INACTIVATION OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* AND PROFILES OF MICROBIAL COMMUNITIES DURING COMPOSTING OF LIVESTOCK MORTALITIES

BY

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A Thesis
Submitted to the Faculty of Graduate Studies of
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MASTER'S THESIS/PRACTICUM FINAL REPORT

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DEDICATION

I would like to dedicate this thesis to my Dad. Your love and respect of animals and the land was passed onto Cassie, Christine and I. Although you may have not imagined a life in agriculture for us, your legacy lives on through everything we do. I love you and miss you more with each passing day.

ABBREVIATIONS

ACE	Abundance-based coverage estimator
BRP	Baker retrieval pyramid
C	Carbon
EC	Electrical conductivity
JD	Johne's disease
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
N	Nitrogen
OM	Organic matter
OTU	Operational taxonomic unit
RDP	Ribosomal database project
VFA	Volatile fatty acids
a_w	Water activity

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ABSTRACT

Tkachuk, Victoria Louise. M.Sc., The University of Manitoba, ----- 2013. Inactivation of *Mycobacterium avium* subspecies *paratuberculosis* and profiles of microbial communities during composting of livestock mortalities. Advisors: K.H. Ominski and T.A. McAllister.

This study explored the use of a biosecure, static composting structure to inactivate MAP. In Experiment #1, it was concluded that composting is unlikely to achieve temperatures necessary to inactivate MAP associated with cattle mortalities and that *M. smegamatis* is an unlikely surrogate for MAP. This study also used the same system to explore changes in the microbial community in mortality compost after exposure to thermophilic temperatures. As high-throughput sequencing technologies advance, it is possible to characterize microbial communities in environments with a high degree of resolution. In Experiment #2, as members of *Clostridia* were present at temperatures > 55°C, it appears that anaerobic conditions existed within regions of the compost. Extreme temperatures and non-homogeneous high moisture conditions resulted in spatial distribution of temperature in a biosecure, static composting system, which failed to meet conditions necessary for complete composting and pathogen reduction.

FOREWORD

This thesis is written in manuscript style, with each manuscript having its own abstract, introduction, materials and methods, results and discussion sections. There is a general introduction and review of the literature prior to the manuscripts, which are followed by a general discussion and conclusions, and literature cited.

1.0 GENERAL INTRODUCTION

Carcass disposal presents many challenges for livestock producers including potential biosecurity risks, possible contamination of feed and depending on the method, expense. Composting, when compared with other mortality disposal methods such as burial, incineration and rendering, can be an environmentally sustainable and inexpensive strategy to turn waste, such as mortalities or manure, into an available source of nutrients for plants. In addition, several studies have demonstrated effective elimination of many pathogens, including *Escherichia coli* (O157:H7), *Campylobacter jejuni* and *Salmonella* spp. (Larney et al. 2003; Grewal et al. 2005; Xu et al. 2009), during composting. However, to the knowledge of the author, no work has assessed the survival of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) during static, biosecure composting initiated at sub-zero temperatures.

Johne's disease (JD) in cattle, as a result of MAP, is difficult to diagnose and irradicate due to the asymptomatic nature of the disease in subclinical stages of infection (McKenna et al. 2005), and its ability to survive in the environment for extended periods (Lovell et al. 1944; Whittington et al. 2004). The disease has a long incubation period of 2-10 years within the animal and requires up to 16 weeks to culture in a laboratory (Whitlock and Buergelt 1996). Tests used to detect early infection, such as milk or serum ELISA or fecal culture, do not have sufficient specificity or sensitivity to detect animals shedding MAP intermittently at low levels through the milk and feces. This has resulted in poor estimates of actual infection rates within Canada (McKenna et al. 2005; Tiwari et al. 2006). A recent review found that about 3% of dairy cattle across Canada are positive

for MAP, but it is estimated that 50% of dairy herds have at least one MAP-infected animal (Tiwari et al. 2006; VanLeeuwen et al. 2006). Significant economic losses to the producer are estimated to be approximately $50 CAD per head per year for dairy (Chi et al. 2002), however costs may be much higher as these prevalence rates are derived using serum ELISA. In addition, MAP has been shown to survive in the environment for up to 385 d (Lovell et al. 1944; Whittington et al. 2004) suggesting that it utilizes a dormancy mechanism (Whittington et al. 2004) such as sporulation (Lamont et al. 2012). Consequently, cattle can be continuously exposed to this pathogen from their surroundings, further contributing to infection and economic losses.

Some studies have suggested that MAP may be linked to Crohn's disease in humans (Chiodini et al. 1984; Hermon-Taylor et al. 1998; Waddell et al. 2008), however, there is no medical consensus regarding this causal association. Studies have demonstrated survival of MAP in milk and milk products following pasteurization (Gao et al. 2002; Hammer et al. 2004; Ellingson et al. 2005; Cerf et al. 2007; Rademaker et al. 2007; Foddai et al. 2010), suggesting that this may serve as a likely route of transmitting MAP from cattle to humans. Voluntary control programs have already been implemented in the United States (United States Department of Agriculture 2010) and Australia (Kennedy and Allworth 2000) and proposed within Canada (McKenna et al. 2006). These programs may become more stringent if a zoonotic connection with Crohn's disease is established.

In addition to assessing the survival of pathogens during mortality composting, there has been increasing interest in characterizing the composting microbial community for a more complete understanding of the composting process. Previous studies

investigating the overall microbial ecosystem of compost used low-throughput, low-resolution, culture-independent techniques such as terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) (Tiquia 2005; Yamamoto et al. 2009; Partanen et al. 2010). These techniques generally only identified the most abundant organisms, providing an initial but not a thorough estimate of overall microbial diversity. To date, a limited number of studies have used high-throughput sequencing to characterize the whole microbial community of compost (de Gannes et al. 2013; Martins et al. 2013), however no work has assessed microbial communities during the composting of livestock mortalitities.

Currently, 454-pyrosequencing has the ability to generate large datasets, with over one million reads per run and an average read length of 700 bp (Glenn 2011; 454 Life Sciences 2013). With higher sequence counts, more microbial biodiversity is accounted for within a selected environment compared with any culture-based or conventional DNA sequencing technique (i.e. Sanger sequencing; Petrosino et al. 2009). The reduced cost and higher resolution of 454-pyrosequencing have made it an attractive sequencing methodology for microbial ecologists when attempting to characterize and estimate which microorganisms are present. Given the variety of initial compost substrates, (Maeda et al. 2010; Partanen et al. 2010; Martins et al. 2013), differences in management including, degree of mixing the compost, (Partanen et al. 2010; de Gannes et al. 2013) and the methodology used to identify different microorganisms in compost (Yamamoto et al. 2009; Maeda et al. 2010; de Gannes et al. 2013), little is known about the microbial communities in mortality compost at mesophilic temperatures or after exposure to thermophilic temperatures.

Recently, a biosecure, static composting system for large-scale cattle disposal was developed (Xu et al. 2009). This system demonstrated that several bacterial (*Campylobacter jejuni* and *E. coli* O157:H7) and viral (Newcastle disease virus) pathogens were rendered non-viable by composting, but MAP was not included in the tests (Xu et al. 2009). Further, there is an emerging need to know which microorganisms are present, including pathogens, to understand how composting conditions (i.e. temperature, moisture, pH, etc.) influence microbial abundance.

The objectives of the first study were to investigate the length of time required to: i) eliminate MAP in static biosecure compost piles which were initiated during subzero temperatures, ii) determine whether MAP organisms reared from lab cultures differ from those in naturally-infected tissues in terms of length of survival, iii) examine if organism survival is a consequence of the compost environment or high temperature and iv) determine survival of *Mycobacterium smegmatis* as a surrogate to MAP, as it is a fast-growing, non-pathogenic species of mycobacteria (Chaturvedi et al. 2007). Using the same system in a second study, the samples were analyzed with 454-pyrosequencing to: i) characterize the microbial community taxonomically and phylogenetically and ii) estimate overall richness and diversity within mortality compost in sites selected for analysis on the basis of either mesophilic or thermophilic temperature exposure.

2.0 LITERATURE REVIEW

2.1 Introduction

Carcass disposal is an issue for livestock producers as it presents many challenges including, biosecurity risks depending on cause of death and depending on the method of disposal, expense. Composting is one of four approved methods, including burial, incineration and rendering, for on-farm disposal of mortalities in Canada. Composting can be an effective way to eliminate pathogens (Senne et al. 1994; Grewal et al. 2005; Xu et al. 2009) and is environmentally sound, turning waste products, such as livestock mortalities or manure into a source of nutrients for plants.

One pathogenic bacterium whose survival rate is unknown during composting is *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the cause of Johne's disease (JD) in ruminants. Prevalence rates of JD are often underestimated, as it is difficult to detect an early MAP infection. In addition, if infected animals are sold or culled from a herd as a result of decreased productivity, they may serve as a reservoir of the pathogen to infect other animals, perpetuating the disease. Finally, as it is speculated that there may be an association between MAP and Crohn's disease in humans (Chiodini et al. 1984; Hermon-Taylor et al. 1998), sale of milk or meat products from MAP-infected animals may enable this pathogen to enter the food chain, increasing the risk of exposure to humans.

In recent years, the area of metagenomics to explore microbial environments has rapidly expanded due to advances in high-throughput sequencing technology. A technique called pyrosequencing now allows us to sequence hundreds of thousands of

DNA sequences. Initially developed for use in clinical medicine, the functionality of this process can now be expanded to any microbial environment.

Pyrosequencing has recently been used to characterize the composting environment. However, to the knowledge of the authors, no studies have examined the microbial community of livestock mortality compost and further, little is known about the mechanisms of succession of the microbial community in this environment. Several studies have compared the succession mechanisms in macro-communities to micro-communities but more research is required for a complete understanding of this process in compost.

2.2 Johne's disease (JD)

Johne's disease (JD) is a chronic, progressive granulomatous enteritis in cattle caused by the bacterium *Mycobacterium avium* subspecies *paratuberculosis* (MAP) (Kahn and Line 2008). This disease is characterized by persistent diarrhea, weight loss and severe loss of body condition in late phases of advanced clinical infection (Whitlock and Buergelt 1996; Kahn and Line 2008). Similar infectious enteritis conditions have been observed in other domestic and wild ruminant species, such as sheep, goats, llamas, bison, alpacas and deer as well as rabbits, foxes, weasels and nonhuman primates (Chiodini et al. 1984a; Whitlock and Buergelt 1996; Kahn and Line 2008).

2.2.1 Stages of infection

Infection with MAP causing JD is characterized by the following four stages: 1) silent infection, 2) subclinical infection, 3) clinical infection and 4) advanced clinical infection, which are differentiated based on severity of clinical symptoms, potential

shedding of MAP into the environment and ease in which the organism can be detected (Whitlock and Buergelt 1996).

The first phase of infection with MAP is the silent infection stage and as the name implies exhibits no visual clinical symptoms, with no way to measure the effects of infection on animals and no cost-effective diagnostic test to detect the disease (Whitlock and Buergelt 1996). Infection with MAP has been confirmed to occur in young animals, as they are more susceptible to disease in utero (Whittington and Windsor 2009), shortly after birth and until two years of age (Larsen et al. 1975). Infection occurs through ingestion of contaminated milk, feed, colostrum or water but animals are most likely to acquire infection via the fecal-oral route from feces of other infected animals (Sweeney 1996). The only accurate diagnostic technique in the silent stage of infection is through culture of MAP from the intestinal tract or through histological observation of granulomatous regions in the intestine or mesenteric lymph nodes (Tiwari et al. 2006). This test can become quite costly with surgical biopsy of targeted tissues, especially if many animals require testing. Fecal culture, which is the most commonly used on-farm diagnostic technique, has been used to identify MAP in animals as young as one month of age (McDonald et al. 1999). However, this may simply be a consequence of the young animal passing the ingested organisms without establishment in the digestive tract (de Lisle et al. 1980b; Sweeney et al. 1992). Samples which are deemed to be false negative using fecal culture may occur as a consequence of intermittent shedding of the organism below culturable levels (de Lisle and Duncan 1981). Testing methods (fecal culture, ELISA, etc.), other than tissue culture, even when used in combination with other techniques to increase the specificity for MAP, are still poor indicators in the silent stage

and therefore make positive verification of infected individuals extremely challenging (McDonald et al. 1999).

Subclinical infection occurs in the second stage of JD infection. Animals appear healthy without typical symptoms of JD, but may be shedding MAP intermittently in feces, and may possess antibodies against MAP or have a detectable altered cellular immune response (Whitlock and Buergelt 1996). Animal variation, in rates of disease progression and organism shedding, as well as dilution of MAP in intestinal contents, can result in false negative samples (de Lisle et al. 1980a). Animals that do test positive may be removed from the herd, however, animals with false-negative tests, may remain in the herd and progress to the next stage of JD, while continuously shedding MAP into the environment and spreading the disease (Whitlock and Buergelt 1996).

The third stage of infection, called clinical infection, follows a prolonged incubation period of 2-10 years after which clinical symptoms of JD are observed (Whitlock and Buergelt 1996). Symptoms are rarely seen in animals less than two years of age and when present include gradual wasting with decreased milk production and diarrhea that may be either persistent or intermittent (Whitlock and Buergelt 1996). However, vital signs such as heart rate, respiratory rate and temperature remain normal (Whitlock and Buergelt 1996). At this stage, most infected animals will consistently test positive through fecal culture and also have increased antibody levels to MAP as detected by rapid, low-cost serologic tests, such as enzyme-linked immunosorbant assay (ELISA) (Whitlock et al. 2000) and agar gel immunodiffusion (AGID) tests (Sherman et al. 1990).

The fourth stage in JD progression is advanced clinical infection. In general, most animals do not progress to this stage because they are most likely culled for other

production related reasons (Whitlock and Buergelt 1996). If animals do advance to this stage, symptoms may include weakness, lethargy, emaciation, intermandibular edema and “pipestream” diarrhea. Death occurs a short time later as a result of dehydration and cachexia (Whitlock and Buergelt 1996).

2.2.2 Ante-mortem detection methods

A key challenge facing producers and food safety officials is the development of an on-farm testing strategy to control and prevent MAP. The first obstacle includes lack of a 100% accurate reference test to consistently detect MAP at any stage of infection. Reference tests for MAP increase in sensitivity and specificity in detection as the number of organisms shed in the feces increases, but only as the animal progresses through the disease process. The second obstacle is cost, which is most often the limiting factor for any on-farm detection technique.

Ante-mortem tissue culture for MAP is often considered the gold standard for a definitive diagnosis. Even in latent stages of infection, sections of terminal ileum and ileocecal lymph nodes show evidence of MAP prior to shedding the bacterium in the feces or the presence of a detectable immune response (McKenna et al. 2004). However, it is suggested that sampling of regional lymph nodes and intestinal mucosa may not be sufficient to confirm a true infection status of the animal due to the commensal nature of the organism (Whitlock and Buergelt 1996). As immune responses to commensal intestinal bacteria are recirculated in the mesenteric arteries and lymph nodes, the mucosal immunity is kept separate from systemic immunity (Macpherson et al. 2005). It is for this reason, Whitlock et al. (1996) propose that up to 100 sites throughout the body

are needed to confirm infection, consequently sacrificing the animal and increasing the cost of a diagnosis.

Fecal culture is the most common test method used for MAP diagnosis. This method has also been considered the standard diagnostic technique due to the difficulties and cost associated with tissue culture (Sweeney et al. 1995; Dargatz et al. 2001; McKenna et al. 2005; McKenna et al. 2005; Tiwari et al. 2006). Although this method does not offer the degree of sensitivity of tissue culture, it is approaching 100% accuracy in terms of its ability to identify subclinical infection (Whitlock et al. 2000). Animals may be tested individually, but due to a cost of $15-$60 per sample (Tiwari et al. 2006; National Institute for Animal Agriculture 2012), and a lengthy incubation of 8-16 weeks, pooling of fecal samples may be more appropriate. If a pooled sample tests positive, each animal used to make up the composite can then be tested individually. A study by Vialard et al. (1993) estimated that herd sensitivities of pooled fecal samples decreased more than 60% when compared to individual culturing. However, when samples are strategically grouped together according to age, pooled samples may have a herd sensitivity of 73% (Kalis et al. 2000). Although this is not a significant increase compared to individual fecal culture, which possesses a sensitivity of 64%, the primary purpose of this pooling strategy is to screen individuals previously thought to be uninfected while reducing the cost involved with testing large herds (Kalis et al. 2000).

With advances in molecular techniques, it is now possible through PCR amplification of specific regions of the MAP genome, to differentiate between closely related species of mycobacteria. The insertion sequence, IS900, is a highly specific region of MAP DNA that is often coupled with tissue or fecal culture to verify MAP

growth. Performing PCR directly from a sample significantly reduces the time required to generate results (less than 8-16 weeks for culturing). Disadvantages of this approach include an inability to distinguish between viable and non-viable cells and the presence of high concentrations of inhibitory substances within feces that may limit detection, especially if low or intermittent numbers of MAP are being shed.

Enzyme linked immunosorbant assay (ELISA) is another approach that is used to measure antibodies to MAP in blood or milk. It is preferred for on-farm use due to the ease of sample collection, rapid test results and low cost ($8-10) per sample (Tiwari et al. 2006; National Institute for Animal Agriculture 2012). However results from ELISA should be interpreted with caution. Whitlock et al. (2000) found that the sensitivity of this test ranged from 15-75% and was positively correlated with number of organisms shed in the feces, thereby generating false-negatives if the number of organisms is low. Another concern is potential of cross reactivity with other organisms. It was estimated that the specificity of this test is 96.8% for all mycobacteria, which would increase the number of false positives in an uninfected herd, if not coupled with a bacteria species-specific diagnostic technique (Dargatz et al. 2001). Without symptoms of disease, it is important to verify positive results obtained with ELISA using another diagnostic test such as fecal culture. If fecal culture tests are negative, each animal that tested positive should be retested every 6-12 months (Tiwari et al. 2006).

Development of a single ante-mortem test is the most challenging aspect in detection of subclinical JD infection. Each test that is commercially available has benefits but cannot stand-alone for a definitive result. That is why tests are used in combination in order to increase the sensitivity and confidence of detection at every stage of infection.

2.2.3 Prevalence of JD

In order to develop and implement prevention and control strategies for JD, the prevalence of disease within a region and its associated economic impact should be considered.

With the increased use of animal health issues as non-tariff trade barriers, the incidence of JD has now caught the attention of policy-makers (McKenna et al. 2006; Furber 2010). In Canada, the Canadian Food Inspection Agency (CFIA) considers JD to be an annually notifiable disease, in which a report indicating presence within the country is submitted to the World Organization for Animal Health (OIE) (Canadian Food Inspection Agency 2010). This classification was chosen because according to the OIE, JD is a List B disease defined as: "Transmissible diseases that are considered to be of socio-economic and/or public health importance within countries and that are significant in the international trade of animals and animal products" (World Organization for Animal Health 2005). Concern arises as some international animal health agencies have contemplated banning importation of animals from exporting countries without JD control programs (McKenna et al. 2006; Furber 2010). To date, Canada does not have a national control program but if a ban occurs, mandatory programs will be implemented in Canada in order to allow the continued export of cattle and cattle products. In the interim, surveillance of disease prevalence and economic impact should continue.

Accurate assessment of the prevalence of JD presents many challenges as a consequence of the difficulties associated with detecting early infection and the testing methodologies. In the Maritime Provinces, a study conducted by McKenna et al. (2004) tested culled dairy cattle for MAP by culturing tissues. They found that 16.1% of the

sampled dairy animals were culture-positive for MAP. This value is likely a close estimate of true prevalence in the culled population due to the high accuracy of tissue culture techniques compared to fecal culture approaches (Tiwari et al. 2006).

