Developing and Testing a Framework to Measure the Sanitation Efficacy on a Random Particle Level in Composting Industry

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Environmental Engineering

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ABSTRACT

Both direct and indirect process validation regulations are being used to ensure the safety of compost products. The North American guidelines specify upper limits for certain pathogenic and indicator microbes, which are presumably achieved by exposing every particle of compost to temperatures ≥55°C for at least 3 consecutive days. Regardless of maintaining high temperatures, there may still be pathogenic microorganisms that survive composting. Hypothetically, this could be because: (1) temperature test methods may not account for spatial and temporal temperature variation in large composting piles, giving the false impression the temperature-contact time condition has been met; and/or (2) the existing temperature contact time criterion may be inadequate.

A temperature probe was developed consisting of a temperature recording circuit and inoculum holder (i.e., cryovial) enclosed in a cylindrical case made of anodized aluminum. Two field trials suggested that this probe behaves like a random particle in compost. Both trials also showed that the aluminum case protects the probe's circuitry and the cryovial from various physical and chemical stresses.

The temperature probe was used to develop a temperature sampling framework for the indirect process validation. It suggested that in a covered aerated static pile the likelihood for a random compost particle to reach and maintain ≥55°C for at least 3 consecutive days varies between 76 – 93% and could increase to 98% after

five pile turnings. Typical cool zones mostly remained at ≤45°C, resulting in questionable thermal inactivation responses for pathogenic microorganisms.

A direct technology validation framework using the temperature probe was developed and proposed. Two experimental runs demonstrated that samples in which temperatures ≥55°C were maintained for at least 3 consecutive days were free of culturable *Salmonella* sp., *Escherichia coli* sp., and phi-S1 bacteriophage. However, a sample that remained in the cool zone throughout one experiment run still had culturable *Salmonella* in appreciable amounts thus reiterating the importance of cool zones in the sanitation process. Molecular analysis of the samples demonstrated that a gradual increase in compost temperature induced a potential viable but not culturable state in *Salmonella* and *E. coli*. It was concluded that the specified time-temperature conditions are likely adequate but more research is needed to study the behavior of VBNC pathogens in compost.

PREFACE

This Ph.D. thesis contains the results of the work undertaken at the Department of Civil and Environmental Engineering of the University of Alberta and supervised by Prof. D.M. McCartney. I acknowledge the Edmonton Waste Management Centre of Excellence and the Natural Science and Engineering Research Council (NSERC) of Canada for funding this research under the latters Collaborative Research & Development (CRD) Grant # 408056. I would like to extend my thanks to the City of Edmonton for support in field activities, and the School of Public Health of the University of Alberta for allocating their laboratories and personnel for this research.

A version of Chapter 2 of this thesis has been published as: Isobaev, P., Wichuk, K. M. & McCartney, D. (2014) Modification and industrial applicability of a temperature probe capable of tracking compost temperature on a random particle level. *Compost Science and Utilization, 22*(2), 93-103. I was responsible for the methodology, data collection and interpretation as well as the manuscript composition.

A version of Chapter 3 of this thesis has been published as: Isobaev, P., Bouferguene, A., Wichuk, K., & McCartney, D. (2014). An enhanced compost temperature sampling framework: Case study of a covered aerated static pile. *Journal of Waste Management*, *34,* 1117-1124. I was responsible for the design of a method, subsequent development of a framework, and the manuscript composition.

A version of Chapter 4 of this thesis has been accepted for publication as Isobaev, P., McCartney, D., Wichuk, K. M., Scott, C., & Neumann, N. (2014). An enhanced direct process temperature validation framework in composting: Case study of a full-scale covered aerated static pile. *Compost Science and Utilization.* I contributed to the design of experiment and to the preparation of microbial inoculum. I also executed the experiment, collected, analysed and interpreted the results. Finally I composed the manuscript.

DEDICATION

To my dearest family for their support and sacrifices during the course of this adventure. They are the most precious what I have in my life.

ACKNOWLEDGEMENT

First and foremost I would like to deeply appreciate my supervisor Dr. Daryl McCartney for the professional guidance and continuous support in every aspect of this research. His belief that I excel as a researcher and encouragement was essential to accomplish this work.

I am sincerely thankful to Dr. Norman Neumann for guiding me through the world of environmental microbiology. His philosophical approach to microbial evolution in the environment still amazes me.

I am so much grateful to Dr. Ahmed Bouferguene for being an excellent coach in finding the best practical solution to problems. His sense of humor and easy-going personality will be missed a lot.

I am indebted to Dr. Christian Felske for giving me the chance to experiment in industrial setting and always helping me with the technical details of the research.

I want to greatly appreciate the valuable help I received from the best research associates Kristine Marie Wichuk and Candis Tammy Scott. They were the best teammates a PhD student can dream of.

I would like to acknowledge the financial support received from NSERC CRD #408056, and the City of Edmonton Waste Management Centre.

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µL	microliter
ANOVA	Analysis of variance
BD	Bulk Density
BHI	Brain heart infusion agar
BNQ	Bureau de Normalisation du Québec
bp	Base pair
CASP	Covered Aerated Static Pile
CCME	Canadian Council of Ministers of the Environment
CFU	Colony forming units
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
HP	Horse power
LB	Luria Bertani
LOD	Limit of detection
LTB	Lauryl tryptose broth
MC	Moisture content
mg	milligram
ml	milliliter
MPN	Most Probable Number
MSRV	Modified semisolid Rappaport Vasilliadis agar
MSW	Municipal solid waste
MTP	Modified Temperature Probe
NH_3	Ammonia
NRC	National Research Council
OD	Optical density
PBS	Phosphate buffered saline solution
PFGE	Pulsed-field gel electrophoresis
PFRP	Process to Further Reduce Pathogens

PFU	Plaque Forming Units
PMA	Propidium monoazide
QMRA	Quantitative microbial risk assessment
RNA	Ribonucleic acid
SDDW	Sterile double distilled water
SPC	Standard plate count
TMECC	Test Method for Examination of Composting and Compost
TNTC	Too Numerous to Count
TSB	Tryptic soy broth
TTC	Time Temperature Criteria
USEPA	United States Environmental Protection Agency
VBNC	Viable But Not Culturable
WBD	Wet Bulk Density
WCS	Working Cell Suspension
WDW	Waste Dry Weight
XLD	Xylose lysine deoxycholate

CHAPTER 1: BACKGROUND INFORMATION AND RESEARCH OBJECTIVES

1.1 Composting

1.1.1 History

The term *composting* is derived from the Latin *compositum*, meaning mixture (Insam and de Bertoldi, 2007), and usually refers to the biological decomposition and stabilization of organic substrates under controlled thermophilic conditions (> 45°C) with sufficient supply of moisture and aeration (Haug, 1993). The final product is believed to be stable and hygienic.

The history of composting can be traced back to the first urban settlements in the Neolithic period. When early civilizations reduced their hunting habits and started to carry out extensive agricultural practices they were found to mix agricultural, animal and human residues with soil to supplement soil nutrient needs (Diaz and de Bertoldi, 2007). Similarly in ancient Rome, the centrally collected urban waste was gathered and applied to agricultural soils (Diaz and de Bertoldi, 2007).

The modern era of composting originated with Sir Albert Howard, the developer of the Indore process, who demonstrated and documented how animal manure and other sources of waste could be composted in the open air (Diaz and de Bertoldi, 2007). The modification of this method was supported in India and China (Diaz and de Bertoldi, 2007) and spread to Europe and North America. Over time, through continuous research into composting and optimization, the benefits of composting were revealed and the simple process of biological decomposition and stabilization graduated into the numerous composting techniques that are being practiced today.

1.1.2 Benefits of composting

One of the practical benefits of composting is its ability to reduce the pressure for landfill space by diverting away organic waste, which is 50-70% biodegradable

(Environment Canada, 2003). This benefit is significant since over the past decades the consumerism-centric lifestyle in society has resulted in increased waste generation. A typical North American citizen annually on a per capita basis generated 219 – 766.5 kg of waste (Farrell and Jones, 2009) while the average annual per capita waste generation in Canada in 2003 was 991 kg (Environment Canada, 2003). Eighty percent of the generated waste is disposed of in landfills. Thus, capturing and composting a fraction of this waste could dramatically conserve the landfill capacity for non-compostable waste.

A global benefit of composting is the abatement of greenhouse gases (GHG). The contribution of the waste sector to the GHG budget was estimated at 5% (Lou and Nair, 2009) with landfills being a major source (Abdushammala, 2009). The landfill gas (LFG) produced during the decomposition of organic content under anaerobic conditions is on average 55% CH_4, 44% CO_2, and 1% other trace organic compounds such as H_2S, N_2O and volatile organic compounds (VOCs) (Humer and Lechner, 1999; Ayalon, 2000). Composting, however, emits mainly CO_2 (which is 24 times less potent GHG than CH_4), H_2O and NH_3 (Chang *et al.*, 2009). Methane is also produced but only in anaerobic zones of the compost and upon reaching the oxygen-rich surface of a compost pile it further oxidizes into CO_2 (Chang *et al.*, 2009; USEPA, 2006).

Another benefit of composting is the agronomic value of the finished product. From a chemical standpoint, land applied compost has consistently demonstrated a better crop yield than commercial fertilizer (Courtney and Mullen, 2008); this is mainly due to the improved availability of macro and micronutrients such as potassium, calcium, magnesium and sodium (Courtney and Mullen, 2008; Perez-Piqueres *et al.*, 2006). From the standpoint of a soil's physical properties, compost was found to significantly enhance the soil's physical properties: such as its structural stability, porosity, water balance and thermal conditions (Pinamonti 1998; Myalavarapu and Zinati., 2009). From a biological standpoint, the positive effect of compost on biotic composition, with subsequent plant disease suppression caused by soil-borne pathogens, has been described (Perez-Piqures *et al.*, 2006; Crecchio *et al.*, 2004). Hoiting and Boehm (1999) explain the

mechanisms of plant disease suppression by compost in five successful steps: 1) parasitism against pathogens by beneficial microorganisms, 2) antibiotic production by beneficial microorganisms; 3) competition for nutrients by beneficial microorganisms; 4) induction of systemic resistance in plants; and 5) improved plant nutrition which enhances disease resistance.

The final practical benefit of composting discussed in this section is product hygienisation. Depending on its nature the composting feedstock can have pathogens and unwanted plant seeds. The heat generated during composting as a result of organic matter biodegradation raises the temperature in the pile (Tchobanoglous, 1993; Christnesen *et al.*, 2002; USEPA, 2003). Pathogen inactivation commences when temperature rises above 50°C; and when the temperature ≥50°C is maintained for a sufficient amount of time, almost all of the known human pathogens are believed to be inactivated. Although heat is the most prevailing factor for pathogen inactivation, some pathogen reduction in composting is also accomplished through competition with indigenous microorganisms, and the exposure to primary or secondary metabolites produced by some fungi and actinomycetes (Wichuk and McCartney, 2007).

1.1.3 Composting technologies

Composting can be carried out using aerobic and anaerobic mechanisms of biodegradation - in both methods the end product is a humus-like, organically stable material. Aerobic composting is preferred because its main byproducts are carbon dioxide, water, and heat; anaerobic composting produces methane, carbon dioxide and many low molecular weight metabolites, which result in an offensive odor. In addition, anaerobic composting releases significantly less heat, thereby operating in the mesophilic range (Tchobanoglous *et al.* 1993), which is not sufficient for sanitation purposes (Tchobanoglous *et al.*, 1993; Haug, 1993; Epstein, 1997).

Aerobic composting technologies can be classified into three types: windrow, static piles (e.g. passively or actively aerated), and reactors (e.g. in-vessel,

channel) (Chiumenti *et al.*, 2005). All of these methods produce good compost provided they are properly managed. Considerations like space requirements, construction and operation costs, process controls and process flexibility usually determine which technology should be used. As a general rule, the cost, operator skills, process and emission control increase as one upgrades from windrow to static piles to reactors; reciprocally space requirement, climate dependency and process flexibility decrease as one follows the same order (Farrell and Jones, 2009).

1.1.4 Abiotic parameters in composting

The following physical and chemical parameters influence the composting process: moisture content (MC), free air space (FAS), temperature, pH, and carbon to nitrogen ratio (C:N) (Agnew and Leonard 2003).

Studies have demonstrated that MC is the critical design and operating factor. It has a direct influence on temperature, chemical properties, microbial activity, and the immobilization of heavy metals in composting (Agnew and Leonard, 2003; Tiquia *et al.*, 1996; Tiquia *et al.*, 1998; Khan *et al.*, 2009). Most of the composting facilities operate at 40-60% MC. These conditions are optimal for microbial activity and meet stabilization and volume reduction objectives (Luo *et al.*, 2008; Khan *et al.*, 2009; Tchobanoglous *et al.*, 1993). Moisture content below 40% reduces microbial activity and above 60% can create anaerobic conditions because of the blocked pore space (Epstein 1997).

FAS is the portion of the total pore space in the system that is not occupied by water (Epstein 1997). It is essential for the proliferation of aerobic microbial communities in the compost pile. According to Epstein (1997), the maximum microbial activity is achieved at 30% FAS. High moisture or compacted feedstock can drastically limit the supply of oxygen to microorganisms, turn the process into an anaerobic one and cause odor nuisances (Agnew and Leonard, 2003). The FAS of a compost pile can be calculated from its bulk density (wet/dry) and particle density through the following relationship (Chang and Chen, 2010):

- FAS(%) = 100 x (1-Bulk density / Particle density)

The problems associated with low FAS can usually be eliminated by adding organic and inorganic bulking agents (e.g. saw dust, wood chips, or cardboard). The added benefits are at least threefold: (a) MC adjustment, (b) provision of structural stability, and (c) enhancement of oxygen transportation through the entire pile (Haug 1993, Adhikari *et al.*, 2008). The composition of bulking agents should be such that they maintain an optimum pH and C:N ratio.

pH affects the growth response of microorganisms in compost. Maximum thermophilic composting occurs at a pH range of 7.5 to 8.5. When pH is acidic the temperature is usually below 55ºC, which is not high enough for pathogen inactivation. pH adjustment is required if wood shavings and wasted animal feed are to be used; otherwise problems in reaching thermophilic temperatures can be encountered.

Carbon and nitrogen are the two most important nutrients. Whereas carbon is utilized as an energy source, nitrogen is used for protein synthesis. Their ratios in the compost affect the composting process, the microbial community and quality of the final product (Epstein, 1997). The optimum carbon to nitrogen ratio (C:N) is believed to be 30:1. This ratio is usually adjusted by adding nitrogen rich substrates into carbon rich feedstock. A lower C:N ratio leads to high initial decomposition of available substrate, rapid increase in temperature and long duration of heating phase, nitrogen volatilization in the form of ammonia, reduced microbial diversity and limited substrate availability for fungal re-colonization and subsequent utilization of recalcitrant fibers such as cellulose, hemicellulose and lignin (Eiland *et al.*, 2001; Epstein, 1997). A high C:N ratio results in slow decomposition of available carbon due to depletion of available nitrogen, slow temperature rise, short duration of heating phase, and inferior sanitation (Eiland *et al.*, 2001; Epstein, 1997).

1.1.5 Biotic parameters in composting

Microbial metabolism is the driving engine in the composting process. The understanding and identification of microorganisms in composting are vital for successful operation. Current knowledge of the compost microbial community is based on either culture-dependent or molecular methods (Chroni *et al.*, 2009). Culture-dependent methods include isolation, identification, and enumeration of microorganisms; whereas molecular methods utilize different approaches, such as phospholipid fatty acid (PFLA) determination (Frostegård *et al.*, 2013), polymerase chain reaction (PCR), and 16S rRNA (Chroni *et al.*, 2009). Through these methods, it is known that a large variety of mesophilic, thermotolerant, and thermophilic microorganisms including bacteria, actinomycetes, and fungi are responsible for the biodegradation of organic substances in compost and other self-heating organic materials (Hassen *et al.*, 2001). When these microorganisms work in consortia, they degrade organic matter more readily than in isolation (Atkinson *et al.*, 1997). Microbial metabolism can be manipulated during the composting process by continuous adjustment of the temperature, MC, pH, oxygen concentration, and C:N ratio.

1.1.5.1 Bacteria

A large variety of bacteria can be isolated from a single composting pile. The new cells that are produced during composting eventually become part of the active biomass. In the beginning phase, when organic rich feed is introduced to the composting process, mesophilic bacteria are the most prevalent. Hassen *et al.* (2001) studied the dynamics of microorganisms in a compost of municipal solid waste. The study demonstrated that the microbial activity during composting was mainly due to the mesophilic bacterial community. Their numbers at the beginning of composting fluctuated between $8.5x10^8$ and $5.8x10^8$ bacteria g^{-1} dry weight (DW). During the thermogenic stage, mesophilic bacteria were partially killed or inactivated (reduced to $1.8x10^7$ g^{-1} DW). They again regained dominance ($1.8x10^8$ cells g^{-1} DW) during the cooling phase when the temperature fluctuated between 30ºC and 40ºC. A similar succession pattern was reported by Chroni *et*

al. (2009) when they analyzed the microbial dynamics of organic waste composted in windrow.

According to Insam and de Bertoldi (2007), the observed fluctuation patterns can be explained by the abundance of soluble sugars and starches in the environment at the beginning of composting. Their biodegradation results in explosive growth of mesophilic bacteria, which outcompete all other microorganisms present in compost. By the time these nutrients become limited, the composting process has already reached the thermophilic stage during which mesophilic bacteria can no longer compete for nutrients with thermophilic, thermotolerant bacteria, thermophilic fungi and actinomycetes and natural die-off occurs. During the thermophilic stage, thermophilic bacteria, in consortia with fungi and actinomycetes, engage in the breakdown of proteins, fats, cellulose, and hemicellulose matter. When the heat generated as a result of microbial biodegradation becomes less than the heat lost to the ambient surroundings by conduction, convection and radiation, the compost starts to cool down. This is called a curing phase, during which the mesophilic bacteria start to recover from thermal shock. However, at this time their growth kinetics are limited by nutrient availability, as lignin and other highly resistant compounds are what is left for the curing phase.

1.1.5.2 Fungi

Different fungi have been isolated from composting. Excluding thermophilic fungi, most of them become inactive when the temperature reaches 50ºC; above 65ºC their activity is further limited. Moisture content and acidity as well as microbial antagonism and antibiosis are the major factors affecting fungal populations in composting (Hassen *et al.*, 2001). Hassen *et al.* (2001) reported that during composting, the number of yeast and filamentous fungi remained stable until the end of the mesophilic phase ($4.5x10^6$ cells g^{-1} DW). It dropped appreciably during the thermophilic phase ($6.3x10^3$ cells g^{-1} DW), and even more towards its end ($2.6x10^3$ cells g^{-1} WDW). Fungal growth detected during the cooling phase was explained by mesophilic temperatures, MC, and an alkaline pH

in the compost material. Chroni *et al.* (2009) also reported a dramatic decline in fungi population at thermophilic temperatures (from $2.5x10^6$ colony forming units (CFU) g^{-1} DW to $3.2x10^2$ CFU g^{-1} WDW) and their regrowth thereafter.

During thermophilic and cooling phases, fungi are highly recognized for their breakdown of proteins, fats, lignin and other recalcitrant organic matter. Most fungi grow in low moisture conditions and tolerate a wide range of pH values. Gaur *et al.* (1982) reported that fungi are key players in the biodegradation of dry, wide C:N ratio materials, and their activity in general improves the quality of the finished product. Therefore, they are important in backyard composting of yard residues and leaves, where the temperature rarely exceeds the mesophilic range.

1.1.5.3 Actinomycetes

Actinomycetes are a group of microorganisms with properties between bacteria and fungi. In composting, actinomycetes remain in high numbers even at elevated temperatures and usually outcompete fungi (Atkinson *et al.*, 1997). They become especially active towards the end of the composting process (Haug, 1993). The most striking characteristic of actinomycetes in composting is their ability to biodegrade highly resistant organic compounds. Some studies have demonstrated that actinomycetes can even break down polyester residues at temperatures >50ºC. It is known that at least 15 isolates of the genus *Streptomyces*, 8 isolates of the genus *Microbispora*, 4 isolates of *Actinomadura*, 3 isolates of genus *Thermoactinomyces*, and one isolate of the genus *Saccharomonospora* are capable of degrading polyesters in compost (Tseng *et al.*, 2007). The literature suggests that there are more than three hundred thermophilic actinomycetes that have been isolated from various environments and which are capable of degrading polyesters and their derivatives (Tseng *et al.*, 2007). Thus, maintaining actinomycetes populations in composting becomes especially important from the perspective of finished compost quality.

1.1.6 Composting feedstock

Traditionally, composting was used to treat farming waste. Nowadays, it is also used in a cost-effective and socially acceptable manner for any solid or semisolid waste with a high biodegradable fraction such as yard trimmings, food waste, municipal solid waste (Deportes *et al.*, 1998) and sludge from municipal treatment facilities (Barrena *et al.*, 2005). Composting facilities are generally designed to compost more than one type of feedstock. Depending on their physical and chemical properties, different feedstock streams could be composted separately or co-composted in order to achieve an optimum recipe.

1.1.6.1 High pathogen feedstock

Much of the waste, predominantly fecal matter, harbors pathogenic microorganisms and is therefore perceived as high pathogen feedstock. Biosolids are a typical example of high pathogen feedstock bound for composting. The term "biosolids" was given by the Water Environment Federation to describe sludge from wastewater treatment plants that had already been treated through one or more controlled processes, such as anaerobic digestion, that reduce but do not eliminate pathogens and attractiveness to vectors (Torri *et al.*, 2012, USEPA, 2003). Since this sludge is comprised of human fecal wastes, contaminants from homes, industries, business, and storm-water (Harrison *et al.*, 1999) its pathogen content is high. According to Sidhu and Toze (2009) the average pathogen level could be in the ballpark of 3.5×10^2 plaque forming units (PFU) g^{-1} dry weight (DW) for enteric viruses; 2.9×10^3 CFU g^{-1} dry weight (DW) for *Salmonella*; 1.5×10^4 g^{-1} dry weight (DW) for *E. coli* and up to 5.6×10^4 cysts g^{-1} dry weight (DW) for *Giardia*. If the aim of composting high pathogen feedstock is the recycling of its nutrients then the proper sanitation through irreversible inactivation of all pathogens before the use of the end product needs to be ensured at all times.

1.1.7 Canadian composting industry profile

According to the most recent survey by Composting Council of Canada (CCC, 2011 unpublished data), centralized composting was practiced in 10 provinces (Table 1-1).

Table 1-1. Nationwide outlook at composting practice in Canada*

Province	# of facilities	Design capacity(tonnes)	% of its Design capacity used
AB	29	684,100	71.41
BC	36	1,001,434	59.34
ON	50	1,254,500	72.05
QB	15	187,075	63.96
SK	13	6,172	17.60
MB	12	31,505	43.84
NS	21	245,100	72.32
NB	18	652,300	66.21
PEI	1	30,000	76.67
NWT	3	7,000	32.70
Nationwide	198	4,099,186	67.22

*The data is based on the numbers provided by the survey conducted by the Composting Council of Canada in 2011 among its member facilities. The actual statistics may vary.

The provinces had a cumulative composting design capacity of 4,100,000 tonnes distributed amongst 198 facilities. These facilities reportedly composted 2.8 million tonnes of biodegradable waste, collectively, thereby utilizing 67.22% of the national design capacity. Prince Edward Island, Ontario, Nova Scotia and Alberta were leaders in utilizing their existing capacities and raising the nationwide average, while Saskatchewan was the province with least capacity and least loading per its capacity. It is believed that these numbers will increase in the coming years.

The single most popular technology used in Canada was windrow composting, reportedly practiced in 75% of facilities. This was followed by in-vessel technology (12%) (Figure 1-1). Other technologies were below 10%.

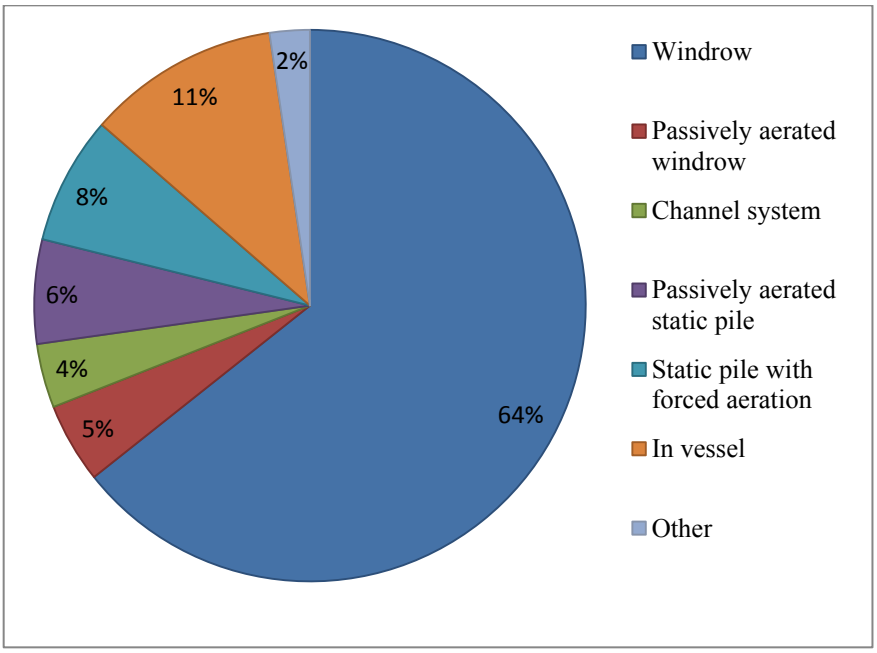

Figure 1-1. The distribution of composting technologies in centralized composting facilities across Canada. Numbers refer to the percentage of total facilities

Windrow composting superseded other technologies in all provinces except Prince Edward Island, which at the time of the survey operated a single plant with the in-vessel technology. In fact, 88% of in-vessel technology was distributed between three provinces: Ontario (38%), Nova Scotia (33%), and British Columbia (17%). Saskatchewan, Manitoba, and New Brunswick did not have in-vessel technology.

The most popular compost feedstock was yard waste that was composted in 81% of the facilities. Other popular waste streams were wood (53%) and food wastes (39%). Institutional waste, which has a high content of biodegradable organic fraction, was composted in 14% of the facilities.

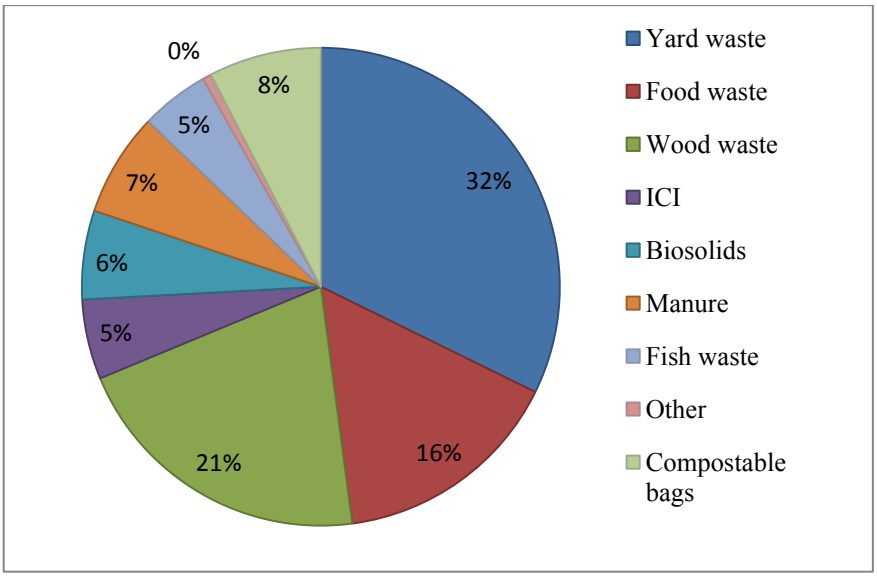

Figure 1-2. The relative popularity of compost feedstock in Canada. The biodegradable waste which was not classified into either class was included into the category "Other".

High pathogen feedstocks included biosolids and animal manure. Biosolids was composted in 30 facilities while manure was composted in 35. The leaders in biosolids composting were the provinces of British Columbia with 10 facilities, followed by Alberta and New Brunswick with 5 facilities each. One facility was

reported for Ontario in the town of St. Mary. Biosolids were not composted in Manitoba, Prince Edward Island and the Northwestern Territories. Although it was not explicitly stated which technology was used for biosolids composting, the data suggests it was either done in windrows or static piles.

1.2 Composting regulations

Composting feedstock could be contaminated with pathogenic microorganisms due to the nature of the feedstock itself or due to the cross-contamination of the feedstock during collection, storage and transportation. The countries where centralized composting is practiced have certain standards that the compost producers have to satisfy in order to ensure proper protection of the public and the environment from pollution with pathogenic microorganisms by offering adequate sanitation. These standards can differ in stringency depending on the jurisdiction. This section provides a review of compost sanitation requirements in North America as well their comparison with regulations and guidelines in Europe and Asia.

1.2.1 Composting regulations in North America

The foundations for composting guidelines in North America were set at the time when the United States faced a need to develop a sophisticated set of rules to utilize biosolids as the Federal Water Pollution Control Act Amendments of 1972 (Epstein, 1997; NBP, 2004) restricted the discharge of sewage into waterways while encouraging its beneficial use. Consequently, in 1977, Congress amended the Act by adding a new section, 405(d), that required USEPA to develop regulations containing guidelines to (NBP, 2004):

- identify alternatives for biosolids use and disposal,
- specify which factors must be accounted for in determining the methods and practices applicable to each of these identified uses, and
- identify concentrations of pollutants that would interfere with each use.

In 1987, Congress once again amended section 405 to establish a timetable for developing biosolids use and disposal guidelines that directed USEPA to (NBP, 2004):

- identify toxic pollutants that may be present in biosolids in concentrations hazardous to public health and the environment,
- promulgate regulations that specify acceptable management practices and numerical concentration limits for these pollutants in biosolids.

Through cooperation with research institutions, the USEPA consequently developed a regulation which specified the different acceptable hygiene levels depending on the ultimate treatment method (including composting) and use. The guidelines resulted in the promulgation in 1993 of "The Standards for the Use and Disposal of Sewage Sludge, Code of Federal Regulations, Title 40, Part 503", collectively referred to as the "Part 503 Rule." (Yanko, 1988; NBP, 2004).

1.2.2 Part 503 Rule

The Part 503 Rule establishes requirements for the final use of biosolids when they are land applied, disposed of, or incinerated (USEPA, 2003). In compliance with the Part 503 Rule, the amount of biosolids that can be land applied depends on the pollutant concentration, pathogen concentration, and vector reduction criteria. Sites where biosolids can or cannot be applied depends on whether biosolids are classified as Class A or Class B. If biosolids contain pathogens (*Salmonella* sp., fecal coliform, enteric viruses, and viable helminthes ova) below detection levels the biosolids meet Class A. In Class B biosolids, the pathogens are still detectable but their levels are not detrimental. Class A biosolids can be applied everywhere, while Class B are site restricted.

Class A or Class B pathogen reduction in biosolids can be achieved using treatment alternatives listed in the Part 503 Rule. These alternatives are: 1) thermal treatment, 2) high pH-high temperature, 3) other processes which inactivate pathogens, 4) unknown processes which inactivate pathogens, 5) process to further reduce pathogens (PFRP), or 6) a process equivalent to PFRP.

1.2.2.1 US Composting guidelines

Composting is recognized under the Part 503 Rule as a PFRP. The end product from composting is considered "Class A" provided the treatment satisfies process and end-product criteria such that:

- if the biosolids had been treated with the in-vessel or aerated static pile composting method the temperature of the biosolids should be maintained at 55°C or higher for three days; **OR** if they had been treated using the windrow composting method, the temperature of the biosolids should be maintained at 55°C or higher for 15 days or longer during which the windrow should be turned a minimum of five times; **AND**
- **either** the density of fecal coliform (FC) in the biosolids should be less than 1,000 most probable numbers (MPN) per gram of total solids DW; **OR** the density of *Salmonella* sp., bacteria in biosolids should be less than 3 MPN per 4 grams of total solids DW.

The treatment alternatives under the Part 503 Rule for Class B are more lenient. The feedstock should either: 1) contain geometric mean fecal coliform density from seven samples collected over a 2 week period below 2,000,000 MPN per gram of biosolids DW, 2) be treated in a process to significantly reduce pathogens (PSRP), or 3) be treated in a process equivalent to PSRP.

Lastly, all pathogen standards established for composting within the framework of the Part 503 Rule are operational rather than risk-based and restricted to fecal coliform and *Salmonella* sp. (NRC, 2002). The inclusion of fecal coliform was due to the fact that they are more abundant in the environment, may be affected by environmental stresses in a fashion similar to most intestinal pathogenic bacteria such as *E. coli* sp., *Shigella* sp., and *Salmonella* sp. (Yanko, 1988; NRC, 2002) as well as many viral enteric pathogens. *Salmonella* sp. was added into the test due to the fact that no indicator organism perfectly replicates the fate of pathogens when exposed to environmental stresses; and due to the elevated concerns over increased *Salmonellosis* outbreaks in the United States (Yanko, 1988). The level of indicator organisms for Class A biosolids and the flexibility to

test either fecal coliform or *Salmonella* sp. levels stems from the study which demonstrated that when the level of fecal coliform falls below 1,000 MPN g^{-1} biosolids the level of *Salmonella* falls below the limit of detection respectively (Yanko, 1988).

After promulgation of the Part 503 Rule, composting requirements were widely adopted (with slight modifications) by many countries (e.g. Canada) as guidelines to ensure not only the biosolid's sanitary conditions, but also the sanitary conditions of any compost from any feedstock (Brinton, 2000).

1.2.3 Overview of Canadian and international compost guidelines and regulations

A summary of the time-temperature requirements in various countries, as well as those in Canada and the United States, is provided in Table 1-2. The guidelines vary between jurisdictions, with many other countries (especially those in Europe) specifying either higher temperatures or longer exposure times (or both) than those required in North America. In Europe, the required time-temperature conditions are also feedstock-dependent. For instance, if animal byproducts (e.g. hides, hooves, horns, feathers, bold, shells, raw milk, hatchery by-products, etc.) from healthy animals are included in the feedstock, there is a requirement to attain 70°C for 1 hour (Barrena *et al.*, 2009). The time-temperature requirements for compost in North America are less rigorous as compared to those elsewhere, with the exception of China, which specifies a lower temperature (50 to 55°C) for a slightly longer period of time (5 to 7 days), and Australia, New Zealand, and Italy, which don't specifically state that any additional composting time is required for windrow systems.

It should be noted that in Canada, a province or territory is able to set its own guidelines (CCME, 2005). Many provinces and territories have adopted the CCME guidelines, in whole or in part, while a few others (including those listed in Table 1-2) have used modified versions. For example, Ontario has specified that both windrows and aerated static piles must attain 55°C for 15 days, while

British Columbia has adopted the USEPA guidelines for Class A and B composts. Nonetheless, these alternate guidelines are still lenient when compared to those in other areas.

Many jurisdictions also require some degree of testing for pathogenic or indicator organisms in the final compost product (Table 1-3). Some areas (e.g. most of Canada (CFIA, 2009; BNQ, 2005; CCME, 2005) and the United States (USEPA, 2003)) require that all compost to be disposed of or used must be tested for indicator organisms, while other areas require periodic testing only (for example British Columbia, Germany, and the United Kingdom) (BC, 2007; BSI, 2005; BioAbfV, 1998).

There are also variations between jurisdictions in terms of whether one or both of the pathogen/indicator tests and indirect process monitoring (time-temperature) criteria are required. In Germany, the United States, and the United Kingdom, compost facilities are required to monitor both time-temperature and the sanitary quality of the finished product. In Ontario (OME, 2004), compost facilities are only required to meet the time-temperature conditions, while in Quebec only a microbiological analysis of the final product is required (Quebec 2008; BNQ 2005). In the rest of Canada (e.g. CCME-adopting provinces and BC), both microbiological and time-temperature criteria are required for compost materials which include feedstock other than yard waste. If compost is produced from yard waste alone, only the time-temperature criteria are required to be met (BC, 2007; CCME, 2005).

Additionally, to add an extra degree of assurance that compost sanitation conditions will be achieved, in some jurisdictions (e.g. United Kingdom and Germany), all new composting facilities (and new processes to be incorporated at existing facilities) must undertake a process validation to ensure that the operating procedures used at that particular facility will be adequate to meet the requirements set out in the standards (e.g. BSI, 2005; BioAbfV, 1998). In Canada, process validation is not required.

Taking into account the above discussion, it is apparent that compost guidelines in Canada are not overly conservative in the world in terms of providing assurance that the compost is hygienic.

Table 1-2. Comparison of compost time-temperature requirements in various jurisdictions.

Jurisdiction	Reference	Composting Technology	Time-Temperature Guidelines	Monitoring Requirements	Comments
Canada[a]	CCME 2005	Aerated static pile OR In-vessel	≥ 55°C for 3 days	not specified	It is preferable for a static pile to be covered with a layer of insulating material to retain heat inside the compost.
		Windrow	≥ 55°C for 15 days		Windrow should be turned at least 5 times during high-temperature period.
Canada - British Columbia (Class A)	BC 2007	Aerated static pile OR In-vessel	≥ 55°C for 3 days	not specified	Aerated static pile should be insulated.
		Windrow	≥ 55°C for 15 days		Windrow should be turned at least 5 times during high-temperature period.
Canada - British Columbia (Class B)	BC 2007	All types	≥ 40°C for 5 days, plus ≥ 55°C for 4 hr	not specified	
Canada - Ontario	OME 2004	Windrow OR Aerated static pile	≥ 55°C for 15 days	Temperature must be recorded daily during high-temperature phase. Monitoring locations must be at least one meter into the pile at enough points to provide a profile of the pile.	• It is preferable for a static pile to be covered with an insulating layer. • Windrows should be turned at least 5 times during the high-temperature period.
		In-vessel	≥ 55°C for 3 days		

Table 1-2. Continued.

Jurisdiction	Reference	Composting Technology	Time-Temperature Guidelines	Monitoring Requirements	Comments
Europe	Barrena *et al.* 2009	All types with ABP[b]	≥ 70°C for 1 hour		
Australia	Hogg *et al.* 2002	All types	≥ 55°C for 3 days		Windrows must be turned at least 3 times. Internal temperatures must reach 55°C for 3 days between turns.
Austria	Hogg *et al.* 2002	All types	not specified	Temperatures must be monitored every working day during thermophilic phase.	
Belgium	Hogg *et al.* 2002	All types	≥ 60°C for 4 days		
China	CEPA 1987	All types	50 to 55°C for 5 to 7 days		
Sweden	Hogg *et al.* 2002	All types	≥ 55°C to 70°C		Exact requirements are dependent upon the compost facility and risk potential of the materials.

[a] CCME sanitation requirements indicate that only one of the time-temperature or indicator organism requirements must be met if the compost includes yard waste only. If the compost contains any other feedstock, both the time-temperature and indicator organism requirements must be met.

[b] In Regulation 1774/2002 of the European Parliament, ABP ("animal by-products", including hides, hooves, horns, feathers, bold, shells, raw milk, hatchery by-products, etc) showing no sign of disease can be treated by composting.

Table 1-2. Continued.

Jurisdiction	Reference	Composting Technology	Time-Temperature Guidelines	Monitoring Requirements	Comments
Denmark	Hogg *et al.* 2002	All types	≥ 55°C for 14 days		
France	Hogg *et al.* 2002	All types	≥ 60°C for 6 days		
Germany	BioAbfV 1998	Windrow OR Static pile	≥ 55°C for 14 days OR ≥ 65°C for 7 days	Temperatures must be recorded at least once per working day, and preferably continuously. Temperatures to be monitored in at least three representative zones.	Designed to eradicate human, animal, and plant pathogens.
		In-vessel	≥ 55°C for 14 days OR ≥ 60°C for 7 days		
Italy	Hogg *et al.* 2002	All types	≥ 55°C for 3 days		
Netherlands	Hogg *et al.* 2002	All types	≥ 55°C for 4 days		
New Zealand	Hogg *et al.* 2002	All types	≥ 55°C for 3 days		

Table 1-2. Continued.

Jurisdiction	Reference	Composting Technology	Time-Temperature Guidelines	Monitoring Requirements	Comments
United Kingdom	BSI 2005	All types	≥ 65°C for 7 days[c,d]	Monitor temperature every working day during sanitation (high-temperature) phase.	• Where applicable, piles should be turned at least 2 times. Insulation may be required for static systems. • Designed to eradicate all human and animal, and most plant pathogens.
United States (Class A)	USEPA 2003	Aerated static pile OR In-vessel	≥ 55°C for 3 days	Temperature monitoring must be done at many locations and a range of depths. Areas likely to be coolest should be included in the monitoring program.	• Temperatures from all points, not just the average, must meet the required conditions. • An insulating layer of at least 0.3 m depth is recommended for static piles.
		Windrow	≥ 55°C for 15 days		Windrow should be turned at least 5 times during high-temperature period. Turning should occur after the core has attained 55°C for 3 consecutive days.
United States (Class B)	USEPA 2003	All types	≥ 40°C for 5 days, plus ≥ 55°C for 4 hr		

[c] The published time-temperature guidelines are a recommendation only. "It is the responsibility of the composter to set critical limits for each sanitation parameter, as appropriate to intended uses of the compost grades, input material types, and capabilities of the composting system." (BSI 2005)
[d] Not necessarily consecutive days.

Table 1-3. Comparison of compost pathogen and indicator organism testing requirements in various jurisdictions.

Jurisdiction	Reference(s)		Fecal coliforms (MPN g^{-1} dry solids)	*E. coli* (MPN g^{-1} dry solids)	*Salmonella* spp.	Comments
Canada	CCME 2005, BNQ 2005, CFIA 2009		<1,000	n/a	Absent[a] OR <3 MPN / 4g dry solids[b]	CCME and BNQ require an additional test for *E .coli* if high levels of fecal coliforms are suspected to be due to false positives.
Canada - British Columbia	BC 2007	Class A	<1,000[c]	n/a	n/a	Analyze 7 representative samples once every 1000 tonnes dry weight or once per year, whichever is first.
		Class B	<2,000,000	n/a	n/a	
Canada - Quebec	Quebec 2008	Category P1	n/a	n/a	not detected in 10 g wet weight	Pathogen criteria in Quebec combine pathogen indicator and biological stability requirements. Category P1 compost must meet the *Salmonella* requirement and pass one of three stability tests. Category P2 compost must meet the *E. coli* requirement and OUR must be ≤ 1500 mg kg^{-1} OM h^{-1}.
		Category P2	n/a	<2,000,000	n/a	
China	CEPA 1987	All types	unclear[d]	n/a	n/a	• Eggs of roundworm must experience a 95% to 100% reduction. Flies and fly larvae must be absent.

Note: n/a, not applicable.
[a] Presence or absence of *Salmonella* is used with the ISO-GRID, AOAC 991.12, and MFLP-75 methods for *Salmonella*, with a 100 g test portion (BNQ 2005).
[b] 3 MPN per 4g compost is the detection limit of the MA. 700 – Sal-tm 1.0 Method for *Salmonella* (BNQ 2005).
[c] Fecal coliform monitoring is not required if compost contains yard waste only.
[d] Units are not specified for the required fecal coliform levels in CEPA 1987.

Table 1-3. Continued

Jurisdiction	Reference(s)		Fecal coliforms (MPN g^{-1} dry solids)	*E. coli* (MPN g^{-1} dry solids)	*Salmonella* spp.	Comments
Europe	Barrena 2009	All types with ABP[e]	n/a	n/a	not detected in 25 g	• Enterobactericeae must be ≤ 300 / g
Germany	BioAbfV 1998		n/a	n/a	not detected in 50 g wet weight.	• Product analysis is required once every 6 months for facilities processing less than 3000 t/yr, and once every 3 months for facilities processing over 300 0 t/yr.
Greece	Lasaridi *et al.* 2006		n/a	n/a	Absent	• Additionally, enterobacteriaceae must be absent • Applicable for composts produced from sewage sludge or municipal solid waste.
United States	USEPA 2003	Class A[f]	<1,000	n/a	<3 MPN / 4g dry solids	Class A compost must meet one of the fecal coliforms or *Salmonella* requirements. All material to be used or disposed must be tested.
		Class B[g]	<2,000,000	n/a	n/a	
United Kingdom	BSI 2005		n/a	< 1,000 CFU/g fresh mass	not detected in 25 g wet weight	Product analysis is required once every 12 months or once for every 5000 m^3 of compost produced, whichever is sooner.

[f] The implicit objective for USEPA Class A products is to reduce the density of all pathogens to below detection limits (e.g. *Salmonella sp.* should be < 3 MPN per 4 g dry solids; enteric viruses should be < 1 PFU per 4 g dry solids; and viable helminth ova should be < 1 viable ova per 4 g dry solids). However, monitoring is required for the indicators organisms listed in Table 1-2 (fecal coliforms or Salmonella) only (USEPA 2003).

[g] Class B products should have reduced numbers of pathogens, but pathogens may not be completely eliminated.

1.3 Pathogen survival during composting

In a previous literature review (Wichuk and McCartney, 2007), it was shown that while pathogens are often eliminated by high-temperature composting, viable pathogenic organisms (bacteria, viruses, protozoa, and helminthes) are sometimes detected in compost that has met the North American-required sanitation conditions. Some potential reasons for pathogen survival were identified (Wichuk and McCartney 2007), including: 1) regrowth from small numbers of surviving organisms (applicable for bacteria only); 2) recontamination of the compost; 3) inadequate time-temperature criteria; and 4) pathogen survival in cool zones of a compost pile, which are undetected due to inadequate temperature monitoring protocols.

Several studies on pathogen survival/inactivation during composting have been done since the Wichuk and McCartney (2007) literature review was conducted. An overview of the relevant features and results of the current key studies is presented in Appendix B. As in the initial literature review, results of different studies were contradictory, with pathogen inactivation occurring in some studies despite temperatures not reaching 55°C for 3 days, while in other studies the same organisms survived for extended periods even when these time-temperature conditions were met. Nonetheless, the results of these recent studies support the previous conclusion that pathogen survival sometimes occurs even when the required time-temperature conditions appear to have been achieved.

For example, Barrena *et al.* (2009) detected *Salmonella* spp. at the end of composting in 100L bins, even though the temperature, monitored at four locations, peaked between 68°C and 70°C. The authors indicated that this survival was most likely due to the existence of cool zones near the surface of the material. Inglis *et al.* (2010) examined the survival of several *Campylobacter* species during windrow composting, and observed that the genes of all the tested species could be detected throughout the high-temperature phase and after curing, despite pile temperatures peaking at approximately 79°C and remaining above 55°C for

over 175 days in some locations. Pourcher *et al.* (2005) detected non-pathogenic *E. coli*, and *Clostridium perfringens* after 4 months of composting and 3 months of curing. During this time, the pile temperature at two of three monitored locations peaked at 65°C and exceeded 55°C for extended periods, though a location at the bottom of the pile did not reach 50°C. Similarly, it took up to four months for *Listeria monocytogenes* to be completely inactivated, during which time two of the three monitored locations exceeded 55°C for extended periods. *Salmonella* spp. were not detectable at the first sampling date (after 1 month of composting), nor were any infectious enteroviruses found, although genomes were still present (Pourcher *et al.* 2005). In a small-scale study involving bin composting of various feedstocks, in which all batches exceeded 55°C for varying amounts of time, both *Salmonella* spp. and *Campylobacter* spp. were detected in a portion of the mature composts. *Cryptosporidium* oocysts were also detected after the high-temperature phase, though they were no longer detectable after maturation (Rao *et al.* 2007). Wéry *et al.* (2008) detected *E. coli*, *C. perfringens*, and *Enterococcus* spp. at the end of the high-temperature phase of aerated static pile composting, though temperatures at the monitored locations exceeded 60°C for 2 to 4 days in all trials. *C. perfringens* and *Enterococcus* spp. were both present even after compost curing. In a study by Xu *et al.* (2009), pathogenic *E. coli* O157:H7 survived for up to 8 days after the temperature at the sampling location in an insulated composting bin exceeded 55°C. However, this organism was inactivated within 28 days even at locations which remained below 50°C. *Campylobacter jejuni* DNA was isolated in the high-temperature areas until day 84, and in the cooler zones until the end of composting.

In contrast, Collick *et al.* (2007) looked at the survival of *Ascaris suum* in a biodrying composting system, in which pathogen inactivation can be attributed to both heating and drying. This organism, which is generally considered to be environmentally resistant, was reduced to below detection limits within the first 4 days of composting, even at one location within the pile which didn't exceed 40°C. Similarly, Szavobá *et al.* (2010) observed the inactivation of inoculated *A. suum* eggs by day 6 of composting, even when temperatures didn't reach 55°C by

this time. In another study, inoculated *Salmonella* spp. were inactivated within 8 days in all trials, regardless of the temperature (the highest temperature observed was 56.8°C, and many trials remained below 55°C) (Erickson *et al.* 2009). In fact, the longest survival was actually observed in the trial which attained 56.8°C. Fourti *et al.* (2008) examined the survival of *Salmonella* sp. and *Staphylococcus aureus* in windrows. In this study, both organisms were present until the average pile temperatures reached 55°C, after which time they could no longer be detected. Kaszewska *et al.* (2006) observed that, during the summer when temperatures in parts of the pile exceeded 53°C to 55°C for up to 16 days, both *E. coli* and *Salmonella seftenberg* W 775 could not be detected within the first 7 to 9 days of composting. In the winter, however, temperatures remained below 50°C and both organisms were still present at the end of the trial. Saidi *et al.* (2008) observed the inactivation of *Salmonella* spp. in windrows once temperatures reached 55°C.

Grewal *et al.* (2006; 2007) performed two experiments which simulated composting by incubating swine manure mixed with saw dust and/or straw at 55°C. In one experiment, naturally-occurring *Listeria* spp., *Escherichia coli*, and *Salmonella* spp., along with inoculated *Mycobacterium avium* subsp. *paratuberculosis*, were monitored (Grewal *et al.* 2006), while in the other *Listeria monocytogenes* and *Salmonella enterica* were inoculated into the compost materials (Grewal *et al.* 2007). The naturally-occurring organisms could not be detected after 3 days of exposure to temperatures at or exceeding 55°C. However, the inoculated organisms survived longer: up to 28 days at 55°C for *S. enterica* and 56 days or more at 55°C for *L. monocytogenes*. *M. avium* was not detected by culturing after 3 days at 55°C, but the genes of this organism could be detected through to 56 days at 55°C, indicating either that genes are present after an organism becomes unviable, or that the organism may be viable but unculturable due to the environmental conditions (Grewal *et al.* 2006). The contrasting results of these two studies imply that differences in survival rates exist even when organisms from the same genus are exposed to very similar conditions. The authors hypothesized that the differences in survival rates may be due to: different

initial concentrations of the organisms, to the differences in specific species present in the different trials, or to differences in the substrate used (Grewal *et al.* 2007). These same factors may help to explain the variation of results seen in other studies as well.

This examination of up-to-date literature leads to the same conclusion that was reached previously (Wichuk and McCartney 2007): compost products appearing to meet the required North American time-temperature guidelines do not consistently achieve a pathogen-free status.

1.3.1 Hygienic Quality of Finished Compost Products

The picture would be incomplete without analyzing those studies that examined the hygienic quality of finished composts. All of them showed that pathogens were present in a portion of products ready for sale. Briancesco *et al.* (2008) looked at the presence of various pathogenic organisms in finished compost products from 20 composting facilities in Italy. *Cryptosporidium* cysts and *Giardia* oocysts were absent from all final products, as were helminth eggs. *Salmonella* spp. reduction from the levels present in the feedstock occurred at most composting plants, but in all 20 products this organism was detected. *Salmonella* levels in finished composts ranged from $< 0.2 \times 10^1$ MPN g^{-1} DW up to 1.6×10^2 MPN g^{-1}. Similarly, non-pathogenic *Escherichia coli* were present in most final products, at levels up to 2.1×10^4 MPN g^{-1}. *Clostridium perfringens* was also present, at levels up to 7.0×10^3 CFU g^{-1}, in all compost products. An interesting conclusion of this study was that composts containing sewage sludge tended to have a better hygienic quality than those containing green waste and municipal solid waste (Briancesco *et al.* 2008). In a similar study in Greece, 23 bagged compost products were tested for pathogens (Lasaridi *et al.* 2006). While *Salmonella* spp. were not detected in any of the sampled materials, the human pathogen *Staphylococcus aureus* was detected in 17% of the products at high levels ($> 10^5$ CFU g^{-1}) and *C. perfringens* was present in all but one of the composts (Lasaridi *et al.* 2006).

In the United States, Brinton *et al.* (2009) examined the pathogen content of 94 point-of-sale composts produced from green waste and excluding sewage sludge. Only one of the 94 composts contained *Salmonella* spp., which was detected at the low level of 1.8 MPN/4g. However, three of the tested composts contained pathogenic *Escherichia coli* O157:H7, 22 out of 47 tested composts contained detectable levels of *Listeria* spp., and 70% of 55 tested products contained *C. perfringens* (up to a level of $10^{4.88}$ CFU g^{-1}). The authors of this study also compared the hygienic quality of composts produced by different technologies and at different-sized plants. They found that composts produced by windrow methods had lower fecal coliform and non-pathogenic *E. coli* concentrations than those produced by other methods. They also saw that smaller facilities tended to have more hygienic products than larger facilities, when evaluated on the basis of non-pathogenic *E. coli* levels (Brinton *et al.* 2009).

Viau and Peccia (2009) also evaluated the sanitary quality of 10 compost products, ready for agricultural application, from several US states. In contrast to those sampled by Brinton *et al.* (2009), the feedstocks used in these composts included biosolids. Neither *Staphylococcus aureus* nor *Clostridium difficile* were detected in any of the product tests. However, *Legionella pneumophila* genomes were detected in 50% of the samples, with a median value of 10^4 genomic units/g, and *L. pneumophila* actually increased during composting in some samples. Adenovirus spp. genomes were detected in 70% of the samples, with a mean value of approximately 104 genomic units/g. The authors indicated that the number of infectious organisms is generally thought to be 2 to 4 orders of magnitude less than the total genomic count (Viau and Peccia 2009); nonetheless, there is likely to be some level of infective adenovirus and *Legionella* in these composts.

1.4 Problem statement

The literature review on pathogen survival during composting suggested that the outlook on composting achievements is not as optimistic as previously assumed. Regardless of maintaining recommended time-temperature conditions, there are still pathogenic microorganisms that survive composting and thrive in the finished product. Pathogens present in finished products have the potential to be transferred to humans via direct or indirect means, such as inhalation or ingestion of compost dust or ingestion of food supplies grown in and contaminated by improperly processed compost (Wichuk, 2007).

Hypothetically, the observed survival phenomenon may be due to either:

1. the non-uniform temperature distributions in large composting piles (Wichuk and McCartney, 2008); consequently temporal and spatial temperature variations falsely give the impression that the time temperature criteria have been met throughout the composting mass although they haven't been; or
2. the fact that the recommended time-temperature conditions are not adequate (the contact time with the desired temperature is shorter than it needs to be for industrial scale operations).

If either hypothesis is true, then the entire recommended thermal inactivation process may need to be revised to ensure that public safety is not threatened by obsolete assumptions. A thorough investigation is needed before any firm conclusions can be drawn. The existing technological capacity did not endeavor to confirm that the existing composting methods ensure the exposure of every particle to 55°C for three consecutive days and to correlate these findings with the survival of key pathogen microorganisms all on a compost particle level.

Previously Wichuk and McCartney (2008) proposed that the best approach to monitor the temperature experience of a random compost particle in compost would be to introduce directly into a process a device which would: a) be self-contained; b) behave like a random particle during pile agitation and settling; c)

be sturdy enough to endure physical and chemical stresses; and d) have the capability to record real time temperature data for an extended period of composting. Since then Wichuk and McCartney (2008) conducted some field testing of a custom made device and a commercial device which was primarily designed to record temperature profiles in aqueous environments. The authors, however, pinpointed that certain characteristics of the commercial device needed to be customized to match the realities of a composting environment.

1.5 Research objectives

The objectives of this research were as follows:

1) to modify the previously developed self-contained tool which could mimic random particle behavior in compost and record the temperature it is exposed to, while withstanding adverse operating conditions (Chapter 2);
2) to validate the modified self-contained tool's physical characteristics and evaluate the rationale of its recovery mechanism using a trommel screen in a field scale operation (Chapter 2);
3) to design and propose the compost temperature sampling framework to account for temporal and spatial temperature variations that inherently exist in open composting systems such as windrow and static piles (Chapter 3);
4) to propose a framework for making an educated guess on the number of temperature monitoring devices needed to account for temporal and spatial temperature variations that inherently exist in open composting systems such as windrow and static piles (Chapter 3);
5) to propose a framework for making an educated guess on the number of turnings needed to increase the probability of every particle's compliance with time temperature criteria (TTC) and also to propose the sensitivity analysis framework (Chapter 3);

6) to investigate from a random compost particle perspective the likelihood that traditionally cool-zones in a compost pile would meet the TTC as a result of turning (Chapter 4);
7) to investigate the effectiveness of TTC to inactivate the indicators of enteric bacteria and enteric viruses whose levels in finished product are regulated by national and provincial regulations (Chapter 4);
8) to analyze whether gradual increases in temperature, as occurs in compost piles, triggers the selected *E. coli* and *Salmonella* strains to enter a viable but not culturable state (VBNC) (Chapter 5);
9) to simulate composting temperature and correlate its effect with the pathogens' survival, their entrance into VBNC, and subsequent potential regrowth from VBNC (Chapter 6).

By achieving these research objectives, this research study will be the first of its kind to analyze the relevance of PFRP requirements for composting from a random particle perspective. The performed study will provide a valuable framework for the optimization of sanitation parameters and direct process validation as required by some jurisdictions, all on an industrial scale.

1.6 Thesis organization

This thesis follows a mixed format with each chapter having its own introduction and bibliography. The general overview of composting process operations, the type of composting feedstock, parameters affecting composting, as well as the regulations to ensure compost sanitation in the US, Canada and other jurisdictions are discussed in Chapter 1. In addition, Chapter 1 reviews the literature pertaining to pathogen survival in compost and proposes two hypotheses to explain the survival mechanism along with the framework of how these hypotheses could be tested.

Chapter 2 presents practical considerations pertaining to the design of the self-contained tool that could test the hypotheses specified in Chapter 1. It also talks about the design of the experiment used to validate the necessary properties of

this tool, such as robustness, random particle movement ability, and readiness to roll-off the pile edge. Finally, Chapter 2 analyzes the applicability of the trommel screen as a means to recover the self-contained tool from the compost mass and provides recommendations for how the recovery could be improved.

Chapter 3 provides information about the trial in which the self-contained tools were used to lay a framework for enhanced temperature sampling by discussing the pros and cons of results from random and systematic temperature sampling approaches. In addition, Chapter 3 uses basic statistics for the framework to calculate the number of temperature monitoring tools needed to address spatial and temporal temperature variations in the composting pile. Using the Markov chain model approach and logistic probability method for sensitivity analysis, Chapter 3 further demonstrates how the gathered temperature data could be used to measure the impact of turning on the likelihood of every particle's exposure to TTC.

Chapter 4 discusses how the self-contained tool was used for direct process validation of covered aerated static pile (CASP) technology which is used for biosolids composting. *Salmonella* sp., *E. coli* sp., and *phi*-S1 phage were used as indicator organisms. In addition, the cool-zones of the CASP were assessed separately. The discussion of results from cool-zones, in particular how they apply to microbial survival and regrowth, are provided.

As another iteration and based on lessons learned from the previous chapter, Chapter 5 again uses CASP technology for direct process validation, but this time it expands the scope by adding the comparison of results from culture based analysis of the compost matrix using USEPA methods 1680 and 1682; the culture based and molecular analysis of indicator *Salmonella* sp., and *E. coli* sp. within the self-contained tool. The concept of VBNC in pathogens as a state to overcome inimical processes during composting is also discussed.

Chapter 6 takes the results on VBNC into a closer look by incubating *Salmonella* sp., and *E. coli* sp. in a temperature chamber with a simulated composting temperature. At discrete time intervals it uses molecular and culture based

methods to quantify the VBNC cells, cells that were still culturable and the cells that were not culturable but resuscitated. The chapter then tries to link these results with the observed regrowth and recontamination that is observed in the finished compost products.

In Chapter 7, the general conclusions of the conducted research as well as a roadmap for the future work are presented. Some of the experimental protocols and supplementary graphs and tables to support the obtained results are presented in the Appendix sections attached at the end of the thesis.

