. coli* O157:H7 (3%). They also found that large composting facilities and those that employed large static piles were more likely to have human pathogen contamination than smaller facilities or those that utilized turned windrows. Additionally, Brinton et al. (2009) reexamined one of the compost facilities with *E. coli* O157:H7 contamination three months later and again found *E. coli* O157, suggesting that contamination can become a persistent issue at composting facilities.

In general, survival of the pathogen is attributed to either the entire pile or pockets within the pile not reaching the sterilizing temperature (Wichuk and McCartney, 2007). These are of particular concern in piles that are not turned or are outside in cold ambient temperatures (Shepherd et al., 2010). Even if the pile is at temperature, there may be pockets that are not up to temperature and can harbor pathogens. Maintaining high

temperatures and evenly mixing the compost are therefore key in ensuring pathogen-kill. Shepherd et al. (2010) found that *E. coli* O157:H7 and *Salmonella* Typhimurium that had been heat-shocked prior to inoculation into the compost survived longer than the strains that had not been heat-shocked. Proteins produced by heat-shocked cells were shown to provide additional protection from extreme desiccation on the surface of the piles. They suggest that optimizing the composting process to minimize the initial mesophilic stage and quickly moving to a prolonged thermophilic stage is more effective to prevent heat-shocking pathogens and allowing them to acclimate to the environment, thereby potentially surviving the composting process.

Moisture is an additional necessity for the survival and, especially, for the regrowth of pathogens. An experiment performed in Australia by Gibbs et al. (1997) demonstrated the regrowth potential of fecal coliforms and *Salmonella spp.* In Australia, at least at the time of the experiment, storage of biosolids for a given amount of time was considered sufficient for the reduction of pathogens so that it could be spread onto the land. Gibbs et al. (1997) tested this theory as well as the survival and regrowth of these same organisms on field plots with the contaminated biosolids incorporated into the surface soil. Both the stockpiled biosolids and the field trials showed an increase of fecal coliforms and *Salmonella spp.* after rainfall. This observation made sense for the field trials because the fields were at less than 1% moisture for several weeks just prior to when rainfall brought the soil moisture up to about 22%. This allowed the fecal coliforms and *Salmonella spp.* to grow from undetectable levels to 1.1×10^5 Most Probable Number (MPN)/g and 0.7 MPN/g respectively, higher than the 6.3×10^4 and 0.09 MPN/g respectively that was initially present at the beginning of the experiment. This same

phenomenon was observed in the stockpiled biosolids, which were open to the elements. However, the rainfall did not affect the moisture content of the piles, which remained between 70-80% moisture for the duration of the experiment. There was no easy explanation for this occurrence in the piles. Gibbs et al. (1997) suggested that there may have been a dissolved nutrient substrate that was moved to other parts of the piles with the rain and allowed for the regrowth in this manner. Recontamination of the piles and fields by wild animals was ruled out since the *Salmonella* observed was the same serotype throughout the experiment. A variety of serotypes would have been expected if wild animals had contaminated the experiment.

Zaleski et al. (2005a) found that recontamination by wild animals was the most likely explanation for the regrowth that was observed in their biosolids piles. Class B biosolids (aerobically or anaerobically digested piles) were placed in concrete bins to evaluate the potential for solar drying as a means to decreasing pathogen populations in biosolids to meet Class A requirements. In both aerobically and anaerobically digested biosolids, *Salmonella* levels were undetectable by week 3 and remained undetectable until week 7 and 8, respectively. It rained on week 7, at which point *Salmonella* levels increased past the Class A threshold and even past the initial levels seen at the beginning of the experiment. Similar trends were seen for fecal coliform populations. When biosolids were applied to soil, *Salmonella* populations increased minimally but remained below the Class A threshold and the detection limit throughout the experiment, the opposite of Gibbs et al. (1997). Zaleski et al. (2005a) also speculated that much of the regrowth seen in the drying beds was due to recontamination by birds at the site, which seemed to frequent the biosolids piles more often after rewetting occurred, again opposite

of the findings by Gibbs et al. (1997). Differences in serotypes found before and after regrowth at their aerobically digested biosolids site supports this hypothesis.

Gibbs et al. (1997) was one of the first experiments to show the regrowth potential of *Salmonella spp.* and also showed very clearly the hazard of assuming that the material is completely free of human pathogens even if the tests show that levels are undetectable. During the dry periods, *Salmonella spp.* and fecal coliforms were undetectable and only once they regrew in correspondence to rainfall did the organisms become detectable again. Clearly, Zaleski et al. (2005a) echoes this sentiment. It should be kept in mind that both Zaleski et al. (2005a) and Gibbs et al. (1997) used digested biosolids, not compost. However, the same principals also apply to contaminated composts. The fact that Gibbs et al. (1997) saw regrowth in their field trials while Zaleski et al. (2005a) did not indicates how complicated regrowth is with a variety of factors beyond moisture likely interacting to support or depress regrowth potentials. Soares et al. (1995) and Zaleski et al. (2005b) noted multiple authors who reported that moisture has a significant role in the regrowth potential of pathogens in composts. The rewetting effect on regrowth is thought to be due to available organic matter that is not completely degraded when the compost is too dry. If the compost is too dry, there is insufficient available water so microorganisms naturally function and grow less, leaving organic matter available. When the compost or soil is rewetted, the microorganisms are able to take advantage of the available organic matter due to the additional moisture, causing a bloom of regrowth (Soares et al., 1995). Kim et al. (2009) found that the minimum moisture content in compost that could support *E. coli* O157:H7 regrowth was 20% moisture, though they noted that different compost compositions will have different water

activities (water availability) even at the same moisture content. In effect, moisture can become a limiting factor in the growth of microorganisms and regrowth can occur when that factor is no longer limiting.

Of course, even if moisture allows the nutrients to become available, there is no guarantee that the pathogens will be able to obtain those nutrients. Competition from indigenous microorganisms in compost and amended soils has been shown to inhibit regrowth of human pathogens (Jiang et al., 2002; Kim and Jiang, 2010; Kim et al., 2011; Paniel et al., 2010; Pietronave et al., 2004; Sidhu et al., 2001). Both the type and quantity of indigenous organisms can affect the suppression or regrowth of pathogens (Kim et al., 2011). Indigenous microorganism populations are dynamic during the composting process and generally follow temperature trends, ranging from mesophilic to thermophilic populations. Paniel et al. (2010) found that the organisms present in the cooling stage of the composting process were instrumental in preventing regrowth of *Salmonella* infantis. Millner et al. (1987) echo this finding. They took samples from composts at different temperatures (and therefore with different communities of microorganisms) and inoculated them with *Salmonella spp.* Samples from compost at 70°C did not suppress growth, samples from 55°C compost suppressed the population by 2-4 orders of magnitude, and samples from 25-40°C compost reduced the inoculated *Salmonella* to undetectable levels. These findings indicate that the organisms present in the compost during thermophilic temperatures do not contribute very much to the suppression of pathogens and that most of the pathogen suppression at these temperatures is the temperature itself. However, the organisms present in the compost at mesophilic temperatures play active roles in the suppression of human pathogens through

competition, allelopathy, or directly killing the pathogens. They also noted that fungi play a relatively minor role in suppression while bacteria and actinomycetes, especially gram-negative bacteria, played a major role in the active suppression of *Salmonella*. However, Sidhu et al. (2001) notes that long term storage of compost material is likely to increase the occurrence of pathogen regrowth, presumably due to a less active microbial community.

Recontamination of a stored pile by wild animals or cross contamination from equipment could allow for growth of pathogens if the pile does not have a sufficiently active microbial community to suppress the introduced pathogens. The incomplete destruction of pathogens during composting, the recontamination of composts via wildlife or contaminated equipment, and the regrowth potential of pathogens in compost all contribute to a contaminated final product. Contaminated composts, even at low amounts, have the potential to transfer pathogens to produce and eventually cause illness (Buck et al., 2003).

Pathogen Transfer to and Survival on/in Produce

Though there have been no established links specifically between the land application of contaminated composts and illness (Zaleski et al., 2005b) there have been links to foodborne illnesses from produce, both circumstantial and confirmed, to runoff from livestock farms, raw/digested manure spread as a fertilizer, and contaminated irrigation water (Mandrell, 2009). Ingram (2009) did note, however, that many of the *Salmonella spp.* isolates he found from several composting facilities were the same serotypes as reported in outbreaks in their respective states in the same year, providing circumstantial evidence of a link between compost and foodborne illnesses.

Human pathogens have been shown to survive in soil and on produce for extended periods of time. *E. coli* O157:H7 and *Salmonella* can survive for several months in compost- or manure-amended soils (Islam et al., 2004a; Islam et al., 2004b; Islam et al. 2004c; Islam et al. 2005; Jones and Martin, 2003; Mandrell, 2009). *E. coli* O157:H7 has been shown to survive longer in the soil after amendment with compost in the fall season as compared to the spring season (Oliveira et al., 2012). This is likely due to a variety of environmental factors such as desiccation and UV radiation related to solar intensity, changes in the microbial community, etc. The type of compost used to amend the soil affected the survival of *Salmonella* and *E. coli* O157:H7 in soil, both surviving longest in poultry compost and the shortest in alkaline-pH-stabilized dairy manure compost (Islam et al., 2004c; Islam et al., 2005). A previous experiment using the same composts did not find a significant difference between compost type and survival time (Islam et al., 2004a). However, the plant being grown in the soil did significantly affect the survival of *E. coli* O157:H7 and *Salmonella* in the soil (Islam et al., 2004a; Islam et al., 2004c; Islam et al., 2005). This shows the need for more research on this topic.

If different plants provide differing levels of survivability in soil to human pathogens, then different crops may have drastically different safety protocols. Islam et al. (2004a) noted that *E. coli* O157:H7 survived in the soil longer after the harvest of parsley than in fields where lettuce was harvested. There was more exposed soil surface after the harvest of the lettuce than in the parsley fields, leading to greater environmental exposure of the *E. coli* O157:H7 in the lettuce fields. The greater cover in the parsley fields allowed the inocula to survive an additional 60 days in the soil as compared to those in the lettuce fields. It is generally true that human enteric pathogens survive longer

in the environment in the presence of plants instead of just soil (Tyler and Triplett, 2008). Greater survival times will allow for greater opportunities for human pathogens to contaminate other crops, perhaps the next rotation if in the same season. It is already known that root crops are more susceptible to contamination by human pathogens simply due to proximity (Islam et al., 2004a; Mandrell, 2009). Leafy green vegetables, though not directly touching the ground, can still become contaminated (Islam et al., 2004a; Islam et al., 2004b; Islam et al., 2005; Oliveira et al., 2012). Survival times of *E. coli* O157:H7 and *Salmonella* on leafy greens and other crops vary greatly from just one or two weeks to several months (Islam et al., 2004a; Islam et al., 2004c; Oliveira et al., 2009; Patel et al., 2009).

Survival of *E. coli* even seems to be somewhat dependent on what animal shed it, with lower survival times in pig feces as compared to cattle and sheep (Avery et al., 2004). Long term farm practices can have a significant impact on the survival of both pathogens. Franz et al. (2008) found that *E. coli* O157:H7 survived longer in soils that had been primarily fertilized with faster release fertilizers such as chemical fertilizers and manure slurries as compared to fields that had a history of being fertilized using slow-release fertilizers such as compost or solid manure. The more oligotrophic the soil and soil community were, the faster the decline of pathogen populations, likely due to an inability to compete for nutrients. Survival for long periods of time allows for many opportunities for pathogens to transfer to plants and animals long after the initial contamination occurred.

Other than being directly applied to the plants by fertilization or irrigation, pathogens can also be transferred through other mechanisms. Human pathogens, just like

some plant pathogens, can be transferred from the soil surface to nearby plants by splash (Monaghan and Hutchison, 2012). Splash can occur whenever there are water droplets hitting the soil surface, such as rain or spray irrigation. Splash can carry microorganisms at least 45cm in all directions, depending mainly on the droplet size (Monaghan and Hutchison, 2012). As might be expected, there is a positive correlation between droplet size and the amount of contamination for a given area around the site of impact. This mode of transportation of pathogens can only be avoided in greenhouses that utilize non-spray irrigation, such as drip irrigation. However, once contaminated, Moyne et al. (2011) found that *E. coli* O157:H7 that was spray inoculated onto the leaves survived at least 28 days on some samples regardless of drip or spray irrigation. The survival times of *Salmonella* and *E. coli* O157:H7 transferred to carrots or radishes by a one-time spray irrigation water contamination are similar to those transferred by contaminated compost (Islam et al., 2004c; Islam et al. 2005). Theoretically this would apply to most other crops as well. Therefore, the initial contamination must be avoided.

Insects and other invertebrates, though less studied, may also be vectors of human pathogens to plants. Flies have been shown to not only be contaminated with *E. coli* O157:H7, but also to directly transfer these bacteria to plants (Berger et al., 2010; Brandl, 2006; Mandrell, 2009). Brandl (2006) points out that given the universal presence of insects in the environment, on both manure and on plants, more research and consideration should be given to the potential of this particular mode of transmission. Additionally, nematodes have also been shown to move *Salmonella* at least 5 cm through the soil (Kenney et al., 2006). These studies show that the transference of pathogens to

produce is a very complex issue and underscore the fact that complete safety is impossible.

To reduce the potential pathogen load acquired from the field, produce products are generally washed before being sold to consumers, often with a dilute chlorine wash meant to not only physically remove soil particles and microbes, but also to chemically sanitize the produce surface and the wash water with the intent of reducing contamination between produce using the same rinse water. However, if the chlorine levels are not closely monitored, the chlorine levels can drop to ineffective levels. When this happens, or chlorine is not used, pathogens and other microbes that are washed off have the opportunity to be transferred to other produce in the same batch, especially since produce is often washed in tubs. Other potential harvest/ post-harvest contamination mechanisms include: contaminated blades used for cutting multiple produce items, unwashed hands of asymptomatic or sick workers, and allowing produce and/or produce containers to touch contaminated soil (Buck et al., 2003; Delaquis et al. 2007).

A complication is that even if produce is washed thoroughly with clean water and with appropriate chlorine levels, contaminated produce can still make it through to the consumer. *E. coli* O157:H7 and *Salmonella spp.* have both been found to be internalized by produce plants (Erickson, 2012; Takeuchi et al., 2000; Takeuchi and Frank, 2000; Auty et al., 2005; Solomon et al., 2002a; Solomon et al., 2002b; Avila-Quezada et al., 2010; Tyler and Triplett, 2008). *E. coli* O157:H7 has been shown to favor colonization of wounded areas (likely due to the release of nutrients from that area) as well as stomata (Takeuchi and Frank, 2000; Erickson2012; Beuchat, 1999; Delaquis et al., 2007; Seo and Frank, 1999). Though *E. coli* O157:H7 has not been shown to enter the plant through the

stomata, they have been found to infiltrate and be contained within the stomata, allowing for protection against sterilization (Seo and Frank, 1999; Beuchat, 1999). *Salmonella* have been shown to attach equally well to the surface of lettuce as to wounded areas (Takeuchi et al., 2000). Beuchat (1999) found that low levels of *E. coli* O157:H7 inocula (10^{0-1} CFU / g), even after being stored at 4°C for 1 or 5 days, are able to avoid chlorine rinses and survive in low numbers on lettuce. Given the low infective dose of *E. coli* O157:H7 and the volume of lettuce usually consumed in a salad, even extremely low levels of contamination are dangerous.

Both organisms have been shown to become internalized into a root system through wounded areas (Takeuchi et al., 2000) and even in unwounded plants (Warriner et al., 2003a). Different organisms, and likely different serotypes of the same organism, have varying abilities to attach and invade plant tissue (Takeuchi et al., 2000). Additionally, susceptibility to invasion has been shown to be widely variable among different plants (Goldberg et al., 2011). Plant defense mechanisms play a large role in preventing internalization, with the age of the plant being a potential factor in susceptibility (Erickson, 2012). Franz et al. (2007) found a negative correlation between sprout weight and leaf contamination. The plants colonized by *E. coli* O157:H7 or either of two *Salmonella* strains were significantly smaller than plants that were not colonized, indicating some sort of interaction between the two. Sprouts have been shown to be particularly susceptible to internalization and have been implicated in a number of outbreaks (Berger et al., 2010; Warriner et al., 2003a), likely due to softer tissues and less active immune responses. Significant adherence of *E. coli* O157:H7 to newly germinated sprouts grown in contaminated soil has been observed in as little as three days (Wachtel

et al., 2002). Wachtel et al. (2002) did not note any internalization into the sprouts. Sprouts may also become contaminated if the seed itself was contaminated. *E. coli* O157:H7 has been shown to preferentially attach to the deep grooves of seed coats and upon germination become associated with the sprout and the root hairs (Wachtel et al., 2002; Warriner et al., 2003a). Warriner et al. (2003b) also noted the same internalization and location trends for *Salmonella,* indicating the susceptibility of young plants to the internalization of human pathogens. Whether *E. coli* O157:H7 adheres (Wachtel et al., 2002) or internalizes (Warriner et al., 2003a; Warriner et al., 2003b) into sprouts is inconsequential, both can cause illness, especially if the sprouts are not cooked or properly washed.

Both *E. coli* and *Salmonella* have been shown to wedge themselves into the intracellular spaces but not actually invading the plant cells (Auty et al., 2005). *E. coli* O157:H7 and *Salmonella spp.* have been shown to penetrate plant tissue to a depth of approximately 60-100 nm (Auty et al., 2005; Seo and Frank, 1999; Takeuchi and Frank, 2000; Takeuchi et al., 2000). Though this depth may seem inconsequential, this is enough to avoid surface sterilization with chlorine water rinses (Takeuchi and Frank, 2000). Different studies have determined that these organisms are internalized through different means. Some studies determined that these organisms were internalized by proxy of an ineffective surface sterilization (Solomon et al., 2002b; Warriner et al., 2003b) while others used Confocal Scanning Laser Microscopy (CSLM) to visualize the organisms inside the plants (Auty et al., 2005; Takeuchi et al., 2000). Those that hypothesized internalization by proxy could have also been explained by hard-to-remove biofilms or even hard-to-reach and/or prevalent crevices and niches in the produce itself, such as

lettuce leafs (Erickson, 2012; Solomon et al., 2002). Nonetheless, surface sterilization and rinses did not remove the pathogens in those studies, a concern in itself regardless of the mechanism of avoidance. Several studies have even demonstrated the potential of the organisms to translocate from the roots to the stems through the vascular system (Solomon et al., 2002; Franz et al., 2007). However, the ability to translocate to the stem and leaves seems to depend on a variety of factors since not all studies have been able to replicate translocation from the roots to the stem and leaves, even at high concentrations and with mechanical or biological wounding from nematodes or disease (Hora et al., 2005).

Given the apparent ease at which they are able to survive inside of plants, there is debate among the scientific community as to whether these human pathogens are opportunistic or facultative endophytes (Erickson, 2012; Tyler and Triplett, 2008). One criticism of many of the internalization studies is the high concentration of organisms at which plants were inoculated. The concentrations used for inoculation were often several logs higher than what would be expected to occur in the field. Thus, the internalization of these human pathogens could be postulated to be an artifact of the unrealistically high inoculation concentrations. One valid counter to this concern would be the realistic scenario of run-off from animal operations contaminating produce, especially if pooling occurs in the field. Regardless, enough studies have shown the potential of these organisms to enter the plant and/or be inaccessible to surface sterilization and rinses so as to warrant concern, especially with the low dosage requirements of *E. coli* O157:H7 and some *Salmonella spp.* serotypes. Once internalized in the plant, the only practical way of avoiding illness is to cook the produce.

Much of the fresh produce grown is eaten with minimal processing and is thus susceptible to contamination at the farm or during its minimal processing (AFF, 2010). Most of the produce-related outbreaks occur in food that is often not cooked, such as salad greens and many fruits and vegetables. Thus, the only way to prevent illness in these cases is to prevent the contamination in the first place, as is always the preference. On farm contamination, though already very low thanks to best management practices and various regulations, is the first step in the chain for the prevention of contamination.