Prevalence rates may be reported at herd level or as true prevalence rates. Herd level prevalence rates are based on the sero-prevalence of one (lenient definition) or two (restrictive definition) positive individual animal samples. Actual true prevalence rates are difficult to determine with available testing methods, so estimates are calculated by correcting for sensitivity and specificity of each test method (McKenna et al. 2005). Several studies have been conducted throughout Canada to determine true prevalence using a serum ELISA test. In all studies, a herd was infected if at least two animals were seropositive for JD. This value was then utilized to estimate true prevalence rates of 16.7% in Nova Scotia, Prince Edward Island and New Brunswick (VanLeeuwen et al. 2001), 24.2% in Saskatchewan (Waldner et al. 2002), 30% in Ontario (Hendrick et al. 2005), 40% in Alberta and as high as 43.1% in Manitoba (VanLeeuwen et al. 2006). Although these values focus on prevalence within Canada, it is important to note that other studies have demonstrated that JD can be found worldwide. A review conducted by Nielsen and Toft (2009) examined prevalence rates of MAP-infected animals throughout Europe. They concluded that no country could claim to be MAP-free due to insufficient information and lack of consistency in analytical methodology (Nielsen and Toft 2009).

Although these estimates of prevalence are valuable to decision makers, it is important to interpret them with caution. Factors such as differences in method to assess infectivity (i.e. herd or individual level), sample size, testing method and lack of

consensus on what level of specificity or sensitivity is adequate to calculate true prevalence rates (Tiwari et al. 2006), all contribute to uncertainity of prediction.

2.2.4 Economic impact of JD

Several studies have been conducted to estimate the economic losses associated with JD including losses related to subclinical infection (Benedictus et al. 1987; Ott et al. 1999; Chi et al. 2002; Tiwari et al. 2008). Costs with a direct impact on animal productivity include those associated with decreased milk yield, increased culling and mortality, decreased fertility and slaughter value, as well as increased incidence of mastitis have been evaluated (Chi et al. 2002; McKenna et al. 2006; Tiwari et al. 2008). Prevalence rates have most often been estimated using serum ELISA. These studies have also assumed that the impact of ELISA false negative animals to be small or null (Tiwari et al. 2008), which may serve to underestimate total losses. A decade ago, Chi et al. (2002) estimated that in the Maritime provinces losses were about $50 CAD per animal, associated with reduced milk production, premature culling, mortality, replacement costs and treatment costs (i.e., veterinary services, medication and extra labour costs). More recently, a study of two large dairy operations in Minnesota estimated losses of $441 USD per animal, based on direct production losses described in previously mentioned studies, as well as the cost of maintaining MAP-infected animals within their herds (Raizman et al. 2009). It should be noted that if estimates of true prevalence rates are not accurately calculated, the associated economic losses also cannot be measured, resulting in an inaccurate accrual of the financial losses due to the disease.

2.2.5 Treatment and vaccination

Antimicrobial treatment of MAP infections is possible but not practical. There are a wide variety of antimicrobial drugs available to treat this disease, but challenges associated with resistance and high cost, make available treatment methods economically impractical.

A comprehensive evaluation conducted by Krishnan et al. (2009) examined the effect of 11 different antimicrobial drugs on MAP. They found that MAP was most susceptible to the macrolides, azithromycin and clarithromycin, whereas ethambutol, isoniazid, rifampicin, dapsone and clofazimine had limited activity against MAP, but have been used historically. Although this study was conducted *in vitro* and provides some guidance regarding an appropriate choice of antibiotic, the results should be interpreted cautiously as often *in vitro* tests with MAP are poor predictors of antibiotic efficacy *in vivo* (Heginbothom 2001). Even if these antibiotics have the potential to treat MAP, most have not been approved in Canada for use in food-producing animals.

Another reason JD treatment is not considered by producers is due to the high cost and efficacy of the drugs. Further, a combination of antibiotics administered daily over a six-month period reduced the expression of clinical symptoms but animals were still positive by fecal culture (Larsen and Vardaman 1953). This indicates that animals cannot be effectively cured, making treatment economically impractical for most producers (Emery and Whittington 2004).

Vaccination is another strategy for on-farm prevention and control however there are several challenges that impede its use in Canada. Firstly, research has demonstrated that MAP vaccines simply delay onset of clinical symptoms (Rosseels and Huygen 2008). Use of either killed or modified live vaccines serve to substantially reduce

shedding of MAP in feces, but does not completely halt shedding (Rosseels and Huygen 2008). A commercially available, killed vaccine for MAP called Mycopar (Fort Dodge Animal Health, Overland Park, Kansas, USA), is currently used in the USA (Spangler et al. 1991) and is only licensed for use in calves less than 30 days old. The vaccine is not recommended for use in adult animals. As the chance of contracting JD in animals over two years of age is substantially reduced, the vaccine is only used to reduce the number of live MAP organisms being shed in herds with high disease prevalence (Rosseels and Huygen 2008). As these vaccines do not completely prevent and eliminate the disease, increases in the number of clinical cases will ultimately be observed following cessation of vaccine use (Emery and Whittington 2004).

Vaccine use and development is also limited as animals develop antibodies that are cross-reactive with other species of mycobacteria (Rosseels and Huygen 2008) including *Mycobacterium bovis*, which causes Bovine Tuberculosis (TB). As TB is a reportable disease in Canada, testing of all animals imported into the country with a *M. bovis* purified protein derivative (PPD) intradermal skin test (Kohler et al. 2001) is required. Research by Kohler et al. (2001) demonstrated that two years after vaccination with a MAP vaccine, reactivity with the *M. bovis* PPD test was still positive, indicating false-positive results for TB infection (Kohler et al. 2001). The high homology between these species of *Mycobacterium* demonstrates the need for higher antigen specificity when developing a vaccine for MAP. More recently, immunization with a recombinant MAP heat shock protein 70 (Hsp70) significantly reduced shedding of MAP through the feces (Koets et al. 2006; Keeble and Walker 2009) and animals did not test positive for TB (Santema et al. 2009). This subunit vaccine shows promise, but more research is

required to investigate whether *M. bovis* contains similar recombinant Hsp70 proteins (Santema et al. 2009).

2.3 *Mycobacterium avium* subspecies *paratuberculosis* (MAP)

2.3.1 Pathogenesis and survival mechanisms

Mycobacterium avium subspecies *paratuberculosis* is an intracellular pathogen that becomes established within the intestinal tract of most ruminant species. Within the host, MAP is an opportunistic pathogen with the ability to modulate host-cell responses to maintain a persistent intracellular infection. Outside the host, the presence of a cell wall structure combined with an ability to enter a dormant state and persist in protozoa (Mura et al. 2006), enables MAP to remain infective for extended periods, contributing to perpetuation of the disease within the herd.

In order to replicate and become pathogenic, MAP requires a host. As mentioned previously, animals acquire MAP through ingestion of feces from contaminated feed or water (Sweeney 1996). Once consumed, MAP penetrates the epithelial layer of the ileum via M cells, which translocate it to the Peyer's patches of the mucosa without degradation (Momotani et al. 1988; Sigurðardóttir et al. 2004). In early infection, MAP survives within subepithelial macrophages in the Peyer's patches where it inhibits maturation of the developing phagosome and resists microbicidal enzymes that impede its proliferation (Sigurðardóttir et al. 2004). In addition, MAP has the ability to up-regulate genes to utilize the iron-chelating substance mycobactin within the macrophage, a prerequisite for iron metabolism by MAP *in vivo* (Zhu et al. 2008). Various immunological chemicals are released that promote aggregation of macrophages and the formation of inflamed

granulomatous lesions found in the gut-associated lymphoid tissues of the intestinal tract (Sigurðardóttir et al. 2004). It is believed that the high concentration of chemicals released from macrophages and a reduction in the sloughing of intestinal mucosa results in the thickened, corrugated appearance typical of MAP-infected tissues (Coussens et al. 2005). As infection progresses into latter stages of clinical disease, circulating immunological agents (T-lymphocytes) become unresponsive to any further stimulation to the MAP antigen (Bassey and Collins 1997).

Within the host, MAP can modulate host-cell responses that promote its persistence as an intracellular pathogen. Outside the host, MAP has also adapted three main functions that aid in its persistence. Firstly, the thick, lipid-rich cell wall structure of MAP resists disinfection, UV radiation and heat. Common disinfectants, such as chlorine, are ineffective against MAP at the standard concentrations used for water treatment (Rowe and Grant 2006; Manning et al. 2008). The cell wall is also responsible for the extreme heat tolerance observed in MAP. Several studies examining the viability of MAP in milk were able to recover it after pasteurization (Gao et al. 2002; Ellingson et al. 2005; Foddai et al. 2010). Further, varying heat treatment regimes have resulted in a significant reduction, but not elimination of viable MAP in milk (Rademaker et al. 2007). Donaghy et al. (2009) examined UV treatment of milk as an alternative to pasteurization. They demonstrated a reduction in the number of viable bacteria in milk, but concluded that this treatment could not completely eliminate MAP.

The second adaptive function is MAP's ability to achieve a dormant state. Studies of MAP survival in soil and manure, first studied in 1944, demonstrated its persistence outdoors for 246 days (Lovell et al. 1944). This research showed that desiccation of MAP

in soil and manure had the greatest impact on viability, but other factors including exposure to sunlight, low iron content and changes in pH may also reduce viability. A similar study by Whittington et al. (2004) looked at those parameters and reported that UV radiation, soil pH, moisture and soil iron content were all unlikely to have an effect on MAP survival, as it was recovered from feces mixed with soil after 385 d under various environmental conditions. They suggested that MAP possesses genes that confer dormancy (defined as a non-spore forming state permitting survival; Whittington et al. 2004), but that the bacterium could later revert back to a pathogenic form. An *in silico* analysis demonstrated that the MAP genome contains dormancy genes that are similar to those that code for resistance to nutritional and oxidative stress in other species of mycobacteria (i.e. *M. smegmatis, M. bovis* and *M. tuberculosis*) (Whittington et al 2004). However, it was concluded that the exact physiological mechanism triggering dormancy was unclear. Sporulation as opposed to dormancy has also been theorized. A MAP spore-like morphotype was produced that possessed the MAP genotype, infected bovine macrophages and remained viable after exposure to heat treatment at 70˚C and post exposure to lysozyme and proteinase K (Lamont et al. 2012). It was concluded that more research was needed to further evaluate the MAP spore-like morphotype in soil, water and post pasteurization (Lamont et al. 2012).

The third adaptive function aiding MAP survival outside the host involves evolutionary adaptation for intracellular growth and survival within free-living protists from soil and water (Finlay and Fenchel 2004). It has been shown that closely related mycobacterial species have evolved mechanisms for intracellular growth and survival within these protozoa (Primm et al. 2004). A study by Mura et al. (2006) demonstrated

that MAP cells were able to survive protozoal internalization. Reduction in MAP numbers was initially observed but subsequently rapidly increased indicating replication despite the absence of mycobactin (Mura et al. 2006).

2.3.2 The connection between JD and Crohn's disease

The first noted similarities between lesions associated with JD in cattle and Crohn's disease (CD) in humans were described in 1913 (Dalzeil 1913). Subsequent studies have attempted to demonstrate that MAP is the etiological agent, although a conclusive connection has never been established. If proven, serious implications regarding mandatory on-farm control and prevention programs will become a global issue as cattle in every country have been exposed to MAP (Nielsen and Toft 2009).

The theory that MAP is the causative agent in chronic intestinal enteritis was discredited for many years as it did not meet the requirements outlined in the classic Koch's postulates for establishing a causal relationship between microorganism and disease (Münch 2003). The classic Koch's postulates are as follows:

1. The organism must be found in all animals with the disease.
2. The organism must be isolated from a diseased animal and grown in pure culture.
3. The cultured organism should produce the same disease when inoculated into a healthy animal.
4. The organism must be re-isolated from an experimentally infected animal.

Failure to meet these standards was attributed to culturing difficulties, as MAP could not be isolated from tissues of infected animals and an agent-specific immune response could not be detected in diseased hosts (Chamberlin et al. 2001). Therefore, MAP was not considered as the causative agent.

More recently, Dalziel's theory (Dalziel 1913) was reconsidered. Chiodini et al. (1984) isolated an unclassified species of *Mycobacterium* from resected sections of terminal ileum of patients with CD. Thereafter, Herman-Taylor et al. (1998) cultured MAP from the lymph nodes of a young boy who developed CD. Richter et al. (2002) isolated MAP from the colon, liver and bone marrow, demonstrating the opportunistic nature of MAP, and that establishment and replication in the intestinal lining, may be an indication of immunosuppressive diseases. Although limited in number, these individual case studies now fulfill a previously unfulfilled postulate, that MAP can be identified and isolated from some patients with CD.

Fulfillment of the remaining postulates, however, can be challenging. The first challenge lies with the inconsistencies in obtaining MAP isolates in patients with CD. Due to the chronic nature of CD and because MAP may be found in the intestinal tract of both healthy and diseased individuals, studies have found MAP DNA to be present in 69% of patients with CD (Sechi et al. 2004). This indicates that the presence of MAP alone may not be enough to establish causality. The next challenge lies in the ability to detect an immune-specific response elicited from MAP infection. Again this may not be a good indicator as challenges with detection methods for MAP in early stages of CD, are similar to the difficulties in cattle with JD.

It has been proposed that Koch's postulates were too rigid and strict adherence to these guidelines was inappropriate in most microorganism-host dependent interactions (Chamberlin et al. 2007). Amendments of the classic Koch's postulates were suggested due to advances in culturing techniques and molecular technologies combined with the realization that some bacterial species cannot be cultured even though they have been

shown to cause disease (i.e *Mycobacterium leprae* and Leprosy). Modifications resulted in the development of host-dependent Koch's postulates, described as follows:

1. The agent is associated with a host-defined immunodeficiency.
2. The organism is isolated from a diseased host and grown in pure culture.
3. Inoculation of a sample of the culture into an animal produces the same disease only in a host that shares a similar immunodeficiency.
4. The organism is then re-isolated from the experimentally infected animal.

The intent of these re-evaluated postulates to a host-defined immunodeficiency is to redefine disease outcomes, such as CD, as a dysfunctional host-pathogen interaction. Many researchers consider CD to be a multifactorial syndrome with unknown environmental factors, genetic susceptibility and autoimmune deregulation (Gibson 1990; Chamberlin et al. 2001; Chamberlin et al. 2007). It is with the guidance of these revised postulates that causality may be established, not only for CD, but also for other conditions with a multifactorial host-dependent origin.

Evidence continues to mount in support of MAP as etiological agent of CD. But until the full clinical spectrum of MAP infection is determined, the true causative sequences of events leading to a diseased state remain inconclusive. It is also recognized that even if a microorganism does not fulfill all of Koch's postulates, it may still be an etiological agent (Chamberlin et al. 2007). With that, it is suggested that even though MAP may not be entirely responsible for CD it certainly plays an associative role in the condition (Chamberlin et al. 2007).

2.3.3 MAP in the food chain

The most likely pathway for human infection with MAP may be from contaminated food products from JD cattle. Currently, JD control programs involve testing and culling. In Canada, results from a survey indicated that a test and cull strategy had highest producer compliance, relative to other strategies including additional bedding to calving areas, removal of newborn from environment with dam immediately after birth and providing colostrum from low-risk animals, as the most reasonable and practical recommendations for disease control (Sorge et al. 2010). However, once infected animals are culled from a herd, they are slaughtered and enter the human food chain or sent to the auction mart and purchased by another producer, thereby continuing the disease cycle.

In Canada, it is estimated that about 32% of herds have at least two cows seropositive for MAP (Tiwari et al. 2009). These animals are usually tested with a milk or serum ELISA and if positive, culled from the herd. Canada currently does not have any regulations regarding the fate of infected animals. Increased concern regarding production losses associated with JD (Tiwari et al. 2008) and the association with MAP and CD, has prompted international agencies to suggest possible restrictions on international movement of cattle and cattle products. It is for these reasons that a Canadian National Voluntary Johne's Disease Control and Prevention Program (CJDPCP) was proposed (McKenna et al. 2006). The mission of this program is to produce best management practices (BMP's) to prevent the transmission and dissemination of MAP, with the intention of controlling the bacteria on-farm and in the food chain (McKenna et al. 2006).

As with every disease control program, there are challenges associated with establishment and implementation. The first challenge is the acceptance and compliance

with the suggested BMP's (Sorge et al. 2010). Sorge et al. (2010) conducted a survey of Canadian dairy farmers to assess their perception regarding the effect of JD on their farm and regarding any recommended changes in management practices resulting from a voluntary control program. Culling of MAP-positive cows had the greatest potential compliance while changes in management practices that were too costly or perceived to be unnecessary had the least (Sorge et al. 2010). Although many producers experienced some economic losses associated with JD, if they concluded that the practice had no perceived benefit, they were unwilling to adopt it even with incentives (Bennett and Cooke 2005).

Another challenge with implementing a BMP program is the ability of MAP to survive for extended periods within the environment and the lack of sensitivity of available tests to detect subclinical infection. As discussed previously, not all infected animals are detectable, and therefore continuously shed MAP. The environment then becomes a sink for the bacteria and a source for other animals to become re-infected. This cycle of disease transmission is very difficult to break making the eradication process extremely slow if not impossible. It is believed that even if test and cull programs were implemented, they would not be sufficient to eradicate the disease as infection routes would not be efficiently closed (Kudahl et al. 2008).

Identification of individuals with CD who possess MAP DNA (Sechi et al. 2004) has provided further evidence for MAP as a causal agent. With increased awareness, consumers may alter their purchasing behaviours for certain high-risk foods including, milk, milk products and meat, which are considered to pose highest risk as a vehicle for the transmission of MAP (Sorge et al. 2010).

In Canada, Gao et al. (2002) tested over 710 retail milk samples for the presence and viability of MAP after regular batch pasteurization and high temperature short time (HTST) pasteurization. They found that 15% of the samples tested positive for MAP DNA but failed to recover any live isolates in culture. Ellingson et al. (2005) tested 702 pasteurized milk samples from the USA and found that 2.8% of the samples did have viable MAP. Jaravata et al. (2007) tested 200 samples of ground beef sold at the retail level, but did not conclusively identify MAP DNA in any of the samples. Even though MAP was disseminated to the diaphragm (one out of 47 animals slaughtered; Alonso-Hearn et al. 2009), ground beef is a less likely route for transmitting MAP to humans.

2.4 Composting as a strategy to eliminate pathogens on-farm

2.4.1 Why compost?

In Canada, there are four approved methods of mortality disposal on-farm, 1) burial, 2) incineration, 3) rendering and 4) composting. Composting mortalities began in the poultry industry in the early 1980's in the USA (Wilkinson 2007) and has gained interest in other industries as it is relatively inexpensive, safe and an environmentally sound way to turn waste, whether household refuse, manure or mortalities, into fertilizer.

Managed composting is an aerobic process involving decomposition of organic matter to a stable final product free from weed seeds and phytotoxic and pathogenic organisms (Zucconi and de Bertoldi 1987). The process of mortality composting has been described by Kalbasi et al. (2005) as the temporary above-ground burial of mortalities in supplemental carbon, aerobically decomposed by microorganisms resulting in the breakdown of tissues at temperatures that kill most pathogens (Kalbasi et al. 2005).