CHAPTER 2: MODIFICATION AND INDUSTRIAL APPLICABILITY OF A TEMPERATURE PROBE CAPABLE OF TRACKING COMPOST TEMPERATURE ON A RANDOM PARTICLE LEVEL[1]

2.1 Introduction

Composting has been accepted as a process to further reduce human and animal pathogens in organic waste-streams to levels that do not endanger consumer safety (Yanko, 1988). Federal (and certain provincial) guidelines and regulations in North America require that for a compost product to have unrestricted use, the level of fecal coliforms should not exceed 1,000 MPN g^{-1} dry solids and *Salmonella* should be present at less than 3 MPN 4 g^{-1} dry solids (Ge *et al.*, 2006; BNQ, 2005; CCME, 2005; USEPA, 2003). Demonstrating this for the entire mass of compost produced at a full-scale facility would mean taking numerous representative samples and analyzing them for pathogenic microorganisms, the process of which is laborious and resource consuming (Turner *et al.*, 2005). Hence, in practice the hygienic level of the end product is usually established through combining the results of a few microbiological assays of the final product with the results of indirect process measurements, such as the minimum time temperature criteria (TTC).

The indirect process monitoring in North America involves ensuring that every particle of compost is exposed to ≥55°C for at least 3 consecutive days. For in-vessel systems and static piles, it is assumed that the preceding requirement can be achieved by ensuring that ≥55°C is maintained for three days throughout the whole pile. For windrows, it is assumed that this condition can be achieved by maintaining 55°C temperatures for at least 15 consecutive days with five pile

[1] A version of this chapter has been published. Isobaev et al. *Compost Science and Utilization, 22*(2), 93-103.

turnings during that period (BNQ, 2005; CCME, 2005; USEPA, 2003). Similar regulations are found in Europe, Australia, and Asian countries (Hogg *et al.*, 2002).

Measuring the temperature pattern over time at specific locations in a compost pile is easy, but assuring that the minimum TTC has been met throughout the pile is challenging. Guidelines and regulations, such as USEPA (2003) and CCME (2005), do not specify which type of temperature monitoring devices should be used, nor do they say where these devices should be located within the compost, how many of them should be used, or how often temperature readings should be taken (Wichuk and McCartney, 2008) in order to claim that the TTC has been met. Since it is neither practical nor possible to measure the temperature of every compost particle during full-scale operation, the temperature in composting facilities is generally monitored using lance-type stationary temperature probes (e.g. Fernandes *et al.*, 1994; Jäckel *et al.*, 2005; Turner *et al.*, 2005), equipped with thermocouples at the tip, or with stationary data logging devices. Such probes are introduced at various spots in the pile, which are often times selected as the bottom, middle and upper strata of the compost (Bhamidimarri and Pandey, 1996; Fischer *et al.*, 1998; Marešová and Kollárová, 2010), with the aim of obtaining data representative of spatial temperature distribution in the pile.

The existing temperature monitoring practice may give the false impression that the time-temperature criterion has been met whereas in reality it hasn't. A literature review by Wichuk and McCartney (2007) revealed that viable pathogenic organisms have sometimes been detected in compost that appeared to comply with the North American TTC. The most commonly cited factors relating to pathogens survival were: (i) failure to detect low temperature zones, which occur as a result of spatial and temporal temperature variability, where microorganisms could have remained intact; and/or (ii) the inadequacy of existing TTC (Barrena *et al.*, 2009; Brinton Jr. *et al.*, 2009; Inglis *et al.*, 2010; Pourcher *et al.*, 2005; Rao *et al.*, 2007; Wéry *et al.*, 2008; Xu *et al.*, 2009). Unfortunately, with existing technological capacity there has, to-date, been no endeavor to confirm that existing composting methods ensure the exposure of every particle to

55°C for three consecutive days and to correlate these findings with the survival of key pathogen microorganisms all on a compost particle level.

2.1.1 Temperature monitoring on a particle level

The idea of monitoring compost temperatures on a particle level was proposed in a study by Wichuk and McCartney (2008). The authors hypothesized that this could be done via introducing a statistically relevant number of devices directly into a composting process. These devices would need to: i) be self-contained; ii) behave like a random particle during pile agitation and settling; iii) be sturdy enough to withstand the physical and chemical stresses of composting; and iv) have the capability to record real time temperature data for an extended period of composting. The majority of temperature logging devices that were commercially available at that time were deemed unsuitable for use in the compost environment for various reasons. One commercial device was selected for testing (the Temp1000 Data Logger, manufactured by MadgeTech, Inc.), which had most of the necessary qualities specified by Wichuk and McCartney (2008) except that its density was about twice that of compost. The authors also designed a custom temperature logging device with all of the desired specifications and compared its performance to that of the Temp1000. The tests performed were:

- material strength evaluation,
- effect of density on movement of probes,
- robustness of circuitry,
- effect of magnetic field on data memory,
- ease of probe recovery from compost heap.

Although both devices had drawbacks, it was concluded that the Temp1000 data loggers had better overall performance than the custom devices. However, certain characteristics of the Temp1000 needed to be improved, including replacing socket-mount parts with surface mount parts, securing the battery more firmly to the circuit board, and enhancing the visibility of the probes within the compost by specifying a case colour easily distinguishable from compost. Finally, though the

authors confirmed that both the custom and commercial probes behaved like random particles, they recommended repeating the experiment to confirm the results.

2.1.2 Modified temperature probe (MTP)

Several modifications were made to the commercial temperature probe (hereafter referred to as the MTP) recommended by Wichuk and McCartney (2008) after discussions with the manufacturer. All socket mount parts were replaced with soldered-on surface mount parts. Each MTP was engraved with a serial number and anodized into a gold color for improved visibility during screening.

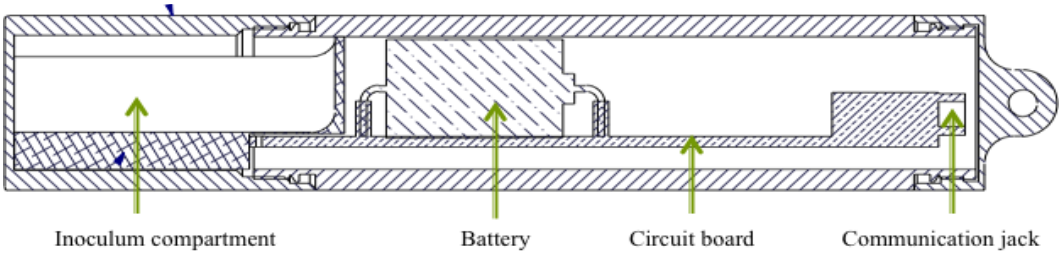

Figure 2-1 Cross sectional perspective of MTP

To enable potential correlation of the TTC in compost with pathogen inactivation at the probe locations, a microbial inoculum compartment (i.e. cryovial holder) was added into the structure of MTP. Having a compartment within the sealed, leak-proof temperature probe served to reduce the possibility of compost contamination with the pathogens in the inoculum in case the probe is was lost or damaged. Screw-on caps were used on both ends of the cylindrical probe for ease of access to both the cryovial compartment and the temperature logger communication jack. The final look of the MTP is provided in the Figure 2-1. The technical characteristics of the MTP were according to Table 2-1.

Table 2-1. Technical characteristics of the MTP

Design parameter	Dimension
Overall length of the probe	141 mm
Outer diameter	25.4 mm
Inner diameter	19.3 mm
Nominal wall thickness	3 mm
Case material	anodized aluminum 6061 grade
Density	1630 kg/m^3
Weight	106 gr
Operating temperature range	-40ºC to +80ºC

2.1.3 Practical considerations

For a technology to be practical among other factors it has to provide accurate readings and be readily available at reasonable fixed and operating cost. The original temperature probe satisfied these requirements: it consistently provided accurate readings and as of September 2013 the manufacturer had the product in stock at a unit cost USD 199 per unit with delivery promise up to 10 business days. Discounts were available for large quantities up to USD 159.80 per unit if more than 100 units ordered. The MTP with the same reading accuracy as its prototype was available within 3 weeks from order date at a differential cost tied to quantity and optional enhancements as specified in Table 2-2.

Table 2-2 Unit price of MTP manufacturing (in US Dollars)*

Feature	1-4	5 to 19	20 to 99	≥100
Manufacturing	751	319	238	216
Engraving	5	5	5	5
Color anodizing	90	21	7.50	3.9
Total	846	345	250.5	224.9

*based on 2010 manufacturer quotation

The MTP could be easily operated via a designated software. However, future use would still be dubious if the unit did not have an effective and efficient recovery framework in place at the end of process. Screening seems to be the unit operation applicable for the recovery of the probes with minimum operational footprint. It is one of the oldest and most widely employed methods to sort large-scale of particle materials according to their size (Yepsen, 2009; Chen *et al.*, 2010). During screening the fraction which is smaller than the mesh size (undersize material) is allowed to escape through its holes leaving the fraction which is larger (oversize material) on the mesh.

Various screening techniques are nowadays being used for size separation in different industrial settings: oscillating, vibrating, trommel and disk screens to name some (Yepsen, 2009). Trommel screens are especially popular in the waste management industry (Chiumenti *et al.*, 2005; Yepsen, 2009). They consist of a rotating perforated drum that is positioned at a slight incline. The material is fed from the upped end; the undersized fraction passes through the mesh and the oversize particles pass through the trommel and removed from the lower end by a conveyor. The advantages of trommeling over other separation techniques, are (Chen *et al.*, 2010): (i) relatively low operating and maintenance cost; (ii) operational simplicity; (iii) ability to alternate driving speed and inclination angle of drum; and (iv) ability to handle hard to untangle materials and materials with broad size range.

It was demonstrated that with trommeling particles of interest can effectively be removed from the compost, either from screen undersize or oversize. For example Lau *et al.* (2005) noted that with a trommel operating at 21.4 rpm and 5° angle they successfully recovered AA batteries from municipal solid waste (MSW) compost. In another study the trommel screen was effective in recovering the metal residues from stabilized MSW (Prechthai *et al.*, 2008). In yet another study the trommel technology was effective in recovering the foreign matter from compost (Page *et al.*, 2005). Finally it was demonstrated that the trommel screen can be adopted as a strategy to recover aluminum tubing from biosolids and mixed biosolids / MSW composts (Wichuk and McCartney, 2008). In that particular experiment operating trommel on one side and visually scanning the overs from the outlet was sufficient to recover 100% of the particles of interest. The authors though neither provided information with regards to the scale of the material that was screened, nor its physical and chemical characteristics.

Trommeling is the primary size separation technique used in the Edmonton Waste Management Centre (EWMC) for its finished Aerated Static Piles and MSW compost products. On an annual basis 31,495 tonnes of biosolids and 47,231 tonnes of MSW compost passes through the trommel.

2.1.4 Objectives of the study

Given the modifications made to the probe, the objective of this study was to analyze how these changes impacted the performance of the MTP. In particular, it was of interest to investigate the following: (i) whether the change in the density and size of MTP changed its ability to move as a random particle of compost would; (ii) whether soldering of circuit components resulted in sufficient robustness of the circuitry; (iii) how the MTP (with or without additional anchors) would behave on the pile edge; and (iv) whether a trommel screen could be recommended as an effective solution to recover the probes from compost.

2.2 Materials and methods

2.2.1 Experiment 1 - Analysis of the spatial distribution and robustness of MTP

Two compost piles, one for each of two trials, were used in this experiment (Table 2-3). The compost was produced from the organic fraction of municipal solid waste. Their bulk density and moisture content were typical of those normally observed in mature compost. Hence, the experimental piles had lower adhesive forces and higher density difference between probes and compost particles when compared to the freshly built piles. As a consequence, this presented a worse-case scenario for the MTPs to settle due to the gravitational force and so if things turned out satisfactorily in these piles, there would likely be fewer problems experienced in fresh piles.

Table 2-3. Compost pile dimensions and characteristics

Factor (dimension)	Experiment 1	
	Trial 1	Trial 2
Length (m)	12	8
Height (m)	1	1
Width at the base (m)	3	3
Width at the crest (m)	2	1
Volume (m^3)	27	16
Density (kg/m^3)*	502	349

*The mean value from 3 consecutive measurements

Four different types of probes, each in the amount 32, were used in the analysis of spatial distribution and robustness (Figure 2-2): (i) dummy probes with a density of 1630 kg/m^3 (probe A); (ii) dummy probes with a density of 1630 kg/m^3 and

amended with anchors (probe B); (iii) dummy probes with a density of 580 kg/m^3 (probe C); and (iv) MTP with a density of 1630 kg/m^3 (probe D). Dummy probes consisted of capped cylindrical tubing of the same diameter (25.4 mm) and length (140 mm, excluding anchors) as probe D. Their density was adjusted by filling them with metal screws, nails, or wires. The dummy probes were made either of the same grade of aluminum as used for probe D (e.g. probes A and B) or of plastic (e.g. probe C). Anchors for probe B were made of a wire that was attached to one end of the probe and bent on two sides. The reasons to add anchors to the structure of the probe were to help the probe compensate its higher than compost density thus preventing its settling to the bottom of the pile and to enhance probe's capability to stay on the pile edge instead of rolling off to the bottom.

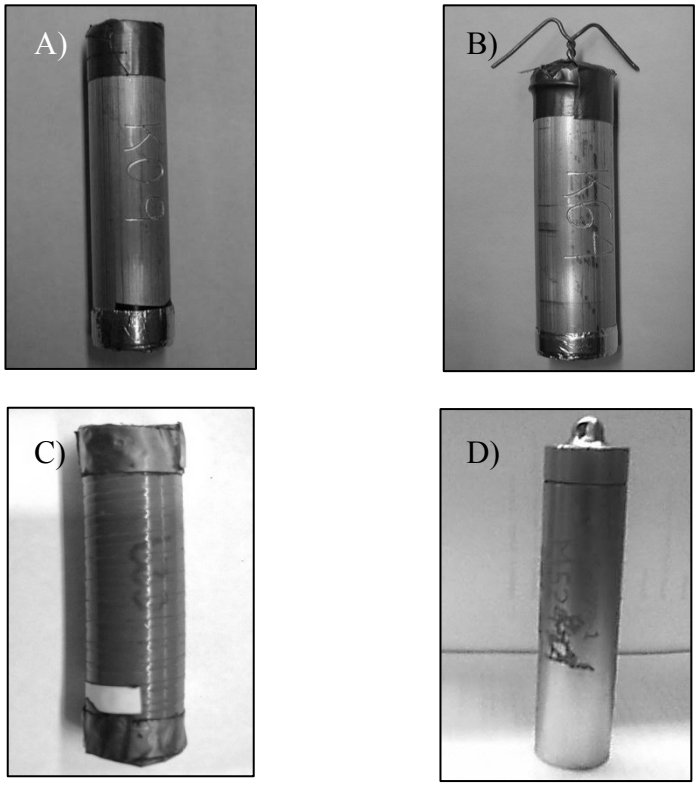

Figure 2-2. Probes and dummy probes used for the random particle movement and robustness analysis: a) Probe A; b) Probe B; c) Probe C; d) Probe D.

Two trials were run to determine the spatial distribution of the probes in compost. In both trials, four trenches were built across the pile. The position along the length of the pile for each trench was decided by generating a random number in R (free statistical calculator) on a scale from 0.1 to 0.9 and multiplying this number with the length of the pile. The depth of the trench in each instance corresponded to the volumetric centroid of the pile (see Wichuk and McCartney 2008), a point on the vertical coordinate at which the volume of the matter above it is equal to the volume beneath it. Four spots along the width of the pile were then randomly selected within each trench. Two probes from A, B, and C were randomly drawn and put into each selected location. After finishing the procedure, the trenches were refilled with compost. Attention was paid to establishing the density across each trench close to the initial bulk density of the compost pile. This was done by compacting the compost with shovels after the probes were introduced until the cover layer attained a texture visually analogous to the rest of compost pile. A self-propelled, straddle turning machine was used to turn the compost pile. The turner was powered by a 140 HP engine and equipped with a high-speed drum with 47 replaceable hardened steel teeth. After turning, workers searched for the probes by carefully digging through the pile with shovels so as to maintain the post-turning vertical distribution of the probes. When any probe was spotted, its height from the ground was measured and recorded for further analysis of spatial distribution. The computed data were fitted to the analysis of variance (ANOVA) model with Bonferroni post-hoc test. The significance was interpreted with SPSS v17 statistical software.

The robustness of the circuitry was evaluated concurrently by batch programming 10 probes from group D to record the pile temperature at 5-minute intervals. The probes were then placed into randomly selected trenches with probes from group A, B and C. Damage to the circuitry would be indicated by any or all of the three following scenarios: (i) failure to connect to software; (ii) discontinuous or failed recording of temperature data; and (iii) temperature measurements that did not make sense and/or were inconsistent with temperatures recorded by other probes. The information from each programmed data logger was downloaded and the

temperature curves analyzed for anomalous patterns and inconsistencies in recording.

The integrity of cryovials within the probes was assessed by adding water into 12 cryovials and subsequently introducing these cryovials into 12 randomly selected probes from group A. The cryovials were left loose inside the probes to ensure that they would experience the worst possible physical stress. After turning the windrow, the cases were opened and the vials visually examined for punctures, leakages, and other performance-affecting signs of wear.

2.2.2 Experiment 2- Analysis of edge effects

An experiment was conducted to determine whether the probes would behave in a similar manner to compost particles if they ended up on the pile boundaries. This test was conducted on a full-scale biosolids compost pile. The moisture content (MC) of the material was approximately 46% and wet bulk density (WBD) was approximately 520 kg m^{-3} (according to the information provided by the site operators). The pile length was approximately 50 m, height approximately 2.6 m, and slope of the compost side was about 47°. The surface of the bed was largely covered with woodchips, and was a dry, relatively flat surface down which round particles were expected to relatively easily roll.

One 20-L bucket of compost/wood chips from the surface of the pile was collected and spray-painted a fluorescent green colour. The paint was allowed to dry to minimize adhesion. The spray-painted compost was mixed with 10 probes from group B and 10 probes from group D. The mixture was loaded back into the bucket. Nine spots were randomly selected along the length of experimental compost bed. The contents of the bucket were discarded onto each spot from the crest of the pile. The process of releasing the bucket contents was video recorded and pictures of the vertical spread of coloured compost particles and the two types of probes were taken for the *a posteriori* analysis.

The retention performance of the MTPs was compared against probes from group B and coloured compost particles. For this, the height of the compost was divided

into three equal segments – 0 to 33% (bottom/toe), 34% to 66% (middle), 67 to 100% (top/upper), with the ground being at 0% and compost crest being at 100% of the pile height. The percentage of the probes coming to rest in each zone was calculated for both groups. The relative quantity of the coloured compost in each zone was inferred visually from pictures and video records. One-way ANOVA and Kruskal-Wallis tests were used in parallel to analyze whether there was a significant difference between these three groups of particles. The Chi Square test was used to see how often one should expect the probes to end up in each vertical segment of compost. For this, instead of relative figures, the exact count of probes B and D in each segment was calculated.

2.2.3 Experiment 3 – Analysis of probe recovery from a full-scale compost pile

Four independent trials were performed to determine the effectiveness probe recovery by screening. These tests were performed in one section of a full-scale biosolids compost heap. Specifically:

1. in the first trial, 23 probes were randomly introduced during pile construction into a biosolids compost pile segment of approximately 48 m^3;
2. in the second trial, 46 probes were randomly introduced into a different biosolids compost pile segment of approximately 264 m^3;
3. in the third trial a 20 m fishing line was attached to each probe through the designated eyelet on probe's one end before 34 probes were randomly introduced into 120 m^3 segment of compost pile; the mentioned length was greatly in excess of the maximum distance that a probe could be buried in a pile in order to not affect its random placement and to analyse if a simple measure such as fishing line can enhance the recovery;
4. in the fourth trial, and analogous to the third trial, a 15 m fishing line was attached to each probe through the designated eyelet before 22 probes were randomly introduced into 132 m^3 segment of compost pile.

In all instances probes were recovered after 6-8 weeks of active composting. In first and second trials a trommel screen with a mesh size of ½-in. (1.27 cm) was used to screen the material and recover the probes. The speed of the drum was adjusted to operate at approximately 19 rpm in order to balance the speed of the process with the ability to recover the probes from the oversize stream. A front-end loader was used to fill the trommel's hopper with compost; new material was added to the hopper when the previous load was approximately 80% screened. Personnel were stationed at the oversize material conveyor and/or near the conveyor discharge to watch for and collect probes. The person stationed at the conveyor belt notified the person at the discharge when a probe was spotted, and the latter person collected the probe from the discharge pile. The procedure was repeated until complete screening of the entire biosolids segment into which probes had been added was done. In the first trial, three people participated in the visual recovery of the probes, while in the second trial only two people participated. In both cases, one of the participants was the front-end loader operator.

During the third and fourth trial the temperature probes were recovered at the end of active composting by pulling the end of the fishing line. The fishing line attached to some of the probes was broken during pile building or pile turning; these probes were recovered from the compost by screening them out with a trommel screen. The set-up of the screening operation was same with previous two.

2.3 Results and discussion

2.3.1 Analysis of spatial distribution

Density plays an important role in determining the position of particles in a given medium, owing to buoyant and gravitational forces. Three scenarios are possible for the object immersed in a medium: (i) when the density of immersed object is significantly higher than the density of medium it is immersed in, the object would tend to sink due to the effects of gravity; (ii) when the density of immersed

object is significantly lower than the density of medium it is immersed into, the object would buoy up due to the effects of buoyant force acting on it; or (iii) when there is no significant difference between densities, the object would tend to remain suspended somewhere in the medium.

Table 2-4 Number of probes (out of the total introduced) retrieved from the compost pile and used in statistical analysis of vertical distribution

Probe type	Number of probes recovered / number of probes used for the analysis		Sample size
	Trial 1	Trial 2	
A	31 / 32	23 / 32	54
B	28 / 32	22 / 31	50
C	31 / 32	28 / 32	59
Total	90 / 96	73 / 95	164

Unfortunately, as is shown in Table 2-4, some probes from all three groups were lost in the experimental compost pile and never recovered. Out of those probes that were recovered, more particles from all three groups tended to move downward rather than upward. Combining the results of the two trials, 97 (59%) of the probes were found below the reference line, whereas 65 (40%) were above reference line, and only 2 (1%) of the probes remained at their starting height (on the reference line). The mean displacement for each group was as follows: -4.85 cm for group A probes; -4.04 cm for group B probes; and -0.38 cm for group C probes. Neither group contained extreme low or high values.

On first glance, it seems that the smaller average downward migration observed in the group C probes was related to their lower density. However, the analysis of variances test from the collected data suggested that there was no significant

difference in the displacement of probes (*F = 0.50, p = 0.61*) between the groups. This observation leads to the conclusion that, in a compost pile with a wet bulk density 502 kg m^{-3}, probes with a density of 1630 kg m^{-3} would behave statistically similar to probes with a density of 580 kg m^{-3}. These findings were in conformance with those of Wichuk and McCartney (2008), who reported that probes with a density in the range from 800 to 2000 kg m^{-3} would exhibit random particle behaviour when introduced to compost with a bulk density of 450 kg m^{-3}.

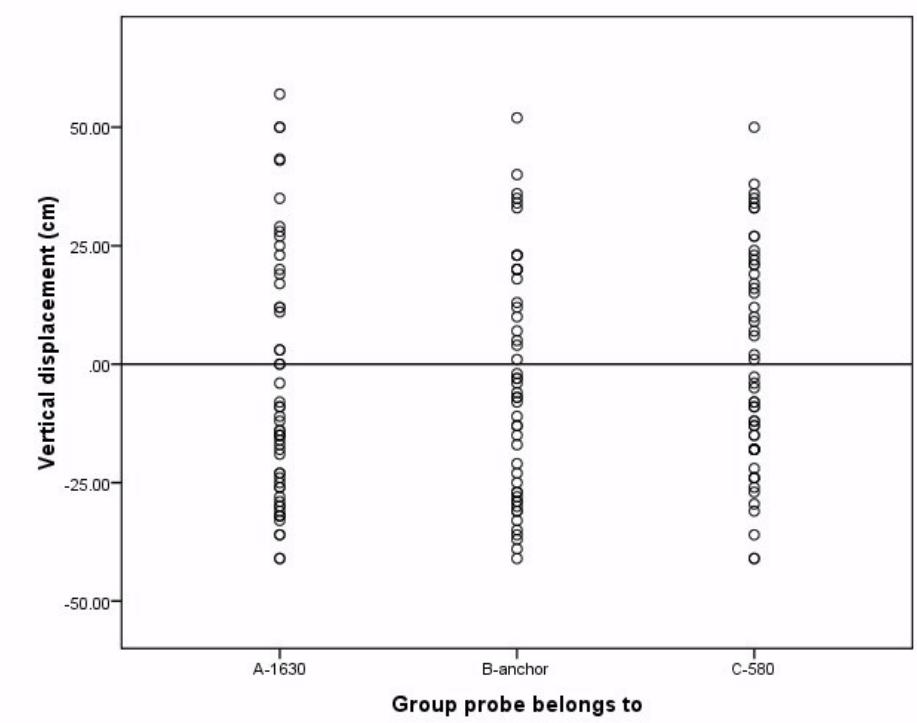

Figure 2-3. Actual displacement of the probes from three different groups

These results also demonstrated that the modification with anchors (group B) did not induce any significant impacts in terms of vertical distribution. This may be due in part to the fact that anchors with acute angles used in the experiment readily clogged with compost and anchors with right angles demonstrated high bending propensity. It cannot be entirely ruled out that anchors of different forms might affect the density factor for probes whose density might otherwise

significantly differ from that of compost. Nonetheless, given the result that there was no significant difference detected between probes of different densities, it appears that anchors would not be necessary for density modification purposes.

2.3.2 Analysis of probe's robustness

Robustness to harsh physical and chemical processes within a compost pile is a vital characteristic, which the temperature probes must possess in order to have a long useful life. Therefore, the case of every probe recovered after pile turning and/or composting was thoroughly visually assessed. Cases did not show any signs of physical or chemical wear. In addition, the probe cases proved to be firm enough to withstand impacts from the turner with the horizontal spinning shaft with paddles. Even when the windrow turner ran over a probe, it did not cause case rupture. Given the fact that the probes survived the physical stresses that were exerted by the windrow turner, it was concluded that aluminum casing is sufficient to protect the probes.

The resilience of probe case to external stresses, though necessary, was not a sufficient on its own to ensure the appropriateness of the probe for use in composting. Therefore, testing was also done to find out how well the circuitry in the probe could stand up to the stresses of routine composting operations. Factors like vibration or impact could theoretically cause failure of the circuit board or its components (by affecting data collection or causing errors in temperature measurement), thereby leading to loss of data and/or wrong inferences. When the information from 10 probes, programmed to record the temperature at 5-minute increments was downloaded, there were no problems with probe functioning, no missing data, and similar patterns in temperature dynamics were observed for all of them. This suggested that the circuitry was sufficiently safeguarded inside the probes.

Finally, the vulnerability assessment of the cryovials showed that they stood up well to physical and thermal stresses. No leaks from the cryovials, deteriorations of the wall or cap, or other failures were observed during visual inspection after

contact with a straddle windrow turner. The high temperature of 80°C for 5 days in a controlled environment did not cause deformations or any other observable damage to the vials. These findings suggest that the use of cryovials in the study of survival dynamics of selected pathogens in any composting technology is appropriate and that any inoculated pathogens will remain contained.

2.3.3 Analysis of edge effects

Once a temperature probe is randomly introduced into a compost pile, it has the potential to end up on the pile edge. Probes may also be placed specifically at the outer edges of a pile in order to monitor the surface temperature profile. When a probe is at the edge of a pile, it always bears a chance of rolling off the pile. This is undesirable, as the data retrieved from outside the pile does not contribute to knowledge of actual pile temperatures.

As demonstrated in Figure 2-4, the distribution varied between trials. In some trials probes and compost particles were evenly spread across the entire side slope of the pile. However, in other trials the spread was skewed such that both the probes and compost particles were concentrated in the upper or lower portions of the pile. This observation seemed to be correlated with the roughness of the slope surface; on surfaces where larger compost lumps predominated (lump diameter $\geq$ 20 cm), more particles stayed in the top and middle strata, while on surfaces where finer compost lumps (lump diameter $\leq$ 10 cm) were prevalent, all of the bucket contents freely made their way to the toe of the pile.

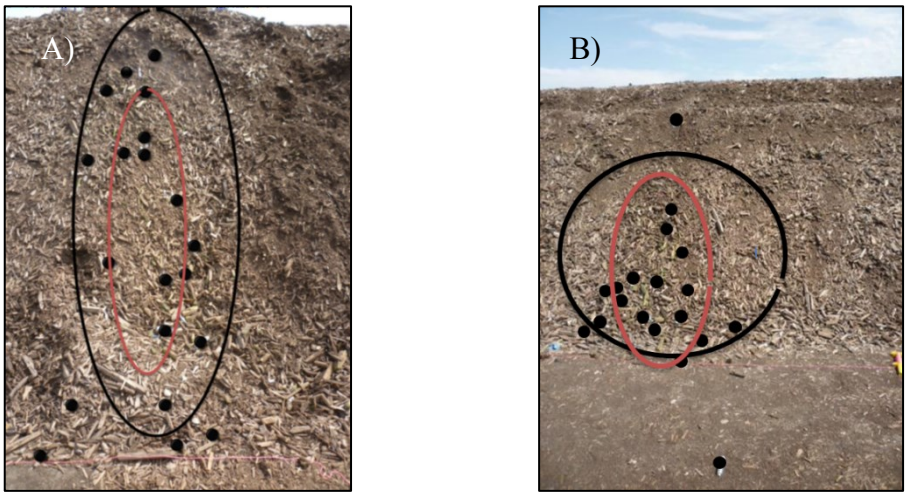

Figure 2-4. Two examples of probe and compost particle distribution on a compost pile edge: a) even distribution; b) skewed distribution. Black dots correspond to the probes. Black and red circles correspond to where ~ 90% of all probes and compost particles, respectively, lie.

Table 2-5 summarizes the percentage of compost particles and two types of probes that remained on the three different strata of compost (average of nine trials). Despite the fact that the probes D (42 ± 8%), on first glance, seem to have rolled to the pile toe more readily than did probes with anchors (group B, 39 ± 9%) or compost particles (30 ± 7%), there is actually no statistically significant difference between the groups. The results of parametric ANOVA and its nonparametric Kruskal-Wallis analogue are presented in

Table 2-6. No difference was found between the retention capabilities of three different materials on the compost edge.

Table 2-5. Distribution of three different particles on the edge of compost pile

Location	% of the material retained		
	Compost (±SE[a])	Probe B (±SE)	MTP (±SE)
Top[b]	26 (±7)	30 (±7)	29 (±10)
Middle[b]	44 (±5)	31 (±5)	29 (±4)
Bottom[b]	30 (±7)	39 (±9)	42 (±8)

[a] SE = standard error; [b] figures were rounded to the nearest integer

Table 2-6. Calculated significance of parametric and non-parametric tests for edge effect analysis

Location	*P* value		Conclusion
	ANOVA	Kruskal - Wallis	
Top	0.921	0.822	Not significant
Middle	0.078	0.142	Not significant
Bottom	0.555	0.557	Not significant

Although the compost particles appeared to have lesser presence in the bottom and higher presence in the middle section of the pile edge, the variation in results between trials, differences in surface roughness, and potential inaccuracies in visually estimating the true amount of the compost in each zone resulted in an inability to show any statistically significant difference between the movement of compost particles and the MTPs on a pile's surface. The results also demonstrated that the enhancement of the tested device's structure with the proposed anchors was not sufficient to achieve a statistically significant advantage. Therefore, it

was postulated that the temperature probes are applicable for use in the composting industry, as they adequately mimicked random particle movement on the pile edge.

2.3.4 Analysis of probe recovery from a full-scale compost pile

The specific objectives of this portion of the study were to assess the recovery against: (i) labour requirements and (ii) the ease in achieving ≥90% recovery efficiency of probes; and if needed (iii) develop recommendations to improve the recovery rate while reducing or keeping the labor constant. The results of four independent trials that were conducted during the current study are given in Table 2-7. Although in the first trial 100% recovery was achieved, the recovery was not flawless. Nearly 4 hours were required to screen the entire segment of compost into which 23 probes were added, and only 19 probes were recovered (82% efficiency). It was necessary to screen the overs once again in order to find the missing MTPs, which required an additional hour.

In the second trial, more probes were placed into a larger section of a compost pile, so there was more material to screen. Nearly, 18 hours was required to screen the entire pile segment, and only 34 out of 46 (79%) of the probes were recovered. The exhibited performance was well below the goal of 90% recovery set for this experiment.

There are a number of reasons why the custom threshold level of 90% was not achieved during trial 2. The first reason could be linked to the moisture content of the screened material. Screening is typically done when the compost is mature and hence has low moisture content. In the current study, the screening of compost was performed when the material was still relatively wet (~50% MC). The wet material both clumped and clogged the screening surface, hence reducing the material throughput rate and increasing the amount of oversize material. This made it more difficult to visually locate probes in the oversize stream.

Table 2-7. The recovery efficiency of MTP from compost pile

Recovery Indicator	Trial 1	Trial 2	Trial 3	Trial 4
Primary recovery by*	TS	TS	FL+TS	FL+TS
Volume screened (m^3)	48	264	120	132
% Recovered before screening	100	79	75	71
Screening time (hr)	5	18	2	3
Number of workers	3	2	2	2
Overall recovery efficiency	100%	79%	97%	100%

*TS = Trommel screen; FL = Fishing line

The second factor, which affected both the recovery rate and efficiency, was the high fraction of lumpy oversize material on conveyor belt that would not pass the ½-in. mesh. In practice, screening is done to remove coarse materials, which normally constitute nearly 30% of the entire volume. In the current study, however, they constituted more than 50% by volume. The loss of 9 MTP with valuable data during the screening can be partially attributed to the increased volume of oversize material. This problem was envisaged early during the preparatory step and was anticipated to be mitigated by painting MTPs an eye-catching color to easily distinguish them from the compost. Half of the MTPs were painted bright gold and half silver. Qualitatively, there was no difference in distinguishing either colour from the rest of the compost mass during visual recovery. The recovery efficiency of silver probes was 74% and that of gold probes was 80%. It is anticipated that the recovery rate and efficiency should improve significantly if the screening is done on finished compost biomass. Finished compost is drier (25 to 35% MC) and contains a larger proportion of fine particles, which can pass through a ½-in. screen.

Finally, the “human factor” was identified as a cause of the slow recovery process and lower than desired recovery rate. Visually scanning the conveyor belt and discharge for several consecutive hours was an exhausting task. It was found that after some time, it became hard to recognize the probes on the conveyor belt, especially when it was accompanied by the large amount of compost. Also, extended periods of visual scanning of the moving conveyor belt caused dizziness in personnel running the experiment. For example, in the second trial the inspector of the conveyor belt failed to spot 7 probes, which were luckily spotted by the overs’ pile inspector. When questioned, the inspector pointed at dizziness and eye fatigue as possible reasons. To further increase the efficiency it is important to reduce the human factor. Something that can communicate back to the operator would significantly improve the recovery efficiency and increase the recovery speed. Therefore methods should be developed which rely more on analytical tools and sensors rather than on the capability of a naked eye to spot probes in the continuously moving mass of oversize material. The tool, which can be used for the probe recovery can be either autonomous, surface mount, or embedded into circuitry. As a necessary condition, the tool, regardless of its type should guarantee the integrity of the probe’s circuitry, inertness to steel, rubber or other components of primary recovery device, be portable and operable by one person. Figure 2-5 further lays down the recommended criteria that recovery equipment should possess.

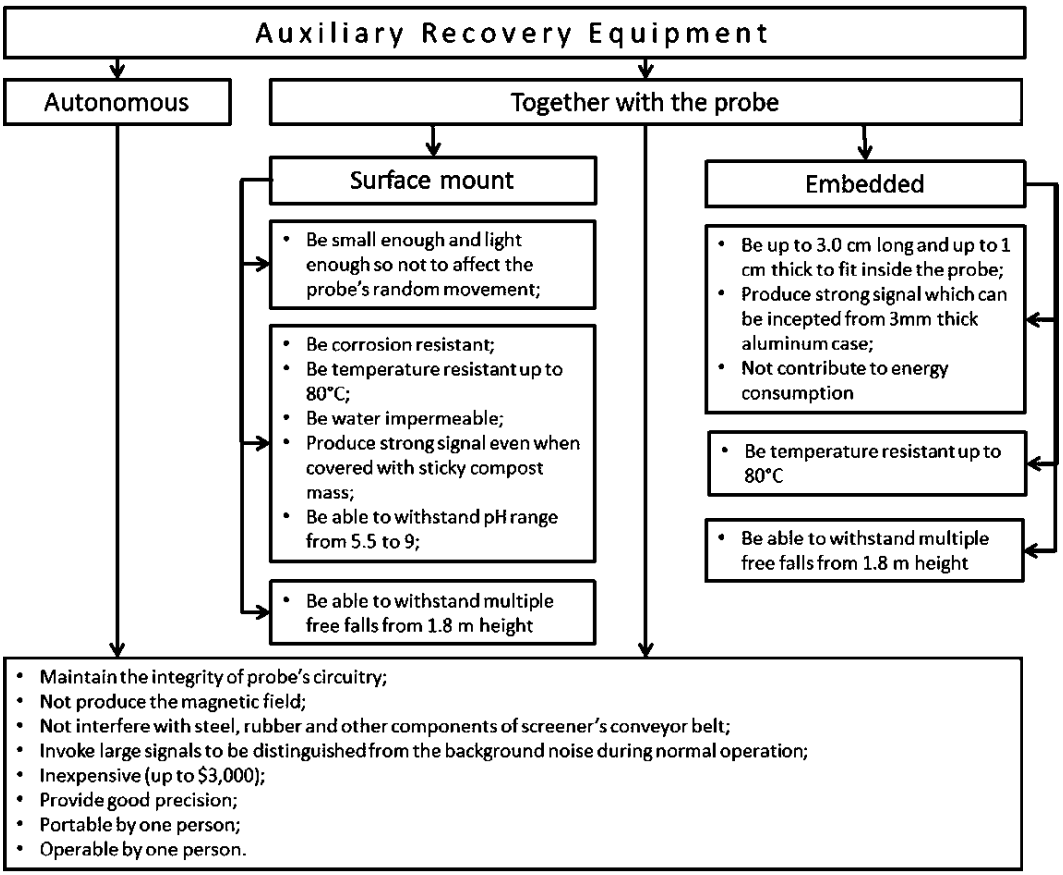

Figure 2-5. Criteria, which an auxiliary recovery device should possess in order to be appropriate for the industrial scale recovery of the temperature probe

The addition of fishing line to the recovery equation in third and fourth trials resulted in reduced screening burden and increased recovery rate but did not completely eliminate the necessity for the screening operation. However there are a few considerations that should be mentioned regarding this amendment. First the addition of fishing line tended to interfere with the idea of random introduction. In the particular study it was challenging to achieve random introduction without having the fishing line tangle with the elements of the conveyor belt. Several blank trials were required before polishing the skill. Second, the fishing line used in the study was marketed to withstand ~70 kg weight. In reality the line broke readily while trying to pull out the probe from

within 1 m. depth of the compost mass. Sometimes the bucket of the front-end loader would accidentally cut the fishing line thus resulting in the loss of the temperature in the pile. Manipulation with different ropes in the fourth trial did not resolve the problem. Therefore more research need to be done to find optimum rope should this approach be pursued.

2.4 Conclusion

A commercial temperature probe (MTP) was modified in accordance with recommendations of a previous study (Wichuk and McCartney 2008). The modified probe is a self-contained device, which is 14.1 cm long, 2.54 cm in diameter, and has a density of 1630 kg m-3 including its anodized aluminum casing. Two field trials showed that the proposed MTP behaved like a random particle in compost. The trials also showed that the probe's aluminum case was sufficient to protect its circuitry and cryovial from various worst-case physical and chemical stresses. If, as a result of moving freely during compost pile turning, the probe ends up on the pile edge, the chance that it would end up in the bottom was estimated as 42%. While this figure seems to be high, it was found that statistically the same chance is applicable to compost particles, thus suggesting that even on the pile edge the probe behaves like a random compost particle. The introduction of primitive anchors to the structure of the probe was found to have an insignificant contribution to the retention of the probe on the pile edge. Overall, based on the results from the field trials, the MTP were deemed applicable for the evaluation of different composting technologies.

Use of a trommel screen was found to be a viable solution for probe recovery from a compost pile. There is still much that needs to be improved both in terms of the material being screened and in terms of equipment selection to achieve high recovery efficiencies in a timely manner. The more particles that pass through the screen, the fewer particles will be on a conveyor belt. Thus it will be easier to visually locate the probes. Also, it will be faster to screen the compost mass as there will be minimal risk of screen clogging. To further increase the recovery

efficiency, it is important to reduce the human factor. A device that could sense an aluminum probe passing through it could signal to the operator when to watch for a probe in the trommel screen discharge stream, and could improve the recovery efficiency to nearly 100% and also increase the recovery speed. Therefore, methods should be developed which rely more on sensors rather than on the capability of a naked eye to spot probes in the continuously moving mass of oversize material.

CHAPTER 3: AN ENHANCED COMPOST TEMPERATURE SAMPLING FRAMEWORK: CASE STUDY OF A FULL-SCALE AERATED STATIC PILE[1]

3.1 Introduction

Composting is an exothermic process (Haug, 1993). During composting, various aerobic microorganisms digest organic matter and release the energy to the ambient environment in the form of heat. The produced heat increases the compost's temperature to thermophilic conditions, which, if sustained for a sufficient period, has the effect of reducing the population of pathogenic microorganisms in the compost by damaging their DNA (Adams and Moss, 2008).

To ensure proper inactivation of pathogens, composting guidelines and regulations in North America (e.g. CCME, 2005; USEPA, 2003) require every particle of compost to be exposed to ≥55°C for at least 3 consecutive days. For in-vessel systems and static piles, it is assumed that the preceding requirement can be achieved by ensuring that ≥55°C is maintained for three days throughout the pile, while for windrows this ≥55°C temperature should be maintained for at least 15 consecutive days with five pile turnings during that period (BNQ, 2005; CCME, 2005; USEPA, 2003). However, the guidelines/regulations do not provide explicit tools or guidance on how to ensure that the specified time-temperature criteria (TTC, ≥55°C for 3 consecutive days) have been met in any particular technology (see Chapter 2). As a result, facility operators are free to choose how to ensure temperature compliance. Their decision is usually dictated by past experience, convenience / ease of implementation or it comes ready with the adopted technology (e.g. Gore-Tex™ composting technology). A review of

[1] A version of this chapter has been published. Isobaev et al. *Waste Management 34,* 1117-1124.

scientific articles that report on compost temperature monitoring lead to the following conclusions about how TTC compliance is evaluated in composting facilities:

- the temperature in composting heaps is mostly monitored with temperature probes (e.g. Fernandes *et al.*, 1994; Jäckel *et al.*, 2005). These are stationary devices equipped with a thermocouple. Probes can vary in size and reading precision. A temperature probe can either come with built in memory or be wired to a recording device (e.g. computer).
- the temperature probes are introduced at various spots, which are often times selected as the bottom, middle and upper strata of the compost heap (Marešová and Kollárová, 2010; Bhamidimarri and Pandey, 1996; Fischer *et al.*, 1998b). This is done with the aim of obtaining a pooled dataset representative of spatial temperature distribution in the pile (Fernandes *et al.*, 1994).
- since there is no specific protocol in place that recommends how many temperature probes should monitor the spatial temperature distribution, their number varies dramatically. Some researchers have used only a few probes for temperature monitoring (e.g. Bhamidimarri and Pandey, 1996), while others have used up to several dozen (e.g. Fernandes *et al.*, 1994). It is not clear whether the decision on the number of probes is based on cost factors, previous experience, internal quality control standards, or any other decision-making tools.
- since there is no consensus on how often the temperature readings should be taken to capture temporal temperature variability, the reported frequency of temperature reading typically varies between minutes and hours. While infrequent readings may reduce the data analysis burden to some degree, more frequent readings enable one to more precisely discern temporal variations on any given timescale. With the ability to automate the recording process with little or no additional cost, the use of more frequent temperature monitoring is recommended.

- there is no general consensus on how to interpret a highly time-correlated, auto-correlated nonlinear temperature profile (Yu *et al.*, 2008), and as a result temperature data is reported in different formats. Often times it is presented as a trend over time (e.g. Bhamidimarri and Pandey, 1996; Marešová and Kollárová, 2010). The descriptive statistics which complement the trend line include the maximum and minimum temperatures, overall mean temperature, the day a particular temperature milestone was reached (e.g. 55°C), time needed to reach this temperature, and the duration of the elevated temperature (≥55°C). As a test of compliance with the TTC, the time (total and consecutive) that the temperature exceeded 55°C should be reported (e.g. Christensen *et al.*, 2002). The temperature variability due to the temporal and spatial effects is usually given as the range between highest and lowest temperatures recorded (e.g. Deportes *et al.*, 1998; Hess *et al.*, 2004).

Temperature monitoring methods and procedures need to be addressed more thoroughly in order to meet the sanitation requirements and ensure that public safety is not compromised. Ideally, decisions about the number of temperature probes placed in a pile, their location, and the frequency of data collection should be based on science such as:

- the locations of the temperature probes should ensure that, over the course of composting, the full spatial and temporal temperature variation in the pile is likely to be detected. The method should further ensure that the operator's bias is minimized as much as possible. There are numerous statistical techniques available that can help decide how this can be achieved (Cochran, 1977). These techniques enable the development of a cost-, time-, and labor-efficient method to retrieve information and draw conclusions about the studied population without studying every member within that population. For example, simple random sampling is one such method, which can easily be adjusted to the composting environment. This method is based on the assumption that each member of the study

population has an equal chance of being selected. A sample of *n* units (where *n* refers to the sample size) is drawn at random out of the entire population *N* and their information is analyzed (Cochran, 1977). The characteristics of a population, such as variance, are then calculated from these *n* units and are considered to be unbiased estimates of the entire population. Thereby, theoretically, if the temperature probes can be included in the compost pile (i.e. population) in a random way and then recovered from it, they should provide an unbiased (operator error-free) estimate of the temperature variability and other parameters of interest.

- the number of temperature probes used for any particular composting technology should be based on the observed temperature variability in the system. In all types of large-scale compost systems, the temperature of the pile varies both temporally and spatially. The variability-inducing factors include wind, solar heating, ambient temperature, forced aeration, and/or substrate availability (Turner *et al.*, 2005; McCartney and Eftoda, 2005; Vinneras *et al.*, 2003; Christensen *et al.*, 2002; Fischer *et al.*, 1998; Hay, 1996).

- the frequency of temperature measurements should be such that any temporal variation is well represented in the data. Due to temporal variations, two or three temperature measurements of □55°C taken a day or two apart, for example, may not necessarily mean that the TTC has been satisfied. The temperature may have fluctuated in between measurements, especially in open systems such as windrows (Strader and Bromhal, 1997).

- the method used to interpret temperature data must be able to demonstrate whether the TTC was satisfied. Mean temperature values from several temperature logging devices that are commonly reported, such as sanitation indicators, are very sensitive to extreme values. The mean also does not reflect the contact time information, as it collapses the time series into a single thermodynamic index (Yu *et al.*, 2008) and hence can be

overly misleading. Therefore, the method to estimate sanitary efficacy should take into the account the time effects. Finally, if pile turning is involved, the interpretation method should also be capable of incorporating this into the sanitary efficacy estimations.

3.1.1 Objectives of the study

The objectives of this study were threefold. The first objective was to compare the results from the traditional systematic temperature sampling approach with the results from random sampling. Of particular interest were: 1) the discrepancy between the two different approaches in statistical information such as central tendency and variance and 2) a comparison of the likelihood of achieving the required TTC using each of the sampling methods. The second objective was to use the gathered information to estimate the number of temperature probes required to capture the observed temperature variability. The final objective was to infer from objectives 1 and 2 the probability of every particle's exposure to temperatures ≥55°C for at least 3 consecutive days, while incorporating the effects of pile turning.

3.2 Materials and methods

3.2.1 Materials

3.2.1.1 Compost pile

The study was conducted during the period from August to October 2010 at the Edmonton Waste Management Centre's composting facility (ECF), which uses Covered Aerated Static Pile (CASP) technology to compost biosolids. According to the technological specifications described in Fichtner *et al.* (2003) the semi-stabilized sludge from the City's wastewater treatment plant was pumped to the ECF after 21 days of stabilization in the anaerobic digester and stored in lagoons located near ECF at a solids concentration of 6% (by weight). The sludge was dewatered by centrifugation to have a final solids concentration of 28%. The dewatered sludge was then mixed with bulking agents (mixture of aged pallet

chips and freshly chipped pallet/wood waste) at a ratio of 2.5 parts bulking agent to 1 part biosolids (by volume) inside a mixing truck fitted with a vertical auger. The mixer content was discharged on the composting pad that is sized for sixteen 50 m long active composting piles with the following approximate dimensions: a length of 50 m; a height of 3 m; and a base width of 6 m. About 264 wet tonnes of feedstock material per pile were composted in two back-to-back stages of approximately 30 days each. Once the pile was built, it was covered with a selective membrane tarp to retain moisture and volatile acids within while preventing the contamination with pathogens and vectors. At the end of each stage the cover was removed, the material remixed with the front-end-loader and placed on another pad and covered for an additional 30 days. The final placing on a cure site in the form of mass beds (4-5 piles per mass bed) for 6 months with 5 mixings of the curing mass which then takes place and does not require covering was excluded from the study.

Although on average one CASP in the cure site is formed from 22 loads of vertical mixer trucks, the given study was limited to a segment of the pile consisting of 4 out of the 5 last loads (Figure 3-1). The practical approach to sample only a segment of a pile was based on the assumption that the segment would be a representative sample of an infinitely long windrow, and that the same biological, physical and chemical processes take place in this segment as in any other segment of the infinitely long pile.

Figure 3-1. The geometry of CASP, showing the segment used for the current study.

3.2.1.2 Temperature probes

In total, 41 temperature probes, which behave like random compost particles and are designed to withstand conditions within a compost pile (Isobaev *et al.* , 2014), were used in the current study. Each probe consisted of a temperature recording circuit enclosed in cylindrical casing made of anodized aluminum 6061 grade and a designated eyelet on one end. Twenty-one probes were designed to monitor temperature only and had the following dimensions: length of 110 mm; diameter of 25.4 mm; and density of 1580 kg m^{-3}. The other 20 probes contained an additional compartment for holding a cryovial, so that microbial inactivation in response to temperature could be studied. The modified probe had a length of 141 mm, a diameter of 25.4 mm, and a density of approximately 1630 kg m^{-3}. Prior to the experiment, the probes were calibrated according to the manufacturer's instructions. After successful calibration, the devices were batch programmed to read and record temperature data with a 10-minute frequency from the day the experiment was slated to begin. Lastly, a 15 m length of fishing line was attached

to the end of 20 probes through the eyelet; this length was greatly in excess of the maximum distance that a probe could be buried in a pile. After placement in the pile, the end of the fishing line remained outside of the pile and was used to easily locate and individually recover probes from the compost environment at the end of the experiment.

3.2.2 Methods

3.2.2.1 Random introduction of temperature probes

Out of the 41 probes, 21 were randomly selected and randomly introduced into the selected segment of CASP at the time when the pile was constructed. It had previously been determined that, on average, the mixer truck used in pile construction required 8 minutes to empty its contents. Assuming that a steady rate on the discharge conveyor is achieved within 10 seconds, and given that 4 loads were used in the experiment, 21 random numbers ranging from 10 to 1920 (representing the number of seconds required to discharge 4 loads) were generated in Excel. Each generated number was assigned to a probe and then removed from the pool eligible for future selection. The timer was started at the moment the mixer began to discharge onto the pile. Each probe was then placed on the mixer discharge conveyor at its preselected random time.

3.2.2.2 Systematic introduction of temperature probes

The remaining 20 probes, which had the attached fishing line, were introduced to the pile systematically. As previously mentioned, part of the compost pile was constructed, and then a section containing four (4) mixer truck loads was used as the experimental section. Before these 4 loads were placed, three of the 20 systematic probes were randomly selected and introduced to the face (i.e. cross-sectional surface) of the pile at approximate heights of 40%, 60% and 80% (of the pile height) from the ground and at an approximate horizontal distance of 40% from the pile surface (Figure 3-2). The mixer then began discharging. After 4 minutes, when the first half of the mixer load was discharged, two probes were manually placed on the new face of the pile, one at 20% and one at 40% of the

pile height from the ground. After the rest of the mixer truck load was discharged, probes were placed at heights of 60% and 80% from the ground. This procedure of placing two probes in the pile after every half mixer load was repeated for each of the 4 loads, as portrayed in Figure 3-2. After the last segment of the compost pile was built, three probes were manually introduced to the end cross-sectional surface of the pile at heights of 20%, 40%, and 60% from the ground. One final mixer truck load was then placed on the pile.

3.2.2.3 Composting

After all the probes were introduced, the pile was covered with a selective membrane. The composting of the biosolids/woodchip feedstock was carried out in 2 back-to-back stages: stage 1, which lasted for 4 weeks; and stage 2, which lasted for 3 weeks. During composting, aeration was accomplished by positive forced aeration through in-ground trenches on a composting pad. Upon the completion of stage 1, the membrane was temporarily removed from the CASP to allow the moving/turning of the entire pile with a front-end loader to begin stage 2.

3.2.2.4 Recovery of temperature probes

The systematically placed probes were manually recovered from the compost pile while it was being moved to stage 2 with a front-end loader. When the loader operator filled the bucket with compost, the fishing line attached to the probes were held. Any probes that ended up in the loader bucket were recovered by pulling the attached ropes. These probes were not reintroduced thereafter and the reasons for this were: 1) inability to preserve the same spatial distribution in stage 2 as it was in stage 1 for a sample size as large as 20; 2) acquisition of sufficient data for the comparison of results from the systematic and random sampling approaches; and 3) uncertainty in the success of the recovery tool. Since the locations of the randomly placed probes were not known, they were moved directly from stage 1 to stage 2 with the rest of the material, and recovered from the pile at the end of stage 2 by passing the composting mass through a trommel screen with ½ inch openings.

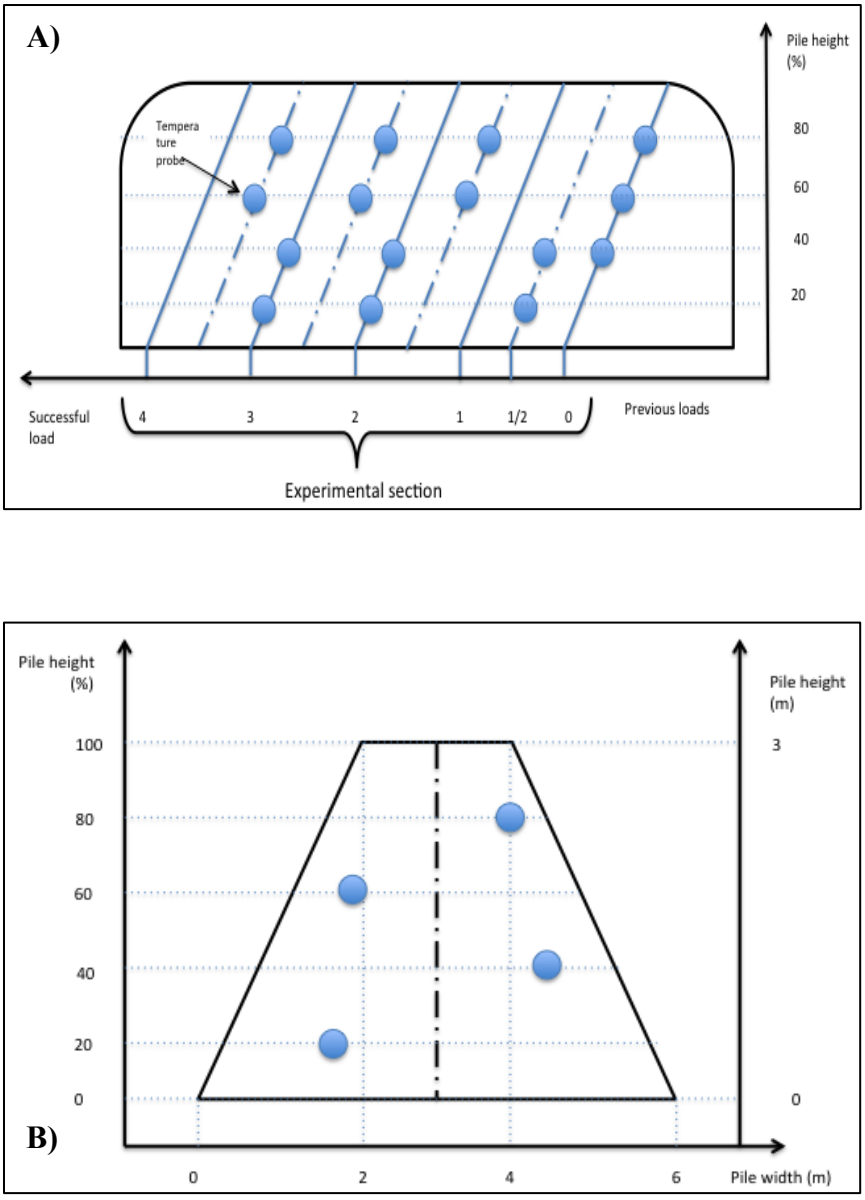

Figure 3-2. Demonstration of (A) longitudinal and (B) cross-sectional projection of the experimental compost pile segment with the systematic placement of the probes within this segment

3.2.2.5 Data analysis

The time-temperature data from each recovered temperature probe was manually downloaded to a computer using software provided by the manufacturer. Not all data from each probe was used in the time-temperature analysis. Only data belonging to the period when the pile was covered with the selective membrane was considered.

3.2.2.6 Comparison of sampling methods

Both sampling approaches (systematic and random) were evaluated against two criteria: (i) the ability to capture daily temperature variance (mean and 95% confidence interval), and (ii) the resulting conclusions about whether the TTC was met. The mean daily temperature and corresponding confidence intervals around each daily mean value in both the systematic and random temperature sampling approach were calculated in Sigmaplot v12. The conclusions about TTC were made by analyzing the temperature profile of each individual temperature probe, counting the total number of the probes in which TTC was met in each sampling approach and subsequently dividing this figure with the total number of temperature probes used in that approach.

3.2.2.7 Estimation of the number of temperature probes required for representative sampling

The method outlined here relies on the assumption that the biochemical composition at each point of the experimental pile is approximately the same (with the exception of pile surface and both its ends) and therefore the temperature of each compost particle at any instance is normally distributed around a central temperature value. Consequently the ratio between sample size variance and the population variance in the collected dataset could be expressed by a chi square distribution (equation [1]), where n is the sample size; S^2 is the sample variance; σ^2 is the population variance, and $\chi^2_{n-1}$is the chi square distribution with n-1 degrees of freedom.

$$\frac{(n-1)\times S^2}{\sigma^2} \sim \chi^2_{n-1} \quad \textbf{[1]}$$

As for the sample variance, it is calculated according to equation [2],

$$S^2 = \frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1} \quad \textbf{[2]}$$

Where $\bar{x}$ is the sample mean. Using the statistic defined in equation [1], the $100\times(1-\alpha)\%$ confidence interval on the parameter σ^2 can be written according to equation [3],

$$A = \frac{(n-1)\times S^2}{\chi^2_{n-1,\frac{\alpha}{2}}} \leq \sigma^2 \leq \frac{(n-1)\times S^2}{\chi^2_{n-1,1-\frac{\alpha}{2}}} = B \quad \textbf{[3]}$$

Equation [3], which concerns a random interval covering σ^2, provides a 100x(1-α)% confidence interval for σ^2.

Selecting upper and lower boundaries of B and A, respectively, equation [3] can be rearranged to yield the following:

$$B - A = (n-1)\times S^2\left(\frac{1}{\chi^2_{n-1,1-\frac{\alpha}{2}}} - \frac{1}{\chi^2_{n-1,\frac{\alpha}{2}}}\right) \quad \textbf{[4]}$$

Rearranging equation [4] for sample size (n) gives:

$$n = 1 + \frac{B-A}{S^2\times\left(\frac{1}{\chi^2_{n-1,1-\frac{\alpha}{2}}} - \frac{1}{\chi^2_{n-1,\frac{\alpha}{2}}}\right)} \quad \textbf{[5]}$$

The n value corresponds to the number of probes needed to account for the observed temperature variability in the pile, assuming that all data comes from simple random sampling where each observation is independent from another.

Because the temperature variability might be different from stage to stage during composting, the necessary number of probes was determined for each stage individually and then the largest number of probes was accepted as the required sample size. In this way, one can be assured that the temperature variability is adequately addressed regardless of the composting stage.