Pathogen Standards

In 1979, federal regulations were put in place to restrict the use of biosolids to help prevent potential illnesses derived from direct contact with the biosolids or from food grown on biosolids-amended soils. In order to apply to land or sell biosolids without restriction, the biosolids had to go through Processes to Significantly Reduce Pathogens (PSRP) and Processes to Further Reduce Pathogens (PFRPs). PSRP methods included aerobic digestion, anaerobic digestion, and lime stabilization. These were designed to reduce the pathogen load by several logs. However, it was known that the final product may still contain high populations of disease-causing organisms. Therefore, biosolids were required to undergo one of the PFRP options which include composting, heat drying, pasteurization, and irradiation. Once biosolids underwent one procedure from each category it was considered safe for land application and distribution.

However, Yanko et al. (1987) found that *Salmonella* was often present in biosolids and biosolids products that had undergone both PSRP and PFRP procedures, indicating that the current regulations may not be enough for the protection of the public from potential contamination. Therefore, the EPA revised the biosolids regulations and

wrote EPA 40 CFR Part 503 (referred to henceforth as Part 503). These new regulations ultimately split biosolids in to two distinct categories: Class A and Class B. Class A biosolids are treated to the point of having no detectable human pathogens while Class B biosolids have large populations still present. Though the actual risk of infection from the land application of Class B biosolids seems reasonably low (Pepper et al., 2008), Class B biosolids have site restrictions and limited public access due to the high probability of having human pathogens present. Only Class A biosolids can be applied to land or distributed to the public without restriction.

Class A biosolids include limits on the populations of fecal coliforms (1000 MPN / g) or *Salmonella spp.* (3 MPN / 4 g) in biosolids and its derivatives to be met before land application or distribution to the public. The specific thresholds used were based on correlations found between the population levels of fecal coliforms and *Salmonella spp.* and other pathogens by Yanko (1987) and Farrell (1992). These two populations were used as the standard thresholds for detection due to the unreasonable expense and effort that would be required to detect all of the pathogens of concern. Therefore it is assumed that if the compost has fecal coliform or *Salmonella spp.* populations below the thresholds, then all of the other human pathogens will also be dead or at non-infectious population levels. These thresholds have been adopted widely throughout the U. S. composting industry (Ingram, 2009).

It should be reiterated that testing for indicator organisms is not measuring the actual pathogen populations and do not guarantee safety. Zaleski et al. (2005a) describes an experiment in which the populations of both fecal coliforms and *Salmonella spp.* were monitored in uninoculated aerobically and anaerobically digested biosolids. The

populations changed over time and regrew at certain points in correlation with rainfall. They noted that at certain points in the experiment if the biosolids had been tested using only the fecal coliform requirement it would have been considered Class A. However, the *Salmonella* populations were far above the Class A standards. Indeed, these particular biosolids had *Salmonella* populations of 10^{4-5} MPN / 4 g during the time it was under the 10^{1} MPN / g fecal coliform threshold. Even in 1974, just after the first regulations on biosolids came into place, fecal coliforms were recognized as not necessarily being the best indicator organism for pathogens in wastewater (Kenner and Clark, 1974). Kenner and Clark (1974) believed that *Salmonella* and/or *Pseudomonas aeruginosa* would be better indicator organisms in wastewater. However, quite often only fecal coliform testing is used simply due to the greater expense and effort required for *Salmonella* testing. A realistic understanding of the fact that indicator organisms are not necessarily accurate, but are only usually so, is necessary when interpreting testing results and when using Class A biosolids or composts that are held to the same standards.

Time-temperature requirements were also made that need to be met before biosolids or any of its derivatives can be considered to have met the PFRP composting option. Using in-vessel or static pile composting methods, the pile must reach 55°C or higher for at least three days while windrows must be turned at least 5 times within 15 days and must reach at least 55°C for at least 15 days (U. S. EPA, 1993). These time-temperature requirements were based on several studies looking at the thermal destruction of *Ascaris,* a parasitic roundworm that has high survivability in the environment and a low infectious dose (Ingram, 2009). It is generally accepted that if the composting process has successfully killed *Ascaris* then all other human pathogens

should have also been killed. These time-temperature standards have also become the accepted standard in the U. S. composting industry due to their well-researched and proven viability for ensuring human pathogen kill in composts (Ingram, 2009).

Currently, only composts containing biosolids must meet federal regulations and use the standard methods stipulated by the EPA. All other compost feedstocks are regulated through state and local governments. Many states and organizations have made their own microbial thresholds for compost. However, they often do not mention, or are vague regarding, any specific protocols to use for sampling and processing. As such, there are no official compost industry standards, however many states and organizations simply refer to the EPA protocols. This has made the EPA methods the most commonly used methods. These are EPA Method 1680 (Fecal Coliforms in Sewage Sludge (Biosolids) by Multiple-Tube Fermentation using Lauryl Tryptose Broth (LTB) and EC Medium) and EPA Method 1682 (*Salmonella* in Sewage Sludge (Biosolids) by Modified Semisolid Rappaport-Vassiliadis (MSRV) Medium). There is an alternate method for fecal coliform isolation, EPA Method 1681 (Fecal Coliforms in Sewage Sludge (Biosolids) by Multiple-Tube Fermentation using A-1 Medium). However, this method is not used as often due to its tendency for false positives (Baker et al. 2005). It is important to note that both the fecal coliform and *Salmonella spp.* methods are designed for use with biosolids, not compost. Compost is often quite variable in its input feedstocks and therefore its final composition as well. Neither EPA Method has been tested for accuracy in non-biosolids compost. The assumption that the EPA methods, or any method, have the same accuracy and viability for every compost type is unfounded and should be verified.

The U. S. Composting Council (USCC) saw the need for the standardization of the composting industry. Thus in 1995, the USCC began to form the Test Methods for the Examination of Composting and Compost (TMECC). According to Wayne Thompson, editor-in-chief of TMECC,

> Standardized methods for monitoring the composting process and analyzing composts can improve production, satisfy regulations and promote quality. In particular, material testing is needed to verify compost product safety and market claims. These methods have been requested by compost producers, state and local regulatory agencies, product marketers, testing laboratories and compost users. In the absence of standard methods for composting, analytical methods used for soils, water, and wastewater residuals have been adapted to composting materials. However, those methods do not always translate well to composting substrates. (Thompson and Rynk, 2002).

The final version of TMECC was published in 2002 after extensive peer-review and editing from almost 200 experts in compost analyses from around the world (Thompson and Rynk, 2002). The pathogen thresholds are the same as the EPA biosolids thresholds, however the methods of detection are different. The two main pathogen testing protocols are TMECC 07.01 (Coliform Bacteria) and TMEC 07.02 (*Salmonella*), which correspond directly to EPA Method 1680 and EPA Method 1682, respectively. TMECC 07.01 includes additional steps compared to EPA Method 1680 for the detection of *E. coli*.

Along with TMECC, the USCC also created the Seal of Testing Assurance (STA) program. This is a voluntary program that requires the participating composting facilities

to adhere to the quality standards outlined in TMECC (which are the same standards found in EPA 40 CFR Part 503) and make publicly available all testing reports (Cotton, 2006). One of the stated goals of the STA program is to move the compost industry toward standardized methods. Currently there are over 200 composting companies enrolled in the STA program. Some states recommend or even require that state funds only purchase compost from facilities that are enrolled in the STA program, indicating that this program and the TMECC methods have begun to be incorporated into the framework of the composting industry in the United States.

Both methods are used in the U. S. composting industry, with TMECC gaining in popularity. This leads to two main sets of protocols being used throughout the industry. However, the EPA and TMECC methods have never been compared to determine which is actually more accurate in composts. Nor have the methods been compared individually between different compost types to determine if each method is capable of being used equally as well in all compost types. Consistency of methods among composting facilities and testing laboratories will allow for more accurate comparisons, greater regulatory enforcement, and greater consumer confidence in the accuracy and validity of the results.

Detection methods, techniques, and technologies have drastically increased in accuracy, sensitivity, and affordability, and entirely new techniques and technologies have emerged, since either the EPA methods or TMECC were written (NRC, 2002). Other methods could be used to easily detect human pathogens directly rather than relying on indicator organisms. Carner et al. (2013) compared EPA Method 1680 and EPA Method 1682 to two rapid tests that only took 1 day to complete. Both rapid tests recovered statistically equivalent levels of their respective target organisms as the EPA

methods but took less time and fewer supplies. Isonhood (2005) describes a method involving immunomagnetic beads and a liquid sample recirculation system called Pathatrix. Immunomagnetic beads are metallic beads coated with antibodies specific to a target organism, in this case *E. coli* O157:H7 and *Salmonella*. This process is a one-day process with an additional PCR step to identify the presence or absence of the target organism. Isonhood (2005) detected *E. coli* O157:H7 from 100% of food samples using this method, even at initial populations of 10^0 CFU / 25 g. *Salmonella* recovery was much lower, though that was attributed to a lower reliability of the immunomagnetic beads than to the process itself. The immunomagnetic beads have likely significantly improved since then. This process shows great potential for the rapid detection of pathogenic organisms if it can be applied to composts.

The addition of protocols that can rapidly detect human pathogens in compost would lead to faster turn-around times for compost providers to receive testing results and therefore decrease any wait time associated with the testing procedures. Additionally, testing facilities could do more samples per any given time period and increase revenue or even add more comprehensive tests. The use of rapid and direct tests for human pathogens in compost could lead to safer compost by increasing the range and decreasing the time of detection. The addition of rapid tests should be considered as additions to both EPA and TMECC methods.

Research Purposes

This research project contained three main purposes. The first purpose was to compare the two most commonly used methods in the U.S. composting industry for fecal coliform and *Salmonella* spp. recovery. Currently there are no independent published papers on the TMECC protocols, at least to my knowledge, and relatively few published papers comparing the EPA methods to other microbial detection methods. Additionally, relatively few published papers have used either EPA method. These published papers more often seem to attempt to validate EPA Method 1682 rather than the fecal coliform detection method (Moncada et al., 2010; Yanko et al., 1995). This research project can be used as another validation study for the under-researched EPA methods as well as one of the first, if not the first, independent validation study of the TMECC protocols.

The second main purpose of this research is to provide evidence that rapid IMS techniques have the potential to be used to detect specific pathogens in compost that current microbial detection protocols might not detect. Specifically, neither EPA Method 1680 nor TMECC 07.01 detect pathogenic *E. coli* but instead detect fecal coliforms. TMECC 07.01 is also able to detect generic *E. coli* through an additional published test (TMECC 07.01C), however, through personal correspondence, this protocol is not often used by the laboratories certified to perform the TMECC protocols for the STA program. The detection methods for *Salmonella* spp. recovery (EPA Method 1682 and TMECC 07.02) are also not often used as indicators unless specifically requested by the composting facility or are required by state law. Thus, quite often, only fecal coliforms are tested for in composts. The use of IMS techniques could allow composts to be quickly tested for specific pathogens rather than, or in addition to, indicator organisms. As

previously mentioned, indicator organisms do not always accurately reflect the populations of pathogens in a given medium (biosolids, composts, etc). The addition of IMS techniques to EPA and TMECC detection protocols to look for specific pathogens in compost would likely benefit public safety and consumer confidence. This research specifically used IMS techniques for the detection of *E. coli* O157:H7 in composts.

The third main purpose of this research is to aid in the ongoing research need to have a reliable determination of the susceptibility of composts to the regrowth of pathogens. Pathogens have been shown to regrow in composts and (an)aerobically digested biosolids (see Survival and Regrowth of Pathogens in Compost section). Currently, there are no methods or indicators able to accurately determine the pathogen regrowth potential in composts. Having an accurate, and preferably simple, predictor of pathogen regrowth would allow compost producers and customers alike to determine the relative susceptibility of any given compost to pathogen regrowth. This, in turn, could impact composting technique, feedstock material, and customer base decisions of compost producers and purchasing decisions of compost buyers.

Chapter 2: Objectives and Methods

Objectives

Objective 1: To compare the recovery of inoculated target organisms (generic *E. coli* and *Salmonella* spp.) by EPA and USCC detection methods, as well as two immunomagnetic bead-based *E. coli* O157:H7detection methods, in compost to determine if one consistently has greater recovery. Comparisons are made in three different compost types (Biosolids, Manure, Yard). Each method was additionally evaluated between compost types to determine if each individual method was capable of equivalent recovery in different compost types. The comparisons that were made were:

a. EPA Method 1680 vs. TMECC 07.01 for detection of inoculated generic (non-pathogenic) *E. coli*
b. EPA Method 1682 vs. TMECC 07.02 for detection of inoculated pathogenic *Salmonella spp.*
c. Modified Elaine Berry method vs. Pathatrix machine (Matrix Microscience, Newmarket, UK) for detection of inoculated pathogenic *E. coli* O157:H7

Objective 2: To evaluate different compost characteristics (% moisture, C:N, volatile solids, Total Organic Carbon (TOC), pH, EC, and maturity as measured by Solvita CO_2 and NH_3 tests) on the re-growth potential of pathogenic *E. coli* O157:H7 and *Salmonella spp.* in compost. Re-growth for *E. coli* O157:H7 was determined using the Modified Elaine Berry method. Re-growth for *Salmonella spp.* was determined using the EPA Method 1682. Objective 1 recoveries of *Salmonella* and *E. coli* O157:H7 by EPA Method 1682 and the Modified Elaine Berry method, respectively, were used as the Day 0 recoveries for Objective 2.

Methods

Detection Methods

Flow charts of EPA Method 1680, EPA Method 1682, TMECC 07.01, and TMECC 07.02 as they were performed in this study are presented in Appendix A of Chapter 3. A description of the direct plating portion of the Modified Elaine Berry method is presented in the "*E. coli* O157:H7 Recovery Method" of Chapter 3. Descriptions of the immunomagnetic portion of the Modified Elaine Berry method and the Pathatrix method are presented in Chapter 4.

Method Deviations and Justifications

The EPA and TMECC methods were modified mainly to reduce redundancy and the workload associated with performing all six methods simultaneously. The following describes how each method differed from the published protocols and the justifications for doing so. A description on the background of the Modified Elaine Berry method, which is based on multiple papers by Dr. Elaine Berry, is also included.

EPA, both methods

a. Both EPA methods require the material to be sieved to 1 cm prior to processing. We used a $3/8^{th}$ inch, or 0.9525 cm, sieve due to availability. The point of sieving was to remove large pieces, especially wood chips, to facilitate the processing procedure. Since this sieve was less than 1 cm, and the difference was minimal, in our judgment we accomplished this goal.
b. Both EPA methods state that the sample combined with the diluent in the blender be blended on high for 1 to 2 minutes. Unfortunately, neither EPA method specified what rpm "high speed" refers to. The blender base used in

this experiment (Waring 700G one-speed, Stamford, CT, USA) was likely too fast (22,000 rpm). We only blended for 30 seconds instead of one minute because after 30 seconds liquid would often leak out of the top of the blender cup around the seal. Especially since we were using un-attenuated pathogens, we felt that this posed an unnecessary safety hazard even while blending in the biological safety cabinet. Due to this, the samples were blended for 30 seconds which still sufficiently homogenized the sample for processing.

c. Both EPA methods required a method blank with each sampling day, a media sterility check for every batch of media, positive and negative controls on every media every day, matrix spikes for each compost, and Ongoing Precision and Recovery (OPR) each week (see official procedures for details). None of these were done due to lack of available personnel and time. In addition, this experiment was essentially performing the matrix spikes and the positive controls for some media, just with different organisms than specified by the EPA.

EPA Method 1680

a. The original method only detected total fecal coliforms. For this reason, EPA Method 1680 only went to an Escherichia coli medium (EC) enrichment step. Since we inoculated with both generic *E. coli* and pathogenic *E. coli* O157:H7, and we did not sterilize the composts before inoculation, an additional step was needed in order to determine the recovery of just the inoculated generic *E. coli*. To that end, we transferred the presumptive positive tubes containing EC to MacConkey's agar (MAC) plates with 80

mg/L Rifampicin for the generic *E. coli* strains. This step was based on the TMECC 07.01 method (see Chapter 3, Appendix A, Flow Chart 3). All MPN/g calculations and all subsequent statistical analyses for EPA Method 1680 were then based off of the MAC positives.

EPA Method 1682

a. The original method was designed to detect *Salmonella spp.* at a MPN range from <0.065 to >16 MPN/g using a 3 dilution by 5 tube MPN scheme. However, we inoculated at 10^{1-2} CFU / g compost (wet weight). Therefore the dilutions used for the actual method needed to be modified in order to appropriately capture the range that we inoculated. EPA Method 1682 states that additional dilutions can be used if the sample is suspected to have greater levels of *Salmonella*. To capture our inocula, five total dilutions would have been needed. Instead of using a 5 dilution MPN scheme, we simply used the two additional dilutions and the last dilution of the normal protocol as our 3 dilution MPN scheme and removed the first two dilutions from the method. This made a 3 dilution by 5 tube MPN scheme with a MPN range of <1.8 to >1600 MPN/g. This allowed for a more appropriate MPN scheme for the recovery of the inocula levels to be used during the experiment and reduced the workload that would have been needed for a five dilution MPN.
b. The original method when transferring biomass from Modified Semisolid Rappaport-Vassiliadis agar (MSRV) to Xylose-lysine Desoxycholate agar (XLD) called for transferring two colonies from MSRV with each being struck onto its own XLD plate. The method then stipulates that one of the

plates is to be used while the other is a back-up. We removed this redundant back-up step to reduce both work and media.

c. In order to facilitate the recovery of the inoculated *Salmonella spp.*, the XLD agar was made with 50 mg / L Nalidixic acid, which the inoculated *Salmonella spp.* is resistant to.

d. The original method requires presumptive positives from the biochemical assay (Triple Sugar Iron agar (TSI), Lysine Iron agar (LIA), and Urea broth tubes) to then be confirmed using Polyvalent O *Salmonella* antiserum test (an agglutination test). Since we assumed that all *Salmonella* present were inocula and not background (we did run background tests for presence of *Salmonella*, all samples were negative), we felt that this step was redundant and unnecessary. To reduce the workload and redundancy, this step was removed.

TMECC, both methods

a. Both methods have a direct plating step that call for plating 50 µL of the 10^{-1} of the Homogenized Sample onto appropriate media (MAC with 80mg/L Rifampicin for TMECC 07.01; Xylose-lysine Tergitol 4 agar (XLT4) with 50 mg / L Nalidixic acid for TMECC 07.02). The upper detection limit for both TMECC methods is 1100 MPN / g. The original method of spiral plating 50 µL has a lower detection limit of 2000 CFU / g. This step is meant as a quick-test to determine if the level of bacteria present is blatantly higher than the detection limit of the TMECC methods. Instead, we spiral plated 100 µL of the 10^{-1} of the Homogenized Sample. This has a lower detection limit of 1000 CFU / g, which overlaps with the upper detection limit of the MPN method.

We felt that this overlap in detection limits would be more beneficial than the original method in detecting bacteria populations greater than the detection limit.

TMECC 07.01

a. This method calls for the use of EC with MUG (ECMUG) as an additional check to determine the presence of *E. coli* apart from fecal coliforms. Those tubes that fluoresce are then transferred to MAC and then to biochemical tests. However, we had issues with reliably detecting fluorescence even on positive controls due to refraction from the glass tubes. Therefore, all those that were positive in ECMUG for gas production and visible growth were transferred to MAC and then to biochemical tests. ECMUG was still used throughout the study for this method but fluorescence was not checked and instead the media was treated as simple EC medium.
b. In order to facilitate the detection of the inoculated generic *E. coli*, the MAC and Eosin-Methylene Blue (EMB) agars were made with 80mg/L Rifampicin, which the inoculated generic *E. coli* is resistant to.
c. The original method says that only if the streak from the positive ECMUG tube is positive on both MAC and EMB can it then be transferred to the biochemical tests (TSI, Motility Indole Lysine agar (MIL)) for further confirmation. Instead, we decided that if it was positive on either one, then we would transfer to biochemical tests. The main reason for this is that both MAC and EMB were made with 80mg/L Rifampicin, which cut down any background that resembles our inoculated *E. coli*. Additionally, some of the

streaks were indeed only positive on the MAC plates. This usually occurred only when there were extremely few colonies on the MAC plate, suggesting a dilution factor caused the missing corresponding colonies on the EMB.