2.4.2 The composting process

Decomposition is a non-steady biological activity, which is dependent on the initial composting matrix (i.e, nutrient balance, pH, particle size and porosity) and management including aeration and temperature (Agnew and Leonard 2003; Bernal et al. 2009). Moisture content of the substrate is also an important factor to consider, optimizing the composting process (Bernal et al. 2009 Agnew and Leonard 2003). Although it is difficult to describe the optimal conditions for all types of compostable substrates and their associated conditions, the basic conditions for optimal composting have been defined (Kalbasi et al. 2005). Composting of organic residues is an aerobic process with highly uniform compost substrates, differing significantly from carcass composting, which tends to consist of anaerobic conditions resulting from the heterogeneous compost substrates (Kalbasi et al. 2005).

Carbon/Nitrogen Ratio

Substrates most commonly composted on-farm includes animal manures and livestock mortalities that are placed in either open-air windrows or biosecure containers (Keener et al. 2000; Stanford et al. 2007; Xu et al. 2010). Each substrate possesses a carbon/nitrogen (C/N) ratio ranging from 25-35:1 (Bishop and Godfrey 1983; Agnew and Leonard 2003), which reflects available energy and the degradable organic carbon (C) and nitrogen (N) required for metabolic activities. It is believed that this ratio is adequate, as most microorganisms require about 30 parts of C to one part N for metabolic activities (Bishop and Godfrey 1983). With a high C/N ratio, OM degradation is slowed due to a lack of available N, thereby inhibiting microbial protein production (Bernal et al. 2009). Conversely, when composting animal mortalities, this ratio may be low due to the excess

inorganic-N from the degradation of nitrogenous tissues within the carcass. With a low ratio, there is not enough available C for microbial energy production, and therefore heating is not observed due to slowed microbial metabolism. In addition, excess N (from the decomposing carcasses) may be lost via conversion to ammonia (NH_3) and volatilized or leached if there is excessive moisture from the compost (Bernal et al. 2009). This ratio may be increased with addition of a C source (e.g. straw, woodchips sawdust, etc.). Over time, the C/N ratio is reduced by approximately half (Kalbasi et al. 2005), as the microorganisms oxidize C-sources to carbon dioxide (CO_2).

pH

The optimum pH range for most compost is reported at values between 6.7 and 8.0, which supports the activity of most microorganisms (de Bertoldi et al. 1983). Although alkaline or acidic environments are not suitable for composting, the pH of most compostable substrates is already within this range. This alkaline pH contributes to ammonia loss from the composting system (Kalbasi et al. 2005).

Particle Size & Aeration

Particle size determines the surface area available for microorganisms to maximize decomposition rates of organic materials (Agnew and Leonard 2003). Optimization of particle size distribution will maintain pile porosity for adequate aeration (Bernal et al. 2009). Recommended optimum particle size for compost varies depending on the material being composted, but the composting matrix should have a porosity of 35-50% (Agnew and Leonard 2003). If the particle size is too large, the accessible surface area for microorganisms to act on will be reduced, increasing the time required for adequate OM degradation. While it may seem that smaller particle sizes will

maximize rates of decomposition more efficiently, compost compaction is more likely to occur, reducing porosity and thus the degree of aeration, possibly leading to anaerobic conditions (porosity < 35%) and a reduced rate of OM degradation (Bernal et al. 2009). Similarly if porosity is too high, exceeding 50%, then the inner compost temperature will remain low due to heat lost through air-filled pore spaces (Bernal et al. 2009).

Aeration is another factor that is dependent on the management of the composting process. Proper aeration can be managed several ways that include actively forcing air through the material, mechanical turning and mixing, passive air exchange or any combination of the aforementioned strategies. As mentioned previously, aeration is influenced by the particle size and porosity of the initial compost matrix. It is suggested that the optimum oxygen concentration should be within the range of 10-20% (Chaw 2001; Kalbasi et al. 2005). If concentrations are properly managed and maintained within this range, compost microorganisms remain active and increase compost temperatures, remove excess moisture (Bernal et al. 2009) and reduce volatile N-losses as a result of nitrification (Kalbasi et al. 2005). Solano et al. (2001) found that mechanical turning of compost lost 25% more N as compared to 4.5% from passive or forced aeration.

Temperature

Temperature change in compost can be divided into two major phases. The first phase is called the biooxidative phase and the second phase is the maturation phase (Kalbasi et al. 2005). Organisms with optimum growth at temperatures ranging between 20-45˚C have been defined as mesophilic while organisms with optimum growth ranging between 50-70˚C have been defined as thermophilic (Misra et al. 2003).

During the biooxidative phase, the temperature profile develops in several stages according to the metabolic activity of the microorganisms. Within the first three days, mesophilic organisms initiate decomposition of OM, increasing the temperature to over 40˚C as a consequence of their metabolic activities (Kalbasi et al. 2005). The next stage is a thermophilic phase, in which thermophilic organisms maximally degrade OM while inactivating many pathogens (Kalbasi et al. 2005). Optimal composting should be maintained within the range of 40-65˚C; temperatures between 52-60˚C are optimum for OM decomposition while those exceeding 55˚C are optimum for inactivation of many pathogens (Miller 1993). Compost may reach temperatures of 70-80˚C. These temperatures serve to inactivate pathogens, although outside the tolerance range of beneficial thermophiles if exposure exceeds 2-3 hours (Miller 1993; Bernal et al. 2009). The final step in the biooxidative phase, is a reduction in temperature due to decreased microbial activity. With a temperature decrease, mesophilic organisms revert back to a more active growth phase and recolonize the compost and degrade any remaining OM (Bernal et al. 2009). Overall within the biooxidative phase, microorganisms utilize O_2 as they rapidly degrade OM to CO_2 and NH_3 with temperatures rising to a level that destroys pathogens.

The maturation phase is a series of slower reactions involving the stabilization and humification of the remaining OM in the compost (Bernal et al. 2009). As this phase progresses the temperature of the compost remains between 10-40˚C. A rise in temperature is no longer observed due to the decomposition of most of the readily available OM (Kalbasi et al. 2005). Humification of OM occurs as a result of lignin and cellulose digestion and the formation of large aromatic molecular compounds from humic

acids, which have a high resistance to microbial degradation (Bernal et al. 2009). Overall, the curing phase produces a mature, sanitized, stable product with humic characteristics beneficial to plant growth.

Moisture

Moisture content is another factor considered important in the initial compost matrix as well as throughout management of the composting process. Optimum moisture content for composting animal mortalities depends again on the initial characteristics of the material, but should typically range between 50-60% wet basis by mass (Keener et al. 2000). If moisture is greater than 60%, compaction may result, reducing porosity and aerobic microbial activity. Conversely, if moisture is less than 40%, decomposition of OM is limited as there is not enough water to support the microbial metabolic activities required to increase the temperature. Optimum moisture content can be maintained through either addition of a dry material or rewetting the matrix if necessary.

2.4.3 Composting mortalities to achieve pathogen reduction

With increasing biosecurity measures utilized on-farm, mortality management via composting seems the most logical solution for safe disposal of carcasses. However, there are few studies that focus on the composting of large livestock mortalities (Fulhage 1997; Fonstad et al. 2003; Mukhtar et al. 2003; Kalbasi et al. 2005; Kalbasi et al. 2006) or the fate of pathogens during mortality composting (Wilkinson 2007; T. Reuter et al. 2008; Xu et al. 2009; Flory and Peer 2010; Guan et al. 2010).

Often mortality composting is accomplished in two stages in order to obtain complete carcass decomposition. The first stage, also called primary composting, is an undisturbed process that results in the decomposition of most soft tissues of the carcass

while thermophilic temperatures are required to kill pathogens (Wilkinson 2007). Only after this phase, is it deemed safe to mechanically mix the compost to allow for secondary composting to decompose any remaining soft tissues or bones (Wilkinson 2007). Studies by Glanville et al. (2007) and Xu et al. (2009) demonstrated that temperatures reached 55-60˚C in passively aerated biosecure systems used for mortality composting. Some undecomposed animal tissues remained in both studies indicating that secondary composting was required to completely decompose all carcass components. In both studies, compost was allowed to reach thermophilic temperatures, cool and was then deemed safe to open and turn (Fonstad et al. 2003; Glanville et al. 2007; Xu et al. 2009). The time required to compost cattle mortalities can range anywhere from 5-9 months to produce a mature and stable product (Kalbasi et al. 2005; Xu et al. 2009; Xu et al. 2010).

The nature of mortality composting, which is characterized by a low C/N ratio, low porosity, high moisture content carcasses, surrounded by a material with a high C/N ratio, good porosity, and a matrix of moderate to low moisture content (Fonstad et al. 2003), has led to concerns regarding the potential survival of pathogens in the event of mass mortalities as a result of a disease outbreak (Kalbasi et al. 2005; Wilkinson 2007). Passively aerated composting to minimize aerosol transmission of any pathogens from disposal of infected animals has been explored (Fonstad et al. 2003). Aeration is often the factor that limits the ability to reach thermophilic temperatures required for pathogen inactivation. Incomplete decomposition and lack of pathogen inactivation can occur due to the heterogeneous nature of composting carcasses.

Several studies have been conducted to examine the inactivation of pathogens associated with livestock mortalities during composting. Some of the first studies

evaluated avian influenza (AI) inactivation in poultry flocks. Senne et al. (1994) was among the first to discover that highly pathogenic strains of AI could not be isolated after 10 days of composting after exposure to temperatures of 55-65˚C (Senne et al. 1994). Studies initiated as a consequence of the outbreak of AI in the USA and Canada all resulted in similar conclusions (Malone et al. 2004; Spencer 2005; Flory and Peer 2010). It was initially suggested that mass mortality composting may only be suitable for small to medium-sized carcasses however, the outbreak of foot-and-mouth disease in the UK, stimulated additional research to determine the ability of this strategy to dispose of any size carcass during an emergency disease outbreak (Wilkinson 2007).

Despite the heterogeneity of large carcass composting, several studies have reported successful carcass decomposition with simultaneous pathogen inactivation (Huang et al. 2007; Xu et al. 2009; Guan et al. 2010; Reuter et al. 2011). For example, prions, responsible for scrapie and bovine spongiform encephalopathy (BSE), are highly resistant to conventional pathogen inactivation practices (Huang et al. 2007). After exposure to thermophilic organisms in compost, there was evidence of prion degradation after 108 days of composting (Huang et al. 2007). Another study by Xu et al. (2009) examined survivability of several pathogens, including *Escherichia coli* O157:H7, *Campylobacter jejuni* and Newcastle disease virus (NDV), in a biosecure passively aerated composting system for cattle mortalities. Both *E. coli* and NDV were inactivated after seven days of composting. Although *Campylobacter jejuni* was still detectable after 147 days of composting, there was a still a significant reduction in the number of viable cells (Xu et al. 2009). Similarly, Larney et al. (2003) found that *E. coli* was inactivated in manure after seven days of windrow composting. This system utilized frequent turning of

the compost, which is not desirable in the event of an infectious disease outbreak. More recently, a study used *Bacillus* species to model *Bacillus anthracis* survival in the event of an anthrax outbreak (Reuter et al. 2011). Although some spores remained viable, it was noted that large portions of the organisms were non-viable after 230 days of composting (Reuter et al. 2011).

Ambient temperature may impact efficient initiation of the biooxidative phase during composting of livestock mortalities. Stanford et al. (2007) demonstrated that composting in open-air windrows at low ambient temperatures (-38˚C four days after construction) was effective, despite using frozen carcasses. The study added aerated, actively heating manure to the compost piles and observed temperatures > 50˚C within the first four days. Stanford et al. (2007) also refers to an unpublished study that used frozen carcasses, small windrows and non-heated manure, in which ambient temperatures > 0˚C were not acheived. These same authors hypothesized that the use of larger windrows would have provided sufficient insulation to maintain temperatures in an open-air system during severe winter conditions and further, may have mitigated the effects of wind speed on declining compost temperatures.

2.5 Use of high-throughput sequencing to characterize microbial communities and their dynamics

2.5.1 History and development of high-throughput sequencing technologies and the field of bioinformatics

Nucleic acid sequencing technologies are increasingly becoming one of the most important tools in the study of biological systems. Recently, there has been a shift from

low-throughput, conventional sequencing techniques to high-throughput sequencing technology. A novel high-throughput sequencing technique, called pyrosequencing, has been applied to the field of metagenomics, with *de novo* assembly of entire genomes.

The history of the evolution of DNA sequencing methods began in the early 1970s. For many years, Sanger DNA sequencing was commonly practiced, by sequencing the 16S rRNA gene for microbial identification. This was the preferred technique because relatively long reads, of about 1000-1500 bp, were produced with reasonable accuracy enabling genus and species identification of bacteria. Initially, taxonomic classification of bacteria was performed manually using enzymatic methods or terminator sequencing chemistry (Sanger and Coulson 1977; Maxam and Gilbert 1977). As sequencing chemistry and instrumentation advanced the speed to which sequences could be generated and classified, primarily facilitated by the Human Genome Project, the number of sequences made available in public reference databases substantially increased (Kolbert and Persing 1999). However, it was soon realized that the entire coding region of the 16S rRNA gene did not have to be sequenced to classify a bacterial species, and shorter targeted regions of the gene were classified with the aid of sequence classifier algorithms to a reference database (Petrosino et al. 2009).

The need to explore the complex nature of microbial environments, such as the human microbiome, has lead to the area of metagenomics. Metagenomics is the study of all genomes within a given environment (Petrosino et al. 2009). Its initiation and development was directed towards clinical medicine to provide a collective understanding of microbial organisms present and their function within a given niche, without the need for them to be cultured (Petrosino et al. 2009). As most organisms have

not been identified with culture-based techniques, a high-throughput, low cost, culture-independent strategy for identifying microbial organisms was needed. As a consequence, pyrosequencing emerged.

Pyrosequencing, also referred to as sequencing by synthesis, is a fast, automated, robust way to generate DNA sequences in parallel (Ronaghi et al. 1996). This method was refined by Ronaghi et al. (1996), as it was apparent that Sanger sequencing would be limiting with regards to throughput and cost per base to sequence entire, highly complex microbial communities (Ronaghi 2001). Limited read lengths was a major limitation in first and second-generation pyrosequencing technology, which rarely exceeded 100-200 bp. The 16S rRNA gene consists of nine conserved regions (C1 to C9) and nine hypervariable regions (V1 to V9) that range from approximately 50-100 bp (Petrosino et al. 2009). Sequencing of multiple hypervariable regions on this gene provides a target region for universal microbial identification, eliminating the need to sequence the entire gene or genome to identify a microorganism (Petrosino et al. 2009). Read lengths that are too short can prevent amplification of multiple regions across the 16S gene, increasing the inaccuracy of microbial identification at the genus or species level.

In 2007, pyrosequencing became a commercialized technique. Third generation pyrosequencing technologies, known as 454-pyrosequencing, were developed and could be used at a reasonable cost per generated base pair (Voelkerding et al. 2009). The 454-pyrosequencing process now had the capacity to generate longer read lengths of more than 400 bp (Voelkerding et al. 2009), enabling amplification of several hypervariable regions and allowing for accurate identification of microorganisms at the genus and even species level. With advances in the read lengths generated by 454-pyrosequencing,

current sequencing systems are generating read lengths of up to 1000 bp (454 Life Sciences 2013). Through refinement of this technology, the entire process is able to generate millions of *de novo* assembled DNA reads from any given microbial environment (454 Life Sciences 2013).

Once generated, the challenge is to analyze the DNA sequences. Since the late 1980's, the field of bioinformatics has used algorithms to analyze proteins, genes and complete collections of DNA, in an effort to define biological systems (Pevsner 2009; Hogeweg 2011). The roots of bioinformatics trace back to the 1960's when many of the basic pattern analysis methods now used in these computational systems were developed. With the advent of data-driven bioinformatics, primarily with the announcement of the Human Genome Project, the challenge was to make sense of the enormous amount of sequence data (Pevsner 2009). The sequencing technology has developed and advanced more rapidly than the computationally intensive systems required for analysis. This has created a bottleneck in overall metagenomic analyses where the time required for sequence processing far exceeds the length of time required to generate the sequences.

2.5.2 The 454-pyrosequencing process

The entire 454-pyrosequencing process is comprised of three main steps. The first step requires the generation of a DNA library. In order to generate this library, universal and conserved regions of the 16S rRNA gene are amplified (Petrosino et al. 2009). Third generation 454-pyrosequencing technologies facilitate sequencing of multiple hypervariable regions of this gene (Petrosino et al. 2009; 454 Life Sciences 2013). The choice of region specific primers is dependent on the utility of that region for universal microbial identification (Petrosino et al. 2009). It has been shown that the V2 and V3

regions are most suitable for distinguishing between all bacterial species down to the genus level of taxonomic classification (Chakravorty et al. 2007). To create this library, the genomic DNA is randomly fragmented, ranging in length from 50-900 bp, fusion primers added, which are specific to a targeted hypervariable region, and then specialized adaptors are added to the ends of the fragments to immobilize them onto DNA capture beads (Margulies et al. 2005). For the purposes of 454-pyrosequencing, the DNA library refers to the total collection of beads carrying a unique single-stranded fragment from the environmental DNA samples (Margulies et al. 2005).

The next step of the pyrosequencing process involves the amplification of the DNA library. Each bead is placed in a PCR-reaction mixture, in an oil emulsion, with each droplet acting as its own microreactor (Margulies et al. 2005). This PCR method clonally amplifies all fragments captured on the beads in parallel while excluding competing or contaminating sequences. Once amplification is complete, millions of copies of one template are captured on each bead. The emulsion is then broken and each bead is deposited into a well of a PicoTitrePlate by centrifugation (Margulies et al. 2005).

The final step in the process is the enzymatic “sequencing by synthesis”. Several enzymes that include DNA polymerase, adenosine triphosphate (ATP), sulfurylase and luciferase, are added to each well in the plate. The plate is then placed into the GS FLX System, and individual nucleotides flow over the wells in a fixed order. Production of inorganic pyrophosphate (PPi) is driven with DNA polymerase as each sequential nucleotide is added (Ronaghi 2001). The PPi is then converted to ATP with ATP-sulfurylase and then the ATP coupled with luciferase is used to convert luciferin to oxyluciferin. This generates a light signal read by a camera that is proportional to the

number of incorporated nucleotides on the template DNA strand (Ronaghi 2001). Before this entire process can begin with another nucleotide, the enzyme apyrase is washed over the wells to degrade any excess nucleotides and ATP (Ronaghi 2001).

However, there are some limitations to 454-pyrosequencing, including the presence of homopolymer regions (three or more consecutive identical bases), which increases the variance associated with the distribution of light intensities, resulting in incorrect base calling and an overall higher error rate (Margulies et al. 2005). The incorrect base-calls form insertions or deletions, result in artificial sequences, and an overestimation of diversity (Kunin et al. 2010). Trimming of these high error regions can be accomplished with computational programs (Schloss et al. 2009).

The second limitation occurs during PCR amplification of the DNA libraries prior to sequencing. Bias may occur in an environmental sample consisting of many 16S rRNA genes, where certain genes may be preferentially amplified (Reysenbach et al. 1992). This bias results in an inaccurate reflection of relative abundance of those sequences within a microbial community (Reysenbach et al. 1992). Chimeras also form when 16S rRNA genes of more than one species are PCR-amplified in a single reaction, and recombine to form a sequence consisting of mixtures of different 16S rRNA genes (Wang and Wang 1996). The amplification process can be optimized to reduce the total number of chimeras, however, they are never completely eliminated (Wang and Wang 1996), resulting in inflated diversity estimates (Kunin et al. 2010). Computational programs such as UChime (Edgar et al. 2011) and Bellerophon (Huber et al. 2004) have algorithms to determine which sequences are chimeras and remove them from a dataset.