3.2.2.8 Likelihood of composting technology compliance to TTC

The probability of compliance with the TTC was determined using the data collected by the randomly introduced temperature probes. The time-temperature data from each individual probe in each composting stage was analyzed to determine whether the temperature in the probe has reached ≥55°C for at least three consecutive days. The probability of any random particle's exposure to the TTC was then estimated by dividing the number of temperature probes that had satisfied the TTC by the total number of probes. The turning effect was incorporated into the picture using a Markov chain model. Sensitivity analysis of the turning effect was done using the logistic probability method described in Doubilet *et al.* (1985).

3.3 Results and discussion

3.3.1 Comparison of sampling methods

3.3.1.1 Temperature variability

Temperature trend lines based on daily mean observations over the course of stage 1 composting for the systematic and random temperature sampling methods are displayed in Figure 3-3. This figure also shows the daily temperature variability. Both methods produced similar results. They both showed that the thermophilic temperature occurred around the 3rd day of composting. Both methods also showed that the temperature started to decrease after the 13th day of composting. The stage 1 mean temperature values from the systematically and randomly sampled data were 56.9°C and 54.8°C, respectively. The maximum daily mean temperature recorded from the systematic sampling method was 69.7°C, while that from the random sampling method was 68.8°C.

There were, however, differences in the information between the two datasets. For instance, the daily temperature variation around the mean value was greater in the randomly sampled probes. Although this difference is not very large during the initial temperature rise, it becomes obvious during the thermophilic and cooling phases. The larger temperature fluctuation in the random set of probes was most

likely induced by the fact that the randomly introduced probes could have ended up anywhere in the compost mass, and the "human factor" (i.e. intentional or unintentional bias) was excluded from any sort of influence on the sampling pattern. It was observed that some probes settled on the surface of the pile, where normally the temperature is lowest and the temperature fluctuation is highest due to interaction with the ambient environment. Other probes likely ended up in the pile core, where the temperature fluctuation is minimal and the temperature tends to be high. Many more were distributed spatially across the pile, thereby supporting the idea of randomization. As a result, the information gathered by the randomly introduced probes captured temperature values from a variety of locations, and therefore provided a more representative picture of the spatial changes in temperature in the pile during the biomass composting. It is therefore recommended to practice random temperature sampling scheme when monitoring the TTC in composting facilities.

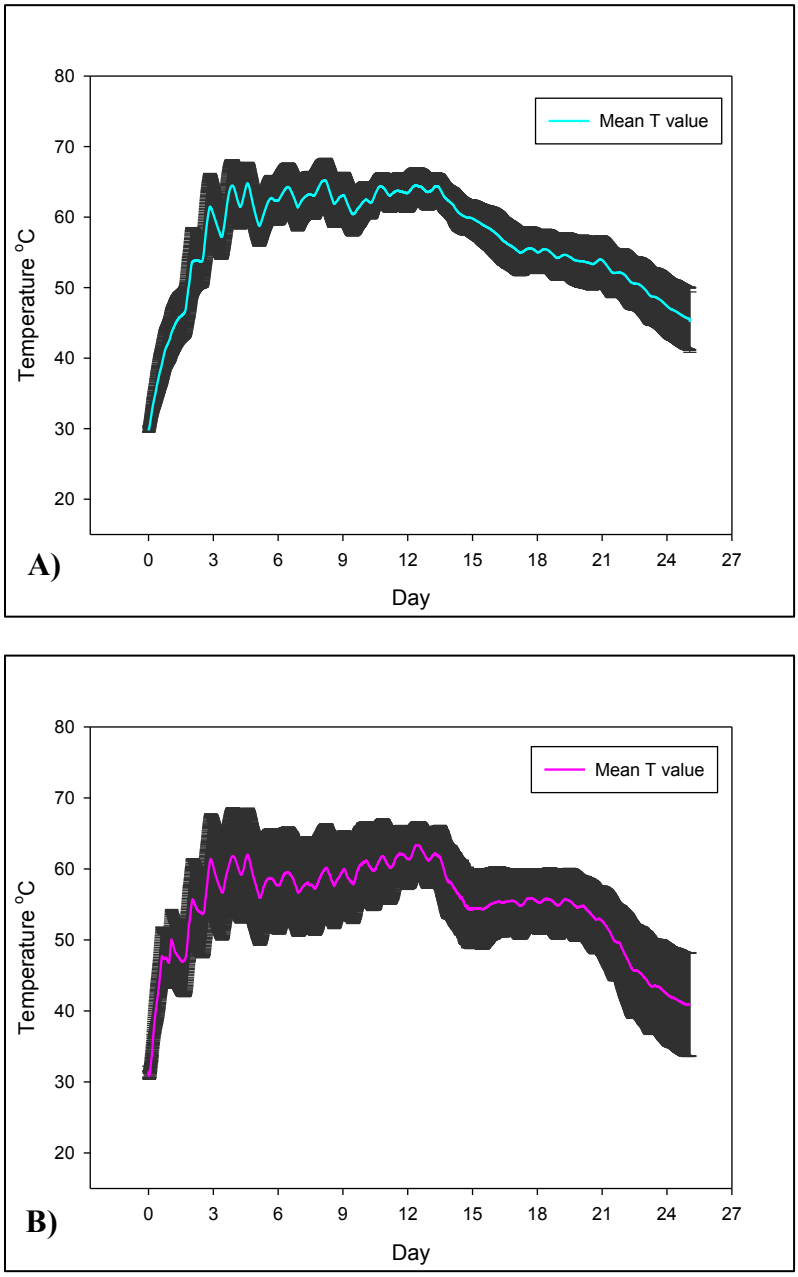

Figure 3-3. Mean daily temperature profiles (colored lines) from two temperature sampling approaches and their 95% confidence intervals (black shading): A) systematic sampling, and B) random sampling.

3.3.1.2 TTC

Descriptive statistics for each data set (e.g. for the systematically and randomly-introduced probes) are presented in Table 3-1. Accordingly, it took about 3 days for the probes in the systematic method to reach T ≥ 55°C, after which the temperature persisted for 17 days. The average time for the randomly introduced probes to heat to ≥ 55°C was the same as that of the systematically placed probes, as was the average number of consecutive days where temperatures ≥ 55°C were maintained.

Table 3-1. Descriptive statistics from data collected from the systematically and randomly introduced probes.

Factor	Systematic method		Random method	
	Mean	±SD	Mean	±SD
T ≥ 55°C reached (d)	3.07	1.82	3.40	3.57
T ≥ 55°C maintained (d)	17.63	6.20	17.20	10.6
% maintained at T ≥ 55°C for more than 3 days	95.00		76.20	

The proportions of temperature probes meeting the TTC were different in the two sampling methods. TTC was met in 95% of the systematically placed probes, but only in 76% of the randomly placed ones. Presumably this difference resulted from the fact that the systematically placed probes were all placed close to the middle section of the pile (as is normally done in practice; it is uncommon for surface temperatures to be monitored). It is known that the middle of the pile is generally hotter than the edges (although there may be cool pockets within). Conversely, the placement of the random probes was not biased in any way; they were free to land anywhere in the pile, including at the surface of the pile, where it is usually cooler. In most scenarios where inferential statistics is involved, 95% is usually the accepted cut-off level for passing the quality criteria, while 76%

would represent a failure to meet the required quality standards. However, it should be noted that for compost production, achieving 95% of particles' exposure to temperature ≥55°C is not sufficient as regulations specifically say that 100% of particles should meet the TTC. A single measurement that does not satisfy the TTC would result in the whole pile being considered out of compliance (UESPA, 2003).

It is interesting to note that, in the scientific literature, pathogens have been reported in finished composts that seemed to meet the TTC requirements (e.g. Brinton *et al.*, 2009). It is possible that if a systematic temperature monitoring method were used, zones in the pile not meeting the TTC may have been missed. The data obtained from this study, showing that randomly introduced probes were less likely to meet the TTC than systematically-placed probes, seem to support this hypothesis. Stating it in another way: the inference from the random sampling approach was more conservative than that from systematic sampling and it is therefore recommended that the random temperature sampling approach should be practiced in the composting industry.

Certainly, demonstrating the advantages of random temperature sampling over systematic sampling is not sufficient for the practical viability of the temperature probe. Obviously it would be too onerous, at this level, to use the random method for routine monitoring, particularly given the efforts that should be spent during the recovery stage of temperature probes. We predict that this method will gain its practical recognition when the technology to enable temperature probe recovery becomes convenient. For the time being, however this approach can be practiced in optimization of composting methods, as well as in indirect and more sophisticated technology validation.

3.3.2 Estimation of the number of temperature probes required for representative sampling

3.3.2.1 Stage 1 composting

The total temperature variation for stage 1 composting was calculated from the information retrieved from the 21 randomly introduced temperature probes. The temperature variation was 172.51. The daily temperature variation dynamics during stage 1 are shown in the (Figure 3-4). The highest 24-hour temperature variation in stage 1 (228.5) was observed on the 28th day of composting, while the smallest (62.1) was recorded on day 15. Assuming simple random sampling, the number of temperature probes needed to account for the spatial and temporal temperature variability in the pile during stage 1 was calculated (using equation [5]) to be at least 41.

3.3.2.2 Stage 2 composting

During stage 2 composting, the overall temperature variation was estimated at 281.15. As shown in Figure 3-4, the highest variation was observed on day 3 (410.9). The lowest variation occurred on day 19, just before the completion of stage 2, and was estimated at 147.7. Consequently, the total number of temperature probes needed to account for the observed variability in stage 2 was calculated to be 47. This value (i.e. n = 47) is recommended as the minimum number of probes to use to monitor this particular composting process from a TTC evaluation standpoint.

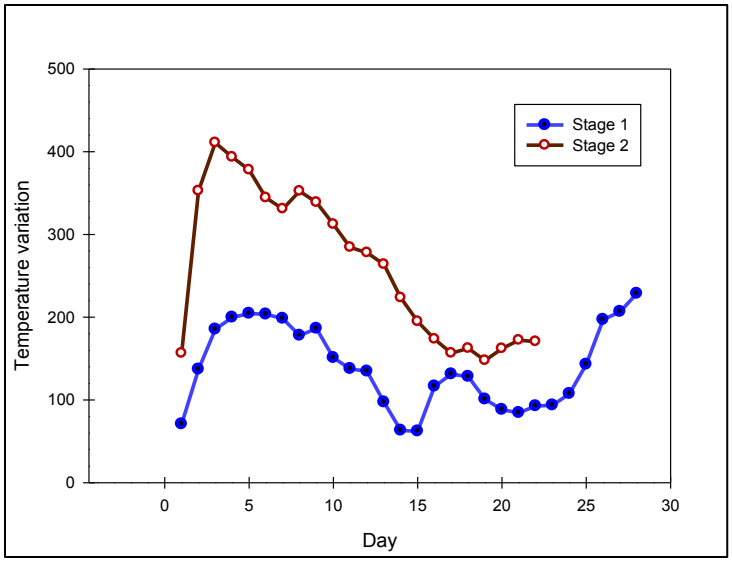

Figure 3-4. Daily temperature variance in the CASP: blue line indicates the squared distance of observed daily temperature values from the mean temperature value at that particular day in randomly introduced probes whereas the red line indicates the same difference but for the stage 2 composting.

The results regarding the number of temperature probes rely on an assumption of the normality of data, which is a requirement for the chi square test. However, the data collected in this study was not normally distributed. In fact, it was nonlinear and highly correlated, which most likely means that the required sample size of 47 temperature probes was underestimated. One can use these results and calculations as a framework to provide rationale when making an educated guess on the approximate number of temperature monitoring devices to be used in a composting plant. Furthermore, it is recommended that this approach should be used with caution; for example, it may be prudent to multiply the results obtained by this method by some reasonable correction factor. Incorporating such a "factor of safety" would produce a greater confidence in inferring the level of sanitation from the temperature probes.

3.3.3 Likelihood of composting technology compliance to TTC

For any random particle in compost sanitation, conditions of either "pass" or "fail" follow the binomial distribution. If a particle was exposed to temperatures ≥55°C for at least three consecutive days, it passes the sanitation criteria. Otherwise, it fails. As was mentioned previously, 21 temperature probes were introduced randomly into the compost pile and recovered after stage 2 of composting. Figure 3-5a shows the percentage of those 21 probes that did satisfy the TTC at some time during either stage 1 or stage 2. It shows, for example, that 76% of the probes met the TTC in stage 1. Since every temperature probe behaved like a random compost particle, we can generalize these results to the entire pile and conclude that during stage 1 of composting 76% of the biomass should have complied with the TTC.

3.3.3.1 Effect of turning

In practice, current regulations (e.g. USEPA, 2003) require that the biomass be turned several times over the course of composting in order to increase the chances of any random particle meeting the TTC for sanitation. In particular, static piles should be turned a minimum of three times over the course of composting while windrows require a minimum of five turns. Whether the specified number of turnings is sufficient was never measured. This section presents a framework that can be used to make an educated guess regarding the number of turnings.

In the current study, it was found that during stage 1 of composting 24% of temperature probes did not meet the sanitation requirements (Figure 3-5a). However, when the compost was moved to the next stage (turning effect), 40% of those that did not meet the requirements in stage 1 did meet them in stage 2. By incorporating this finding with the information from stage 1, it can be said that the single turning increased the probability for any random particle to comply with the sanitation requirements up to 85%.

Using the assumption that the fractions depicted in Figure 3-5a are the results of multiple repeated observations, the Markov's chain method can be applied to calculate how many successful turnings are required in order to achieve the desired sanitation level. It is necessary to know both the initial state distribution matrix (S_0) (i.e. probability of exposure without any turning) and the transition probability distribution matrix (P) (i.e. probability of what would happen after turning takes place). For the given case based on a single observation, S_0 and P could be expressed as follows:

$$S_0 = [0.76 \quad 0.24];\ P = \begin{bmatrix} 1.0 & 0.0 \\ 0.4 & 0.6 \end{bmatrix}$$

The S_0 matrix expresses that without turning there will be a 76% probability that any particle will pass the TTC. The P matrix states that all particles which passed previously will maintain their passing status, and those which didn't pass in stage 1 have a 40% chance of passing (and 60% of failing) in the subsequent stage. The first state distribution matrix (S_1) corresponding to the probability of temperature contact time compliance as a result of a single turning is the product of these two matrices i.e.

$$S_1 = S_0 \times P = [0.85 \quad 0.15]$$

where the first term (0.85) corresponds to the probability (85%) of compliance to the sanitation criteria. Note that this is the same probability which was obtained before for the entire pile after stage 2 of composting. This procedure can be repeated to estimate the exposure probability after two or more turnings by multiplying each state matrix (S_0, S_1, …, S_n) by the transition probability matrix (P).

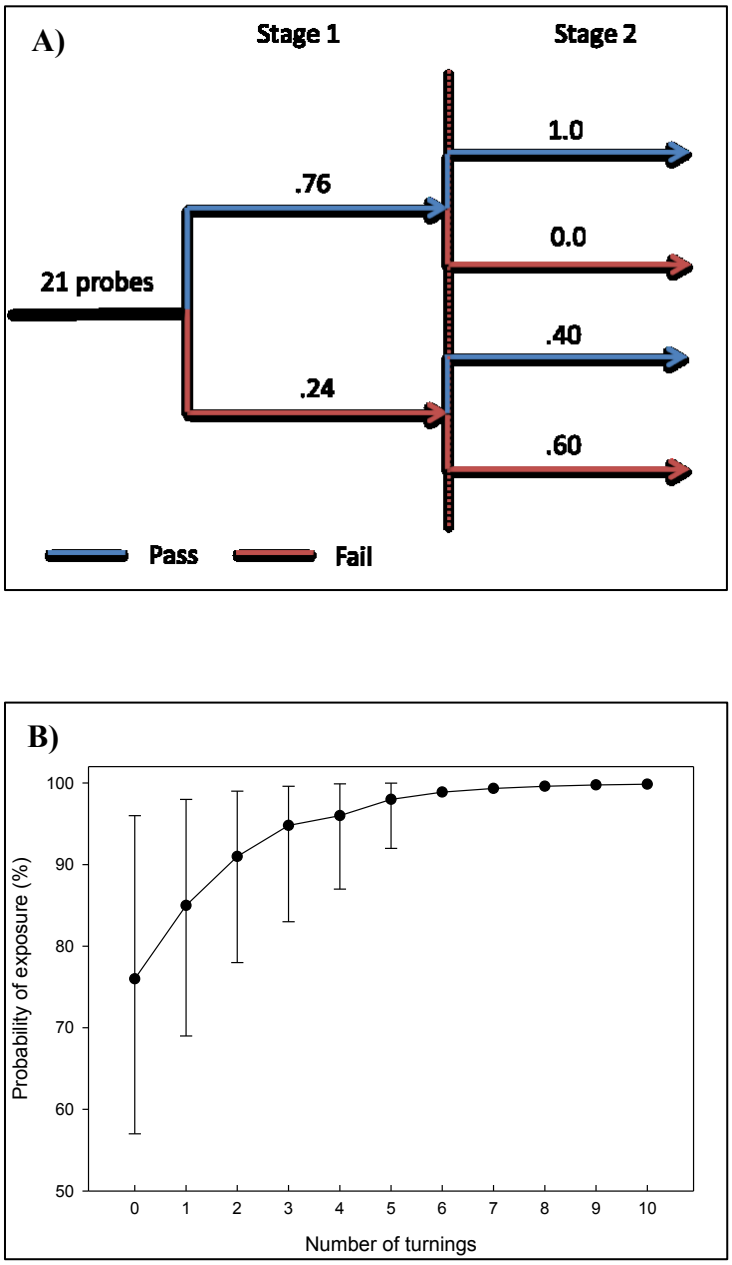

Figure 3-5. The transition diagram showing the percentage of temperature probes which either passed or failed to pass the TTC in the CASP: the blue branches indicate the fraction which did pass and the orange branches the fraction which did not; B) the plot of sanitation efficacy as a function of successful turning of biomass in CASP. Error bars represent 95% confidence interval.

3.3.3.2 Sensitivity analysis

The transition probabilities in Figure 3-6a were assumed to be constant, whereas in real life they are likely to vary due to changes in physical and chemical conditions such as feedstock composition, weather and other uncertainties surrounding composting technology. The ideal case to tackle these uncertainties would be to run multiple repetitive observations (trials) and derive the transition probability with accompanied confidence intervals from those observations. This however could not be done given the complexity and time commitment needed to complete each trial. The sensitivity analysis is a typical approach used to address the uncertainties given time and resource constraints.

The expected increase in the probability of meeting the TTC (i.e. a "pass") as related to the number of pile turnings

Figure 3-5b. The probabilistic sensitivity analysis (95% confidence range) in the Figure 3-5b is based on the method from Doubilet *et al.* (1985). In brief, although the method used herein was designed for use in biostatistics, the conditions in clinical studies were assumed to resemble those in the compost pile. Like in clinical trials, the inference on TTC, in the current study, was collected from the small sample size and the transition probability used for the calculation of baseline values for pass or fail was collected by individually monitoring each station (analogous to interviewing patients at each station). The sensitivity analysis, which is defined by its mean and standard deviation, further assumed a logistic-normal distribution for each transition probability (Dubilet *et al.*, 1984) which can be characterized by two parameters: mean and standard deviation. To define the mean of logistic-normal distribution the normal approximation for a binomial (pass-fail) distribution was used to calculate the lower bound of its 95% confidence range using Equation [6]

$$p_{0.95} = \hat{p} - 1.96\sqrt{\frac{\hat{p}(1-\hat{p})}{n}} \qquad \textbf{[6]}$$

where $\hat{p}$ represents the baseline value provided in state distribution matrix and n corresponds to the sample size.

To determine the population parameters (i.e. mean-μ and standard deviation-σ) of the normal distribution approximating the logistic-normal distribution Doubilet *et al.* (1985) provides following equations:

$$\mu = \frac{B - E \times \sqrt{B^2 - M^2 + (M \times E)^2}}{1 - E^2} \quad \textbf{[7]}$$

where:

- $M = \log(\frac{\hat{p}}{1-\hat{p}})$;
- $B = \log(\frac{p_{0.95}}{1-p_{0.95}})$, and
- $E = \frac{1.96}{\phi^{-1}(\hat{p})}$,where $\phi^{-1}(\hat{p})$ is the inverse of the standard normal distribution.

The standard deviation σ was calculated using equation [8]

$$\sigma = \frac{|\mu - B|}{1.96} \quad \textbf{[8]}$$

After estimating the distribution parameters the implicit upper bound of 95% confidence range was calculated satisfying equation [9]

$$Implicit\ upper\ bound = \frac{e^{(\mu + 1.96\sigma)}}{1 + e^{(\mu + 1.96\sigma)}} \quad \textbf{[9]}$$

One interesting observation from the Figure 3-5b is that the first five turnings provide the most significant impact on the probability of meeting the TTC when using CASP technology. After the 5th turning, the probability of every particle's exposure is approximately 98%. This agrees with the theory, which is normally applied to windrows, that five turnings should be sufficient to comply with the TTC. It can further be seen that the curve becomes asymptotic after approximately the 7th or 8th turn. In probability this is called as "asymptotically almost surely" and means that as number of turnings approaches large number (e.g. infinity) the likelihood of compliance apparently converges to 100%. However, the cost of turning beyond 6 or 7 times likely outweighs the benefits.

Last, but not least, it should be mentioned that the sensitivity analysis was based on 21 observations. This sample size is typically considered small for the binomial distribution to approximate the normal distribution. In addition, the values in the transition probability distribution matrix were held constant whereas in reality they are likely to change. That's to say, if after the first turning 40% of the samples passed the TTC, then after the second and subsequent turnings this proportion would be different, which would subsequently affect the values in the state distribution matrix and the sensitivity analysis curve by changing the confidence interval width. The authors, therefore, once again recommend that the methods and consecutive results presented here be viewed as a framework for making educated guesses.

3.4 Conclusion

In the current study, the importance of proper temperature sampling was discussed. It was demonstrated that whenever possible random temperature sampling should be practiced. Random sampling captures more temperature variability, both temporally and spatially, than does systematic sampling. Therefore, logically, the inferences made based on random temperature sampling should be more conservative and more representative of actual conditions in the compost pile when compared with the traditional systematic temperature monitoring practice.

Furthermore, if the random temperature monitoring approach is used to monitor the temperature development in the pile (either for process validation or routine monitoring purposes), the number of the temperature probes needed to obtain representative data can be statistically estimated. For example, in the given study the information gathered with the random temperature monitoring was plugged into a chi square distribution to estimate the number of temperature probes needed to ensure the adequate capturing of the spatial and temporal temperature variability. The results demonstrated that at least 47 probes should be used for obtaining meaningful inferences from CASP technology. This number is much

greater than what is normally used in practice. Given the benefits of the random approach, it may be justifiable to use this method for routine monitoring, or at least for process validation.

Finally, the information collected by random sampling was used in combination with the Markov chain equation to gauge the effect of pile turning. If temperature probes are introduced randomly into the compost, they have the same chances of settling anywhere within the biomass as an ordinary compost particle. Likewise, when the pile is turned the temperature probes have the same chance as a random particle of compost of being moved into another spot. Pile turning should increase the chances of a compost particle (or temperature probe) being exposed to temperatures ≥55°C for three consecutive days. It was found that before turning the CASP, 76% of the particles would comply with the TTC. It was also found that turning the pile did, in fact, increase the probability of meeting the TTC. The first five turnings are likely to have the most profound impact on increasing the chance of compliance. In the current study, after one turning the chance of meeting the TTC increased to 85% and 98% after 1 or 5 turnings, respectively.

There were several limitations to the current study. Firstly, assumption of the normality of data is applied to the chi square test. However, the data collected in this study was not normally distributed, which would most likely mean that the required sample size was underestimated. It is recommended that this approach should be used with caution; for example, it may be prudent to multiply the results obtained by this method by some reasonable correction factor. Incorporating such a "factor of safety" would produce greater confidence in inferring the level of sanitation from the temperature probes.

A second limitation of this study is that only data from a single turning event was available to estimate the effects of multiple turnings. It is possible that the period during which the monitoring was conducted was somehow abnormal, or that subsequent turnings of the pile would not produce exactly the same effect as the first turning.

To address the above concerns, it is highly recommended to conduct repetitive monitoring and to build a representative pool of reference data for any process being evaluated by the methods proposed herein. This data pool will not only increase the robustness of the proposed methods against underlying assumptions, but it will also increase the accuracy of future inferences.

CHAPTER 4: AN ENHANCED DIRECT PROCESS VALIDATION FRAMEWORK IN COMPOSTING: CASE STUDY OF COVERED AERATED STATIC PILE[1]

4.1 Introduction

Composting is the degradation of organic matter by microorganisms. The indigenous microorganisms found in compost feedstock transform biodegradable organic materials into a biologically stable state, which can then be used as a soil conditioner (Haug, 1993). It is estimated that more than 1.7 million tonnes of organic matter is composted in centralized composting facilities in Canada and 58.7 million tonnes in the US per year (Elliott, 2008; USEPA, 2011). Although these figures may indicate society's shift towards sustainable resource utilization, the raw feedstock for composting often times can be contaminated with pathogenic microorganisms. Producers therefore are obliged to ensure the efficient sanitation of the end product before it is released to the consumer market.

In order to ensure that the finished compost product is hygienic, the direct enumeration of pathogenic and indicator microorganisms is typically carried out. In addition, the minimum process criteria (i.e., TTC) must be verified (NRC, 2002; USEPA, 2003). The indicator organisms typically used as a measure of a treatment's efficiency include: 1) helminthic ova and enteric viruses, due to their hardiness and resistance (NRC, 2002); 2) total and fecal coliforms and fecal streptococci, as they may be affected by environmental stresses similarly to most bacterial pathogens and therefore can serve as useful indicators of predominantly pathogenic *Escherichia coli*, as well as *Shigella* and *Salmonella* sp. (Yanko 1988; NRC, 2002). Federal and provincial regulations in North America require that for

[1] A version of this chapter has been accepted for publication. Isobaev et al. *Compost Science and Utilization* (March 2014).

unrestricted compost the level of fecal coliforms should not exceed 1,000 MPN g^{-1} dry solids and that of *Salmonella* should be less than 3 MPN 4 g^{-1} dry solids (Ge *et al.*, 2006; BNQ, 2005; CCME, 2005; USEPA, 2003). In terms of process criteria, the North American guidelines require every particle of compost to be exposed to ≥55°C for at least 3 consecutive days. This combination of process and end-product criteria have become common practice for the past two decades to ensure that hygienic compost products are produced (Morales *et al.*, 2005). Similar regulations exist in Europe, Australia, and some Asian countries (Hogg *et al.*, 2002).

A recent literature review by Wichuk and McCartney (2007) revealed that viable pathogenic organisms (bacteria, viruses, protozoa, and helminths) have sometimes been detected in compost that appeared to comply with the North American time-temperature criteria (TTC). Among other factors, the authors hypothesized that the reported pathogen survival could be due to: 1) failure to detect low temperature zones where microorganisms proliferated; and/or 2) inadequacy of the existing time-temperature criteria. More recent literature regarding compost sanitation supported the findings of Wichuk and McCartney (2007) (e.g. Barrena *et al.,* 2009; Inglis *et al.,* 2010; Wery *et al.,* 2008; and Xu *et al.,* 2009 in Section 1.3)

Although the time-temperature criteria extend to every compost particle, it is neither practical nor possible to monitor the fate of every particle in compost during full-scale operation. Moreover, existing regulations, such as those of the USEPA (2003) and CCME (2005), do not provide guidelines on how to ensure that the contact time-temperature criteria have been properly satisfied in any particular technology (see Section 3.1).

To cover this gap Wichuk and McCartney (2008) hypothesized that the best approach to monitor the temperature experience of a random compost particle in compost would be to introduce directly into a process a device which would: a) be self-contained; b) behave like random particle during pile agitation and settling; c) be sturdy enough to endure physical and chemical stresses; and d) have the

capability to record real time temperature data for an extended period of composting. Wichuk and McCartney (2008) and then Isobaev *et al.* (2014) modified a commercial temperature data logger (probe), so that it would be better adapted to a composting environment. The field trials demonstrated that the temperature probe behaved like a random particle inside the compost pile and on its surface (Isobaev *et al.*, 2014). Moreover, the aluminum case was sufficient in protecting the probe's circuitry from various physical and chemical stresses. Finally it was demonstrated that after being randomly introduced into the compost pile the probe can be recovered from it by screening the pile at the end of composting with a trommel screen.

4.1.1 Objectives of the study

Specific objectives of the study were to investigate from a random compost particle perspective: 1) the likelihood that a compost product would meet the TTC in a full-scale operation; 2) the likelihood that traditionally cool zones would meet the TTC as a result of turning and 3) the effectiveness of TTC to inactivate the indicators of enteric bacteria and enteric viruses whose levels in finished product are regulated by national and provincial regulations. So far, no similar study aimed at assessing the TTC from a random particle perspective has been undertaken to-date.

4.2 Materials and methods

4.2.1 Materials

4.2.1.1 Compost pile

The study was conducted during the period from July to September 2011 at the Edmonton Waste Management Centre's composting facility (ECF), which uses CASP technology to compost biosolids (see Section 3.2.1.1).

4.2.1.2 Temperature probes

In total, 34 temperature probes were used in this portion of the study. Each probe consisted of a temperature recording circuit enclosed in cylindrical casing made of anodized aluminum 6061 grade (see Section 2.1.2). Seventeen probes were designed to monitor temperature only and had the following dimensions: length = 110 mm, diameter = 25.4 mm. Another seventeen of these probes contained a designated built-in compartment for holding a microbial inoculum, in order that microbial inactivation in response to temperature could be studied, hence the length was extended to 141 mm. Prior to the experiment, the probes were calibrated and batch programmed to read and record temperature data with a 15-minute frequency from the day the experiment was slated to begin. Lastly, a 20 m length of fishing line was attached to the end of each probe; this length was greatly in excess of the maximum distance that a probe could be buried in a pile in order to avoid affecting its random placement. The fishing line was used to improve the recovery efficiency of the probes; after placement in the pile the end of the line was kept outside of the pile. At the end of the experiment, the line was used to locate and recover the probes during pile deconstruction.

4.2.1.3 Microbial inoculum

Pure cultures of *Escherichia coli* ATCC 29425 (*E. coli*), *Pseudomonas* fluorescens ATCC 27663 (*Pseudomonas*), and *P. fluorescens* phage phi-s1 ATCC 27663-B1 (phi-s1) were obtained from Cedarlane Labs (Burlington, ON Canada). A strain of *Salmonella enterica* var. Meleagridis (*Salmonella*) was supplied by the Provincial Laboratory of Public Health (Edmonton, AB Canada). Stock solutions of these microorganisms were prepared 3 days before the experiment. To prepare stock solutions of *E. coli* and *Salmonella*, their pure cultures were individually seeded into tryptic soy broth (TSB). The two solutions were then incubated overnight at 36°C and their optical density (OD) was monitored over time with a spectrophotometer set at 600 nm wavelength. When the microbial growth in each stock solution reached the stationary phase, which was defined as having no change in their OD for over a 3 hour period, the concentration of cells in each

stock solution was estimated using a standard plate counting method on standard plate count (SPC) agar plates.

The phi-s1 was resuscitated in the laboratory, following the supplier's instructions, and 0.5 mL was transferred into a test tube containing 15 ml of *Pseudomonas* stock previously grown overnight. The solution was incubated at 26°C for 24 hours and then filter-sterilized through a 0.22 μm filter to separate the phage from the bacterial cells. The resulting aliquot contained an enriched stock solution of phi-s1. The phage concentration was estimated via a soft *Pseudomonas* agar overlay plaque forming dilution assay.

The final solution ("inoculum") was prepared one day before the experiment start date, after the concentration of microorganisms in their stock solutions was defined. Calculations were done to determine the volume of each stock solution required to obtain 24 mL of a final solution containing a mixture of organisms in the following concentrations: *E. coli* and *Salmonella* at 10^6 colony forming units (CFU) mL^{-1} and phi-s1 at 10^6 plaque forming units (PFU) mL^{-1}. The calculated volumes were added into a sterile plastic test tube, topped to 24 mL with phosphate buffered dilution water and shaken 25 times. One mL of the inoculum was then transferred into each of 24 cryovials, which were sealed and stored at 4°C until being inserted into the temperature probes.

4.2.2 Methods

4.2.2.1 Temperature probe introduction and recovery

The night before the experiment, 17 cryovials were inserted into the 17 temperature probes with cryovial compartments. The cryovial compartment of each probe was sealed to prevent any uncontrolled release into the environment. Two cryovials were also designated as controls – one was stored at 4°C and another at room temperature (~22°C). All 34 probes (17 with cryovials and 17 without cryovials) were transferred to ECF and introduced into the experimental compost pile. The 17 probes with cryovials and 7 probes without cryovials were introduced randomly into the pile: at randomly selected times, they were thrown

onto the discharge belt of the mixing truck used to construct the pile. The remaining 10 probes were introduced into cool zones - close to the pile's surface which remains cool relative to the bulk of the material.

The composting process involved two back-to-back stages. In stage 1, the pile was covered and aerated periodically for 30 days. After stage 1 the cover was removed from the pile and the entire mass turned with the front-end loader. All probes inside the pile were allowed to move with the bulk of the material during the turning process. Then the pile was covered again and left to compost for another 26 days (stage 2).

At the end of stage 2 twenty-six temperature probes were recovered by holding onto the fishing lines as the pile was being moved to form mass beds. The fishing line attached to 8 of the probes was broken during pile building or pile turning; these probes were recovered from the compost by screening the compost mass with a trommel screen. The recovered probes were brought to the laboratory for microbial survival and temperature analysis.

4.2.3 Analysis of temperature data

The temperature profile from each temperature probe was downloaded using the manufacturer's software and then imported into a spreadsheet. The data corresponding to the time before the compost pile was covered, as well as the data from when the cover was removed after stage 2 composting were deleted from the dataset. Graphical analysis was done using the graphical software SigmaPlot v12.

4.2.4 Microbiological analysis

One day prior to probe recovery, *E. coli, Salmonella* and *Pseudomonas* were grown overnight in their selective broths for use as controls. Analysis of microbial viability in elevated temperatures was begun within 24 hours of the probes being removed from the compost pile. When not in use, the samples were stored either in a 4°C fridge or on ice on the laboratory bench. Unless otherwise indicated, all serial dilutions were done in triplicates.

Phi-s1 viability was determined using a soft *Pseudomonas* agar overlay plaque forming dilution assay. The cryovial was vortexed and 0.1 mL of its inoculum pipetted into a centrifuge tube containing 0.9 mL of ultrapure water. The centrifuge tube was vortexed and its inoculum filter sterilized to remove any bacterial traces. Soft *Pseudomonas* agar was prepared by adding 5 $g \cdot L^{-1}$ of agar into freshly prepared *Pseudomonas* broth. Three mL of the soft agar was transferred into sterile tubes and kept warm in a 45°C water bath. 0.3 mL of *Pseudomonas* and 0.1 mL of the diluted and filter-sterilized inoculum was then added into each tube. The test tubes were removed from the water bath, rolled between the palms for 2-3 seconds to mix the contents, and the soft agar poured on the surface of a *Pseudomonas* agar plate. The plates were allowed to harden at room temperature and then incubated inverted overnight at 26°C. Plaque formation was counted after 24 hours of incubation.

Salmonella was detected and quantified by culture using chromogenic xylose lysine deoxycholate (XLD) agar. 100 µL of inoculum from a cryovial was whole plate spread on an XLD plate. The inoculum was allowed to absorb on the agar surface. All plates were then incubated inverted overnight at 36°C. After 24 hours the concentration of viable *Salmonella* was determined by counting black colony formations on XLD.

E. coli concentrations in the cryovials were determined using both XLD agar and enzyme-substrate method (IDEXX QuantiTray™ 2000). All QuantiTrays™ were incubated at 35°C for 24 ± 0.5 hours. After 24 hours, the concentration of viable *E. coli* was determined by counting the wells that had turned yellow. These counts were converted to most probable number (MPN) using the MPN chart provided by IDEXX. For quality control, some QuantiTrays™ were also inoculated with *Salmonella* to ensure that this organism would not show positive results that might interfere with *E. coli* enumeration.

4.3 Results and discussion

4.3.1 Analysis of temperature data

4.3.1.1 Stage 1 composting

According to the data from the randomly introduced probes, during stage 1 the temperature within the compost pile started to rise immediately and as can be seen from Figure 4-1a, the rising pattern was very steep. The mean temperature remained within the thermophilic range (>45°C) until the end of stage 1. The maximum temperature in the pile, as experienced by one of the probes, was 79°C, and it was recorded on the 2nd day of composting. As the temperature in the pile was rapidly rising, so was its spatial gradient. Large differences between recorded maximum and minimum temperature values at any particular time were observed, beginning on day 2 and eventually peaked at 64°C on day 3. Thereafter, the spatial temperature gradient fluctuated between 20-40°C.

Data from the probes that were introduced into the cool zones of the compost pile also showed this rising tendency (Figure 4-1b). While the mean temperature in the cool zones throughout stage 1 mostly remained in the mesophilic range (20°C to 44°C), there were some locations where the temperature was thermophilic. The maximum temperature in sampled cool zones rose to 61.7°C on day 5, while the largest difference of 40.3°C between same time maximum and minimum temperature values was detected on day 2. Obviously, temperature in the cool zones was prone to external factors and fluctuated more than it did within the random locations in the pile.

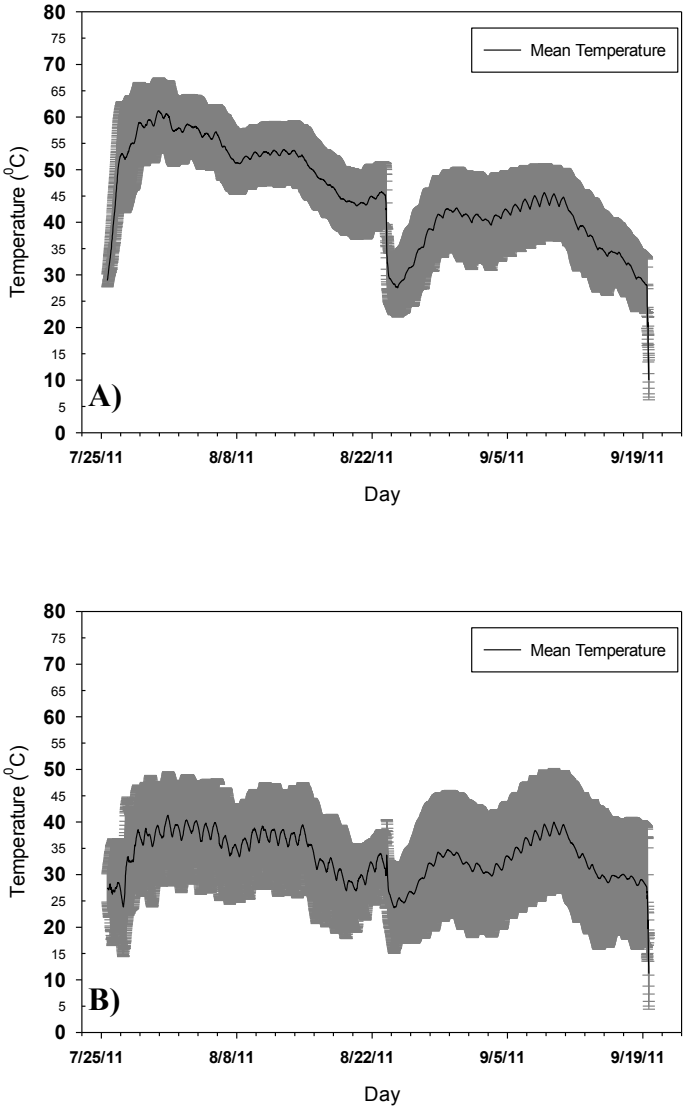

Figure 4-1. The temperature profile of: a) randomly introduced probes; and b) probes introduced into cool zones. The black line in the centre indicates the mean temperature at that particular time while the gray area around mean temperature shows the 99% confidence interval of temperature distribution from all probes at that particular time.

4.3.1.2 Stage 2 composting

The end of stage 1 / beginning of stage 2 composting was characterized by an unambiguous transition of the pile from the thermophilic to mesophilic domain due to pile turning. When the pile was rebuilt after turning, its temperature started to rise again. The mean temperature profile throughout stage 2 composting in the pile was lower than that in stage 1, whereas the temperature fluctuation was higher. As Figure 4-1a, and Figure 4-1b demonstrate the mean temperature profile from the probes that were initially randomly introduced into pile was around 45°C, which is the lower thermophilic boundary. The mean temperature profile of the probes from cool zones, on the other hand, was completely mesophilic. The maximum temperature detected in either set of probes during stage 2 composting was 62.4°C, which was recorded on the day 6 of stage 2.

4.3.1.3 TTC

The information on time-temperature from each probe is summarized in Table 4-1 and Table 4-2. Approximately 63% of the probes within the pile reached 55°C in the first 3 days of composting. At the end of stage 1, the TTC was met in 80% of the randomly introduced temperature probes. When 55°C or higher temperatures were reached within the pile, they typically persisted from 4 to 20 consecutive days. Therefore, provided one accepts that: 1) the temperature probes used in this study mimic compost particles (Wichuk and McCartney, 2008), and 2) any random compost particle is a member of a larger population (compost pile) then it can be concluded that the likelihood of any random particle being exposed to ≥55°C for three consecutive days during stage 1 of composting is 80%.

During stage 2 composting two additional randomly introduced probes satisfied TTC, thus increasing the overall compliance likelihood from 80% to 87%. It should be noted that stage 2 was initiated after turning the entire pile and covering it with the membrane. One of the objectives in pile turning is to provide a chance for the particles that did not experience TTC to experience it by moving the mass inward towards the hot zone. The turning that resulted in a 7% increase towards

TTC compliance from status quo might partly imply that it was not carried out effectively. Pile turning, which is aimed at increasing the chances of temperature-contact time compliance, should ideally be done such that a majority of the particles that did not experience TTC migrate towards the pile's core where the temperature is at its maximum.

Unlike randomly introduced probes, none of the probes that were initially placed in the cool zones complied with TTC during stage 1 composting. In only 2 out of 10 probes the temperature exceeded 55°C. In the first probe, the temperature reached 55°C on day 4 and remained above this temperature for 11 hours, during which time the temperature peaked at 60°C for about 4 hours. In the second probe, temperatures above 55°C were observed on day 2 for 6.5 hours. The maximum temperature in the remaining 8 probes ranged from 37 to 49°C. After turning only one cool zone probe exceeded 55°C; this was for a period of 17 days. All other probes remained in the mesophilic range during stage 2. This again, could potentially be an indication that the pile was not turned thoroughly.

In this particular case, the finding that some particles do not reach the necessary temperature contact time is not a significant concern. After composting in the covered aerated static piles, the material is cured in mass-beds for another 5 or so months (stage 3) under the thermophilic temperature with a one month turning frequency of the entire bed (Wichuk *et al.*, 2011). These conditions might be sufficient to eradicate most of known pathogens of concern in scenarios, like the one with the cool zones, that compost particle might experience during active composting. This assumption, however, would need to be confirmed via further experiments.

Table 4-1. Description of temperature in the compost pile as recorded by randomly introduced temperature probes with cryovials*

	Stage 1				Stage 2			
Probe #	Days to reach 55°C	Days 55°C persisted	Max Temp (°C)	Min Temp (°C)	Days to reach 55°C	Days 55°C persisted	Max Temp (°C)	Min Temp (°C)
1	1	29	79.3	29.5	NA	NA	37.9	17.3
2	NA	NA	52.1	22.9	NA	NA	49.0	29.0
3	5	13	72.4	36	NA	NA	53.8	33.4
4	1	20	71.1	40.1	NA	NA	51.6	19.6
5	1	28	73.6	41.8	NA	NA	41.3	28.8
6	4	3	68		NA	NA	41.6	20.7
7	2	4	57.4	41.2	3	15	59.7	26.2
8	1	20	67.8	41.5	NA	NA	53.5	38.1
9	1	18	76.9	48.2	12	9	58.6	16.0
10	NA	NA	50.2	28.5	NA	NA	33.0	12.8
11	1	22	74.8	43.9	3	7	57.2	25.2
12	2	27	71	61.1	NA	NA	52.1	29.3
13	NA	NA	40.5	17.9	NA	NA	34.5	0.5
14	NA	NA	48.2	28.4	6	12	55.4	38.6
15	4	5	55.3	33.8	2	7	56.0	27.7
16	NA	NA	47.4	29.2	7	12	57.3	51.8

*NA in this context stands for “Not Applicable”.

Table 4-2. Description of temperature in the compost pile as recorded by randomly introduced temperature probes without cryovials*

	Stage 1				Stage 2			
Probe #	**Days to reach 55°C**	**Days 55°C persisted**	**Max Temp (°C)**	**Min Temp (°C)**	**Days to reach 55°C**	**Days 55°C persisted**	**Max Temp (°C)**	**Min Temp (°C)**
17	1	4	75.6	37.1	NA	NA	44.7	32.6
18	2	11	67.7	40.7	NA	NA	35.8	15.0
19	1	19	71.9	51.9	NA	NA	49.3	36.1
20	1	20	72.7	37.5	NA	NA	53.8	39.0
21	2	22	66.2	36.2	NA	NA	33.5	17.9
22	1	14	72.2	48.5	3	5	57.0	27.0
23	3	20	60.8	31.2	6	2	55.0	31.5
24	1	23	77	46.7	NA	NA	34.1	15.5

*NA in this context stands for “Not Applicable”.

Table 4-3. Description of temperature in the compost pile as recorded by temperature probes from cool zones*

Probe #	Stage 1				Stage 2			
	Days to reach 55°C	Days 55°C persisted	Max Temp (°C)	Min Temp (°C)	Days to reach 55°C	Days 55°C persisted	Max Temp (°C)	Min Temp (°C)
25	NA	NA	49.1	25.4	NA	NA	43.6	34.4
26	4	1	61.7	35.6	NA	NA	40.3	24.7
27	NA	NA	39.1	16.6	NA	NA	39.0	31.1
28	NA	NA	40.8	13.4	NA	NA	43.5	36.7
29	NA	NA	37.3	20.1	NA	NA	28.3	7.9
30	NA	NA	38	13.9	NA	NA	51.7	14
31	NA	NA	45.9	34.7	NA	NA	39.9	29.1
32	NA	NA	41.3	20.8	NA	NA	29.4	22.8
33	2	1	57	16.2	4	17	62.4	51.8
34	NA	NA	40.1	15.6	NA	NA	32.5	13.6

*NA in this context stands for “Not Applicable”.

4.3.2 Microbiological analysis

The survival of the three studied microorganisms in the cryovials are presented graphically in Figure 4-2. The temperatures that these microorganisms were exposed to are provided in Table 4-1. Discussion of these findings is provided in the following sections.

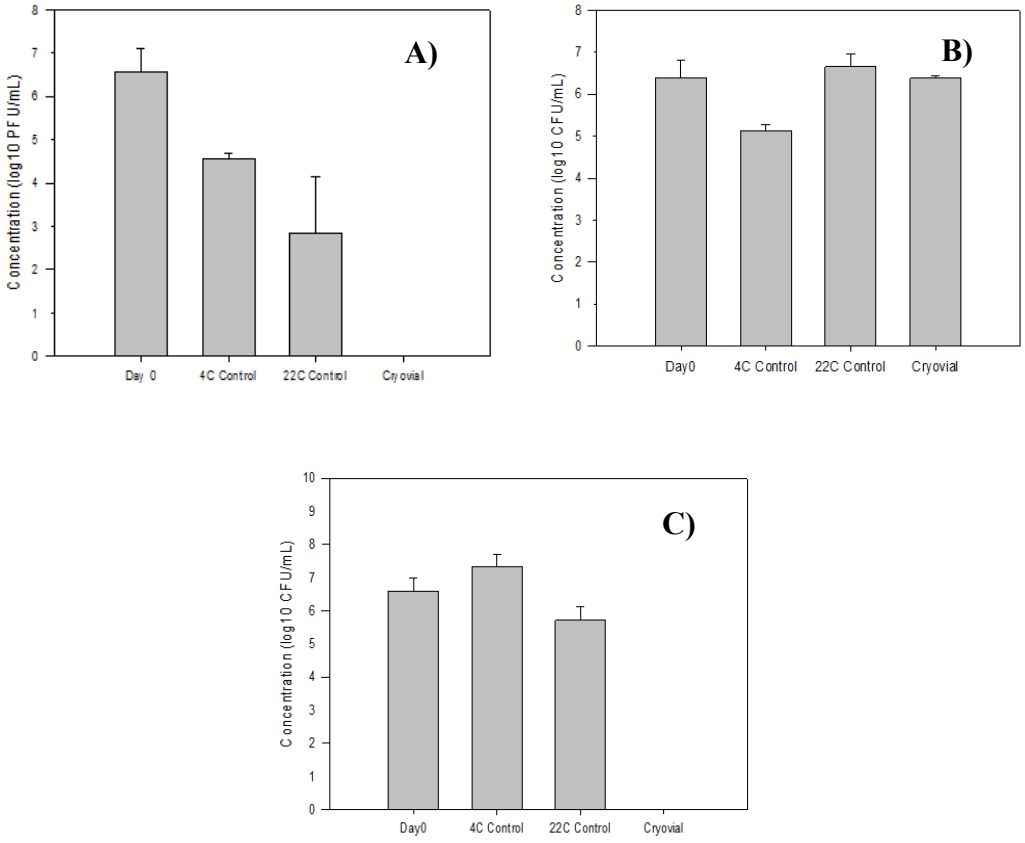

Figure 4-2. Concentration of three different microorganisms at different conditions: a) phi-s1; b) *Salmonella*; and c) *E. coli*. All numbers and their confidence interval bars are based on triplicate readings. For the *Salmonella* the cryovial readings corresponds to the only probe which was *Salmonella* positive at the end of composting

4.3.2.1 Survival of phi-s1

As can be seen from Figure 4-2a there were no plaque formations in any cryovial samples following thermal treatment inside the aerated static pile. Thus it was concluded that the temperature-contact time in the pile was sufficient to inactivate the phi-s1. In fact the phi-s1 was found to be very unstable even at its near optimum temperatures. For example the optimum growth temperature for phi-s1 temperature is the one at which its host *Pseudomonas* fluorescens thrives, i.e. around 26°C. Nevertheless, according to the Figure 4-2a, the concentration of phi-s1 declined at 22°C, near optimum temperature, by 3.8 logs. This in turn was found to be a significant change over the initial concentration *($n=3$, p=0.002)*. Moreover, over the timespan of the experiment another control which was stored at 4°C also dropped in concentration by 2 Log_{10}, which was also statistically significant (*$n=3$, $p=0.011$*). Therefore, given evidence of natural instability of the bacteriophage used herein, the findings from this study should be very cautiously extrapolated to all enteric viruses that may potentially be present in biosolids.

4.3.3 Survival of Salmonella

Figure 4-2b summarizes how the concentration of the culturable *Salmonella* changed in 56 days under different temperature scenarios. Generally speaking, culturable *Salmonella* were <1 CFU mL^{-1} in cryovials where the TTC were met. This observation thus supports the adequacy of the TTC as minimum process criteria in full scale composting operations.

According to the Table 4-2 there were three probes (i.e. 2, 10 and 13) with inoculums in which the temperature never reached 55°C. The temperature profile of each of these probes is provided in the Figure 4-3. The maximum temperature in the probe number 2 reached 52.1°C during stage 1 and 49°C during stage 2. In the probe number 10 the maximum temperatures during both stages were 50.2°C and 33°C respectively. Despite these probes' failure to achieve the TTC, the culturable *Salmonella* was still absent in their respective inoculums. Both of these probes were exposed to the temperature ≥45°C for at least 5 consecutive days.

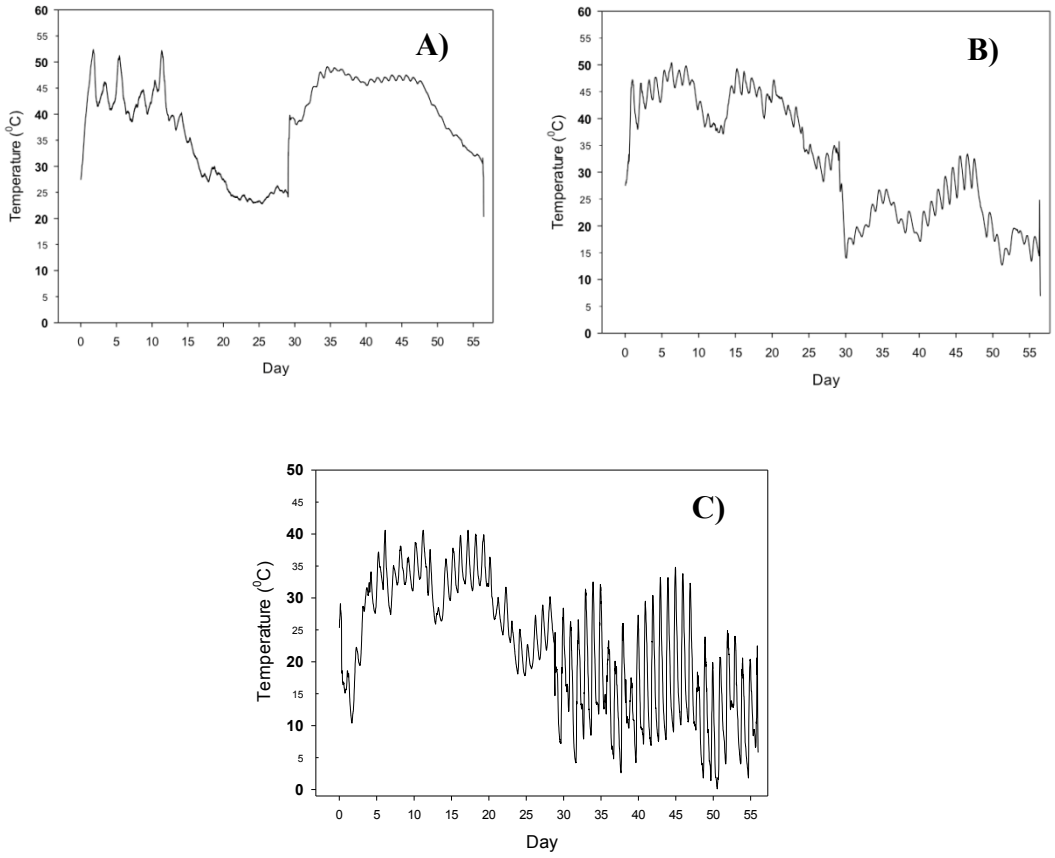

Figure 4-3. Temperature profile of the probes listed in Table 4-1 which did not meet the TTC: a) probe 2; b) probe 10; and c) probe 13. Although the TTC was not met in all of these probes, culturable *Salmonella* was detected in probe 13 only.

Salmonella was culturable in the third probe (number 13 in Table 4-1) that failed to be exposed to thermophilic condition (Figure 4-3c). The probe experienced significant temperature fluctuations throughout the compost experiment and the maximum temperature reached by this probe was only 40.5°C (see Figure 4-3). Consequently, *Salmonella* from its inoculum not only developed into well-defined colonies, but was statistically insignificantly reduced from its initial concentration (*n=3, p=0.95,*Figure 4-2b). Based on the data obtained from the 3 probes, and the fact that the strain of *Salmonella* used in this experiment was obtained from clinical samples (i.e., Provincial Laboratory), it is unknown whether exposure to T≥45°C for at least 5 consecutive days could be sufficient to reduce the culturable population of autochthonous *Salmonella* to <1 CFU mL^{-1}.

The survival of *Salmonella* was explained by the genetic adaptation of the wild strain to known stresses. Typically, microorganisms in a batch system (such as those conditions likely experienced by the microbe within the cryovial) microorganisms commonly go through a series of distinct stress response phases including: stress-induction, adaptation, and death (if the stressor overwhelms the stress response). Many physical, chemical and biological factors can affect the survival and the stress response in bacteria (Hibbing *et al.*, 2009; Colwell, 2009). The current study was limited to addressing microbial stress events related to: 1) temperature; 2) microbial competition (i.e., *Salmonella* and *E. coli* in the same cryovial); and 3) nutrient availability (microbes suspended in water). This particular *Salmonella* strain is typically found in birds that possess core body temperatures of around 40°C. Hence, no detrimental effect from this temperature for at least a portion of the 56 days was observed. The other two events are known activators of the generalized *RpoS* stress response within bacteria that possess these genetic elements (Dodd *et al.*, 1997) owing to which *Salmonella* probably survived these stressed conditions upwards of 56 days with no appreciable loss in viability.

The survival and subsequent regrowth of pathogenic microorganisms during compost curing and storage following thermophilic composting has been reported (Grewal *et al.*, 2007; Higgins *et al.*, 2007; Domingo and Nadal, 2009). This

observation can, in part, be explained by the ability of *Salmonella* to survive temperatures < 40.5°C.

Provided the temperature is the only variable for inactivation, then, according to the Table 4-3 at the end of composting the temperature in 30% of the probes from the cool zone never exceeded 40.5°C. As such, there was likely no decline in *Salmonella* numbers in 30% of the biomass from the cool zones of a compost pile. Furthermore, all temperature probes that were sampled the cool zones were buried about 0.5 m deep inside the pile. The dimensions of the pile are given in Figure 4-4; according to which the volume of the compost in cool zones comprised ~34% of the total. Hence ~10% of the initial compost mass did not achieve *Salmonella* reduction. If the biosolids feedstock in this particular case is assumed to be loaded with *Salmonella* at concentrations as high as 2.9×10^3 cells per gram of dry solids as suggested by Sidhu and Toze (2009), and the compost pile at the end of 56 days is assumed to be mixed such that all the surviving *Salmonella* is equally distributed throughout the pile, then the *de facto Salmonella* reduction would be one $\log_{10}$. Consequently, the resulting biomass could have a final *Salmonella* concentration at levels as high as 10^2 cells per gram of dry solids.

The existence of cool zones in aerated static piles has long been recognized. Due to naturally occurring conductive and convective heat loses into the ambient surroundings, it has been found that points within 0.3 m of the surface of an aerated static pile may be unable to reach TTC (USEPA, 2003). Therefore, in accordance with the Biosolids Part 503 Rule, 0.3 m of insulating material, which is often times finished pathogen free compost, should cover the entire surface of an aerated static pile (USEPA, 2003) after pile construction. The CAPS technology examined herein did not have its surface covered with a dedicated insulation layer. The membrane used as a cover was not a substitute for the insulator. The data derived from the current study shows that increasing the insulating material from 0.3 m to beyond 0.5 m could reduce the occurrence of cool zones within the pile.

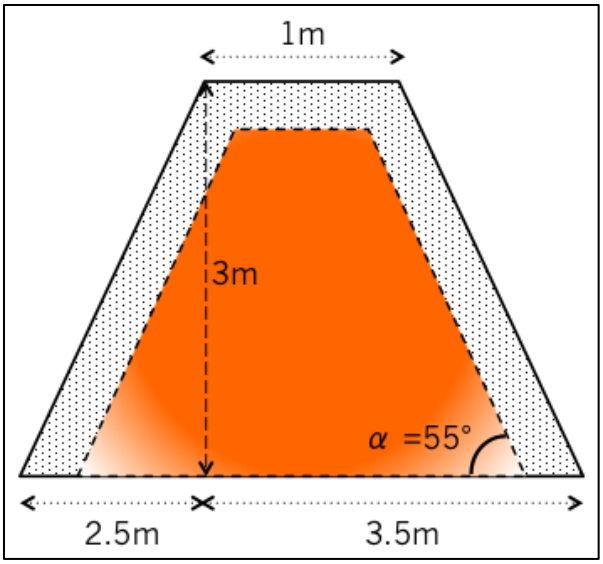

Figure 4-4. Cross section of CASP with dimensions which were used in calculating the approximate mass which remained in the cool zones. The pile resembled a trapezoid with the following dimensions: base width = 6m; crest width = 1m; height = 3m; and length = 50m. The temperature probes that sampled the cool zones were buried about 0.5 m deep inside the pile. Dotted pattern models the cool zone along pile surface

4.3.3.1 Bacterial Survival – *E. coli*

The compost temperature was sufficient to inactivate *E. coli* in all of the cryovials in which thermophilic conditions were reached (Figure 4-2c). In the probe that reached a maximum temperature of 40.5°C (and in which culturable *Salmonella* was detected) culturable *E. coli* was not detected. Two separate methods of recovery and isolation (i.e., Colilert and plating on XLD) demonstrated the absence of culturable *E. coli* within all cryovials after these time periods. Three possible hypotheses are proposed to explain why *E. coli* from that single probe did not grow on its bacteriological media: 1) the organism was outcompeted by *Salmonella* in the co-shared microflora; 2) *E. coli* self-destructed; and/or 3) the

bacteria entered a viable but not culturable state and therefore failed to grow on routine bacteriological media.