TMECC 07.02

a. The original method requires presumptive positives from the biochemical assay (TSI and MIL tubes) to then be confirmed using Polyvalent O *Salmonella* antiserum test (an agglutination test). Since we assumed that all *Salmonella* present were inocula and not background (we did run background tests for presence of *Salmonella*), we felt that this step was redundant and unnecessary. To reduce the workload and redundancy, this step was removed.
b. In order to facilitate the detection of the inoculated *Salmonella spp.*, the XLT4 agar was made with 50 mg / L Nalidixic acid, which the inoculated *Salmonella spp.*is resistant to.
c. The original method prepares the iodine supplement for Tetrathionate (TT) broth differently from the media bottles prescribe. TMECC 07.02 prescribes mixing 6g Iodine and 5g Potassium Iodide into 20mL of distilled water and then using this solution at a rate of 20mL/L of TT broth. Acumedia does not prescribe a ratio of Iodine to Potassium Iodide but does say to use the solution at a rate of 41.7mL/L of TT broth. The Difco formulation of 5g Iodine to 8g Potassium Iodide in 40mL of distilled water was used at the Acumedia prescribed ratio of 41.7mL/L of TT broth. The TMECC formulation was not used because we were unaware that it differed from the Difco formulation until half-way through the experiment. To be consistent, we continued with

the Difco formulation. The Acumedia Iodine solution:TT broth rate was used because we used the Acumedia TT broth formulation, which does differ from the Difco TT broth formulation.

Pathatrix

a. The original method is meant to be done in one day. The sample is supposed to be enriched for 5 hours and then immediately run through the Pathatrix machine. However, instead of running the sample the same day, the sample was placed at 4°C overnight after enrichment and then processed the next day. This was done for two reasons. First, setting up and running the Pathatrix the same day would have been at least a 12 hour work day due to other aspects of the project that were also required to be done on the same day. Second, the Modified Elaine Berry had, as part of the procedure, placing the enriched sample at 4°C and then processing the next day. Putting both in the cold and then running through the Pathatrix machine the next day removed the additional factor of cold storage from the methods comparison between Modified Elaine Berry and Pathatrix.

Modified Elaine Berry

The Modified Elaine Berry method was based on several papers involving Dr. Elaine Berry and her colleagues who were primarily recovering *E. coli* O157:H7 from feedlot cattle feces (Barkocy-Gallagher et al., 2002; Barkocy-Gallagher et al., 2005; Berry et al., 2010; Berry and Wells, 2008; Berry and Wells, 2012; Brown-Brandl et al., 2009). After reviewing several of her and her colleagues' methods, a conglomerate of the various procedures was created for the recovery of *E. coli* O157:H7 from finished

compost. This new procedure included two separate portions, both using the same sample homogenate. One portion is a simple direct plating method to be used for the determination of *E. coli* O157:H7 regrowth in compost. This portion is described in Chapter 4. The second portion is a dilution enrichment plus immunomagnetic bead recovery portion to be used for the comparison of immunomagnetic bead recovery to the Pathatrix method. This portion is described in Chapter 3.

Strains

Seven total strains were inoculated into each compost sample.

a. Three generic (non-pathogenic) *E. coli* (TVS 353, TVS 354, TVS 355). All three strains are resistant to 80 mg/L of Rifampicin. All three strains were isolated from Salinas, CA from various sources (Tomas-Callejas et al., 2011).
b. Two *Salmonella spp.* (SAL 2133, SAL 2353). Both strains are pathogenic and resistant to 50 mg/L of Nalidixic acid. SAL 2133 is a *Salmonella* Newport strain isolated from Virginia creek sediment. SAL 2353 is a *Salmonella* Saintpaul strain isolated from Jalapeno peppers from Mexico.
c. Two *E. coli* O157:H7 (RM 4407, RM 5279). Both strains are pathogenic and resistant to 50 mg/L of Nalidixic acid.

Processing Overview

Compost samples were obtained from across the country (Figure 2.1). The compost samples were categorized as follows: Biosolids (n=10), Manure (n=4), or Yard (n=15). Those placed in the Biosolids category contained biosolids. Some of the composts had very little biosolids (<5%) while others were a majority biosolids (≥50%). Regardless of the proportion of biosolids, all composts that had any biosolids were placed in the

Biosolids category. Similarly, those placed in the Manure category contained some amount of animal manure, but no biosolids. Once again, some composts had very little manure (<5%) while others were a majority manure (≥50%). Those composts placed in the Yard category contained no biosolids or manure. Crop residue was also considered Yard waste.

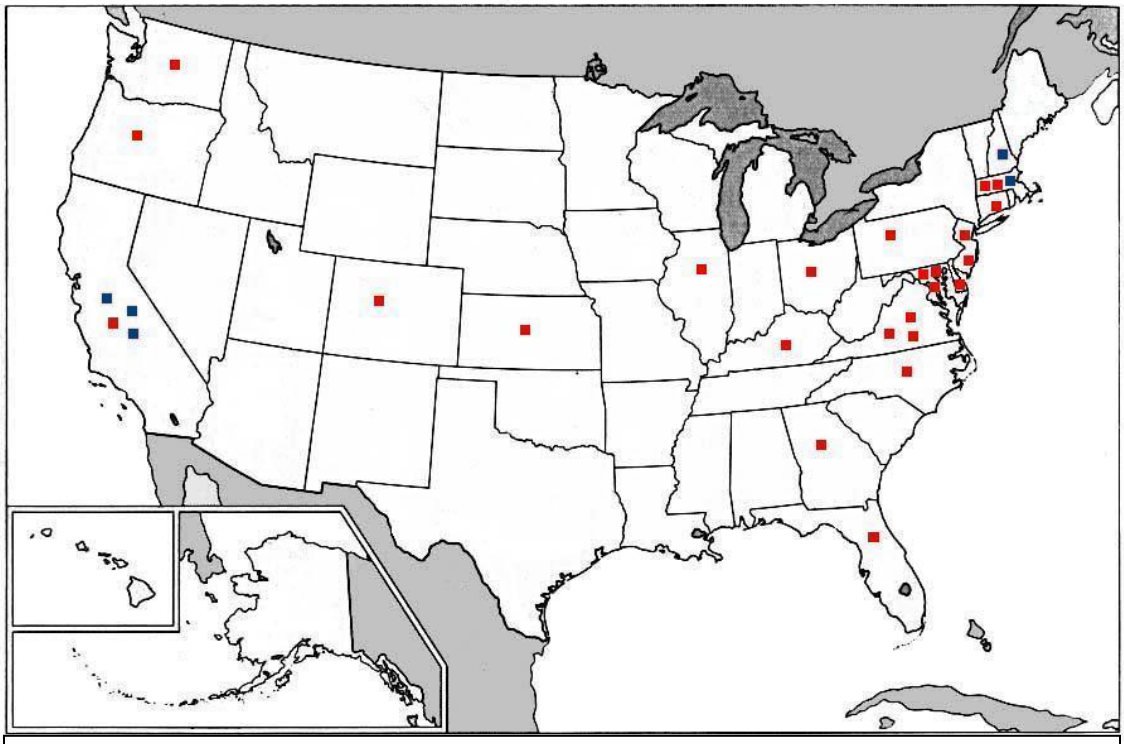

Figure 2.1: Locations of compost facilities from which compost samples were obtained. One dot represents one compost facility. Red dots represent compost facilities that are STA certified, n=24; blue dots represent compost facilities that are not STA certified, n=5.

A Chain of Custody form along with guidelines for sampling was sent to each participating compost facility. We had no direct control over the compost facilities' adherence to the sampling guidelines, however it is assumed for this experiment that the guidelines were properly adhered to.

Approximately one 5-gallon bucket of compost was obtained from each compost facility and shipped to the Beltsville Agriculture Research Center by overnight shipping. Upon delivery, the compost sample was immediately placed in 4°C until processing (generally five days to two weeks).

Three days prior to compost inoculation, each strain was streaked onto a new plate (of appropriate media and antibiotics) and incubated at 37°C for 22±4 hours. Two days prior to compost inoculation, one-half loop-full of each strain was harvested from their respective plates and deposited into 20mL of a Milorganite extract (see Box 1), which was then incubated at 37°C and shaken at 250rpm for 44±4 hours. This allowed each strain to have a final concentration of 10^{8-9} of compost-acclimated cells on the day of compost inoculation.

Box 1: Milorganite® Extract

Each strain was individually grown in 20mL of a sterile Milorganite extract to acclimate the strains to nutrient conditions of compost before inoculation into the compost samples. EPA Methods 1680 and 1682 use Milorganite as a biosolids control. Therefore, Milorganite extract was assumed to be appropriate for acclimation of the strains to general compost conditions. The Milorganite extract was made using these steps:

1. Obtained one 36 pound bag of 5-2-0 Milorganite fertilizer from local retailer
2. Added one part Milorganite fertilizer to five parts distilled water (ex. 400g Milorganite into 2000mL distilled water)
3. Stirred the solution with a stirring rod for 10 minutes
4. Passed the solution through two layers of cheese cloth into an autoclavable container
5. Autoclaved the extract for 30 minutes on a liquid cycle to sterilize

On the day of compost inoculation, the compost sample was sieved to 0.9525cm ($3/8^{th}$ inch) and then homogenized by hand in a sterile bin. Three 400g (wet weight) replicates were weighed from the sieved compost and put into Ziploc bags. Each 400g replicate was inoculated with a cocktail of all seven strains. The strains were serial diluted from the Milorganite extract to Buffered Peptone Water (BPW) to a concentration of 10^4 in the final dilution. One mL of the final dilution from each strain was combined into a 7mL cocktail and inoculated into one compost replicate at a rate of 10^{1-2} CFU/g (wet-weight) per strain. Each replicate was inoculated with one 7mL cocktail. Immediately after pouring the 7mL cocktail into the compost sample, the Ziploc bag was sealed and the inoculated compost sample was externally hand massaged while in the Ziploc bag for 5 minutes to homogenize the inocula throughout the sample.

Using the final serial dilution that was used to make the cocktail, each strain was also spiral plated onto two plates of appropriate media at a rate of 50 µL per plate in order to determine the initial inoculation concentration of each strain. All of the plates were placed at 37°C for 24±2 hours and then counted. The *Salmonella* strains were spiral plated onto two plates each of both XLD and XLT4. However, XLT4 often smeared and so most of the counts reported in this study for the *Salmonella* are based off of only the XLD plates. Regardless of strain, if a plate was unreadable, it was not counted nor replated because the counts from a replate could not be trusted to be an accurate gauge of the initial concentration after 24 hours at 4°C. Initial concentration amounts were therefore based on the counts of the remaining plate(s). All of the replicates of a given sample were assumed to have been inoculated with the same level of inocula because the

same dilution tubes were used to create each of the individual cocktails used to inoculate each replicate.

Immediately after homogenization, each compost replicate was processed using EPA Method 1680, EPA Method 1682, TMECC 07.01, TMECC 07.02, Modified Elaine Berry method, and Pathatrix. This sampling day is termed "Day 0". The compost sample replicates were then incubated at room temperature overnight, re-sampled and re-processed using EPA Method 1682 (for *Salmonella* recovery) and the Modified Elaine Berry method (for *E. coli* O157:H7 recovery) for three consecutive days ("Day I", "Day II", and "Day III"). Since each method requires more than one day to complete, and the samples were re-sampled every day for four consecutive days, the procedures overlapped throughout the week (see Figure 2.2). Only one MPN/g or CFU/g value was obtained from each sampling day.

The MPN/g or CFU/g values obtained from each method on Day 0 were used for Objective 1 calculations (% recovery of inoculated strains) as well as providing the baseline for Objective 2 (Day 0 of re-growth). Only EPA Method 1682 and the Modified Elaine Berry method were repeated on Days I, II, and III to determine the re-growth of *Salmonella spp.* and *E. coli* O157:H7 respectively. The compost samples were incubated at room temperature (22-26°C) each night.

Sampling Day	Saturday	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday	Monday	…
Day 0	Inocula		Day1	Day2	Day3	Day4	Day5	Inocula		Day1	…
Day I				Day1	Day2	Day3	Day4	Day5			…
Day II					Day1	Day2	Day3	Day4	Day5		…
Day III						Day1	Day2	Day3	Day4	Day5	…

Figure 2.2: Weekly processing procedure for each sample. "Inocula" denotes when the seven strains of inocula were inoculated into an enrichment broth of Milorganite extract to grow and acclimate to compost conditions. Compost samples were inoculated with the acclimated strains on Day1 of Day 0 (Monday). "Day1" denotes the first day of the detection methods, each of which requires more than one day to complete (EPA Method 1680 requires 4 to 5 days; EPA Method 1682 requires 5 days; TMECC 07.01 requires 5 to 6 days; TMECC 07.02 requires 5 days; Modified Elaine Berry requires 3 days; Pathatrix requires 2 days). Each compost sample was sampled on four consecutive days ("Sampling Day") after incubation at room temperature. All six methods were performed on Day 0. Only EPA Method 1682 and Modified Elaine Berry were performed on Days I, II, and III.

To reduce redundancy, work load, and variability to some degree, consolidations between the methods were done. Since both EPA methods require the same amount of initial sample, are homogenized the same way, and the initial dilutions are made the same way and in the same diluent, those steps were combined for both EPA methods. In other words, both EPA methods used the same compost sample to make the same homogenized sample and the same dilutions. The methods then went into their respective MPN tubes from these same dilutions.

In the same way, both TMECC methods require the same amount of initial sample, are homogenized the same way, and the initial dilutions are made the same way and in the same diluent. Therefore, both TMECC methods used the same compost sample to make the same homogenized sample and the same dilutions. The methods then went into their respective MPN tubes from these same dilutions. This means that instead of six different subsamples being taken from the inoculated sample replicate (one for each method) only four subsamples were taken (Figure 2.3).

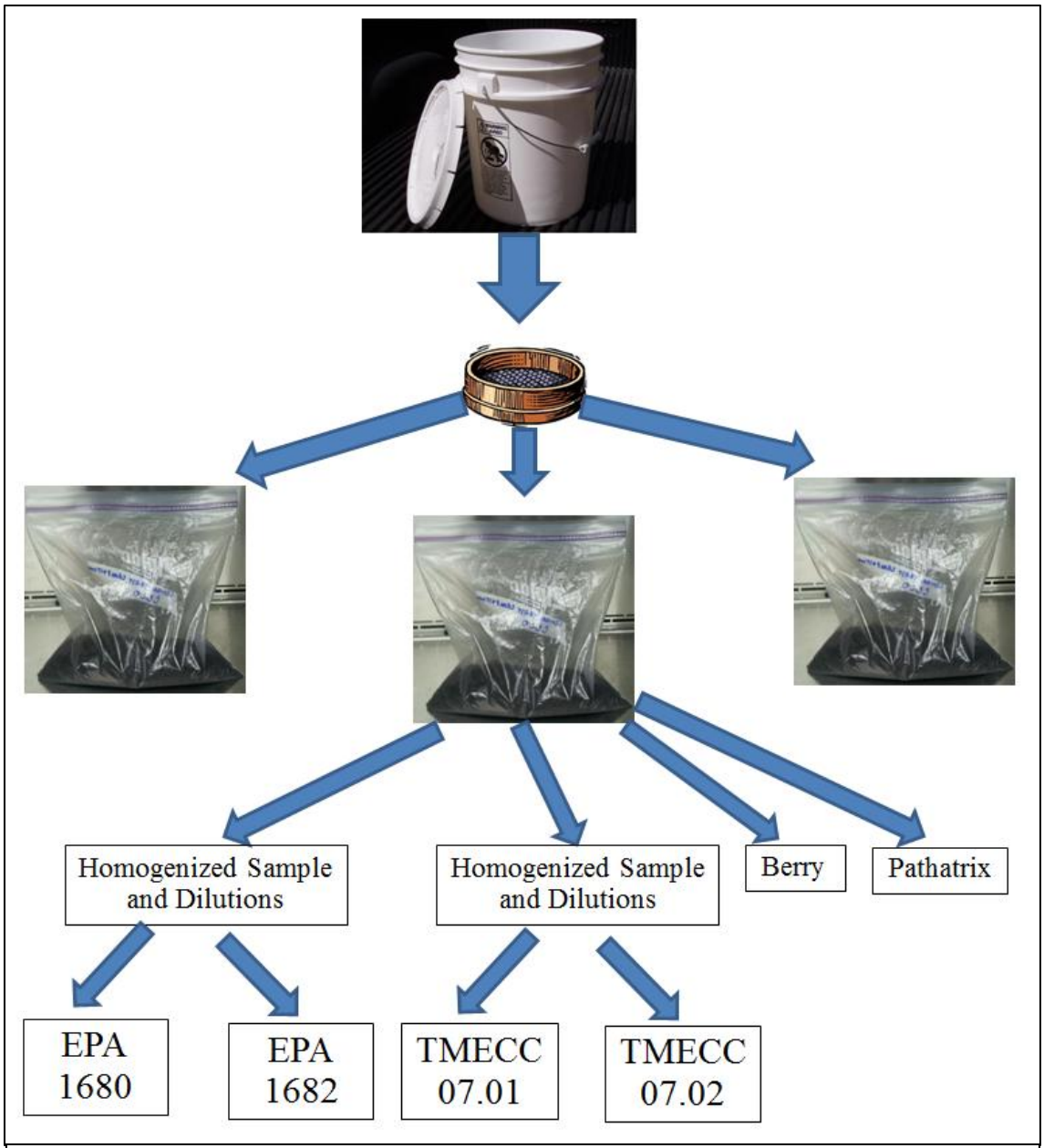

Figure 2.3: General Processing Overview. The samples were received in a 5 gallon bucket or equivalent volume. The samples were placed at 4^0C until processing. Prior to inoculation, each sample was sieved to 0.9525 cm. Three 400 g replicates were weighed from the sieved material. Each replicate was inoculated with all seven control strains. Each replicate was subsampled for each detection protocol. There were four subsamples per replicate, one each for the EPA methods (EPA Method 1680, EPA Method 1682), TMECC methods (TMECC 07.01, TMECC 07.02), Modified Elaine Berry method, and Pathatrix method.

The remaining uninoculated sieved compost sample was used to determine background characteristics: background fecal coliform populations, background *Salmonella spp.* populations, C:N, total organic carbon (TOC), % moisture, % volatile solids, pH, electrical conductivity (EC), and maturity (Solvita index).

EPA Method 1680 and EPA Method 1682 were used to test for the background fecal coliform and *Salmonella spp.* populations, respectively. Instead of using 30 g of compost into 270 mL of phosphate buffered saline (PBS) diluent as prescribed in both methods, 50 g of compost was diluted with 450 mL PBS instead to potentially capture more background populations. EPA Method 1680 had an additional MAC plating step as described in Chapter 3 to look for background *E. coli* populations. EPA Method 1682 was performed completely.

The background characteristics were measured using standard protocols. C:N was analyzed using a Elementar Vario Max CN machine (Elementargroup, Hanau, Germany). TOC was analyzed using a Phoenix 8000 Infrared Spectrometer (Teledyne Tekmar, Mason, OH). Percent moisture was analyzed by drying 10 g of compost in a conventional drying oven. Percent volatile solids was analyzed by ashing the dried compost material from the % moisture step. pH and EC were analyzed using a Spectrum MW802 pH/EC meter (Spectrum Technologies, Inc., Aurora, IL). Maturity was analyzed using Solvita CO_2 and NH_3 detection panels and the associated maturity index (Solvita, Mt. Vernon, ME).

Chapter 3
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Comparison of U.S. Environmental Protection Agency and U.S. Composting Council microbial detection methods in finished compost and regrowth potential of *Salmonella spp.* and *E. coli* O157:H7 in finished compost.