Other sequencing platforms, such as Illumina, have also been used for targeted high-throughput sequencing of the 16S rRNA gene (Claesson et al. 2010; Degnan and Ochman 2012). Initially this platform was only able to generate reads of approximately 36-76 bp in length with quality deteriorating as read length increased (> 60 bp) (Claesson et al. 2010). Further, shorter reads were insufficient in length for accurate microbial identification resulting in overestimates of diversity (Youssef et al. 2009). However, the base-calling accuracy of the Illumina platform surpasses the 454-sequencing platform with its terminal restriction dye method (method not discussed here) resulting in fewer homopolymers and base call errors (Luo et al. 2012). As the most current Illumina platforms generating read lengths of 250 bp (MiSeq platform; Logares et al. 2012), the lengths now span more of the 16S rRNA gene, and Illumina utilization is expected to increase in targeted high-throughput sequencing studies.

2.5.3 Importance of biogeography to understand microbial succession

The dynamics of microbial community structure is a controversial area within the ecological research community. The debate revolves around whether one successional pattern for all microbial communities can be delineated. This debate has subsequently given rise to the study of microbial biogeography, which aims to explain the distribution of microorganisms over time, taxonomically, disclosing which microorganisms are present, their abundance, function and how diversity is maintained over successive generations (Martiny et al. 2006). This field of study becomes more complicated given that the majority of the microbial diversity on the planet remains unaccounted for, which begs the question whether one common successional pattern can be accurately demonstrated. It has been hypothesized that the biogeography of microorganisms can be

compared to the biogeography of macroorganisms (i.e. plants and animals) although little research is available in this area (Fierer et al. 2010). Due to recent advances in molecular sequencing and phylogenetic tools, and identification of many previously unknown microorganisms, it is now possible to make inferences about how communities change and how they are fundamentally and functionally related to one another. These technological advances have given rise to a branch of microbial biogeography known as trait-based microbial biogeography, which attempts to discover ecological strategies based on the functions or traits of microorganisms to explain why they live where they do and how they form functional communities (Green et al. 2008).

The Baas-Becking hypothesis states that "everything is everywhere: but the environment selects" (Baas-Becking 1934). This hypothesis implicitly assumes that all microorganisms have great dispersal capabilities to exist in all environments, but environmental selection is not limited to the abiotic or biotic factors within that habitat. A review by Martiny et al. (2006) discusses the need to consider abiotic and biotic conditions of the current habitat as well as spatial arrangement and historical events of these communities in order to discover biogeographic influences.

It has been suggested that our knowledge of succession in macro-communities can be extrapolated to succession within microbial communities (Green et al. 2004; Martiny et al. 2006; Green et al. 2008; Fierer et al. 2010). As with macroorganism biogeography, there was an initial adoption of a taxa-based approach to understand microbial community structure. Now as theories consider functionality, interpretation of taxonomic patterns in terms of how they affect a community may be misleading and should not be the sole consideration (Green et al. 2008). Weiher and Keddy (1995)

introduced the idea that traits not taxonomy should be considered as the fundamental units of biogeography arguing that organisms with similar function will eventually sort into similar environments with similar adaptive requirements. However, current knowledge of microbial function in nature is limited due to their diverse metabolic and physiologic capabilities (Green et al. 2008). The greatest challenge is then to determine a measureable trait (i.e. lifetime pattern of growth, reproduction, genome size, microbial resistance to viruses, mutation rates, etc.) that can be directly linked to the fitness or performance of microorganisms (Green et al. 2008). If a trait can be deduced, temporal patterns and consequences of environmental change could be tested, which are more directly related to properties important to overall ecosystem function (Green et al. 2008). As more information regarding macroorganism biogeography is known, compared with the limited information regarding microorganism biogeography, it is hypothesized that similar patterns will occur.

There are several mechanisms of macroorganism biogeography that may also apply to microbial biogeographic patterns. Fierer et al. (2010) suggest two mechanisms; the first mechanism refers to the role of dispersal in succession. Just as seed dispersal capabilities are not the same, not all microorganisms have similar dispersal capabilities within or to new substrates. This affects the types and quantities of microorganisms that can successfully colonize a substrate and further, influences the succession pattern of a given community (Vargas-Garcia et al. 2007; Fierer et al. 2010). For example, throughout composting, a relationship may be observed between microorganism distribution and their relative appearance throughout succession. If dispersal capabilities were similar for abundant and rare species in a given environment, succession of rare species within a

community would be lower and less influential, simply because they are not as widely distributed (Fierer et al. 2010).

The second mechanism addresses resource limitation during succession. As succession progresses, dispersal limitations become less relevant, and become more influenced by resource limitation (Fierer et al. 2010). As with plants and animals, resource availability is likely to be a fundamental driver of microbial succession and is determined by the resource capture abilities and tolerance of low nutrient avaibility to the microorganisms. Grouping microorganisms according to which resource is most limiting allows us to understand the function of the community and how it influences succession. For instance, in early stages of the biooxidative phase of composting, the microbial community is comprised of predominantly copiotrophic bacteria (i.e. survive in an abundance of nutrients) that consume available C. As composting progresses, the microbial community shifts to oligotrophic bacteria, which survive with limited C. However, as composting shifts from the biooxidative phase to the maturation phase, defining the limiting resource becomes problematic due to the complex physiologic diversity of the community. The pattern of succession is altered as the microorganisms respond to the limited availability of resources to meet their nutritional requirements for growth and cell division. This further reinforces the need to understand a community at the functional level.

Although resource availability is the major factor dictating phylogenetic diversity throughout succession, environmental stresses also contribute to these changes. Similar to plants, a variety of environmental stresses, such as pH, water availability, UV radiation, salinity and temperature, coupled with resource limitation increase the difficulty in

predicting changes in microbial diversity (Fierer et al. 2010). Fierer et al. (2010) suggest that environmental stresses may actually be more influential to phylogenetic diversity in earlier stages of succession, but as a community progresses, the microorganisms may be able to modify their surroundings to become less sensitive or resistant to these changes (Besemer et al. 2007). However, if an environmental factor is of a sufficient magnitude (i.e. mechanical turning of compost), successional dynamics can be completely altered, preventing the normal pattern of successional progression. Fierer et al. (2010) conclude their review by stating that studies of succession must consider both environmental conditions and resource supply to completely understand functional and phylogenetic changes.

Although these mechanisms may be applied to macro- or microbial communities, there are major differences in these systems that make direct comparisons challenging. Some of these challenges include dormancy, physiological diversity and evolutionary rates, which are all higher or more prevalent in microbial communities compared with macro communities. These factors also influence successional dynamics making similar conclusions difficult to replicate or observe in any macro-community (Fierer et al. 2010).

2.6 Summary

Challenges associated with eliminating MAP on farm include accurate detection methods and determination of prevalence rates, treatment, vaccination and a lack of understanding regarding survival tactics in the environment. The biochemical processes of composting rely on microbial interactions to achieve the thermophilic conditions required to mitigate pathogens during composting, including MAP.

Inactivation of MAP has been assessed under variable environmental conditions and under simulated composting conditions, but to date, MAP viability has not been assessed in a farm-scale biosecure composting system. Further, composting in a biosecure, static system, associated with a disease outbreak, has not been performed in Manitoba. Additional research is required to characterize the composting conditions as well as the microbial community in order to effectively control disease on-farm.

2.7 Hypotheses and Objectives

Hypotheses

1) On-farm composting of livestock mortalities can inactivate MAP.
2) The compost microbial community is primarily influenced through changes in temperature during the composting of livestock mortalties.

Objectives

The general objectives of the thesis research were to:

1) Determine the temperature and length of time required to eliminate MAP during livestock mortality composting.
2) Determine if inactivation arises from temperature, the compost environment or both.
3) Determine whether *M. smegmatis* is an appropriate surrogate for MAP.
4) Describe the phylogentic, taxonomic, richness and diversity changes in the mortality compost microbial community after exposure to mesophilic or thermophilic temperatures.

3.0 Assessing the inactivation of *Mycobacterium avium* subspecies *paratuberculosis* during composting of livestock carcasses

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3.1 Abstract

Mycobacterium avium subspecies *paratuberculosis* (MAP) causes Johne's disease (JD) in ruminants with substantial economic impacts to the cattle industry. Johne's disease is known for its long latency period and difficulties in diagnosis are due to insensitivities of current detection methods. Eradication is challenging as MAP can survive for extended periods within the environment, resulting in new infections in naïve animals (Xu et al. 2009). This study explored the use of a biosecure, static composting structure to inactivate MAP. *Mycobacterium smegmatis* was also assessed as a surrogate for MAP. Two structures were constructed to hold three cattle carcasses each. Naturally-infected tissues and ground beef inoculated with laboratory cultured MAP and *M. smegmatis* were placed in nylon and plastic bags to determine effect of temperature and compost environment on viability over 250 (days) d. After removal, samples were cultured and growth of both organisms assessed after 12 weeks (wks). After 250 d, MAP was still detectable by PCR, while *M. smegmatis* was not detected after 67 d of

composting. Furthermore, MAP remained viable in both implanted nylon and plastic bags over the composting period. As the compost never reached a homogenous thermophilic (55-65°C) state throughout each structure, an *in vitro* experiment was conducted to examine viability of MAP after exposure to 80°C for 90 d. Naturally-infected lymph tissues were mixed with and without compost. After 90 d, MAP remained viable despite exposure to temperatures typically higher than that achieved in compost. In conclusion, it is unlikely composting can be used as a means of inactivating MAP associated with cattle mortalities.

3.2 Introduction

Composting can be used as a means to inactivate pathogenic bacteria (Senne et al. 1994; Grewal et al. 2005; Xu et al. 2009). Most research regarding pathogen inactivation has examined composting of municipal waste, manure, and garden refuse, with considerably less information available regarding the composting of livestock mortalities (Kalbasi et al. 2005). Compared to other carcass disposal methods such as burial or incineration, composting has proven to be an inexpensive, safe and an environmentally sustainable way to eliminate pathogens associated with livestock mortalities (Larney et al. 2003; Xu et al. 2009). Several studies have evaluated the survival of *Escherichia coli* (O157:H7) and *Salmonella* spp. during composting (Larney et al. 2003; Grewal et al. 2005), but to date, no studies have been conducted to assess the survival of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) during composting.

Mycobacterium avium subspecies *paratuberculosis* causes Johne's disease (JD) in cattle, a condition that is difficult to diagnose due to the asymptomatic nature of the

disease, fastidious nature of the causative agent, an incubation period of 2-10 years and requires up to 16 weeks (wks) to culture within a laboratory (Whitlock and Buergelt 1996). Tests typically used to detect early infection, such as milk or serum ELISA or fecal culture, do not have sufficient specificity or sensitivity to detect MAP in cattle shedding intermittently or at low levels in milk or feces (McKenna et al. 2005). This bacterium can also survive for extended periods in the environment (Lovell et al. 1944; Misra et al. 2003; Xu et al. 2009), with survival demonstrated in the soil for at least 385 d (Xu et al. 2009), thereby exposing cattle to this pathogen from their surroundings. The mechanism by which survival may be achieved includes dormancy (Whittington et al. 2004) and sporulation (Lamont et al. 2012). A recent review reported that 3% of dairy cattle across Canada are positive for MAP (Tiwari et al. 2006), but it is estimated that 50% of dairy herds have at least one MAP-infected animal (VanLeeuwen et al. 2006).

It has been suggested that MAP may be linked to Crohn's disease in humans (Chiodini et al. 1984; Hermon-Taylor et al. 1998; Waddell et al. 2008). Although there is no medical consensus regarding the causal association between MAP and Crohn's disease, several possible sources of MAP have been identified. Studies have shown that MAP can survive pasteurization in milk and milk products (Ellingson et al. 2005; Cerf et al. 2007; Rademaker et al. 2007; Foddai et al. 2010), which may serve as vectors for the transmission of MAP from cattle to humans. Meat products contaminated with MAP from infected cattle may also be an infectious source (Brady et al. 2008; Alonso-Hearn et al. 2009), but MAP has not been detected in retail ground beef (Jaravata et al. 2007). Most MAP-infected cattle are culled as a result of decreased milk yield, fertility or weight gain, all of which contribute to significant economic losses for the producer (Chi

et al. 2002). The costs of direct losses are estimated to be approximately $50 CAD per head per year for dairy, based on prevalence levels derived from serum ELISA results (Chi et al. 2002). Significantly greater losses of $441 US per head per lactation have been estimated based on prevalence estimates obtained by direct fecal culture (Raizman et al. 2009). As most cattle are culled as a result of decreased productivity prior to the onset of clinical symptoms, many are likely to enter the food chain. Voluntary control programs have already been implemented in the United States (United States Department of Agriculture 2010) and Australia (Kennedy and Allworth 2000) and proposed within Canada (McKenna et al. 2006), but more stringent on-farm programs may become mandatory if a zoonotic connection with Crohn's disease is established.

Recently, a biosecure, static composting system for large-scale cattle carcass disposal was developed (Xu et al. 2009; Xu et al. 2010). It has been demonstrated that *Campylobacter jejuni*, *Escherichia coli* (O157:H7) and Newcastle disease virus were rendered non-viable by composting using this system, but the survivability of MAP under these conditions has not been assessed. The purpose of this study was to determine if static composting of cattle carcasses inactivates MAP in a biosecure composting system and further, to determine whether *Mycobacterium smegmatis* could be used as a surrogate for MAP, as it is a fast-growing species that is much easier to culture (Chaturvedi et al. 2007) and has no association with human disease.

3.3 Materials and methods

3.3.1 Experiment #1: Biosecure, static composting of MAP

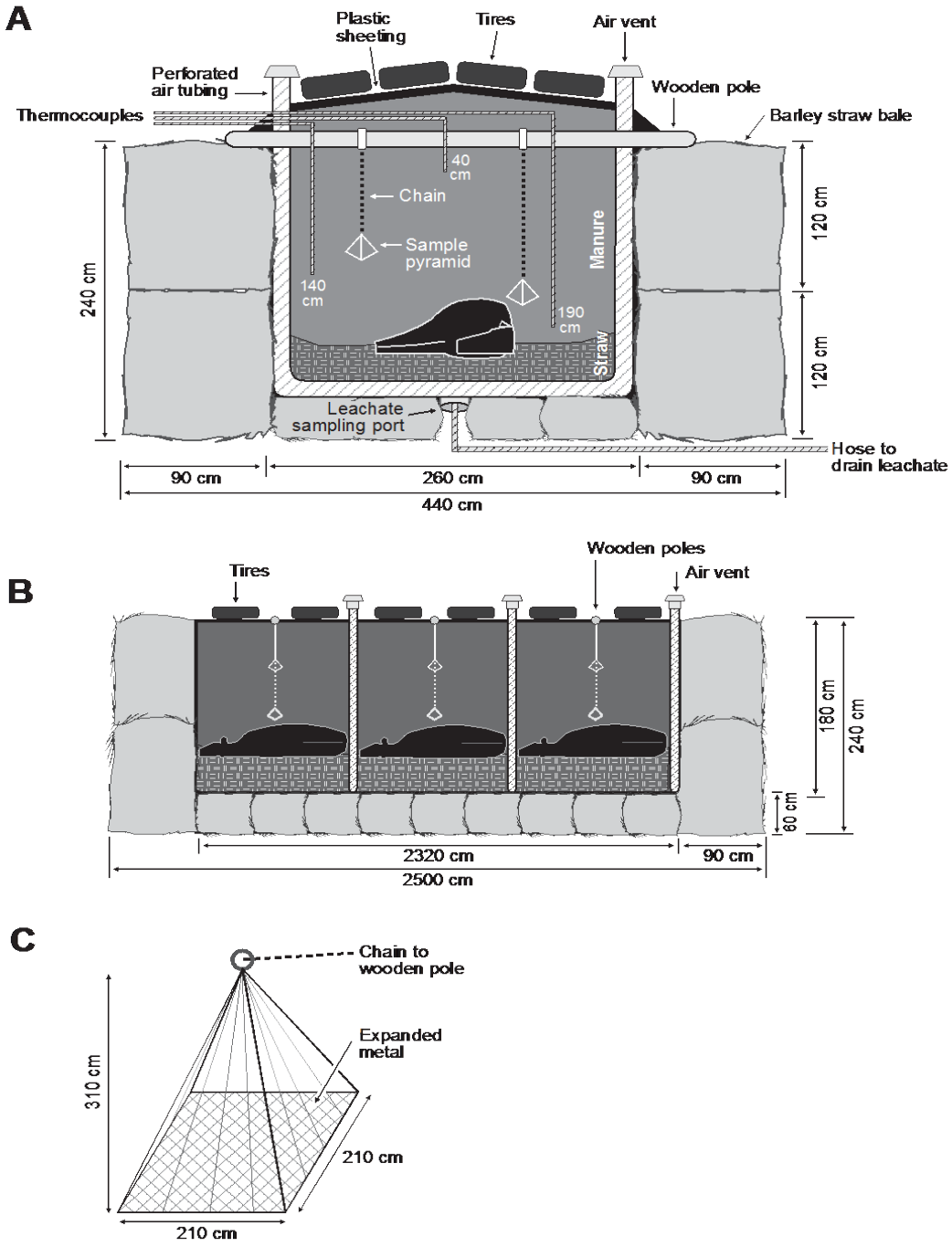
3.3.1.1 Biocontainment structures

In March 2008, two bio-containment composting structures (Fig. 3.1), Pile 1 and Pile 2, were constructed on a concrete pad at the Glenlea Research Facility at the University of Manitoba (Winnipeg, MB, Canada). The structures were 25 m long, 4.4 m wide and 2.4 m high (Fig. 3.1A and 3.1B). The outer walls were made of large barley straw bales, with a dimension of 2.0 m X 0.9 m X 1.2 m. Small straw bales were used to create the floor in each structure. The inside of each structure was lined with heavy black plastic sheeting. Perforated air tubing was used to drain leachate into a stainless steel basin with a drain that was positioned in the middle of the straw floor. Wire mesh was placed on top of the basin to prevent large particles from blocking the drain. A hose attached to the bottom of the basin ran from underneath the pile so that it was accessible as a leachate sampling port (Fig. 3.1A). Within the structure, loose straw was spread on the bottom of the plastic sheeting to form a 60 cm layer. Three cull JD negative Holstein cows were euthanized on site and laid head to tail on top of the straw layer. Next, a 120 cm layer of mixed, cattle manure was laid over the carcasses. Flexible perforated plastic pipes were embedded in between each carcass in the straw with air vents at the ends of the pipes passing through the plastic to enable passive aeration of the compost (Fig. 3.1A and 3.1B). The plastic was overlapped and old tires were placed on top to limit inward seepage of moisture (Fig. 3.1A and 3.1B). On day 194, both piles were opened to turn and aerate the compost.

3.3.1.2 Sample preparation

The four treatments examined included 1) a control using extra-lean ground beef, 2) lab-cultured MAP (ATCC 19698), grown in Middlebrook 7H9 broth with ADC

Figure 3.1 Biocontainment structures used for composting cattle mortalities in Experiment #1. Diagrams adapted from Xu et al. (2009). Structures include straw outer walls, three carcasses per pile, perforated air tubing, leachate sampling ports and inner thermocouple wires. Cross-section and dimensions are indicated along the width (A) and length (B) of each structure. Baker Retrieval Pyramid's (BRP; Reuter et al. 2008) used for sampling MAP, and its dimensions (C), were inserted in the vicinity of each carcass.



enrichment, inoculated onto ground beef, 3) lab-cultured *M. smegmatis* (mc2 155; ATCC 700084), grown in the same type of culture media as MAP, also inoculated onto ground beef and 4) lymph tissues (ileal, mesenteric and ileocecal) collected from six cows infected with MAP as determined by serum ELISA (Hendrick et al. 2005). For each treatment, tissues (15 g) were mixed and placed into either a heat sealed nylon bag (pore size 50 μm; ANKOM rumen *in situ* bags; Ankom Technology, Macedon, NY, USA), to assess biochemical and microbial effects of composting on MAP viability, or into a sterile plastic bag to test the effects of temperature only.