The hypothesis regarding microbial competition as a stressor for the enhanced survival of *Salmonella* over *E. coli* is supported by data observed in our co-cultured control experiments (see Figure 4-2b and 4-2c). For example in the context of the 22°C stored control, the concentration of culturable *Salmonella* rose to 4×10^6 CFU mL^{-1} from the initial stock concentration of 1×10^6 CFU mL^{-1}, whereas at 4°C the concentration of *Salmonella* declined to approximately 1.5×10^5 CFU mL^{-1} (see Figure 4-2b). Conversely, the concentration of *E. coli* at 4°C exceeded 10^7 CFU mL^{-1} and at 22°C declined to nearly 5×10^4 CFU mL^{-1} (see Figure 4-2c). Hence, in mesophilic and thermophilic conditions *Salmonella* clearly dominated the co-culture environment, whereas at cooler temperatures *E. coli* dominated. Dodd *et al.* (1997) also observed that the presence of a live competitor (such as *E.* coli) was shown to induce RNA polymerase sigma factor RpoS (σ^S) in *S. typhimurium* under co-culture conditions thus enabling a later overcoming of external stresses.

Regarding the second hypothesis, the self-destruction of *E. coli* by oxidative bursts as a result of superoxide production has been evidenced by Bloomfield *et al.* (1998). Bloomfield *et al.* (1998) observed that when *E. coli* was transferred from a nutrient-deprived state into a nutrient-rich state with subsequent incubation at a temperature normally optimal for its enzymatic activity, the strain did not have time for phenotypic adaptation to these environmental conditions. This evoked a metabolic imbalance in the microorganism, resulting in the production of superoxide. Due to the lack of oxidative stress-protective genes (e.g. superoxide dismutase), *E. coli* could not scavenge the radical and as a result the cell, which was otherwise metabolically active, dies.

Finally, strains of *E. coli* are known to enter the viable but nonculturable state (VBNC), during which they reduce their metabolic activity, and thus do not develop into colonies on routine bacteriological media (Oliver, 2010; Liu *et al.*, 2009). Different suboptimum physical and chemical conditions can induce the

VBNC in bacteria. For *E. coli*, well-known inducers are prolonged starvation (Aertsen and Michiels, 2004, Oliver, 2010) and the exposure to above or below optimum temperature (Liu *et al.* 2009, Trevor 2011, Zeng *et al.* 2013). For the given study, since it cannot be concluded that the nutrients in the cryovial remained in abundance throughout the entire composting period, and given that the temperature in that cryovial was only 4°C above optimum (which is not likely to kill the organism) it is logical to assume that the inability of *E. coli* to develop into colonies on XLD agar or demonstrate metabolic activity in the vicinity of the Colilert system may be due to the induced VBNC state in the microorganism. Chapter 6 addresses how composting conditions associated with elevated temperatures, nutrient deprivation and microbial co-culture affect VBNC states in both *E. coli* and *Salmonella* using molecular tools.

4.4 Conclusion

In this experiment 34 probes were used to investigate the temperatures within a full-scale covered, aerated static pile and to answer two questions: 1) how likely is it that a random compost particle would experience 55°C for at least three consecutive days; and 2) how effective is this TTC in eradicating pathogenic microorganisms commonly present in compost feedstock. *Salmonella*, *E. coli*, and phi-s1 were used as microbial surrogates to assess the effectiveness of the temperature-contact time criteria.

Results demonstrated that composting in a covered, aerated static pile, which takes place in two back-to-back stages, allowed more than 85% of particles within the compost pile to experience a thermophilic temperature >55°C for at least three consecutive days. It was also found that pile turning significantly increased the likelihood of exposure and was therefore confirmed as a practical measure to meet temperature-contact time compliance. However, it should be noted that turning methods are also an important consideration. For example, particles were found to be in cool zones before and after turning. At this facility; however, several turning events are also completed as part of the curing process using a mass bed turner.

This should be sufficient to assure all particles are meeting the temperature-contact time criterion. Further investigation is required to confirm the relative importance of turning.

With regards to the inactivation of pathogens, there is still much to be understood in the behavior of the microorganisms used for the study. Although it was found that all probes meeting the TTC had no culturable microorganism in their inoculums, firm conclusions on the efficiency of the temperature-contact time criteria specified in North American guidelines could not be made. Additional tests need to be conducted and it is in the scope of the current study to conduct molecular tests to see what, in fact, happened to microorganisms in the probes. Also, it was not clear if the absence of culturable *E. coli* was due to the temperature, the presence of *Salmonella*, which suppressed its growth, or eventual cell necrosis. Likewise, it was not explicit why the control inoculum, which was stored at room temperature for 56 days, had a three-log decline in the concentration of *Pseudomonas* phi-s1 phage. These facts should certainly be taken into account by researchers who wish to conduct a similar experiment. For example, it can be recommended that different strains of *E. coli* and *Salmonella* be used together as indicator microorganisms and that their relationship be studied. Also, it is recommended that more thermotolerant phages and bacteria strains be selected and that the spectrum of microorganisms be expanded to include parasites.

CHAPTER 5: SANITARY ASSURANCE AT BIOSOLIDS COMPOSTING FACILITIES: ASSESSING THE TEMPERATURE CONTACT TIME CRITERION IN COVERED AERATED STATIC PILE[1]

5.1 Introduction

Biosolids composting is on the rise in North America. According to the most recent BioCycle survey, in total there are 258 operational biosolids composting facilities spread across 44 states in the United States. These cumulatively account for composting of 562,000 dry tons year of biosolids, mostly in aerated static piles (Beecher and Goldstein, 2010). The raw biosolids matrix can contain high levels of human pathogenic microorganisms (Sidhu and Toze, 2009). Their inactivation in aerated static piles is dependent upon assuring that every particle of compost is exposed to a temperature of 55°C or higher for at least 3 consecutive days (CCME, 2005; USEPA, 2003). In addition, the levels of indicator organisms in the finished compost must not exceed the allowed limits of <1,000 most probable number (MPN) g^{-1} total solids (TS) for fecal coliforms or < 3 MPN g^{-1} TS for *Salmonella* spp. in order for the product to classify as Class A (USEPA, 2003)

A review of the literature illustrated that, regardless of maintaining high temperatures, there may still be pathogenic microorganisms that survive composting (Brinton *et al.*, 2009; Wichuk and McCartney, 2007). One potential hypothesis is that pathogen survival during apparent high-temperature composting is actually the result of inadequate temperature monitoring, where spatial and temporal temperature variations in large composting piles are not captured. This could give the false impression that the temperature-contact time condition has been met.

[1] A version of this chapter has been submitted for publication. Isobaev et al. *Compost Science and Utilization* (March 2014).

Another hypothesis regarding the reason for pathogen survival in the compost matrix could be the inadequacy of the existing time-temperature criteria (TTC). Microbial behavior in the environment is complex. The microorganisms continuously evolve and demonstrate an extraordinary ability to adapt to diverse environmental conditions within their ecological reservoirs. Gradually changing chemical, biochemical and physical conditions in a reservoir (such as gradual temperature rise) may trigger pathogenic bacteria to evolve towards a viable but non-culturable (VBNC) state. While switching into VBNC, bacteria exhibit dwarfing and reduced nutrient transport, respiration rate and macromolecular synthesis (Oliver, 2005). During VBNC, bacteria also upregulate stress response proteins which help them overcome hostile processes (such as high temperature) (Oliver, 2005). These physical and biochemical changes during VBNC prevent microbial growth during routine culturing procedures in standard bacteriological media. Pathogenic strains of *Escherichia coli* and of *Salmonella,* the levels of which are regulated in compost, are known to enter VBNC. Consequently, they can escape detection via routine culture-based bacteriological procedures (e.g. USEPA, 2010; USEPA, 2006) and resuscitate when favorable conditions are regained.

5.1.1 Previous work

Relevant to hypothesis (1), a novel temperature probe that behaves like a random compost particle was researched and developed (Isobaev *et al.*, 2014). Field trials confirmed that the temperature probe behaved like a random particle in compost, and the statistically significant number of probes needed to capture temperature variability within a compost pile was determined. Pertaining to hypothesis (2), in a recent trial 17 temperature probes containing *Salmonella meleagridis*, *E. coli* ATCC 29425, and phi-S1 bacteriophage were introduced into a CASP. After composting, no colony formation was detected, using culture-based methods, in probes where the temperature-contact time condition was met. *S. meleagridis* was still culturable in a probe in which maximum temperature was 40.5°C, while no culturable *E. coli* or bacteriophage were detected for the same probe. Although it

appeared that the recommended TTC was likely to be adequate to reduce pathogenic organisms to below detectable levels in a CASP, it was not clear whether this reduction was due to the transition of microorganisms into VBNC or due to microorganism inactivation. Hence, it was recommended that more observations be made with an increased sample size in addition to using microbial strains that are more robust to environmental stresses than those in the first trial.. In addition, evaluation of pathogens via both culturing and molecular methods would help to identify organisms that may have entered the VBNC state.

5.1.2 Study objectives

The long-term goal of this research is to develop a method of compost sanitation assurance based on particle-level monitoring, which can effectively be used in a variety of composting technologies. The particular objectives of the current study were: 1) to demonstrate how the two hypotheses mentioned above can be tested with minimal bias, taking into account the results and recommendations from previous work; and 2) to analyze whether gradual increases in temperature, as occurs in compost piles, triggers the selected *E. coli* and *Salmonella* strains to enter VBNC.

5.2 Materials and methods

5.2.1 Materials

5.2.1.1 Compost pile

The study was conducted during the period from August to October 2012 at the Edmonton Waste Management Centre's composting facility (ECF), which uses CASP technology to compost biosolids (see Figure 5-1). The description of CASP is provided in section 3.2.1.1. The physical and chemical properties of the CASP are provided in Table 5-1.

Figure 5-1. A view of experimental pile: a) the process of the aerated static pile building with the vertical mixer; b) the final set-up of the pile after it is covered with the selective membrane

Table 5-1. Summary of physical and chemical properties of experimental pile*

Parameter	Feedstock	Stage 1	Stage 2
Bulk density (kg m^{-3})	460	410	403
Moisture content (%)	61.25	48.80	44.67
Electrical conductivity (μS cm^{-1})	2732	3283	4653
pH	8.06	7.38	6.78
Organic matter	72.23	69.60	57.84

*The figures are mean values from triplicate readings

5.2.1.2 Temperature probes

In total, 22 temperature probes, designed to withstand conditions within a compost pile (see section 2.1.2), were used in the current study. Prior to the experiment, the probes were calibrated according to the manufacturer's instructions. After successful calibration, the devices were batch programmed to read and record temperature data with a 15-minute frequency, starting from the day the experiment was slated to begin. Lastly, a 15 m length of fishing line was attached to the end of each probe; this length was greatly in excess of the maximum distance that a probe could be buried in a pile in order to not affect its random placement. The fishing line was used to improve the recovery efficiency of the probes; once a probe was placed in the pile, the end of the line was placed on the ground outside of the pile. At the end of experiment, during pile deconstruction, the line was used to locate and recover the probes.

5.2.1.3 Microbial inoculum

Environmental strains of *E. coli* and *Salmonella* were used in this experiment. A *Salmonella spp* was previously isolated from compost materials, and *E. coli strain* 43031 (*E. coli)* previously isolated from a wastewater treatment facility were used in this study. Both strains were supplied by the Provincial Laboratory of Public Health (Edmonton, Alberta, Canada). To prepare stock solutions of *E. coli* and

Salmonella, pure cultures were individually seeded into tryptic soy broth. The two solutions were then incubated overnight at 36°C and their optical density (OD) was monitored over time with a spectrophotometer set at 600 nm wavelength. When the microbial growth in each stock solution reached the stationary phase, which was defined by no change in OD over a 3 hour period, the concentration of cells in each stock solution was estimated using a standard plate counting method on standard plate count (SPC) agar plates. The next day, after appropriate dilution steps, one mL of *Salmonella* and one mL of *E. coli* were seeded into 1.2 mL cryovials, each at a concentration 10^8 CFU mL^{-1} (13 cryovials were prepared per organism: 11 to go with the probes into compost and 2 as controls). All cryovials were sealed and stored at 4°C for several hours until being inserted into temperature probes.

5.2.2 Methods

5.2.2.1 Introduction and recovery of temperature probes

The night before the experiment, 22 cryovials (11 containing *Salmonella* and 11 containing *E. coli*) were inserted into the 22 temperature probes. The cryovial compartment of each probe was sealed to prevent any leakage into the environment. Concurrently, two controls for each organism were also set – one was stored at 4°C and another at room temperature (~22°C). All 22 probes were transferred to the composting facility, where they were introduced randomly into the experimental compost pile: at randomly selected times, they were thrown onto the discharge belt of the mixing truck used to construct the pile. The composting process involved two back-to-back stages. In stage 1, the pile was covered and aerated periodically for 36 days. After stage 1, the cover was removed from the pile and the entire mass turned with the front-end loader. The pile was covered again and left to compost for another 20 days (stage 2). At the end of stage 2 composting, the temperature probes were recovered, while the pile was being deconstructed, by pulling the end of the fishing line. The fishing line attached to some of the probes was broken during pile building or pile turning; these probes were recovered from the compost by screening them out with a trommel screen.

The recovered probes were brought to the laboratory for microbial survival and temperature analysis.

5.2.2.2 Sampling of the compost matrix

The compost feedstock was sampled by filling a previously disinfected 10 L bucket with the feedstock mass being discharged from the vertical mixer truck. In total, samples were collected from 10 truckloads and formed into a composite feedstock sample. Prior to sample collection, a random number was generated for each truckload, which corresponded to the time (in minutes) when the sample from that particular load was to be collected. It was assumed that the load in the vertical mixer underwent thorough mixing and was homogenous upon discharge. Hence, 2 shovels of compost from any location of the newly formed pile were taken upon the arrival of its random time. Between truckloads and when being transported to the laboratory facility, the bucket was kept on ice with a closed lid to prevent contamination from the surroundings, and to minimize changes in the sample's original physical, chemical, and microbiological characteristics.

Composite samples were each comprised of 10 shovel loads randomly collected from various locations at the end of Stage 1 and Stage 2 composting into three separate previously disinfected 10 L buckets. This was done in order to have three independent observations of compost quality after each stage. During the collection, samples were stored with a closed lid and transported to the facility the same day, where they were immediately placed into a walk-in fridge at 4°C. After each sample collection event, a representative sample was obtained from each bucket for the physical and microbiological methods within 24 hours after collection by emptying the bucket content onto a disinfected plastic tarp, thoroughly mixing it with a disinfected shovel, and using the quartering method to obtain a sub-sample size of about 4 kg after sieving through a 9.5 mm sieve.

5.2.2.3 Analysis of temperature-contact time compliance

The temperature profile from each temperature probe was downloaded using the manufacturer's software and then imported into a spreadsheet. The data corresponding to the time before the compost pile was covered, as well as the data

subsequent to when the cover was removed after stage 2 composting, were deleted from the dataset. To test whether the compost pile complied with the TTC, the temperature profile from each probe was individually analyzed to determine whether, during either stage of composting, that particular probe had experienced 55°C for at least 3 consecutive days. The likelihood of compliance of the CASP technology was estimated by dividing the total number of probes which satisfied the TTC with the total number of temperature probes evaluated. Graphical analysis of temperature profiles was done using SigmaPlot v12.2.

5.2.2.4 Microbiological analysis of compost matrix

The quantitative analysis of fecal coliforms was performed for every collected sample in accordance with USEPA method 1680 (USEPA, 2010). The sieved (9.5 mm) feedstock and compost from stage 1 were analyzed for compliance with Biosolids Class B requirements while the compost from stage 2 was analyzed for compliance with Biosolids Class A requirements. In accordance with the USEPA classification Class B biosolids has a maximum fecal coliform density of 2 million MPN g^{-1} ds and Class A biosolids has a fecal coliform density of <1,000 MPN g^{-1} ds or *Salmonella* density < 3 MPN $4g^{-1}$ ds (USEPA, 2003).

The quantitative analysis of *Salmonella* was performed in accordance with USEPA method 1682 (USEPA, 2006), also on sieved samples. The analysis included following steps: 1) enrichment in tryptic soy broth (TSB); 2) selection phase on Modified Semisolid Rappaport Vassiliadis (MSRV) media; 3) selection phase on xylose lysine deoxycholate media (XLD); and 4) bio-confirmation with urea, lysine iron, and triple sugar iron agar slants.

5.2.2.5 Microbiological analysis of cryovial contents

Analysis of microbial viability within the cryovials was begun within 24 hours after the probes were removed from the compost pile. When not in use, samples were stored either in a 4°C fridge or on ice on the laboratory bench. Unless otherwise indicated, all serial dilutions were done in triplicates. *E. coli* concentrations in the cryovials were determined by the IDEXX QuantiTray™ 2000 method as per manufacturer instructions. All QuantiTrays™ were incubated

at 35°C for 24 ± 0.5 hours. After 24 hours, the concentration of viable *E. coli* was determined by counting wells that turned yellow and fluoresced under UV light. These counts were converted to most probably number (MPN) using the MPN chart provided by IDEXX.

Salmonella was detected and quantified by culture using XLD. 100 µL of inoculum from a cryovial was whole plate spread on an XLD plate. The inoculum was allowed to absorb on the agar surface. All plates were then incubated inverted overnight at 36°C. After 24 hours, the concentration of viable *Salmonella* was determined by counting black colony formations on XLD.

To check for VBNC cells, an aliquot of 0.5 mL was removed from each cryovial and treated with propidium monoazide (PMA) according to the method by Nocker et al (2007). A aliquot of 1.25 µL of PMA working stock solution was used for treatment for a final concentration of 50 µM followed by incubation for 5 minutes of dark and 5 minutes of light. Once PMA treated, the samples were centrifuged in a microcentrifuge for 5 minutes at maximum speed. The supernatant was discarded leaving the sample pellet in the bottom of the tube. The pellet was then washed with 0.5 mL of phosphate buffered saline solution (PBS). The pellet was re-spun for 5 minutes and the supernatant was removed. The remaining pellet was processed for DNA using Qiagen's DNeasy Blood & Tissue Kit following the protocol for gram-negative bacterial pellet. The DNA was stored at 4°C prior to molecular testing.

Quantitative polymerase chain reaction (q-PCR) was used for evaluation of VBNC states. Plasmid standards were generated for the *invA* gene (*Salmonella*) and *uidA* gene (*E. coli*) using the TOPO TA cloning kit (Invitrogen). PCR products were produced using the primers below and these products were ligated into the pCR2.1-TOPO vector and transformed into chemically competent *E. coli* TOP10F´ cells. These standards were used for generating standard curves on the Applied Biosystems 7500 Fast Real-Time PCR System. Quantitative PCR (qPCR) was performed using the above system along with the standard curves as reference samples for running the unknown DNA concentrations obtained from

the cryovials. The cycling conditions were ran as the default conditions at 95°C for 20 seconds followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Primers and probe for the *invA* gene (104 bp portion) were from Daum *et al.* (2002) and were as follows: invA-F (forward primer): 5′-GCG TTC TGA ACC TTT GGT AAT AA-3′; invA-R (reverse primer): 5′-CGT TCG GGC AAT TCG TTA-3′; invA-P (probe):5′-FAM-TGG CGG TGG GTT TTG TTG TCT TCT-MGBFQ-3′. Primers for the *uidA* gene (143 bp portion) were as follows: uidA-F (forward primer): 5′-CGC AAG GTG CAC GGG AAT A-3′; uidA-R (reverse primer): 5′-CAG GCA CAG CAC ATC AAA GAG A-3′. The probe used was from Taskin *et al.* (2011) and was as follows: uidA-P (probe): 5′-FAM-ACC CGA CGC GTC CGA TCA CCT-MGBFQ-3′(FAM, 6-carboxyfluorescein; MGBFQ, dihydrocyclopyrroloindole tripeptide).

5.3 Results and discussion

5.3.1 TTC compliance

The temperature profile in the experimental compost pile is shown in Figure 5-2. The temperature started to rise almost immediately after pile formation. During the period of steep temperature rise in first 3 days of composting, the temperature gradient (i.e. the range in temperatures) in the pile was minimal. This was in part because the microbial activity was sufficient to compensate for the heat loss to the ambient. However, as composting progressed, the temperature gradient increased. It was most pronounced during stage 2.

Approximately 71% of the probes reached their maximum temperature during the first 10 days of composting. The absolute maximum temperature recorded for the pile was 78°C, which was recorded on the 6^{th} day of composting. The mean temperature for the entire period of composting during both stages was in the thermophilic range (>45°C).

When the pile was turned between stages 1 and 2, the temperature transitioned from the thermophilic to mesophilic domain. However, after the pile was rebuilt,

the temperature started to rise again. The mean temperature profile throughout stage 2 was lower than that in stage 1, whereas the temperature fluctuation was higher. As Figure 5-2 shows, the mean temperature profile was mesophilic for much of stage 2. The maximum temperature detected during stage 2 composting was 73°C, which occurred soon after pile was rebuilt.

The novelty of the temperature probes used in this study lies in their ability to mimic random compost particles (Wichuk and McCartney, 2008). Moreover, if the entire compost pile is assumed to be a population and each compost particle is a member comprising that population, then randomly introduced probes should represent randomly sampled particles of compost. Thus, the conditions monitored by the probes should shed light on how long a random particle would be likely to experience thermophilic conditions. In this sense approximately 55% of the temperature probes had reached 55°C within first 3 days, and at the end of stage 1 composting the percentage had increased to 92%. When 55°C or higher temperatures were reached within the pile, they generally persisted for 4 to 32 consecutive days. The remaining 8% of probes did not reach 55°C in stage 1. Based on these numbers, it can be stated that in the experimental pile, given the composting conditions, the likelihood for any random particle to be exposed to 55°C for three consecutive days was 92%. This was higher than what was observed in analogous studies conducted by the researchers previously (Isobaev *et al.*, 2012).

One of the objectives of pile turning is to increase the chances that every particle will comply with the temperature-contact time criteria. Although, during stage 2, the probability that every compost particle will comply with the process criteria was increased to 93%, the findings herein show that the TTC were not met in stages 1 and 2, as the USEPA clearly requires that 100% of particles meet the TTC.

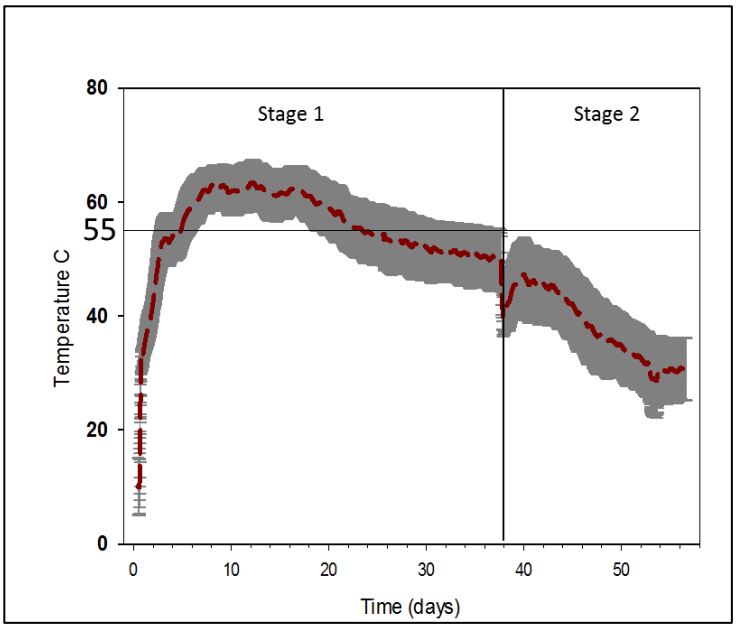

Figure 5-2. The temperature profile of randomly introduced temperature probes. The dashed line in the centre indicates the mean temperature at that particular time, while the gray area around the mean (n=22) temperature shows the 95% confidence interval of temperature distribution from all probes at that particular time.

The finding that some particles did not achieve the TTC does not assert the inefficiency of aerated static piles to meet PFRP requirement. It "has been found that points within 0.3 m of the surface of aerated static pile may be unable to reach PFRP temperatures" (USEPA, 2003) due to naturally occurring conductive and convective heat loses into the ambient. Therefore, in accordance with the Biosolids Part 503 Rule 0.3 m of insulating material, which is oftentimes a finished Class A compost, should cover the entire surface of an aerated static pile (USEPA 2003) after pile construction. The CAPS technology examined herein does not require its entire surface to be covered with a dedicated insulation layer and hence did not have it during experiment. The membrane, which is used in CASP technology was not a substitute to the insulator as it is used to control odor and moisture. In fact the need for insulating layer was demonstrated in the

previous study by Isobaev *et al.* (2012). A subsequent evaluation of the TTC of identical composting technology found that the temperature at the "cool zones" of the pile predominantly remained mesophilic for the most of the stage 1 although there were a few locations where the temperature reached and for some time maintained thermophilic conditions. As outlined in the previous chapter, it was recommended that an insulating layer to be at least 0.5m thick be applied to the compost pile. In addition the temperature fluctuation in the cool zones was very high with largest difference of 40.3°C between same time maximum and minimum temperature values detected on day 2. Hence the absence of the insulating layer probably resulted in higher temperature loses and subsequently the probes which remained within 0.3 m reach of the pile surface reported insufficient performance from the TTC perspective.

Like it was stated in the previous chapter, the inability of all particles to achieve the TTC was not a significant concern for the particular facility where the experiment was run since, after composting in the covered aerated static piles, the material is cured in mass beds for approximately 5 more months (stage 3). Thermophilic temperatures are maintained within the mass beds for much of this time, and the beds are turned on an approximately monthly basis (Wichuk *et al.*, 2011). These conditions should be sufficient to eradicate most known pathogens of concern. It should also be noted that the mass bed material is tested for pathogen indicator organisms prior to being accepted as finished compost.

5.3.2 Microbial survival from the compost matrix

Under USEPA Part 503 Rule for the quality assurance of the end product in addition to continuous temperature monitoring the end product should also comply with the specified levels of pathogens indicator organisms- fecal coliform or *Salmonella* sp. The combination of two, in theory should ensure that consistently pathogen free product reaches the consumer market.

5.3.2.1 Fecal coliforms

Fecal coliforms are described as facultative anaerobic, rod-shaped, gram-negative bacteria which produce acid and gas from lactose within 48 hours at 44±0.5°C (Tortorello, 2003). Chosen as an indicator organism for biosolids (USEPA 2003), fecal coliforms are predictors of the possible presence of pathogenic microorganisms in finished compost products. Typically, the presence of fecal coliforms at concentrations <1,000 MPN g^{-1} ds indicates that the likelihood of presence of pathogenic organisms is so low that it is not likely to present a public health risk.

The concentrations of fecal coliforms in the compost feedstock and in the compost after stage 1 and stage 2 are presented in Figure 5-3a. The feedstock material contained fecal coliforms in excess of $6x10^4$ MPN g^{-1} ds, but these levels were still below what is recommended for biosolids Class B. The samples obtained from stage 1 after 30 days of thermophilic composting demonstrated a slight rise in fecal coliforms from the initial levels, with concentrations of approximately $9x10^4$ MPN g^{-1} ds, though this rise was not statistically significant ($n = 3$; $p = 0.93$). It was hypothesized that during sampling from stage 1 more samples were taken from cooler zones in which fecal coliform apparently were able to survive hence resulting in unchanged concentration. After nearly 60 days of composting, almost 2-logs reduction in fecal coliform was achieved ($8x10^2$ MPN g^{-1} ds), meaning the compliance of the biosolids matrix with the USEPA Class A specifications for fecal coliforms.

5.3.2.2 *Salmonella* sp.

The USEPA requires quantification of either *Salmonella* sp. as a pathogen surrogate or fecal coliforms as a predictor of *Salmonella* and other potential pathogens (USEPA, 2003). However, since no indicator organism is perfect and since the use of fecal coliforms as indicator organisms has been criticized (Tortorello, 2003), a parallel test to quantify *Salmonella* spp. in the same matrix sample was run in the current study.

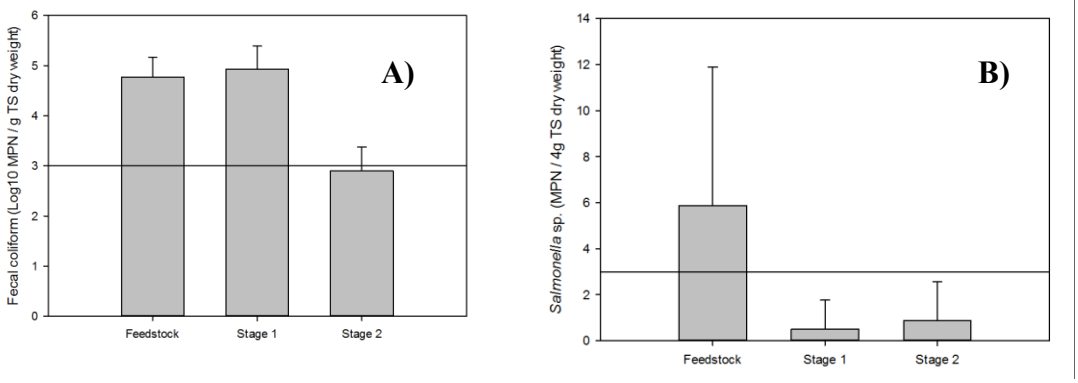

Figure 5-3. The concentration of indicator organisms in the compost matrix as geometric mean from a sample size of 3: A) concentration of fecal coliform estimated with the USEPA 1680 method; B) concentration of *Salmonella* spp. estimated with the USEPA 1682 method. The horizontal lines represent the allowed limit for the product to be classified as class A.

The quantitative analysis of *Salmonella* spp. in the biosolids matrix is presented in Figure 5-3b. In particular, it shows that the initial feedstock contained 5.8 MPN 4 g^{-1} ds. Although the limit for *Salmonella* sp. is not specified for Class B biosolids, this level is high enough that the raw biosolids should not be approved for unrestricted use (Class A). As composting time progressed, the high temperatures within the compost pile caused levels of *Salmonella* spp. deteriorate to <1 MPN $4g^{-1}$ ds after stage 1, which was in compliance with Class A product.

It should be noted that the studied bisolids compost was in compliance with Class A from a *Salmonella* sp. perspective as early as after the end of stage 1, while from a fecal coliform perspective the biosolids reached Class A compliance requirements only after stage 2 composting. Successful recovery is both time and labour intensive as it is a multi-step process that involves recovery from high solids matrix where large number of competing bacteria such as *Proteus* and *Pseudomonas* spp. co-exist (Yanko, 1995). Each step introduces bias, both random (which affects the precision of results) and systematic (which affects the accuracy of the results), so every additional step increases the bias. In this sense,

the fecal coliform quantification is a two-step multiple-tube fermentation procedure that uses culture-specific quantification. The quantitation of *Salmonella,* on the other hand, is a four step performance-based method that includes enrichment on TSB, two stage isolation, and finally bio-confirmation. Consequently, the extra steps in the *Salmonella* test might have affected the accuracy of the results, leading to an underestimate the real *Salmonella* density in the biosolids compost pile. Thus, the differences in quantification procedures could be one of the reasons why *Salmonella* levels were lower than fecal coliform levels.

5.3.3 Microbial survival from cryovial samples

Microbial survival assessment from cryovial samples was determined to be superior to the indicator methods for several reasons: a) cryovials were seeded with surrogate organisms which directly mimic the fate of pathogens in the compost pile, which therefore eliminated the need to make inferences from indicators; b) the enumeration of surrogate microorganisms in the samples was a one-step versus a two- or four-step procedure; and c) analysis of each sample is cheaper and allows for more samples to be processed. Results of microbial survival from the cryovials are provided below.

5.3.3.1 Survival of *E. coli* and *Salmonella:* culture based methods

According to the results from Colilert in Quantitrays, at the end of stage 2 composting there were no fluorescing yellow wells. In the Quantitray method, the absence of any fluorescing yellow wells is typically enough to conclude that the sample is *E. coli*-free (Figure 5-4a). It was observed that 2-stage composting of biosolids was sufficient to reduce the levels of *E. coli* from 10^8 MPN mL^{-1} to <1 MPN mL^{-1}. The concentrations in the controls, on the other hand, remained stable, thus suggesting the inimical effect of temperature on reducing nearly 10^8 MPN mL^{-1} of *E. coli* to below the detection level of this culture-based procedure.

Similarly, there were no culturable *Salmonella* at the end of stage 2 composting in any samples (Figure 5-4b). This result suggests that the temperatures in the

experimental compost pile were adequate to eradicate this pathogenic microorganism. Given that biosolids, on average, harbor *Salmonella* at a concentration of 10^2 CFU g^{-1} ds (Sidhu and Toze, 2009) that the results herein demonstrated a 9-log reduction performance may further suggest the effectiveness of composting as a process to further reduce pathogens.

5.3.3.2 Survival of *E. coli* and *Salmonella:* molecular method

The culture-based methods have a drawback in that they account only for viable cells that can develop into colonies on nutrient rich media. Failure to develop into colonies on nutrient rich media does not necessary signal absence or eradication of organisms. Among other pathogens, *E. coli* and *Salmonella* are now known to induce the VBNC survival strategy upon exposure to harsh environment (Oliver, 2009).

According to the results from molecular assays (Figure 5-4c and Figure 5-4d) an appreciable amount of *E. coli* and *Salmonella* remained in VBNC-state, thus were unaccounted for in the culture-based method. Furthermore, the *E. coli* concentration was reduced by nearly 6- log after 13 days of continuous exposure to >55°C and then remained stable. There was no observable pattern in *Salmonella* reduction as a function of temperature-time exposure. In one sample after nearly 44 days of exposure to >55°C the concentration of VBNC *Salmonella* was 10^6 cells mL^{-1}, while in another sample after 34 days of exposure to >55°C, there were no cells mL^{-1}. In agreement with these results are the findings in Brinton and Droffner (1994). Although the authors did not quantify the concentration of survived organism they showed that after 44 days of continuous exposure of compost matrix to 62°C there still were viable *Salmonella* sp. Likewise viable *Shigella* and *E. coli* were detected in the food compost matrix after 9 days of exposure to the temperature ranging from 60-70°C (Brinton and Droffner, 1994).

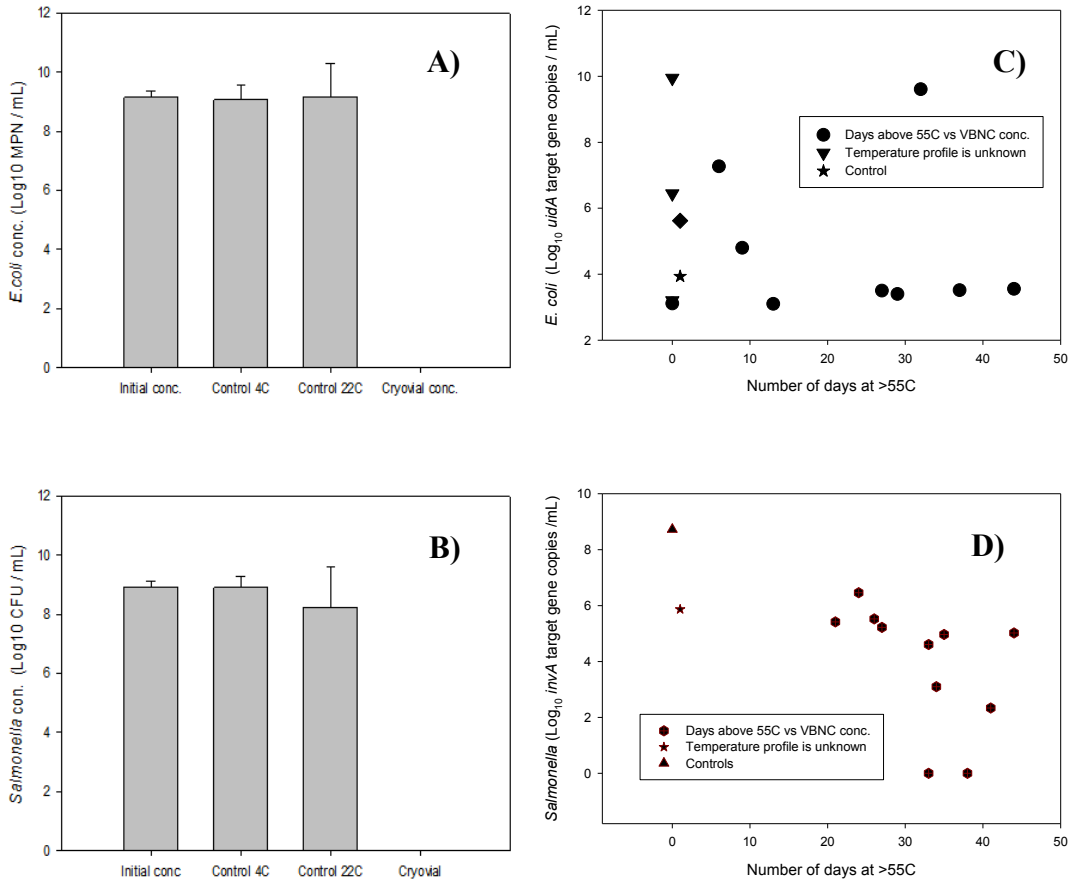

Figure 5-4. Concentration of *E. coli* and *Salmonella* as determined by culture- and molecular based methods: A) summary of culturable *E. coli* concentrations under different scenarios; B) summary of culturable *Salmonella* concentration under different scenarios; C) distribution of VBNC *E. coli* as determined by *uidA* target gene copies as a function of exposure length to 55°C; D) distribution of VBNC *Salmonella* as determined by *invA* gene copies as a function of exposure length to 55°C. Note: the cryovial concentration in panels A) and B) is the average of all cryovials after 2-stage composting.

The possible induction by *Salmonella* and *E. coli* into VBNC states, as determined by the abundance of quantifiable gene copies in samples after PMCA treatment (i.e., for *Salmonella*), demonstrates that the TTC as a factor alone may not be sufficient to inactivate the studied pathogenic microorganisms. It is possible that due to their adaptive ability to deal with environmental stressors such as elevated temperature through entry into VBNC states, bacteria may eventually resuscitate, a possibility that is also in agreement with conclusions made earlier by Brinton and Droffner (1994).

This fact however should be interpreted very carefully when inserting VBNC pathogens into microbial risk assessment models used as decision-making tools. First of all the temperature in compost was still sufficient to attain 6-$\log_{10}$ reduction in cryovial. If the sampled matrix had initial concentration of *Salmonella* at 6 cells $4g^{-1}$ ds then 6-fold reduction should have resulted in the final concentration of 6 *Salmonella* VBNC cells per kg of compost off stage 2 composting.

Although VBNC bacteria were found to maintain their virulence and pathogenicity (Pinto *et al.*, 2011; Colwell, 2009), the concentration of 6 *Salmonella* cells per kg of compost is hardly significant to cause gastroenteritis and other health issues in humans given its minimum effective dose of $10^3 – 10^9$ cells (Epstein, 1997). Second, VBNC organisms do not regrow in compost and indeed need specific stimuli to resuscitate (Pinto *et al.*, 2011) in comparison to non-stressed organisms. This fact further diminishes the risk. Therefore CASP could be concluded as an effective biosolids sanitation process. However, as a recommendation it is believed that the inclusion of insulating cover into CASP would result in enhanced sanitation of cool zones while still maintaining the benefits of selected membrane.

Furthermore this study was designed such that the factor, which affected microbial integrity, was limited to temperature. It is known that other physical chemical and microbiological factors in composting system such as pH, toxic compounds, and microbial antagonism in combination with high temperature

might have more profound impact on pathogens inactivation. With regards to pH, Dlusskaya *et al.* (2011) isolated and described *E. coli* strain which was capable of withstanding temperature above 70°C but the slight pH alteration in the system resulted in uncompromised death. Highly volatile ammonia (NH_3) was long known to be toxic to non-spore forming organisms such as *Salmonella* and *E. coli* (e.g. Himmathongham and Riemann, 2006). Indigenous microorganisms naturally generate NH_3 when they rapidly utilize carbon that is not bound to complex carbohydrates and insufficient to stabilize all of the nitrogen in the system thereby leading to NH_3 generation. Although the actual killing mechanism of NH_3 has not been explicitly elucidated some believe that NH_3 crosses cell membrane by diffusion and causes rapid alkalization of the cytoplasm and subsequent reduction of proton concentration formed at lower intracellular pH by changing into ammonium (NH_4^+) is (Park *et al.*, 2003). At least 30 mmol L^{-1} of NH_3 was found to be sufficient to cause 3-log reduction in *Salmonella* in 4 days (Park *et al.*, 2003) while at 46 mmol L^{-1} NH_3 concentration and pH 7.9 the 5-log *Salmonella* reduction after 4.2 days was reported (Ottoson *et al.*, 2008). Microbial antagonism, something that is naturally present in biosolids but what has been omitted in the cryovial is another crucial factor affecting survival and regrowth of pathogenic microorganisms. A different array of bacteria, actinomycetes, protozoa, and fungi (natural inhabitants of biosolids matrix) when added into sterile compost initially spiked with *Salmonella* were found to immediately suppress its growth at temperature below 70°C (Epstein, 1997). Likewise Hussong *et al.* (1985) reported on *Salmonella* regrowth in sterile compost while no *Salmonella* was detected in non-irradiated compost after 7 days. Zaleski *et al.* (2005) referred to microbial antagonism as the most important factor suppressing *Salmonella* growth when they noted that *Salmonella* growth increased significantly in the absence of indigenous microorganisms whereas in the presence of live competitors it was quickly suppressed. Hence it is recommended that more studies be conducted which would evaluate the combined effect of temperature and other inimical factors affecting microbial survival and growth in

the compost before drawing early conclusions about effectiveness of composting technology as PFRP.

5.4 Conclusion

The objectives of the current study were: 1) to demonstrate how the two hypotheses mentioned above can be tested with minimal bias, taking into account the results and recommendations from previous work; and 2) to analyze whether gradual increases in temperature, as occurs in compost piles, triggers the selected *E. coli* and *Salmonella* strains to enter VBNC. With regard to TTC, it was found that the likelihood for any random particle to be exposed to 55°C for three consecutive days was 92%. During stage 2, the probability that every compost particle would comply with the process criteria was increased to 93%. The findings herein show that the TTC were not met in stages 1 and 2, as the USEPA clearly requires that 100% of particles meet the TTC.

The results also revealed that on average 6-log reduction in indicator organisms was achieved. This was enough to hypothetically to reduce the density of indicator *Salmonella* to final concentration of 6 VBNC cells per kg of compost off stage 2 composting. The result has been found to constitute low risk given the minimum effective dose for *Salmonella* sp of 10^3 – 10^9 cells to cause gastroenteritis in human and specific stimuli for cells already in VBNC to resuscitate. In addition, this study was designed such that the only factor, which affected microbial integrity, was temperature. Hence, it is likely that other physical, chemical, and microbiological factors (such as pH, toxic compounds and microbial antagonism) that exist in compost piles in combination with high temperature might have had a more profound impact on pathogen inactivation. All of these factors in combination may reduce the number of organisms entering the VBNC state.

CHAPTER 6: EXAMINING THE VBNC INDUCTION AND REGROWTH POTENTIAL IN *E. COLI* AND *SALMONELLA* EXPOSED TO A SIMULATED THERMOPHILIC CONDITION FROM A COMPOSTING PILE[1]

6.1 Introduction

One of the goals of composting is sanitization of organic matter with respect to pathogenic microorganisms present in the feedstock. These organisms belong to five major categories: bacteria, enteric viruses, fungi, protozoa and helminth (Haug, 1993). If the process is managed properly, sanitation is accomplished by thermal inactivation (USEPA, 2003) in conjunction with one or more of the following factors: 1) microbial competition/predation, 2) production of toxic substances within compost such as ammonia, or primary / secondary metabolites by some fungi and actinomycetes, and 3) natural die-off (Wichuk and McCartney 2007; Zaleski *et al.*, 2005).

The survival of pathogenic microorganisms in low numbers during thermophilic composting and curing phases (Grewal *et al.*, 2007; Veen *et al.*, 2009; Orsburn *et al.*, 2008; Domingo and Nadal, 2009) and their subsequent regrowth to high levels under favorable conditions (Kim *et al.*, 2009; Castro-del Campo *et al.*, 2007; Higgins *et al.*, 2007; Zaleski *et al.*, 2005; Burge *et al.*, 1997) has been reported. Bacteria, as a part of their survival strategy, are well known for the ability to sense and adapt to hostile environments. Depending on the type of stress experienced within a hostile environment, a bacterial cell may choose to compete (e.g. for nutrients), cooperate (e.g. engage in quorum sensing), or cheat (e.g. while in quorum sensing) one another (Hibbling *et al.*, 2009). It has also been demonstrated that bacteria experience increased mutation rates during stress, the

[1] A version of this chapter has been submitted for publication. Isobaev et al. *Applied and Environmental Microbiology* (April 2014).

results of which often lead to beneficial mutations (Jain *et al.*, 1999; Thomas and Nielsen, 2005) enabling them to adapt to changing physical (Fotadar *et al.*, 2005,), chemical (Orr and Unckless, 2008), and/or host-generated (Chiu *et al.*, 2005) stresses.

The microbial 'viable but not culturable' (VBNC) state has been viewed as one of the microbial stress responses during composting. Higgins *et al.* (2007) and Viau and Peccia (2009) described the presence of bacterial pathogens in a VBNC state in biosolid matrices. The reactivation and regrowth phenomena of fecal coliforms (FC) in centrifugally dewatered biosolids from an anaerobic digester suggested that FC enter a VBNC state and resume culturability immediately after high-speed centrifugation (Higgins *et al.* 2007, Qi *et al.* 2007). *Salmonella*, which is known to enter the VBNC state, can be found in the raw sludge at a range from 100 to 3.4×10^4 MPN g^{-1} (Jacobsen & Bech, 2012), and Castro-del Campo *et al.* (2007) reported regrowth of *Salmonella* to 10^6 CFU g^{-1} from VBNC in a Class A biosolids stored under anaerobic conditions.

It is not known which genes initiate the VBNC state (Aertsen and Michels, 2004). Once in the VBNC state, the bacterial cell does not grow on routine microbiological media (Colwell, 2009). More than 60 bacterial species are now known to enter into a VBNC state (Zeng *et al.*, 2013; Mukamolova *et al.*, 2003). Among them, pathogenic *Escherichia coli* O157:H7 (Liu *et al.*, 2009), *Vibrio spp., Enterococcus faecalis, Pseudomonas spp., Shigella dysenteriae 1* (Trevors, 2011), *Salmonella* spp. (Zeng *et al.*, 2013), and *Listeria monocytogenes* (Commichau *et al.*, 2013) are notable pathogens. Entry into the VBNC state can be induced by changes in temperature (Liu *et al.*, 2009; Trevor, 2011; Zeng *et al.*, 2013, Koyunoglu, 2010), pH (Trevor, 2011), limited substrate concentration (Higgins *et al.*, 2007; Trevor, 2011, Zeng *et al.*, 2013), toxic chemicals (Kunte *et al.* 2000; Puchajda and Olezkewicz, 2006), and dehydration (Trevor, 2011; Gruzdev *et al.*, 2012).

Induction of the VBNC state in a bacterial cell greatly lowers its metabolic activity (Oliver, 2009) and the cell subsequently undergoes certain morphological

changes (Colwell, 2009). Starvation-induced VBNC cell dwarfs due to *in situ* carbohydrate consumption, catabolism of proteins and sometimes RNA (Trevor, 2011). According to Colwell (2009) a 10-fold size reduction in environmental isolates of *Vibrio cholera* was typical. Zeng *et al.* (2013) reported that cells in a VBNC state were smaller than the normal cells; some were clustered together under fluorescent microscopy by the acridine orange direct count (AODC) method suggesting changes in surface membrane charge also occur during the transition to a VBNC state. Under atomic force microscopy the same researchers reported the shape of the VBNC cells changed from short rods to coccoids. The average coccoid VBNC cell was 0.5 μm in diameter with a volume of 0.785 μm^3, shrinking from an initial rod size of 2 × 1 μm and 1.57 μm^3. Su *et al.* (2013) found that following one year of storage of *Mycobacterium luteus,* the bacterial cell diameter shrank from 1.4 μm to 0.3 μm and various other morphological and physiological changes were also observed.

The rate at which microorganisms enter into VBNC depends on the stress they have been exposed to. Liu *et al.* (2009) studied how fast different stressors induce the state of VBNC in *E. coli* O157: H7. By exposing the bacterial cells to a variety of stressful environments (i.e., chloraminated water, river water and starving them all at different temperature and osmotic pressure), Liu *et al.* (2009) found that chloramination successfully induced VBNC in 90% of the studied population within 15 minutes, whereas river water required 14 weeks to induce VBNC in 14% of *E.coli* O157:H7 population. In comparison it took as long as 1.5 years of starvation at low temperature to induce VBNC in the same microorganism. Islam *et al.* (1993) reported that *Shigella dysenteriae* Type 1 induced VBNC after 2-3 weeks following inoculation into various water microcosms. Similarly, Cho and Kim (1999) studied the time needed for *Salmonella typhi* to enter VBNC in groundwater microcosm. None of the cells could be grown in laboratory media after 27 days of incubation though they still could be enumerated using epifluorescence microscopy. Zeng *et al.* (2013) suspended 10^7 CFU mL^{-1} *Salmonella* Typhi into sterilized water and stored them in dark for 120 days at -20°C. All cells entered VBNC state after 48 hours of

incubation. Gruzdev *et al.* (2012) dehydrated 50 µL of *Salmonella* bacteria (approximately 10^8 CFU) in 96 well polysterene plates for 22 hr at 25°C and 40% relative humidity in a climate controlled incubator. Samples were analyzed for viability as the total viable count (CFU) of surviving bacteria after dehydration and storage at 4°C for 2, 4, 8 12 and 100 weeks. Researchers found that after as little as 22 hours of dehydration there were no culturable bacteria present in the samples. The percentage of culturable bacteria as shown by LIVE/DEAD™ staining kit was about the same in all samples, thus suggesting that cells entered VBNC within 22 hours.

The VBNC state in bacteria may be reversible depending on the magnitude of cellular damage (Nystrom, 2003) and the duration in which they have been in a VBNC state (Aertsen and Michelis, 2004). The VBNC state becomes irreversible when bacteria consume their DNA as an energy source; hence if bacteria persist in VBNC for a long period of time the DNA content would decline to a threshold amount, after which the recovery of the cell is not possible and death is inevitable (Trevors, 2011; Nystrom, 2003). VBNC bacteria can resuscitate when provided with necessary nutrients under favorable environmental conditions (Jiang *et al.*, 2012). Resuscitation commences with RNA synthesis followed by protein synthesis, cell enlargement, replication of DNA and, finally, cell division (Trevors, 2011; Colwell, 2009; Oliver, 2005). The VBNC *Salmonella* and several other VBNC-state bacteria were resuscitated with a simple temperature increase and adding catalase or Tween 20 to prevent the accumulation of hydrogen peroxide in VBNC cells and to provide more carbon (Zeng *et al.* 2013). Gruzdev *et al.* (2012) looked at resuscitation efficiency of different rich media, in particular Luria-Bertani broth (LB), tryptic soy broth (TSB) and brain heart infusion (BHI) agar as compared to sterile double distilled water (SDDW) at different temperature scenarios (25°C and 37°C). Overall recovery at 37°C was statistically better than at 25°C while in rich media the resuscitation improved recovery by up to 1 $\log_{10}$ (BHI) compared with SDDW. To recover VBNC cells Liu *et al.* (2009) used auto-inducers produced by *E. coli* O157:H7 itself in a serum-based medium.

A resuscitation promoting factor (Rpf) was discovered by Kaprelyants and Kell (1993) in the Gram-positive coccus, *Micrococcus luteus*. The Rpf was a peptidoglycan hydrolase, an enzyme involved in the complex process of cell wall digestion in order to allow cell division to occur (Su *et al.*, 2013). The Rpf was subsequently found by various researchers to stimulate resuscitation of a number of Gram-positive as well as some Gram-negative organisms (Su *et al.*, 2013).

Pathogens entering into the VBNC state were found to maintain their virulence and therefore capable of causing infection upon successful resuscitation. Liu *et al.* (2008) showed that VBNC *E. coli* O157:H7 preserved the expression of Shiga-like toxins gene. Zeng *et al.* (2013) confirmed the virulence of VBNC cells by *in vivo* administration of 10^6 CFU mL-1 *Salmonella typhi* cells in a Kunming mouse model and subsequent isolation of this strain from the ascites fluid of mice that developed diarrhea.

The collective data on VBNC suggests that treatment efficiency of composting technology should be assessed against inactivation of pathogens having the potential to enter into VBNC states during composting, especially pathogenic bacteria such as *Salmonella* and *E. coli* which are used in direct process validation of compost efficiency (e.g. USPEA 2003; CCME, 2005; BNQ, 2005). In a VBNC state these indicator organisms/pathogens may go undetected by culture-based methods, thus providing a false impression on sanitation level. Under favorable conditions they may resuscitate and cause infections in animals and humans.

The objectives of this study were to investigate whether *E. coli* and *Salmonella* strains isolated from compost could enter into VBNC states and withstand the thermal temperatures associated with composting.

6.2 Materials and methods

6.2.1 Materials

Based on our previous work using novel temperature probes for assessing composting temperatures in full scale, a representative temperature compost curve

was derived as the average of 24 temperature profiles from individual temperature probes randomly introduced into covered aerated static pile (CASP) during one of the sampling seasons (July 25, 2011 – September 19, 2011) (Isobaev *et al.*, 2014). This representative time-temperature compost curve represented a compromise between the best and the worst-case temperature scenarios of thermal inactivation of particles within a compost pile. The original temperature data from CASP was read on 15 minute intervals. Consequently, in the model curve the readings were further averaged to one per every four hours (16 readings); hence one temperature reading in the model temperature curve was the 4 hour average from 24 temperature probes.

A programmable temperature incubator (Incucenter IC80, Cole Palmer) was used to simulate the model temperature curve. One program in the incubator could store up to 15 different temperature values including start/end time of each temperature value as well as the rate of temperature change over time. To accommodate 335 separate temperature values comprising the model temperature curve the values were fed manually into 33 separate programs.

Escherichia coli and *Salmonella* sp. were used in the current experiment as model pathogenic organisms of study. Importantly, the strains used in the current study were originally isolated from composted biosolids, the intent of which was to try and isolate microbial strains that may possess the necessary genetic elements for surviving stressful compost conditions. The *Salmonella* sp. was originally isolated from biosolids compost using the USEPA Method 1682 protocol (USEPA, 2006). Similarly, the *E. coli* strain used in this study was isolated from a biosolids compost pile using selective enrichment on Eosin-methylene blue (EMB) agar, and re-cultured on MacConkey agar. Both isolates were confirmed as *Salmonella* and *E. coli* using an automated bacterial identification system (Vitek, Biomerieux). Pure cultures of *E. coli* and *Salmonella* were stored on sheep blood agar at 4°C.

Luria Bertani (LB) broth was prepared by diluting LB powder (Fisher) in 1 L of ultrapure water and autoclaving at 121°C. The broth was always kept capped and

stored at 4°C in a clean refrigerator. Xylose lysine deoxycholate (XLD) plates were supplied from Dalynn Biologicals (Calgary, Alberta). Enzyme substrate reagents and vessels (Colilert™/Quantitrays) were supplied by IDEXX Laboratories. Propidium monoazide (PMA) was procured from Biotium and stored at -20°C. All DNA extractions were done using Qiagen's DNeasy Blood & Tissue kit according to manufacturer's protocols.

6.2.2 Methods

6.2.2.1 Sample preparation

E. coli and *Salmonella* monocultures were grown for 4 hours at 37°C in 50 mL single strength TSB solution. Samples were then washed with phosphate buffered saline and re-suspended in 50 mL 1% TSB solution overnight at 35°C. The final concentration of the stock solution was estimated using whole XLD plate count dilution assay for *Salmonella* and IDEXX Colilert/QuantiTray™ 2000 method for *E. coli*. The working cell suspension (WCS), which also corresponded to the zero time concentration of each microorganism, was prepared by diluting the stock monoculture solution to a final concentration of ~10^8 CFU mL^{-1}. Next, 2 mL of WCS was individually dispensed into 2 mL micro-centrifuge tube. In total, 30 samples of each monoculture of *E. coli* and *Salmonella* were prepared.

6.2.2.2 Sampling frequency

Prepared samples were placed in plastic micro-centrifuge holders and incubated under simulated compost temperature conditions within the programmable incubator. During the acclimatization phase of the simulated temperature experiment, in which temperatures gradually increased to 55°C during the first ~3 days, samples of *Salmonella* and *E. coli* were withdrawn from the incubator at a frequency of 1 sample per day. When 55°C was first reached the withdrawal frequency changed to 1 sample every 12 hours for the next three consecutive days. Thereafter, and until the end of the experiment, the sampling frequency from each category was reduced to one sample per every 5 days.

6.2.2.3 Model temperature curve

In order to verify the accuracy of the temperature curve inside the programmable incubator, two temperature probes were placed into the incubator at the time of experiment. Each temperature probe (Temp100, Madge Tech, Inc.) was programmed to take readings at 15 minutes interval. At the end of experiment the data was extracted from the probes using dedicated software. This profile was then used in analysis of microbial response to heat shock.

6.2.2.4 Analysis of culturable cell count

To quantify culturable *Salmonella* a chromogenic XLD agar was used. 100 µL of bacterial inoculum from each sample was serially diluted and whole plate spread on an XLD plate. After the inoculum was absorbed on the agar surface all plates were then incubated for 24 hours at 36°C. The concentration of culturable *Salmonella* was determined by counting black colony formations on XLD surface.

The concentration of culturable *E. coli* was determined using the enzyme substrate (IDEXX Colilert®/QuantiTray™ 2000) methods. Samples were removed from the incubator and vortexed for 15 seconds to uniformly suspend pellets. One-hundred µL of the *E. coli* suspension was added to 99.9 mL sterile water in a 100 ml sample vessel containing Colilert®, mixed vigorously, and the sample poured into a QuantiTray™ 2000. All QuantiTrays™ were incubated at 36°C for 24 ± 0.5 hours. After 24 hours, the concentration of viable *E. coli* was determined by counting the wells that had turned yellow and fluoresced under 365 nm UV light. These counts were converted to most probable number (MPN) using the MPN chart provided by IDEXX.

6.2.2.5 Analysis of regrowth/ resuscitation potential of bacteria

To analyze the regrowth/resuscitation potential of heat-stressed *Salmonella* and *E.coli*, 100 µL from each sample was individually seeded into a 15mL LB broth and incubated at 36°C for 1 month. To exclude the potential for cross-contamination for all samples for which growth was observed in LB medium (i.e., turbidity in the culture broth), bacteria were plated on blood sheep agar and incubated for 24 hours at 36°C. A random colony was picked from each incubated

plate and subject to DNA analysis using pulsed-field gel electrophoresis (PFGE), and performed at the Provincial Laboratory for Public Health, in order to ensure that the resuscitated isolates were of the same clonal origin as the original microbes (i.e., no contamination of culture) used to seed the monocultures.

6.2.2.6 Analysis of VBNC state in cells

To check for VBNC cells, thermal treated samples were exposed to PMA according to the methods of Nocker et al (2007). PMA binds to double stranded DNA (i.e., naked DNA or cellular DNA from microbes for which the cell membrane has been damaged), impairing the ability of DNA polymerases to replicate DNA by polymerase chain reaction (PCR) (Nocker *et al.*, 2007). For bacterial cells in which the outer membrane remains intact (i.e., vegetative or VBNC cells), PMA cannot cross the cell membrane. Consequently, PMA can be used to evaluate DNA integrity in bacteria cells entering the VBNC state (Taskin *et al.*, 2011).

For each of the thermal treated samples, an aliquot of 500 µL was removed from the bacterial suspension and 1.25 µL of PMA (20nM working stock in dimethylsulfoxide [DMSO]) was added to the suspension resulting in a final concentration of 50 µM. Five minutes of incubation in the dark was followed by 5 minutes of light exposure (intense visible - 600 W halogen light) for cross-linking PMA to DNA. Once the samples were PMA treated, they were pelleted in a micro-centrifuge for 5 minutes at maximum speed. The supernatant was discarded leaving the sample pellet in the bottom of the tube. The pellet was washed with 500 µL of phosphate buffered saline solution (PBS) and the suspension centrifuged for 5 minutes and the supernatant removed. The remaining pellet was processed for DNA using Qiagen's DNeasy Blood & Tissue Kit following the protocol for Gram-negative bacteria. All DNA was stored at -20°C prior to molecular testing.