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Comparison of U.S. Environmental Protection Agency and U.S. Composting Council Microbial Detection Methods in Finished Compost and Regrowth Potential of *Salmonella spp.* and *E. coli* O157:H7 in Finished Compost

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Abstract

Bacterial pathogens may survive and regrow in finished compost due to incomplete thermal inactivation during or recontamination after composting. Both the U.S. Environmental Protection Agency's (EPA) and U.S. Composting Council's (USCC) methodologies are used to recover fecal coliforms and *Salmonella spp.* in finished composts. Twenty-nine finished composts were obtained from 19 U.S. states. EPA and USCC methods were compared for their sensitivity to recover generic *E. coli* (fecal coliforms) and *Salmonella spp*. Regrowth of *Salmonella* spp. and *E. coli* O157:H7 in finished composts was also evaluated. EPA methods recovered significantly ($p=0.0001$) more generic *E. coli* and statistically equal ($p=0.27$) amounts of *Salmonella* inocula compared to USCC methods. Physicochemical parameters (C:N, % moisture, total organic carbon) were unable to serve as predictors of regrowth *Salmonella spp.* and *E. coli* O157:H7 in finished composts, although the combination of C:N ratio, total organic carbon, and moisture content influenced pathogen regrowth potential.

Keywords: compost, methods, testing, pathogen, regrowth

1. Introduction

Approximately one in six people in the United States experience some form of foodborne illness, resulting in an estimated 48 million cases of foodborne illness each year (Scallan et al., 2011). The source of these illnesses vary greatly including undercooked or improperly stored food, unsanitary practices by food handlers, and contaminated produce. The Centers for Disease Control and Prevention (CDC) estimated that 46% of cases of foodborne illness in the United States resulted from the consumption of contaminated produce from 1998-2008 (Painter et al., 2013). Contamination of produce on the farm is possible through contaminated water, wildlife intrusitions, use of contaminated fertilizers such as animal manure, and potentially unfinished or recontaminated compost (Zaleski et al., 2005; Buck et al., 2003; Doyle et al., 2006).

Compost is organic material that has been degraded into a nutrient stabilized humus-like substance. During composting, microbial activity from degrading the feedstocks can generate sufficiently high (thermophilic) temperatures ($\geq 55^{o}$C) to kill enteric bacterial pathogens originally present in the feedstocks, assuming proper carbon:nitrogen ratios (C:N), moisture, and aeration levels are maintained. Enteric bacterial pathogens die when exposed to thermophilic temperatures for extended periods of time. To ensure sufficient pathogen inactivation through achieving thermophilic temperatures within a compost pile, the EPA and United States Department of Agriculture (USDA) National Organic Program (NOP) recommend maintaining the pile at >55°C for at least three consecutive days for static and aerated piles. For windrow composting, temperatures must be maintained at >55°C for 15 days and be turned at least 5 times within that time period. However, low levels of bacterial pathogens can survive

the composting process, often due to sections of the compost pile not getting up to the required temperature (Wichuk and McCartney, 2007). Pockets within the pile or 'toes' on the edges of the compost pile not reaching thermophilic temperatures are issues for all composting methods, especially when composting in cold ambient temperatures. For example, certain methods for composting do not "turn", or mix, the compost pile, increasing the likelihood of low temperature pockets within the compost pile that do not reach a thermophilic temperature, potentially allowing pathogens to survive for the duration of the composting process. Several studies have found human pathogens in finished compost which has supposedly achieved > 55^{o}C temperature requirement (Ingram, 2009; Brinton et al., 2009; Shepherd et al., 2010; Wichuk and McCartney, 2007). In addition to microbial hardiness, unsanitary conditions at composting facilities can contribute to the survival of human pathogens through the composting process.

It should be noted that the detection of human pathogens in finished compost (compost that has previously reached thermophilic temperatures) is rare but has been documented. In an evaluation of 15 different composting facilities from across the U.S., Ingram (2009) only found 1 sample out 105 (< 1%) positive for shiga-toxigenic *E. coli* (STEC) and only 6 samples out 105 (< 6%) that contained *Salmonella spp.* All samples that were positive for *Salmonella spp.* as well as the STEC isolate were isolated from facilities that composted biosolids. Ingram noted that the some of the *Salmonella* serotypes found in the compost were the same serotypes which were associated with produce outbreaks that same year. In an evaluation of 94 non-sludge composting facilities, Brinton et al. (2009) found only one finished compost sample containing *Salmonella spp.* (1%) and three that were contaminated with *E. coli* O157:H7 (3%).

Furthermore, *E. coli* O157:H7 was detected at one of the sites three months after initial pathogen testing, indicating the environmental persistence of the pathogen or the lack of adherence to good composting practices within the facility. These authors found that compost produced at large composting facilities which utilized static (unturned) piles were more likely to contain pathogen contamination than smaller facilities or those which utilized windrow composting (Brinton et al., 2009). Persistence of contamination from composting facilities can be attributed to several factors: contaminated feedstocks, composting at sub-thermophilic temperatures, recontamination of finished piles by wildlife, or cross-contamination within the composting facility.

Even if compost piles are properly maintained and achieve pathogen inactivation, the finished compost can subsequently become recontaminated. Wild birds, have been shown to carry *Salmonella spp.* and *E. coli* O157:H7 (Gorski et al., 2011; Mandrell, 2009; Stephens et al., 2007; Teplitski et al., 2012). Proper vector reduction measures, such as compost coverings and turning, can help to reduce the likelihood of transferring enteric pathogens from wildlife to composts. Cross-contamination within the composting facility can potentially occur from machinery used on multiple compost piles, run-off water from contaminated piles, and the practice of blending composts.

Salmonella spp. and *E. coli* have shown the ability to regrow in finished compost (Zaleski et al., 2005, Wichuk and McCartney, 2007), making survival and recontamination of compost with pathogens an issue of concern. Finished compost that has few indigenous microorganisms is particularly susceptible to regrowth of *Salmonella spp.* and *E. coli* O157:H7 (Zaleski et al., 2005; Millner et al., 1987; Sidhu et al., 2001). Composts may have low levels of indigenous microorganism due to overheating

(excessive high temperatures for an extended periods of time) which subsequently kills many of the microorganisms present in the compost, or when compost has been purposefully sterilized (Zaleski et al., 2005; Sidhu et al., 2001; Kim et al., 2011). Regrowth of *Salmonella spp.* populations in composts with high levels of microorganisms (as would generally be the case if proper composting conditions and temperatures were met) is much less likely and indeed often shows an increase followed by a rapid die-off, suggesting that the competing microorganisms in compost may prevent extended regrowth of human pathogens (Zaleski et al., 2005; Russ and Yanko, 1981). However, the potential for pathogen regrowth indicates that compost that is spread as a fertilizer is still a potential vector of pathogens onto produce crops which are often consumed raw or with minimal processing. Identifying non-microbiological indicators (physicochemical factors) which could predict the regrowth of human pathogens in compost, such as *Salmonella spp.* and *E. coli* O157:H7, would aid composting operations in choosing feedstocks, composting methods, as well as aid produce growers in determining the suitability of using compost as fertilizer.

The U.S. Environmental Protection Agency (EPA) created microbiological standards through the Part 503 rule for biosolids (human waste), which includes compost made from biosolids feedstocks (U. S. EPA, 1993). EPA methods 1680 and 1682 were designed specifically to test biosolids for fecal coliforms and *Salmonella*, respectively. All other feedstocks (animal manure, yard waste, food waste, etc.) are regulated at the state level and not subject to EPA microbiological standards. Microbiological standards pertaining to compost vary from state to state. In order to help provide standards for the composting industry, the U.S. Composting Council (USCC) established the Seal of

Testing Assurance (STA) program. Under this voluntary program, composts made from non-biosolid feedstocks are held to the same Part 503 microbiological standards for biosolids and all facilities in the program must make their compost testing results publicly available. The USCC employs microbiological methods, termed Testing Methods for the Examination of Composting and Compost (TMECC), which uses the same microbiological thresholds and standards for fecal coliforms and *Salmonella* spp. as the EPA Part 503 rule. EPA microbiological standards to indicate the presence of pathogens for compost are < 1000 MPN / g for fecal coliforms or < 3 MPN/4 g for *Salmonella* spp. As of summer 2013, approximately 200 composting facilities across the U.S.A. are in the STA program and thus are tested using the TMECC protocols. Many states base their compost microbiological standards on the EPA Part 503 rule but do not use STA while other states require all of their composting facilities to be in the STA program. This has led to both EPA and TMECC methods being used to test finished compost across the U.S.

The EPA methodologies to test for fecal coliforms and *Salmonella* spp. were originally designed for testing for biosolids, whereas the TMECC protocols were designed specifically for compost made from all feedstocks. Despite both EPA and TMECC testing methodologies currently being used by the composting industry, no direct comparison between these two testing methods has been performed to determine which is more sensitive in recovery of the target organism.

The first objective of our study was to compare the recovery of generic *E. coli* (as a surrogate for fecal coliforms) and *Salmonella spp.* from multiple commercial, point-of-sale, finished composts made from either biosolids, manure, or yardwaste feedstocks,

using both EPA and TMECC methods. Methods were additionally evaluated to determine which method was capable of equivalent recovery of target organisms from different compost types (biosolids, manure and yardwaste). The comparisons that were made were: a) EPA Method 1680 vs. TMECC 07.01 for detection of inoculated generic (non-pathogenic) *E. coli*; b) EPA Method 1682 vs. TMECC 07.02 for detection of inoculated *Salmonella spp.*

The second objective of our research study was to evaluate if different physicochemical compost characteristics (% moisture, C:N, volatile solids, Total Organic Carbon (TOC), pH, electrical conductivity (EC), and maturity as measured by Solvita CO_2 and NH_3 tests) were able to predict the re-growth potential of pathogenic *Salmonella spp.* and pathogenic *E. coli* O157:H7 in compost.

2. Methods

2.1 Composts used

Finished, commercial point of sale composts from 29 composting facilities from 19 different states were obtained for this experiment. Twenty-four of the 29 compost samples obtained were STA-certified. When the composting facility was close enough geographically, samples were obtained at the composting site and brought back to the Environmental Microbial and Food Safety Laboratory (EMFSL) in Beltsville, MD for analyses. Otherwise a composite sample (from several different locations in the compost pile) was obtained according to TMECC or EPA sampling protocols by a commercial composting facility, and was shipped overnight at 4^{o}C to EMFSL. All samples were placed at 4^{0}C upon arrival and held there until processed. Each sample was approximately one 5-gallon bucket in volume.

Each compost sample was categorized based on the feedstock used into one of three broad categories: biosolids (n=10), manure (n=4), or yardwaste (n=15). Regardless of the proportion of biosolids used in the feedstock, all composts that had any biosolids content were classified as biosolids; a similar standard was used for compost classified as having a manure feedstock. Those composts placed in the yardwaste category contained no biosolids or manure. Feedstocks containing crop residue were also considered as yardwaste.

2.2 Bacterial strains used and culture conditions

Three non-pathogenic *E. coli* (TVS 353, TVS 354, TVS 355), resistant to 80 mg / L Rifampicin, were obtained from Dr. Trevor Suslow at the University of California-Davis. These *E. coli* were originally isolated from various produce-growing environments (Tomas-Callejas et al., 2011). *Salmonella* Newport, originally isolated from creek sediment from the Virginia eastern shore, and *Salmonella* Saintpaul, originally isolated from jalapeno peppers in Mexico, were obtained from the US Food and Drug Administration Center for Food Safety and Applied Nutrition (CFSAN). Both *Salmonella* isolates were made resistant to 50 μg / ml of Nalidixic acid through spontaneous mutation and laboratory culture methods. *E. coli* O157:H7 RM 4407 was isolated from a 2006 spinach outbreak; *E. coli* O157:H7 RM 5279 was originally isolated from a bagged vegetable outbreak. *E. coli* O157:H7 strains were obtained from Dr. Robert Mandrell (USDA-ARS Albany, CA).

Three days prior to compost inoculation, each strain of non-pathogenic *E. coli*, *Salmonela spp.*, and *E. coli* O157:H7 was isolated on MACR (MacConkey agar (Neogen, Lansing, MI) with 80 μg / mL Rifampicin (Sigma-Aldrich, St. Louis, MO)), XLDN

(Xylose Lysine Desoxycholate (Neogen) agar with 50 μg / mL Nalidixic acid (Sigma-Aldrich) or CHROMN (CHROMagar O157 (DRG, Springfield, NJ) with 50 μg / mL Nalidixic acid), respectively, and incubated at 37°C for 22±4 hours. Two days prior to compost inoculation, one-half loop-full (5 μL) of a colony of each bacterial isolate was taken from the selective medium and deposited into 20mL of a Milorganite (Milorganite, Milwaukee, WI) extract (see section 2.3), which was then incubated at 37°C and shaken at 250 rpm for 44±4 hours. EPA Methods 1680 and 1682 use Milorganite as a biosolids control. Therefore, Milorganite extract was assumed to be appropriate for the acclimation of the strains to general compost nutritional conditions. After incubation in the Milorganite extract, each strain achieved a final populations of 10^{8-9} CFU / mL.

Individual cultures were then diluted to ca. 10^4 CFU / mL in buffered peptone water (BPW) (Neogen). Diluted cultures of each strains were then combined to form a 7 mL multi-strain inoculum (1 mL per strain) to be added to compost samples. Cultures of all seven individual bacterial strains which were diluted in BPW were spiral plated (Don Whitley, Microbiology International, Frederick, MD) onto two plates of appropriate selective media (50 μL, in duplicate), incubated at 37°C for 24±2 hours in order to determine the initial population of each strain.

2.3 Preparation of the Milorganite Extract

Each bacterial isolate was individually grown in 20 mL of Milorganite extract before inoculation into compost samples. Milorganite was diluted 1:5 in distilled water and stirred for 10 minutes, filtered through a double layer of cheesecloth, and then sterilized by autoclave. The sterilized extract was then stored at room temperature until used. The extract was stirred again before use due to settling of particles.

2.4 Inoculation of compost

On the day of compost inoculation, the finished point-of-sale commercial compost sample was sieved to 9.51 mm (The W. S. Tyler Company, Cleveland, OH) and then manually homogenized in a sterile bin. Three 400g (wet weight) replicates of each compost sample were weighed from the sieved compost and put into Ziploc (SC Johnson & Sons, Racine, WI) bags. Each 400g replicate was inoculated with 7 mL of the multi-strain inoculum of *E. coli*, *Salmonella spp.*, and *E. coli* O157:H7, yielding an approximate population of 10^{1-2} CFU / g (wet weight) per organism. Immediately after adding 7mL of the bacterial inoculum into the compost sample, the Ziploc bag was sealed and the inoculated compost sample was hand- massaged for 5 minutes to evenly distribute the inocula throughout the sample.

After homogenization, each compost replicate was processed according to the protocols listed below. All of these methods, with the exception of the one used to recover *E. coli* O157:H7, were previously published, and were used in our experiment with minor changes (see Appendices 3.A.1 through 3.A.4 for the methods as performed in this experiment). The initial inocula population of each group of bacteria (generic *E. coli*, pathogenic *Salmonella spp*., and pathogenic *E. coli* O157:H7) was determined by combining the CFU / g populations of each group of isolates: non-pathogenic *E. coli*, *Salmonella* spp., and *E. coli* O157:H7.

2.5 Microbiological recovery methods used

The following microbiological recovery methods were used for this work. The details of the procedures are presented in the listed figures.

EPA Method 1680 "Fecal Coliforms in Sewage Sludge (Biosolids) by Multiple-Tube Fermentation using Lauryl Tryptose Broth (LTB) and EC Medium" *see Appendix 3.A.1*

EPA Method 1682 "*Salmonella* in Sewage Sludge (Biosolids) by Modified Semisolid Rappaport-Vassiliadis (MSRV) Medium" *see Appendix 3.A.2*

TMECC 07.01 (A, B, and C) "Coliform Bacteria. Three Methods" *see Appendix 3.A.3*

TMECC 07.02 (B and C) "Salmonella. Three Methods" *see Appendix 3.A.4*

2.5.1 *E. coli* O157:H7 recovery method.

The following protocol was used to determine regrowth of *E. coli* O157:H7 populations.Ten grams of inoculated compost was combined with 90 mL Tryptic Soy Broth (TSB) in a filter WhirlPak (Nasco, Ft. Atkinson, WI) bag and homogenized for at least 30 seconds. Homogenates (200 μL, in duplicate) were spiral-plated onto CHROMagar O157 (CHROMagar, Paris, France). The plates were incubated at 37°C for 24±2 hours before populations were determined and expressed in CFU / g (dry weight) for analyses. Regrowth of *E. coli* O157:H7 in finished composts were assessed in this manner on days 0, 1, 2, and 3 after inoculation. Inoculated compost was stored at 25°C between sampling days.

2.6 Variations in EPA Method 1680

EPA Method 1680 is specific to recover all fecal coliforms, while TMECC 07.01 is specific to recover *E. coli*. To directly compare these recovery methods, it was necessary extend the procedure for the EPA 1680 methods beyond the final EC broth inoculation used to determine the Most Probable Number (MPN) of fecal coliforms (see Appendix A.1). Ten microliters of culture from EC tubes which were positive for fecal coliforms were isolated on MACR to recover the Rifampicin-resistant generic *E. coli* inoculated into the compost. This also excluded any background *E. coli* that might have been already present in the compost. Calculations for MPN / g and all subsequent analyses for EPA Method 1680 were then based on the recovery of Rifampicin-resistant *E. coli*.

2.7 Evaluation of physicochemical parameters of finished point-of-sale compost

Uninoculated compost from each of the 29 samples were analyzed for the following characteristics by using standard laboratory methods listed, followed by the equipment used: Carbon:Nitrogen ratio (C:N), Elementar Vario Max CN (Elementargroup, Hanau, Germany); Total Organic Carbon (TOC), Phoenix 8000 Infrared Spectrometer (Teledyne Tekmar, Mason, OH); % moisture, Drying oven; % volatile solids, ashing oven; pH and Electrical Conductivity (EC), Spectrum MW802 (Spectrum Technologies, Inc., Aurora, IL); Maturity, Solvita CO2 and NH3 tests and maturity index (Solvita, Mt. Vernon, ME).

2.8 Background fecal coliform and *Salmonella spp.* testing

Before inoculation of composts occurred, uninoculated compost from each of the 29 compost samples were analyzed for background populations of fecal coliforms and *Salmonella spp.* using EPA Method 1680 and EPA Method 1682, respectively.

2.9 Microbial recovery and statistical analyses

The recovery percentages of the respective inocula recovered by the EPA and TMECC methods were determined using the following formula.

$$\text{Recovery \%} = (\text{MPN / g / CFU / g}) \times 100\%$$

Where the MPN / g is the recovery of the respective pathogen by either EPA or TMECC methods (calculated using MPN Calculator Build 23 VB6 version, http://i2workout.com/mcuriale/mpn/index.html), and CFU / g is the initial population of the respective pathogen in compost. Both populations (MPN / g and CFU / g) were calculated using the dry weight (g) of compost. Each compost recovery method was carried out three times on each compost sample.

The calculated recovery percentage values for non-pathogenic *E. coli* and for *Salmonella spp.* were divided by the largest recovery percentage from a single inoculated compost replicate so that the range of values used in the statistical analyses were rescaled to the (0, 1) proportion range as a Beta distribution. Statistical analysis was performed using Statistical Analysis Software (SAS, Cary, NC). Generalized linear mixed effects models (using the PROC GLIMMIX function), using Beta distributions and the logit link functions, were fit to the (0, 1) proportion recovery data. A two-way ANOVA model using compost type and microbiological method was fit to the data for each pathogen separately, specifying a compound symmetric covariance structure to model correlation

between methods. Type refers to the feedstock (biosolids, manure, and yardwaste) and method refers to the recovery method (EPA or TMECC) evaluated. Using 66.67% confidence intervals, differences in proportional recovery percentages by EPA or TMECC methods were determined. This confidence interval was adequate to determine significant differences because, in essence, Beta distributed data requires two standard deviations between the means being compared to show significant differences.

2.10 Regrowth potential of *Salmonella spp.* and *E. coli* O157:H7 in compost

To determine the regrowth potential of *Salmonella spp.* and *E. coli* O157:H7 (Objective 2), three replicates of inoculated compost was held at room temperature (25°C) for three consecutive days after inoculation. On days 0, 1, 2, and 3 populations of *Salmonella spp.* and *E. coli* O157:H7 were determined by EPA Method 1682 and the *E. coli* O157:H7 plating method, respectively. Recovered *Salmonella* spp. and *E. coli* O157:H7 populations from each replicate compost sample were averaged for each day and growth curves were constructed. To standardize the growth curves to represent the change in population over the 3-day period, the average recovery on Day 0 was subtracted from the average recoveries on days 1, 2, and 3.