Subsamples were cultured to ensure MAP and *M. smegmatis* were present in all tissues (Day 1) prior to exposure to compost (culture method described below). To further ensure mycobacterial presence, a suspension was created with 2 g of tissue from each bag and 30 mL of Middlebrook 7H9 broth (Becton, Dickinson and Company (BD), Franklin Lakes, NJ, USA) with albumin, dextrose and catalase (ADC) enrichment (BD). The suspension was centrifuged at 1700 × *g* for 20 min, the supernatant decanted and 500 μL of the re-suspended pellet was added to Mycobacteria Growth Indicator Tubes (MGIT; BD) with oleic acid, albumin, dextrose and catalase (OADC) enrichment (BD) and a mixture of antibiotics consisting of polymyxin B, amphotericin B, naladixic acid, trimethoprim and azlocillin (PANTA) (BD). A pure culture of *M. smegmatis* was used as the positive control. Tubes were allowed to incubate at 37˚C for 7 d and mycobacterial presence determined by fluorescing MGIT tubes under UV light.

Survivability of MAP in the nylon and plastic bags was investigated using Baker Retrieval Pyramids (BRP) (Fig. 3.1C; Reuter et al. 2008). A mixture of manure and straw was used to pack eight samples (each treatment within each bag type) into a BRP in a

manner so that there was no contact between bags. Two BRP's were suspended within the vicinity of each cow at 100 and 180 cm depths from a stainless steel cable anchored to wooden poles at 1.5 m intervals along the length of the pile (Figures 3.1A and 3.1B). A total of five pyramids in Pile 1 and six pyramids in Pile 2 were embedded as each pile was constructed. One BRP per pile was removed after 35, 67, 96, 131 and 250 days of composting. For removal, the stainless steel cable holding the suspended BRP was unclamped from the suspension pole and attached to the front-end loader of a tractor and pulled from the pile, leaving the remainder of the plastic sheet intact.

After the BRP's were extracted they were transported from the Glenlea Research Facility to a biosafety level 2 microbiology lab at the University of Manitoba. Sample bags were recovered from the manure in the BRP and one half of the tissue in each bag was stored at -20˚C while the remaining half was used for culturing purposes. Compost was collected by sampling 10 different areas from each pile on days 1, 96, 131, 194, 203 and 250. The compost samples from each day were composited, mixed and stored at -20˚C until further analysis.

3.3.1.3 Temperature monitoring

Ambient and internal composting temperatures were monitored for each compost pile. At each carcass within each structure, three type-T thermocouples were embedded at depths of 40, 140 and 190 cm (Fig. 3.1A) resulting in nine locations at which temperature was measured. Temperature was measured once daily with a hand-held digital thermometer (Digi-Sense Thermometer; Cole-Parmer Canada Inc., Montreal, QC, Canada), for the first 150 d and then once weekly for the remainder of the composting

experiment (total experimental period of 250 d). The thermocouple temperature within the piles was averaged at each site on each measurement day throughout the 250 d composting period. Temperature monitors (Hobo U12-015 Stainless Temperature Data Loggers; Onset Computer Corporation, Bourne, MA, USA) were also attached to the outside of each composting structure and to each BRP to measure ambient and inner temperatures hourly, and averaged for each day.

3.3.1.4 Chemical analysis

The moisture content of the compost was determined by drying at 60˚C to a constant weight for 50 h (method 942.05; Association of Official Analytical Chemists 1995). Oven-dried samples were then ground to pass through a 1-mm screen for analysis of total carbon (C), nitrogen (N) and sulfur (S) (method 968.06; Association of Official Analytical Chemists 1995). Ash content was determined by placing the dried compost in a muffle furnace at 600˚C for 2 h (method 942.05; Association of Official Analytical Chemists 1995). Macromineral (Na, K, P, Mg and Ca) content was determined by digestion of the ash with 1% HNO_3 5N HCl and analyzed using inductively coupled plasma (ICP) mass spectrophotometry (Vista-MPX CCD Simultaneous ICP-OES; Agilent Technologies Canada Inc., Mississauga, ON, Canada). Organic matter (OM) was determined using the equation: OM (g/kg) = 31.1 + 1.797(total C g/kg) (Larney et al. 2005). The pH and electrical conductivity (EC) of the compost (12.5 g) were determined after compost was allowed to stand in 50 ml of distilled water for 2 h and measured with an Accumet Research AR2 Dual Channel pH/Ion Meter (Fisher Scientific, Pittsburgh, PA, USA). Volatile fatty acid (VFA) profile was determined by shaking a 10 g sample of

compost with 0.1N HCl for 12 h, mixing with 1 ml of meta-phosphoric acid and centrifuging at 3000 rpm for 20 min. The supernatant (2 ml) was analyzed using gas chromatography following the column conditioning instructions for the 80/100 Chromosorb WAW (Supelco, Bellefonte, PA, USA). Analysis of chloride (Section 04.05-CL; Thompson 2002), ammonia (Section 04.02-C; Thompson 2002) and nitrate (Section 04.02-B; Thompson 2002) were conducted at A & L Canada Laboratories Inc. (London, ON, Canada). Recommended conditions for optimal composting (mortality and non-mortality) were compared with the biochemical results from this study (Table 3.1).

3.3.1.5 DNA analysis of bacteria

To identify MAP from composted tissues, a digestion/decontamination step was performed following the manufacturer's protocol in the BBL MycoPrep specimen digestion/decontamination kit (BD). Samples were then centrifuged at 3000 × *g* for 20 min and the pellet re-suspended in BBL MycoPrep phosphate buffer (BD). Next, 200 µl of the re-suspension was inoculated onto BBL Herrold's Egg Yolk Agar slants with mycobactin J, amphotericin, naladixic acid and vancomycin (BD) and incubated at 37˚C for 12 wks, with growth recorded weekly. At the end of 12 wks, all growth from the surface of the slants was removed and DNA extracted.

The DNA from the colonial growth on the surface of the slants was extracted using the Fungal/Bacterial DNA Kit (Zymo Research Corp., Orange, CA, USA) following the manufacturer's protocol. Survival of the cultured MAP and *M. smegmatis* in samples was verified with PCR. Primers used for verification are outlined in Table 3.2. A final volume of 25 µl containing 12.5 µl of PCR Master Mix (Promega Corp. Madison,

Table 3.1 Literature data for rapid composting in comparison with conditions from the present study over 250 days of composting of livestock mortalities.

Condition	Range from the present study	Optimal range[a]	Reference
C/N ratio	10:1 – 22:1	13:1 – 50:1	(Bishop and Godfrey 1983; Rynk 1992; Kalbasi et al. 2005; Xu et al. 2009)
Moisture content (%)	48 - 80	40 - 65	(Bishop and Godfrey 1983; Keener et al. 2000; Kalbasi et al. 2005)
pH	7.5 – 9.1	5.5 – 9.5	(Rynk 1992; Langston et al. 2002; Bernal et al. 2009 ; Xu et al. 2009)
Temperature (˚C)	10 – 64	40 - 65	(Rynk 1992; Kalbasi et al. 2005; Bernal et al. 2009)

[a] Recommended conditions for effective mortality composting during the biooxidative phase.

Table 3.2 Primer sets for PCR amplification and differential identification of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and *Mycobacterium smegmatis*.

Species	Orientation	Sequence (5'-3')	Size (bp)
MAP	Forward	GGGTTGATCTGGACAATGACGGTTA	569[a]
	Reverse	AGCGCGGCACGGCTCTTGTT	
Smeg	Forward	GTGCGCTACCTCGTCATGATG	624[b]
	Reverse	CTAGTTCATGTTCCAGGGCTCG	

[a] Primer set from Vansnike et al. (2004) targeting the IS *900* gene in MAP.
[b] Primer set designed using NCBI BLAST targeting the MSMEI_5897 gene in *M. smegmatis* (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; 2008).

WI, USA), 10.5 μl of nuclease free water (Promega Corp.), 0.5 μl of the forward primer (25 μM), 0.5 μl of the reverse primer (25 μM) and 1 μl of genomic DNA was used to perform PCR. Tubes were placed in a thermocycler (C1000 Thermal Cycler; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and amplified as follows: one cycle of denaturation at 94˚C for 4 min followed by 40 cycles of denaturation at 94˚C for 45 s, annealing at 61˚C for 45 s and extension at 72˚C for 45 s and a final extension at 72˚C for 10 min (Vansnick et al. 2004). Bacterial DNA isolated from MAP and *M. smegmatis* were both used as positive controls to differentiate these closely related species based on size of the amplicons. Water served as the negative control. The final PCR products were electrophoresed and visualized on a 1% (w/v) agarose gel stained with 2 μl ethidium bromide.

3.3.2 Experiment #2: *In vitro* incubation of MAP

The *in vitro* incubation of MAP included the following three treatments: 1) a control consisting of compost not mixed with MAP infected tissues, 2) lymph tissues (ileal, mesenteric and ileocecal) from cattle positive for MAP determined by serum ELISA (Hendrick et al. 2005) and 3) a combination of MAP positive tissues and compost mixed 1:4, respectively. Cured compost sampled at the completion of Experiment #1 was provided as the compost substrate for the control and 1:4 mixtures. Tissues were mixed together and 100 g of homogenate was placed into eight plastic bags and mixed by hand for 5 min. Two bags of each treatment were then placed in separate incubators at 4, 45, 60 and 80˚C. Samples were collected after 1, 2, 4, 8, 17, 30, 60 and 90 d and analyzed for

viable MAP as outlined in Experiment #1. Again, to ensure MAP was present in all treatments, tissue from each bag was cultured prior to incubation.

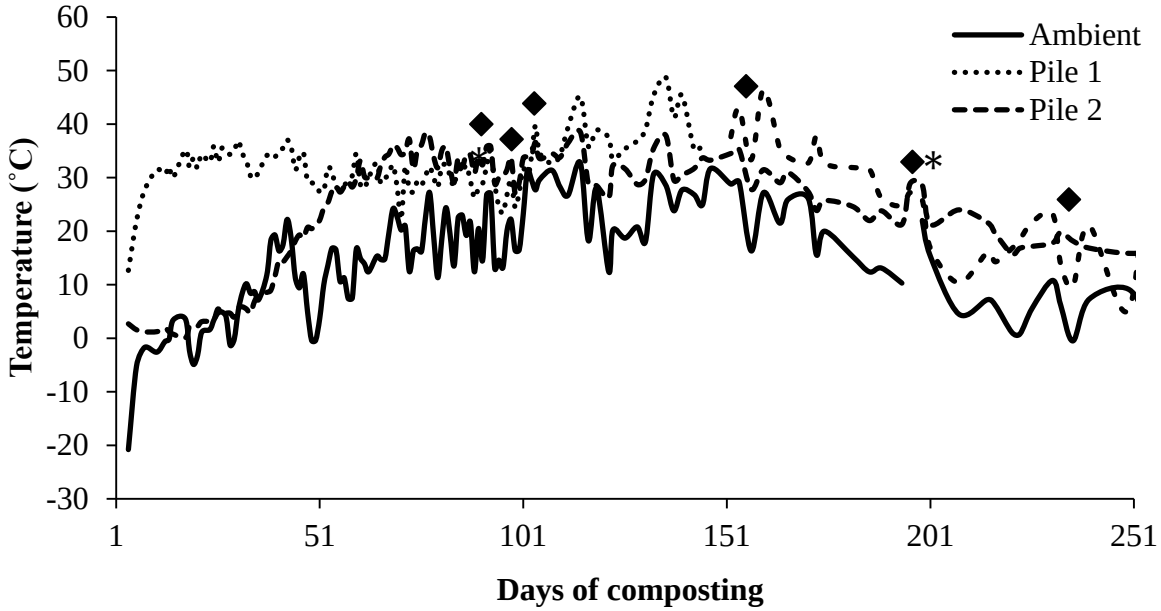
3.4 Results

3.4.1 Experiment #1: MAP survival in composting piles

During construction of the two composting piles, daily average ambient temperatures were -25°C (Fig. 3.2), with a windchill averaging -36˚C (www.climate.weatheroffice.gc.ca). Despite these cold temperatures, the composting piles did not freeze as the internal temperatures of both piles remained above 0°C (Fig. 3.2) throughout the trial. Nonetheless, thermophilic conditions were not achieved when considered as a homogeneous system, as the temperatures throughout either pile did not exceed 55°C. However, as the ambient temperature increased, so did the interior temperature of the piles. On day 194, both piles were turned and aerated, but temperature of the compost did not notably increase in either pile (Fig. 3.2). During this mixing event, the carcasses were not readily identifiable, but some soft tissues and bones were observed. On day 250, large bones, such as the skull, pelvis and long bones were still present, but all soft tissues had decomposed.

Temperature data collected from the Hobo data loggers (Onset Computer Corporation) attached to each BRP indicated that spatial variability did occur and a thermophilic state was achieved at several locations. Within Pile 1, the temperature of BRP 2 (extracted on day 67) was > 50˚C for 35 d with a peak temperature of 64˚C on day 25 (Fig. 3.3A). All other BRP locations within Pile 1 remained between 35-40˚C for the entire 250 d composting period except for BRP 1 (extracted on day 35), which only

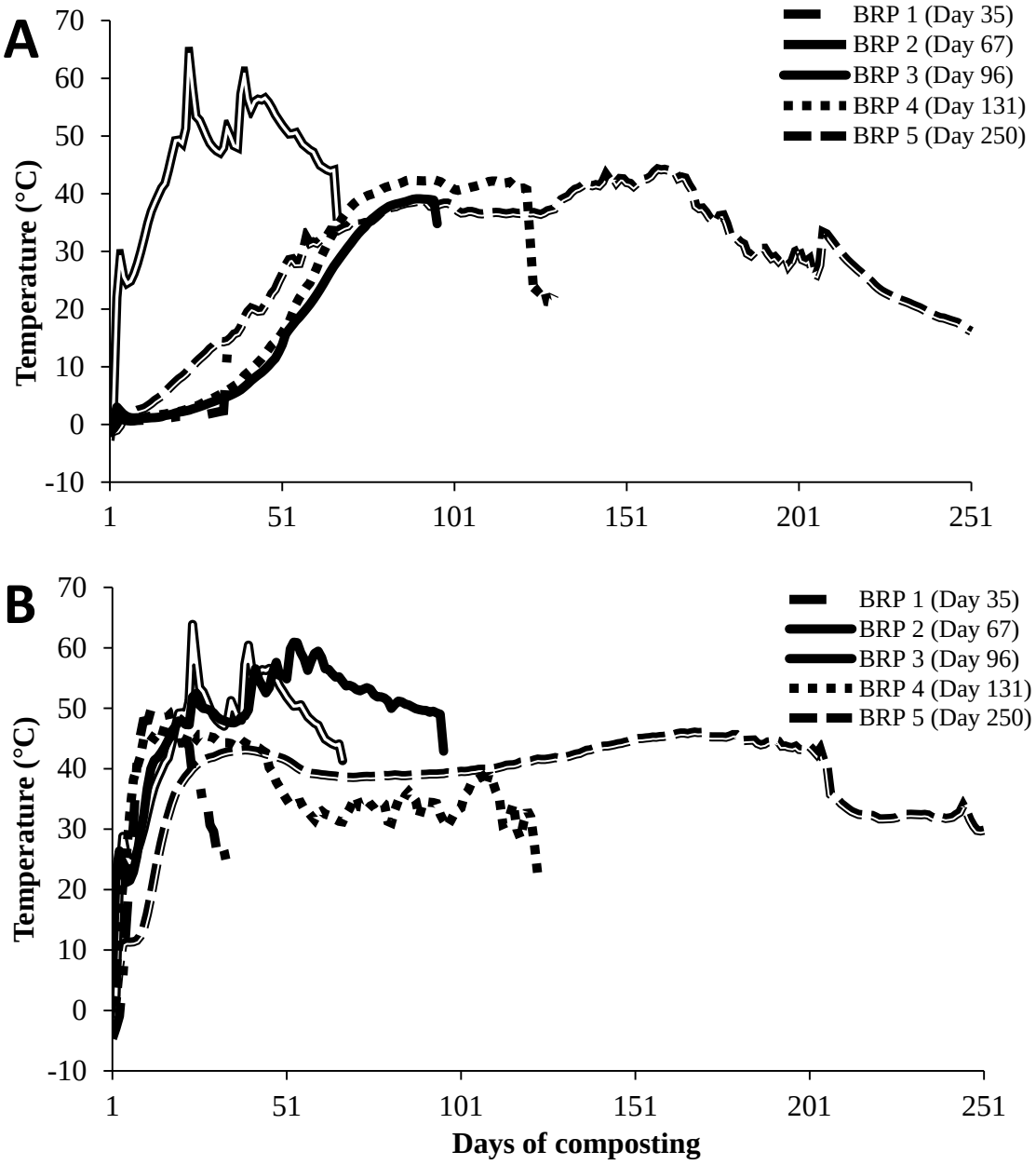
Figure 3.2 Ambient and internal composting temperature profiles of Pile 1 and Pile 2 over 250 days of composting.



* Turning events of both compost piles.

◆ Major rain events throughout composting experiment (> 25mm/day; http://climate.weatheroffice.gc.ca/climateData).

Figure 3.3 Internal composting temperature profiles of Pile 1 (A) and Pile 2 (B) for each individual chamber location over 250 days of composting.



reached up to 15˚C (Fig. 3.3A). Within Pile 2, temperature at all BRP locations was > 40˚C by day 25 (Fig. 3.3B). In Pile 2, BRP 1 increased to 52˚C by day 15 then declined to 20˚C and remained at that temperature until extraction on day 35 (Fig. 3.3B); BRP 2, extracted on day 67, increased to 65˚C on day 25 and remained > 50˚C for approximately 40 d (Fig. 3.3B); BRP 3, extracted on day 96, remained between 50-62˚C for about 60 d (Fig. 3.3B); BRP 4 increased to 47˚C by day 20 then remained between 25-40˚C until removal on day 131 (Fig. 3.3B); and BRP 5 remained between 35-45˚C for the entire 250 d composting period (Fig. 3.3B).

Moisture content ranged between 480-800 g/kg and was relatively constant throughout the experiment except between days 101 to 201 (Fig. 3.4A) (major rain events indicated in Fig. 3.2). This period coincided with the summer months, where warmer ambient temperatures (between 10-30˚C) and fewer rain events resulted in desiccation of the piles. Large volumes of leachate were collected from both piles (data not shown) prior to day 101 and after day 201. Prior to day 101 leachate volumes were attributed to the high moisture losses associated with the decomposition of carcasses. After day 201, an association between rain events and volume of leachate collected was observed (data not shown), which increased moisture content to 800 g/kg (Fig. 3.4A). The pH of compost ranged from 7.5 to 9.0 (Fig. 3.4B). Total C rapidly declined from 450 to 370 g/kg until day 100 and plateaued thereafter to day 250 (Fig. 3.5A). As expected, organic matter (OM) exhibited the same slight decline as observed for total C (Fig. 3.5B). Nitrogen increased until day 194 (Fig. 3.5A), when the compost was turned. Following an initial decline until day 94, the C/N ratio remained relatively stable at 12:1 over the 250 d composting period (Fig. 3.5C). Compost temperatures in the present study had a

Figure 3.4 Moisture (A) and compost pH (B) measured over 250 days of composting.