Quantitative polymerase chain reaction (q-PCR) was used for evaluation of VBNC states. Its steps are described in section 5.2.2.5

6.2.2.7 Statistical analysis

Graphs and statistical significance analysis were produced with Sigmaplot v12. The analysis of difference in the concentration between three or more samples was done using Analysis of variance (ANOVA). The difference in concentration in control from the initial concentration was analyzed using two-tailed paired T-test. In all tests the cut-off value for α was set at 0.05.

6.3 Results and discussion

6.3.1 Model temperature curve

The simulated temperature profile for the incubator is shown in Figure 6-1. In general, the curve resembled the pattern typically described for composting processes (e.g. Epstein, 1997). The temperature in the incubator commenced at 23°C, analogous to the average daily ambient temperature observed in Edmonton in July. Over the next few days the temperature increased at an average rate of 0.4°C per hour. The target 55°C temperature in the incubator was reached after 2.5 days (60 hours). Over the next ~3 days (68 hours) the temperature reached 62°C which was the maximum temperature reached in the experiment. After remaining at this temperature for 1 day (26 hours), the temperatures slowly started to decline. After another 5 days (128 hours) the temperature fell below 55°C and up until the 29th day the modeled temperature ranged between 54°C and 45°C. To mimic turnover of the compost pile, as is required by USEPA (2003) for biosolids composting, the incubator was programmed for a rapid temperature drop to 30°C on days 29-32. Over the next 5 days temperatures were programmed to gradually increase to 40°C and then for an additional 10 days to reach 45°C, at which the temperature remained for the next 3 days. The temperature then gradually declined until day 56 when it reached 28°C, and at which time the experiment was concluded.

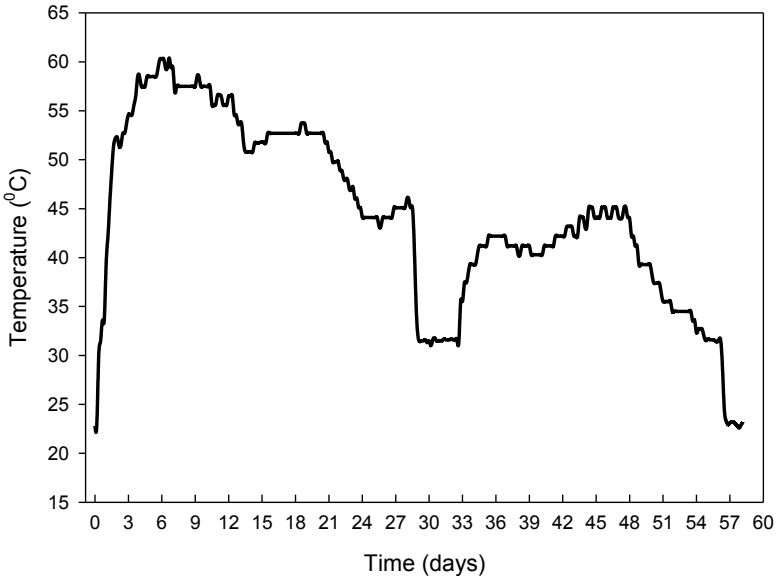

Figure 6-1. The model temperature compost curve obtained in the programmable incubator

6.3.2 Analysis of culturable cells

Regulations such as USEPA (2003) and CCME (2005) require every compost particle to be exposed to ≥55°C for at least three consecutive days. Arguably, this should be sufficient to inactivate pathogenic microorganisms such as *E. coli* and *Salmonella,* bringing their concentrations in biosolids below the limit of detection (LOD) for culture-based assays. From a temperature perspective, this criterion was satisfied within first 6 days of the experiment, and consequently, it was hypothesized that culturable cells should be absent beyond the 8th sample in the series taken from the incubator.

6.3.2.1 Analysis of culturable *E. coli*

According to Figure 6-2a there was no significant change in concentration of culturable *E. coli* (*n = 3; F = 1.05; p = 0.40*) after the first 48 hours of the experiment during which the temperature rose from 23°C to 52°C. The next 16 hours of continuous exposure to ≥52°C reduced the *E. coli* concentration by 5.5

$\log_{10}$ MPN mL^{-1}. By the time 55°C was reached the concentration of culturable cells had been reduced to <1 MPN mL^{-1}. Consequently, all subsequent samples taken from the incubator resulted in the inability to culture *E. coli* using the enzyme substrate test (i.e., Colilert®). The concentration of *E. coli* in the control sample, incubated at room temperature, did not change significantly over the entire 56 day period (*n = 3*; *p = 0.10*)

6.3.2.2 Analysis of culturable *Salmonella*

During the first 2 days of incubation the concentration of culturable *Salmonella* fluctuated but was always above 8 $\log_{10}$ CFU mL^{-1} (Table 6-1b). However, by the time the 55°C temperature was reached culturable *Salmonella* was below the limit of detection (LOD) (< 1 $\log_{10}$ CFU mL^{-1}). None of the subsequent *Salmonella* samples were culturable on XLD after the 55°C temperature was reached. The concentration of culturable *Salmonella* cells in the control sample did not change significantly from the initial seeding concentration over the course of 56 days when stored at room temperature. (*n=3; p=0.23*0).

6.3.3 Analysis of regrowth potential of bacterial cells

Failure of a microorganism to develop into visibly distinct colonies during routine culture was formerly viewed as a proof of inactivation. However, VBNC may dominate the population of bacterial cells, and as such, may not be readily detected by routine culture-based methods, requiring extended periods in the right conditions to resuscitate and initiate re-growth. Re-growth of cultures was carried out by extending the incubation phase in nutrient rich media to assess the potential for resuscitation of VBNC followed by active growth.

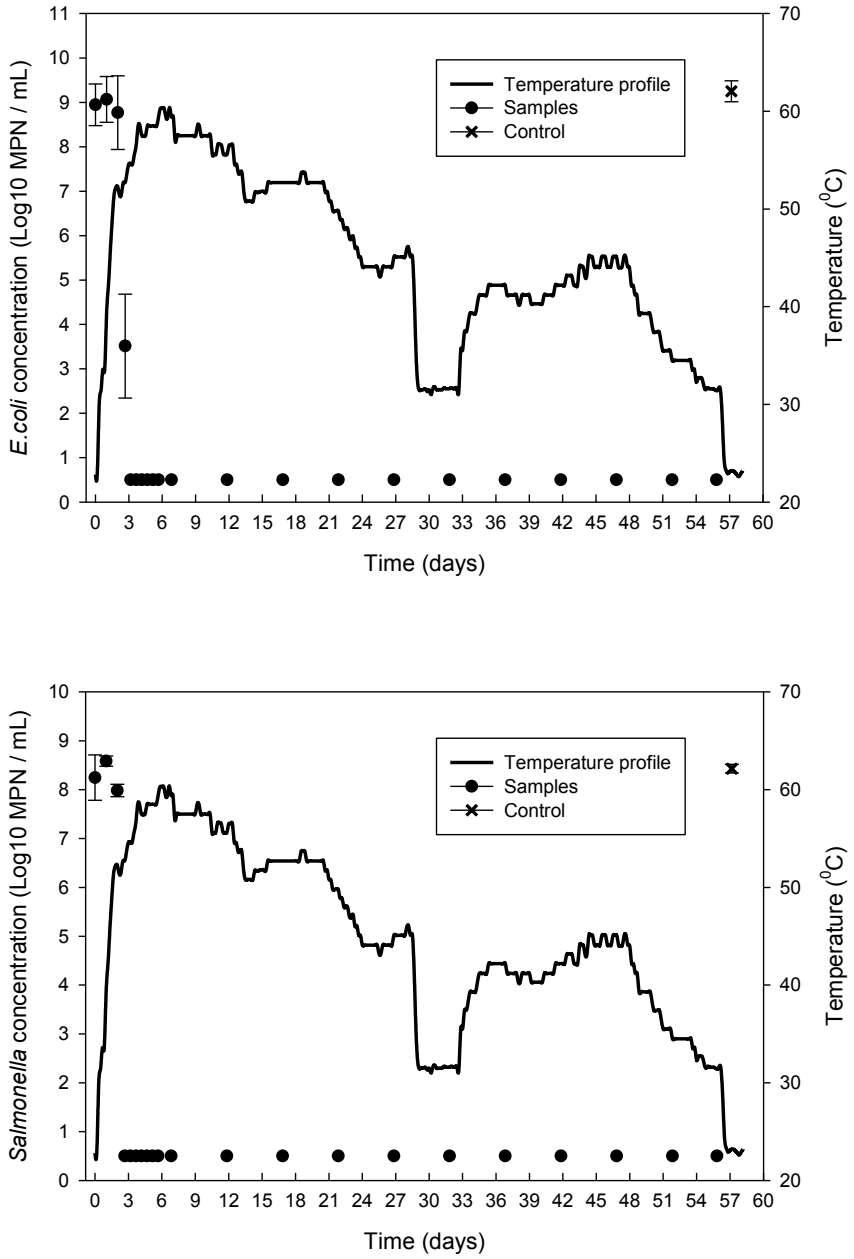

Figure 6-2. The change in the concentration of *E. coli* and *Salmonella* with the temperature over time: A) *E. coli* samples; B) *Salmonella* samples. N=3.

6.3.3.1 Analysis of *E. coli* regrowth

The results of *E. coli* regrowth in LB broth are provided in Table 6-1a. Although *E. coli* could not be cultured by enzyme-based substrate methods after reaching 55^{o}C (see sample 3 in Figure 6-2a) the microbes were still metabolically active and could be resuscitated after 24 hours in LB broth. The last *E. coli* sample removed from the incubator and in which regrowth occurred in LB broth was from sample 8. The cells from this sample were continuously subject to an elevated temperature ≥55°C for 2.7 days, with a maximum temperature reaching 62^{o}C. Samples taken after the 8th sample did not resuscitate in LB broth even after incubation for 30 days.

To ascertain that *E. coli* sp. which successfully regrew in LTB were the identical clones of the original control (lane labeled E0 in **Figure 6-3**a) the samples from LTB were analysed using PFGE technique. The results, as demonstrated in **Figure 6-3**a confirmed that regrowth in samples E3, E4, E6, E7 and E8 was the same clonal isolate of *E.coli* sp. that was used to seed original monocultures (see **Figure 6-3**a).

6.3.3.2 Analysis of *Salmonella* regrowth

Salmonella consistently regrew in LB broth until the temperature reached 55°C. This regrowth was in agreement with what was observed regarding routine culturability of the cells on XLD agar. There was no regrowth in samples 3 through 6, but unexpectedly, regrowth resumed in sample 7 corresponding to 2.2 days of exposure to ≥55°C and then in sample 13 corresponding to 9.7 days at ≥55^{o}C.

Table 6-1. The regrowth of *E. coli* and *Salmonella* following heat stress

Sample #	Days ≥55°C	Days to resuscitate in LB broth[$]	
		E. coli	*Salmonella*
0	0	1	1
1	0	1	1
2	0	1	1
3	0.2	1	-
4	0.7	1	-
5	1.2	1	-
6	1.7	1	-
7	2.2	1	4
8	2.7	1	-
9	3.2	-	-
10	3.7	-	-
11	9.7	-	-
12	9.7	-	-
13	9.7	-	2
14	9.7	-	-
15	9.7	-	-
16	9.7	-	-
17	9.7	-	-
18	9.7	-	-
19	9.7	-	-
20	9.7	-	-
Control[*]	0	1	1

*Control stored at room temperature for 56 days; N=3.

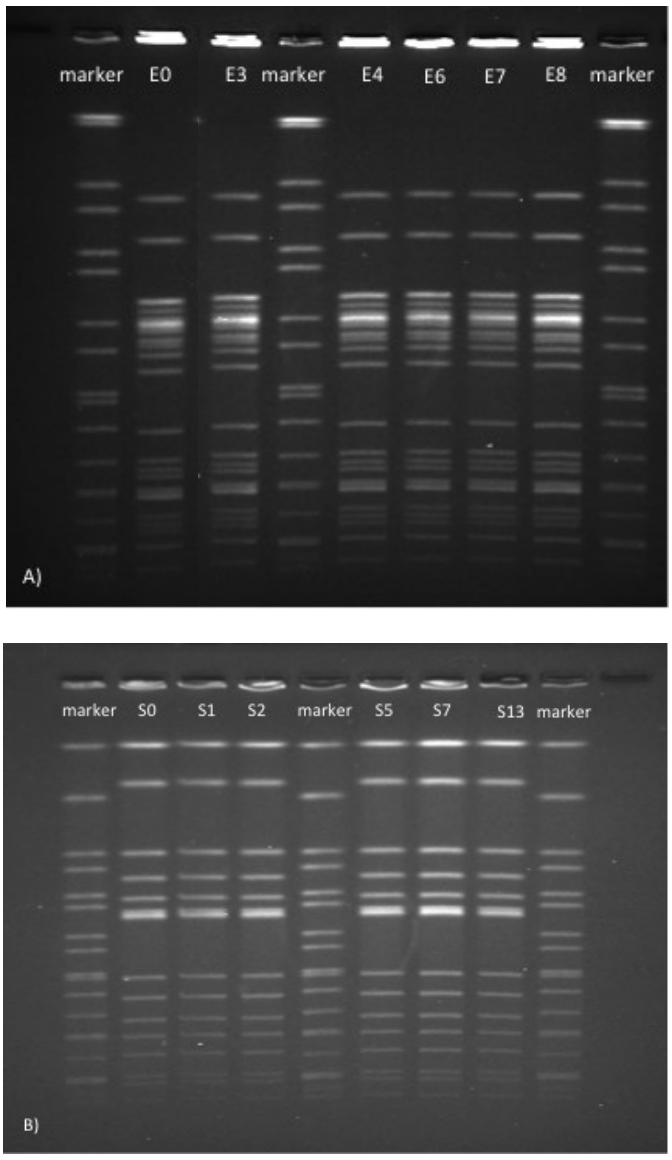

Figure 6-3. Analysis of the clonal relatedness of *E. coli* (Panel A) and *Salmonella* isolates (Panel B) as determined by PFGE. The lanes labeled with 'E' and 'S' encoded numbers represent samples identified in Table 6-1**. Data suggests that resuscitated *E. coli* (E3, E4, E6, E7 and E8) were clonally similar to control samples (E0) ruling out the possibility of accidental contamination of resuscitated cultures. Similarly, all resuscitated samples of *Salmonella* (S1, S2, S5, S7, S13) were clonally identical to the control (S0).**

The maximum temperature reached in these samples was 62^{o}C. Furthermore, resuscitation required 4 and 2 days of incubation in LB broth for samples 7 and 13, respectively. No regrowth was observed in samples taken after sample 13. When samples from LB resuscitated cultures of 7 and 13 were transferred onto XLD they developed into colonies with typical *Salmonella* morphology. Furthermore, all *Salmonella* samples which regrew in LB were shown to be identical to the original control strain (lane labeled S0 in **Figure 6-3**b**Error! Reference source not found.**) by PFGE, thereby confirming that regrowth was the same clonal isolate of *Salmonella* sp. that was used to seed original monocultures.

6.3.4 Analysis of VBNC state in bacterial cells

As mentioned previously, the VBNC state becomes irreversible when bacteria consume their DNA as an energy source (Trevors, 2011; Nystrom, 2003). Consequently, DNA integrity can be used to assess what proportion of the bacterial population may be in a VBNC state (i.e., steady state concentration of microbial DNA) and when the population enters into an irreversible phase of VBNC that eventually results in death and necrosis (i.e., decline in DNA concentration). PMA is a selective nucleic acid intercalating dye that is able to penetrate through compromised cell's membrane and intercalate with DNA via a photo-inducible azide group so that the DNA cannot be amplified during PCR (Taskin *et al.*, 2011). Since cells in the VBNC state maintain the integral structure of their membrane (Colwell, 2008; Zeng *et al.*, 2013) the PMA dye cannot penetrate and bind their DNA. Subsequently, whatever DNA is amplified in the sample during real-time PCR the DNA originates from a viable population of bacterial cells even though the bacterial cells themselves may fail to develop colonies on agar and/or do not exhibit regrowth in LB (i.e., a full VBNC state). When VBNC cells enter the irreversible phase necrosis of the cell ensues, compromising the integrity of the membrane structures thereby facilitating binding of PMA to DNA and loss of PCR amplification. In addition, necrotic cells degrade DNA resulting in a general loss of amplifiable DNA by PCR.

6.3.4.1 Analysis of VBNC *E.coli* in the samples

The change in concentration of *uidA* gene copies in PMA-treated *E. coli* samples over time is presented in Figure 6-4a. It can be noted that the *uidA* target concentration did not change significantly during the first 48 hours of incubation (see E0, E1 and E2 in Figure 6-4a) and corresponded with data demonstrating that cells at this stage were readily culturable with no significant loss in their numbers (see Figure 6-2**Error! Reference source not found.**). However, after another 24 hours when the temperature rose to ~52°C a significant proportion of the cells appeared to enter the VBNC state (see E3, Figure 6-4a). This was based on the observation that a ~5.5 $\log_{10}$ (i.e., >99.999%) reduction in culturable *E. coli* numbers was accompanied with only a ~ 1 $\log_{10}$ (i.e., 90%) reduction in *uidA* gene copy numbers in PMA-treated *E. coli*, and for which these cells could be resuscitated. When the temperature reached ≥55°C in the next 12 hours the *E. coli* was no longer culturable by routine methods (Figure 6-2a), but could still be readily resuscitated (Table 6-1) and with *uidA* gene copies remaining stable in PMA-treated *E. coli* (Figure 6-4a). Similar resuscitation behavior was observed in all cells exposed to ≥55°C for up to 2.7 days, and corresponded with stable *uidA* gene copy numbers from PMA treated cells during this time. The data suggests that although total culturable *E. coli* numbers declined by ~9 $\log_{10}$ (i.e., 99.9999999%) after 3 days of thermal treatment at ≥55^{o}C (i.e., sample 9 in Table 6-1), VBNC data suggested a moderate 1.5 $\log_{10}$ reduction in *E. coli* concentrations (i.e., 93%) during this same time period. Under prolonged thermal stress *E. coli* could no longer be resuscitated, and *uidA* gene copy numbers declined by an additional 1.5 $\log_{10}$ after 9.7 days exposure to ≥55^{o}C. However, even though cells were no longer culturable and could not be resuscitated using LB broth, *uidA* gene copy numbers remained relatively constant (~6.5 $\log_{10}$ copy number) up until 42 days of incubation. Between 42 and 56 days of thermal treatment there was an additional 1.5 $\log_{10}$ decline in the concentration of the *uidA* gene targets in PMA-treated samples. Conversely, control samples maintained at room temperature for 56 days had no appreciable loss in culturable cell concentrations or *uidA* gene copy numbers in PMA-treated cells. Overall, the

concentration of *uidA* gene copies at the beginning of the experiment was 9.46 $\log_{10}$ mL^{-1} declining to 5.42 $\log_{10}$ gene copies mL^{-1} by 56 days of thermal stress.

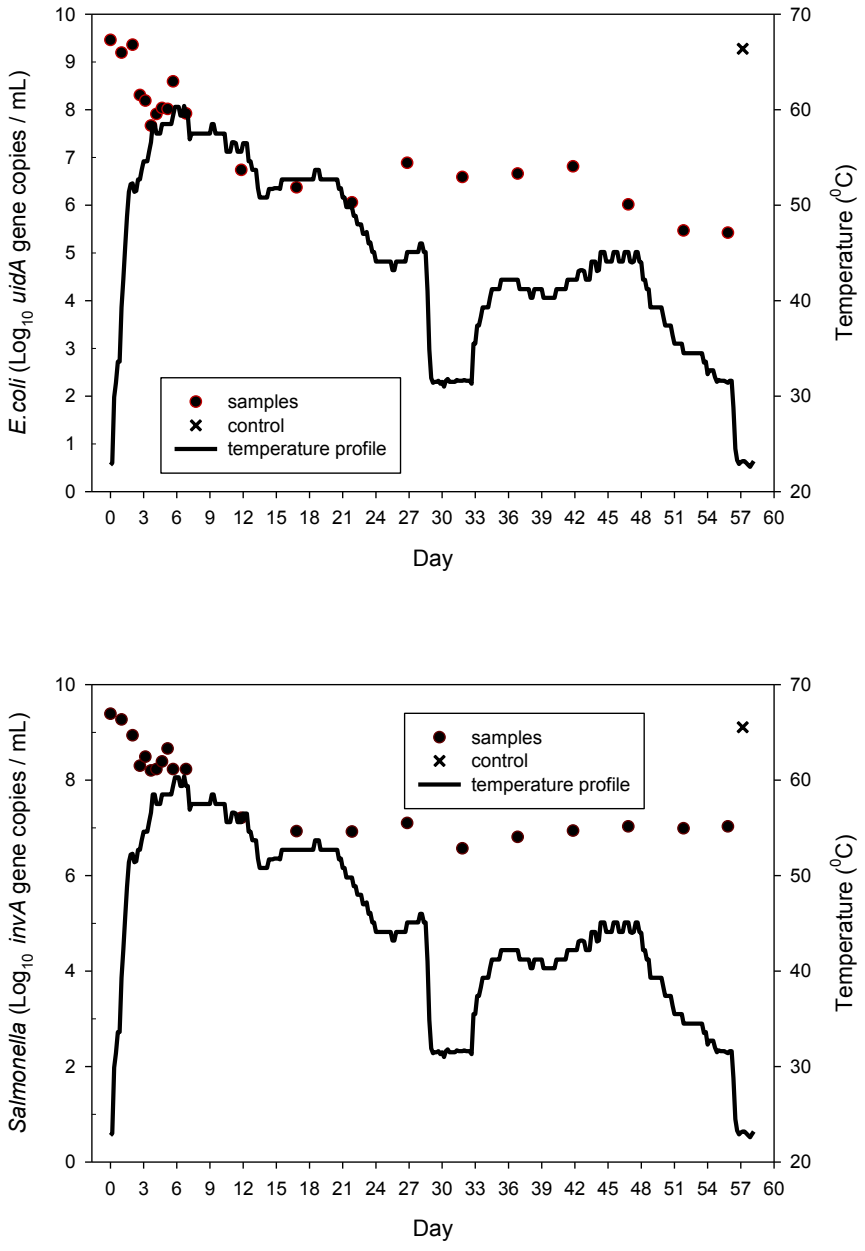

Figure 6-4. Analysis of VBNC cells as determined by the real-time PCR in PMA treated samples: A) *E. coli* and B) *Salmonella*. N=2.

6.3.4.2 Analysis of VBNC Salmonella in the samples

Salmonella also responded to the elevated temperature by inducing the VBNC stress response mechanism (Figure 6-4b). The response was much faster than that observed in *E. coli*, and with *Salmonella* appearing to have greater survival in a VBNC state than *E. coli*. In particular as the temperature increased from 22°C to 52°C and was sustained for nearly 24 hours the number of culturable cells dropped below the LOD but the number of *invA* gene copy numbers decreased from 9.39 $\log_{10}$ mL^{-1} to 8.30 $\log_{10}$ mL^{-1}. Thus a 9 $\log_{10}$ drop in culturable numbers of *Salmonella* was accompanied by only a 1 $\log_{10}$ decline in *invA* gene copy number in PMA exposed cells. In addition to the loss in culturability during this time, *Salmonella* could not be resuscitated. Interestingly, in sample 7, a monoculture exposed to temperatures $\geq$55^{o}C for 2.2 days and in which a maximum temperature had peaked at 62^{o}C, *Salmonella* could not be cultured on XLD plates, but could be resuscitated after extended periods in LB broth (4 days), and for which the number of intact *invA* gene copies remained constant (8.3 Log_{10} mL^{-1}). Similarly, in sample 13, a monoculture exposed to temperatures >55^{o}C for 9.7 days and in which a maximum temperature had peaked at 62^{o}C, *Salmonella* could not be cultured on XLD plates, but could be resuscitated after extended periods in LB broth (2 days), and for which the number of *invA* gene copies was still 7.03 $\log_{10}$ mL^{-1}. The number of intact *invA* gene copy numbers declined within the first 48 hours of thermal stress from 9.39 $\log_{10}$ mL^{-1} to 8.30 $\log_{10}$ mL^{-1} but remained at this level until day 7. In between days 7 and 12, the number of intact *invA* gene copy numbers declined to 7.03 $\log_{10}$ mL^{-1}, but remained at this concentration up until day 56 suggesting a prolonged ability of *Salmonella* to remain in a reversible VBNC state. Theoretically then, the analysis of *Salmonella* concentrations in these thermal composting conditions, based on the VBNC state resulted in only a 2.3 $\log_{10}$ reduction in *Salmonella* occurrence compared to a 9 $\log_{10}$ drop in culturable numbers of *Salmonella*.

6.3.5 Discussion of results

The thermal resistivity of *E. coli* and *Salmonella* has long been recognized (Fotadar, *et al.*, 2005; Bronikowski *et al.*, 2001; Brinton and Droffner, 1994). Nystrom (2003) suggested that similar to spore forming Gram-positive bacteria, the ability to enter VBNC in Gram-negative bacteria might in part explain the survival of these organisms beyond their typical temperature niches. Indeed the current study supports the concept of VBNC as a potentially important aspect to consider in the composting of human biosolids.

The present study examined the role that temperature plays in the transitioning of culturable bacteria (*Salmonella* and *E. coli*) into the VBNC state. Its novelty was at least twofold. Firstly, this study simulated an unbiased composting temperature profile whereas other similar studies (e.g. Weil *et al.*, 2013; Singh *et al.*, 2011) usually ramp the temperature to a certain degree (e.g. 55°C) and maintain their samples at that temperature for certain time interval. By so doing we tried to minimize the bias in our findings via incorporating natural temperature fluctuations, as well as replicate the rate at which temperature increases in a typical composting system. The latter is particularly important as it might affect the microbial acclimatization to changing environment and subsequent expression of stress-response factors. Secondly, the strains of bacteria used in this study originated from composted human biosolids. The isolation of *Salmonella* and *E. coli* strains from composted human biosolids was intended to select for isolates that had the potential to enter into VBNC states (i.e., they survived the biosolids composting conditions). The composting process represents a highly stressful and challenging environment with temperature, pH, nutrient deprivation, humidity, toxic metabolites and microbial competition/predation acting as selective forces on microbial survival. These same stressors are known to induce VBNC states in bacteria (Liu *et al.*, 2009; Trevor, 2011; Zeng *et al.*, 2013; Trevor, 2011; Higgins *et al.*, 2007; Gruzdev *et al.*, 2012).

The VBNC state is characterized by an inability to culture a particular microbe on standard media. It is believed that when a microbe persists in this state for an

extended period of time the metabolism of key molecules (carbohydrates, lipids, proteins, nucleic acids [including DNA]) results in eventual death and autolysis of the organism (Trevors, 2011; Nystrom, 2003). In our study, evidence of the VBNC persistence of *E. coli* and *Salmonella* strains in response to thermal stress was observed through the following sequence of events:

- an inability to culture *E. coli* and *Salmonella* upon exposure to increasing levels of thermal stress, but for which the organisms could be resuscitated under nutrient rich conditions,
- the gradual inability to resuscitate stressed organisms in nutrient rich broth under ongoing thermal stress, but;
- for which the integrity of cellular DNA was maintained during this VBNC transitioning period, and
- upon continued exposure to the thermal stressor a measurable loss in DNA integrity was observed.

Whereas culture-based assays yielded a ~9 $\log_{10}$ reduction for both *E. coli* and *Salmonella* after thermal stress conditions (i.e., those mimicking the composting of biosolids) an assessment of inactivation based on VBNC suggested that only a 4.4 $\log_{10}$ reduction in *E. coli* and a 2.3 $\log_{10}$ reduction in *Salmonella* inactivation was observed. The focus of this work related to the role of temperature in thermal inactivation of bacteria, and did not examine other factors affecting microbial survival in a full-scale composter such as pH, humidity, microbial predation, toxic metabolites, etc. Nevertheless, the widely disparate outcome on thermal composting efficiency between culture-based and VBNC-based methods warrants further examination of the true level of bacterial pathogen inactivation during composting.

The VBNC state in pathogenic bacteria such as *E. coli* and *Salmonella* is especially important for the composting industry since current methods examining compost efficiency rely on culture-based assays. Non-culturable bacteria in the VBNC state may be reversible depending on the magnitude of cellular damage (Nystrom, 2003) and the time cells have been in the VBNC state (Aertsen and

Michelis, 2004). Liu *et al.* (2009) demonstrated that even 10-month-old VBNC cells of *E.coli* O157:H7 could be successfully resuscitated. Gruzdev *et al.* (2012) reported that dehydrated *Salmonella* was still VBNC even after 100 weeks of storage at 4°C. According to Dhiaf *et al.* (2010) *Salmonella typhimurium* could be successfully resuscitated following 20 years of starvation in seawater and soil microcosms. *Citrobacter freundii* strain WA1 was also resuscitable after 11 years of starvation in seawater and soil (Dhiaf *et al.*, 2008). Our results show that the VBNC in both *E. coli* and *Salmonella* was reversible to a certain degree. In the *Salmonella* strain used in this study we observed resuscitation after 22 days in a sample which was exposed to ≥55°C for more than 9.7 days (and which peaked at 62^{o}C), which is significantly longer than is required by certain jurisdictions (e.g. USEPA, 2003; CCME, 2005). For *E. coli* the resuscitation was still possible after 6 days of composting with 2.7 days of exposure to ≥55°C, which is slightly less than the composting requirements in jurisdictions in North America, but still making this particular *E. coli* strain abnormally thermotolerant. No decline in culturable numbers or DNA integrity was observed in *E. coli* or *Salmonella* samples maintained at room temperature for 56 days. We cannot definitively conclude that the failure of the remaining *E. coli* and *Salmonella* cells to resuscitate after thermal stress was due to irreparable damage or the failure to provide all necessary nutrients and favorable environmental conditions. Being of enteric origin the best resuscitation condition for VBNC cells is *in vivo* which besides temperature includes proper supply of macro and micro-nutrients including different resuscitation promoting factors. Nonetheless, DNA integrity persisted over the course of the 56 days as assessed by qPCR and PMA treatment of cells. The ratio of copy numbers of the *invA* (*Salmonella*) and *uidA* (*E. coli*) genes to culturable bacterial numbers for both species approximated 1:1 in seeding monocultures and remained constant in control cultures stored at room temperature for 56 days. Under 56 days of thermal stress conditions *invA* gene copy numbers for *Salmonella* were maintained at 7.03 $\log_{10}$ mL^{-1} and for *E. coli* the *uidA* gene copy number was still 5.42 $\log_{10}$. Moreover, during the course of the 56 days, DNA integrity was observed to decline in a stepwise fashion for both

E. coli and *Salmonella*. In the case of *E. coli*, an initial ~1.5 $\log_{10}$ decline in DNA integrity was observed after temperatures reached $\geq 55^{o}C$, but which remained stable for an addition 3 days before declining by another ~1.5 $\log_{10}$. Subsequently, DNA integrity remained at this level from day 12-42, before going through an additional and gradual ~1.0 $\log_{10}$ decline to day 56. For *Salmonella*, a stepwise 2.43 $\log_{10}$ loss in DNA integrity was observed in the first 12 days of culture, but DNA integrity remained stable for the remainder of the experiment (up to 56 days under thermal stress). The data suggests that DNA integrity persisted under the VBNC state but that extended thermal stress conditions resulted in a gradual loss in overall DNA integrity in sample, an outcome predicted by the eventual and irreversible nature of the bacteria remaining in the VBNC state.

The rate at which microorganisms enter VBNC depends on the stress they have been exposed to. If the temperature profile used in the experiment is considered as a good representation of compost temperature then it could be said that the *Salmonella* entered VBNC after 2 days of composting when the temperature reached 52°C while for *E. coli* it took nearly 2.5 days to do so and by that time the temperature had exceeded 54°C. We don't know whether different susceptibility to elevated temperature or the rate at which VBNC was induced resulted in higher survival of *Salmonella* than *E. coli.*

On one side it might appear that at the end of composting the product is safe since the VBNC cells require special conditions before they regain the capacity to revert the VBNC and regrow. On other hand some hypothesize that the curing and storage phases in composting can provide these favorable conditions (Grewal *et al.*, 2007; Higgins *et al.*, 2007). While this argument could be subject of scientific debates the sure thing is that the VBNC still poses risk from public health perspective. A number of papers have shown that VBNC state cells retain their potential for virulence (Su *et al.*, 2013). Consequently, if land applied compost contains VBNC *Salmonella* the pathogen may regrow *in vivo*. Indeed, in incidents where a salmonellosis outbreak occurred with as low as 3 cells of bacteria per gram of food, has lead to speculation that VBNC cells may in fact be involved in outbreaks of disease (Gruzdev *et al.*, 2012). Having stated this more studies are

surely needed to better understand the potential virulence of VBNC human pathogens during their transition from the sludge matrix to the soil environment (Torri *et al.* 2012).

Finally, more studies are needed to go above and beyond the effect of temperature and see how chemical, physical and microbiological factors that originally present in compost matrix induce the stress-response mechanisms and subsequently affect the concentration of VBNC cells.

6.4 Conclusion

This study demonstrated that *E. coli* and *Salmonella* randomly isolated from the biosolids compost pile lose their culturability upon exposure to ≥52°C, which typically occurs on the 3rd day of active composting. The loss of culturability was demonstrated to be due to VBNC induction in these microorganisms. The VBNC state helps both *E. coli* and *Salmonella* survive at appreciable concentration throughout the 56 days long composting cycle. The VBNC at the early state in *E. coli* and *Salmonella* can be reverted when optimum growth conditions are supplied. Neither organism could be resuscitated after 22 days of composting. It needs to be examined if the loss of culturability is due to irreparable cell damages or failure to provide optimum conditions.

CHAPTER 7: GENERAL CONCLUSIONS AND RECOMMENDATIONS

7.1 Thesis overview

Composting is a promising waste management strategy in working towards a sustainable future within a rapidly increasing consumer-oriented society. Composting reduces the pressure for landfill space by diverting away organic waste, reduces GHG emissions and improves soil fertility. The biodegradable feedstock, after adjusting certain physical and chemical properties, can be composted in windrows, static piles and reactors.

Ensuring adequate sanitation during composting is critical. Many countries have developed guidelines and regulations, which specify how a composting facility should ensure proper sanitation of its end product (see Section 1.2.3 and Table 1-2). Although the regulations vary between the jurisdictions in values, they are similar in that they require both indirect process monitoring and direct validation of the end product's hygienic properties before it is sold, distributed or land applied.

In North America the direct validation requires that the level of fecal coliforms should not exceed 1,000 MPN g^{-1} dry solids and *Salmonella* should be present at less than 3 MPN 4 g^{-1} dry solids (see Section 1.2.2.1). This sanitation level is believed to be achieved by ensuring that every particle of compost is exposed to ≥55°C for at least 3 consecutive days. For in-vessel systems and static piles, it is assumed that the preceding requirement can be achieved by ensuring that ≥55°C is maintained for three days throughout the whole pile. For windrows, it is assumed that this condition can be achieved by maintaining 55°C temperatures for at least 15 consecutive days with five pile turnings during that period. The indirect process validation is a demonstration of compliance to this TTC.

The necessity to conduct the given study arose after discovering that, regardless of maintaining the recommended TTC, pathogenic microorganisms could still be

found in the finished compost. This lead to the formulation of two hypotheses: 1) the non-uniform temperature distributions in large composting piles falsely give the impression that the time temperature criteria have been met throughout the composting mass, although they haven't been; or 2) the recommended time-temperature criteria are not adequate (the contact time with the desired temperature is shorter than it needs to be for industrial scale operations).

In order to test both hypotheses, a commercial temperature probe was modified (MTP) (see Section 2.1.2). It was a 14.1 cm long and self-contained device with a dedicated compartment into which an inoculum with pathogenic microorganisms could be added. Two field trials showed that the MTP behaved like a random compost particle. The trials also showed that the probe's aluminum case was sufficient to protect its circuitry and the microbial inoculum from various worst-case physical and chemical stresses.

Since the information from the randomly introduced MTP could be retrieved only after it was successfully recovered from the compost pile, two recovery mechanisms were tested (see Section 2.3.4). The first method used the trommel screen only. This proved to be a viable but time consuming solution. The second method used fishing line and the trommel screen. This approach was less laborious and more efficient in terms of recovery. It consistently recovered 97% of MTPs. Neither method, however, was fully sophisticated for the mass adoption in the composting industry. Both heavily rely on the human factor and are prone to the physical properties of the mass being screened.

Once the recovery method was developed and tried, the next step towards testing both hypotheses was to design and propose the compost temperature sampling framework (see Chapter 3). Traditionally, the temperature sampling involves sampling at preset locations within the compost pile. However, it was experimentally confirmed that random sampling captured more temperature variations, both temporally and spatially, than did systematic sampling.

To beat the temporal and spatial temperature variations within the pile a certain number of samples need to be extracted from the studied population, and their

response analyzed. Subsequently, a framework was developed for deriving an estimate on a number of temperature monitoring devices (see Section 3.2.2.7). The method calculated that at least 47 MTPs should be used to obtain meaningful inferences from CASP technology. This number is much greater than what is normally used in practice.

The pile turning is required by most regulations to increase the probability of every particle experiencing time temperature criteria. Not much had been done to quantify the effect of turning since almost all temperature monitoring devices have to be removed before the pile is turned, and reintroduced at the end of turning. The framework was developed and proposed for making an educated guess on the number of turnings (see 3.3.3.1).

Two trials to test both hypotheses were executed (see Chapter 4 and 5). *E. coli* and *Salmonella* sp. served as indicator organisms. A microbial inoculum with the binary culture (Chapter 4) or monoculture (Chapter 5) was added inside MTP. The MTP was randomly introduced into the biosolids CASP and recovered at the end of composting following the recommendations developed in previous chapters. The conclusions pertaining to both hypotheses as well as general conclusions and recommendations on how to improve the sanitation assurance framework and eventually graduate it into the validation protocol are summarized below.

7.2 Conclusions and recommendations

1. Regarding the first hypothesis, during composting in CASP with single turning, the likelihood of every particle's compliance to TTC typically achieves 76 to 93% compliance.

2. Pile turning is a significant step towards assuring the particle's compliance with TTC. In particular the first five turnings have the most profound impact on increasing the chance of compliance. However, since the data from a single turning event was available to estimate the effects of

multiple turnings, it is possible that subsequent turnings of the pile would not mirror the same effect. It is recommended that the effect of turning be further studied using composting technologies that involve multiple turning stages.

3. The CASP surface, if not insulated, remains in the mesophilic temperature range (≤45°C) up to 50cm depth and provides a good environment for microbial proliferation. After a single turning with the front-end loader, 40% of the particles in the cool zones still remain mesophilic. It is recommended that: 1) the insulating layer up to 50cm in thickness be used to cover the pile and ensure that the traditionally cool zones reach thermophilic temperatures; and 2) the cool zones be adequately handled during the mixing step to ensure that what was in the cool zone gets into the pile core after mixing.

4. A large number of MTPs are required to address the temporal and spatial temperature variations in the composting pile. Therefore, for the proposed temperature sampling framework to graduate into a stand-alone protocol it is recommended to: 1) address the assumptions of the normality and linearity of data which was applied to the chi square test when estimating the sample size; and 2) conduct more research in the probe recovery and develop methods which would replace a naked eye in spotting probes in the continuously moving mass of oversize material as well as minimize the need to use the screening unit operation.

5. Regarding the second hypothesis, no culturable indicators of enteric bacteria and enteric viruses, whose levels in finished product are regulated by national and provincial regulations, were found in samples that met the TTC. However, it is recommended to repeat the experiment in which indigenous to compost matrix isolates of indicator organisms be used.

6. Certain species/strains of *Salmonella* (i.e., *Salmonella enterica* var. Meleagridis) can remain culturable at 40.1°C. This temperature was

observed in 30% of samples from cool zones after one pile turning and may explain the appearance of pathogenic microorganisms at the end of composting, as well as reiterate the importance of proper handling of composting mass from the cool zones.

7. Gradual exposure to TTC induces a VBNC state in *E. coli* and *Salmonella*. The VBNC state helps both *E. coli* and *Salmonella* survive at appreciable concentration throughout the 56 days long composting cycle. With certain constraints the VBNC at the early state in *E. coli* and *Salmonella* can be reverted when optimum growth conditions are supplied.

8. Since bacterial cells in a VBNC state are still metabolically active and can regrow in the environment upon attaining favorable conditions, it is recommended that the behavior of VBNC state pathogens when they are in the compost matrix itself be also studied to address the following concerns: 1) the speed of VBNC induction in the compost matrix; 2) the effect of physical, chemical, and microbiological factors (such as pH, toxic compounds and microbial antagonism) in combination with high temperature on inactivation and VBNC state in pathogens in the compost matrix; 3) the effect of duration of stress on microbial ability to recover from VBNC; and 4) the effect of different media and resuscitation promoting factors on microbial ability to recover from VBNC.

7.2.1 Practical implication of results

The results of this dissertation, as they apply to the context of CASP technology tested herein, convey several messages to the composting practitioners and regulatory authorities.

First of all, several pile turnings don't ensure 100% compliance with TTC. Each additional turning increases the chances of every particle to meet the required TTC. However, after 6^{th} turn the probability of compliance increases to 98%; thereafter the impact of turning becomes negligible and practically unattractive. Furthermore, the effect of turning is significantly profound during active

composting stage. Since, on average, the CASP requires more than three days for its temperature to reach 55°C we recommend not to turn the pile until seven days have passed from its construction, and every three days thereafter.

Next, it is not recommended to view the temperature as an effective stand-alone sanitation factor. According to the collected evidences, pathogens like *E. coli* and *Salmonella* can survive thermophilic conditions, similar to those in the composting pile. The cells, when exposed to 55°C for more than 3 consecutive days can induce stress-response mechanism and subsequently transit into VBNC state. During direct process validation the organisms in VBNC successfully skip culture-based detection methods and pose the risk to regrow during storage and transportation. The stakeholders should always keep that in mind when distributing the product. At least the existing direct process validation methods should be amended to incorporate the pathogens in VBNC.

Since TTC can never be reached and pathogens can survive in particles that met TTC, the practitioner may ask if the compost is a safe product at all? At this point it is hard to answer this question. On one side there are chances that pathogens in VBNC will reach the consumer. On the other side there are lots of unknowns that remain to be assessed and incorporated into the sanitation equation before a clear statement about the safety of compost product can be made. First of all, besides temperature there are other physical, chemical and microbiological factors, such as pH, ammonia, and competition, which likely provide the synergistic effect onto the microbial inactivation in the system. Their aggregate effect needs to be measured. Moreover, those factors, which correlate well with inactivation and which parameters can be easily acquired can further be combined with temperature and used to enhance the indirect process validation.

Second, although when in VBNC pathogens maintain their virulence, the fate of VBNC cells in compost released to environment has not been studied. The quantitative microbial risk assessment (QMRA) framework needs to be conducted to gauge the risk of exposure to VBNC and the consequences of this exposure. There are different risk assessment approaches under QMRA. They can be

classified under three domains: exponential, beta-Poisson, and mixed linear. Exponential and beta-Poisson models are based on the same assumptions but utilize different distributions, while the mixed linear model also discriminates between outcomes whether it is infection or illness. The exponential and beta-Poisson models, however, are easier to use when compared to mixed linear model. Once the associated health outcomes via QMRA are known one can decide if compost is safe. But even then the outcome from QMRA would need validation that can be done using the data from epidemiological studies, which is a challenge by itself given the limited information of epidemiological studies associated with compost. So, there are many unanswered questions that require further research.

7.2.2 Author's contribution

The contribution of the author to each chapter presented in this thesis was as follows:

- In chapter 1 the author conducted the literature review, updated the information on microbial survival in composting and contributed to the comparison of composting sanitation assurance regulations.
- In chapter 2 the author designed a method to validate the properties of modified temperature probe in full industrial setting, collected and interpreted field data.
- In chapter 3 the author designed a method to compare two temperature sampling approaches, both on compost particle level as well as proposed a framework how the data from temperature probes can be used to judge about the likelihood of composting technology compliance to TTC.
- In chapter 4 the author contributed to the design of experiment and to the preparation of microbial inoculum which contained *Salmonella enterica* var. Meleagridis*, E. coli* K-12 and pseudomonas phi-S1 phage. The author executed the experiment, collected, analysed and interpreted the results.

- In chapter 5 the author contributed to the design of experiment. He also executed the experiment, analysed the concentration of culturable organisms in compost matrix and in the cryovial using culture-based methods. The author also treated all collected samples for subsequent quantification of VBNC cells.
- In chapter 6 the author designed and ran the experiment. He also analysed the concentration of culturable organisms using culture-based methods. Finally the author treated each collected sample for subsequent quantification of VBNC cells in it.

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Appendix A **EXPERIMENTAL PROCEDURES**

A.1 **Analysis of density and anchors on particle movement**

1. Equipment

1.1. Compost pile with the density between 450 – 600 kg m^{-3} and MC of 40-45%;

1.2. Two to three assistants for the recovery of the probe from the compost pile;

1.3. Windrow turner to turn the windrow pile.

1.3.1. Note: Adjust the length and height of the experimental compost pile according to the technical characteristics of the windrow turner.

2. Procedure

2.1. Use three sets of probes (one with d = 1630 kg m^{-3}, another with d = 600 kg m^{-3} and the final with d = 1630 kg m^{-3} and anchors) each with the sample size of 32. A sample size of 32 is adequate given the scope of labor involved to recover particles after the bed turner, and assuming the recovery efficiency of 80 -90%. Also equal size across the groups makes calculations easier and increases the power of the test;

2.2. Label every probe: the one with the density close to compost -"C"; those similar to newly manufactured probes - "NC"; and newly manufactured probes with anchors - "AC" respectively;

2.3. Place all probes at the hypothetical volumetric center of the compost pile so that there is equal volume of mass above and below the probe. This is their starting point before turning. Record the height of the starting point from the ground;

2.4. Turn the compost pile three times with the windrow turner;

2.5. Recover probes manually using shovels and record their height with regards to the ground;

2.6. Place all recovered probes in the cardboard box for storage and further use;

2.7. Analyze the collected data for extreme values and outliers. This can be done by plotting boxplots; Use SPSS v17.0 or similar statistical software;

2.8. Compare the departure values of each cohort from the reference point by using analysis of variance test. This test is usually used to compare the significance between several different treatments. Interpret the results at 95% confidence level;

1) Ho: There is no significant difference between three different groups of probes i.e. probes behave same in the compost environment regardless of their density and existence of anchors ($\sigma_C = \sigma_{CN} = \sigma_{AN}$)
2) Ha: There are at least two groups of probes which show different distribution pattern in the compost after turning ($e.g.\ \sigma_C \neq \sigma_{CN}$)

A.2 **Analysis of the robustness of circuitry**

1. Equipment

1.1. Original temperature data logger (a.k.a. probe, MTP);

1.2. Compost pile with the BD between 450 – 600 kg m^{-3} and MC of 40-45%;

1.3. Two to three assistants to recover the probe from the compost pile;

1.4. Windrow turner to turn the windrow pile.

1.4.1. Note: Adjust the length and height of the experimental compost pile according to the technical characteristics of the windrow turner.

2. Procedure

2.1. Set up 10 original probes to record temperature data;

2.2. Place the probes in the middle of a windrow;

2.3. Turn the windrow using a windrow turner;

2.4. Open up cases and examine the circuit board for visible damage;

2.5. Download data from each device to see if recording is consistent during the test, take note of abnormalities in the temperature profile pattern.

A.3 **Analysis of the Robustness of Pathogen Cryovials**

1. Equipment

1.1. Original cryovials to remove any bias;

1.2. Compost pile with the BD between 450 – 600 kg m^{-3} and MC of 40-45%;

1.3. Two to three assistants for the recovery of the probe from the compost pile;

1.4. Windrow turner to turn the windrow pile.

1.4.1. Note: Adjust the length and height of the experimental compost pile according to the technical characteristics of the windrow turner.

2. Procedure

2.1. Set up original probe with cryovial;

2.2. Place the device in the middle of a windrow;

2.3. Turn the windrow using a windrow turner;

2.4. Open up cases and visually examine the vials and probe inner walls for punctures, leakages and other performance affecting signs of wear.

A.4 Analysis of shape on particle roll-off frequency from a compost edge

1. Equipment

1.1. Compost pile with the BD of 450 – 600 kg m^{-3} and MC of 40-45%;

1.2. Reserve 1-2 assistants to run the experiment;

1.3. Two sets of probes (dummy probes with d = 1630 kg m^{-3}, and dummy probes with d = 1630 kg m^{-3} and anchors) each with the sample size of 10;

2. Procedure

2.1. Spray paint a bucket of compost with a distinguishing color. Allow the paint to dry so that it doesn't affect the adhesive properties of experimental compost during experiment;

2.2. Mix the probes with the spray painted compost and load back into the bucket;

2.3. Divide the height of the compost pile into three equal strata starting from the ground (0 to 33% - toe; 34 to 66% - middle; and 67 to 100% - top);

2.4. Randomly choose the spot along the length of experimental pile;

2.5. Climb up on the pile crest and discard the contents of bucket onto slope. Try to imitate the emptying procedure by the front-end loader;

2.6. Ask one assistant to film and take pictures of the vertical spread of painted compost particles and the two types of probes

2.7. Repeat as necessary (generally the more the better);

2.8. Calculate the percentage of the probes coming to rest in each stratum;

2.9. Using visual materials infer about the percentage of coloured compost particles in each stratum;

2.10. Use ANOVA to analyse the significant difference between the three groups in each stratum

2.11. Use Chi Square test to see how often one could expect the probes to end up in each vertical segment of compost pile.

A.5 **Random Temperature Sampling of a Compost Pile**

1. Materials

1.1. Self-contained data-logging temperature probes

1.2. Compost pile

1.3. Pile building equipment with operator

1.4. Personal protective equipment

2. Procedure

2.1. Obtain from site supervisor the expected length of the pile (L), the expected number of loads (N) (e.g. mixer loads, bucket loads, etc.) and the time needed to empty one load (T_L).

2.2. Divide the length of entire pile into set of 20 segments with equal length (l) and number each segment from 1 to 20. Randomly select 5 out of 20 segments. These will be the pile segments where the temperature probes will be introduced.

2.2.1. Note: Deciding which segments to use without bias can be accomplished using a random number generator. For example, to generate 5 random digits from 1 to 20 using MS Excel 2010 select 5 cells you want to generate random numbers into, type the =RANDBETWEEN(1,20) function into the "Formula" text box and press CTRL+ENTER.

2.3. Calculate the average number of loads needed to build one such segment (n) by dividing total number of loads (N) by 20.

2.4. Calibrate temperature probes per manufacturer instructions

2.5. Set the start time for the probes on the same date the building of compost pile is slated with a 15 min. reading frequency.

2.6. Cut 40 meters of neoprene cord. Fold it into two and attach one end to the temperature probe though the designated eyelet in its cap. Repeat this for all temperature probes to be used in experiment.

2.7. Store the probes such that their cords don't tangle (consider coiling the cords individually).

2.8. Divide the available temperature probes into 5 equal groups. To each group assign its pile segment.

2.9. For each temperature probe within selected pile segment, generate two random numbers: **x** - the load it will be introduced to $\{1 \leq x \leq 20\}$; and **y** - the time (in seconds) it will be introduced into that load $\{0 \leq y \leq T_L\}$.

2.10. Clearly mark the segments of the compost pile which will be monitored, for example with flags or spray paint.

2.11. Observe the process until it comes to the first marked segment.

2.12. When it comes to build selected segment fully uncoil the cord from the probe and introduce each probe for that segment to its respective load in its respective time (see example calculation in Appendix C). Ensure that sufficient amount of cord is remained outside of the pile.

2.13. Repeat the step 2.12 until all probes are introduced into their respective segments.

2.14. When all probes have been introduced into the pile, record the pile location in your logbook for future reference. Also record the date and time that pile building commenced and was completed.

A.6 **Recovery of Temperature Probes from a Compost Pile**

1. Materials

1.1. Front-end loader, with operator

1.2. Trommel screen

1.3. Compost pile

1.4. Temperature probes

1.5. One person to recover probes

1.6. Personal protective equipment for all on-site personnel, including coveralls, dust masks, safety glasses, safety vests, leather gloves, hardhats.

2. Procedure

2.1. Cut 40 meters of neoprene cord. Fold it twice and attach one end to the temperature probe though the designated hole in its cap

2.2. Repeat the procedure for all temperature probes to be used in experiment.

2.3. Store the probes such that their cords don't tangle (consider coiling the cords individually)

2.4. Fully uncoil the cord from the probe

2.5. Randomly introduce temperature probes into the compost pile (see SOP on random temperature sampling of a compost pile)

2.6. Ensure that sufficient amount of cord is remained outside of the pile

2.7. Ask the operator responsible for recovery to be prepared whenever the pile biomass is slated for moving

2.8. During the recovery process front-end loader operator should grab bucketful of the composting material and the operator responsible for recovery firmly hold the free end of the cord and pull it at the time when the probe is detected in the bucket of the front-end loader

2.9. Signal the front-end loader operator when it is impossible to recover the probe by pulling the cord. Instruct the operator to start gradually emptying the contents of the bucket until the probe can be pulled

2.10. Repeat steps 2.8 and 2.9 until all probes are recovered

2.11. If the pile should be turned before recovery of the probes then ask the recovery operator to hold the ends of the cord and escort the material to the location where it would undergo further composting. At any instance the recovery operator is responsible for ensuring that enough cord is staying outside of the pile.

Note: In some instances the cord may break or be cut by the bucket of the front-end loader. In this case proceed to step 2.12. through 2.21

2.12. Secure a flat and safe area for the installation of screener

2.13. Turn the screener on and adjust the rotational speed of a drum to 19 – 23 rpm (based on experience but can be changed depending on the feedstock properties). For the steps to achieve this please refer to the equipment's user manual for further instructions.

2.14. After the required rotational speed has achieved steady state feed the screener with 2 bucketsful of compost using front-end loader.

2.15. Introduce each additional load comprised of 2 bucketsful at least 3 minutes from its predecessor.

2.16. **First worker:** stay at the outlet of conveyor belt with "overs" and visually screen the falling particles and the forming pile. When the eye spots the probe recover it as soon as possible from the pile of falling overs. There is no need to stop the screening process or alter it somehow when retrieving the spotted probe.

2.17. Once the probe is recovered from the stream of "overs", put it aside in a safe place and write its serial number for further records.

2.18. **Second worker:** continuously observe the conveyor belt itself for items resembling temperature probes. When anything like temperature probe is spotted inform about it the worker who is at the outlet of the

conveyor belt. **First worker** expect the probe's arrival with the "overs" and prepare for its retrieval in advance.

2.19. Workers exchange your places every 15 to 30 minutes in order to prevent the development of dizziness and nausea symptoms.

2.20. Let the front-end loader operator remove the pile with "overs" aside when its height has reached 1.2 m. This is critical because with pile getting higher it becomes harder to embrace it visually.

2.21. Give yourself a 15 minute break after every 2 hours of operation.

A.7 Method Used to Collect, Transport and Store Compost Sample (TMECC 02.01)

1. Equipment

1.1. Straight edged shovel, scoop, or trowel

1.2. Sanitized container with lid or sealable container

2. Procedure

2.1. Collect compost samples in plastic containers to contain enough material for all desired tests. It is a good idea to collect at least 4 times as much as may be needed for all tests (including replicates), in order to account for oversized material that will be sieved out of the sample, and to have enough for backup.

2.2. Fill the sample container(s) to overflowing and then seal.

2.3. Label each container with the following information, where relevant:

2.3.1. sample ID

2.3.2. sample location

2.3.3. date of collection

2.3.4. name of person collecting the sample

2.4. Transport compost samples in a cooler (or other insulated container) containing several cool packs. Upon arrival in the laboratory, store the compost samples under appropriate conditions until analysis.

2.5. Analyze compost samples within 24 to 48 hours of collection, unless special steps have been taken to preserve the samples.

2.6. Store samples at 4°C for up to 48 hours. If the sample must be held longer than 48 hours before analysis, store frozen at -20°C.

A.8 **Method Used to Reduce Samples of Compost to Testing Size (ASTM C 702-98)**

IMPORTANT! Sample mixing and splitting should be done as quickly as possible so as to minimize sample moisture changes and loss of volatile compounds.

1. Equipment

1.1. Straight edged shovel, scoop, or trowel.

1.2. Broom or brush

1.3. Canvas blanket, tarp, or plastic sheet (for Option 2)

2. Procedure

2.1. *Quartering on a Hard Surface*

2.1.1. Place the entire original sample on a hard, clean, level surface.

2.1.2. Mix the material thoroughly by turning the entire sample over three times using a shovel.

2.1.3. With the last turning, shovel the entire sample into a conical pile by depositing each shovelful onto the top of the preceding one.

2.1.4. Carefully flatten the pile to a uniform thickness and diameter by pressing down on the apex. The diameter should be approximately four to eight times the thickness.

2.1.5. Using the shovel, divide the sample into four equal sections and remove two diagonal corners (these will be "rejects").

2.1.6. Brush the space clean. Repeat steps "2.1.2." through "2.1.5" until the required amount of compost is attained.

2.2. *Quartering on a Canvas Blanket*

2.2.1. If a clean hard, level floor surface is unavailable, the Canvas Blanket method can be used.

2.2.2. Place the entire compost sample on a canvas blanket, tarp, or plastic sheet. Mix with shovel, as in Option 1b, or by alternately

lifting each corner of the canvas and pulling it over the sample toward the opposite end.

2.2.3. Flatten pile as in 2.1.4.

2.2.4. Insert a long stick underneath the blanket in the middle of the pile and then lift the stick to get two equal piles. Leave a small fold of material in between the piles to separate them. Turn the stick 90° and repeat this process. (Alternatively, if the surface under the blanket is relatively flat, you can use the shovel for quartering as per 2.1.5.)

2.2.5. Remove the two diagonally opposite corners (these will be the "rejects")

2.2.6. Repeat steps "2.2.2." through "2.2.5." until the required amount of compost is attained.

Note: The "rejects" (excess material) can be retained for use in other tests. This excess material should be stored at 4°C for use within 24 to 48 hours. If the excess is to be stored for longer than 48 hours, it should be placed into frozen storage (-20°C)

A.9 **Method Used to Obtain Under 9.5 mm Material for Compost Analysis (TMECC 02.02B)**

This method describes how to sieve an as-received compost sample to an appropriate size range for use in analysis. It has been determined (TMECC 2002) that all compost analyses (on finished and in-process compost samples) should be performed on samples which have been sieved to less than 9.5 mm.

1. Equipment

1.1. Stainless steel sieve (9.5 mm opening size)

1.2. Solid pan or sieve with very small openings (e.g. <100 µm) to catch the undersized material; the pan should be stackable with the 9.5 mm sieve.

1.3. Sieve cover

1.4. Mechanical shaker (optional)

2. Procedure

2.1. Collect compost sample as described in the document summarizing TMECC method 02.01.

2.2. Sieve the sample as soon as possible after collection. If it is not possible to sieve samples immediately, they should be stored at 4°C for no longer than 24 hours.

2.3. Determine the amount of material that should be sieved, according to the analyses to be performed. Aim to obtain at least 3 times the required amount (including replicates) of sieved material. This will provide enough for all analyses and for backup.

2.3.1. Sieve either the entire collected sample, or perform sample mixing and splitting (as per ASTM C702) prior to sieving in order to obtain only the desired amount (to obtain the "aliquot to be sieved"). It is recommended to split the sample prior to sieving, as this will reduce

the workload. The unsieved portion of the as-received sample can be stored at -20°C for future use.

2.4. Stack the 9.5 mm sieve on the collection pan.

2.5. Transfer an aliquot of approximately 250 cm^3 (250 mL) of as-received compost onto the 9.5 mm sieve.

2.6. Place the cover onto the sieve and collection pan, and secure them all together.

2.7. Shake the sieves for 1 to 5 minutes, either on a mechanical shaker or by hand.

2.7.1. Note that excessively moist material may clump and compact during the sieving process. This material can be screened by moving it back and forth across the screen with a clean scoop or other flat implement.

2.8. The oversized material (i.e. that material retained on the 9.5 mm screen) can be discarded in an appropriate manner.

2.8.1. Consider that this material may need to be disinfected by autoclaving prior to final disposal.

2.9. The undersized material (i.e. that material passing through the 9.5 mm screen) should be placed in a clean, sterile container and immediately sealed in order to minimize changes in moisture content and loss of volatile compounds.