Regrowth patterns of *Salmonella* and *E. coli* O157:H7 populations in various compost were organized into clusters for statistical analysis based on similarities in the changes in populations during regrowth experiments. Each compost physicochemical characteristic was compared to the clusters of growth curves to determine if characteristics could accurately predict regrowth of *Salmonella spp.* or *E. coli* O157:H7 populations in finished compost.

The *Salmonella spp.* and *E. coli* O157:H7 regrowth data were also combined and analyzed together using a boosted regression analysis of covariance (ANCOVA) model. Only the population changes from Day 0 to Day 1 were considered in the boosted regression model. The regressors in the model were: C:N, TOC, % moisture, CO_2, Solvita maturity index values, pH, EC, compost type, and pathogen (*Salmonella spp.* or *E. coli* O157:H7). To analyze these data, the generalized boosted models (gbm) package in the statistical package *R* (GNU Operating System, Free Software Foundation) was used. J.H. Friedman's gradient boosting method was used to fit a boosted regression model to the data. The model-fitting process was performed a total of 30 times to check the stability of the model. The models were then compared to determine which physicochemical factors influenced regrowth of *E. coli* O157:H7and *Salmonella spp*. populations.

Due to the highly variable nature of compost combined with the very broad categories used (Biosolids, Manure, Yardwaste), α=0.10 was used for all reports of significance for both the comparison of microbiological detection methods and regrowth experiments. For all statistical analyses, it should be noted that one compost sample was replicated six times rather than three. Thus, 29 compost samples and 90 replicates were analyzed. Regrowth of *E. coli* O157:H7 populations were only observed in 27 compost samples, resulting in 84 replicate samples being analyzed.

3. Results and Discussion

3.1 Background fecal coliform and *Salmonella spp.* testing

Using the methods described in Section 2.8, no compost sample had background fecal coliforms or *Salmonella* populations over Part 503 limits. Therefore, no additional microbial background testing was done.

3.2 Objective 1: Fecal Coliform Recovery Methods (EPA Method 1680, TMECC 07.01)

Overall, EPA Method 1680 for fecal coliforms recovered significantly more generic *E. coli* inocula than TMECC 07.01 (p=0.0003) (Table 3.1). There was a significant interaction between the method used (EPA or TMECC) and the compost type (biosolids, manure or yardwaste) (p=0.0001). On average, EPA Method 1680 recovered 68.7% of the inoculated generic *E. coli* while TMECC 07.01 recovered 48.1% from inoculated finished composts (Table 3.1). EPA Method 1680 recovered significantly more *E. coli* than TMECC 07.01 from both biosolids and manure composts, but not from yardwaste composts.

EPA Method 1680 recovered a significantly (p=0.06) greater percentage of inoculated generic *E. coli* from biosolids composts than from yardwaste composts. However, TMECC 07.01 had the opposite trend, recovering a significantly greater percentage of inocula from yardwaste composts than from either biosolids (p=0.02) or manure composts (p=0.096) (Table 3.1).

Table 3.1: Average percent recovery of inoculated generic *E. coli* by EPA and TMECC pathogen detection methods in different compost types. The p-values of the methods comparison (EPA 1680 vs TMECC 07.01) are shown in the "EPA vs. TMECC" column. Within each column (recovery method), average recovery values followed by the same capital letter indicate that recovery values are not significantly ($p < 0.10$) different based on compost type. Within each row (compost type), recovery values followed by the same lowercase letter indicate there are no significant differences based on the method of recovery ($p < 0.10$).

Compost Type	EPA 1680	TMECC 07.01	EPA vs. TMECC
Biosolids, n=10	87.7% Aa	29.4% Ab	p<0.0001
Manure, n=4	71.8% ABa	31.1% Ab	p=0.03
Yard, n=15	56.0% Ba	64.1% Ba	p=0.44
All Types, n=29	68.7%a	48.1%b	—

3.3 Objective 1: *Salmonella spp.* recovery methods (EPA Method 1682, TMECC 07.02)

Overall, EPA Method 1682 recovered 89.1% and TMECC 07.02 methods recovered 72.4% of inoculated *Salmonella* spp. from finished composts (Table 3.2). These recovery percentages were statistically equivalent (p=0.54) (Table 3.2). There was not a significant interaction between the method used (EPA or TMECC) and the compost type (p=0.27). Despite this, interesting trends in the simple effects are presented for discussion. EPA Method 1682 recovered a significantly greater percentage of *Salmonella* from biosolids compost (85.1%) compared to TMECC 07.02 methods (39.5%). However, recovery of *Salmonella* spp. by EPA and TMECC methods from manure and yardwaste composts did not significantly differ. EPA Method 1682 showed no significant statistical differences in recovery percentages between compost types. TMECC 07.02 recovered significantly more *Salmonella* from yardwaste (97.3%) composts than from biosolids (39.5%) composts (p=0.09).

Table 3.2: Average percent recovery of inoculated *Salmonella spp.* by EPA and TMECC pathogen detection methods in different compost types. The p-values of the methods comparison (EPA vs TMECC 07.02) are shown in the "EPA vs. TMECC" column. Within each column (recovery method), average recovery values followed by the same capital letter indicate that recovery values are not significantly ($p < 0.10$) different based on compost type. Within each row (compost type), recovery values followed by the same lowercase letter indicate there are no significant differences based on the method of recovery ($p < 0.10$).

Compost Type	EPA 1682	TMECC 07.02	EPA vs. TMECC
Biosolids, n=10	85.1% Aa	39.5% Ab	p=0.07
Manure, n=4	39.9% Aa	54.6% ABa	p=0.66
Yard, n=15	103.8% Aa	97.3% Ba	p=0.78
All Types, n=29	89.1%a	72.4%a	—

3.4 Regrowth of *Salmonella spp.* and *E. coli* O157:H7 in finished composts

In most compost samples, *Salmonella spp.* populations showed initial growth on Day 1 and then were static or decreased on Days 2 and/or 3 (Figure 3.1). In general, the *Salmonella spp.* populations in the compost on Day 3 were either greater than or roughly equal to the recovery on Day 0 (Figure 3.1). Twelve of the 29 (41%) samples were determined to have *Salmonella spp.* populations on Day 3 that were approximately equal to their corresponding recovery on Day 0 (± 1 log MPN / g dry weight). Six of the 29 (21%) samples showed an increase of 2 log MPN/g in average population on Day 3 compared to Day 0 recoveries; eight samples (28%) showed an increase of 3 log MPN / g dry weight over three days. Only three of the 29 (10%) samples showed a > 2 log MPN / g decrease in *Salmonella spp.* populations from Day 0 to Day 3. No association between compost type and levels of regrowth could be ascertained.

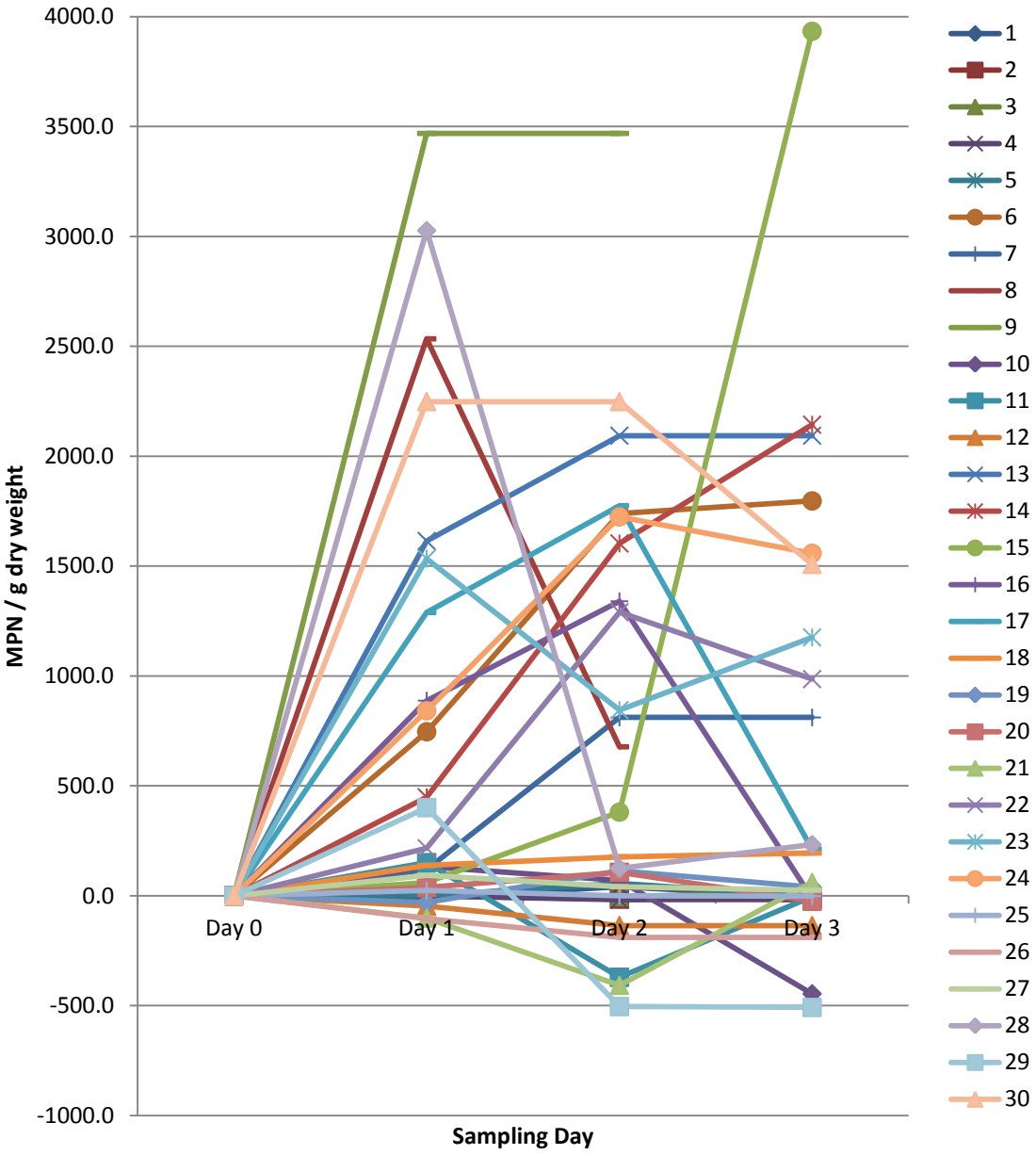

Figure 3.1: Changes in populations of *Salmonella spp*. (MPN / g dry weight), relative to day 0, when reinoculated into commercial compost sample (n=29) stored at 25^{o}C over three days. *Salmonella* populations were determined using EPA 1682. The key refers to each compost sample listed numerically in the order in which it was processed. Each data point represents the average of three replicates. Note that samples 12 and 26 are the same compost sample that was processed twice.

E. coli O157:H7 populations were static in six of the 27 (22%) compost samples (Figure 3.2). *E. coli* O157:H7 populations increased by 2 log CFU / g in six of 27 (22%) compost samples on Day 3 as compared to Day 0; populations increased by 3 log CFU / g in 6 of 27 (40%). Conversely, five out of the 27 (19%) compost samples had decreases of 2 log CFU / g *E. coli* O157:H7 populations on Day 3 as compared to Day 0, while 2 of 27 (7%) of compost samples showed E. coli O157:H7 populations which decreased by at least 3 log CFU / g dry weight.

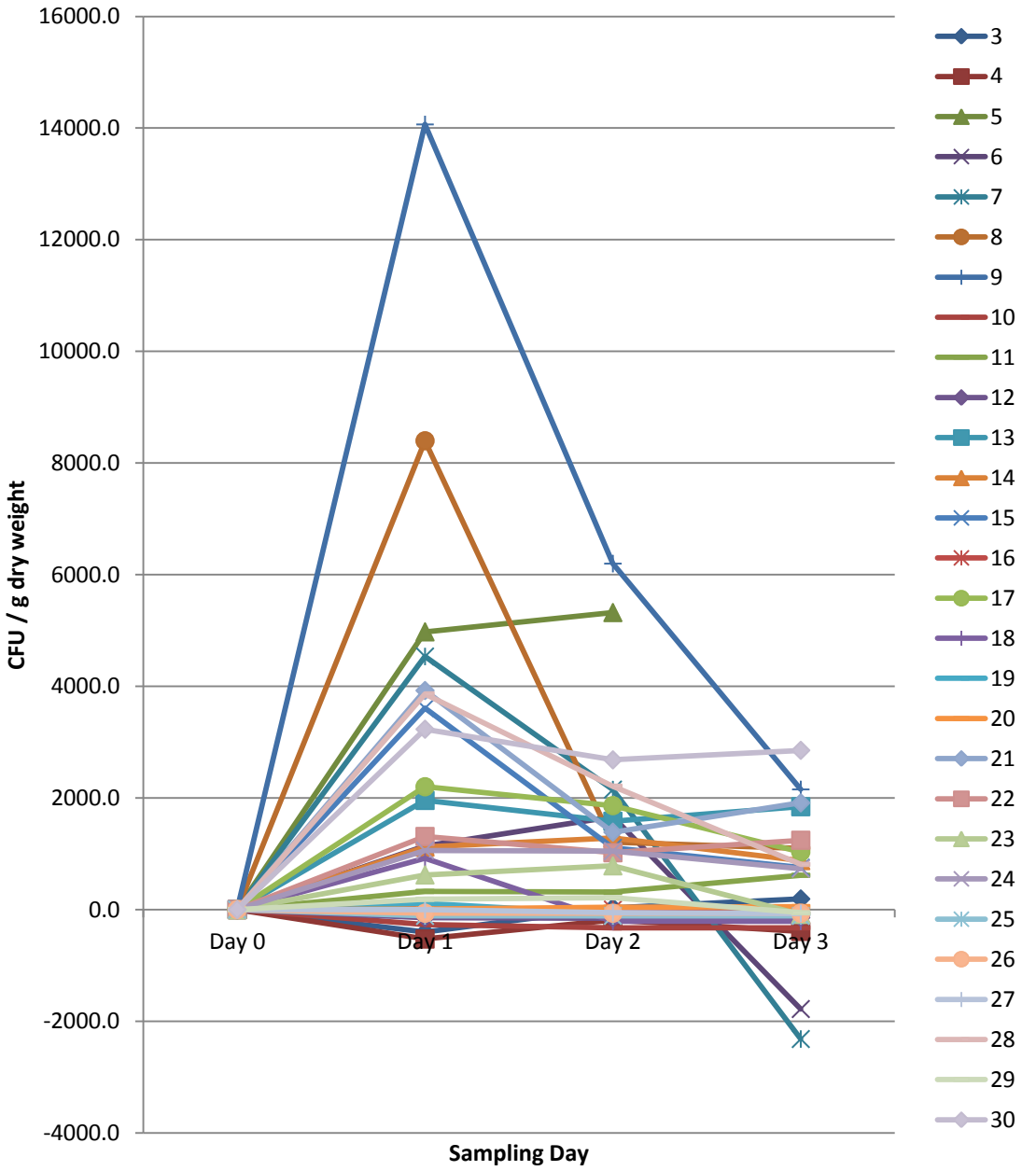

Figure 3.2: Changes in populations of *E. coli* O157:H7 (CFU / g), relative to day 0, when reinoculated into commercial compost sample (n=27) stored at 25°C over three days. *E. coli* O157:H7 populations were determined by a direct plating method. The key refers to each compost sample listed numerically in the order in which it was processed. Each data point represents the average of three replicates. Note that samples 12 and 26 are the same compost sample that was processed twice. Also, note that compost samples 1 and 2 were not analyzed for *E. coli* O157:H7 regrowth.

Growth curves of *Salmonella* spp. and *E. coli* O157:H7 were clustered based on regrowth patterns. Cluster analysis was used to correlate physicochemical characteristics of the compost to regrowth patterns. However, no single compost characteristic could accurately predict the clusters despite the use of multiple clustering techniques.

A boosted regression analysis of covariance (ANCOVA) model was then performed combining both the *Salmonella spp.* and the *E. coli* O157:H7 regrowth data using the physicochemical characteristics as regressors in the model. The analysis was repeated 30 times using only the differences in populations from Day 1 and Day 0 for each compost sample. Once again, no single characteristic was able to predict the potential for regrowth of either pathogen in finished compost. However, this model identified three physicochemical characteristics which contributed the most to the potential regrowth of these pathogens in compost: C:N, TOC, and % moisture.

3.5 Discussion

Three of four methods evaluated – EPA Method 1680 (fecal coliforms), TMECC 07.01 (*E. coli*), and TMECC 07.02 (*Salmonella* spp.) – had significantly different recovery percentages of the target pathogen from yardwaste composts as compared to biosolids composts. EPA Method 1680 recovered significantly less *E. coli* from yardwaste composts compared to biosolids, while both TMECC methods recovered significantly higher percentages from yardwaste composts compared to biosolids composts. All four methods (EPA Method 1680, TMECC 07.01, EPA Method 1682, TMECC 07.02) had statistically equivalent recoveries from manure composts as compared to biosolids composts, and three of four methods (both EPA methods and TMECC 07.02) had statistically equivalent recoveries from manure and yardwaste

composts. Both EPA Methods recovered significantly more inocula of the target pathogen than their TMECC counterpart from biosolids composts. The two EPA methods were designed to be used with biosolids, so the recovery methodologies may be more suited to recover pathogens from composts with biosolids and manure as feedstocks.

One possible explanation for the differences in recoveries between compost types is that some composts did not facilitate homogenization of the inoculum. Clumping of the inocula may have hindered the even dispersion of the inocula through the compost replicate and may be a partial explanation for the observed variability in recovery percentages of target pathogens among replicates of the same sample and perhaps between compost types.

The larger amount of compost initially used and the larger transfer and serial dilutions volumes used were responsible for the significantly higher recovery percentage of *E. coli* by EPA Method 1680 compared to TMECC 07.01. For the recovery of fecal coliforms, EPA Method 1680 and TMECC 07.01 are similar in their techniques, media and solutions used (see Appendices A.1 and A.3 for a detailed flowchart of each method). Both methods follow the same progression of steps as well as use identical media, along with the same scale of dilutions (1:10 dilution of compost to diluent). However, there are differences in the initial amount of compost used and in the amount of volume used in serial dilutions in the two methods. EPA Method 1680 uses more compost in its initial homogenate (30g compost) in 270 mL phosphate buffered saline (PBS) and larger volumes in its serial dilutions (10 mL homogenate into 90 mL PBS) compared to TMECC 07.01. TMECC 07.01 uses 20 g of compost in 180 mL buffered peptone water (BPW) to make the initial homogenate, and then adds 1 mL of the homogenate into 9 mL

Lauryl Tryptose Broth. In both the EPA and TMECC methods, the effective concentration of compost in the initial homogenate is 0.1 g / mL. However, in EPA Method 1680, a total of one gram of compost (10 ml of 0.1 g compost)/ mL) is transferred in the serial dilutions of homogenate to 2X LTB broth. In the TMECC 7.01 method, 0.1 g (1 mL of 0.1 g compost/mL) is transferred to make serial dilutions. The larger amounts of compost being transferred using the EPA methods results in higher *E. coli* populations being recovered from the compost, especially when compost may have very low levels of *E. coli* or other fecal coliforms present. Overall EPA Method 1680 enriched and tested a total of 5.555 g of compost while TMECC 07.01 only tested 0.333 g of compost total. The larger amounts of compost being transferred for each serial dilution for EPA Method 1680 results in higher *E. coli* populations being recovered from the compost compared to the TMECC 07.01. These procedural differences between the two methods (compost amount, volume used in serial dilutions, and MPN scheme size) are the most likely explanations for the differences in recoveries of the generic *E. coli* inocula between EPA Method 1680 and TMECC 07.01.