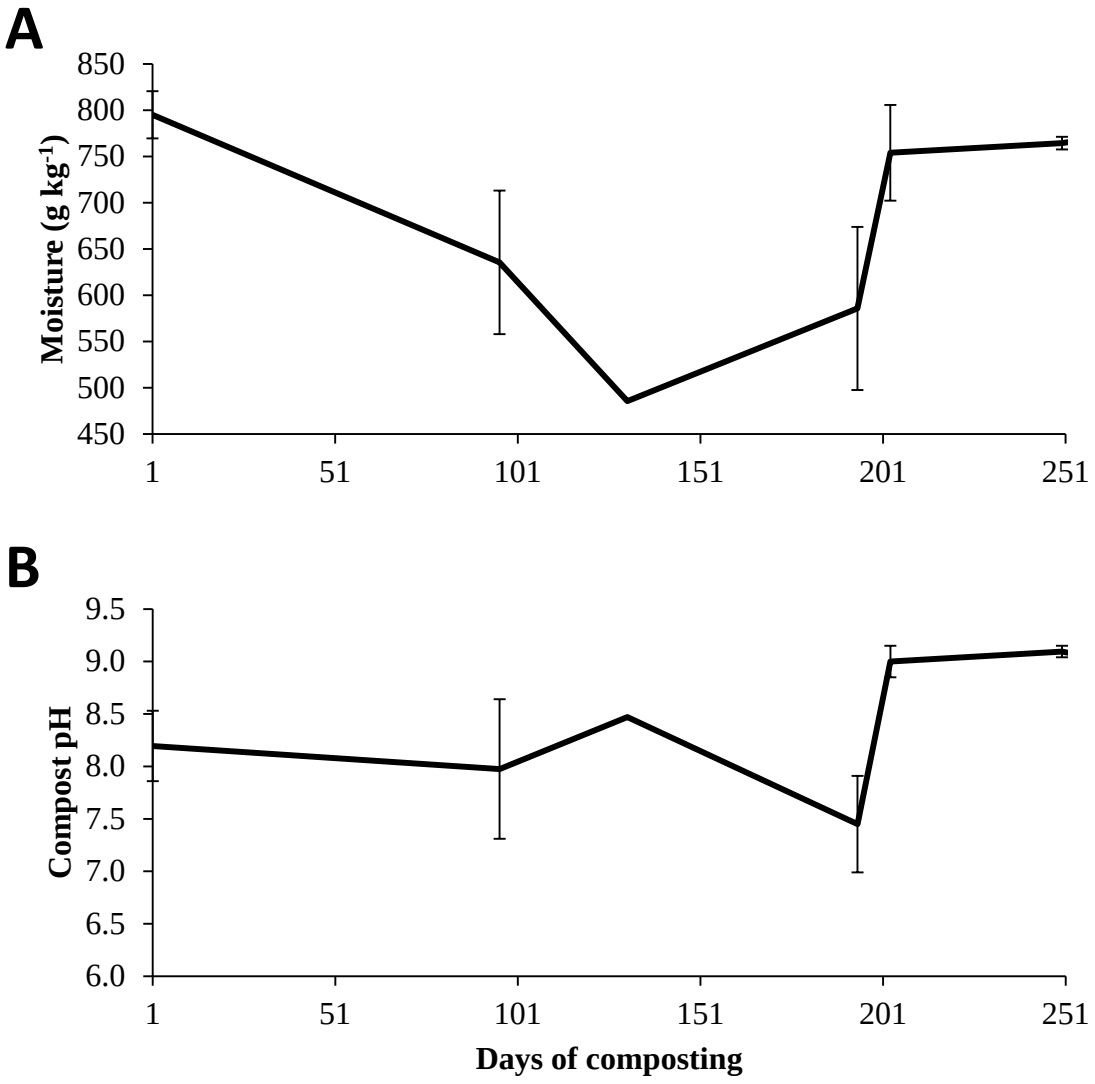
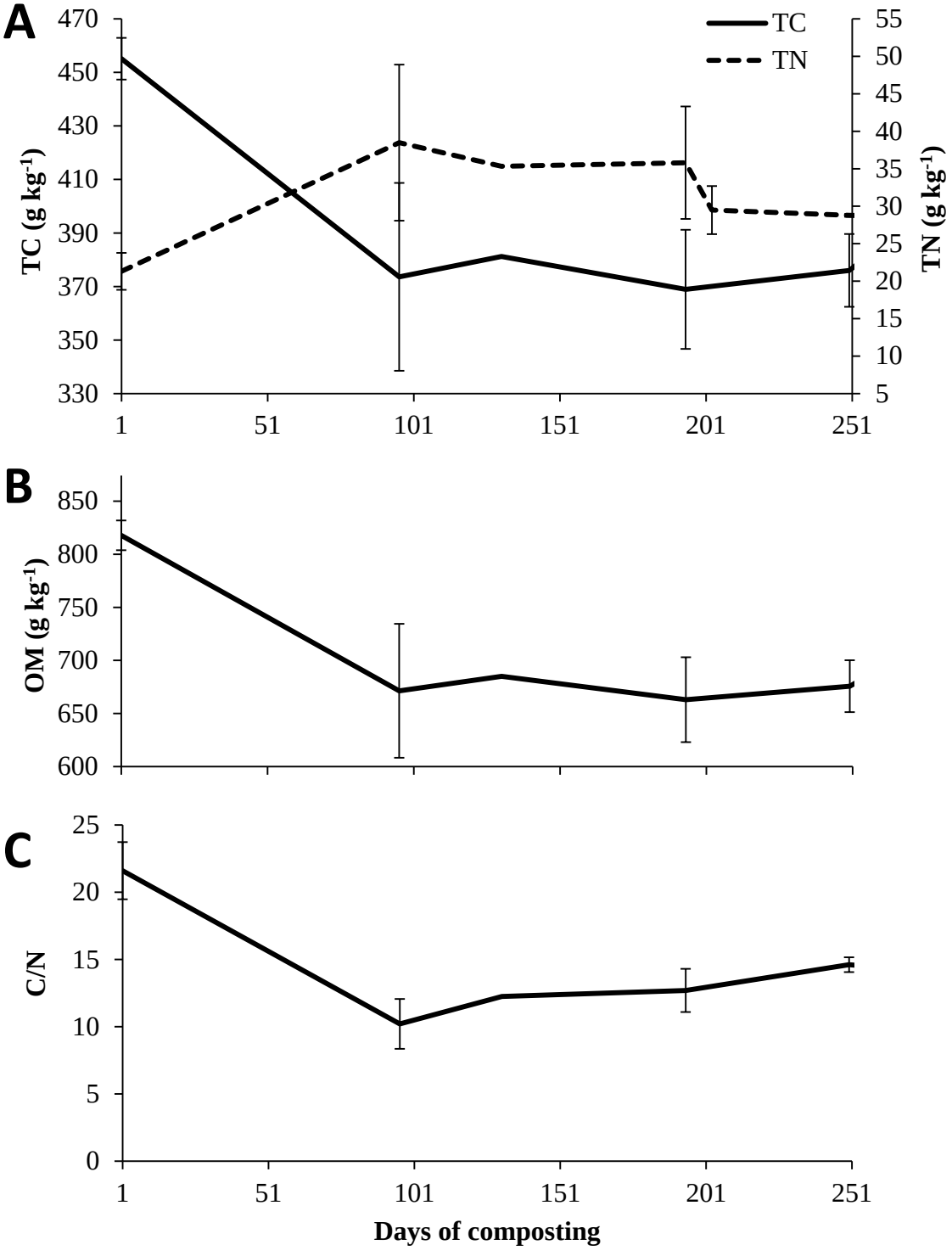


Figure 3.5 Total carbon (TC) and total nitrogen (TN) (A), organic matter (OM) (B) and the C/N ratio (C) over 250 days of composting.



range of 10-64˚C (Fig. 3.2), a much broader range than the optima of 40-65˚C for the biooxidative phase (Table 3.1). However, according to the temperature readings from the data loggers attached to each BRP, several locations were within acceptable levels, indicating heterogeneity of the compost.

Survival of MAP and *M. smegmatis* from both piles after 1, 35, 67, 96 and 250 days of composting are presented in Table 3.3. After day 67, *M. smegmatis* was not detected, however MAP, irrespective of the source or sample bag, was isolated over the entire 250 d of composting.

3.4.2 Experiment #2: *In vitro* survival of MAP

The *in vitro* incubation experiment revealed that MAP was recoverable from compost mixed with infected tissues held at 80°C for 90 d (Table 3.4). Although MAP was not consistently identified in compost samples collected on all days (i.e. MAP not identified in the 1:4 mixture on days 8 and 60 at 60˚C), we attribute this to the inability of PCR to amplify DNA due to sampling variability as MAP spiked tissues were mixed with compost in a 1:4 ratio (Table 3.4).

3.5 Discussion

A biosecure, static structure for composting livestock mortalities was constructed to assess inactivation of MAP and *M. smegmatis* during the decomposition of organic matter. Throughout the 250 d experimental period, temperature and various environmental conditions were monitored in an attempt to characterize those factors that may influence bacterial survival.

Table 3.3 Viability of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and *Mycobacterium smegmatis* (Smeg) from both piles after 250 days of composting.

	Days of composting									
	1		35		67		96		250	
	MAP [e]	Smeg [f]	MAP	Smeg	MAP	Smeg	MAP	Smeg	MAP	Smeg
	----------Nylon bag----------									
Control[a]	-/-*	-/-	-/NA**	-/NA	-/-	-/-	-/-	-/-	-/-	-/-
Smeg[b]	-/-	+/+	-/NA	+/NA	-/-	-/+	-/-	-/-	-/-	-/-
MAP [c]	+/+	-/-	+/NA	-/NA	+/+	-/-	+/+	-/-	+/+	-/-
LN[d]	+/+	-/-	+/NA	-/NA	+/+	-/-	-/+	-/-	+/+	-/-
	----------Plastic bag----------									
Control	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
Smeg	-/-	+/+	-/-	+/-	-/-	-/+	-/-	-/-	-/-	-/-
MAP	+/+	-/-	+/+	-/-	+/+	-/-	-/+	-/-	+/+	-/-
LN	+/+	-/-	+/+	-/-	+/+	-/-	-/+	-/-	+/+	-/-

*First value in cell indicates survival of samples from Pile 1; Second value in cell indicates survival of samples from Pile 2.
**No samples available for analysis from Pile 2 at 35 days of composting.
[a] Ground beef as a control not inoculated with MAP.
[b] Lab cultured *Mycobacterium smegmatis* (Smeg) inoculated into ground beef.
[c] Lab cultured *Mycobacterium avium* subspecies *paratuberculosis* (MAP) inoculated into ground beef.
[d] MAP positive mesenteric lymph tissue.
[e] Primer set for MAP to detect viability by PCR in all samples (Table 3.1).
[f] Primer set for Smeg to detect viability by PCR in all samples (Table 3.1).

Table 3.4 Viability of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) *in vitro* over 90 days of incubation.

	Sample days							
Temperature (°C)	1	2	4	8	16	30	60	90
	----------Compost (control)[a]----------							
4, 45, 60 and 80	-	-	-	-	-	-	-	-
	----------Lymph tissue[b]----------							
4, 45 and 60	+	+	+	+	+	+	+	+
80	+	+	+	+	+	+	+	-
	----------Compost mixed with lymph tissue[c]----------							
4	-	+	+	+	+	+	+	+
45	+	+	+	-	+	+	+	+
60	+	-	+	-	+	+	-	+
80	-	-	+	+	+	+	+	+

[a] *Mycobacterium avium* subspecies *paratuberculosis* (MAP) negative compost as control.
[b] MAP positive mesenteric lymph tissue.
[c] MAP positive mesenteric lymph tissue and compost (1:4 ratio, respectively).

During composting, temperature changes associated with aerobic metabolic activity of the composting microorganisms can be described by two phases, the biooxidative phase and the maturation phase (Kalbasi et al. 2005; Bernal et al. 2009). The biooxidative phase generally begins within the first few days of composting when primarily mesophilic organisms (optimum growth at temperatures between 20-45˚C; Misra et al. 2003) decompose OM, resulting in an increase in temperature to over 40˚C (Kalbasi et al. 2005; Bernal et al. 2009). As the temperature increases, thermophilic organisms proliferate (optimum growth at temperatures between 50-70˚C; Misra et al. 2003) and decompose OM and inactivate many pathogens (Kalbasi et al. 2005; Bernal et al. 2009). Optimal composting during this phase should result in temperatures between 40-65˚C (Rynk 1992). Temperatures between 52-60˚C are optimal for OM decomposition and temperatures >55˚C inactivate a number of pathogens (Miller 1993; Kalbasi et al. 2005). As the remaining available OM is degraded, the temperature declines due to reduced microbial activity (Bernal et al. 2009) and the compost enters the maturation phase. During this phase, the compost temperature remains between 10-40˚C while the remaining OM, which includes recalcitrant cellulose and lignin, undergoes humification (Bernal et al. 2009).

Although internal temperatures measured with the thermocouples averaged 30˚C over the 250 d of composting in both piles (Fig. 3.2), temperatures measured with Hobo data loggers (Onset Computer Corporation) at the site of sample implantation were far more variable ranging from 0˚C to 60˚C (Figures 3.3A and 3.3B). Xu et al. (2009) used a similar composting system and observed rapid heating of the compost, with temperatures reaching 55-65°C within 20 d and remaining in this range for 35 d in both structures.

However, these piles were exposed to ambient temperatures that remained around 20°C over the 147 d composting period. As mentioned previously, the average ambient temperature was as low as -25°C (Fig. 3.2) with the windchill resulting in average ambient temperatures of -36˚C throughout construction of the compost structures. An initial temperature spike was not observed in this study, and due to the low ambient temperature the average internal temperatures (20˚C) for both piles over the first 35 d (Fig. 3.2) were below the recommended range (Table 3.1). Construction of the composting system at sub-zero temperatures (-20˚C) precluded the rapid increase in compost temperature and impeded microbial activity at a key point in the composting process. However, upon closer evaluation of the internal temperatures, each BRP was subject to a unique temperature profile (Figures 3.3A and 3.3B) as a consequence of the heterogeneous nature, which is characteristic of a carcass-composting matrix. It is plausible that the composting pile temperatures observed were only in part, a consequence of the low ambient temperature.

Biochemical compost parameters from this study were within the recommended ranges for effective composting (Table 3.1). Mortality compost is a heterogeneous mixture of animal carcasses with high moisture (leading to lowered porosity), high N and low C, mixed with materials of low to medium moisture (leading to higher porosity), low N and high C (Kalbasi et al. 2005). Such mixtures tend to become imbalanced in their C/N ratio as C is oxidized and N is concentrated. This leads to decreased microbial activity and a transition from the biooxidative to the mature composting phase (Bishop and Godfrey 1983; Kalbasi et al. 2005). However, in the current study, one location in Pile 1 and three locations in Pile 2 had temperatures > 50˚C, but heating was not

homogeneous throughout each pile. The ambient temperature during construction was likely the limiting factor for a uniform heating throughout the compost piles.

Under the described conditions, MAP remained viable after 250 d of composting in large scale composters designed for the disposal of cattle mortalities despite internal composting temperatures adequate for pathogen inactivation (Rynk 1992; Kalbasi et al. 2005; Bernal et al. 2009; Xu et al. 2009) being reached in specific locations within each pile for several weeks (Figures 3.3A & 3.3B). Several sites in both piles achieved temperatures between 50-65°C for at least 3 wks with MAP recoverable at all sites, regardless of the temperature. Furthermore, MAP was still recovered in an *in vitro* study after sustained temperatures of 80˚C over 90 days, suggesting compost conditions are not sufficient to inactivate MAP from infected carcasses. As indicated in Table 3.3, MAP still remained viable after 90 d at this temperature suggesting that even effective composting conditions are unlikely to be a suitable method for disposal of cattle infected with MAP. Firstly, it is unlikely that temperatures during carcass composting will be homogeneous throughout the pile, as demonstrated in this study, or that temperatures as high as 80°C will be achieved during the composting process (Kalbasi et al. 2005). Secondly, if such temperatures were attained, they would have a deleterious effect on composting, by inactivating many beneficial organisms essential to the process, as temperatures greater than 80˚C, are not within the optimal range of 40-65˚C for composting (Rynk 1992). Temperatures greater than 72˚C, have been shown to markedly reduce microbial activity during composting (Bernal et al. 2009).

It has been observed that MAP possesses several characteristics that may account for its environmental fitness under extreme conditions. The thick, lipid-rich cell wall is

responsible for its extreme tolerance to heat (Gao et al. 2002; Cerf et al. 2007; Rademaker et al. 2007; Whittington et al. 2010), and resistance to desiccation (Lovell et al. 1944; Whittington et al. 2004) and UV radiation (Donaghy et al. 2009). Thermal tolerance of MAP demonstrated in this study has been observed elsewhere. Studies examining resistance concluded that although numbers of MAP were reduced by pasteurization (72˚C), chlorination of water (2 ppm; Rowe and Grant 2006) and exposure to 24 min of UV radiation in milk (Donaghy et al. 2009), viable cells could still be isolated after these treatments. Turbulent-flow pasteurization has also been shown to significantly reduce but not eradicate MAP in milk (Hammer et al. 2004; Rademaker et al. 2009; Whittington et al. 2010). In the current study, viability of MAP was not observed on day 8 and day 60 at 60˚C (Table 3.4). It was speculated that sampling variability rather than thermal intolerance was responsible as MAP was detected on day 90 at 60˚C and 80˚C. However, if previous studies did not completely eliminate MAP it is possible that viable cells (cultured on HEYA but not quantified in this study) were still present, but at numbers too low to be detected by PCR.

Survival of MAP has been demonstrated for extended periods of time in various environments (Lovell et al. 1944; Whittington et al. 2004; Grewal et al. 2005; Rowe and Grant 2006). As early as 1944, Lovell et al. (1944) demonstrated that feces infected with MAP placed in an open bowl exposed to atmospheric conditions, survived for 246 d after exposure to freezing and desiccation at temperatures between -3˚C to 23˚C. Whittington et al. (2004) recovered viable MAP cells after 385 d in feces applied to soil in a fully shaded environment. In addition, the survival of MAP was not influenced by soil pH (5.7 to 7.4; Whittington et al. 2004). Similarly, compost pH did not influence MAP survival in

the current study (Fig. 3.4B). Whittington et al. (2004) concluded that dormancy, defined as a non-spore forming state permitting survival, was the most likely survival mechanism as MAP was recovered after 24 wks when previous attempts to culture fecal and soil samples at 18 wks were negative. However, those aforementioned studies did not examine viability in a composting environment. Grewal et al. (2005) examined the persistence of MAP in four-liter vessels designed to simulate liquid manure storage and a composting environment. Temperatures of 55˚C were maintained in the liquid manure and composting environments throughout a 56 day-period. Viable cells were isolated using standard culture techniques from the liquid storage treatment on all sample days (0, 3, 7, 14, 28 and 56) and from the compost on days 14, 28 and 56 but not on days 3 and 7. Further, MAP DNA was detectable in all samples collected from both the liquid storage and compost. In addition, MAP DNA was also detectable in samples taken on day 175 in the liquid storage. Grewal et al. (2005) speculated that since MAP was not observed in culture on day 3 or 7, the cells were either dead or below detectable levels at that time. Conversely, the large-scale composting piles in the current study demonstrated that MAP was not inactivated after exposure to both microbial and biochemical compost conditions. The observed differences regarding the presence of MAP may be attributed to differences in the methodology used in the two studies. In the current study, MAP DNA was extracted directly from the bacterial cultures and confirmed positive with PCR. Thus all samples positive for MAP were considered viable. Grewal et al. (2005), however, analyzed MAP via culture and PCR separately, and therefore MAP was only considered viable if observed in culture.