2.10. Repeat steps 2.4. through 2.9. until the entire "aliquot to be sieved" has been sieved.

The sieved material is now ready to use for compost testing. If testing cannot be performed immediately after sieving, the sample should be stored under appropriate conditions (see the summary of TMECC 02.01 – Sample Collection, Transport, and Storage).

A.10 **Analysis of Compost Bulk Density (TMECC 03.01A)**

1. Equipment

1.1. Unsieved, compost (in-process or finished) or feedstock materials.

1.2. Graduated Beaker with Handle - 2000-mL low-form polypropylene, straight-wall (not tapered) beaker.

1.3. Balance - top loading balance, accurate to ±0.1 g with at least 0.1 to 1.0 kg range.

1.4. Desiccator with Desiccant.

1.5. Rubber Mat - mat constructed from four (4) stacked layers of closed-cell polyethylene foam, 6-mm (¼-in.) thick per layer.

1.6. Funnel - approximately 2.5-cm (~1-in.) diameter delivery stem, 15 cm (6 in.) mouth.

2. Procedure

2.1. Bulk density should be determined within 7 days of sample collection (within 48 hours is ideal).

2.2. Label a page in the log book with the test name (Bulk Density, Unsieved), date, and operator name. Create columns for: Sample ID; m_{beaker} (g) and $m_{beaker + compost}$ (g); and $TS_{as\ rec'd}$(%).

2.3. Determine the total solids content of the as-received/unsieved sample on a parallel aliquot, using method TMECC 03.09 or using a moisture balance ($TS_{as\ rec'd}$).

2.4. Weigh and record the tare weight of the empty 2000-mL graduated beaker, ±0.01 g (m_{beaker}).

2.5. Fill graduated beaker with compost:

2.5.1. Transfer a 600 cm^3 aliquot of as-received compost into the modified 2000-mL graduated beaker using the funnel if desired.

2.5.2. To ensure uniform packing of compost throughout the modified graduated beaker, allow beaker containing compost to fall freely onto

a rubber mat once from height of 15 cm (6 in). Carefully maintain the beaker in an upright position at all times.

2.5.3. Repeat the filling with 600 cm^3 and free falling operation two more times (three times total).

2.5.4. After the third free-fall drop, fill the graduated beaker to the 1800 mL mark with compost ("topping off"). Do not repeat free-fall drop after topping off. Topping off should be limited to 2-3 cm.

2.6. Determine the as-received weight of the sample:

a. Weigh and record the weight of the 2000-mL graduated beaker containing 1800 cm^3 of as-received compost, ±0.01 g ($m_{beaker+compost}$).

3. Calculations

(1) $m_{wet,\ 1800} = m_{beaker\ +\ compost} - m_{beaker}$

(2) $m_{dry,\ 1800} = m_{wet,\ 1800} \cdot (TS_{as\ rec'd}/100)$

(3) $BD_{dry} = (m_{dry,\ 1800} / V_{compost,\ 1800}) = (m_{dry,\ 1800} / 1800\ cm^3)$

(4) $BD_{wet} = (m_{wet,\ 1800} / V_{compost,\ 1800}) = (m_{wet,\ 1800} / 1800\ cm^3)$

Where:

$m_{wet,\ 1800}$ = mass of 1800 mL of compost, moist/wet basis (as-received weight)

$m_{dry,\ 1800}$ = mass of 1800 mL of compost, dry basis

$TS_{as\ rec'd}$ = total solids [%], as determined on a parallel aliquot of as-received/unsieved compost by method TMECC 03.09 or using a moisture balance

100 = conversion factor to convert TS from percent to a fraction

$V_{compost,\ 1800}$ = volume of compost in 1800 mL = 1800 mL = 1800 cm^3

BD_{dry} = bulk density of the sample, dry basis [$g \cdot cm^{-3}$]

BD_{wet} = bulk density of the sample, wet basis [$g \cdot cm^{-3}$]

A.11 **Analysis of Compost Total Solids and Moisture Content (TMECC 03.09)**

1. Equipment

1.1. compost or feedstocks that have been sieved to 9.5mm, as per TMECC 02.02B;

1.2. Balance—capable of weighing to at least 100 g, with an accuracy of ±0.01 g;

1.3. Desiccator Cabinet—vacuum with desiccant tray containing color indicator of moisture concentration or an instrument indicator;

1.4. Evaporation Dish— any heat-resistant dish with a capacity of 150 mL;

1.5. Drying Oven—vented, set at 70±5°C (do not microwave);

2. Procedure

2.1. TS and MC analyses should be started within 24 h of sample collection

2.2. Label a notebook page with the test name (Total Solids and Moisture Content), date, and operator name. Note whether the analyses are being done on sieved or unsieved materials. Create columns for: Sample ID; dish ID; m_{dish} (g); $m_{dish + compost, wet}$ (g); and $m_{dish + compost, dry}$ (g).

2.2.1. All samples should be analyzed in triplicate.

2.3. Tare the balance.

2.4. Measure and record the evaporation dish weight (m_{dish}).

2.5. Transfer an aliquot of compost material to the evaporation dish (obtain the aliquot by the quartering method, ASTM C702). For finished compost samples, an aliquot of ~50 cm^3 is adequate. You may wish to use a larger aliquot for feed stock materials or in-process samples, depending on the material's heterogeneity. A volume up to 4000 cm^3 may be used, in an appropriately-sized container.

2.6. Measure and record the weight of the compost aliquot and the dish ($m_{dish + compost, wet}$).

2.7. Place the uncovered dish containing the as-received moist sample aliquot into a drying oven preheated to 70±5°C. Dry the sample for approximately 18 h to 24 h, until the weight change due to moisture loss diminishes to nil.

2.8. Place the oven-dried sample in a desiccator and cool to ambient laboratory temperature.

2.9. Weigh and record the gross weight of the cooled dish and dry sample ($m_{dish + compost, dry}$).

3. Calculation

(1) $$mw = m_{dish+compost,wet} - m_{dish}$$

(2) $$dw = m_{dish+compost,dry} - m_{dish}$$

(3) $$TS = \frac{dw}{mw} \times 100$$

(4) $$MC = \left(1 - \frac{dw}{mw}\right) x100 = 100 - TS$$

Where:

- TS = total solids; percentage solid material in sample, wet basis [% $g \cdot g^{-1}$]
- MC = moisture content; percentage moisture in sample, wet basis, [% $g \cdot g^{-1}$]
- dw = dry weight; net sample weight after drying in an oven at 70 ± 5°C [g]
- mw = moist weight; net sample weight at as-received moisture [g]

A.12 **Electrometric pH Determination for Composts, 1:5 Slurry Method (TMECC 04.11)**

Appendix A. Equipment

A.1. The compost (in-process or finished) sieved to 9.5mm, as per TMECC 02.02B;

A.1.1. If pH analysis is to be done on feedstock materials, sieving may not be appropriate, and size reduction may be necessary. This should be determined on a case-by-case basis

A.2. pH meter - with electrode (e.g., Accutech XL20)

A.3. Stirring rod - approximately 15-cm length, glass or plastic

A.4. Sample bottles - 250-mL, plastic or glass bottles, with screw-cap lid

A.5. Shaker - capable of shaking a sample flask at a rate of $\geq$ 60 rpm

A.6. Ultrapure water—ammonia-free, carbonate-free deionized water with a minimum resistivity of 17 $M\Omega \cdot cm^{-1}$

A.7. Reference solutions – commercial pH buffers, pH 4.0, 7.0, and 10.0.

Appendix B. Procedure

B.1. pH analysis should be done within 48 hours of sample collection, on samples that have been stored at 4°C.

B.2. Label a page in a log book with the test name (Electrometric pH), date, and operator name. Set up columns for sample ID, mw (g), V_{water} (mL), and pH.

B.3. Calibrate the pH meter according to the manufacturer's instructions. Do a three-point calibration using pH 4.0, 7.0, and 10.0 buffer solutions. The slope after calibration should be between 90 and 110%.

B.3.1. Check the pH of all three buffer solutions to confirm that the meter is working and the calibration was acceptable. Recalibrate if necessary.

B.4. Estimate the total solids (TS) content of a parallel aliquot of compost.

B.4.1. Use a moisture balance to get a quick estimate of TS. This value will be used to determine the required mass of as-received compost (mw) equivalent to 40 g of dry-weight compost. This is the amount required for the extraction step.

B.5. Prepare the compost sample slurries (1:5 compost:water).

B.5.1. Replicate samples—for every twelve samples, prepare at least one sample slurry in duplicate or triplicate to monitor precision. If desired, prepare duplicate or triplicate slurries for each sample.

B.5.2. Determine the weight of the as-received compost (mw, moist weight) required to give an aliquot of approximately 40.0f dry weight of compost, using equation (1). Record this weight in the log book.

(1) $$mw = \frac{dw}{TS \cdot \frac{1}{100}} = \frac{40}{TS \cdot \frac{1}{100}}$$

Where:

- mw = mass of as received moist compost aliquot [g]
- dw = dry weight equivalent of sample = 40.0g
- TS = sample total solids content [% wet weight basis]
- 100 = factor to convert TW from a percentage to a fraction [unitless]

B.5.3. Determine the volume of ultrapure water (V_{water}) required to bring the liquid fraction of the 1:5 slurry to an equivalent of 200 mL. This step is based upon the assumption that 1 mL of water is equivalent to 1 g of the as-received compost liquid fraction, and that 1 mL of water is equivalent to 1 g of water. Use equation (2) to determine the required volume. Record this volume in the log book.

(2) $$V_{water} = V_{total} - (mw - dw) \cdot \left(\frac{1mL}{1g} \right) = 200mL - (mw - 40g) \cdot \left(\frac{1mL}{1g} \right)$$

Where:

- V_{water} = volume of ultrapure water to be added to the as-received compost sample, mL
- V_{total} = target volume of the liquid fraction of the 1:5 slurry, 200 mL
- mw = moist weight, the required weight of as-received compost equivalent to 40 g dry weight
- dw = dry weight, the total solids fraction of the compost aliquot, 40 g

B.5.4. Weigh out the required mass of as-received compost (as determined above) into a 250mL sample bottle. Label the sample bottle with the sample ID, date, and operator name or initials.

B.5.5. Add the required amount ultrapure water (determined above) to the as-received moist compost aliquot using a graduated cylinder.

B.5.6. Repeat steps 2.5.2. though 2.5.5. for all samples and replicates.

B.5.6.1. Note that for replicate samples, the total solids (TS) content only needs to be determined once.

B.5.7. Place all of the prepared sample bottles on a shaker for 20 minutes at ~60rpm. Maintain the slurry at room temperature (20-23°C)

NOTE: Steps 2.4. and 2.5. are the same for pH and electrical conductivity, so they only need to be done once for each sample for both tests.

A.13 **Culturing Bacteria in Broth Media**

1. Materials:

Equipment	Supplies	Media/Reagents
Incubator(s) Fume hood (optional) Biological Safety Cabinet	Pure culture on blood plate, in skim milk, or in glycerol. Colony picks/inoculating loops Gloves Test tubes with loose fitting caps. Serological pipettes (5 mL) Pipette aid	Broth media conducive to organism to be grown • e.g. TSB, *Pseudomonas* broth, enriched nutrient broth

2. Specimen: Culture to be grown in broth media.

3. Procedure:

Step	Action
1	Label test tubes with culture name and date.
2	Remove cap from first tube.
3	Transfer 5 mL's of broth into the test tube using serological pipette and aid.
4	Using an inoculating loop or colony pick, pick pure colony from plate and transfer to TSB tube. Alternately, scrape a small amount from skim or glycerol and transfer to TSB tube.
5	Place loose fitted cap back on tube. Gently shake.
6	Repeat steps 2 - 5 for each culture.
7	Incubate for desired amount of time in appropriate incubator. • eg. 20 ±4 hours at 36 ±1°C

A.14 **Culturing Bacteria on Plate Media**

1. Materials:

Equipment	Supplies	Media/Reagents
Incubator(s) Fume hood (optional) Biological Safety Cabinet	Pure culture(s) in broth. Inoculating loops Pipette (200 µL) Pipette tips (200 µL) Bent glass rod spreaders Gloves	Plated media conducive to organism to be grown • e.g. Tryptic Soy Agar, *Pseudomonas* agar, enriched nutrient agar, blood plate

2. Specimen: Culture(s) to be grown on plated media.

3. Procedure:

Step	Action
	For whole plate spread method:
1	Label media plate(s) with culture name and date.
2	Mix broth culture well.
3	Remove cap from the first liquid culture.
4	Pipette out 100 µL of turbid culture and onto the center of the matching plate.
5	Put lid back onto the culture tube.
6	Using a bent glass rod spreader, spread the inoculum over the entire plate until all the inoculum has been absorbed by the plated media.
7	Repeat steps 1 - 6 for all cultures.
8	Invert plate(s) and incubate at recommended temperature for the recommended time. • e.g. *E. coli* at 35 - 37 ° C for 16 - 24 hours.
	For isolation spread method:
9	Label media plate(s) with culture name and date.
10	Mix broth culture well.
11	Remove cap from the first liquid culture.
12	Gently immerse the inoculating loop inside the broth media and swirl gently.
13	Pull the inoculating loop out of liquid culture and tap off excess liquid on the inside of the test tube.
14	On one quarter of the plate, gently rub the inoculating loop to form a

Step	Action
	confluent lawn.
15	Using a new inoculating loop, gently pull some inoculum from the first quarter and spread in a 'S' pattern onto the second quarter of the plate.
16	Using a new inoculating loop, gently pull some inoculum from the second quarter and spread in a 'S' pattern onto the third quarter of the plate.
17	Using a new inoculating loop, gently pull some inoculum from the third quarter and spread in a 'S' pattern onto the fourth quarter of the plate.
18	Invert plate(s) and incubate at recommended temperature for the recommended time. • e.g. *E. coli* at 35 - 37 ° C for 20 ± 4 hours. • colonies will form along the lines on inoculum, getting further apart as the inoculum gets more dilute.

A.15 **Standard Plate Count Method**

1. Materials:

Equipment	Supplies	Media/Reagents
Incubator Vortex Used tip crock Bleach crock Biohazard bin	Pipette (200 µL & 1000 µL) Pipette tips (200 µL & 1000µL) 1.5 mL Microcentrifuge tubes Bent glass rods (sterile) Gloves	Nutrient broth • eg. TSB, LB Sterile water Plated media • eg. SPC plates (pure cultures) • eg. XLD (mixed *Salmonella* and *E. coli*)

2. **Specimens:** Culture in broth requiring enumerating (from culturing in broth SOP).
3. **Procedure:**

Step	Action
Day 1	
1	Remove broth culture(s) from incubator. • Culture(s) grown overnight in broth • See SOP for growing cultures in broth
2	Label 10 - 1.5 mL centrifuge tubes (per culture) with: • side: culture name • top: ST, -1, -2, -3, -4, -5, -6, -7, -8, -9
3	Pipette 900 µL of sterile water into all the tubes labeled with a number (not ST tubes)
4	Vortex test tube with first culture.
5	Pipette 1 mL of culture into tubes labeled ST and culture name.
6	Repeat steps 4 & 5 for remaining cultures.
7	Vortex first centrifuge tube labeled ST and culture name.
8	Pipette out 100 µL and into the centrifuge tube labeled -1 and culture name.

Step	Action
9	Dispose of tip in used tip crock.
10	Repeat steps 7 - 9 for remaining dilutions for the first culture. • -2, -3, -4, -5, -6, -7, -8, -9
11	Set tubes aside.
12	Repeat steps 4 - 11 for remaining cultures.
13	Change gloves
14	Label plated media (in triplicate) with: • culture name • dilutions -4, -5, -6, -7, -8, -9 • 18 plates per culture total (6 dilutions in triplicate)
15	Line up labeled plates with the first culture.
16	Vortex -9 dilution tube.
17	Pipette 100 µL of inoculum out of -9 tube onto the center of the first -9 plate.
18	Dispose of used tip in used tip crock.
19	Whole plate spread inoculum using a bent glass rod. • see SOP for culturing on plated media (whole plate spreading)
20	Dispose of glass rod in bleach crock.
21	Repeat steps 16 - 20 for second and third plate of the -9 dilution.
22	Repeat steps 16 - 21 for remaining dilutions for first culture. • -4, -5, -6, -7, -8
23	Set plates and tubes to the side.
24	Repeat steps 15 - 23 for remaining cultures.
25	Incubate plates, inverted, in incubator for required time. • eg. 36 °C for 22 ± 2 hours
26	Place all biohazardous materials in biohazard bin.
27	Put away all supplies in their designated spots. • eg. cultures in fridge
28	Clean counter with 10% bleach.
29	Place all used gloves in biohazard bin.
Day 2	
30	Put on a pair of gloves.
31	Remove all plates from incubator(s).

Step	Action
32	Count colonies on plates and record on enumerating culture chart below. • Plates greater than 300 colonies are TNTC (too numerous to count)
33	Calculate CFU per culture using calculations formula below.
34	Place all biohazardous materials in biohazard bin.
35	Put away all supplies in their designated spots.
36	Clean counter with 10% bleach.
37	Place all used gloves in biohazard bin.

4. Example Calculations:

Dilution	Replicate 1	Replicate 2	Replicate 3
-4	TNTC	TNTC	TNTC
-5	304	277	289
-6	29	31	33
-7	3	3	2
-8	1	0	0
-9	0	0	0

$(277 + 289 + 31 + 33) / (10^{-5} + 10^{-5} + 10^{-6} + 10^{-6}) = 630 / 2.2x10^{-5} = 28\ 636\ 363.64$ CFU/100 µL

multiply by 10 = 286 363 636.4 CFU/1 mL

A.16 **High Titre Assay of Phage**

1. Materials:

Equipment	Supplies	Media/Reagents
Incubator Vortex Water bath (45°C) Magnetic stirrer with heat. Weight scale BSC (level 2 organism)	Host Phage Test tubes Erlenmeyer flask Magnetic stir bar Serological Pipette(s) - 5 mL Pipette aid Micropipette(s) - 1000 µL & 200 µL Pipette tips - 1000 µL & 200 µL Weighing boat Disposable spoon 1.5 - 2 mL centrifuge tubes Centrifuge tube holder Paper towels Gloves 0.22 nm Filter Syringe 15 mL centrifuge tube.	Media broth selective for host. Plated hard agar selective for host. Agar Sterile buffered water

2. Specimen: Phage and its host.

3. Procedure:

Step	Action
Day 1	
1	Grow host according to instructions from supplier (ATCC). a) Open cryovial. b) Rehydrate with 0.5 - 1.0 mL of selective broth. c) Pipette up and down to mix well. d) Pipette 2 x 10 mL of selective broth into a sterile test tubes. e) Transfer 1/2 the content of cryovial into each test tube. f) Incubate host for 4 - 8 hours at recommended temperature for one test tube and overnight for the other test tube.
2	Rehydrate phage according to instructions from supplier (ATCC). a) Open cryovial. b) Rehydrate with 1 mL of selective broth.

Step	Action
	c) Mix by pipetting up and down until entire contents have been hydrated.
3	Add 200 µL of phage into the tube containing the host (4 - 8 hour growth).
4	Incubate phage/host inoculum overnight (18 - 24 hours) at recommended temperature. • temperature dependant on phage/host • eg. phi-S1 and its' host (*Pseudomonas fluorescens* ATCC 27663) incubate at 26°C.
5	Pipette remainder of original phage suspension into a clean, sterile centrifuge tube and store at 4°C.
Day 2	
6	Remove phage/host inoculum from incubator, mix well. • Appearance should be relatively clear. • Any turbidity is a result of phage resistant bacteria.
7	Remove host inoculum from incubator, mix well. • Appearance should be turbid due to bacterial growth.
8	Filter sterilize the phage/host inoculum. a) Dispense the well mixed inoculum into a sterile syringe. b) Screw on a sterile filter to end of syringe. c) Gently push the inoculum through the filter unit and into a clean centrifuge tube. d) Label tube.
9	Test concentration of high titre using dilutions and a double layer plaque assay (see plaque assay SOP).
Day 3	
10	Count plates to get a Plaque Forming Unit (PFU) per mL of original high titre solution.
11	Repeat above steps for a higher titre solution.
12	Dispose of waste according to biosafety guidelines.

4. Calculations:

Take an average of the plates for each dilution. Enumeration will be in plaque forming units (PFU) / mL.

5. Interpretation of Results:

The counts should give close to a 10 fold count starting from the lowest readable dilution. If a 10 fold count is not seen then the following errors could have occurred:

- samples were not properly vortexed
- pipetting error

If no plaques are seen at any dilution the following error(s) could have occurred:

- rehydration error (e.g. wrong media was used)
- wrong host was used
- incubation temperature was wrong
- phage not viable
- bacteria was not removed during the filter sterilization step

A.17 **Plaque Assay of Phage**

1. Materials:

Equipment	Supplies	Media/Reagents
Incubator • temperature dependant on organism Vortex Water bath (45°C) Magnetic stirrer with heat. Weight scale BSC (level 2 organism)	Host and Phage Test tubes Erlenmeyer flask Magnetic stir bar Serological Pipette(s) - 5 mL Pipette aid Micropipette(s) - 1000 µL & 200 µL Pipette tips - 1000 µL & 200 µL Weighing boat Disposable spoon 1.5 - 2 mL centrifuge tubes Centrifuge tube holder Gloves	Media broth selective for host. Plated hard agar selective for host. Agar Sterile buffered water Media blank in the incubator to check for sterility

2. Specimen: Phage and its host.

3. Procedure:

Step	Action
Day 1	
1	Grow host according to instructions from supplier (ATCC). a) Open cryovial. b) Rehydrate with 0.5 - 1.0 mL of selective broth. c) Pipette up and down to mix well. d) Pipette 5 - 10 mLs of selective broth into a sterile test tube. e) Transfer entire content of cryovial into test tube. f) Incubate according to recommended instructions.
Day 2	
2	Take plated media (hard agar) out of fridge and allow to warm up to room temperature. Media must be free of condensation therefore may need to be taken out the day before. • Label 27 plates as -1 through -9 and a - c for each for a total of 27 plates and 1 plate labelled blank. • Note: May want to do each dilution 5 times rather than in triplicate. If so adjust amount of plates, media broth, agar, and test tubes. Label accordingly.

Step	Action
3	Rehydrate phage according to instructions from supplier (ATCC). a) Open cryovial. b) Rehydrate with 1 mL of selective broth. c) Mix by pipetting up and down until entire contents have been hydrated.
4	Serial dilute the phage. a) Label 9 centrifuge tubes (-1, -2, -3, ... , -9). b) In the -1 through -9 tubes, pipette in 900 µL of sterile buffered water. c) Pipette 100 µL from the phage suspension into centrifuge tube -1. d) Close lid and vortex. e) Pipette 100 µL from -1 dilution into -2. f) Close lid and vortex. g) Repeat until all dilutions are made.
5	Pipette remainder of original phage suspension into a clean, sterile centrifuge tube and store at 4°C.
6	Turn on water bath and place test tube holder with 27 empty but labelled (-1a, -1b, -1c, etc.) test tubes (9 dilutions in triplicate) in it.
7	Make 'soft' agar. a) Measure 200 mL of selective broth in an Erlenmeyer flask (more if >4 repetitions are being performed for each dilution) . b) Measure agar to make a 5% soft agar (e.g. 5% for 1L would be 5 grams). c) Place the broth on the magnetic stirrer with heat and turn on. d) Add the agar, heat and mix until all the agar is completely dissolved (the solution should appear clear). e) Allow solution to boil for at least 1 minute.
8	Pipette 5 mLs of 'soft' agar into each of the 27 test tubes. Leave the test tubes in the water bath at all times to avoid agar from hardening.
9	Set out plates right side up in order of pouring (e.g. set up equivalent to the test tubes for quick pouring).
10	Mix the host test tube culture well, vortex the -9 phage dilution well.
11	Remove one -9 test tube from the water bath, dry test tube, and pipette in 300 µL of host and 100 µL of phage.
12	Roll the test tube between palms for 3 - 5 seconds in order to mix.
13	Pour 'soft' agar mixture on the hard agar plate labelled -9a.
14	Gently swirl the plate in order to get agar confluent and to the edges.
15	Put lid back on plate and allow to sit about 1 hour or until 'soft' agar

Step	Action
	has completely hardened.
16	Repeat steps 10 - 15 for remainder of replicates and dilutions.
17	Place plates inverted in moisture controlled incubator for 18 - 24 hours. • temperature dependant on phage/host • eg. phi-S1 and its' host (*Pseudomonas fluorescens* ATCC 27663) incubate at 26°C.
Day 3	
18	Take plates out of the incubator and count the plaque forming units.
19	Dispose of plates in biohazard waste.

4. Calculations:

Take an average of the plates for each dilution. Enumeration will be in plaque forming units (PFU) / mL.

5. Interpretation and results:

The counts should give close to a 10 fold count starting from the lowest readable dilution. If a 10 fold count is not seen then the following errors could have occurred:

- samples were not properly vortexed
- pipetting error

If no plaques are seen at any dilution the following error(s) could have occurred:

- rehydration error (e.g. wrong media was used)
- wrong host was used
- incubation temperature was wrong
- phage not viable

A.18 **Cryovial Preparation**

1. Materials:

Equipment	Supplies	Media/Reagents
Incubators • 26 & 35°C BSC Vortex	*E. coli* (K12 or equivalent) *Salmonella melaegridis* *Pseudomonas* Phage phi-S1 Colony picks/Inoculating loops Gloves Test tubes with loose fitting caps. Cryovials	Ultrapure water.

2. Specimen:

- *E. coli* (K12 or equivalent)
- *Salmonella melaegridis*
- *Pseudomonas* Phage phi-S1

3. Procedure:

Step	Action
Day 1	
1	Culture organisms according to culturing SOP - broth, plaque assay SOP (step 1), and/or high titre SOP (steps 1 - 5).
	E. coli
Day 2	
2	Using the standard plate count SOP method (& dilutions), set up dilutions on plated media and incubate over night (steps 5 - 18).
Day 3	
3	Count plates to get enumeration of original culture suspension.
4	Adjust original culture suspension to 10^6 cells/100 µL. a) If original culture enumeration is too concentrated, dilute with enough sterile water to have a 10^6 cells/100 µL concentration. b) If original culture enumeration is not concentrated enough: • redo step 1 with more culture then redo steps 2 & 3. **OR** • spin down culture and pipette out the required amount of broth to reach the 10^6 cells/100 µL concentration.
6	Pipette 100 µL of *E. coli* into each cryovial.

Step	Action
	Salmonella
Day 2	
7	Using the standard plate count SOP method (& dilutions), set up dilutions on plated media and incubate over night (steps 5 - 18).
Day 3	
8	Count plates to get enumeration of original culture suspension.
9	Adjust original culture suspension to 10^6 cells/100 µL. a) If original culture enumeration is too concentrated, dilute with enough sterile water to have a 10^6 cells/100 µL concentration. b) If original culture enumeration is not concentrated enough: • redo step 1 with more culture then redo steps 2 & 3. • spin down culture and pipette out the required amount of broth to reach the 10^6 cells/100 µL concentration.
10	Pipette 100 µL of *Salmonella* into each cryovial.
	***Pseudomonas* phage phi-S1**
Day 2	
11	Prepare high titre phage inoculum according to SOP high titre (steps 6 - 9).
Day 3	
12	Count plates to get a Plaque Forming Unit (PFU) per mL of original high titre solution.
13	Adjust original culture suspension to 10^7 viruses/100 µL. a) If original culture enumeration is too concentrated, dilute with enough sterile water to have a 10^7 viruses/100 µL concentration. b) If original culture enumeration is not concentrated enough: • Repeat above steps for a higher titre solution. • This would require an extra 2 days.
14	Pipette 100 µL of *Pseudomonas* phage phi-S1 into each cryovial.
15	Dispose of waste according to biosafety guidelines.

4. Calculations:

Step	Action
1	Take an average of the plates for each dilution. Enumeration will be in plaque forming units (PFU) / mL for *Pseudomonas* phage phi-S1 and colony forming units (CFU) for *E. coli sp.* and *Salmonella sp.*
2	Take an average of the 5 plates for each dilution of *E. coli* and *Salmonella*. Colony count *10 = colonies/mL for that dilution.

5. Interpretation and results:

The counts should give close to a 10 fold count starting from the lowest readable dilution. If a 10 fold count is not seen then the following errors could have occurred:

- samples were not properly vortexed
- pipetting error

If no plaques are seen at any dilution the following error(s) could have occurred:

- rehydration error (e.g. wrong media was used)
- wrong host was used
- incubation temperature was wrong
- phage not viable
- bacteria was not removed during the filter sterilization step

A.19 **Detecting Fecal Coliforms in Biosolids Compost Matrix (USEPA Method 1680)**

1. Materials:

Equipment	Supplies	Media/Reagents
Incubators 35° C & 44.5° C Vortex Magnetic stirrer with heat. Weight scale BSC Blender Autoclave (if making media)	100 mL vessels with lids Inoculating loops Pipet (1000 µl) and tips Serological pipet (10 ml) Pipette aid Test tubes Test tube racks Stir bars 500 mL & 1000 mL bottles or flasks Large tray Measuring scoops (if making media)	LTB with durham tube - single strength EC with durham tube Sheep blood agar plates Non-selective plated media • eg. HIA, SPC, TSB Ice Sterile phosphate buffered water Precept or 10% bleach For all recipes refer to EPA Method 1680, section 7.

2. **Specimen:** *Pseudomonas aeruginosa* ATCC 27853*, Escherichia coli* ATCC 25922*, Enterobacter aerogenes* ATCC 13048.

3. **Procedure:**

Step	Action	
1	Request organisms from QC on blood plate.	
	If	Then
	QC has organism(s)	Proceed to step 2
	QC does not have organism(s)	Proceed to step 3
2	Place organisms in fridge (4°C) and proceed to step 5 procedures.	
3	Order specimen(s) from ATCC through Cedarlane Laboratories.	
4	Rehydrate organisms according to instructions from supplier (ATCC). • Open cryovial. • Rehydrate with 500 µL of selective broth. • Pipette up and down to mix well. • Pipette 10 mL of selective broth into a sterile test tubes. • Transfer 450 µL of the content of cryovial into the test tube.	

Step	Action
	• Plate remaining inoculum on a blood plate. • Incubate overnight at recommended temperature. • Skim liquid stock culture and freeze at -70°C • Place blood plate in fridge (4°C)
Day 1	**Two days prior to sample testing.**
5	Remove organisms (isolated on blood plates) from fridge and allow to warm up to room temperature.
6	Remove sterile blood plates from fridge and allow to warm up to room temperature (1 - 1.5 hours) prior to use.
7	Label the bottom of 3 blood plates with *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, and *Enterobacter aerogenes* ATCC 13048.
8	Streak to isolation *Pseudomonas aeruginosa* on the blood plate labelled as so. • See culturing SOP - plating, steps 9 - 17 and diagram.
9	Repeat step 8 for *Enterobacter aerogenes*.
10	Repeat step 8 for *Escherichia coli*.
11	Invert plates and incubate at 35° C for 24 ± 2 hours.
Day 2	**One day prior to sample testing.**
12	Take streaked blood plates out of the incubator and confirm growth.
13	Prepare a 1 % solution of Lauryl Tryptose Broth (LTB) • Section 15.2.2 of EPA method 1680 • Measure out 99 mL of phosphate buffered dilution water and dispense into a sterile screw cap bottle or vessel. • Add in 1 mL of LTB (single strength). • Shake well.
14	Prepare the **undiluted spiking suspension (USS).** • Section 15.2.3 of EPA method 1680 • Transfer a small loopful of the **stock culture** to 1 % LTB. • Shake minimum 25 times. • Incubate at 35°C for 20 ± 4 hours.
15	Put streaked blood plates in fridge.
16	Prepare a media sterility check and a blank for the phosphate buffered dilution water. • LTB - 35°C for 48 ± 3 hours • EC - 44.5°C for 24 ± 2 hours

Step	Action
	• Phosphate water, 20 mL & non-selective media - 35°C for 24 ± 2 hours
17	Label all media and wares for the following day. • See table 1 and table 2 below. • Note: This media and ware's count and labelling is for 1 complete sample. If more than 1 sample is being processed, label sample 1, sample 2, etc (in addition to the rest of the labels from the tables below. If samples are being processed in triplicate, label samples a, b, c (eg. 1a - sample 1, triplicate a). The only exception is SSA - SSD vessels. Spiked sample will only be performed on one samples and/or triplicate.
Day 3	**Sample arrival day.**
18	Take all media needed for the day and allow to warm up to room temperature (1 - 1.5 hours). • non-selective media must be dry and free of condensation so may need to be put in 35°C incubator for a short period of time.
	Prepare homogenized sample (section 11.1.2) and dilutions (section 11.2.2.1)
19	Weigh out 30 grams of sample into a blender.
20	Add 270 mL of phosphate buffered water to the sample in the blender.
21	Blend for 1 minute.
22	Transfer the entire content to a 500 mL bottle or flask (labelled unspiked, homogenized sample) containing a sterile stir bar.
23	Place on magnetic stirrer and adjust pH to 7.0 - 7.5 with either 1.0N HCl or 1.0N NaOH. • Do not use more than 5% of HCl or NaOH to pH sample. • This is the **homogenized sample**.
24	Repeat steps 19 - 23 for additional samples
25	Line up the vessels for dilutions (HA-2, HB-3) that match the sample.
26	Measure out 99 mL of phosphate dilution water and dispense into HA-2.
27	Pipette out 11 mL's of the well mixed homogenized sample into HA-2.
28	Mix well by shaking a minimum of 25 times.
29	Measure out 99 mL of phosphate dilution water and dispence into

Step	Action
	HB-3.
30	Pipette out 11 mL's of the well HA-2 and dispense intoHB-3.
31	Mix well by shaking a minimum of 25 times.
32	Set aside the homogenized sample and the 2 dilutions by placing them in the large tray with ice.
33	Repeat steps 25 - 32 for any additional samples.
	Inoculate unspiked samples (section 11.2.2.2 a - d)
34	Line up all the LTB tubes (unspiked) needed to process the first sample.
35	Pipette 1 mL of dilution B (HB-3) into each of the tubes labelled A - E, 1UB.
36	Swirl gently to mix.
37	Pipette 1 mL of dilution A (HA-2) into each of the tubes labelled A - E, 1UA.
38	Swirl gently to mix.
39	Pipette 1 mL of the **homogenized sample** into each of the tubes labelled A - E, 1U.
40	Swirl gently to mix.
41	Pipette 10 mL of the **homogenized sample** into each of the tubes labelled A - E, 10U.
42	Swirl gently to mix. • Note: All the samples must be immersed into the LTB. If not, use an inoculating loop to carefully immerse.
43	Repeat steps 34 - 42 for remaining samples and/or triplicates.
44	Inoculate the positive and negative control. • *E. coli* ATCC 25922 positive control • *P. aeruginosa* ATCC 27853 negative control
	Making the spiking suspension. (section 15.3)
45	Shake the USS (*E. coli*) a minimum of 25 times to mix well.
46	Pipette 99 mL's of phosphate buffer water into each of the vessels labelled SS A-E
47	Pipette 1 mL of the USS into vessel SS-A and shake well. • 1 mL of SS-A is 10^{-2} of the USS
48	Pipette 11 mL of SS-A into SS-B, shake well. • 1 mL of SS-B is 10^{-3} of the USS
49	Pipette 11 mL of SS-B into SS-C, shake well.

Step	Action
	• 1 mL of SS-C is 10^{-4} of the USS
50	Pipette 11 mL of SS-C into SS-D, shake well. • 1 mL of SS-D is 10^{-5} of the USS
51	Pipette 11 mL of SS-D into SS-E, shake well. • 1 mL of SS-E is 10^{-6} of the USS
	Spiking suspension enumeration (section 15.4)
52	Line up the dry, room temperature non-selective plates.
53	Shake the vessel labelled SS-E well.
54	Pipette 100 µL (10^{-7} of the USS) onto each of the 3 plates labelled SS-E, 1 - 3.
55	Whole plate spread and set plates aside so the inoculum can completely absorb into the agar. • See culturing SOP - plating (step 6) for further instruction on how to whole plate spread.
56	Repeat steps 53 - 55 for SS-D (10^{-6} of the USS) and SS-C (10^{-5} of the USS).
57	Invert plates and incubate at 35°C for 24 ± 4 hours.
	Spiking Class A biosolid samples (section 15.5.2)
58	Change the label on the unspiked, homogenized sample to spiked, homogenized sample.
59	Pour sample back in clean, sterile blender.
60	Spike the sample based on spiking calculations below. • using spiking suspension B
61	Blend 1 - 2 minutes in blender. This is the **spiked, homogenized sample**.
62	Pipette 99 mL of phosphate buffered water into each of the vessels labelled SH A - D.
63	Pipette 11 mL of the **spiked, homogenized sample** into vessel SHA and mix well.
64	Pipette 11 mL of SHA into SHB, mix well.
65	Pipette 11 mL of SHB into SHC, mix well.
66	Pipette 11 mL of SHC into SHD, mix well.
	If greater than one sample is processed, this only needs to be performed on 1 sample though can be done in triplicate.
	Inoculating spiked samples (section 11.2.2.2 c-f)
67	Line up all the LTB tubes (spiked) needed to process the first sample.

Step	Action	
68	Pipette 1 mL of SHD into each of the tubes labelled A - E, 1SD.	
69	Swirl gently to mix.	
70	Pipette 1 mL of SHC into each of the tubes labelled A - E, 1SC.	
71	Swirl gently to mix.	
72	Pipette 1 mL of SHB into each of the tubes labelled A - E, 1SB.	
73	Swirl gently to mix.	
74	Pipette 1 mL of SHA into each of the tubes labelled A - E, 1SA.	
75	Swirl gently to mix. • Note: All the samples must be immersed into the LTB. If not, use an inoculating loop to carefully immerse.	
76	Repeat for triplicate if necessary. • steps 67 - 75	
	Presumptive phase (section 12.3.1.4)	
77	Incubate all the unspiked and spiked LTB tubes at 35°C for 24 ± 2 hours.	
Day 4		
78	Remove LTB and EC media out of fridge and allow to warm up to room temperature (1 - 1.5 hours).	
	Spiking Suspension Enumeration (section 15.4.6)	
79	Take non-selective plated media out of the incubator and count colonies between 30 & 300. • below 30: TFTC (too few to count) • greater than 300: TNTC (too numerous to count)	
80	Record counts.	
	Confirmation Phase	
81	Remove LTB control from incubator and read results. • Positive (*E. coli* ATCC 25922): Turbid appearance (growth) with gas in durham tube. • Negative (*P. aeruginosa* ATCC 27853): Clear appearance (no growth) with no gas in durham tube.	
82	Remove all LTB tests from incubator, read, & record results. • See results and Interpretation below (step 83). • if there are negative samples, new LTB controls need to be set up.	
83	If	Then

Step	Action	
	Growth & Gas	Positive for Fecal, write a + on the cap, & proceed to step 84 (confirmation phase).
	Growth & No Gas	Put back in incubator for another 24 hours.
	Gas & No Growth	Put back in incubator for another 24 hours.
	No Growth & No Gas	Put back in incubator for another 24 hours.
	Confirmation Phase	
84	Label the EC tubes exactly as the positive LTB tubes are labelled.	
85	Transfer a small amount of sample from the LTB tube to the matching EC tube using an inoculating loop.	
86	Repeat step 85 for all remaining positive LTB tubes.	
87	Inoculate the positive and negative controls for EC. • *E. coli* ATCC 25922 positive control • *Enterobacter aerogenes* ATCC 13048 negative control	
88	Incubate all EC tubes at 44.5°C for 24 ± 2 hours.	
Day 5		
89	Remove EC media out of fridge and allow to warm up to room temperature (1 - 1.5 hours).	
90	Remove the EC & LTB controls from the incubator and read results. • Positive EC (*E. coli* ATCC 25922): Turbid appearance (growth) with gas in durham tube. • Negative EC (*E. aerogenes* ATCC 13048): Clear appearance (no growth) with no gas in durham tube. • Positive LTB (*E. coli* ATCC 25922): Turbid appearance (growth) with gas in durham tube. • Negative LTB (*P. aeruginosa* ATCC 27853): Clear appearance (no growth) with no gas in durham tube.	
91	Remove LTB tubes from incubator and read results. • See results and Interpretation below (step 92) • New EC controls need to be set up if there are any positive LTB tubes (see step 87)	
92	If	Then
	Growth & Gas	Positive for Fecal, write a + on the cap, & proceed to step 112 (confirmation phase).

Step	Action	
	Growth & No Gas	Negative for fecal, write a - on the cap, samples will NOT be processed further.
	Gas & No Growth	Negative for fecal, write a - on the cap, samples will NOT be processed further.
	No Growth & No Gas	Negative for fecal, write a - on the cap, samples will NOT be processed further.
93	Repeat steps 84 - 88 for remaining positive LTB tubes.	
94	Remove the EC tubes from the incubator from the previous day and read results. • See Results and Interpretation below (step 95)	
95	If	Then
	Growth & Gas	Confirmed positive for fecal.
	Growth & No Gas	Negative, LTB positive result was false.
	Gas & No Growth	Negative, LTB positive result was false.
	No Growth & No Gas	Negative, LTB positive result was false.
Day 6		
96	Remove all the remaining EC from the incubator and read results. • including controls (see step 90) • See Results and Interpretation below. • See step 88.	
97	Proceed to Data Analysis and Calculations (section 14.0)	

4. Labeling

4.1. Media Labelling per Sample

Media	**Number of tubes**	**Label**
Tube Media		
Double strength LTB (2x) - 10 mL	5	A - E, 10U
Single strength LTB (1x) - 10 mL	5	A - E, 1U
Single strength LTB (1x) -10 mL	5	A - E, 1UA
Single strength LTB (1x) - 10 mL	5	A - E, 1UB
Single strength LTB (1x) - 10 mL	5	**A - E, 1SA**
Single strength LTB (1x) - 10 mL	5	**A - E, 1SB**
Single strength LTB (1x) - 10 mL	5	**A - E, 1SC**
Single strength LTB (1x) - 10 mL	5	**A - E, 1SD**
Single strength LTB (1x) - 10 mL	2 - 4	2 each of *E. coli* & *P. aeruginosa*
EC - 10 mL	2 - 4	2 each of *E. coli* & *E. aerogenes*
EC - 10 mL	Up to 40	Do not label until needed
Plate Media		
Non-selective plated media	3	SSC1 - 3, SSD1 - 3, SSE1 - 3
Sheep blood plates	3	*E. coli, P. aeruginosa, E. aerogenes*

Note:

- U = unspiked, 1UA = 1 mL unspiked dilution A, S = spiked, 1SA = 1 mL spiked dilution A
- SS = spiking suspension enumeration, SSC1 = SS dilution C triplicate 1

4.2. Ware's Labelling per Sample

Ware	Number	Label
100 mL vessel	2	HA-2, HB-3
100 mL vessel	5	SSA, SSB, SSC, SSD, SSE
100 mL vessel	4	SHA, SHB, SHC, SHD
100 mL vessel	1	USS (*E. coli*)
500 mL bottle or flask (or 1 L)	1	Unspiked Homogenized Sample

Note:

- HA = homogenized dilution A
- SSA = spiking suspension dilution A
- SHA = spiked homogenized dilution A
- USS = undiluted spiking suspension

5. Calculations:

Spiking calculations:

For every 100 mL of sample remaining, add in 1 mL of the spiking suspension B.

a. Started with 300 mL - 11 mL for dilutions - 55 mL (165 mL for triplicate) for inoculating LTB = 234 mL (124 mL for triplicate) (± pH solution, not included in calculations)
b. ≈ 2.34 mL of spiking suspension B
c. ≈ 1.24 mL of spiking suspension B for triplicate.

6. LTB & EC tube test interpretation

- gas production in durham tube & turbid appearance = positive sample
- gas production only = negative sample
- turbidity only = negative sample
- clear & no gas production = negative sample

A.20 **Detecting *Salmonella* in Biosolids Compost Matrix (USEPA Method 1682)**

1. Materials:

Equipment	Supplies	Media/Reagents
Incubators • 35°C • 42°C Vortex Magnetic stirrer Analytical scale BSC Blender(s) • 1 per sample or ability to sterilize between sample uses. Autoclave pH meter Used tip crock Crock with 10% bleach Biosafety bin	100 mL vessels with lids Inoculating loops Pipet and tips • 1000 µl ART • wide bore and regular Serological pipet (25, 10, & 1 mL) Pipette aid Test tubes Test tube racks Stir bars 500 mL bottles or flasks Large tray Measuring scoops (if making media) Glass spreaders (sterile) Transfer pipettes Colony picks Metal weigh boats	TSB broth • triple strength • single strength Non-selective plated media • eg. HIA, SPC, TSB MSRV plated media XLD plated media TSI, LIA, Urea tube media Ice Sterile phosphate buffered water Precept or 10% bleach HCl/NaOH (1N)

2. Specimen: *Escherichia coli* ATCC 25922, *Salmonella meleagridis,* & *Proteus vulgaris* ATCC 13315

3. Procedure:

Step	Action	
1	Request organisms from QC on blood plate.	
	If	Then
	QC has organism(s)	Proceed to step 2
	QC does not have organism(s)	Proceed to step 3
2	Place organisms in fridge (4°C) and proceed to step 5 procedures.	
3	Order specimen(s) from ATCC through Cedarlane Laboratories.	
4	Rehydrate organisms according to instructions from supplier (ATCC). • Open cryovial. • Rehydrate with 500 µL of selective broth. • Pipette up and down to mix well. • Pipette 10 mL of selective broth into a sterile test tubes. • Transfer 450 µL of the content of cryovial into the test tube. • Plate remaining inoculum on a blood plate. • Incubate overnight at recommended temperature. • Skim liquid stock culture and freeze at -70°C • Place blood plate in fridge (4°C)	
Day 1	**Two days prior to samples arriving.**	
	Remove organisms (isolated on blood plates) from fridge and allow to warm up to room temperature.	
5	Remove sterile blood plates from fridge and allow to warm up to room temperature (1 - 1.5 hours) prior to use.	
6	Label the bottom of 2 blood plates with *Escherichia coli* ATCC 25922, and *Salmonella meleagridis*.	
7	Streak to isolation *E. coli* ATCC 25922 on the blood plate labelled as so. • See culturing SOP - plating, steps 9 - 17	
8	Repeat step 7 for *Salmonella meleagridis*.	
9	Invert plates and incubate at 35° C for 24 ± 2 hours.	
Day 2	**One day prior to samples arriving.**	
10	Take blood plates out of the incubator and confirm growth.	
11	Prepare a 1 % solution of Tryptic Soy Broth (TSB) • Section 14.2.1.2 of EPA method 1682 • Measure out 99 mL of phosphate buffered dilution water and dispense into a sterile screw cap bottle or vessel. • Add in 1 mL of TSB (single strength).	

Step	Action
	• Shake well.
12	Prepare the **undiluted spiking suspension (USS).** • Transfer a colony from the blood plate that is streaked with *Salmonella meleagridis* to 1 % TSB. • Shake minimum 25 times. • Incubate at 35°C for 20 ± 4 hours.
13	Prepare a media sterility check and a blank for the phosphate buffered dilution water. • TSB (3x & 1x), XLD, TSI, LIA, Urea, & non-selective media - 35°C for 24 ± 2 hours • MSRV - 42°C for 24 ± 2 hours • Phosphate water - 20 mL at 35°C for 24 ± 2 hours
14	Put blood plates in fridge until the next day.
15	Label all media and wares for the following day. • See table 1 below. • Note: This media and ware's count and labelling is for 1 complete sample (unspiked + spiked). If more than 1 sample is being processed, label sample 1, sample 2, etc (in addition to the rest of the labels from the tables below. If samples are being processed in triplicate, label samples a, b, c (eg. 1a - sample 1, triplicate a). The only exception is SSA - SSD vessels. Spiked sample will only be performed on one samples and/or triplicate.
Day 3	**Day samples arrive.**
16	Take all media needed for the day and allow to warm up to room temperature (1 - 1.5 hours). • non-selective media must be dry and free of condensation so may need to be put in 35°C incubator for a short period of time.
17	Remove blood plates, streaked with organisms, out of fridge and allow to warm up to room temperature.
18	Take media and phosphate water out of incubator and check for sterility.
19	Set up controls in TSB (1x and/or 3x) for MSRV controls on day 4. • inoculate TSB with *Salmonella meleagridis*

Step	Action
	• inoculate TSB with *E. coli* ATCC 25922 • incubate at 35°C for 24 ± 2 hours
	Sample spiking dilutions
20	Remove the USS out of the incubator and mix vigorously.
21	Shake the USS (*S. meleagridis*) a minimum of 25 times to mix well.
22	Pipette 99 mL's of phosphate buffer water into each of the vessels labelled S A-D
23	Pipette 1 mL of the USS into vessel SA and shake well. • 1 mL of SA is 10^{-2} of the USS
24	Pipette 1 mL of SA into SB, shake well. • 1 mL of SB is 10^{-4} of the USS
25	Pipette 11 mL of SB into SC, shake well. • 1 mL of SC is 10^{-5} of the USS
26	Pipette 11 mL of SC into SD, shake well. • 1 mL of SD is 10^{-6} of the USS
27	Set vessels aside (on ice) until ready to spike.
	Sample preparation and homogenization
28	Weigh out 30 g of the sample and pour into blender.
29	Add 270 mL of phosphate buffered water to the sample in the blender.
30	Blend for 1 minute.
31	Transfer the entire content to a 500 mL bottle or flask (labelled unspiked, homogenized sample) containing a sterile stir bar.
32	Place on magnetic stirrer and adjust pH to 7.0 - 7.5 with either 1.0N HCl or 1.0N NaOH. • Do not use more than 5% of HCl or NaOH to pH sample. • This is the **homogenized sample**.
33	Repeat steps 28 - 32 for remaining samples (unspiked). • repeat one more time and pour contents into bottle or flask labeled spiked, homogenized sample.
	Spike samples
28	Pour spiked, homogenized sample back in blender.

Step	Action
29	Mix vessel SSD well.
30	Pipette out 500 µL of SSD into spiked, homogenized sample.
31	Blend for 1 - 2 minutes.
32	Pour sample back into 500 mL bottle or flask, labelled accordingly. • spiked, homogenized sample
	Inoculate samples into TSB tubes (section 11.2.2 and 12.2)
33	Line up all the TSB tubes needed for the sample in a test tube holder
34	Mix the corresponding homogenized sample well
35	Pipette out 20 mL of homogenized sample and dispense into TSB tube (10 mL) labelled unspiked A20. • this is 3x TSB
36	Repeat step 35 for unspiked B - E 20.
37	Pipette out 10 mL of homogenized sample and dispense into TSB tube (5 mL) labelled unspiked A10. • this is 3x TSB
38	Repeat step 37 for unspiked B - E 10.
39	Pipette out 1 mL of homogenized sample and dispense into TSB tube (10 mL) labelled unspiked A1. • this is 1x TSB
40	Repeat step 39 for unspiked B - E 1.
41	Repeat steps 33 - 40 for all unspiked samples and/or triplicates.
42	Repeat steps 33 - 40 for spiked homogenized samples and/or triplicates.
43	Incubate all TSB samples at 35°C for 24 ± 2 hours.
	Enumeration of spiking solution (section 14.4)
44	Line up the dry, room temperature non-selective plated media.
45	Shake the vessel labelled SD well.
46	Pipette 100 µL (10^{-7} of USS) onto each of the 3 plates labelled SD 1 - 3 .
47	Spread inoculum by whole plate method and set plates aside so the inoculum can completely absorb into the agar. • See culturing SOP - plating (step 6) for further instruction on how to whole plate spread.
48	Repeat steps 45 - 47 for SC (10^{-6} of USS) and SB (10^{-5} of USS).
49	Invert plates and incubate at 35°C for 24 ± 2 hours.

Step	Action	
Day 4		
50	Take MSRV out of fridge and allow to warm up to room temperature (about 1 - 1.5 hours). MSRV must be dry and free of condensation.	
	Enumeration of spiking solution	
51	Take HIA plates out of the incubator and count colonies between 30 & 300. • below 30: TFTC (too few to count) • greater than 300: TNTC (too numerous to count)	
52	Record counts.	
53	Remove TSB tubes from incubator (including controls) and check for turbidity. • both controls should be turbid as they are not TSB controls but MSRV controls.	
	If	Then
	Samples are turbid	Write an + on the top of the tube. Samples will be processed further.
	Samples are not turbid	Write an - on the top of the tube. Samples will not be processed further.
	Proceed to step 54.	
54	Record results from step 53.	
55	Proceed to selection phase.	
	Selection phase	
56	Set up controls for the MSRV plates (Positive - *Salmonella meleagridis* and negative - *E. coli*) • Using the TSB controls set up the day before, inoculate, using a clean sterile transfer pipette, the negative control plate with 3 - 6 drops of *E. coli* (spaced evenly around the plate). • Inoculate the positive control plate the same, using the *Salmonella*. • Use a new transfer pipette for each sample	
57	Allow inoculum to absorb into the media (approximately 1 hour) in the dark.	
58	Repeat steps 56 & 57 for all the turbid TSB samples (positive). • plates will be labeled along the top of petri dish (along the	

Step	Action	
	sides) and labeled exactly as the corresponding TSB tubes.	
59	Incubate all MSRV plates in a humidity controlled incubator at 42°C for 16 - 18 hours. • Do NOT invert plates. • if humidity controlled incubator is not available, place a pan of water in the incubator as a replacement	
Day 5		
60	Remove the XLD plates from the fridge and allow to warm up to room temperature. XLD plates must be dry and free of condensation.	
61	Remove all MSRV plates from incubator.	
62	Read the controls and the samples. • *Salmonella* spp. produces halos on MSRV media which indicates motility. • record results	
63	If	Then
	Whitish halo is seen	Positive for *Salmonella*, proceed to XLD streaking
	No whitish halo is seen	Negative for *Salmonella*.
	Selection phase continued	
66	Label 2 XLD plates for every 1 positive MSRV plate. Label exactly as the MSRV plates, adding in plate 1 & 2.	
67	Stab into a halo from the outer edge of the target positive colony using an inoculating loop.	
68	Streak to isolation on the corresponding XLD plates. • See culturing SOP - plating, steps 9 - 17.	
69	Repeat steps 67 & 68 for remaining positive MSRV plates and the controls. • Plate only one plate for each control (*Escherichia coli & Salmonella meleagridis*) • Include an extra plate control which is plated with *Proteus vulgaris*	
70	Invert plates and incubate at 35°C for 18 - 24 hours.	
71	Place all MSRV media in fridge (4°C) until the next day.	
Day 6	**Biochemical confirmation**	
72	Remove the XLD sample plates and controls from the incubator.	

Step	Action
73	Read and record the results of the controls. • see step 74 for interpretation
74	Read and record the results of the sample plates • Typical colonies - pink to red with black centers (H_2S positive) • Atypical colonies - translucent pink to red (H_2S negative) • Negative - other than above.
75	Take TSI, LIA, and Urea slants out of fridge and allow to warm up to room temperature prior to use. • 1 - 1.5 hours
	Label the LIA, TSI, & Urea control tubes as follows: • LIA positive - *Salmonella meleagridis* • LIA positive - *Proteus vulgaris* • LIA negative - *Escherichia coli* • TSI positive - *Salmonella meleagridis* • TSI positive - *Proteus vulgaris* • TSI negative - *Escherichia coli* • Urea positive - *Proteus vulgaris* • Urea positive - *Escherichia coli* • Urea negative - *Salmonella meleagridis*
76	Pick the center of a well isolated pure colony of *Salmonella melaegridis* using an inoculating pick.
77	Inoculate the LIA slant by piercing the butt of the slant, twice, to the bottom of the tube, then streaking up the slant in a side to side motion. This the positive control.
78	Repeat steps 76 & 77 for the other positive control (*Proteus vulgaris* ATCC 13315) using a new inoculating pick.
79	Repeat steps 76 & 77 for the negative control (*Escherichia coli* ATCC 25922) using a new inoculating pick.
80	Pick the center of a well isolated pure colony of *Salmonella melaegridis* using an inoculating pick.
81	Inoculate the TSI slant by piercing the butt of the slant 3/4 of the way to the bottom of the tube, then streaking the slant in a side to side motion. Label this the positive control.
82	Repeat steps 80 & 81 for the other positive control (*Proteus vulgaris* ATCC 13315) using a new inoculating pick.
83	Repeat steps 80 & 81 for the negative control (*Escherichia coli*

Step	Action
	ATCC 25922) using a new inoculating pick.
84	Pick the center of a well isolated pure colony of *Proteus vulgaris* ATCC 13315 using an inoculating pick.
85	Inoculate the Urea slant by streaking the surface using a side to side motion. Label this the positive control.
86	Repeat steps 84 & 85 for the other positive control (*Escherichia coli* ATCC 25922) using a new inoculating pick.
87	Repeat steps 84 & 85 for the negative control (*Salmonella melaegridis*) using a new inoculating pick.
88	Set controls aside.
89	Sort the XLD plates with suspect *Salmonella* (positive) from those that appear negative.
90	Line up a LIA tube, a TSI tube, & an Urea tube.
91	Label all tubes exactly the same as the first positive XLD plate. • Do not need to run the duplicate XLD plate • Label exactly as XLD plate, noting if the colony came from plate 1 or 2.
92	Find an isolated colony on the plate, this colony will be used to inoculate all 3 slants.
93	Pick from the center of the colony and inoculate the LIA slant according to step 77.
94	Using the same inoculating pick and the same colony, inoculate the TSI slant according to step 81.
95	Using the same inoculating pick and the same colony, inoculate the Urea slant according to step 85.
96	Dispose of inoculating pick and set the 3 tubes aside.
97	Repeat steps 90 - 96 for remaining samples.
98	Incubate all tubes for 24 ± 2 hours at 35°C.
99	Put all XLD plates in fridge until next day.
Day 7	**Reading biochemical confirmation tests**
100	Take tubes out of incubator for reading.
	LIA
101	Read the controls. • Positive - purple slant with purple or black butt • Negative - any other color combination (see table 1 below)
102	Read all the LIA sample tubes and use controls/table 2 below as comparisons.
103	Mark the top of the positive samples with a '+'.
	TSI

Step	Action
104	Read the controls. • Positive - red slant with yellow or black butt (with/out gas production) • Negative - any other color combination (see table 2 below)
105	Read all the TSI sample tubes and use controls/table 2 below as comparisons.
106	Mark the top of the positive samples with a '+'.
	Urea
107	Read the controls. • Positive - any growth or color change • Negative - no color change or growth to slant or butt
108	Read all the Urea sample tubes and use controls/table 2 below as comparisons.
109	Mark the tops of the positive samples with a '+'.
110	Record all results.
111	Dispose of all contaminated materials according to biohazard specifications.

4. Media and ware's labelling per sample.

Media	Amount needed	Label
TSB (3x) 10 mL	5	Unspiked, A – E, 20
TSB (3x) 5 mL	5	Unspiked, A – E, 10
TSB (1x) 10 mL	5	Unspiked, A – E, 1
TSB (3x) 10 mL	5	**Spiked, A – E, 20**
TSB (3x) 5 mL	5	**Spiked, A – E, 10**
TSB (1x) 10 mL	5	**Spiked, A – E, 1**
TSB (1x) 10 mL	2	1 - *E. coli,*1 - *Salmonella*
Non-selective plated media	9	3 – B(-5), 3 – C(-6), 3 – D(-7)
500 mL bottles	2	**1 – spiked**, 1 – unspiked
Vessels	4	SS A - D

Note: Perform spiking on one sample (or in triplicate if triplicates are performed).

5. Color indicator of LIA, TSI, and Urea biochemical confirmation tests.

Color change	Positive/Negative	Likely Organism
LIA indicator		
purple slant (alkaline) with purple butt (alkaline) with or without H_2S production (blackening)	Positive	*Salmonella* spp.
red slant with yellow butt (acid)	Positive	*Proteus* spp.
purple slant (alkaline) with yellow butt (acid) or neutral butt (no change)	Negative	*E. coli*
TSI indicator		
red slant(alkaline) with yellow (acid) or black butt (H_2S production) with/out gas production	Positive	*Salmonella* spp.
red slant (alkaline) with yellow (acid) or black butt (H_2S production) with/out gas production	Positive	*Proteus* spp.
yellow (acid) slant with yellow (acid) butt, with gas production without H_2S production	Negative	*E. coli*
Red (alkaline) slant without color change on butt, no gas or H_2S production	Negative	*Pseudomonas aeruginosa*
Urea indicator		
growth and intense pink color change	Positive	*Proteus* spp.
growth and yellow or no color change	Positive	*E. coli*
No growth or color change	Negative	*Salmonella* spp.