Both EPA methods (1680 and 1682) also have more a more expansive MPN-testing scheme when compared to TMECC methods. EPA Method 1680 used a four sequential 1:10 dilution (4-dilution) by 5-tube (replicate) scheme, while EPA Method 1682 has a series of three 1:10 (3-dilution) by 5-tube (replicate) MPN scheme. Both TMECC methods use a series of three 1:10 dilutions by 3-tube (replicate) MPN scheme. The additional tube per dilution in the EPA MPN schemes, and the additional dilution for EPA Method 1680, further increase the sensitivity of the microbiological test for the target organism as well as increasing the accuracy of the test.

There was no statistical difference between the average overall recovery percentages between the EPA Method 1682 and TMECC 07.02 *Salmonella spp.* recovery methods, even though EPA Method 1682 uses larger amounts of compost and increased transfer and serial dilution volumes compared to TMECC 07.02 methods. Unlike the fecal coliform recovery methods, the two *Salmonella spp.* recovery methods used different media (see Appendix A.2 and A.4 for procedures, respectively). Both *Salmonella spp.* recovery methods used non-selective enrichment media (TSB and BPW) for pre-enrichments while both fecal coliform recovery methods used selective enrichment media. The non-selective, initial pre-enrichment step employed in both *Salmonella* spp. recovery methods may have allowed for potentially physiologically-injured populations of *Salmonella* to recover and grow to higher populations of *Salmonella* before MPN analysis and culture confirmation. It is likely the pre-enrichment accounted for the higher average recovery percentages of *Salmonella* spp. 89.1% and 72.4%, for EPA 1682 and TMECC 0.7.02, respectively as compared to the fecal coliform recovery methods - 67.8% and 48.1% for EPA 1680 and TMECC 07.01, respectively). The lack of statistically significant differences in the EPA 1682 and TMECC 7.02 recovery percentages of *Salmonella* spp. was due to the greater variability in recovery of *Salmonella* from different compost types when compared to the recovery of *E. coli*. For EPA 1682 and TMECC 7.02, the largest difference in the average recovery of *Salmonella* spp. from the highest and lowest recovery percentage from the three compost types ranged from 63.9% and 57.8%, respectively. The largest difference between the highest and lowest average recovery percentages of EPA 1680 and TMECC 7.01 methods to recover *E. coli* from the three compost types ranged from 31.7% and 35.3%, respectively.

The use of the initial non-selective pre-enrichment step to increase the *Salmonella* populations seems to minimize the expected increase in recovery from using larger amounts of compost and greater transfer and dilution volumes in the EPA Method 1682 when compared to the TMECC 07.02 method. However, this pre-enrichment step may also account for the increased variability observed with the recovery of *Salmonella* populations from these finished composts. Non-pathogenic mesophilic and thermophilic bacteria present in the finished compost samples may also grow well at 37^{o}C in the enrichment broths used by EPA Method 1682 and TMECC 07.02 (TSB and BPW, respectively). Several studies have shown that various groups of microorganisms in compost piles can directly compete with and inhibit the growth of human pathogens (Hussong et al., 1984; Millner et al., 1987; Kim et al., 2011; Pietronave et al., 2004). Novinscak et al. (2009) showed that finished compost made from biosolids feedstock and aged for 24 months had a variety of bacterial species from the *Proteobacter* phylum, including *Enterobacter*, *Erwinia*, *Pantoea*, and many others. These species will grow and compete with *Salmonella spp.* at 37°C in a nutrient-rich environment, such as TSB or BPW. Hussong et al. (1984) found that *Salmonella spp.* populations were inhibited by the indigenous microorganisms present in finished compost from a sewage sludge feedstock. In another study, Miller et al. (2013) showed that high levels of indigenous bacteria (ca. 7 log CFU / g) in composted fish emulsion limited the increase of inoculated *E. coli* O157:H7 and *Salmonella* spp. populations to ca. 1 log CFU / g with 24 h. Millner et al. (1987) found that *Salmonella spp.* growth was directly inhibited by gram-negative bacteria, especially in combination with mesophilic *Actinomycetes*. These organisms are typically found in the mesophilic stage of compost, i.e. finished compost. This microbial

competition may affect the levels of *Salmonella* populations in the enrichment broths, and may account for the wide variability of recovery of *Salmonella* populations by both EPA 1680 and TMECC 7.02. Thus, it would be unsurprising if the unique indigenous microbial community present in each compost affected the quantitative recovery of *Salmonella* populations by both recovery methods. It should also be noted that methods incorporating the non-selective pre-enrichment of buffered peptone water recovered significantly more *Salmonella* spp. from biosolids samples than other methods which did not include the non-selective pre-enrichment step (Yanko et al., 1995). Furthermore, methods containing the non-selective pre-enrichment most frequently recovered the highest number of *Salmonella* (as measured by MPN / g) than other methods which did not include the enrichment step (Yanko et al., 1995). These results indicate the necessity of including the pre-enrichment when quantitatively recovering *Salmonella* spp. populations from finished composts samples.

Our data attempting to predict the regrowth potential of *Salmonella spp.* and *E. coli* O157:H7 in finished compost indicates that it is a phenomenon which involves multiple physicochemical factors. Overall regrowth potential of pathogens is still poorly understood and likely cannot be predicted by a single compost characteristic.

As previously mentioned, background microflora play a significant role in the suppression of human pathogens, including *Salmonella spp.* and *E. coli* O157:H7. Compost extracts diluted up to 1:10 in sterile water have been shown to contain nutrients for significant growth of *E. coli* O157:H7 and *Salmonella spp.* (Kim et al., 2009). Compost that has been overheated, purposefully sterilized, or has been in storage for a long time tend to have much lower levels of background populations of microorganisms

(Zaleski et al., 2005). This lack of competition may allow any bacterial pathogens to regrow or survive for longer durations in finished composts.

In our study, the compost was held at 25^{o}C in sealed Ziploc bags for three days (opening the sample once per day for microbiological analysis). Compost that is bagged for commercial sale would face similar conditions, depending on the weather and storage conditions. Though bagged commercial composts are less likely to contain pathogens compared to unbagged composts (Yanko et al.,1995; Brinton et al., 2009), the potential for contamination still exists. The process of bagging compost may homogenize any contaminants which may then be able to survive at low numbers or potentially grow for at least three days (depending on the particular compost). Increased relative humidity, as would be found in sealed bags, has been shown to aid bacterial growth and survival in finished stored composts (Pietronave et al., 2004). *Salmonella* Arizonae and *E. coli* populations inoculated into commercial finished compost were inactivated more slowly at 40% and 80% humidity than at 10% when stored over a 30-day period (Pietronave et al., 2004). Moisture contents in our finished compost samples ranged from 21.0 – 60.0% (Table 3.3), indicating that the compost samples had moisture content percentages which would support pathogen survival; however, moisture content alone could not predict the regrowth of *Salmonella* spp. or *E. coli* O157:H7 in finished composts.

Our statistical analysis revealed that C:N ratio, total organic carbon, and moisture content all strongly contributed to the regrowth of pathogens in finished compost more than other physicochemical factors. Previous work has shown that C:N ratios in compost feedstock should be between 20:1 to 40:1 to achieve sufficient microbial growth to achieve thermophilic temperatures (Rynk et al., 1992). Other studies have proposed

adjusting C:N ratios in compost feedstock to promote shorter lag times until thermophilic temperatures are reached during the composting process (Berry et al., 2013). In finished composts, it is expected that the C:N ratio would be lower since many of the carbon-based nutrients would be utilized by the indigenous bacteria present in the compost while nitrogen is cycled more efficiently through the pile. The C:N ratio in our finished compost samples ranged between 6.6 – 14.2 (Table 3.3). These relatively low levels, in combination with the indigenous microorganisms present in the finished compost, may not offer enough nutrients for extensive pathogen regrowth to occur, but cannot be used as the sole indicator of pathogen regrowth in finished compost. Total Organic Carbon (TOC) levels for finished compost samples ranged between 15.6 – 290.4 ppm (Table 3.3), although it is unclear if *Salmonella* and *E. coli* O157:H7 were able to use any of the carbon compounds measured by TOC to regrow in finished composts.

Table 3.3: Commercial compost samples organized by compost type by location. Three major compost physicochemical characteristics (% moisture, carbon:nitrogen ration, C:N, and total organic carbon (TOC)) are presented.

Compost Type	Compost Code Number	State of Origin	% Moisture	C:N	TOC (ppm)
Biosolids	1	GA	38.2	9.8	78.2
	6	MD	36.3	7.9	87.7
	7	IA	36.4	10.5	87.8
	10	VA	43.8	9.7	96.3
	14	MA	27.8	9.6	72.7
	16	VA	35.0	8.1	54.6
	19	CA	33.7	7.7	41.4
	20	CO	21.7	7.3	15.6
	21	NH	50.7	9.7	122.8
	27	OH	27.0	10.7	24.4
Manure	2	CA	25.8	6.1	71.8
	4	CA	21.1	7.4	61.6
	18	PA	52.8	10.6	69.9
	23	KA	23.4	14.1	34.9
Yardwaste	3	CA	21.0	9.4	47.5
	5	MD	32.5	14.2	26.6
	8	VA	48.3	10.7	17.0
	9	MD	55.1	10.8	43.7
	11	NJ	33.5	7.9	67.7
	12	DE	36.0	12.3	216.6
	13	MA	26.3	9.5	26.3
	15	MA	60.0	10.1	14.1
	17	NC	45.8	10.7	90.4
	22	OR	25.5	8.2	27.8
	24	KY	30.8	12.1	15.3
	25	CT	48.9	11.1	19.1
	28	FL	50.6	11.4	25.4
	29	WA	41.4	11.2	290.4
	30	NJ	31.6	6.6	42.5

Our study has shown the potential for *Salmonella spp.* and *E. coli* O157:H7 to decrease, increase, or remain at a steady-state population over a three-day period in 29 different composts. Understanding which non-microbiological parameters influence pathogen regrowth could lead to new formulations of feedstock materials, and potentially provide compost producers with a rapid test to determine if enteric pathogen regrowth is likely to occur in their compost. Our results indicate that a combination of physicochemical factors, rather than any single factor, is likely responsible for the regrowth potential of pathogens in finished compost. A fuller understanding of the mechanisms and interactions that play important roles in the regrowth of human pathogens, including *Salmonella spp.*, will lead to safer compost and better composting regulations and recommendations.

4. Conclusions

EPA methods recovered significantly higher percentages of inoculated *E. coli* and than TMECC method due to the use of larger transfer and dilution volumes. EPA methods should be used in microbiological testing of finished compost to ensure the most sensitive quantitative recovery method of pathogens is being used to comply with federal and state standards. The regrowth potential of *Salmonella spp*. and *E. coli* O157:H7 populations in composts could not be predicted by any one physicochemical characteristic measured, though C:N, TOC, and % moisture were reconfirmed as major contributors, indicating pathogen regrowth in compost is influenced by multiple factors.

5. Acknowledgements

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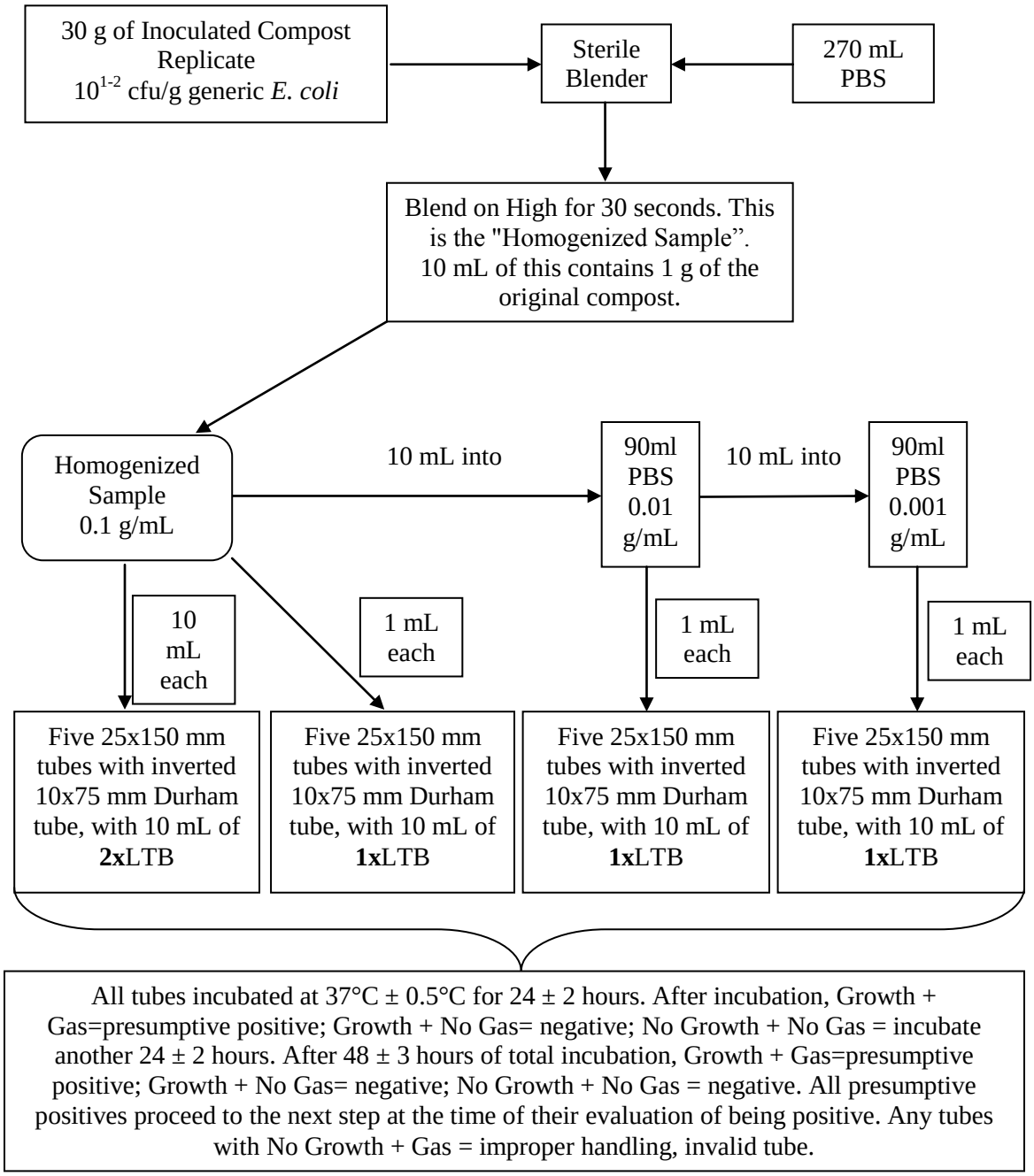
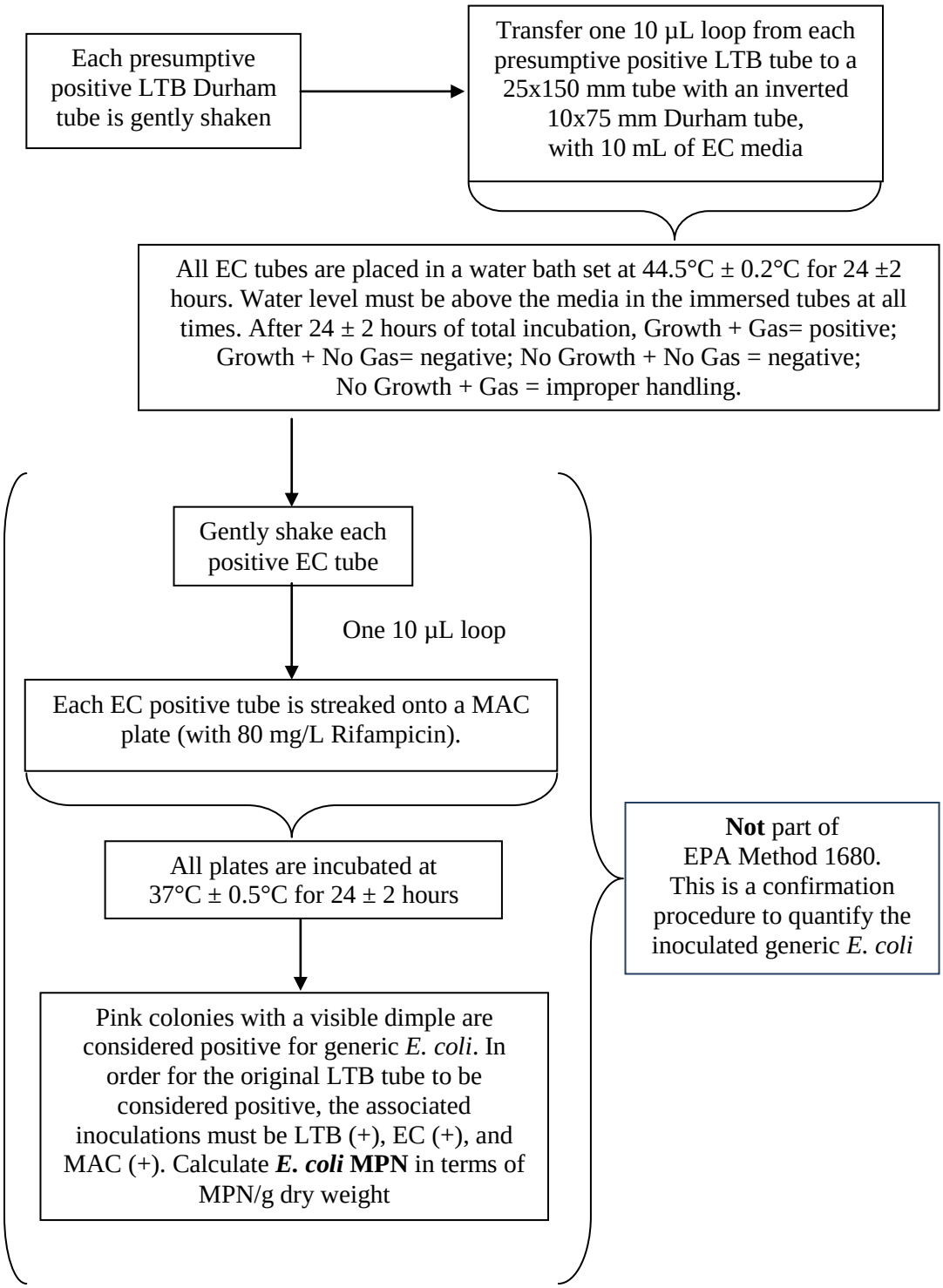
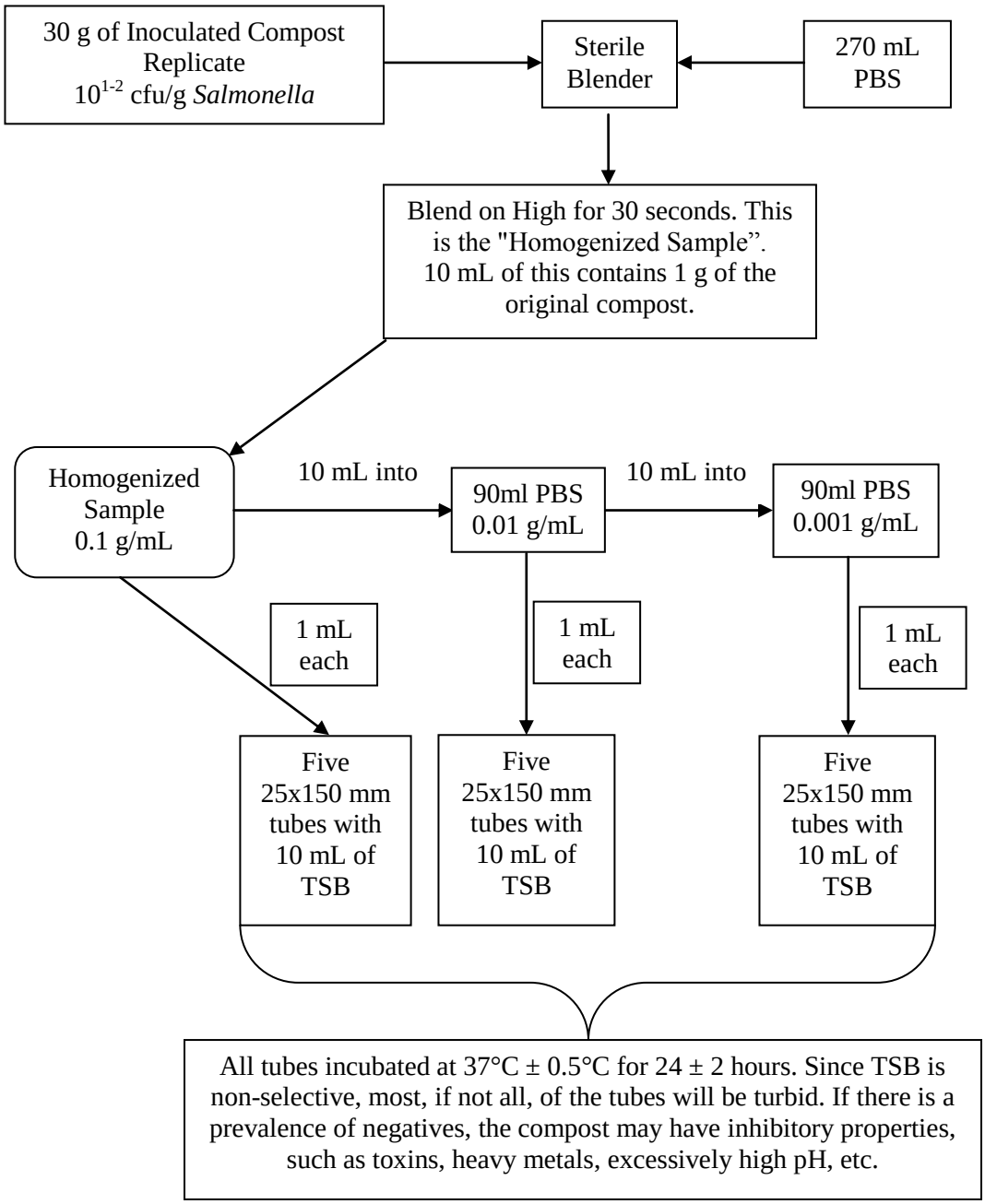
Appendix 3.A.1
EPA Method 1680 for Fecal Coliforms,
plus *E. coli* Inoculation Confirmation Procedure