Other adaptive functions of MAP have been suggested to explain why it can survive for extended periods of time in the environment. In an *in silico* analysis by Whittington et al. (2004), the MAP genome contained dormancy genes similar to those coding for resistance to nutritional and oxidative stress in other species of mycobacteria (i.e. *M. smegmatis*, *M. bovis* and *M. tuberculosis*). However, the exact physiological mechanism triggering dormancy was unclear (Whittington et al. 2004). More recently, sporulation has been suggested as a means for MAP environmental persistence (Lamont et al. 2012). A MAP spore-like morphotype was produced possessing the MAP genotype with the ability to infect bovine macrophages and remained viable after exposure to heat treatment at 70˚C and post exposure to lysozyme and proteinase K (Lamont et al. 2012). Although sporulation was induced by nutrient deprivation in that study, the authors of this study speculated that temperature-induced sporulation may be the mechanism by which MAP survived at > 55°C in the compost piles and at 80°C *in vitro* (Lamont et al. 2012). It is apparent that more research is needed to further evaluate the MAP spore-like morphotype in soil, water and post pasteurization (Lamont et al. 2012). Intracellular growth and survival in environmental protozoa (Mura et al. 2006; Rowe and Grant 2006), biofilm formation or aerosolization (Rowe and Grant 2006) have also been proposed as survival tactics. However, all studies concluded that more research is required to determine the exact cause of MAP's resilience in the environment.

Mycobacterium smegmatis possesses desirable characteristics as a surrogate for MAP. It is a fast-growing species of mycobacteria (Chaturvedi et al. 2007) with no linkage to human disease and possesses dormancy genes similar to those in MAP (Whittington et al. 2004). These characteristics have led to its use as a surrogate for

Mycobacterium tuberculosis (Whittington et al. 2004). However, our data suggests that *M. smegmatis* is not a suitable surrogate for MAP, as it could not be detected after 67 d of composting. Further, MAP may have a resistance mechanism, such as sporulation, that is not active or present in *M. smegmatis* under composting conditions.

In conclusion, MAP could not be inactivated in a biosecure, static composting system for cattle mortalities. Neither temperatures of 60ºC in several sites nor biochemical conditions, which prevailed within the compost, rendered MAP non-viable. An *in vitro* incubation experiment demonstrated MAP survival after 90 days at 80˚C in the presence of a compost matrix, making it unlikely that MAP could ever be completely inactivated through composting. Further it was concluded that *M. smegmatis* is not a suitable surrogate for MAP. Given the possible role of MAP as a causative agent in Crohn's disease and its resistance to inactivation in many different environments, an alternative means for disposal of JD infected cattle is necessary if biocontainment is to play a role in arresting the spread of this disease. Additional research into traits such as dormancy or sporulation is also required to further understand MAP's mechanism of survival within the environment.

4.0 Targeted 16S rRNA high-throughput sequencing to characterize the microbial community in compost of livestock mortalities

4.1 Abstract

Manipulation of the microbial community is necessary to ensure a significant reduction in pathogens during the composting process. To gain a better understanding of changes occurring in the microbial community during mortality composting, two biosecure, static composting systems containing cattle carcasses were constructed and sampled after 35, 67, 96, 131 and 250 d of composting. Temperature at each sampling site was measured continuously and samples were classified as either thermophilic (> 55°C) or mesophilic (< 50°), based on temperature exposure. High-throughput 454-sequencing of the 16S rRNA gene was used to characterize the bacterial communities within each sample. Clustering of bacterial communities was not observed within piles or sampling day, but thermophilic and mesophilic samples clustered separately. Neither richness nor diversity differed between temperature groups. *Firmicutes* was the most abundant phylum within both temperature groups, but the numbers were higher in thermophilic samples ($p < 0.05$), whereas *Proteobacteria* was more pronounced in mesophilic samples ($p < 0.05$). In thermophilic samples, members of the groups *Thermobifida*, UO (*Actinomycetales*) and UO (*Bacillales*), which all played an important role in organic matter degradation, were significantly higher ($p < 0.05$) at higher temperature. Members of *Clostridia*, were also prominent at temperatures > 55°C ($p < 0.05$), suggesting that anaerobic pockets existed within the composting piles. Substantial spatial diversity exists within bacterial communities in field- scale compost piles. Lack of

uniform distribution of air and nutrients resulting in uneven heating in the composting pile is the likely explanation for this observation.

4.2 Introduction

Microbial ecology involves defining the types, frequency and function of microorganisms within a variety of environments (Ward 2002; Jessup et al. 2004). It is estimated that less than 1% of all microorganisms in the natural world have been identified and even less known about their function (Fuhrman 2009). The advent of high-throughput sequencing has increased the capacity and rate at which functional genes and novel sequences of previously unknown microorganisms are discovered (454 Life Sciences 2013). Currently, third generation 454-sequencing has the ability to generate large datasets, with over one million reads per run and an average read length of 700 bp (454 Life Sciences 2013). With a higher sequence count and longer read lengths, a more comprehensive picture of the microbial biodiversity within a given environment is observed, compared to that using culture-based or conventional DNA sequencing technique (Petrosino et al. 2009; 454 Life Sciences 2013). The reduced cost of next generation sequencing has also made it an attractive methodology for microbial ecologists (Petrosino et al. 2009; 454 Life Sciences 2013).

Composting is as an environmentally sound way to dispose of organic material including household waste and livestock mortalities (Senne et al. 1994; Xu et al. 2009). The composting process is affected by a number of environmental variables (e.g. temperature, moisture, oxygen, pH, C/N ratio) that undoubtedly exert their influence by altering the microbial communities involved in the decomposition of organic matter.

Consequently, it is relevant to study the effect of these variables on the frequency and composition of microbial communities, including pathogens during the composting process.

The process of mortality composting has been described by Kalbasi et al. (2005) as the temporary, above-ground burial of mortalities in supplemental carbon, aerobically decomposed by microorganisms at temperatures required to breakdown tissues and kill most pathogens. This process has been shown to inactivate several foodborne pathogens, including *Esherichia coli* and *Camplobacter jejuni* (Xu et al. 2009), and viruses, including avian influenza (Flory and Peer 2010) and foot and mouth disease (Guan et al. 2010). Further, there is evidence that the risk of disease spread from infected carcasses could be reduced with the use of composting to either eliminate or significantly reduce the concentration of a number of disease-causing agents (Huang et al. 2007; Flory and Peer 2010; Reuter et al. 2010).

Several studies have previously characterized the microbial community of compost. However, direct comparisons between studies is challenging due to differences in compost matrices (e.g. manure (Maeda et al. 2010), organic waste (Martens et al. 2013), municipal biowaste (Partanen et al. 2010)), the composting scale (i.e. field (Yamamoto et al. 2009) vs. pilot (Schloss et al. 2003)) and molecular methods utilized to identify microorganisms (Yamamoto et al. 2009; Maeda et al. 2010; de Gannes et al. 2013; Martins et al. 2013).

Across studies, substantial spatial variation in temperature and oxygen availability has been reported in the composting process (Fernandes et al. 1994; Maeda et al. 2010; Xu et al. 2010). In all composting systems, the abundance and activity of microorganisms

are mainly affected by temperature, moisture, oxygen availability and nutrient availability (Kalbasi et al. 2005; Bernal et al. 2009). However, if any of the previously mentioned variables fall outside specific ranges and oxygen in particular is limited, microbial activity is inhibited and the raw material will fail to heat or sustain optimum temperatures for rapid decomposition (Fernandes et al. 1994). Even within piles of uniform compost substrates, temperature can vary significantly within the pile depending on aeration method (Fernandes et al. 1994). In actively aerated systems, oxygen limitation is overcome through mechanical turning of the compost (Xu et al. 2010). Conversely, static systems are typically not mixed until uniform temperatures exceeding 55°C have been achieved (Xu et al. 2010), to ensure adequate inactivation of weed seeds and phytotoxic and pathogenic organisms (Zucconi and de Bertoldi 1987). As temperature development is a good indicator of composting performance, temperature variation at different pile locations are indicators of uneven distribution of air and nutrients that needs to be addressed to improve the process.

As temperature plays a key role in pathogen activation (Xu et al. 2009; Reuter et al. 2010), and uneven heating in a mortality composting pile is common, an understanding of the spatial variation in temperature, which is observed within the pile, is essential in order to improve the composting process. To further understand changes in temperature, the microbial community in a biosecure composting structure was characterized using 454-pyrosequencing.

4.3 Materials and methods

4.3.1 Composting experiment

As previously described in a study by Tkachuk et al. (2013), a large-scale static composting experiment of cattle mortalities was conducted at the University of Manitoba Glenlea Research Station (Winnipeg, Manitoba, Canada). In brief, tissue samples were packed into (Baker Retrieval Pyramid's) BRP's and suspended into two biosecure composting structures (Pile 1 and Pile 2), as outlined in Manuscript I. Samples were placed in nylon bags (pore size 50 μm; ANKOM rumen *in situ* bags; Ankom Technology, Macedon, NY, USA), for a total of eight samples per BRP and a total of five BRP's in Pile 1 and six BRP's in Pile 2 were embedded as each pile was constructed. Pyramids were removed on day 35, 67, 96, 131 and 250 of composting. For removal, the stainless steel cable holding the suspended BRP was unclamped from the suspension pole and attached to the front-end loader of a tractor and pulled from the pile, leaving the remainder of the pile intact. After the BRP's were extracted they were transported from the Glenlea Research Facility to a biosafety level 2 microbiology laboratory at the University of Manitoba. Sample bags were recovered from the manure in the BRP and one half of the tissue in each bag was stored at -20˚C while the remaining half was used for the DNA extraction. Temperature monitors (Hobo U12-015 Stainless Temperature Data Loggers; Onset Computer Corporation, Bourne, MA, USA) were attached to the outside of each composting structure and to each BRP to measure ambient and inner temperatures hourly, and averaged for each day. Samples were considered thermophilic if the compost had been exposed to temperatures greater than 55°C, and subsequently declined to temperatures < 50°C at the time of sampling and site of sample implantation, and mesophilic if compost had only been exposed to temperatures < 50°C (Kalbasi et al. 2005; Xu et al. 2009; Tkachuk et al. 2013). In addition to temperature, other biochemical

parameters measured included moisture, pH, total nitrogen (N), total carbon (C), C/N ratio and total organic matter (OM) were reported (Tkachuk et al. 2013).

4.3.2 Sample preparation for pyrosequencing

Approximately 200 μg of tissue from each nylon bag were removed and total genomic DNA extracted using the MO BIO PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA), following the protocol outlined in the kit. Each sample was analyzed by PCR using the primer set 27F (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342R (5'-CTGCTGCCTCCCGTAG-3') to guarantee amplification of a 352 bp-region across the V1 to V3 region of the 16S rRNA gene. Each DNA sample absorbance was measured with a spectrophotometer (Beckman Coulter DU 800 Spectrophotometer; Beckman Coulter, Brea, CA, USA) at wavelength 260 nm. The DNA concentration was subsequently calculated from the absorbance and a minimum DNA concentration of 20 μg/ml was required from each sample for 454-pyrosequencing. The DNA quality was also measured at an OD ratio of 260:280 nm and maintained within the range of 1.1-1.2. Tubes were placed in a thermocycler (C1000 Thermal Cycler; Bio-Rad Laboratories Inc., Hercules, CA, USA) and PCR was performed in a final volume of 25 μl containing: 2.5 μl of 10x iTaq Buffer (Bio-Rad Laboratories), 0.75 μl $MgCl_2$ (50 mM; Bio-Rad Laboratories), 0.13 μl iTaq DNA polymerase (5 units/μl; Bio-Rad Laboratories), 0.5 μl dNTP mix (10 mM each x 200 μl; Bio-Rad Laboratories), 18.12 μl of nuclease free water (Promega Corporation, Madison, WI, USA), 0.5 μl of the forward primer (25 μM), 0.5 μl of the reverse primer (25 μM) and 1 μl of DNA template. Amplification was as follows: one cycle of denaturation at

94˚C for 3 min followed by 36 cycles of denaturation at 94˚C for 60 s, annealing at 60˚C for 60 s and extension at 72˚C for 60 s and a final extension at 72˚C for 5 min. Water was used as template for the negative control. The final PCR products were analyzed by electrophoresis on a 1% (w/v) agarose gel stained with ethidium bromide and visualized under UV light. If a band could not be visualized on the agarose gel, the genomic DNA was either concentrated or diluted until a product was observed.

4.3.3 454-pyrosequencing of the V1-V3 region of the 16S rRNA genes

Samples were sent to the Research and Testing Laboratory in Lubbock, Texas, USA (http://www.researchandtesting.com) and analyzed using the Genome Sequencer FLX Titanium Series Instrument (454 Life Sciences, Roche Diagnostics Corporation, Branford, CT, USA) according to the methodology reported by Dowd et al. (2008). The V1-V3 region (491 bp) of the 16S rRNA gene, for read lengths less than 700 bp, was targeted as it has been deemed the most suitable for distinguishing bacterial species ranging from the phylum to the genus level (Petrosino et al. 2009; Kim et al. 2011). A standard flowgram format (sff) file was generated after targeted sequencing with the primers 28F (5'-GAGTTTGATCNTGGCTCAG-3') and 519R (5'-GTNTTACNGCGGCKGCTG-3'), which included information regarding the light signal strength from each sequenced sample, along with the intensity of the signal proportional to the number of bases added throughout each flow and plotted as a function of flow (nucleotide) order (454 Life Sciences 2013).

4.3.4 Sequence-processing pipeline

Mothur (version 1.29.0; April 2013) was used to process and analyze the 454-pyrosequences (Schloss et al. 2009; Schloss et al. 2011). The first step in the sequence-processing pipeline included removing low quality reads due to sequencing error from the raw data output file. The PyroNoise algorithm identified an idealized form of each flowgram from each sample, requiring a minimum length of 450 flows, resulting in de-noised data translated into 199,947 total DNA sequences (Appendix 1) (Quince et al. 2011). The DNA sequences were merged into a FASTA file, formatted to contain tentative consensus sequences, and the number of reads integrated into each consensus (Dowd et al. 2008). Sequences were removed from the dataset if there were greater than one mismatch with the barcode, greater than two mismatches to the primers, ambiguous bases, a homopolymer length of at least eight nucleotides and if reads were less than 200 bp (Schloss et al. 2011). The remaining 98,189 redundant sequences (Appendix 1) were aligned to the curated Silva bacterial reference database (http://www.mothur.org/wiki/Silva_reference_files; Pruesse et al. 2011). Sequences were aligned to their closest match and the Needleman-Wunsch algorithm was used to make a pairwise alignment compatible between the unknown candidate sequence and the database template sequences (Schloss et al. 2011). After the alignment, the sequences were screened and filtered to ensure that all of the sequences overlapped the same region of the gene, which resulted in 95% of the reads with a length between 227-260 bp (Schloss et al. 2011). The precluster algorithm merged sequences within two bp mismatch to the more abundant sequence to further reduce sequencing error, due to the likelihood that a one bp mismatch per every 100 bp arises as a result of pyrosequencing error (Huse et al. 2010). Next, chimeras were removed with the Uchime detection

program (Edgar et al. 2011) using the most abundant sequences as references (Schloss et al. 2011). Finally, potential contaminants (i.e. chloroplasts, mitochondria, sequences not classified at the Kingdom level) were identified, by classifying the sequences to a reference dataset provided in Mothur, and removed from further analysis (Schloss et al. 2011).

4.3.5 Preparing sequences for operational taxonomic unit (OTU)-based analysis

A total of 92,679 sequences were used to calculate pairwise distances and build a distance matrix. The matrix was used to cluster sequences with the average neighbour clustering algorithm at several genetic distances (unique, 0 and incrementally up (+ 0.01) to 0.06). For the operational taxonomic unit (OTU)-based approach, sequences with 3% genetic distance were considered to be species and defined at this distance for the remainder of the analysis (Appendix 1). A shared file was generated that included the number of times an OTU was observed in each sample using the count parameter in Mothur. The number of OTU's per sample was determined and one sample (Pile2_Day35_GLN(rep1)) was removed from further analysis, due to low sequence coverage. After removal of the sample, 89,822 sequences remained. Subsampling down to the lowest number of reads per sample, was used to equalize the number of OTU's across all samples to reduce bias (under or over estimates of diversity) related to sample size and alpha (α) and beta (β) diversity analyses (Gihring et al. 2012). Finally, a representative sequence was classified from each OTU and assigned a taxonomy using the Ribosomal Database Project (RDP) database with an 80% bootstrap cutoff (Schloss et al. 2011). Taxa unclassified at the genera level were classified to the nearest classification

level (UF = unclassified, Family classification; UO = unclassified, Order classification; UC = unclassified, Class classification; UP = unclassified, Phylum classification).

4.3.6 α-diversity measurements

The shared file and subsample parameters in Mothur were used to calculate the coverage, richness and diversity. Good's non-parametric coverage estimator (Good 1953; Esty 1986) was used to estimate the percentage of total species sequenced in thermophilic (> 55°C) and mesophilic (< 50°C) samples. Community richness indices, Chao1 and abundance-based coverage estimator (ACE) were calculated to estimate the number of species within each temperature classification. The diversity within each temperature classification was estimated with the Simpson and non-parametric Shannon indices. Effective species were calculated to estimate a better representation of the true diversity within the composting environment (Jost 2006). The effective species for the effective Shannon (diversity) value was calculated from the exponential value of non-parametric Shannon while the effective species for the inverse Simpson was calculated by dividing the Simpson index by one (Jost 2006). The effective Shannon provides equal weighting to abundant and rare species, while the inverse Simpson provides more weighting to abundant species (Jost 2006). Therefore, rare species within the composting environment were estimated to be the difference of the inverse Simpson from the effective Shannon. Rarefaction curves for temperature groups were generated in Mothur with a re-sampling frequency of 500 sequences without replacement.

4.3.7 β-diversity measurements

The shared file and the subsample parameter were used in Mothur to generate a dendogram. Samples were clustered together using the unweighted pair group method with arithmetic mean (UPGMA) algorithm and the traditional Jaccard index to estimate the distances among communities, resulting in a phylogenetic tree describing community membership. To determine clustering significance within the tree, samples were grouped by pile, day and temperature to allow pairwise comparisons to be made between all samples according to their grouping. The unweighted UniFrac (Lozupone and Knight 2005) command in Mothur was used to generate an unweighted score and a *p*-value for all pairwise comparisons. Next, a non-metric multidimensional scaling (NMDS) ordination plot was generated in Mothur to describe the relationship of community structure based on the temperature achieved in compost. Pairwise distances between all samples, representing each sampled community, were calculated with the Yue and Clayton theta similarity coefficient (Yue and Clayton 2005) and statistical significance of sample clustering was determined with the analysis of the molecular variance (AMOVA) (Martin 2002; Schloss 2008) test in Mothur. Spearman's rank correlation coefficient was calculated in Mothur to measure the correlation of the relative abundance of each individual OTU greater than 1%, across all communities, with the two axes in the NMDS dataset and superimposed on the ordination plot.

4.3.8 Statistical analysis

The UNIVARIATE procedure in SAS (version 9.2; SAS Institute Inc., Cary, NC, USA) was used to test the normality of residuals for α-diversity measurements. Non-normally distributed data were log transformed (log10) in SAS (SAS Institute Inc.).