6. Results table for biochemical confirmation test

Sample ID	LIA	TSI	Urea	Salmonella Confirmation
1	+	+	+	No
2	+	+	-	Yes
3	+	-	-	No
4	+	-	+	No
5	-	-	+	No
6	-	-	-	No

A.21 **Initial Precision and Recovery (for USEPA Method 1680 & 1682)**

1. Purpose:

The purpose of the IPR is to demonstrate precision and recovery within the acceptable range. The acceptable range is 0 - 254 % for mean percent recovery and 92% (as maximum relative standard deviation) for precision.

2. Materials:

Equipment	**Supplies**	**Media/Reagents**
Incubators • 35°C • 42°C • 44.5°C Vortex Magnetic stirrer Analytical scale BSC Blenders • 2 minimum Autoclave pH meter Used tip crock Crock with 10% bleach Biosafety bin Drying oven • 103 - 105°C	100 mL vessels with lids Inoculating loops Pipet and tips • 1000 µl ART • wide bore and regular Serological pipet (25, 10, & 1 mL) Pipette aid Test tubes Test tube racks Stir bars 500 mL bottles or flasks Large tray Measuring scoops (if making media) Glass spreaders (sterile) Transfer pipettes Colony picks Metal weigh boats (can go in drying oven)	TSB broth • triple strength • single strength LTB with durham tube - single strength EC with durham tube Sheep blood agar plates Non-selective plated media • eg. HIA, SPC, TSB MSRV plated media XLD plated media TSI, LIA, Urea tubed media Milorganite or equivalent Ice Sterile phosphate buffered water Precept or 10% bleach

3. **Specimen:** *Escherichia coli* ATCC 25922, *Salmonella meleagridis, Pseudomonas aeruginosa* ATCC 27853*, Enterobacter aerogenes* ATCC 13048*, &* *Proteus vulgaris* ATCC 13315**,** Media blank in the incubator to check for sterility

4. **Procedure:**

Step	Action	
1	Request organisms from QC on blood plate.	
	If	Then
	QC has organism(s)	Proceed to step 2
	QC does not have organism(s)	Proceed to step 3
2	Place organisms in fridge (4°C) and proceed to step 5 procedures.	
3	Order specimen(s) from ATCC.	
4	Rehydrate organisms according to instructions from supplier (ATCC). • Open cryovial. • Rehydrate with 500 µL of selective broth. • Pipette up and down to mix well. • Pipette 10 mL of selective broth into a sterile test tubes. • Transfer 450 µL of the content of cryovial into the test tube. • Plate remaining inoculum on a blood plate. • Incubate overnight at recommended temperature. • Skim liquid stock culture and freeze at -70°C • Place blood plate in fridge (4°C)	
Day 1		
	Setting up the stock cultures.	
5	Remove organisms (isolated on blood plates) from fridge and allow to warm up to room temperature.	
6	Remove blood plates from fridge and allow to warm up to room temperature (1 - 1.5 hours) prior to use.	
7	Label the bottom of 5 blood plates with date & *Escherichia coli* ATCC 25922, *Salmonella meleagridis, Pseudomonas aeruginosa* ATCC 27853, *Enterobacter aerogenes* ATCC 13048, & *Proteus vulgaris* ATCC 13315.	
8	Streak to isolation *Escherichia coli* on the blood plate labelled as so. • See culturing SOP - plating, steps 9 - 17.	

Step	Action
	• This is the **stock culture** for method 1680.
9	Repeat step 8 for *Salmonella meleagridis*. • This is the **stock culture** for method 1682.
10	Repeat step 8 for *Pseudomonas aeruginosa* ATCC 27853, *Enterobacter aerogenes* ATCC 13048, & *Proteus vulgaris* ATCC 13315. • These cultures are needed as controls.
11	Invert plates and incubate at 35 ° C for 20 ± 4 hours.
12	Prepare media sterility checks and a blank for the phosphate buffered dilution water. • LTB - 35°C for 48 ± 3 hours (at least 24 hours) • EC - 44.5°C for 24 ± 2 hours • TSB, XLD, LIA, TSI, & Urea - 35°C for 24 ± 2 hours • MSRV - 42°C for 16 - 18 hours • Phosphate water (20mL) - 35°C for 24 ± 2 hours
Day 2	
	Preparing the undiluted spiking suspension.
13	Remove blood plates from incubator and check for growth.
14	Prepare 1 % solution of Lauryl Tryptose Broth (LTB) • EPA method 1680. • Measure out 99 mL of phosphate buffered dilution water and dispense into a sterile screw cap bottle or vessel. • Add in 1 mL of LTB (single strength). • Shake well.
15	Prepare the **undiluted spiking suspension (USS) for method 1680.** • Transfer a small loopful of the ***E. coli*** **ATCC 25922 stock culture** to 1 % LTB (≈ 10 µL). • Shake minimum 25 times. • Incubate at 35°C for 20 ± 4 hours.
16	Prepare 1 % solution of Tryptic Soy Broth (TSB) • EPA method 1682. • Measure out 99 mL of phosphate buffered dilution water and dispense into a sterile screw cap bottle or vessel.

Step	Action
	• Add in 1 mL of TSB (single strength). • Shake well.
17	Prepare the **undiluted spiking suspension (USS) for method 1682.** • Transfer a small loopful of the ***Salmonella meleagridis*** **stock culture** to 1 % TSB (≈ 10 µL). • Shake minimum 25 times. • Incubate at 35°C for 20 ± 4 hours.
18	Check media and water sterility tests.
19	Place the 5 blood plates streaked with organisms at 4°C.
20	Label all media and wares for the following day. • See table 1, table 2, and table 3 below. • Note: This media and ware's count and labeling is for 1 complete sample. • IPR requires 4 samples to be processed for both 1680 & 1682 therefore labeling should also be sample 1, sample 2, etc.
Day 3	
	Prepare Milorganite for spiking.
21	Weight out 30 grams of sample into a blender.
22	Add 270 mL of phosphate buffered water to blender.
23	Blend for 1 minute.
24	Transfer the entire content to a 500 mL bottle or flask (labelled spiked homogenized sample) containing a sterile stir bar.
25	Rinse out blender with distilled water.
26	Place on magnetic stirrer and adjust pH to 7.0 - 7.5 with either 1.0N HCl or 1.0N NaOH if needed. • Do not use more than 5% of HCl or NaOH to pH sample. • This is the **homogenized sample**.
27	Repeat steps 21 - 26 for additional samples. • should have 4 samples for 1680 and 4 samples for 1682. • should also have 1 extra sample weighed out (10 - 30 g) for drying (calculation of dry weight at end). Do not re-suspend in buffered water (see step 103)
28	Fill large tray with ice and set samples in ice to keep cool.
	Making the spiking solution for 1680 (*E. coli*).
29	Shake the USS (*E. coli*) a minimum of 25 times to mix well.
30	Pipette 99 mL's of phosphate buffer water into each of the vessels

Step	Action
	labelled SS A-E
31	Pipette 1 mL of the USS into vessel SS-A and shake well. • 1 mL of SS-A is 10^{-2} of the USS
32	Pipette 11 mL of SS-A into SS-B, shake well. • 1 mL of SS-B is 10^{-3} of the USS
33	Pipette 11 mL of SS-B into SS-C, shake well. • 1 mL of SS-C is 10^{-4} of the USS
34	Pipette 11 mL of SS-C into SS-D, shake well. • 1 mL of SS-D is 10^{-5} of the USS
35	Pipette 11 mL of SS-D into SS-E, shake well. • 1 mL of SS-E is 10^{-6} of the USS
	Spiking suspension enumeration for 1680 (*E. coli*).
36	Line up the dry, room temperature non-selective plates.
37	Shake the vessel labelled SS-E well.
38	Pipette 100 µL (10^{-7} of the USS) onto each of the 3 plates labelled SS-E, 1 - 3.
39	Whole plate spread and set plates aside to the inoculum can completely absorb into the agar. • See culturing SOP - plating (step 6) for further instruction on how to whole plate spread.
40	Repeat steps 37 - 39 for SS-D (10^{-6} of the USS) and SS-C (10^{-5} of the USS).
41	Invert plates and incubate at 35°C for 24 ± 4 hours.
	Making the spiking suspension for 1682 (*S. meleagridis*).
42	Shake the USS (*S. meleagridis*) a minimum of 25 times to mix well.
43	Pipette 99 mL's of phosphate buffer water into each of the vessels labelled S A-D
44	Pipette 1 mL of the USS into vessel SA and shake well. • 1 mL of SA is 10^{-2} of the USS
45	Pipette 1 mL of SA into SB, shake well.

Step	Action
	• 1 mL of SB is 10^{-4} of the USS
46	Pipette 11 mL of SB into SC, shake well. • 1 mL of SC is 10^{-5} of the USS
47	Pipette 11 mL of SC into SD, shake well. • 1 mL of SD is 10^{-6} of the USS
	Spiking suspension enumeration for 1682 (*S. meleagridis*).
48	Line up the dry, room temperature HIA plates.
49	Shake the vessel labelled SD well.
50	Pipette 100 µL (10^{-7} of USS) onto each of the 3 plates labelled SD 1 - 3 .
51	Whole plate spread and set plates aside to the inoculum can completely absorb into the agar. • See culturing SOP - plating (step 6) for further instruction on how to whole plate spread.
52	Repeat steps 49 - 51 for SC (10^{-6} of USS) and SB (10^{-5} of USS).
53	Invert plates and incubate at 35°C for 24 ± 4 hours.
	Spiking Milorganite for 1680 (*E. coli*).
54	Place first sample (with stir bar) on magnetic plate and turn on stir function.
55	Spike the sample with 3 mL of well mixed spiking suspension B. • 1 mL spiking suspension for every 100 mL sample.
56	Stir on high speed for approximately 5 minutes. This is the **spiked homogenized sample.**
57	Pipette 99 mL of phosphate buffered water into each of the vessels labelled SH A - D.
58	Pipette 11 mL of the **spiked homogenized sample** into vessel SHA and mix well.
59	Pipette 11 mL of SHA into SHB, mix well.
60	Pipette 11 mL of SHB into SHC, mix well.
61	Pipette 11 mL of SHC into SHD, mix well.
62	Place spiked sample back on ice.
63	Repeat steps 54 - 62 for all 1680 samples (4 samples + 4 dilutions each = 16 total).
	Spiking Molorganite for 1682 (*S. meleagridis*).
64	Place first sample (with stir bar) on magnetic plate and turn on stir function.

Step	Action
65	Pipette 500 µL (0.5 mL) from a well mixed SD into sample.
66	Stir on high speed for approximately 5 minutes. This is the **spiked sample.**
67	Place spiked sample back on ice.
68	Repeat steps 64 - 67 for all 1682 samples (4 samples total).
	Inoculating samples for 1680 (*E. coli*).
69	Line up all the LTB tubes needed to process the first sample. • sample 1
70	Pipette 1 mL of SHD into the tube labeled A-1SHD (sample 1).
71	Swirl gently to mix. • Do not allow air up into the durham tube while swirling
72	Repeat steps 70 & 71 for samples (B-E)-1SHD (sample 1).
73	Dispose of tip in used tip crock.
74	Pipette 1 mL of SHC into the tube labeled A-1SHC (sample 1).
75	Swirl gently to mix. • Do not allow air up into the durham tube while swirling
76	Repeat steps 74 & 75 for samples (B-E)-1SHC (sample 1).
77	Dispose of tip in used tip crock.
78	Pipette 1 mL of SHB into the tube labeled A-1SHB (sample 1).
79	Swirl gently to mix. • Do not allow air up into the durham tube while swirling
80	Repeat steps 78 & 79 for samples (B-E)-1SHB (sample 1).
81	Dispose of tip in used tip crock.
82	Pipette 1 mL of SHA into the tube labeled A-1SHA (sample 1).
83	Swirl gently to mix. • Do not allow air up into the durham tube while swirling
84	Repeat steps 72 & 83 for samples (B-E)-1SHA (sample 1).
85	Repeat steps 69 - 88 for remaining samples. • samples 2, 3, & 4 • Note: All the samples must be immersed into the LTB. If not, use an inoculating loop to carefully immerse.
	Inoculating samples for 1682 (*S. meleagridis*).
86	Line up all the TSB tubes needed for the first sample in a test tube

Step	Action
	holder. • sample 1
87	Mix the first sample well.
88	Pipette out 20 mL of the sample and put into the test tube labeled A20 (sample 1) • use a 25 mL serological pipette
89	Repeat step 88 for B - E 20 (sample 1)
90	Dispose of serological pipette in biohazard bin.
91	Pipette out 10 mL of the sample and put into the test tube labeled A10 (sample 1) • use a 10 mL serological pipette
92	Repeat step 91 B - E 10 (sample 1).
93	Dispose of serological pipette in biohazard bin.
94	Pipette out 1 mL of the sample and put into the test tube labeled A1 (sample 1) • use 1 mL serological pipette or 1000P wide bore ART tip
95	Repeat step 94 for B - E 1 (sample 1)
96	Dispose of serological pipette in biohazard bin or wide bore tip in used tip crock
97	Repeat steps 86 - 96 for remaining samples. • samples 2, 3, & 4
	Controls (for use with both 1680 & 1682)
98	Inoculate the 2 LTB tubes and 2 TSB (1X) tubes with the following: • LTB - *E. coli* ATCC 25922 positive control • LTB - *P. aeruginosa* ATCC 27853 negative control • TSB - *E. coli* ATCC 25922 • TSB - *Salmonella melaegridis*
99	Incubate all the TSB and LTB tubes at 35°C for 24 ± 2 hours
100	Place all biohazardous materials in biohazard bin.
101	Put away all supplies in their designated spots.
102	Clean counter with 1000 ppm precept or 10% bleach.
103	Place final 30 g sample of Milorganite in drying oven.

Step	Action	
	• 103°C - 105°C, overnight	
Day 4		
104	Remove MSRV, LTB, and EC media from fridge and allow to warm up to room temperature.	
105	Remove all non-selective media plates from incubator, count and record colonies. • below 30: TFTC (too few to count) • greater than 300: TNTC (too numerous to count)	
	1680 results (*E. coli*)	
106	Remove LTB control from incubator and read results. • Positive (*E. coli* ATCC 25922): Turbid appearance (growth) with gas in durham tube. • Negative (*P. aeruginosa* ATCC 27853): Clear appearance (no growth) with no gas in durham tube.	
107	Remove all LTB tests from incubator, read, & record results. • See results and Interpretation below (step 108). • if there are negative samples, new LTB controls need to be set up.	
108	If	Then
	Growth & Gas	Positive for Fecal, write a + on the cap, & proceed to step 112 (confirmation phase).
	Growth & No Gas	Put back in incubator for another 24 hours.
	Gas & No Growth	Put back in incubator for another 24 hours.
	No Growth & No Gas	Put back in incubator for another 24 hours.
	1682 results (*S. meleagridis*).	
109	Remove TSB tubes from incubator (including controls) and check for turbidity. • both controls should be turbid as they are not TSB controls but MSRV controls.	
	If	Then
	Samples are turbid	Write an + on the top of the tube. Samples will be processed

Step	Action	
		further.
	Samples are not turbid	Write an - on the top of the tube. Samples will not be processed further.
	Proceed to step 110.	
110	Record results from step 109.	
111	Proceed to step 117	
	Confirmation phase for 1680 (*E. coli*).	
112	Label the EC tubes exactly as the positive LTB tubes are labelled.	
113	Transfer a small amount of sample from the LTB tube to the matching EC tube using an inoculating loop (≈ 10 µL).	
114	Repeat step 113 for all remaining positive LTB tubes.	
115	Inoculate the positive and negative controls for EC. • *E. coli* ATCC 25922 positive control • *Enterobacter aerogenes* ATCC 13048 negative control	
116	Incubate all EC tubes at 44.5°C for 24 ± 2 hours.	
	Selection Phase for 1682 (*S. meleagridis*).	
117	Set up controls for the MSRV plates (Positive - *Salmonella meleagridis* and negative - *E. coli*) • Using the TSB controls set up the day before, inoculate, using a clean sterile transfer pipette, the negative control plate with 3 - 6 drops of *E. coli* (spaced evenly around the plate). • Inoculate the positive control plate the same, using the *Salmonella*. • Use a new transfer pipette for each sample	
118	Allow inoculum to absorb into the media (approximately 1 hour) in the dark.	
119	Repeat steps 117 & 118 for all the turbid TSB samples.	
120	Incubate all MSRV plates in a humidity controlled incubator at 42°C for 16 - 18 hours. • Do NOT invert plates.	
121	Place all biohazardous materials in biohazard bin.	
122	Put away all supplies in their designated spots.	
123	Clean counter with 1000 ppm precept or 10% bleach.	
124	Remove dried Milorganite from drying oven and weigh on analytical scale.	
125	Record results.	
Day 5		

Step	Action	
126	Remove EC media and XLD plates out of fridge and allow to warm up to room temperature (1 - 1.5 hours).	
	Confirmation Phase continued (*E. coli*).	
127	Remove the EC controls from the incubator and read results. • Positive (*E. coli* ATCC 25922): Turbid appearance (growth) with gas in durham tube. • Negative (*E. aerogenes* ATCC 13048): Clear appearance (no growth) with no gas in durham tube.	
128	Remove LTB control from incubator and read results. • Positive (*E. coli* ATCC 25922): Turbid appearance (growth) with gas in durham tube. • Negative (*P. aeruginosa* ATCC 27853): Clear appearance (no growth) with no gas in durham tube.	
129	Remove all LTB tests from incubator, read, & record results. • See results and Interpretation below (step 130)	
130	If	Then
	Growth & Gas	Positive for Fecal, write a + on the cap, & proceed to step 112 (confirmation phase).
	Growth & No Gas	Negative for fecal, write a - on the cap, samples will NOT be processed further.
	Gas & No Growth	Negative for fecal, write a - on the cap, samples will NOT be processed further.
	No Growth & No Gas	Negative for fecal, write a - on the cap, samples will NOT be processed further.
131	Remove the EC tubes from the incubator from the previous day, read & record results. • See Results and Interpretation below (step 132).	
132	If	Then
	Growth & Gas	Confirmed positive for fecal.
	Growth & No Gas	Negative, LTB positive result was false.
	Gas & No Growth	Negative, LTB positive result was false.

Step	Action	
	No Growth & No Gas	Negative, LTB positive result was false.
	Confirmation Phase for 1682 (*S. meleagridis*).	
133	Remove all MSRV plates from the incubator	
134	Read and record results of the controls. • *Salmonella* spp. produces halos on MSRV media which indicates motility.	
135	Read and record results of MSRV sample plates.	
136	Label 2 XLD plates for every 1 positive MSRV plate. Label exactly as the MSRV plates, adding in plate 1 & 2.	
137	Stab into a halo from the outer edge of the target positive colony using an inoculating loop.	
138	Streak to isolation on the corresponding XLD plates. • See culturing SOP - plating, steps 9 - 17.	
139	Repeat steps 136 - 138 for remaining positive MSRV plates and the controls. • Plate only one plate for each control (*Escherichia coli & Salmonella meleagridis*) • Include an extra plate which is plated with *Proteus vulgaris*	
140	Invert plates and incubate at 35°C for 18 - 24 hours.	
141	Place all MSRV media in fridge (4°C) until the next day.	
142	Place all biohazardous materials in biohazard bin.	
143	Put away all supplies in their designated spots.	
144	Clean counter with 1000 ppm precept or 10% bleach.	
Day 6		
	Biochemical confirmation (*S. meleagridis*).	
145	Remove LIA, Urea, & TSI tubes from the fridge and allow to warm up to room temperature.	
146	Remove the XLD sample plates and controls from the incubator.	
147	Read and record the results of the controls. • See step 148 for interpretation	
148	Read and record the results of the sample plates • Typical colonies - pink to red with black centers (H_2S positive) • Atypical colonies - translucent pink to red (H_2S negative) • Negative - other than above.	

Step	Action
149	Label the LIA, TSI, & Urea control tubes as follows: • LIA positive - *Salmonella melaegridis* • LIA positive - *Proteus vulgaris* • LIA negative - *Escherichia coli* • TSI positive - *Salmonella melaegridis* • TSI positive - *Proteus vulgaris* • TSI negative - *Escherichia coli* • Urea positive - *Proteus vulgaris* • Urea positive - *Escherichia coli* • Urea negative - *Salmonella melaegridis*
150	Pick the center of a well isolated pure colony of *Salmonella melaegridis* from the XLD control using an inoculating pick.
151	Inoculate the LIA slant by piercing the butt of the slant, twice, to the bottom of the tube, then streaking up the slant in a side to side motion. Set tube aside.
152	Repeat steps 150 & 151 for the other positive control (*Proteus vulgaris* ATCC 13315) & the negative control (*Escherichia coli* ATCC 25922) using a new inoculating pick.
153	Pick the center of a well isolated pure colony of *Salmonella melaegridis* from the XLD control using an inoculating pick.
154	Inoculate the TSI slant by piercing the butt of the slant 3/4 of the way to the bottom of the tube, then streaking the slant in a side to side motion. Set tube aside.
155	Repeat steps 153 & 154 for the other positive control (*Proteus vulgaris* ATCC 13315) & the negative control (*Escherichia coli* ATCC 25922) using a new inoculating pick.
156	Pick the center of a well isolated pure colony of *Proteus vulgaris* ATCC 13315 from the XLD plate using an inoculating pick.
157	Inoculate the Urea slant by streaking the surface using a side to side motion. Set tube aside.
158	Repeat steps 156 & 157 for the other positive control (*Escherichia coli* ATCC 25922) & the negative control (*Salmonella melaegridis*) using a new inoculating pick.
159	Label one each of LIA, TSI, & Urea tubes for each positive sample. • Do not need to run the duplicate XLD plate • Label exactly as XLD plate, noting if the colony came from plate 1 or 2.
160	Find an isolated colony on the plate, this colony will be used to inoculate all 3 slants.
161	Pick from the center of the colony and inoculate the LIA slant according to step 151.

Step	Action
162	Using the same inoculating pick and the same colony, inoculate the TSI slant according to step 154.
163	Using the same inoculating pick and the same colony, inoculate the Urea slant according to step 157.
164	Dispose of inoculating pick and set the 3 tubes aside.
165	Repeat steps 159 - 164 for remaining samples.
166	Incubate all tubes for 24 ± 2 hours at 35°C.
167	Place all biohazardous materials in biohazard bin.
168	Put away all supplies in their designated spots.
169	Clean counter with 1000 ppm precept or 10% bleach.
Day 7	
	Reading biochemical confirmation tests
170	Take all tubes out of incubator for reading.
	LIA
171	Read the controls. Positive - purple slant with purple or black butt Negative - any other color combination (see table 4 below)
172	Read all the LIA sample tubes and use controls/table 4 below as comparisons.
173	Mark the top of the positive samples with a '+'.
174	Record results on result table • see table 5 below for example result table
	TSI
175	Read the controls. Positive - red slant with yellow or black butt (with/out gas production) Negative - any other color combination (see table 4 below)
176	Read all the TSI sample tubes and use controls/table 4 below as comparisons.
177	Mark the top of the positive samples with a '+'.
178	Record results on result table • see table 5 below for example result table
	Urea
179	Read the controls. Positive - any growth or color change

Step	Action
	Negative - no color change or growth to slant or butt (table 4 below)
180	Read all the Urea sample tubes and use controls/table 4 below as comparisons.
181	Mark the tops of the positive samples with a '+'.
182	Record results on result table • see table 5 below for example result table
183	Dispose of all contaminated materials according to biohazard specifications.
184	Place all biohazardous materials in biohazard bin.
185	Put away all supplies in their designated spots.
186	Clean counter with 1000 ppm precept or 10% bleach.

5. Labeling

5.1. Ware's Labeling per Sample for 1680

Ware	Number	Label
100 mL vessel	1	USS (E. coli)
100 mL vessel	5	SSA, SSB, SSC, SSD, SSE
100 mL vessel	4	SHA, SHB, SHC, SHD
500 mL bottle or flask (or 1 L)	1	Spiked *E. coli* Sample

Note: SSA = spiking suspension dilution A, SHA = spiked homogenized dilution A, USS = undiluted spiking suspension

Note: The amounts are per sample. 4 samples will be processed for each method therefore labeling needs to reflect the number of samples.

5.2. Media Labeling per Sample for 1680

Media	Number of tubes	Label
Tube Media		
Single strength LTB (1x) - 10 mL	5	A - E, 1SHA
Single strength LTB (1x) - 10 mL	5	A - E, 1SHB
Single strength LTB (1x) - 10 mL	5	A - E, 1SHC
Single strength LTB (1x) -	5	A - E, 1SHD

10 mL		
Single strength LTB (1x) - 10 mL	2 - 4	2 each of: *E. coli, P. aeruginosa*
EC - 10 mL	2 - 4	2 each of: *E. coli, P. aeruginosa*
EC - 10 mL	Up to 20	Do not label until needed
Plate Media		
Non-selective plates	3	SS - C1, SS - C2, SS - C3
Non-selective plates	3	SS - D1, SS - D2, SS - D3
Non-selective plates	3	SS - E1, SS - E2, SS - E3
Sheep blood plates	2	*E. coli, P. aeruginosa*

Note: S = spiked, 1SA = 1 mL spiked dilution A, SS = spiking suspension enumeration, SSE - C1 = SSE dilution C triplicate 1. 4 samples will be processed therefore labelling needs to reflect the number of samples.

Note: The amounts are per sample. 4 samples will be processed for each method therefore labeling needs to reflect the number of samples.

5.3. Media and Ware's labeling per Sample for 1682.

Media	**Amount needed**	**Label**
TSB (3x) 10 mL	5	A – E, 20
TSB (3x) 5 mL	5	A – E, 10
TSB (1x) 10 mL	5	A – E, 1
TSB (1x) 10 mL	2	1 - *E. coli,*1 - *S. meleagridis*
Non-selective plates	9	3 SB(1-3), 3 SC(1-3), 3 SD(1-3)
Sheep blood plates	3	*S. meleagridis, E. coli, P. vulgaris*
MSRV plates	Up to 15 + controls	Do not label until needed
XLD plates	Up to 15 + controls	Do not label until needed
TSI slant (5 mL)	Up to 15 + controls	Do not label until needed
LIA slant (5 mL)	Up to 15 + controls	Do not label until needed
Urea broth (5 mL)	Up to 15 + controls	Do not label until needed
Ware's	**Amount needed**	**Label**
500 mL bottles	1	Spiked *S. meleagridis* sample
100 mL Vessels	4	SA, SB, SC, & SD
100 mL Vessels	1	USS (*S. meleagridis*)

Note: S = spiked, SA = spiking suspension dilution A, SB1 = spiking suspension dilution B triplicate 1, USS = undiluted spiking suspension

Note: The amounts are per sample. 4 samples will be processed for each method therefore labeling needs to reflect the number of samples.

6. Recipe:

All recipes refer to EPA Method 1680, section 7 and EPA Method 1682, section 7.

7. Calculation:

All calculations refer to EPA Method 1680, section 14 and EPA Method 1682, section 14.

A.22 PMA Treatment of Organisms (Nocker *et al.*, 2007; Liang et.al., 2011)

1. Materials:

Equipment	Supplies	Media/Reagents
BSC Centrifuge Light Source (>600 W halogen) Used tip crock	Centrifuge tubes, clear screw cap (1.5 mL) Pipette & tips(10 µL, 100 µL, 1000 µL) Ice container Aluminum foil	PBS PMA Ice

2. Specimen: Samples (organisms) to PMA treat

3. Procedure:

Step	Action
1	Label 1.5 mL clear, screw cap tubes. • one for every sample
2	Pipette 500 µL of sample into first 1.5 mL clear, screw cap tube.
3	Repeat step 1 for remaining samples.
4	Prepare pan for light exposure the following way: • Line pan with aluminum foil. • Cover aluminum foil with ice.
5	Remove PMA from -20°C freezer and allow to thaw on ice.
6	Pipette 1.25 µL of PMA into each tube that requires PMA treatment (final concentration of 50 µM).
7	Mix tubes well.
8	Place all PMA treated tubes in dark for 5 minutes, shaking occasionally.
9	Remove from dark and lay tubes on their sides in the pan with ice.
10	Turn on light source.
11	Place pan near light source so that samples are 20 cm away from light.
12	Expose samples to light for 5 minutes, shaking samples occasionally. Note: Do not allow samples to heat up.
13	Shut off light source.
14	Spin down all samples in centrifuge at highest speed for 5 minutes.
15	Remove supernatant.

Step	Action
16	Wash each pellet with 500 µL PBS.
17	Re-spin and remove supernatant.
18	Proceed to genomic extraction using the DNeasy Blood & Tissue Kit or freeze at -80°C until ready to extract.

A.23 **Extracting Genomic DNA (DNeasy, 2006)**

1. Materials:

Equipment	Supplies	Media/Reagents
Water baths/thermomixer • 56°C Microcentrifuge Vortex	Pipettes and tips • 1000 µL, 200 µL, and 20 µL Timer 1.5 mL LoBind centrifuge tubes Gloves Spin Columns Collection tubes	Ethanol (96-100%) DNeasy Blood & Tissue Kit • Buffer ATL • Proteinase K • Buffer AL • Buffer AW1 • Buffer AW2 • Buffer AE

2. Specimen: Samples PMA treated including controls

3. Procedure:

Step	Action
1	Turn on thermomixer/waterbath and set to 56°C.
2	Remove centrifuge tubes with sample pellets that were PMA treated from the -20°C freezer. • these will include the controls (pure cultures of organisms used to seed the cryovials*)* which were also PMA treated. • See PMA SOP
3	Allow to warm up to room temperature.
4	Resuspend the pellets with 180 µL of Buffer ATL.
5	Pipette in 20 µL of proteinase K and mix thoroughly by vortexing.
6	Incubate in thermocycler at 56°C for 1 hour. • if a thermocycler is not available, periodic vortexing is necessary throughout the incubation to disperse sample.
7	Remove samples from thermomixer and vortex for 15 seconds.
8	Add 200 µL of Buffer AL to each sample, vortex to mix well.
9	Add 200 µL of ethanol to each sample, vortex to mix well.
10	Label the DNeasy spin column tubes with the ID from the centrifuge tubes.
11	Place the spin column tubes on collection tubes.

Step	Action
12	Pipette in the mixture from the centrifuge tube into the corresponding spin column (include any precipitate that may have formed).
13	Centrifuge at ≥ 6000 x g for 1 minute.
14	Discard the flow through and collection tube.
15	Place spin column on new collection tube.
16	Pipette 500 µL of Buffer AW1 into each spin column.
17	Centrifuge at ≥ 6000 x g for 1 minute.
18	Discard the flow through and collection tube.
19	Place spin column on new collection tube.
20	Pipette 500 µL of Buffer AW2 into each spin column.
21	Centrifuge at 20,000 x g for 3 minute (to dry the membrane).
22	Discard the flow through and collection tube.
23	Label 1.5 mL centrifuge tubes as: • side: ID, date, DNeasy • top: ID
24	Place the spin columns on the labeled centrifuge tube.
25	Pipette 100 µL of Buffer AE directly onto the membrane.
26	Allow to incubate for 1 minute at room temperature.
27	Centrifuge at ≥ 6000 x g for 1 minute to elude the DNA from the membrane.
28	Repeat steps 25 - 27.
29	Discard the spin column.
30	The final elution volume is 200 µL, this is the DNA. • if DNA is not going to be processed using PCR/qPCR immediately, freeze DNA at ≥ -20°C.

A.24 **General qPCR Method**

1. Materials:

Equipment	**Supplies**	**Media/Reagents**
RT-PCR (ABI 7500 fast) Dead box Centrifuge (micro) Centrifuge with 96 well plate adapter Vortex QIAgility (optional)	Pipettes and tips • 1000µL, 200µL, 20µL, 2.5µL 1.5 mL LoBind microcentrifuge tubes 1.5 mL O-Ring centrifuge tubes Centrifuge tube rack PCR strip tubes & rack Gloves MicroAmp fast optical 96-well reaction plate with barcode MicroAmp Optical adhesive film Tinfoil or small dark box Cooler Ice	TaqMan Fast Universal PCR MasterMix (2x), No AmpErase UNG • Life Technologies • Cat. No. 4366073 Primers • forward • reverse Probe Nuclease free water TE buffer (optional) BSA (10mg/mL) - optional Plasmid prep standards • 500,000 copies/5µL

2. Specimen: unknown samples, plasmid prep standards

3. **Procedure A: Performing sample draft of well set up and calculations for mastermix.**

Step	Action
1	Draft out the set up of the wells on well set up and calculation sheet. See table 1: sample draft below.
2	Calculate the amount of mastermix needed to run calibrator samples on the bottom of the well set up and calculation sheet (see calculations below). • Note: 20x primer/probe mix is made in # of reactions + 10% + 10% so that there is enough primer/probe mix to add into 1.33x mastermix. • eg 38 reactions + 10% + 10% = 46 reactions (46 µL) • only 42 µL will be used (remaining will be disposed of) in the 1.33x mastermix.
3	Fill cooler halfway with ice.
4	Proceed to clean room (procedure B). • Remember to bring calculation sheet and cooler with ice

Calculations:

Master Mix set up: (step 2 from above)

Number of reaction:

unknown samples (in duplicate - 10x2)

\+ 3 no template control

\+ 15 standard curve reactions (plasmid)

= 38 reactions

\+ 10% extra needed

= 41.8 (round up) = 42 reactions worth of master mix is needed

For total number of reactions + 10% more (see step 2 above):

- Primer concentration in final reaction = 900 nM
    - 2 primers total (forward and reverse)
- Probe concentration in final reaction = 250 nM

- 1 probes total

- Make a 20x Primer/Probe mix
  - primers are 100 µM and need to be 900 nM (0.9 µM)
  - probe is 100 µM and needs to be 250 nM (0.25 µM)
  - final [primer] * 20 = x µM (.9 * 20 = 18 µM)
  - 100 µM / 18 µM = 5.56 fold dilution
  - 46 reactions / 5.56 = 8.27 µL of each primer
  - final [probe] * 20 = x µM (.25 * 20 = 5 µM)
  - 100 µM / 5 µM = 20 fold dilution
  - 46 reactions / 20 = 2.3 µL of probe
  - H_2O volume = reactions - primers (2) - probes (1)
    - 46 - (8.27 * 2) - (2.3) = 27.16 µL H_2O
- Make a 1.33x master mix (for each reaction):
  - 2x TaqMan Master Mix 10 µL
  - 20x Primer/Probe Mix 1 µL
  - BSA 0.3 µL

  } multiple by number of reactions (e.g. 42)

  - H_2O 3.7 µL
  - Total per reaction 15 µL
  - 2x TaqMan Master Mix 10 µL * 42 reactions = 420 µL
  - 20x Primer/Probe Mix 1 µL * 42 reaction = 42 µL (from above)
  - BSA 0.3 µL * 42 reactions = 12.6 µL
    - BSA final concentration of 150 µg/mL (for a 20x reaction)
    - this is optional, if not using add 0.3 µL to H_2O = 4.0 µL
  - H_2O 3.7 µL * 42 reactions = 155.4 µL
  - Total Master Mix 15 µL * 42 reactions = 630 µL
  - Mix all together in one tube, place on ice

4. **Procedure B: Preparing the primers and probes and setting up the mastermix in the clean room (Room 2B4.61 of the ProvLab).**

Step	Action	
1	Put on a fresh pair of gloves.	
2	Put all supplies needed into the deadbox. • located in cupboard next to sink • pipette's and tips, 1.5 mL tubes	
3	Place the TE buffer (optional) and nuclease free water in the dead box.	
4	If	Then
	Primers are new and need rehydrating • forward • reverse	Rehydrate primers with nuclease free water or TE buffer (see calculations below). Proceed to step 5.
	Primers have previously been rehydrated • forward • reverse	Remove frozen stock (aliquots) from primer/probe box in the -20°C freezer. Proceed to step 13.
5	Place lyophilized primers in centrifuge tube rack. • forward • reverse	
6	Pipette known amount (from calculation below) of TE buffer or nuclease free water into the tube.	
7	Gently pipette up and down a couple times to rehydrate primer.	
8	Dispose of tip in used tip crock and cap primer tube.	
9	Vortex tube briefly.	
10	Repeat steps 5 - 9 for any other primers which need rehydrating.	
11	Centrifuge primers for 3 seconds to pull any primers down that may be trapped in the cap.	
12	Aliquot primers into smaller volumes. • Label 2 - 1.5 LoBind centrifuge tubes per primer with name of primer • Aliquot 100 µL of primers into each of the tubes with the appropriate label. • The aliquoted primers will be used as working primer tubes and the master stock will not be used until these working tubes are empty or a problem arises such as contamination.	
13	Remove probes from box labeled primers/probes in -20°C freezer.	

	• Entero1Probe • Probe may have been previously aliquoted into a smaller volume (in dark tube). If so, use the aliquoted working tube first before going back into master stock. • Keep covered as probe is light sensitive
14	Place in centrifuge tube rack in dead box.
15	Remove BSA from box labeled PCR reagents in -20°C freezer.
16	Place in centrifuge tube rack in dead box.
17	Remove a 1.5 mL tube of TaqMan Fast Universal PCR MasterMix (2x), No AmpErase UNG from the box labeled PCR reagents 4°C from the 4°C fridge.
18	Place in dead box.
19	Place an empty bag for used tips and tubes on the bag rack.
20	Label the side of empty 2 - 1.5 mL LoBind centrifuge tube as primer/probe mix & 1.33x mastermix.
	Make the 20x primer/probe mix
21	Pipette known amount of nuclease free water into the 1.5 mL LoBind centrifuge tube labeled primer/probe mix (from calculations above). • 27.16 µL
22	Dispose of used tip in used tip bag.
23	Cap bottle and set aside.
24	Set pipette to volume for the primers (from calculations above). • 8.27 µL
25	Vortex both primers for 1 second each to mix them.
26	Place both primers in the microcentrifuge and briefly centrifuge to pull down any primers from the lid.
27	Pipette known amount of forward primer into the 1.5 mL LoBind centrifuge tube labeled primer/probe mix. • forward = 8.27 µL
28	Dispose of used tip in used tip bag.
29	Cap forward primer and set to side.
30	Pipette known amount of reverse primer into the 1.5 mL LoBind centrifuge tube labeled primer/probe mix. • reverse = 8.27 µL
31	Dispose of used tip in used tip bag.
32	Cap reverse primer and set to side.

33	Set pipette to volume for the probe (from calculations above). • 2.3 µL
34	Vortex probe for 1 second to mix.
35	Place probe in the microcentrifuge and briefly centrifuge to pull down any probe that may have got caught up in the lid. • balance centrifuge with an empty tube
36	Pipette known amount of probe into the 1.5 mL LoBind centrifuge tube labeled primer/probe mix. • Probe = 2.3 µL
37	Dispose of used tip in used tip bag.
38	Cap probe and set to side. • Keep covered as probe is light sensitive
	Make the 1.33x mastermix
39	Pipette known amount of nuclease free water into the 1.5 mL LoBind centrifuge tube labeled 1.33x mastermix (from calculations above). • 155.4 µL • no BSA = 168.0 µL water
40	Dispose of used tip in used tip bag.
41	Cap bottle and set aside.
42	Pipette known amount of 20x primer/probe mix into the 1.5 mL LoBind centrifuge tube labeled 1.33x mastermix (from calculations above). • 42.0 µL
43	Dispose of used tip and remaining unused 20x primer/probe mix in used tip bag.
44	Pipette known amount of BSA into the 1.5 mL LoBind centrifuge tube labeled 1.33x mastermix (from calculations above). • 12.6 µL • optional, see step 39
45	Dispose of used tip in used tip bag.
46	Cap tube and set aside.
47	Pipette known amount of TaqMan Fast Universal PCR MasterMix (2x), No AmpErase UNG into the 1.5 mL LoBind centrifuge tube

	labeled 1.33x mastermix (from calculations above) • 420 µL
48	Dispose of used tip in used tip bag.
49	Cap bottle and set aside.
50	Cap 1.5 mL LoBind centrifuge tube and place on ice.
51	Re-freeze primers & probe at -20°C. • in box labeled primers & probes
52	Re-freeze BSA at -20°C. • in box labeled PCR reagents
53	Place TaqMan Fast Universal PCR MasterMix (2x), No AmpErase UNG back in the fridge in box labeled PCR reagents 4°C.
54	Place all supplies and nuclease free water back in cupboard next to sink.
55	Spray inside of deadbox with 50% ethanol.
56	Wipe deadbox using a kimwipe.
57	Remove all waster from dead box and place in biohazard box lined with yellow bag.
58	Place cover on dead box and turn on UV light to 1on the timer (side of dead box).
59	Dispose of gloves in biohazard box lined with yellow bag.
60	Dispose of gown in used laundry bag.
61	Proceed to SAB 342 at the U of A (procedure C).

Calculations:

For reconstituting each primer: (step 2 from above)

Primer's nmol (from data sheet) x 10^3 = pmol

100 pmol/µL = 100 µM

Add water/TE to get 100 pmol/µL

eg. data sheet states nmol of Entero-EPA-F is 75.6

Therefore, adding 756 µL of either TE buffer or nuclease free water to lyophilized primer will give a 100 µM stock concentration.

5. Procedure C: Setting up the 96 well plate for processing (SAB 342).

Step	Action	
1	Put on a fresh pair of gloves.	
2	Remove plasmid standard from freezer in SAB 344. • see SOP plasmid prep & calculations on how to make plasmid standard and calculate concentration.	
3	Remove extracted calibrator samples from fridge in SAB 344.	
4	If	Then
	Setting up the reactions using the QIAgility	Proceed to step 5.
	Setting up the reactions by hand in the dead box	Proceed to step 15.
5	Turn on the QIAgility, the computer, and open the QIAgility software.	
6	Set up QIAgility program specific to your draft 96-well plate (Table 1 above). • dilutions (1:10) of the plasmid (50000, 5000, 500, 50, 5) in triplicate • 10 unknown samples in duplicate • NTC in triplicate • 38 wells total	
7	Pipette required amount of water into a 1.5 mL centrifuge tube. • QIAgility program will state volume of water required.	
8	Place plasmid DNA, mastermix (from procedure A), water, and calibrator samples in the QIAgility. • Make sure the right tubes go in the right spots reserved for them in the QIAgility.	
9	Place the plastics needed in the QIAgility to support the program. • 8 Strip tube, optical plate	
10	Fill up the tip racks with enough 50 and/or 200 µL tips that the program can be supported.	
11	Check to make sure the tip dispenser box is empty and attached to the QIAgility.	
12	Start run. • Software will prompt user to save program	

Step	Action
	• Save program as descriptive name & date in a folder with user's name
13	Remove plate from machine when run finishes.
14	Proceed to step 30.
15	Place all supplies & media/reagents in the dead box. • 96 well plate & film, pipette and tips, strip tubes & rack, centrifuge tube rack, water, plasmids, unknown samples, & 1.33x master mix (from procedure B).
16	Pipette 45 µL of water into 6 wells of the PCR strip tube.
17	Briefly vortex the plasmid prep standard to mix
18	Pipette 5 µL of the plasmid into the first well. • This is dilution 1 (50000 copies/5µL)
19	Pipette up and down gently to mix. • Try not to aerosolize the plasmid.
20	Pipette 5 µL of dilution 1 into the second well. • This is dilution 2 (5000 copies/5µL)
21	Repeat steps19 & 20 for all the dilutions • dilution 3 (500 copies/5µL), 4 (50 copies/5µL), & 5 (5copies/5µL)
22	Set strip tubes to the side.
23	Pipette 15 µL of 1.33x mastermix to the first 38 wells on the 96-well optical plate. • see plate set up above
24	Pipette in 5 µL of water into each of the 3 wells labeled no template control (NTC) • see plate set up above • use a new tip for every well
25	Line up all unknown samples.
26	Pipette in 5 µL of unknown sample 1 into each of the 2 wells labeled unknown sample 1 (unk1)

Step	Action
	• see plate set up above • use a new tip for every well
27	Repeat step 26 for remaining unknown samples • 2, 3, 4, 5, 6, 7, 8, 9, & 10
28	Pipette in 5 µL of plasmid standard dilution 5 (5 copies/5µL) into each of the 3 wells labeled standard 5 (ST5) • see plate set up above • use a new tip for every well
29	Repeat step 28 for remaining dilutions • 50, 500, 5000, & 50000
30	Apply optical adhesive cover to top of plate. • Carefully peel back one side of the white protective backing of the film and stick the one side to the plate. • Using the grey square applicator, seal the film by moving the applicator across the film while peeling off the remaining protective backing. • When film is completely sealed, hold the applicator to each edge and remove remaining film at the perforated edges. • Run the applicator over the film to ensure it is completely sealed.
31	Spin down the 96 well plate in the appropriate centrifuge to ensure all reagents and DNA are at the bottom of the wells. • located in SAB 344 • 2 minutes at 1200 rpm
32	Proceed to procedure D.

6. Procedure D: Loading and setting up the program on the ABI 7500 Fast (SAB 344).

Step	Action
1	Turn on the ABI 7500 and the computer connected to the system.
2	Open the 7500 software from the desktop screen.
3	Click on new experiment.
4	Open door of RT-PCR. • Place finger on divot and gently push • Guide the door open, do not allow it to open too quickly • do NOT push anywhere else on the door except the divot
5	Gently place the plate in the machine and close the door • Place finger on divot and gently push until the door closes. • do NOT push anywhere else on the door except the divot
6	Click on tab that is labeled experimental properties.
7	Create a name for the experiment that is being ran. Eg. June 26,2013InvAtargetABAgsamples
8	Fill in user name and comments (optional).
9	Click on instrument using as 7500 fast (96 wells).
10	Click on type of experiment as Quantitation (standard curve).
11	Click on reagents to detect the target sequence as TaqMan reagents.
12	Click on ramp speed used in the instrument run as fast, ~ 40 minutes.
13	Click on tab labeled plate set up.
14	Go to define targets & samples tab.
15	Define target by name by clicking in space that says 'target 1'. • eg. Change to say InvA
16	Choose reporter as FAM from the drop down menu.
17	Choose quencher as TAMRA from the drop down menu.
18	Leave color as default.
19	Define samples by naming each sample being used remembering to add new sample for every sample. • clicking in space 'sample 1' and change it to say STANDARD • click on Add New Sample and 'sample 1' will re-appear. Change to unk1. • Repeat for remaining samples • eg. STANDARD, unk1, unk2, unk3, unk4, unk5, unk6, unk7, unk8, unk9, unk10, NTC

Step	Action
20	Leave color as default.
21	Go to assign targets & samples tab. • On the left side of the screen will show list of target and samples. • On the right side of the screen will show a view of the 96 wells which will be set up to look similar to the sample draft of well set up (from above).
22	Click on define & set up standards. • left side of screen, below targets • new screen will open
23	Select target from the dropdown menu. • eg. InvA
24	Define the standard curve for the target by choosing 5 for # of points and 3 for # of replicates.
25	Choose the starting quantity for the standards. This is based on the working stock concentration from the plasmid DNA calculations. Put the value for the highest concentration that is being used for the standards. • In the above calculations, 50,000 copies/5 µL is the starting concentration since plasmid stock is 500,000 and the dilutions were made so that 50,000 would be the starting concentration.
26	Choose serial factor (dilution) of 1:10 since 1:10 dilutions were made.
27	Select & arrange wells for the standards by choosing "let me select wells".
28	Select arrange standard in columns of plate set up for the standards is in columns. If it is in rows select rows (above sample plate set up draft is in rows).
29	Click apply then close. • previous screen from step 21will re-appear • the first 15 wells will be filled in (A1-12, B1-3)
30	Highlight wells B4 - 6 by left clicking on them with the mouse (control click to highlight all 3 wells) • Select target (InvA) from target list on left side of screen. • Select N from sample task bar to indicate negative control • Select NTC from sample list (below target list on left side of

Step	Action
	screen)
31	Highlight wells B7 & B8 by left clicking on them with the mouse (control click to highlight all 3 wells) • Select target (InvA) from target list on left side of screen. • Leave the U selected in the sample task bar to indicate unknown sample. • Select unk1 from sample list (below target list on left side of screen)
32	Repeat step 31 for remaining calibrator samples. • example: o B9 & B10, InvA, U,unk2 o B11 & B12, InvA, U, unk 3 o C1 & C2, InvA, U, unk 4 o C3 & C4, InvA, U, unk 5 o C5 & C6, InvA, U, unk 6 o C7 & C8, InvA, U, unk 7 o C9 & C10, InvA, U, unk 8 o C11 & C12, InvA, U, unk 9 o D1 & D2, InvA, N, unk 10
33	Select ROX as the dye to use as the passive reference.
34	Click on run tab.
35	Type 20 µL in reaction volume/well.
36	Select 95°C for 20 seconds for the holding stage.
37	Select 95°C for 3 seconds for step 1 of the cycling stage.
38	Select 60°C for 30 seconds for step 2 of the cycling stage.
39	Select 45 for number of cycles.
40	Start run. • Program will prompt to save experiment. • click yes and save in appropriately labeled folder. o name of user, project name, and/or year
41	Proceed to procedure E for analysis

7. Procedure E: Analyzing the data.

Step	Action
1	Look at the graph under the tab amplification curve.

Step	Action	
2	If	Then
	C_T has previously been established for this set of plasmids. • LOD_{95}	Adjust the threshold level to previously set C_T. Use this threshold for all calibrator sample analysis using this particular plasmid standard. • Eg. if the threshold is 0.1 when the LOD_{95} was performed, then the threshold must be adjusted to 0.1 every time that particular standard is used. As well, if the C_T value at 50,000 copies is 21±0.3 then it should always be 21±0.3. If not, there is an error in the standards and data is unreliable.
	C_T not been previously established for this set of plasmids. • LOD_{95}	LOD_{95} needs to be run in order to determine C_T. Once determined, C_T can be set for the calibrator samples.
3	Check the standard curve to make sure that the slope is a negative value (-3.32 is absolute perfect and gives 100% PCR efficiency).	
4	Check the standards and no template control (NTC) to make sure there are no outliers amplification in NTC that will drastically skew the results.	
	If	Then
	Outliers present	Right click on the well, hit omit well, then re-analyze the data.
	No outliers present	Go to step 6.
	Amplification in NTC	Right click on the well, hit omit well, then re-analyze the data.
	No amplification in NTC	Go to step 6.
	Amplification in 2 or 3 of the NTC	Contamination may have occurred. Calibrators need to be re-run with new standards and mastermix.
6	Check unknown samples to see if amplification occurred and if so what the number of targets is at the threshold level.	

Expected Values:

The values of the unknown samples should fall within the standard curve. If any samples exceed the range, re-running those samples in dilutions so that they fall within the standard range will give a more accurate quantification.

Primer/Probe Details:

Enterococcus primer and probe set:

Forward primer (Entero1F): 5'-GAGAAATTCCAAACGAACTTG

Reverse primer (Entero1R): 5'-CAGTGCTCTACCTCCATCATT

TaqMan® probe (Entero1P): [6-FAM]-5'-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA

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Appendix B **MATERIALS USED IN CHAPTER 1**

B.1 **Summary of Microbial Survival Literature Used in Chapter 1**

Reference	Composting Type	Composting Scale	Material composted	Peak Temp.	Time > 55°C	Temperature monitoring details	Pathogens analyzed & details	Results
Barrena *et al.* (2009)	Bins	small (100L)	animal by-products (poultry and rabbit entrails, carcasses, and feathers), wood chips, and yard waste.	68 to 70°C at varying locations	~5 to 9 days, depending on location	Temperature was monitored at four evenly-spaced locations in each bin.	*Salmonella* sp.	*Salmonella* was not detected in sampled of the raw materials. However, at the end of composting, this organism was present. The sample used at the beginning of composting was likely not representative of all the material. Survival of this organism through temperatures reaching up to 70°C was attributed, most probably, to survival in cooler zones near the surfaces of the material. Recontamination is another possible cause.
Briancesco *et al.* (2008)	various. compost from 20 Italian facilities sampled. (End product evaluation)	full	various combinations of green waste, sewage sludge, municipal solid waste, agricultural wastes.	unknown	unknown	unknown	indicators (faecal coliforms, enterococci); *Escherichia coli* (non-pathogenic); *Salmonella* spp.; *Clostridium perfringens; Cryptosporidium Giardia;* helminth ova;	*E. coli* was reduced by 2 to 5 orders of magnitude, but was still detectable in many finished composts. *Salmonella* was detected in all final products, with some plants showing very low densities and others showing little reduction from initial levels in the feestocks. *Cryptosporidium* and *Giardia* were not detected in any final products. *Clostridium perfringens* spores were present at

Reference	Composting Type	Composting Scale	Material composted	Peak Temp.	Time > 55°C	Temperature monitoring details	Pathogens analyzed & details	Results
								varying levels in all final composts, from < 1.5 x 10^1 CFU/g (dry weight) up to 1.7 x 10^4 CFU/g (dry weight).
Brinton *et al.* (2009)	various (94 market-ready composts from three USA states) (End product evaluation)	full	green waste. Feedstocks do NOT include sewage sludge.	unknown	unknown	unknown	indicators (fecal coliforms and fecal streptococci) *Escherichia coli* (non-pathogenic) *Escherichia coli* O157:H7 *Salmonella* spp. *Listeria* spp. *Clostridium perfringens*	Only one sample out of the 94 composts had detectable *Salmonella* (at 1.8 MPN/4g). *E. coli* O157:H7 were found in composts from 3 facilities (6% of tested), all of which were large facilities. *C. perfringens* was detected in 70% of the 55 tested composts at levels up to $10^{4.88}$ CFU/g. *Listeria* spp. were detected in 22 out of 47 sampled composts, with one of these samples having a very high level ($10^{4.6}$ MPN/g)
Bustamante *et al.* (2008)	4 static piles and 3 turned piles.	small	combinations of grape stalk, grape marc, sewage sludge, manure (cow and poultry)	only one pile (#6) exceeded 55°C, and peaked at ~65°C	pile #6 exceeded 55°C for about 2 days	unknown	*Salmonella* sp. *Staphylococcus aureus* *E. coli* (non-pathogenic)	In piles where *Salmonella* was detected at the beginning of composting, it was still detected after maturation of the piles. In both of these piles, temp. remained below 50 °C. However, in pile 6, which reached 65°C, *Salmonella* was not detected at the beginning, but was detected after maturation. *Staphylococcus aureus* was present in all but one of the piles at some point during

Reference	Composting Type	Composting Scale	Material composted	Peak Temp.	Time > 55°C	Temperature monitoring details	Pathogens analyzed & details	Results
								composting. Where testing was done, this organism was not eliminated during the thermophilic phase. In some piles it was not detectable after maturation, while in others (including the highest-temperature pile) it was still present. *E. coli* presence was detected in all piles at some point after the thermophilic phase, and in 6 out of 7 piles (including the highest-temperature pile) after maturation.
Collick *et al.* (2007)	biodrying composting system (dries compost using forced aeration and natural heat generation)	full (existing on-farm pile)	manure, animal bedding, and alfalfa hay	~65°C in the middle of the pile at the end.	~14 days for all thermocouples except the one at 45 cm above the bottom, which didn't ever reach 40°C.	Four vertical locations (16, 45, 74, and 94 cm) were monitored at a single location, plus two centre locations at the front and back ends of the pile.	*Ascaris suum*	All 120 chambers containing *Ascaris suum* had no viable eggs from the first sampling day (day 4) on. (Temperature increased to 55°C, except in the noted location, within the first day). Inactivation was attributed to both the high temperatures and to drying.
Erickson *et al.* (2009)	Reactors	lab (15 L)	manure, wheat straw, and cottonseed meal	~56.8°C was the highest average temperature in any trial, with many mixtures remaining under 55°C	unclear	Temperatures recorded at 30 minute intervals at 4 locations (top and bottom edges and centres).	*Salmonella* spp. (inoculated)	*Salmonella* was inactivated within 8 days in all trials, regardless of temperature. The longest survival was actually observed in the trials with the highest temperatures. There was a correlation in this study between degree-days required for pathogen kill and C:N ratio (lower degree-days

Reference	Composting Type	Composting Scale	Material composted	Peak Temp.	Time > 55°C	Temperature monitoring details	Pathogens analyzed & details	Results
								were required for lower C:N ratios)
Fourti *et al.* (2008)	windrows	full (3m x 1.5m x 7.5m)	solid waste, and solid waste plus sewage sludge	Average pile temperature peaked between 60 and 65°C	>10 days (average temperature)	Temperatures monitored daily at three depths and averaged.	*Salmonella* spp. *Staphylococcus aureus*	*Salmonella* and *Staphylococcus aureus* were detected from days 1 to 30, after which time they could no longer be isolated. It should be noted that average temperatures reached 55°C by day 30, so after treatment at 55°C, these organisms were inactivated.
Grewal *et al.* (2006)	Thermophilic composting (at 55°C) was simulated by incubating materials	lab scale (4 liter capacity vessel (LxD = 30x15 cm) made of PVC pipe placed in incubator set at 55C)	Dairy manure; sawdust and straw	55°C	Up to 65 d. due to self heating	Incubator set at 55°C (BioCold Environmental, Inc., Fenton, MO)	*Mycobacterium avium* subsp. *paratuberculosis* (inoculated) *E. coli* (non-pathogenic, naturally occurring) *Salmonella spp.* (naturally occurring) *Listeria spp.* (naturally occurring)	The incubated compost had no detectable *E. coli*, *Salmonella*, or *Listeria* organisms after 3 days No *M. paratuberculosis* was detected with culturing method after 3 days of composting In contrast the PCR method detected *M. paratuberculosis* DNA through day 56 in all samples. Verdict: *M. paratuberculosis* may be viable but unculturable due to the severe physicochemical condition and/or microbial competition
Grewal *et al.* (2007)	simulated composting environment by incubating materials	lab	swine manure and sawdust	~64°C	incubated at 55°C for 56 days	Temperatures within the vessels were recorded every 12 minutes	*Listeria monocytogenes* (inoculated) *Salmonella enterica* (inoculated)	*Listeria* was detected in some of the incubated samples even after 56 days (though levels were reduced from initial) *Salmonella* was detected in some of the incubated samples even after 28 days of exposure to 55°C (after 42 days, all were below

Reference	Composting Type	Composting Scale	Material composted	Peak Temp.	Time > 55°C	Temperature monitoring details	Pathogens analyzed & details	Results
								the detection limit) Both organisms were significantly reduced after 3 days, however.
Hanajima *et al.* (2004)	static piles	unclear	manure and rice straw ("control"), or manure and tofu residue ("tofu")	> 70°C in both piles	>3 d in control pile, > 10 d in tofu pile	recorded hourly at the centre of the piles	*E. coli* (indigenous)	Indigenous *E. coli* was decreased to below the detection limit during the thermophilic phase, but re-grew later, and subsequently fell below the detection limit again after storage.
Inglis *et al.* (2010)	Windrow composting	Medium scale (LxWxH = 12, 2.5, 2)	Bovine manure	~78.9°C at the depth of ~20 cm	~ 175 days at the depth of ~ 20cm	thermocouples were placed at the centre and top (~10cm from the surface) of the windrow	*Campylobacter* spp. (*coli*, *fetus*, *hyointestinalis*, *jejuni*) Survival analysis was done by quantitative and qualitative PCR	The DNA of *Campylobacter* species persisted throughout active and curing phases of composting (~7.7 months). No spatial difference in survival was observed *C. jejuni* did not decrease significantly over the active composting period
Karpowicz *et al.* (2010)	Open air windrow piles	Medium scale (15m x 3m x 3m)	Biosolids mixed at a 1:1 ratio with wood debris	unknown	unknown	unknown	*Clostridium perfringens* Samples were taken from six different compost piles aged 1,4,8,13,18 and 24 months at the depth of 50 cm.	The results suggested that the windrow type composting did not decrease *C. perfringens* numbers, even after a period of 2 years. They were higher than recommended for adequate sanitation of biosolids (>3000 cells/g dry weight)
Kaszewska *et al.* (2006)	turned piles	unclear (probably small-scale)	sewage sludge, straw, and sawdust	Summer: 57°C (upper layer), ~55°C (middle), ~53°C (bottom) Winter: 49.5°C (upper), ~40°C (middle), 33°C (bottom)	Summer: ~16 d total in upper part of pile, 1 d. in middle. Temperatures peaked by day 4. Winter: n/a	unknown	*Escherichia coli* (inoculated) *Salmonella seftenberg W775* (inoculated)	Summer: *E. coli* was eliminated within samples from all layers within 9 days of composting in summer trials. *Salmonella* was also eliminated in the summer pile (by the 7th

Reference	Composting Type	Composting Scale	Material composted	Peak Temp.	Time > 55°C	Temperature monitoring details	Pathogens analyzed & details	Results
								day), though they were eliminated faster in the cooler bottom part of the pile than in the higher-temp. middle. Winter: Both *E. coli* and *S. seftenberg* were still detected at the bottom of the pile in the winter trials at the end of the experiment (day 33). In the warmer top and middle sections, *E. coli* was eliminated between d. 20 and d 33. The temperature in the top and middle sections peaked at around day 24.
Koné *et al.* (2007)	Windrow composting	small (two compost heaps of 3 m^3)	Dewatered fresh public sludge and septage mixed at a 1:2 ratio	68°C (observed in the inner layer of compost)	~28 days in the middle of compost, None in the outer layer of compost (up to 53°C)	Temperature measured daily at different locations in the centre and in the upper layers of the heaps	Helminth eggs (*Ascaris* and *Trichuris* eggs)	An optimum composting period (T>45°C) of at least 2 months was necessary to reduce the load of Ascaris to 1 Ascaris egg/g total solids Ascaris viability was reduced to <10% with a max. count of <5 eggs/g total solids in the final product
Lasaridi *et al.* (2006)	various (23 Greek compost products attained in packaging for sale) (End product evaluation)	full	various.	unknown	unknown	unknown	indicator organisms (fecal coliforms and fecal streptococci) *Salmonella* spp. *Staphylococcus aureus* *Clostridium perfringens*	*Salmonella* was not detected in any final products *Staphylococcus aureus* (a human pathogen transmitted via contaminated food consumption) was present in 4 of the 23 composts tested (17%) *Clostridium perfringens* was detected in all but one of the composts

Reference	Composting Type	Composting Scale	Material composted	Peak Temp.	Time > 55°C	Temperature monitoring details	Pathogens analyzed & details	Results
								(96%).
Pourcher *et al.* (2005)	Bins (turned monthly for four months, then cured for 3 months, unturned.)	small	sewage sludge and straw	> 65°C at inlet and outlet. At bottom, temperature peaked at 49.8°C.	inlet: 5 to 16 days between each turning outlet: difficult to see, but >3 days between each monthly turning. bottom: n/a	measured hourly at three locations	enteroviruses *Clostridium perfringens* *E. coli* (non-pathogenic) *Listeria monocytogenes* *Salmonella* spp.	enterovirus genomes were detected until the third month, though no infectious viruses were found at the end of the first month. *E. coli* were not totally inactivated , and were detected even after 4 months of composting and 3 months of maturation. *Clostridium perfringens* were still detected in significant numbers even after curing (though levels did decrease). *C. perfringens* is a spore-forming bacteria. *Salmonella* spp. was inactivated within the first month of composting. *Listeria monocytogenes* was detected even at month 3 of composting, though it was undetectable after month 4. No *Listeria* species were detected in mature composts.
Rao *et al.* (2007)	Bins	small	various combinations of pig slurry solids, poultry litter, spent mushroom compost, wood shavings, straw, shredded newspaper,	not reported, but some batched exceeded 70°C.	All batches exceeded 55°C for varying amounts of time.	temperatures measured at 12 locations (4 points at top, middle, and bottom locations) every 10 minutes.	*Salmonella* spp. *Campylobacter* spp. *Cryptosporidium* spp.	None of the three pathogens present in the raw materials was found in the pelletized product. However, *Salmonella* and *Campylobacter* were detected in some of the final batches of mature compost. *Cryptosporidium* oocysts were detected in composts after 3

Reference	Composting Type	Composting Scale	Material composted	Peak Temp.	Time > 55°C	Temperature monitoring details	Pathogens analyzed & details	Results
			and cocoa shells					months, but not after 6 months of maturation.
Saidi *et al.* (2008)	Windrow composting	Small scale (LxWxH = 2, 1.5, 1.5 m^3)	Combination of vegetable residues, Posidonia oceanica weed and organic fraction of MSW	~80-garden waste ~85-garden waste and posidonia ~70-MSW	70 days (garden waste); 85 days (garden waste with Posidonia) 150 days (MSW)	Not specified	*Salmonella* The detection of Salmonella was determined as recommended by Standard methods of American Public Health Association.	Salmonella sp. was not detected in C1 and C2 composts. The salmonella sp. (S. munchen, and S. corvalis) appeared at the beginning of composting in C3, but were destroyed when the temperature reached 55C
Sharma *et al.* (2009)	Windrow composting	Small scale (LxWxH = 12x1.5x1.5 m)	Livestock manure	66.2 – Control 54.9 – TY 52.8 - TS	8 – Control 8- TY 0 - TS	Thermocouples and data loggers recorded the temperature from the top and the middle of each windrow on an hourly basis	Total *E coli*; Ampicillin resistant *E coli*; and Tetracycline resistant *E. coli* were assayed in this experiment	At the end of composting time (18 weeks) total E. coli persisted only in TY. Amp. Resistant E.coli was not detected in any compost type. Tetracycline resistant E. coli were detected in up to week 18 in TY.
Szabová *et al.* (2010)	Aerobic composting with forced aeration	The study was performed in industrial conditions of the Industrial Composting Plant	Agricultural waste, crop products from beer production, and sewage sludge from WWTP	<u>In winter:</u> 65C on day 6 in the beginning of pile, 64C in the middle of pile on day 4 and 64C in the end of pile on day 7 <u>In summer:</u> 71C in the middle of pile on day 4;	In winter the from day 6 to day 30. In summer from day 4 to approximately day 41	No information provided. It is only known that the temperature was measured from 3 locations (beginning, middle and end of pile)	Non-embryonated *Ascaris suum* eggs were used in the experiment. They have the highest tenancy and viability during composting process. Compost was artificially contaminated with parasite germs. Parasites were inoculated into polyurethane carriers at a dose of 2,000 eggs per one carrier. The carriers were placed into plastic nets (3 carriers per net) and introduced into the middle of pile (7 in	Within the mentioned temperatures, annihilation of *A. suum* eggs deposited into the composting pile was reached on day 6. Authors conclude upon their results that resulting product of composting process is hygienically safe and poses no risk for pathogen transmission to the environment when used in agriculture.