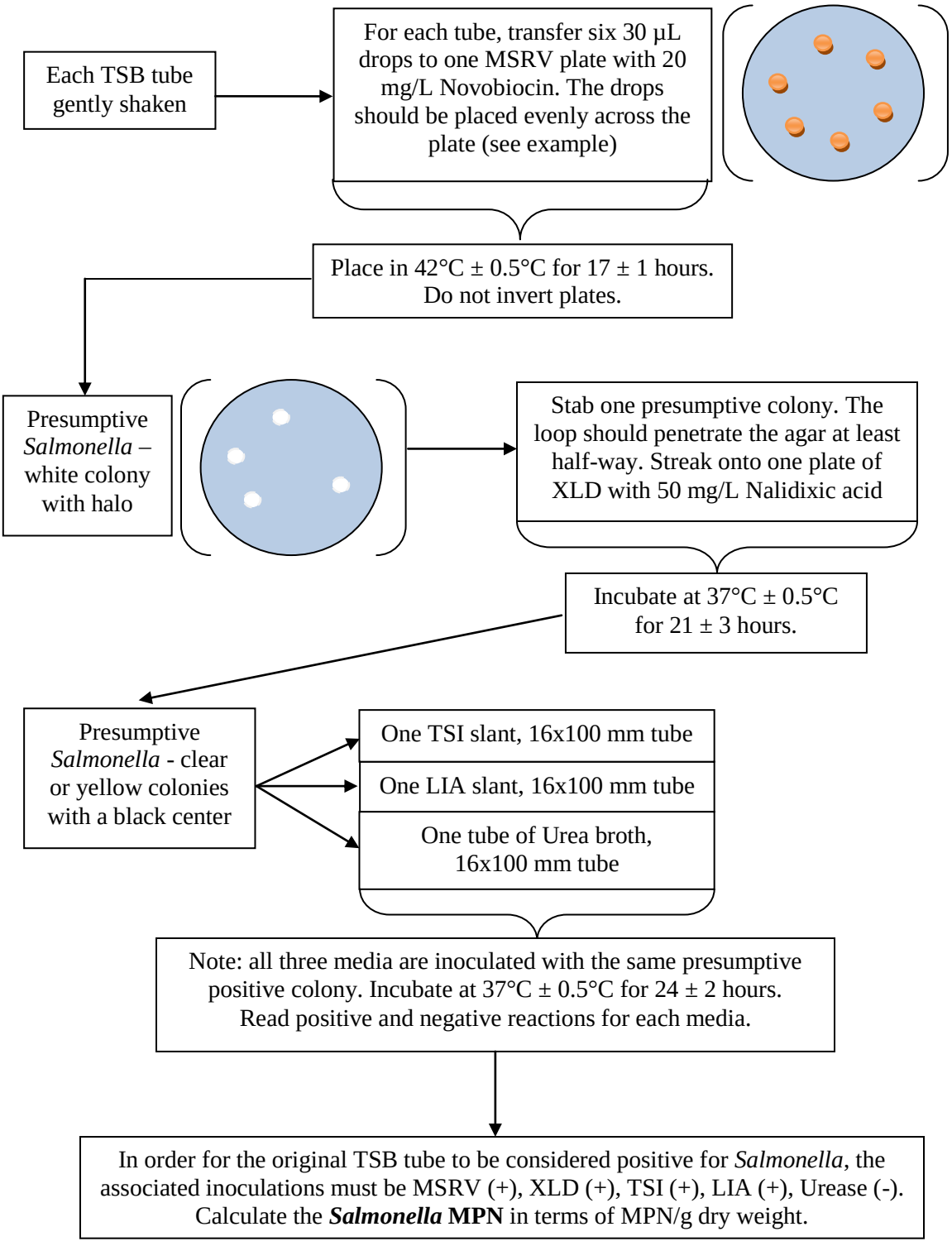
Appendix 3.A.1
EPA Method 1680 for Fecal Coliforms,
plus *E. coli* Inoculation Confirmation Procedure *continued*



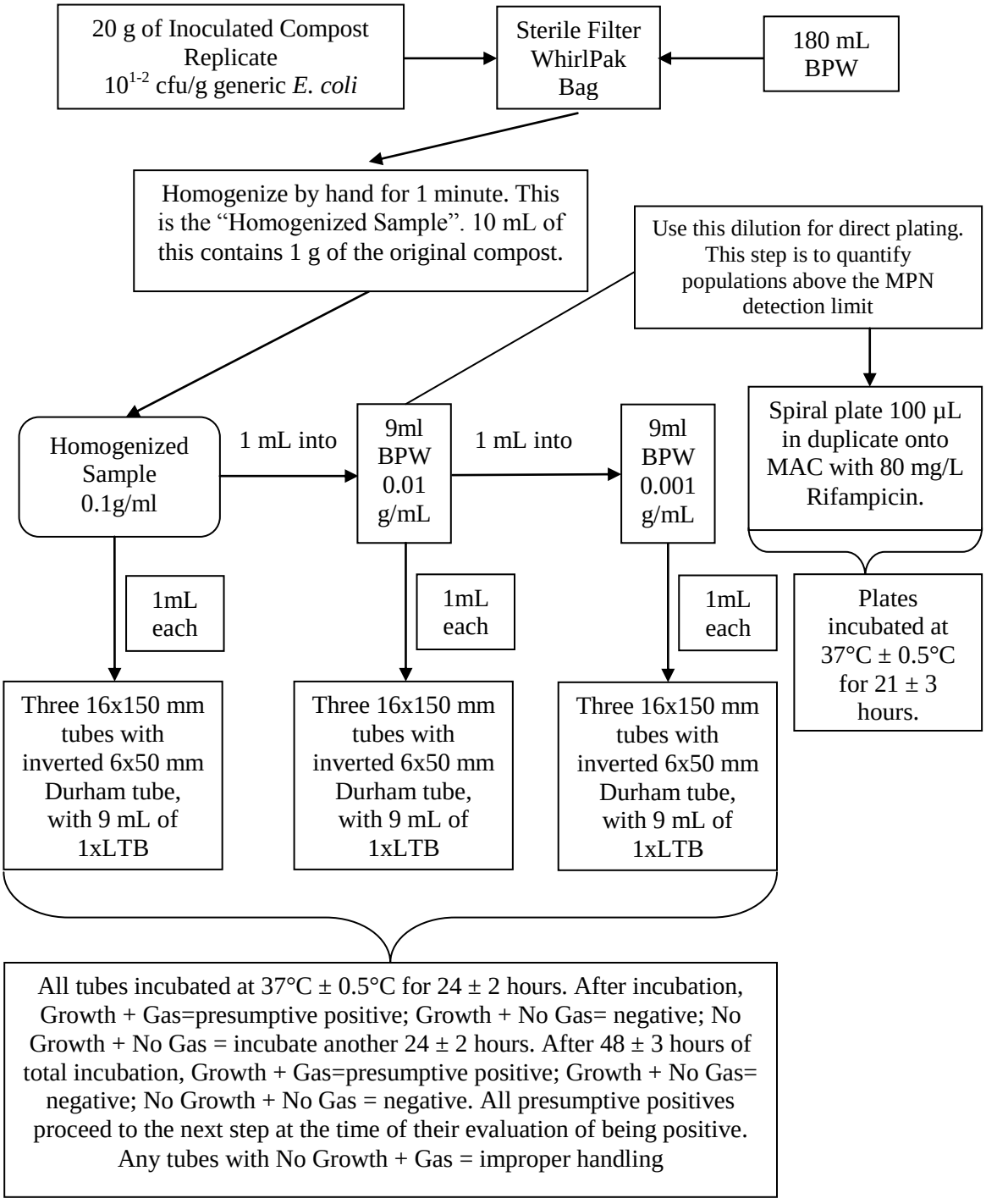
Appendix 3.A.2
EPA Method 1682, *Salmonella*



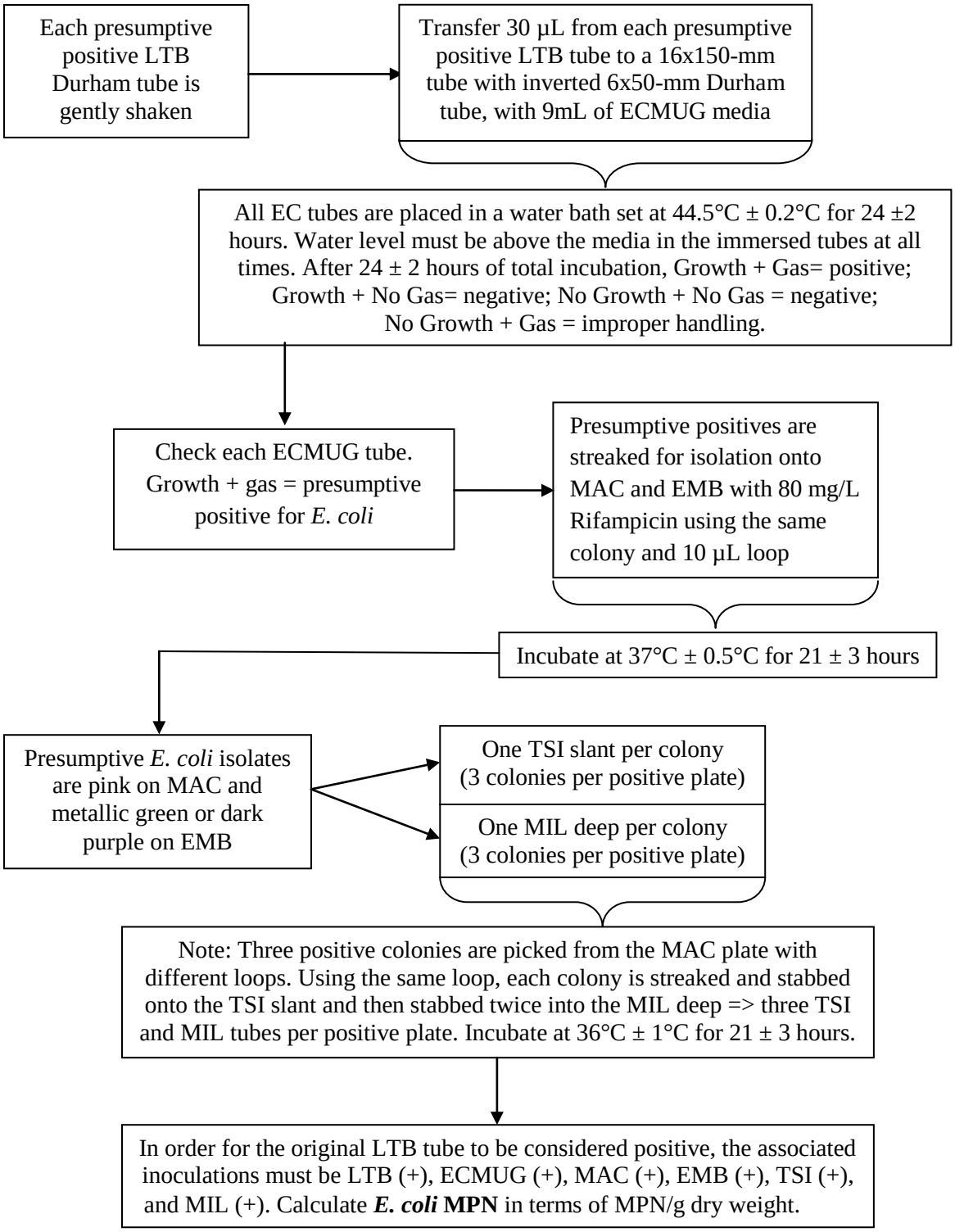
Appendix 3.A.2
EPA Method 1682, *Salmonella continued*



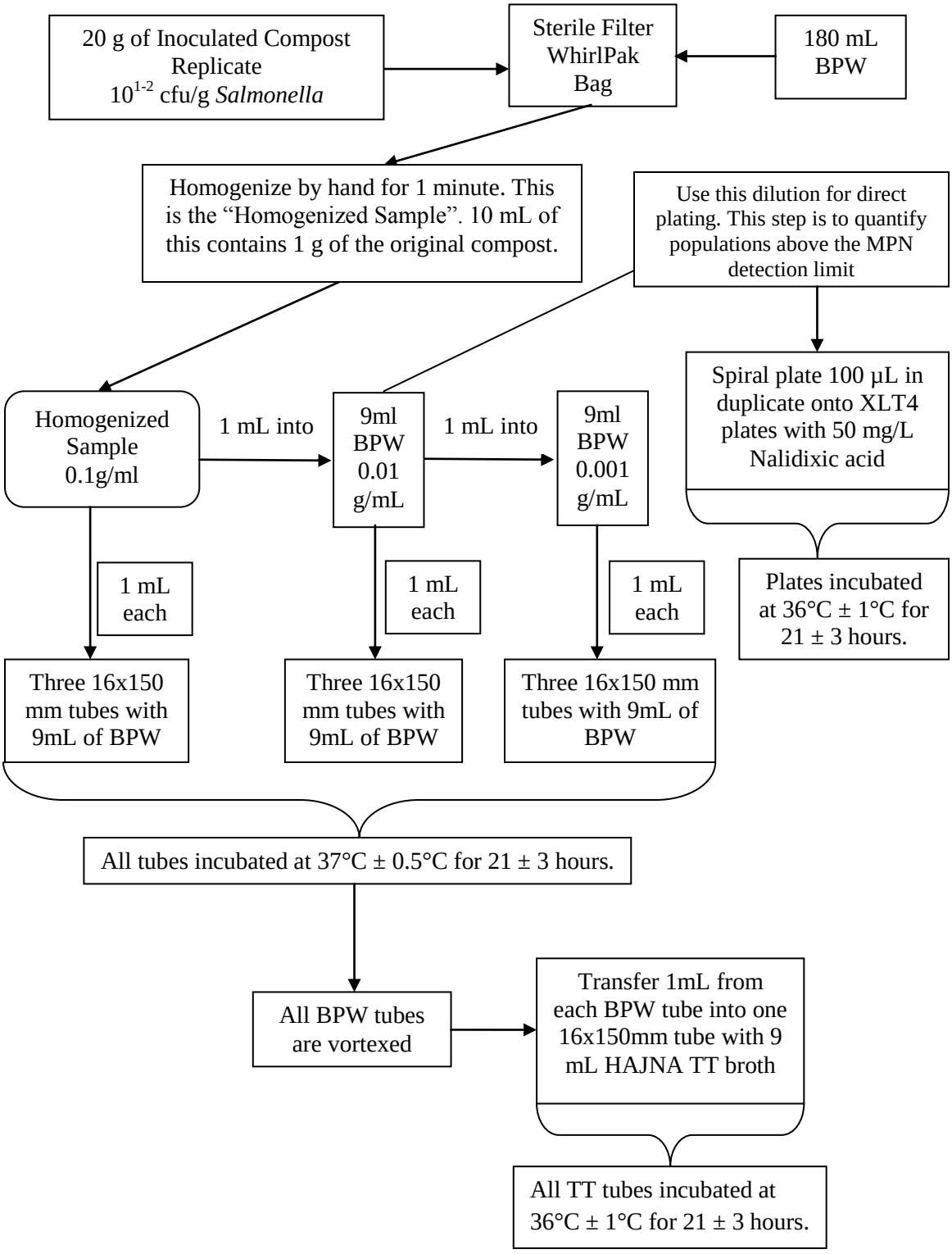
Appendix 3.A.3
TMECC 07.01 A, B, C Total Coliforms, Total Fecal Coliforms, Total *E. coli*



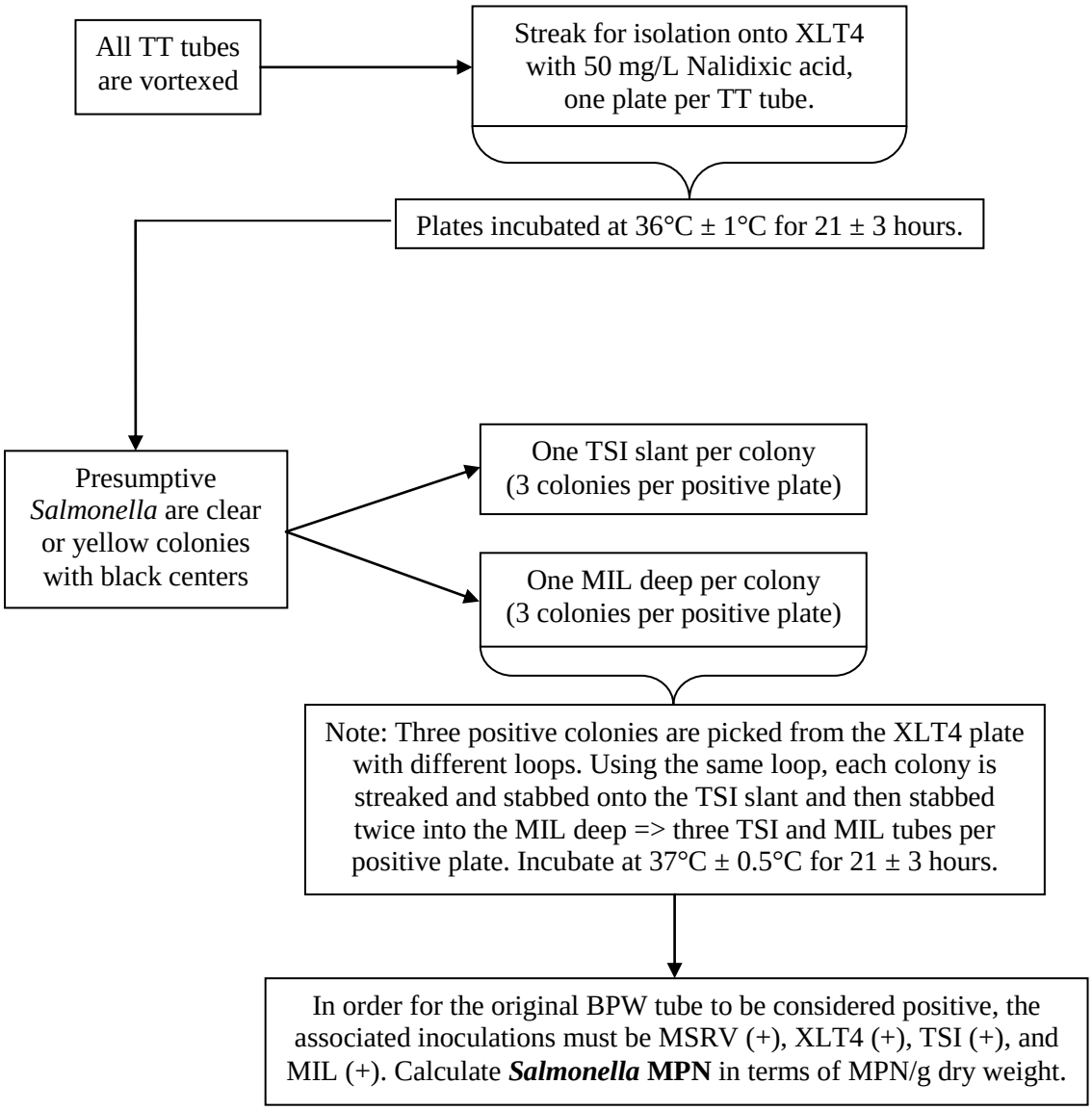
Appendix 3.A.3
TMECC 07.01 A, B, C Total Coliforms, Total Fecal Coliforms, Total *E. coli*
continued



Appendix 3.A.4
TMECC 07.02 B, C Total *Salmonella spp.*



Appendix 3.A.4
TMECC 07.02 B, C Total *Salmonella spp. continued*



Chapter 4
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Evaluation of Two Immunomagnetic Separation Techniques for *E. coli* O157:H7 Recovery in Finished Compost

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Abstract

Two immunomagnetic separation protocols were investigated for their efficacy in recovering inoculated pathogenic *E. coli* O157:H7 from 90 replicates of 29 different commercially-produced mature compost samples. Both protocols identified all inoculated compost samples as positive with 3-4% false negatives among replicates.