Reference	Composting Type	Composting Scale	Material composted	Peak Temp.	Time > 55°C	Temperature monitoring details	Pathogens analyzed & details	Results
				70C in the beginning and the end of the pile.			winter and 8 in summer). Samples were collected for parasitological analysis on days 0,1,4,5,6,7,10,11, and 62 in winter; and 0,3,4,5,6,7,10,11, and 62 in summer.	
Tønner-Klank *et al.* (2007)	composting toilet bins	small	fecal materials plus ryegrass, sucrose, and fertilizer (Trial C).	Trial C – 57°C	Trial C – 30.2 to 52.3 hours	Temperatures monitored every 10 minutes at pathogen inoculation/sampling locations (top, middle, and bottom).	*Ascaridia galli* eggs *Salmonella typhimurium* phage 28B (surrogate/indicator for viruses) *Salmonella senftenberg* 775W (heat resistant strain of Salmonella)	*Salmonella seftenberg* and the bacteriophage were both detectable after composting for 21 days at all locations (though there was some degree of reduction). Levels were lowest at the top; as temperatures at the top were lower than elsewhere, it was hypothesized that dessication was responsible for this reduction. Some of the *A. galli* eggs remained viable in one of the containers, even at the location where temperature was highest (>55°C for 1.25 days).
Viau and Peccia (2009)	various (8 windrows and 2 in-vessel systems) (End product evaluation)	full	biosolids composts with various amendments (e.g. sawdust, green waste, wood chips, paper, or no amendment)	unknown	unknown	unknown	fecal coliforms male-specific coliphages human adenovirus species (qPCR) *Legionella pneumophila* (bacteria) (qPCR) *Staphylococcus aureus* (bacteria) (qPCR) *Clostridium difficile* (clostridium)	fecal coliform levels in all composts were below the USEPA Class A limit of 10^3 CFU/g, with many of the samples actually showing fecal coliform levels below the detection limits. male-specific coliphages (investigated as potential indicator organisms) were detected generally at

Reference	Composting Type	Composting Scale	Material composted	Peak Temp.	Time > 55°C	Temperature monitoring details	Pathogens analyzed & details	Results
							(qPCR)	higher levels than fecal coliforms. *Staphylococcus aureus* and *Clostridium difficile* genomes were not detected in any of the finished composts. *Legionella pneumophila* genomes were detected in 50% of compost samples, with a median value of ~ 10^4 genomic units/g. *Legionella* actually increased during composting in some samples. Adenovirus spp. genomes were detected in 70% of compost, with a mean value of ~10^4 genomic units/g.
Wéry *et al.* (2008)	aerated static pile (turned once during active composting) Study took place in France.	full	dewatered sludge, green waste, wood chips, and refuse	60-70°C	T> 60°C for 4 days in 2004 T> 60°C for 2-4 days in 2005	unknown	*Escherichia coli* *Salmonella spp.* *Clostridium perfringens* *Enterococcus spp.* Pathogen sampling took place in both 2004 and 2005 piles. for each sampling event, 40 1L samples were taken from various locations in the pile and mixed.	While reductions were seen during the active phase, all four tested organisms were detected at the end of the high-temperature phase in both years, with the exception of *Salmonella* in 2004 and 2005. After the maturation phase, again only *Salmonella* spp. was not detected in both years. E. coli was not detected in 2004, but was detected in 2005. *C. perfringens* and *Enterococcus* were both detectable after maturation. After storage, *Enterococcus* spp. were still detectable (by both PCR and culture methods), while *C.*

Reference	Composting Type	Composting Scale	Material composted	Peak Temp.	Time > 55°C	Temperature monitoring details	Pathogens analyzed & details	Results
								perfringens was detectable by culture but not PCR.
Xu *et al.* (2009)	bin, insulated with straw bales	small	cattle mortailities, straw, and manure	80cm, Bin 1: 64.8°C 80 cm, Bin 2: 61.3°C 160cm, Bin 1: 48.3°C 160 cm, Bin 2: 47.4°C	80cm, Bin 1: 35 d (from d. 6 to d. 41) 80 cm, Bin 2: 43 d (from d. 8 to d. 51) 160cm, Bin 1: 0 d 160 cm, Bin 2: 0 d	temperatures monitored at each sampling location once per hour (averaged daily); temperatures also monitored twice daily at the mouth of each of the 16 carcases in the compost bin (max temperatures were between 40.3 and 56.0°C. Only one of these locations exceeded 55°C).	*Escherichia coli* O157:H7 *Campylobacter jejuni* Newcastle disease virus (representative of viruses such as avian influenza) All three pathogens were inoculated into fresh manure in nylon mesh bags. E. coli and NDV were also inoculated into sterilized manure in sealed vials to isolate temperature effects. Samples all placed at each of the sampling locations and analyzed periodically.	*E. coli* O157:H7 survived for between 7 and 14 d at all 80cm locations, and at all but one 160 cm location. When isolated from all effects but temperature, survival was up to 14 days at 80cm and 28 days at 160cm. *C. jejuni* DNA from this organism was detected at 80 cm until day 84, and at 160 cm at all sampling times (declined after d. 28). "Although the presence does not necessarily equate to the viability of cells, it likely reflects intact cells." "Campylobater are likely to be more thermotolerant than E. coli". Newcastle disease virus (NDV) was destroyed by day 7 in all inocuolated samples exposed (in vials and in nylon bags).

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Appendix C **MATERIALS USED IN CHAPTER 2**

C.1 **Data Used for Random Movement Analysis**

Density	Trench	Trial	Net displacement	Relative displacement %
A-1630	Head	Trial 1	-36	-90
A-1630	Head	Trial 1	-25	-62.5
A-1630	Head	Trial 1	-30	-75
A-1630	Head	Trial 1	-32	-80
A-1630	Head	Trial 1	-15	-37.5
A-1630	Head	Trial 1	3	7.5
A-1630	Head	Trial 1	-14	-35
A-1630	Head	Trial 1	-18	-45
A-1630	Head	Trial 2	43	119.44
A-1630	Head	Trial 2	0	0
A-1630	Head	Trial 2	-23	-63.89
A-1630	Head	Trial 2	-11	-30.56
A-1630	Head	Trial 2	-26	-72.22
A-1630	Mid 1	Trial 1	-9	-22.5
A-1630	Mid 1	Trial 1	-19	-47.5
A-1630	Mid 1	Trial 1	-32	-80
A-1630	Mid 1	Trial 1	-41	-102.5
A-1630	Mid 1	Trial 1	-15	-37.5
A-1630	Mid 1	Trial 1	20	50
A-1630	Mid 1	Trial 1	25	62.5
A-1630	Mid 1	Trial 1	28	70
A-1630	Mid 1	Trial 2	-12	-33.33
A-1630	Mid 1	Trial 2	-31	-86.11
A-1630	Mid 1	Trial 2	17	47.22
A-1630	Mid 1	Trial 2	-26	-72.22
A-1630	Mid 1	Trial 2	0	0
A-1630	Mid 1	Trial 2	-4	-11.11
A-1630	Mid 2	Trial 1	50	125
A-1630	Mid 2	Trial 1	11	27.5
A-1630	Mid 2	Trial 1	-24	-60
A-1630	Mid 2	Trial 1	-8	-20
A-1630	Mid 2	Trial 1	-41	-102.5
A-1630	Mid 2	Trial 1	57	142.5
A-1630	Mid 2	Trial 1	12	30
A-1630	Mid 2	Trial 2	43.3	120.28
A-1630	Mid 2	Trial 2	23	63.89
A-1630	Mid 2	Trial 2	-30	-83.33
A-1630	Mid 2	Trial 2	-15	-41.67

Density	Trench	Trial	Net displacement	Relative displacement %
A-1630	Mid 2	Trial 2	29	80.56
A-1630	Mid 2	Trial 2	-14	-38.89
A-1630	Mid 2	Trial 2	-29	-80.56
A-1630	Tail	Trial 1	-9	-22.5
A-1630	Tail	Trial 1	3	7.5
A-1630	Tail	Trial 1	27	67.5
A-1630	Tail	Trial 1	-17	-42.5
A-1630	Tail	Trial 1	12	30
A-1630	Tail	Trial 1	-23	-57.5
A-1630	Tail	Trial 1	-16	-40
A-1630	Tail	Trial 1	50	125
A-1630	Tail	Trial 2	35	97.22
A-1630	Tail	Trial 2	19	52.78
A-1630	Tail	Trial 2	-36	-100
A-1630	Tail	Trial 2	-33	-91.67
A-1630	Tail	Trial 2	-32	-88.89
A-1630	Tail	Trial 2	-28	-77.78
B-anchor	Head	Trial 1	-4	-10
B-anchor	Head	Trial 1	-36	-90
B-anchor	Head	Trial 1	-3	-7.5
B-anchor	Head	Trial 1	-35	-87.5
B-anchor	Head	Trial 1	-11	-27.5
B-anchor	Head	Trial 1	-17	-42.5
B-anchor	Head	Trial 1	-29	-72.5
B-anchor	Head	Trial 1	-37	-92.5
B-anchor	Head	Trial 2	33	91.67
B-anchor	Head	Trial 2	-23	-63.89
B-anchor	Head	Trial 2	-28	-77.78
B-anchor	Head	Trial 2	-13	-36.11
B-anchor	Head	Trial 2	-27	-75
B-anchor	Mid 1	Trial 1	34	85
B-anchor	Mid 1	Trial 1	23	57.5
B-anchor	Mid 1	Trial 1	-31	-77.5
B-anchor	Mid 1	Trial 1	-39	-97.5
B-anchor	Mid 1	Trial 1	-30	-75
B-anchor	Mid 1	Trial 1	35	87.5
B-anchor	Mid 1	Trial 1	52	130
B-anchor	Mid 1	Trial 2	13	36.11
B-anchor	Mid 1	Trial 2	-27	-75
B-anchor	Mid 1	Trial 2	-29	-80.56

Density	Trench	Trial	Net displacement	Relative displacement %
B-anchor	Mid 1	Trial 2	4	11.11
B-anchor	Mid 1	Trial 2	20	55.56
B-anchor	Mid 1	Trial 2	-33	-91.67
B-anchor	Mid 1	Trial 2	-31	-86.11
B-anchor	Mid 2	Trial 1	23	57.5
B-anchor	Mid 2	Trial 1	23	57.5
B-anchor	Mid 2	Trial 1	36	90
B-anchor	Mid 2	Trial 1	-13	-32.5
B-anchor	Mid 2	Trial 1	-21	-52.5
B-anchor	Mid 2	Trial 1	-15	-37.5
B-anchor	Mid 2	Trial 1	-41	-102.5
B-anchor	Mid 2	Trial 2	20	55.56
B-anchor	Mid 2	Trial 2	-7	-19.44
B-anchor	Mid 2	Trial 2	-6	-16.67
B-anchor	Mid 2	Trial 2	10	27.78
B-anchor	Mid 2	Trial 2	5	13.89
B-anchor	Mid 2	Trial 2	12	33.33
B-anchor	Tail	Trial 1	40	100
B-anchor	Tail	Trial 1	-3	-7.5
B-anchor	Tail	Trial 1	1	2.5
B-anchor	Tail	Trial 1	7	17.5
B-anchor	Tail	Trial 1	-8	-20
B-anchor	Tail	Trial 2	-2	-5.56
B-anchor	Tail	Trial 2	20	55.56
B-anchor	Tail	Trial 2	18	50
B-anchor	Tail	Trial 2	-25	-69.44
B-anchor	Tail	Trial 1	-7	-17.5
C-580	Head	Trial 1	12	30
C-580	Head	Trial 1	-9	-22.5
C-580	Head	Trial 1		-102.5
C-580	Head	Trial 1	-12	-30
C-580	Head	Trial 1	-24	-60
C-580	Head	Trial 1	-9	-22.5
C-580	Head	Trial 1	-13	-32.5
C-580	Head	Trial 2	33	91.67
C-580	Head	Trial 2	27	75
C-580	Head	Trial 2	-29.5	-81.94
C-580	Head	Trial 2	9	25
C-580	Head	Trial 2	16	44.44
C-580	Head	Trial 2	1	2.78

Density	Trench	Trial	Net displacement	Relative displacement %
C-580	Head	Trial 2	-8	-22.22
C-580	Mid 1	Trial 1	23	57.5
C-580	Mid 1	Trial 1	-18	-45
C-580	Mid 1	Trial 1	-41	-102.5
C-580	Mid 1	Trial 1		-102.5

C.2 **Data Used in Edge Effect Analysis**

Trial	Zone	Material	Amount (%)
1	1	Compost	20
1	1	Probe with anchors	30
1	1	Temp 1000	40
1	2	Compost	60
1	2	Probe with anchors	70
1	2	Temp 1000	40
1	3	Compost	20
1	3	Probe with anchors	0
1	3	Temp 1000	20
2	1	Compost	30
2	1	Probe with anchors	30
2	1	Temp 1000	20
2	2	Compost	50
2	2	Probe with anchors	20
2	2	Temp 1000	30
2	3	Compost	20
2	3	Probe with anchors	50
2	3	Temp 1000	50
3	1	Compost	10
3	1	Probe with anchors	10
3	1	Temp 1000	0
3	2	Compost	60
3	2	Probe with anchors	30
3	2	Temp 1000	40
3	3	Compost	30
3	3	Probe with anchors	60
3	3	Temp 1000	60
4	1	Compost	10
4	1	Probe with anchors	30
4	1	Temp 1000	30
4	2	Compost	30
4	2	Probe with anchors	30
4	2	Temp 1000	30
4	3	Compost	60
4	3	Probe with anchors	40
4	3	Temp 1000	40
5	1	Compost	15
5	1	Probe with anchors	10

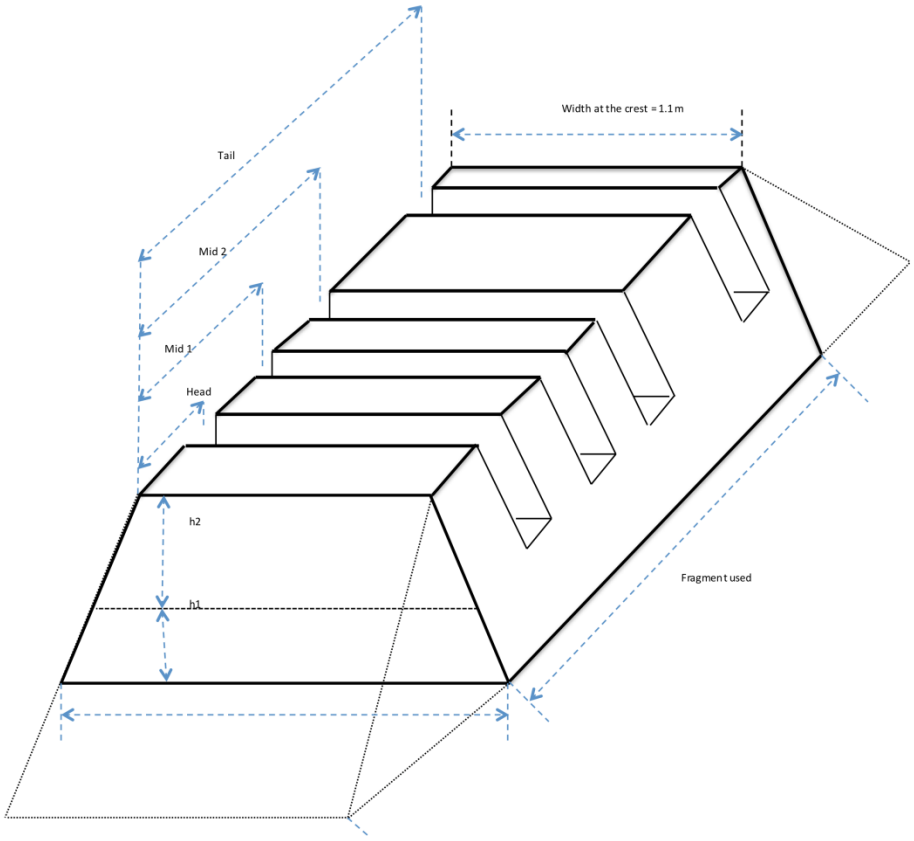
Trial	Zone	Material	Amount (%)
5	1	Temp 1000	0
5	2	Compost	25
5	2	Probe with anchors	20
5	2	Temp 1000	30
5	3	Compost	60
5	3	Probe with anchors	70
5	3	Temp 1000	70
6	1	Compost	10
6	1	Probe with anchors	10
6	1	Temp 1000	0
6	2	Compost	40
6	2	Probe with anchors	30
6	2	Temp 1000	30
6	3	Compost	50
6	3	Probe with anchors	60
6	3	Temp 1000	70
7	1	Compost	45
7	1	Probe with anchors	30
7	1	Temp 1000	20
7	2	Compost	35
7	2	Probe with anchors	20
7	2	Temp 1000	40
7	3	Compost	20
7	3	Probe with anchors	50
7	3	Temp 1000	40
8	1	Compost	70
8	1	Probe with anchors	70
8	1	Temp 1000	90
8	2	Compost	27
8	2	Probe with anchors	30
8	2	Temp 1000	10
8	3	Compost	3
8	3	Probe with anchors	0
8	3	Temp 1000	0
9	1	Compost	20
9	1	Probe with anchors	50
9	1	Temp 1000	60
9	2	Compost	70
9	2	Probe with anchors	30
9	2	Temp 1000	10

Trial	Zone	Material	Amount (%)
9	3	Compost	10
9	3	Probe with anchors	20
9	3	Temp 1000	30

Legend:

- Compost – painted compost particles
- Probe with anchors – the modified temperature probe with the anchor to improve its retention on the surface of compost pile
- Temp 1000 – Temperature data logger (aka MTP, Temperature probe) used in every study
- Zone – the section where the particles landed
    - 1 – top
    - 2 – middle
    - 3 - bottom

C.3 **Sketch of Experimental Pile Used in Random Movement Analysis**



Appendix D **MATERIALS USED IN CHAPTER 3**

D.1 Temperature Data Used in Development of Sampling Method, Estimating Sample Size and Effect of Pile Turning

Due to the large volume, the original temperature profiles data cannot be presented in the Appendix in their original form. However the data can be reached from

http://goo.gl/NmgkIX

D.2 Sample Size Calculation of Temperature Probes for Indirect Composting Process Validation

Statement:

Assume the following:

a) 30 temperature probes were randomly introduced into the compost pile and recovered 60 days after;
b) The probes were programmed to read the temperature every 30 minutes;
c) The highest daily variance from all 30 probes throughout composting was observed at 228.5 on day 28;
d) The lowest daily variance from all 30 probes throughout composting was observed at 62.1 on day 15;
e) The overall daily variance was 172.51

Calculate the required minimum number of temperature probes?

Solution:

To calculate the required number of probes let's use the equation [1].

$$n = \frac{B-A}{S^2 \times \left(\frac{1}{\chi^2_{n-1,1-\frac{\alpha}{2}}} - \frac{1}{\chi^2_{n-1,\frac{\alpha}{2}}} \right)} \qquad \text{[Eq. 1]}$$

From the problem statement let's assume with 95% confidence (α/2 =0.025; 1-α/2 = 0.975) that the true population variance lies between highest and lowest observed daily temperature variance (i.e. between B = 228.5 and A = 62.1). Let's also assume that the calculated overall temperature variance is a true sample variance (i.e. S^2 = 172.51). Then the unknown that needs to be calculated is the number of probes (i.e. "n"). It should be noted that the chi square value which is in the equation [1] on the right hand side is a function of "n" and the specified confidence level. So the problem should be solved by trial and error method. The results of the trial and error are given in the Table D1.

Table D1. Finding required number of probes by trial and error

Hypothetical	$\chi^2_{n-1,1-\frac{\alpha}{2}}$	$\chi^2_{n-1,\frac{\alpha}{2}}$	**Calculated**

“n”			“n”
30	16.05	45.22	24
35	19.81	51.97	31
40	23.65	58.12	38
42	25.21	60.56	42

Conclusion:

According to the Table D1 the minimum number of probes that should be introduced into the compost pile should be at least 42. This number however can be higher depending on the credibility of assumptions underlying calculation.

Appendix E **MATERIALS USED IN CHAPTER 4**

E.1 **Temperature Data**

Due to the large volume, the original temperature profiles data cannot be presented in the Appendix in their original form. However, the data can be reached from

a) Random probes with cryovials:
http://goo.gl/eDS9IY

b) Random probes without cryovials:
http://goo.gl/e9SFh1

c) Probes from cool spots:
http://goo.gl/5zxYSB

E.2 **Concentration of Cryovial Content**

E.2.1. Concentration of cryovial content at Day-0

a) Phi S-1 phage (PFU / 100 μL)

Sample	**dilution (10^)**							
	-1	**-2**	**-3**	**-4**	**-5**	**-6**	**-7**	**-8**
1	TNTC	TNTC	TNTC	21	1	1	1	0
2	TNTC	TNTC	TNTC	48	3	1	0	0
3	TNTC	TNTC	TNTC	46	10	1	0	0

b) *Salmonella meleagridis* (CFU / 100 μL)

Sample	**dilution (10^)**							
	-1	**-2**	**-3**	**-4**	**-5**	**-6**	**-7**	**-8**
1	TNTC	TNTC	TNTC	TNTC	TNTC	105	5	0
2	TNTC	TNTC	TNTC	TNTC	TNTC	44	8	0
3	TNTC	TNTC	TNTC	TNTC	TNTC	122	8	0

c) *E. coli* K12 (CFU / 100 μL)

Sample	**dilution (10^)**							
	-1	**-2**	**-3**	**-4**	**-5**	**-6**	**-7**	**-8**
1	TNTC	TNTC	TNTC	TNTC	TNTC	101	6	0
2	TNTC	TNTC	TNTC	TNTC	TNTC	40	5	0
3	TNTC	TNTC	TNTC	TNTC	TNTC	25	9	0

E.2.2. Concentration of cryovial content at Day-56

a) Concentration of survived *Salmonella meleagridis* (probe 13 in Table 14)

Sample	dilution (10^)							
	-1	-2	-3	-4	-5	-6	-7	-8
1	TNTC	TNTC	263	21	4	0	0	0
2	TNTC	TNTC	231	18	7	1	0	0
3	TNTC	TNTC	265	5	1	1	0	0

b) *Salmonella meleagridis* control (CFU / 100 µL at 22°C)

Sample	dilution (10^)							
	-1	-2	-3	-4	-5	-6	-7	-8
1	TNTC	TNTC	TNTC	43	19	1	0	0
2	TNTC	TNTC	TNTC	63	22	0	0	0
3	TNTC	TNTC	TNTC	37	13	1	0	0

c) *Salmonella meleagridis* control (CFU / 100 µL at 4°C)

Sample	dilution (10^)							
	-1	-2	-3	-4	-5	-6	-7	-8
1	TNTC	157	11	2	0	0	0	0
2	TNTC	143	10	0	0	0	0	0
3	TNTC	124	9	0	0	0	0	0

d) *E. coli* K12 control (CFU / 100 μL at 22°C)

Sample	dilution (10^)							
	-1	**-2**	**-3**	**-4**	**-5**	**-6**	**-7**	**-8**
1	TNTC	TNTC	70	12	4	0	0	0
2	TNTC	TNTC	34	9	1	0	0	0
3	TNTC	TNTC	61	17	1	0	0	0

e) *E. coli* K12 control (CFU / 100 μL at 4°C)

Sample	dilution (10^)			
	-1	**-2**	**-3**	**-4**
1	TNTC	TNTC	TNTC	300
2	TNTC	TNTC	TNTC	154
3	TNTC	TNTC	TNTC	217

f) Phi S-1 phage (22°C)

Sample	dilution (10^)			
	-1	**-2**	**-3**	**-4**
1	2	2	0	0
2	2	0	0	0
3	0	0	0	0

g) Phi S-1 phage (4°C)

Sample	dilution (10^)			
	-1	**-2**	**-3**	**-4**
1	TNTC	32	0	0
2	TNTC	41	1	0
3	TNTC	37	3	0

Appendix F **MATERIALS USED IN CHAPTER 5**

F.1 **Temperature Data**

Due to the large volume, the original temperature profiles data cannot be presented in the Appendix in their original form. However the data can be reached from

http://goo.gl/D7Rlxs

F.2 **Concentration of Cryovial Content**

F.2.1. Concentration of culturable cells at Day-0

a) *E. coli* 43031 (MPN / mL)

Sample	Description	Dilution (10^)			
		-5	**-6**	**-7**	**-8**
1	Big wells	49	49	46	13
	Small wells	48	45	14	1
	Conc (mL-1)	**>2419.6**	**1733**	**167**	**16**
2	Big wells	49	49	43	14
	Small wells	48	42	4	4
	Conc (mL-1)	**>2419.6**	**1300**	**96**	**21**
3	Big wells	49	49	47	10
	Small wells	47	41	6	1
	Conc (mL-1)	**2420**	**1203**	**140**	**12**

b) *Salmonella meleagridis* (CFU / 100 µL)

Sample	**dilution (10^)**							
	-1	**-2**	**-3**	**-4**	**-5**	**-6**	**-7**	**-8**
1	TNTC	TNTC	TNTC	TNTC	TNTC	90	7	1
2	TNTC	TNTC	TNTC	TNTC	TNTC	68	9	0
3	TNTC	TNTC	TNTC	TNTC	TNTC	98	13	0

F.2.2. Concentration of culturable cells at Day-56

a) *E. coli* 43031 (MPN / mL at 4°C)

Sample	Description	Dilution (10^)			
		-4	-5	-6	-7
1	Big wells	49	49	49	47
	Small wells	48	48	37	11
2	Big wells	49	49	49	44
	Small wells	48	48	35	8
3	Big wells	49	49	49	46
	Small wells	48	48	46	14

b) *E. coli* 43031 (MPN / mL at 22°C)

Sample	Description	Dilution (10^)			
		-4	-5	-6	-7
1	Big wells	49	49	49	49
	Small wells	48	48	48	18
2	Big wells	49	49	49	36
	Small wells	48	48	24	7
3	Big wells	49	49	49	49
	Small wells	48	48	47	15

c) *Salmonella meleagridis* (CFU / 100 μL at 22°C)

Sample	Dilution (10^)				
	-4	**-5**	**-6**	**-7**	**-8**
1	TNTC	67	16	8	0
2	157	80	20	3	0
3	TNTC	TNTC	73	6	0

d) *Salmonella meleagridis* (CFU / 100 μL at 4°C)

Replicate	Dilution (10^)				
	-4	**-5**	**-6**	**-7**	**-8**
1	TNTC	67	16	8	0
2	157	80	20	3	0
3	TNTC	TNTC	73	6	0

F.3 **Physical and Chemical Test of Compost Matrix**

F.3.1. Day – 0

a) Bulk density

Sample ID	Mbeaker (g)	Mbeaker+ compost (g)	TS %	Mwet 1800,g	Bdwet (g/cm^3)
1	142.59	1053.8	37.24	911.21	0.51
2	142.8	927.8	37.24	785	0.44
3	144.7	929.7	37.24	785	0.44
4	144	950	37.24	806	0.45

b) Moisture content

Sample ID	Mdish (g)	Mdish+compost (gwet)	Mdish+ compost(gdry)
1	7.6913	57.0937	26.5594
2	7.677	57.1403	27.467
3	7.7797	57.1675	26.545

c) EC and pH

Sample ID	TS %	MW (g)	V water (mL)	EC (mS cm-1)	pH
1	37.24	107.5	132.5	2.442	8.21
2	37.24	107.4	132.6	2.826	8.14
3	37.24	107.4	132.6	2.507	8.18

c) Organic matter

Sample ID	Mdish (g)	Mdish+ compost (g)	Mdish+ compost (70C)	Mdish+ compost (550C)
1	55.58	100.48	77.84	77.966
2	55.93	103.02	77.16	68.457
3	55.76	103.45	79.20	70.265

F.3.2. End of Stage – 1

a) Bulk density

Sample ID	Mbeaker (g)	Mbeaker+ compost (g)	TS %	Mwet 1800,g	Bdwet (g/cm^3)
1	142.53	875.8	51.20	733.27	0.41
2	142.53	839.3	51.20	696.77	0.39
3	142.53	886.8	52.20	744.27	0.41

b) Moisture content

Sample ID	Mdish (g)	Mdish+compost (gwet)	Mdish+ compost(gdry)
1	13.1	178.8	96.7
2	13.0	181.5	99.4
3	13.0	181.6	97.4

c) EC and pH

Sample ID	TS %	MW (g)	V water (mL)	EC (mS cm-1)	pH
1	50.59	80.85	159.15	3713	7.42
2	50.59	80.85	159.15	3737	7.38
3	50.59	80.85	159.15	3736	7.34

c) Organic matter

Sample ID	Mdish (g)	Mdish+ compost (g)	Mdish+ compost (550C)	OM
1	126.38	144.56	77.966	62.596
2	125.94	146.62	68.457	55.464
3	119.94	143.27	70.265	55.465

F.3.3. End of Stage – 2

a) Bulk density

Sample ID	Mbeaker (g)	Mbeaker+ compost (g)	TS %	Mwet 1800,g	Bdwet (g/cm^3)
1	142.53	883.3	55.33	740.77	0.41
2	142.53	886.9	55.33	744.37	0.41
3	142.53	866.2	55.33	723.67	0.40

b) Moisture content

Sample ID	Mdish (g)	Mdish+compost (gwet)	Mdish+ compost(gdry)
1	13.15	157.48	94.06
2	13.16	152.13	89.8
3	13.11	182.56	104.15

c) EC and pH

Sample ID	TS %	MW (g)	V water (mL)	EC (mS cm-1)	pH
1	55.33	80.85	159.15	4923	6.92
2	55.33	80.85	159.15	4971	6.84
3	55.33	80.85	159.15	5244	6.89

c) Organic matter

Sample ID	Mdish (g)	Mdish+ compost (g)	Mdish+ compost (550C)	OM
1	143.93	163.29	150.74	64.824
2	122.56	142.8	127.2	77.075
3	125.36	146.54	132.37	66.903

F.4 **Analysis of Fecal Coliform in Compost Matrix (USEPA 1680)**

F.4.1. Day – 0 results

a) LTB test

Sample	Dilution	Replicate				
		1	2	3	4	5
1	B	1	1	1	1	1
	C	1	1	1	1	1
	D	1	1	1	1	1
	E	1	1	1	1	1
2	B	1	1	1	1	1
	C	1	1	1	1	1
	D	1	1	1	1	1
	E	1	1	1	1	1
3	B	1	1	1	1	1
	C	1	1	1	1	1
	D	1	1	1	1	1
	E	1	0	1	1	1
S1	D	1	1	1	1	1
	E	1	1	1	1	1
	F	1	1	0	0	1
	G	1	0	0	0	0
S2	D	1	1	1	1	1
	E	1	1	1	1	1
	F	1	0	0	0	0
	G	0	0	0	0	0

1-positive; 0-negative; yellow marker – positive after 48 hours; S – spiked

b) EC test

Sample	Dilution	Replicate				
		1	2	3	4	5
1	B	0	0	1	1	0
	C	0	1	0	0	1
	D	0	0	0	0	0
	E	0	1	0	0	0
2	B	1	1	1	1	1
	C	1	1	1	1	0
	D	1	1	1	1	1
	E	1	1	0	1	0
3	B	1	1	1	1	1
	C	0	1	1	1	1
	D	1	1	1	1	1
	E	0	0	0	1	0
S1	D	1	0	0	0	0
	E	0	0	0	0	0
	F	0	0	0	0	0
	G	0	0	0	0	0
S2	D	1	0	0	0	0
	E	1	1	0	0	0
	F	0	0	0	0	0
	G	0	0	0	0	0

1-positive; 0-negative; yellow marker – positive after 48 hours; S – spiked

c) Matrix calculation (as per USEPA Method 1680)

Sample	Largest volume tested	Positives	MPN Index mL	95% Lower CL	95% Upper CL	MPN/mL	% TS	MPN/g TS	Geometric mean
1	1.00E-04	2-0-1	0.68	0.06	1.64	**6.80E+03**	4.70E+01	**1.45E+04**	**5.87E+04**
2	1.00E-04	4-5-3	6.39	2.09	15.7	**6.39E+04**	4.70E+01	**1.36E+05**	
3	1.00E-04	4-5-1	4.83	1.64	12.56	**4.83E+04**	4.70E+01	**1.03E+05**	
S1	1.00E-05	1-0-0	0.2	0.03	0.68	**2.00E+04**	4.70E+01	**4.25E+04**	**7.43E+04**
S2	1.00E-05	1-2-0	0.61	0.03	1.51	**6.10E+04**	4.70E+01	**1.30E+05**	

d) Matrix recovery calculation

Calculation of E.coli (CFU / mL) in USS (EC undilute spike)				
Replicate	**-5 plate**	**-6 plate**	**-7 plate**	***E.coli* CFU / mL**
1	136	12	0	**1.09E+07**
2	94	11	5	
3	97	10	1	

1. Volume of USS per unit (g) of spiked biosolids sample	
Description of spiked sample	V spiked per unit biosolids
Class B solid	**1.00E-03**

2. Calculation of Spiked EC (wet weight)		
EC undiluted Spike (CFU/mL)	V spiked per unit biosolids	Spiked EC wet weight
1.09E+07	1.00E-03	**1.09E+04**

3. Conversion to true spiked EC CFU / g TS (DW)		
Description of spiked sample	% TS	True spiked EC CFU / g TS

Class B solid	4.70E+01	**2.32E+04**

4. Percent recovery				
Matrix	Ns	Nu	T	% Recovery
Class B solid	7.43E+04	5.87E+04	2.32E+04	**67.38**

F.4.2. Results at the end of Stage 1

a) LTB test

Sample	Dilution	Replicate				
		1	2	3	4	5
1	B	1	1	1	1	1
	C	1	1	1	1	1
	D	1	1	1	1	1
	E	1	1	1	1	1
2	B	1	1	1	1	1
	C	1	1	1	1	1
	D	1	1	1	1	1
	E	1	1	1	1	1
3	B	1	1	1	1	1
	C	1	1	1	1	1
	D	1	1	1	1	1
	E	1	0	1	1	1
S1	D	1	1	1	1	1
	E	1	1	1	1	1
	F	1	0	1	1	0
	G	0	1	1	1	0
S2	D	1	1	1	1	1
	E	1	1	1	1	1
	F	1	1	0	0	1
	G	0	0	0	0	0

1-positive; 0-negative; S – spiked

b) EC test

Sample	Dilution	Replicate				
		1	**2**	**3**	**4**	**5**
1	B	1	1	1	1	1
	C	1	1	1	1	1
	D	1	1	1	1	0
	E	0	0	1	0	0
2	B	1	1	1	1	1
	C	1	0	1	1	1
	D	0	1	0	1	1
	E	0	0	0	0	0
3	B	1	1	1	1	1
	C	1	1	1	0	0
	D	1	1	1	0	0
	E	0	0	0	0	0
S1	D	1	1	1	1	1
	E	1	1	1	1	1
	F	1	0	1	1	1
	G	0	0	0	0	0
S2	D	1	1	1	1	1
	E	1	1	1	1	1
	F	1	1	0	0	1
	G	0	0	0	0	0

1-positive; 0-negative; S – spiked

c) Matrix calculation

Sample	Largest volume tested	Positives	MPN Index mL	95% Lower CL	95% Upper CL	MPN/mL	% TS	MPN/g TS	Geometric mean
1	1.00E-04	5-4-1	17.24	4.29	49.75	**1.72E+05**	5.04E+01	**3.42E+05**	
2	1.00E-04	4-3-0	2.71	0.9	8.09	**2.71E+04**	5.13E+01	**5.28E+04**	**8.53E+04**
3	1.00E-04	3-3-0	1.72	0.49	4.77	**1.72E+04**	5.01E+01	**3.43E+04**	
S1	1.00E-05	5-4-0	12.99	3.48	31.08	**1.30E+06**	5.04E+01	**2.58E+06**	**1.99E+06**
S2	1.00E-05	5-3-0	7.92	2.47	18.86	**7.92E+05**	5.13E+01	**1.54E+06**	

d)Matrix recovery calculation

Calculation of E.coli (CFU / mL) in USS (EC undilute spike)				
Replicate	**-5 plate**	**-6 plate**	**-7 plate**	***E.coli* CFU / mL**
1	TNTC	52	11	
2	177	19	5	**2.16E+07**
3	211	36	8	

1. Volume of USS per unit (g) of spiked biosolids sample	
Description of spiked sample	V spiked per unit biosolids
Class B solid	**1.00E-01**

2. Calculation of Spiked EC (wet weight)		
EC undiluted Spike (CFU/mL)	V spiked per unit biosolids	Spiked EC wet weight
1.09E+07	1.00E-01	**2.16E+06**

3. Conversion to true spiked EC CFU / g TS (DW)		
Description of spiked sample	% TS	True spiked EC CFU / g TS

Class B solid	5.09E+01	**4.25E+06**

4. Percent recovery				
Matrix	Ns	Nu	T	% Recovery
Class B solid	1.99E+06	8.53E+04	4.25E+06	**44.88**

F.4.3. Results at the end of Stage 2

a) LTB test

Sample	Dilution	Replicate				
		1	2	3	4	5
1	U1	1	1	1	1	1
	A	1	1	1	1	1
	B	1	1	1	1	0
	C	0	0	1	1	1
2	U1	1	1	1	1	1
	A	1	1	1	1	1
	B	0	1	0	1	1
	C	0	0	1	0	0
3	U1	1	1	1	1	1
	A	1	1	1	0	0
	B	1	1	1	0	0
	C	0	1	1	0	0
S1	A	1	1	1	1	1
	B	1	1	1	1	1
	C	1	1	1	1	1
	D	0	1	1	1	0
S2	A	1	1	1	1	1
	B	1	1	1	1	1
	C	1	1	1	0	1
	D	0	0	1	1	0

1-positive; 0-negative; S – spiked

b) EC test

Sample	Dilution	Replicate				
		1	**2**	**3**	**4**	**5**
1	U1	1	1	1	1	1
	A	1	1	1	1	1
	B	1	1	1	1	0
	C	0	0	1	1	1
2	U1	1	1	1	1	1
	A	1	1	1	1	1
	B	0	1	0	1	1
	C	0	0	1	0	0
3	U1	1	1	1	1	1
	A	1	1	1	0	0
	B	1	1	1	0	0
	C	0	1	1	0	0
S1	A	1	1	1	1	1
	B	1	1	1	1	1
	C	1	1	1	1	1
	D	0	1	1	1	0
S2	A	1	1	1	1	1
	B	1	1	1	1	1
	C	1	1	1	0	1
	D	0	0	1	1	0

1-positive; 0-negative; S – spiked

c) Matrix calculations

Sample	Largest volume tested	Positives	MPN Index mL	95% Lower CL	95% Upper CL	MPN/mL	% TS	MPN/g TS	Geometric mean
1	1.00E-02	5-4-1	17.24	4.29	49.75	**1.72E+03**	5.61E+01	**3.08E+03**	
2	1.00E-01	5-4-3	27.81	8.82	86	**2.78E+02**	5.51E+01	**5.04E+02**	**7.96E+02**
3	1.00E-01	5-3-3	17.5	4.34	51.31	**1.75E+02**	5.37E+01	**3.26E+02**	
S1	1.00E-03	5-4-0	12.99	3.48	31.08	**1.30E+04**	5.61E+01	**2.32E+04**	**1.82E+04**
S2	1.00E-03	5-3-0	7.92	2.47	18.86	**7.92E+03**	5.51E+01	**1.44E+04**	

d) Matrix recovery calculation

Calculation of E.coli (CFU / mL) in USS (EC undilute spike)				
Replicate	**-5 plate**	**-6 plate**	**-7 plate**	***E.coli* CFU / mL**
1	TNTC	83	15	
2	TNTC	54	3	**7.20E+07**
3	TNTC	79	7	

1. Volume of USS per unit (g) of spiked biosolids sample	
Description of spiked sample	V spiked per unit biosolids
Class B solid	**1.00E-04**

2. Calculation of Spiked EC (wet weight)		
EC undiluted Spike (CFU/mL)	V spiked per unit biosolids	Spiked EC wet weight
1.09E+07	1.00E-04	**7.20E+03**

3. Conversion to true spiked EC CFU / g TS (DW)		
Description of spiked sample	% TS	True spiked EC CFU / g TS

Class A solid	5.56E+01	**1.29E+04**

4. Percent recovery				
Matrix	Ns	Nu	T	% Recovery
Class A solid	1.82E+04	7.96E+02	1.29E+04	**134.73**

F.5 **Analysis of *Salmonella* spp. in Compost Matrix (USEPA 1682)**

F.5.1. Day – 0 results

a) MSRV test

Sample	Dilution	Replicate				
		A	**B**	**C**	**D**	**E**
1	20	+	+	+	+	+
	10	+	+	+	+	+
	1	+	+	+	+	+
2	20	+	+	+	+	+
	10	+	+	+	+	+
	1	+	+	+	+	+
3	20	+	+	+	+	+
	10	+	+	+	+	+
	1	+	+	+	+	+
S1	20	+	+	+	+	+
	10	+	+	+	+	+
	1	+	+	+	+	+
S2	20	+	+	+	+	+
	10	+	+	+	+	+
	S2-1	+	+	+	+	+
S3	20	+	+	+	+	+
	10	+	+	+	+	+
	1	+	+	+	+	+

S – spiked;

b) XLD test

Sample	Dilution	Replicate				
		A	**B**	**C**	**D**	**E**
1	20	-	-	+	+	+
	10	-	-	-	+	+
	1	-	-	+	-	-
2	20	-	+	+	+	+
	10	-	-	+	+	+
	1	-	-	-	+	+
3	20	-	-	-	+	+
	10	-	+	+	+	+
	1	-	-	-	-	-
S1	20	-	-	-	-	+
	10	-	-	-	+	+
	1	-	-	-	+	+
S2	20	-	+	+	+	+
	10	+	+	+	-	+
	S2-1	-	-	-	-	-
S3	20	+	+	+	+	+
	10	+	+	+	+	+
	1	+	-	-	+	+

S – spiked;

c) Bio-confirmation test

Sample	Dilution	Replicate				
		A	B	C	D	E
1	20	-	-	+	+	+
	10	-	-	-	+	+
	1	-	-	+	-	-
2	20	-	+	+	+	+
	10	-	-	+	+	+
	1	-	-	-	+	+
3	20	-	-	-	+	+
	10	-	+	+	+	+
	1	-	-	-	-	-
S1	20	-	-	-	-	+
	10	-	-	-	+	+
	1	-	-	-	+	+
S2	20	-	+	+	+	+
	10	+	+	-	-	+
	S2-1	-	-	-	-	-
S3	20	+	+	-	+	+
	10	-	+	-	+	+
	1	+	-	-	+	+

S – spiked; + are the samples which were positive in Bio-confirmation test as well as in VITEK assay

d) Matrix calculation

Sample	Positive tubes	MPN Index*	Lower 95%	Upper 95%
1	3-2-1	0.555	0.171	1.112
2	4-3-2	1.086	0.441	2.228
3	2-4-0	0.547	0.168	1.098
S1	1-2-2	0.379	0.085	0.795
S2	4-3-0	0.797	0.295	1.579
S3	4-3-3	1.245	0.52	2.656

*The MPN index is adjusted for 1:10 dilution factor of solid sample

e) MPN / 4g (dry weight) of compost matrix calculation

Sample	Volume of Homogenized sample used to inoculate TSB			MPN / mL (wet weight	% Total Solids	MPN / 4g (dry weight)	Lower 95%	Upper 95%
	20mL	10mL	1mL					
1	3	2	1	0.555	47.01	4.72	1.46	9.46
2	4	3	2	1.086	47.01	9.24	3.75	18.96
3*	2	4	0	0.547	47.01	4.66	1.43	9.34
S1	1	2	2	0.379	47.01	3.23	0.72	6.77
S2	4	3	0	0.797	47.01	6.78	2.51	13.44
S3	4	3	3	1.245	47.01	10.60	4.43	22.60

*Was subsequently used for matrix spiking

f) % recovery calculation

1. Calculations of *S. meleagridis* spiking suspension concentration				
Replicate	CFU / plate (triplicate analysis) from HIA plates			Salmonella CFU / mL in undiluted spiking suspension (S. undiluted spike)
	-6 plates	-7 plates	-8 plates	
1	53	9	0	5.40E+07
2	50	9	2	
3	59	6	1	

2. Volume of undiluted spiking suspension per unit (g) of spiked biosolids samples (Vspiked per unit biosolids)	
Description of spiked sample	V spiked (mL) per unit (g) biosolids wet weight
Class A solid	1.67E-08

3. Calculation of Spiked Salmonella wet weight		
Salmonella undiluted spike	V spiked	Spiked Salmonella (wet weight CFU / g)
5.40E+07	1.67E-08	9.02E-01

4. Conversion to true spiked Salmonella CFU / 4g TS (dry weight)		
Replicate	Total solids	True spiked Salmonella CFU / 4g dry weight
1	47.01	7.67E+00
2	47.01	7.67E+00
3	47.01	7.67E+00

5. Percent recovery			
S. MPN / 4g (dw) spiked sample	S. MPN / 4g (dw) unspiked sample	True spiked salomenlla CFU / 4g dw	Percent recovery (%R)
3.23	4.66	7.67E+00	-18.63
6.78		7.67E+00	27.72
10.60		7.67E+00	77.40

F.5.2. At the end of Stage – 1

a) MSRV test

Sample	Dilution	Replicate				
		A	B	C	D	E
1	20	+	+	+	+	+
	10	+	+	+	+	+
	1	+	+	+	+	+
2	20	+	+	+	+	+
	10	+	+	+	+	+
	1	+	+	+	+	+
3	20	+	+	+	+	+
	10	+	+	+	+	+
	1	+	+	+	+	+
S1	20	+	+	+	+	+
	10	+	+	+	+	+
	1	+	+	+	+	+
S2	20	+	+	+	+	+
	10	+	+	+	+	+
	S2-1	+	+	+	+	+
S3	20	+	+	+	+	+
	10	+	+	+	+	+
	1	+	+	+	+	+

S – spiked;

b) XLD test

Sample	Dilution	Replicate				
		A	**B**	**C**	**D**	**E**
1	20	-	-	-	-	-
	10	-	-	-	-	-
	1	-	-	-	-	-
2	20	-	-	-	-	-
	10	-	-	-	-	-
	1	-	-	-	-	-
3	20	-	-	-	-	-
	10	-	-	-	-	-
	1	-	-	-	-	-
S1	20	-	-	-	+	-
	10	-	-	-	+	-
	1	-	-	-	-	-
S2	20	-	+	+	+	+
	10	-	-	-	+	+
	S2-1	-	-	-	+	-
S3	20	-	-	-	+	-
	10	+	-	-	+	+
	1	+	+	-	-	-

S – spiked;

c) Bio-confirmation test

Sample	Dilution	Replicate				
		A	**B**	**C**	**D**	**E**
1	20	-	-	-	-	-
	10	-	-	-	-	-
	1	-	-	-	-	-
2	20	-	-	-	-	-
	10	-	-	-	-	-
	1	-	-	-	-	-
3	20	-	-	-	-	-
	10	-	-	-	-	-
	1	-	-	-	-	-
S1	20	-	-	-	+	-
	10	-	-	-	+	-
	1	-	-	-	-	-
S2	20	-	+	+	+	+
	10	-	-	-	+	+
	S2-1	-	-	-	+	-
S3	20	-	-	-	+	-
	10	+	-	-	+	+
	1	+	+	-	-	-

S – spiked;

d) Matrix calculation

Sample	Positive tubes	MPN Index*	Lower 95%	Upper 95%
1	0-0-0	6.47E-02	0.00E+00	2.23E-01
2	0-0-0	6.47E-02	0.00E+00	2.23E-01
3	0-0-0	6.47E-02	0.00E+00	2.23E-01
S1	1-1-0	1.44E-01	1.20E-02	3.77E-01
S2	4-2-1	7.48E-01	2.69E-01	1.48E+00
S3	1-3-2	4.75E-01	1.32E-01	9.67E-01

*The MPN index is adjusted for 1:10 dilution factor of solid sample

e) MPN / 4g (dry weight) of compost matrix calculation

Sample	Volume of Homogenized sample used to inoculate TSB			MPN / mL (wet weight	% Total Solids	MPN / 4g (dry weight)	Lower 95%	Upper 95%
	20mL	10mL	1mL					
1	0	0	0	0.06	50.40	0.51	0.00	1.77
2	0	0	0	0.06	51.30	0.50	0.00	1.74
3*	0	0	0	0.06	50.10	0.52	0.00	1.78
S1	1	1	0	0.14	50.40	1.14	0.10	2.99
S2	4	2	1	0.75	51.30	5.83	2.10	11.53
S3	1	3	2	0.48	50.10	3.79	1.05	7.72

*Was subsequently used for matrix spiking

f) % recovery calculation

1. Calculations of *S. meleagridis* spiking suspension concentration				
Replicate	CFU / plate (triplicate analysis) from HIA plates			Salmonella CFU / mL in undiluted spiking suspension (S. undiluted spike)
	-6 plates	-7 plates	-8 plates	
1	154	47	5	2.45E+07
2	217	64	8	
3	TNTC	81	8	

2. Volume of undiluted spiking suspension per unit (g) of spiked biosolids samples (Vspiked per unit biosolids)	
Description of spiked sample	V spiked (mL) per unit (g) biosolids wet weight
Class A solid	1.67E-08

3. Calculation of Spiked Salmonella wet weight		
Salmonella undiluted spike	V spiked	Spiked Salmonella (wet weight CFU / g)
2.45E+07	1.67E-08	4.09E-01

4. Conversion to true spiked Salmonella CFU / 4g TS (dry weight)		
Replicate	Total solids	True spiked Salmonella CFU / 4g dry weight
1	5.04E+01	3.24E+00
2	5.13E+01	3.19E+00
3	5.01E+01	3.26E+00

5. Percent recovery			
S. MPN / 4g (dw) spiked sample	S. MPN / 4g (dw) unspiked sample	True spiked salomenlla CFU / 4g dw	Percent recovery (%R)
1.14	5.14E-01	3.24E+00	19.39
5.83	5.05E-01	3.19E+00	166.86
3.79	5.17E-01	3.26E+00	100.46

F.5.3. At the end of Stage – 2

a) MSRV test

Sample	Dilution	Replicate				
		A	**B**	**C**	**D**	**E**
1	20	+	+	+	+	+
	10	+	+	+	+	+
	1	+	+	+	+	+
2	20	+	+	+	+	+
	10	+	+	+	+	+
	1	+	+	+	+	+
3	20	+	+	+	+	+
	10	+	+	+	+	+
	1	+	+	+	+	+
S1	20	+	+	+	+	+
	10	+	+	+	+	+
	1	+	+	+	+	+
S2	20	+	+	+	+	+
	10	+	+	+	+	+
	S2-1	+	+	+	+	+
S3	20	+	+	+	+	+
	10	+	+	+	+	+
	1	+	+	+	+	+

S – spiked;

b) XLD test

Sample	Dilution	Replicate				
		A	**B**	**C**	**D**	**E**
1	20	-	-	-	-	-
	10	-	-	-	-	-
	1	-	-	-	-	-
2	20	-	-	-	-	-
	10	-	-	-	-	-
	1	-	-	-	-	-
3	20	+	-	-	-	+
	10	+	-	+	-	+
	1	-	-	-	-	-
S1	20	-	+	-	+	-
	10	-	-	-	-	-
	1	-	-	-	-	-
S2	20	-	-	-	+	-
	10	+	-	-	+	-
	S2-1	-	-	-	-	-
S3	20	-	+	+	+	+
	10	-	-	+	+	+
	1	+	+	-	+	+

S – spiked;

c) Bio-confirmation test

Sample	Dilution	Replicate				
		A	B	C	D	E
1	20	-	-	-	-	-
	10	-	-	-	-	-
	1	-	-	-	-	-
2	20	-	-	-	-	-
	10	-	-	-	-	-
	1	-	-	-	-	-
3	20	+	-	-	-	+
	10	+	-	+	-	+
	1	-	-	-	-	-
S1	20	-	+	-	+	-
	10	-	-	-	-	-
	1	-	-	-	-	-
S2	20	-	-	-	+	-
	10	+	-	-	+	-
	S2-1	-	-	-	-	-
S3	20	-	+	+	+	+
	10	-	-	+	+	+
	1	+	+	-	+	+

S – spiked;

d) Matrix calculation

Sample	Positive tubes	MPN Index*	Lower 95%	Upper 95%
1	0-0-0	0.06	0.00	0.22
2	0-0-0	0.06	0.00	0.22
3	2-3-0	0.43	0.11	0.89
S1	2-0-0	0.16	0.01	0.40
S2	1-2-0	0.22	0.02	0.52
S3	4-3-4	1.41	0.60	3.22

*The MPN index is adjusted for 1:10 dilution factor of solid sample

e) MPN / 4g (dry weight) of compost matrix calculation

Sample	Volume of Homogenized sample used to inoculate TSB			MPN / mL (wet weight	% Total Solids	MPN / 4g (dry weight)	Lower 95%	Upper 95%
	20mL	10mL	1mL					
1	0	0	0	0.06	56.06	0.46	0.00	1.59
2	0	0	0	0.06	55.15	0.47	0.00	1.62
3*	2	3	0	0.43	53.73	3.21	0.82	6.60
S1	2	0	0	0.16	56.06	1.11	0.09	2.88
S2	1	2	0	0.22	55.15	1.62	0.12	3.79
S3	4	3	4	1.41	53.73	10.53	4.48	23.96

*Was subsequently used for matrix spiking

f) % recovery calculation

1. Calculations of *S. meleagridis* spiking suspension concentration				
Replicate	CFU / plate (triplicate analysis) from HIA plates			Salmonella CFU / mL in undiluted spiking suspension (S. undiluted spike)
	-6 plates	-7 plates	-8 plates	
1	TNTC	45	2	5.17E+07
2	TNTC	61	5	
3	TNTC	49	11	

2. Volume of undiluted spiking suspension per unit (g) of spiked biosolids samples (Vspiked per unit biosolids)	
Description of spiked sample	V spiked (mL) per unit (g) biosolids wet weight
Class A solid	1.67E-08

3. Calculation of Spiked Salmonella wet weight		
Salmonella undiluted spike	V spiked	Spiked Salmonella (wet weight CFU / g)
5.17E+07	1.67E-08	8.63E-01

4. Conversion to true spiked Salmonella CFU / 4g TS (dry weight)		
Replicate	Total solids	True spiked Salmonella CFU / 4g dry weight
1	5.61E+01	6.16E+00
2	5.51E+01	6.26E+00
3	5.37E+01	6.42E+00

5. Percent recovery			
S. MPN / 4g (dw) spiked sample	S. MPN / 4g (dw) unspiked sample	True spiked salomenlla CFU / 4g dw	Percent recovery (%R)
1.11E+00	4.62E-01	6.16E+00	10.46
1.62E+00	4.69E-01	6.26E+00	18.58
1.05E+01	3.21E+00	6.42E+00	156.69