Keywords: Immunomagnetic, compost, *E. coli* O157:H7, Pathatrix

Research Note

Compost is organic matter that has degraded into a nutrient-stable humus-like material. Compost has a variety of uses including waste management, fertilizer, and a soil enhancer. Currently, compost is mostly regulated at the state level. Only compost that includes biosolids (human waste) has additional federal regulations. Safety standards vary between states and often between compost feedstocks. Generally, fecal coliform populations are used as indicators of the total pathogen load of compost, though some states also require *Salmonella spp.* populations to be measured. Fecal coliform testing requires less work and cost than testing for *Salmonella spp.*, thus making fecal coliform testing the preferred method in the industry. Fecal coliform testing requires 3-4 days and *Salmonella spp.* testing requires 5 days.

Besides occasional *Salmonella spp.* testing, no other specific pathogens are regularly tested for, mainly due to cost and practicality. Rapid tests that allow for faster turn-around of pathogen content tesing as well as more target pathogens to be processed in a given time are imperative to the advancement of public safety measures in determining the safety of compost for public use. Thus, this study compared two immunomagnetic separation techniques for the recovery of *E. coli* O157:H7, a human

pathogen implicated in several food-borne illness outbreaks in the last two decades, in compost.

Commercial compost samples were collected and shipped to the U.S. Department of Agriculture Beltsville Agriculture Research Center via overnight postal service from 29 different composting facilities across the United States. The compost samples varied greatly in feedstock type (combinations of biosolids, animal manure, food waste, yard waste, etc.) and thus provided a reasonable cross-section of the variability within the composting industry. Each compost sample was split into three 400g replicates. Each replicate was inoculated with two strains of pathogenic *E. coli* O157:H7 resistant to 50 mg / L Nalidixic Acid at 10^{1-2} cfu/g (wet weight). Each compost replicate was then processed using both methods.

The first method is referred to as the Modified Elaine Berry method. Dr. Elaine Berry has done numerous experiments involving the immunomagnetic separation and capture of *E. coli* O157:H7 from bovine samples, mainly manure. The method used in this experiment hybridizes her methods from several of her papers and proceeds as follows.

Ten grams of the inoculated replicate sample was combined with 90 mL of Tryptic Soy Broth (TSB) in a filter WhirlPak bag and manually homogenized for at least 30 seconds. From this solution, 1 mL was combined with 9 mL Buffered Peptone Water (BPW) to make a 10^{-1}. A 10^{-2} and 10^{-3} were made the same way. These dilutions were incubated at room temperature for 2 hours, transferred to 42°C for 6 hours, then to 4°C overnight.

The next day, 500 µL of each dilution was combined with 500 µL BPW and 10 µL of Dynabeads *E. coli* O157:H7 immunomagnetic beads in a 42-well deep well plate. These solutions were shaken at 100 rpm at 37°C for 30 minutes. After shaking, a magnetic PickPen was used to remove the immunomagnetic beads and place them in 100 µL of BPW in a 1.5 mL eppendorf tube. From there, all 100 µL were streaked onto one CHROMagar O157 plate with 50 mg / L Nalidixic Acid. The plates were incubated at 37°C for 24±2 hours and checked for positive isolates. Plates with questionable colonies were confirmed using *E. coli* O157:H7 DrySpot cards. Since this was from enriched cultures, counts were not done, only a positive/negative confirmation for the presence of *E. coli* O157:H7. Eighty seven out 90 replicates were positive for the presence of *E. coli* O157:H7 on at least one dilution. The three false negatives were in samples that were accidently inoculated below the detection limit of the test. Even so, the Modified Elaine Berry protocol captured *E. coli* O157:H7 in half of the replicates below its calculated detection limit.

The second method used a Pathatrix machine (Matrix Microscience, Newmarket, UK). Twenty five grams of the inoculated compost replicate was combined with 225 mL modified Enterohemorrhagic Escherichia coli (mEHEC) broth in a filter WhirlPak bag and homogenized for at least 30 seconds. This bag was then incubated at 37°C while shaking at 125 rpm for 5 hours. After 5 hours, the solution was placed at 4°C overnight. The next day, the enriched homogenized sample was allowed to warm for approximately half an hour. The entire homogenized sample was then put through the Pathatrix machine protocol. Briefly, this involves recirculating the liquid enrichment over a magnet that has the immunomagnetic beads attached to it. This allows the entire enrichment to flow over

the beads and separate the target organism from the sample. After 30 minutes of recirculation, the beads were washed with BPW and resuspended in 100 µL of Peptone Buffered Saline (PBS) in a 1.5mL eppendorf tube. This eppendorf tube was then placed at -20°C until real-time PCR (RT-PCR) could be performed for the *Stx*2 gene. At first, RT-PCR resulted in all negatives. After one round of DNA purification (Zymo) and up to four separate rounds of purification using Zymo's Inihibitor Purification Kit, 86 out 90 samples came back as positive for the presence of the *Stx*2 gene. Those that were negative occurred in three samples with high or medium total organic carbon (TOC) measurements (87.7, 216.6, and 290.4 ppm). Likely, either more purification was needed to remove PCR inhibitors or the DNA had been lost during transfer after four rounds of purification.

Both methods can be improved upon. The Modified Elaine Berry method could easily be turned into an MPN scheme by adding more dilution series, creating a rapid detection test that gives a population estimate. The Pathatrix method currently can be done in approximately 11 hours (including one inhibitor purification and one DNA purification step). A two hour incubation time at 42°C may be enough to allow for recovery and cut the necessary time down to an 8 hour work day. Both of these methods could easily be used with other immunomagnetic beads and other minor changes to target other pathogens, making both methods very flexible. In conclusion, both methods have the potential to be very useful in the compost industry as rapid tests for the detection of human pathogens.

Chapter 5: Final Thoughts

Additional Discussion of Immunomagnetic Recovery Methods

Both the Modified Elaine Berry and Pathatrix methods could be improved upon to further increase recovery efficacy in composts. The dilution enrichment and immunomagnetic capture portion of the Modified Elaine Berry method has a relatively high detection limit of 200 CFU / g in its current iteration. The detection limit can be brought down to a more reasonable 20 CFU / g simply by using 10 mL of the original homogenate as part of the enriched dilutions. Additionally, using more than 10 g of compost in the original homogenate and/or using a 1:5 dilution for the original homogenate instead of 1:10 can further increase the likelihood of recovery and decrease the detection limit to levels that would be able to capture the low levels of *E. coli* O157:H7 that may actually be found in finished composts. This part of the method could also be easily turned into a 3 dilution by 3 tube MPN scheme (or larger) to allow for quantification of *E. coli* O157:H7 instead of just detection. This method currently stipulates a total of 8 hours of incubation before capture with immunomagnetic beads and plating. This inevitably leads to placing the enriched dilution tubes at 4^0C until the following day for plating which then takes an additional 18-24 hours of incubation. If the enrichment only took 6 hours then the plating could be done on the first day and the whole test would be done by the second day. Making these changes could reduce the time to a one-day quantification test for *E. coli* O157:H7 in compost and reduce the detection limit ten-fold.

The Pathatrix (Matrix Microscience, Newmarket, UK) also has some issues that need to be resolved in terms of recovery of pathogens from finished composts. The largest issue with the Pathatrix was the need to remove PCR inhibitors from the washed beads before being able to run RT-PCR. Unfortunately, it is impossible to tell how many rounds of purification need to be done on any given sample in order for RT-PCR to work. While it seems to be somewhat correlated to the total organic carbon (TOC) content of the compost, there is no definitive correlation, rather only that 3 of the 4 replicates that were not positive had the highest TOC content of all of the samples and the fourth replicate that was negative was from a compost sample that had the 8th highest TOC content. Human error may have been involved as well. Perhaps the beads were not adequately washed in the Pathatrix machine which may have allowed for high TOC particles to cling to the beads and subsequently interfere with RT-PCR. Regardless of the source or how it occurred, the uncertainty in the number of purifications needed to recover via RT-PCR is a major deterrent from using this Pathatrix method. Only 77% of samples were positive after one round of inhibitor purification.

To avoid this entire issue, the recommendation is to simply plate half of the beads onto a selective medium such as CHROMagar O157 and use the other half of the beads for a confirmation RT-PCR if there is no growth on the selective media. A pilot study successfully recovered *E. coli* O157:H7 from beads directly after Pathatrix. Directly plating the beads onto selective agar would save a considerable amount of labor and expense, and in fact give a more accurate depiction of the presence of viable *E. coli* O157:H7 cells in the compost as compared to using RT-PCR. The original intent of using RT-PCR directly after recovery using Pathatrix was to create a one-workday recovery

method of *E. coli* O157:H7. However, given the prevalence of PCR inhibitors in finished compost and the uncertain number of purifications needed, it may be more accurate and less time consuming overall to simply do one direct plating step rather than running RT-PCR multiple times on the same sample in hopes of a positive. Making this change would make for a rapid, reliable, one-day detection method with a very low detection limit for *E. coli* O157:H7 in compost.

Issues of Practicality of Methods

EPA and TMECC methods

EPA Methods 1680 and 1682 and TMECC 07.01 and 07.02 all used borosilicate glass tubes. Instead of discarding the tubes after one use, each tube was reused. The tubes were autoclaved, rinsed with hot soapy water, and dish-washed before refilling with media or placed in storage. This process took a significant amount of time and the tubes that needed to be cleaned would often back up while other parts of the project were on-going due to lack of adequate time and personnel. When repeating this experiment, this cleaning stage needs to be taken into account when planning, either by budgeting time to clean or budgeting money for the disposal and replacement of tubes.

EPA, both methods

Both EPA Method 1680 and 1682 utilized a blender for the homogenization of the initial dilution. Neither EPA method specifies the precise speed that the blenders are supposed to operate, but instead only say “Cover and blend on high speed for one minute”. This leaves the specific “high speed” up to the discretion of the experimenter. The blenders used in these experiments were likely too fast (22,000rpm) because after one minute the homogenate would consistently begin to leak out of the top of the blender.

Therefore, the time was changed to 30 seconds to maintain a sanitary work environment. The shorter time appeared to be sufficient to mix the homogenate. Upon repeating this experiment, a small pilot study should be conducted to ensure that the specific blender and time used will be sufficient in both homogenization and sanitation.

TMECC, both methods

Both TMECC 07.01 and 07.02 call for the use of 16x150mm screw cap tubes for multiple steps. The height of the tube made it extremely difficult or impossible to functionally use a micropipetor because it simply could not reach the liquid in the tubes. Therefore pipette guns were needed which led to an increase in the waste generated as well as time used per transfer. This is also the reason why in TMECC 07.01 when 30 µL of the LTB MPN tubes are transferred to ECMUG MPN tubes that three 10 µL loop-fulls are used rather than a pipette. It is highly recommended that the USCC change TMECC 07.01 and 07.02 to not recommend these tubes but instead use 16x125mm tubes because the 16x150mm tubes are an inconvenience at best and provide no benefit over shorter tubes.

Final Statement on Practicality of Methods

In regards to these specific issues, it is recognized that laboratories are not restricted to using only the equipment (tubes, blenders, etc.) specified by the protocols as literally as was followed here. Even the STA certified laboratories that run the testing for USCC do not follow the protocols this precisely (personal correspondence). Therefore, it is probable that EPA laboratories also deviate from the protocol when necessary/convenient. The purpose of following the protocols as literally as possible was

to test the protocols *as written*. These are the possible issues that a fledgling laboratory or novice to these protocols might encounter if they were trying to follow these procedures.

Conclusions

Very little research has been published on the efficacy of the EPA or TMECC microbial detection methods in compost. In fact no published data of any relevance on TMECC 07.01 or TMECC 07.02 was discovered. Apparently, this is the first published test of TMECC 07.01 and TMECC 07.02 independent of the USCC's tri-annual Compost Analysis Proficiency testing program, which is a quality assurance program designed to assess the uniformity of testing results derived from its STA testing laboratories. While the results of these quality assurance tests are available to the public, they do not appear to be substantial enough to truly assess the effectiveness of the protocols. They are only suitable for assessing the homogeneity of the STA laboratories' responses, which is what it was designed to do. This project compared these two TMECC microbial methods to their corresponding EPA methods in the first comparison of the two most commonly used fecal coliform/*E. coli* and *Salmonella* detection methods in the U. S. composting industry. Additionally, this appears to be the first published study to compare the proficiency of either EPA or TMECC procedures in multiple compost types (biosolids, manure, and yardwaste).

Both EPA methods recovered statistically more or equivalent levels of inocula as their corresponding TMECC methods (at α=0.10) overall and in every compost type. The greater amount of compost used initially, the higher dilution volumes (though still 1:10), and the larger MPN scheme used by both EPA methods are the most likely explanation for the higher or equivalent recoveries. The greatest differences were seen between the

fecal coliform methods, which were essentially the same method other than the procedural differences noted above. Both *Salmonella* recovery methods had more variable recovery than the fecal coliform methods. This increased variability explains why the *Salmonella* methods had mostly statistically equivalent recoveries, with EPA Method 1682 having greater recovery than TMECC 07.02 only in biosolids compost and TMECC 07.02 only having greater recovery in yardwaste compared to biosolids composts. It is possible that the use of a non-selective pre-enrichment step for both methods contributed to the increased variability. The non-selective pre-enrichment would allow many fast growing microorganisms to rapidly grow to levels that may out-compete any *Salmonella* present in the MPN tube. The fecal coliform methods both used a selective pre-enrichment and had much less variation in recovery. Both EPA and USCC should consider using a more selective pre-enrichment step for the *Salmonella* methods to allow for more accurate recoveries. USCC should consider using a greater amount of compost in their initial homogenates, higher dilution volumes, and larger MPN schemes to increase the recovery of TMECC 07.01 and TMECC 07.02.

Current compost testing does not generally include any actual tests for pathogens besides *Salmonella*, and even that is generally not done due to increased expenses unless required by state regulations. More rapid tests that check for specific pathogens could decrease the expense per test, increase the number of tests that could be performed in a given time period, reduce wait time for results, and increase confidence in the safety of the compost. The use of fecal coliforms as indicator organisms generally works well, however there are documented cases where the compost is clearly not Class A for other pathogens, such as *Salmonella*, when the fecal coliform counts would have allowed it to

be classified as such. This study reviewed two immunomagnetic bead-based methods for the rapid detection of *E. coli* O157:H7. The Modified Elaine Berry and Pathatrix methods recovered *E. coli* O157:H7 in all but 3 or 4 replicates, respectively, out of 90. The Modified Elaine Berry method missed three replicates because the *E. coli* O157:H7 was inoculated at levels well below the detection limit of the test (inoculated at 10^{-1} CFU / g). The Pathatrix method recovered *E. coli* O157:H7 from all of the low inoculation level replicates that the Modified Elaine Berry method missed. Conversely, the Modified Elaine Berry recovered all other replicates on the first attempt while the Pathatrix method required up to four rounds of PCR inhibitor purification but still was unable to recover the inocula in four compost replicates that had high total organic carbon content.

Both methods could be improved upon and further research is needed to do so. The use of immunomagnetic beads as the method of capture make both methods very flexible. Both methods can capture any pathogen for which a reliable immunomagnetic bead has been designed. Understanding the need for PCR inhibitor purification and taking into account detection limits are issues with these methods that must be resolved before they can be fully reliable. However, both methods have shown considerable promise.

The regrowth potential of human pathogens in compost, specifically *Salmonella* and *E. coli* O157:H7, is still poorly understood. Understanding the factors that influence regrowth potential and how to use them to predict the regrowth of human pathogens in compost will increase public safety by reducing the number of pathogens applied to food crops which can subsequently infect human consumers. Though this study did not identify any single factor that could accurately predict regrowth of either *Salmonella* or

E. coli O157:H7, moisture content, total organic carbon, and the C:N ratio were determined to have the greatest influences on regrowth among the factors measured. This also indicates that there is no simple relationship between compost characteristics and the regrowth potential of human pathogens in finished compost. More in-depth research is needed to understand the interrelationships between compost characteristics and human pathogens.

Appendix 5.A

Average percent recovery of each compost sample (three replicates per sample) .

		Fecal Coliform Methods		*Salmonella spp*. Methods	
Sample	Type	EPA 1680	TMECC 07.01	EPA 1682	TMECC 07.02
1	Biosolids	61.6%	0.0%	47.6%	0.0%
6		73.9%	31.6%	7.2%	75.6%
7		121.0%	25.0%	74.5%	60.9%
10		48.3%	24.8%	79.9%	10.1%
14		64.5%	30.2%	271.1%	59.0%
16		237.2%	36.3%	21.8%	11.7%
19		128.3%	70.0%	41.8%	68.1%
20		58.8%	32.1%	88.3%	80.0%
21		49.4%	18.1%	203.7%	20.8%
27		34.0%	26.3%	15.3%	9.2%
Average	B	87.7%	29.4%	85.1%	39.5%
2	Manure	17.0%	0.0%	56.9%	0.0%
4		32.0%	23.5%	6.5%	143.2%
18		156.8%	18.6%	51.3%	21.3%
23		81.5%	82.5%	44.8%	53.8%
Average	M	71.8%	31.1%	39.9%	54.6%
3	Yardwaste	24.2%	41.0%	5.3%	37.6%
5		22.5%	14.1%	1.1%	1.4%
8		49.0%	36.1%	196.9%	80.6%
9		110.2%	46.0%	28.7%	308.4%
11		41.2%	75.8%	140.3%	77.3%
12		28.0%	153.7%	66.0%	52.4%
13		52.1%	20.8%	42.2%	39.5%
15		75.7%	44.7%	144.9%	493.3%
17		52.9%	88.9%	9.1%	12.2%
22		52.5%	86.6%	42.4%	41.7%
24		19.3%	26.7%	74.6%	40.6%
25		64.7%	38.4%	1.5%	2.0%
26		26.4%	50.7%	53.9%	38.1%
28		136.1%	167.5%	139.2%	133.9%
29		107.5%	74.0%	451.1%	55.5%
30		34.2%	59.8%	263.9%	124.3%
Average	Y	56.0%	64.1%	103.8%	97.3%
Average	All	68.7%	48.1%	89.1%	72.4%

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