Normalized data was used to assess the effect of temperature exposure ($p < 0.05$) using the MIXED procedure in SAS (SAS Institute Inc.). Piles were considered replicates and tissue replicates were treated as random in the model.

Phylum and genus sequence counts were transformed to the associated percentage of each taxon within each sample to evaluate statistical differences between temperatures achieved in compost. As described above, samples were considered thermophilic if the compost was exposed to temperatures greater than 55°C, and subsequently declined to temperatures < 50°C at the time of sampling, and mesophilic if compost had only been exposed to temperatures less than 50°C (Kalbasi et al. 2005; Xu et al. 2009). Taxa were considered abundant if greater than 1% of the total species and considered of low abundance if greater than 0.1% of the total species. Taxa below 0.1% were not analyzed further. The UNIVARIATE procedure in SAS (SAS Institute Inc.) was used to test the normality of the residuals at each taxonomic level. Data, not normally distributed, was transformed using arcsin transformation and the MIXED model used to assess the effect of temperature. If the percent abundance data was still not normally distributed after the transformation, Poisson distributions were fitted with the GLIMMIX procedure in SAS (SAS Institute Inc.) to assess temperature effects. For the data that did not converge, either the exponential or Gaussian distribution was fitted to the model.

4.4 Results

4.4.1 454-sequence analysis

A total of 199,947 454-pyrosequencing reads were generated from 38 samples. After removal of barcode and primer mismatches, homopolymers, ambiguous bases,

chimeras and contaminants, one sample due to low coverage, screening, filtering and preclustering, 89,822 redundant sequences were used for the remainder of the analysis (Appendix 1).

4.4.2 Determination of phylogenetic relatedness

A phylogenetic tree was constructed based on the similarity of the OTU's, at a 3% genetic distance, within each sample (Fig. 4.1). No clear clustering patterns were observed between pile and day of BRP removal, despite differences among days (unweighted UniFrac: $p < 0.001$). Samples classified based on exposure to thermophilic (> 55°C) or mesophilic temperatures (< 50°C) (Kalbasi et al. 2005; Xu et al. 2009) were clustered (unweighted UniFrac: $p < 0.001$), however, thermophilic samples still clustered separately between piles (Fig. 4.1), due to the differing taxonomic groups of organisms within each pile. Piles were still analyzed as replicates, as it was speculated that the function of the organisms at those given temperatures, despite the taxonomic class, should be similar.

4.4.3 Bacterial α-diversity

Several α-diversity means, at a 3% OTU distance, were calculated between thermophilic and mesophilic samples (Table 4.1). No statistical differences ($p > 0.05$) were observed for coverage, richness (ACE and Chao1) or diversity (Simpson and Shannon) estimates. Good's coverage estimates were 94% of bacterial species in samples

Figure 4.1 Phylogenetic tree depicting similarity in community membership of samples over 250 days of mortality composting. The Jaccard Index was used to calculate the dissimilarity between samples. Coloured lines indicate samples that were exposed to different temperature profiles (black lines: < 55°C and; red lines: > 55°C) as previously reported by Tkachuk et al. (2013). Significance of sample clustering between each temperature group determined with unweighted UniFrac ($p < 0.01$).

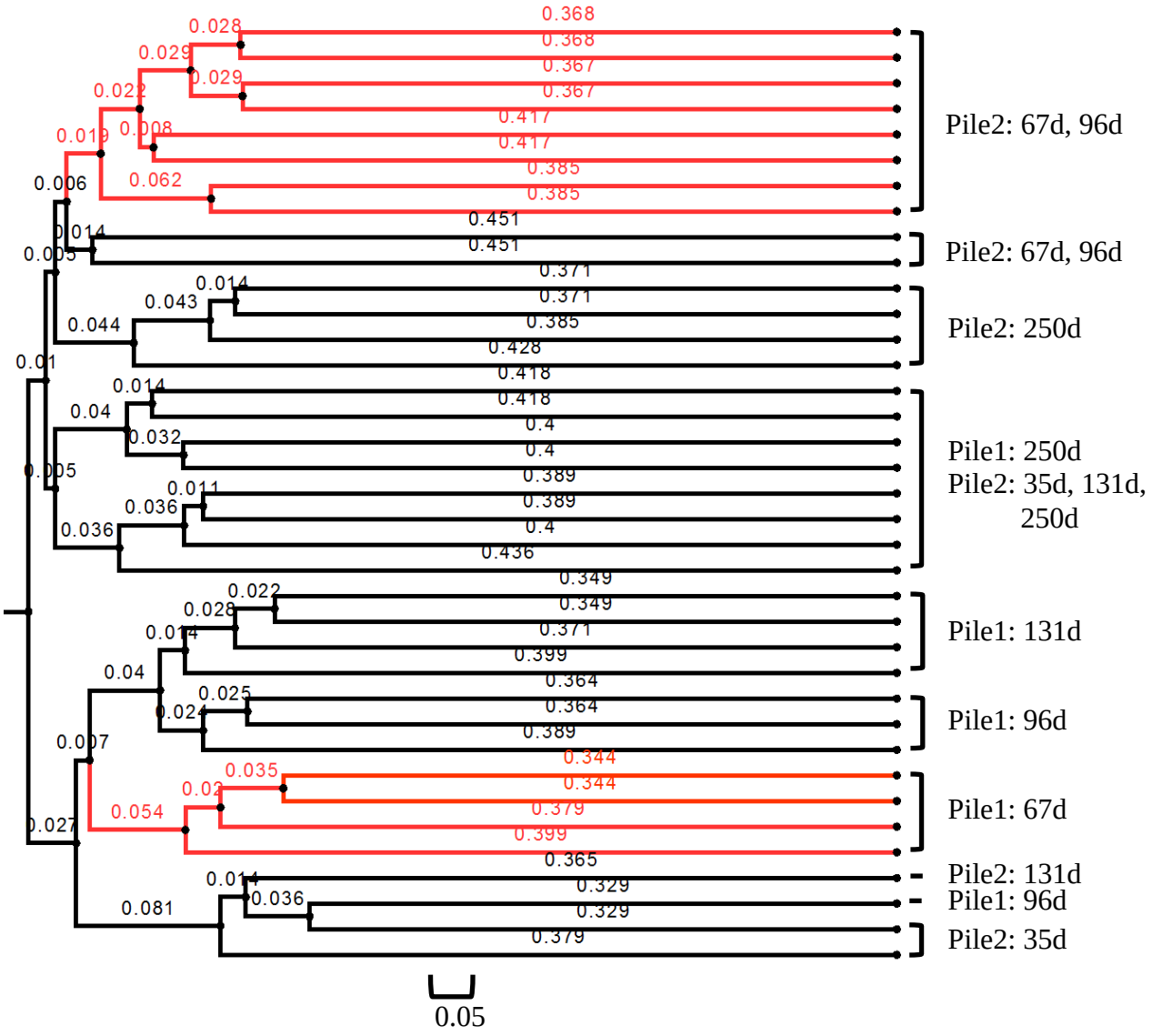


Table 4.1 Summary of alpha diversity indices of 16S rRNA gene sequences at a 3% OTU genetic distance grouped by temperature after exposure to mesophilic (< 50°C) temperatures and after exposure to thermophilic (> 55°C) temperatures following a return to mesophilic temperatures in compost.

	Means							
		Richness		Diversity				
						Effective species		
Temperature	Coverage[1] (%)	Ace[2]	Chao1[3]	Simpson[4]	Shannon[5]	Effective Shannon[6]	Inverse Simpson[7]	Rare species[8]
>55°C	93.6	272.7	185.4	0.15	3.20	32.0	14.3	17.8
<50°C	91.2	390.0	254.8	0.16	3.29	36.2	11.7	24.5
SEM	**0.58**	**27.61**	**16.16**	**0.027**	**0.139**	**4.04**	**1.42**	**2.83**
	P-value							
Pile	0.7660	0.2312	0.1312	0.1389	0.1535	0.1534	0.1385	0.1435
Temperature	0.5731	0.1167	0.1155	0.3708	0.9398	0.9399	0.3760	0.7020
Pile × Temperature	0.6861	0.1867	0.1489	0.8893	0.7631	0.7636	0.8974	0.5699

[1] Coverage indicates Good's coverage for number of observed operational taxonomic units (OTU's).
[2] ACE indicates the abundance-based coverage estimator (ACE) of richness for number of observed OTU's.
[3] Chao indicates the Chao1 richness estimate for number of observed OTU's.
[4] H' indicates the non-parametric Shannon diversity index for number of observed OTU's.
[5] Simpson indicates the Simpson diversity index for number of observed OTU's.
[6] Effective Shannon is a conversion of the non-parametric Shannon diversity index to true diversities (Shannon entropy=exp(H')) (Jost 2006).
[7] Inverse Simpson is a conversion of the Simpson diversity index to true diversities (Inverse Simpson =1/(Simpson)) (Jost 2006).
[8] Rare species indicates the difference between the true diversities (Rare species=Effective Shannon – Inverse Simpson) (Jost 2006).

> 55°C and 91% in samples < 50°C ($p > 0.05$) indicating that coverage was similar across temperature classifications (Table 4.1). Adequate coverage of the bacterial community was also demonstrated by rarefaction, as the number of OTU's, averaged for samples in each respective temperature group, both approached an asymptote (Fig. 4.2).

4.4.4 Bacterial community composition in compost

A total of five bacterial phyla were found in all samples classified at a 3% OTU distance. Highly abundant phyla (> 10% of total OTU's) included: *Actinobacteria*, *Firmicutes* and *Proteobacteria*, whereas low-abundant phyla (> 1% of total OTU's) consisted of *Bacteroidetes* and *Synergistetes* (Fig. 4.3). The phyla *Chlorobi*, *Chloroflexi*, *Fibrobacteres*, *Fusobacteria*, *Spirochaetes*, *SR1*, *Tenericutes*, *TM7* and *Verrucomicrobia* were all < 0.1% of total OTU's and as result they were removed from further analysis. All phyla differed ($p < 0.05$) between thermophilic and mesophilic populations, except *Synergistetes*. *Firmicutes* was the most abundant phylum in both temperature classifications, but was more prominent (63.6%) in samples exposed to thermophilic-mesophilic temperatures compared to those only exposed to mesophilic temperatures (36.8%) (Fig. 4.3). The remaining order of abundance in thermophilic-mesophilic samples was *Actinobacteria* (16.8%), *Proteobacteria* (8.6%) and *Bacteroidetes* (2.4%) (Fig. 4.3). In the mesophilic samples, after *Firmicutes*, *Proteobacteria* (33.7%), *Actinobacteria* (8.4%) and *Bacteroidetes* (4.3%) predominated (Fig. 4.3). Unclassified sequences at the phylum level (> 55°C: 8.3%; < 50°C: 11.8%; $p > 0.05$) were not included in further analysis.

Figure 4.2 Rarefaction curves of average OTUs (grouped at a 3% level) in samples after exposure to mesophilic (< 50°C) temperatures and after exposure to thermophilic (> 55°C) temperatures following a return to mesophilic temperatures during mortality composting.

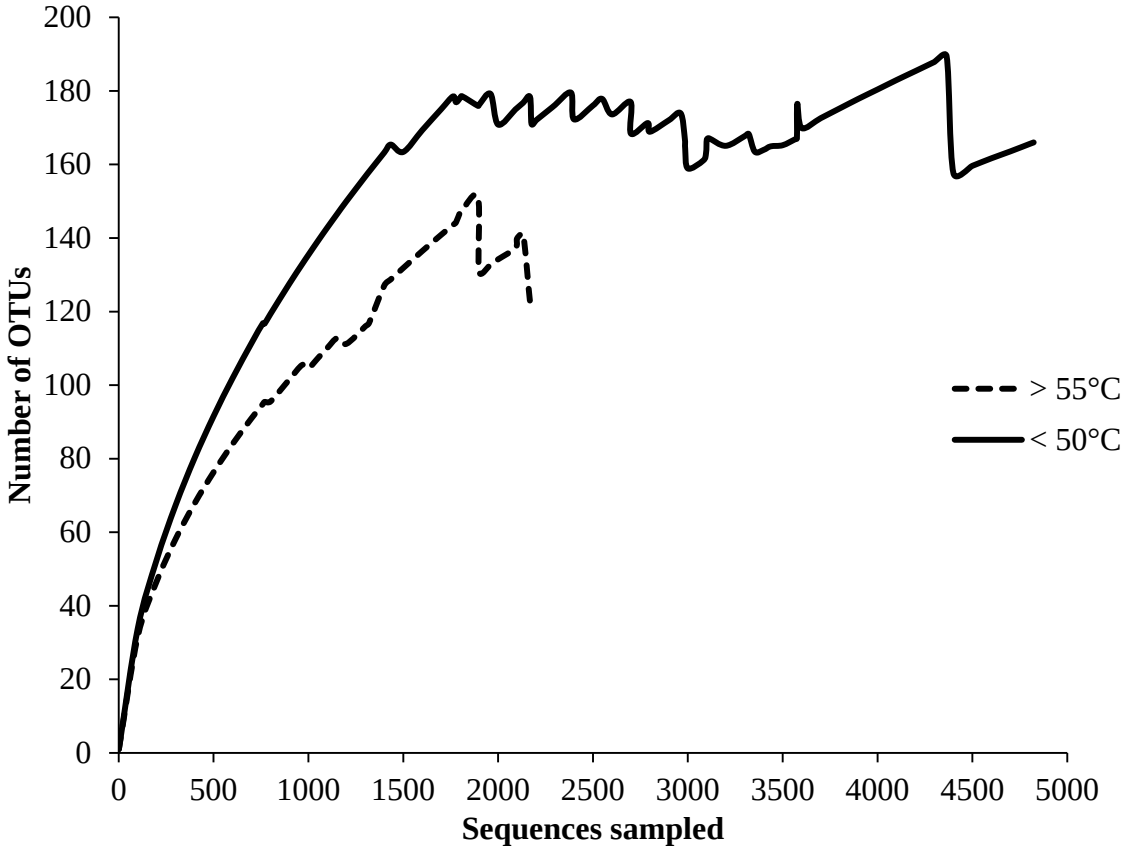
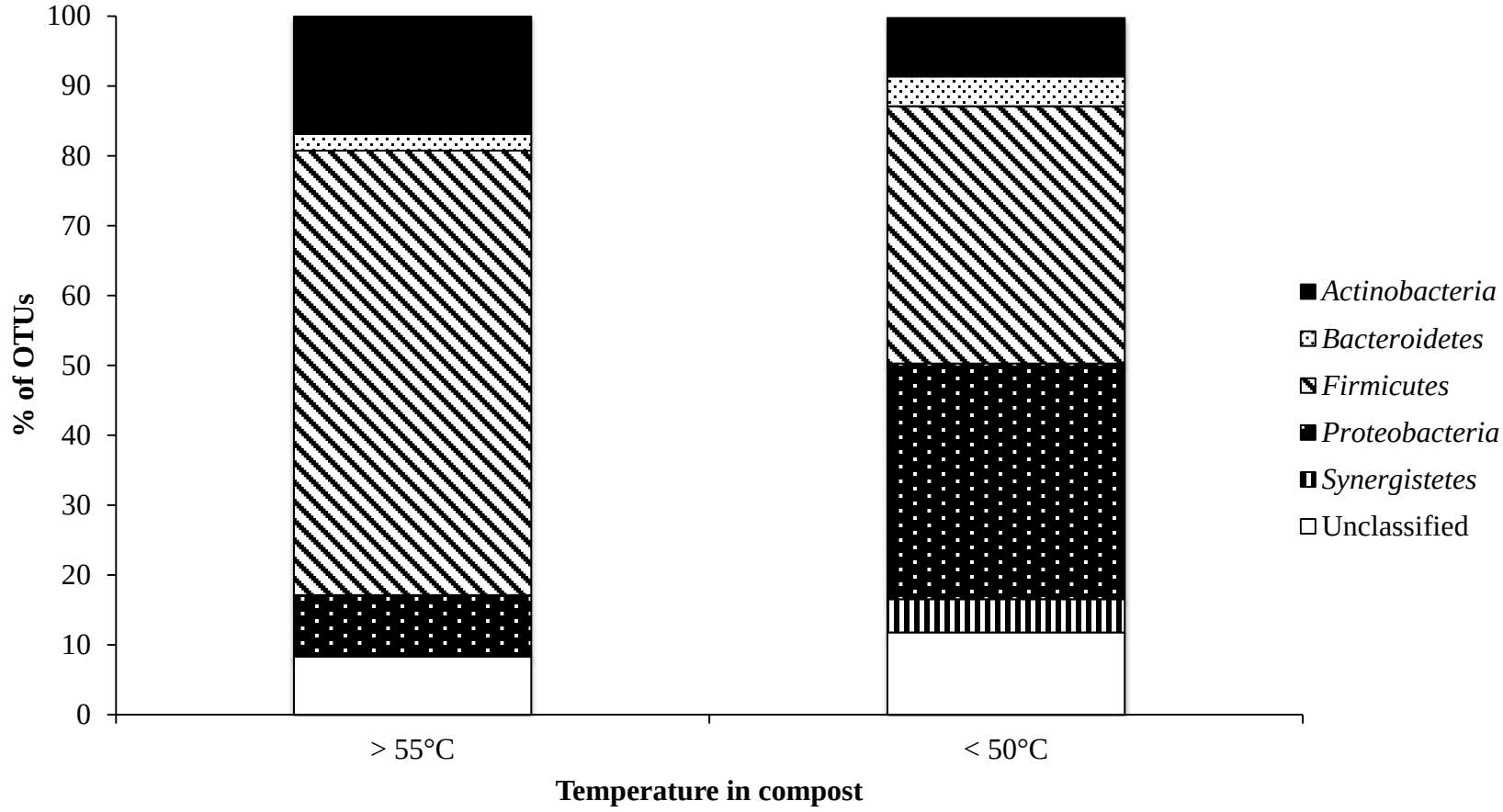


Figure 4.3 Abundant phyla (> 10%; *Actinobacteria*, *Firmicutes* and *Proteobacteria*) and low-abundance phyla (> 1%; *Bacteroidetes* and *Synergistetes*) after exposure to mesophilic (< 50°C) temperatures and after exposure to thermophilic (> 55°C) temperatures following a return to mesophilic temperatures in compost defined at a 3% OTU genetic distance (pooled SEM = 1.62).



In the phylum *Actinobacteria*, *Thermobifida* (3.54%) and UO (*Actinomycetales*) (12.29%), were more ($p < 0.05$) abundant in > 55°C samples than < 50°C (Fig. 4.4). In contrast, in the phylum *Bacteroidetes*, UP (*Bacteroidetes*; Fig. 4.4) (1.70%) and UO (*Flavobacteriales*; Fig. 4.5) (0.26%) were more abundant at < 50°C ($p < 0.05$) than in > 55°C samples. In the phylum *Firmicutes*, several genera, including: *Clostridium sensu stricto* (3.64%), *Clostridium* XI (0.59%), *Proteiniclasticum* (0.40%), *Proteocatella* (2.16%), *Sporobacter* (1.03%), *Syntrophomonas* (0.21%), *Tepidimicrobium* (0.57%), UF (*Bacillaceae* 2) (3.77%), UF (*Clostridiaceae* 1) (5.29%), UF (*Clostridiales Incertae Sedis* XI) (6.20%), UF (*Peptostreptococcaceae*) (0.63%), UF (*Thermoanaerobacteraceae*) (2.18%), UO (*Bacillales*) (19.49%) and UP (*Firmicutes*) (1.88%) were also more ($p < 0.05$) abundant in > 55°C samples compared to < 50°C samples (Figures 4.4 and 4.5) where *Syntrophomonas* predominated (0.31%) (Fig. 4.5). In the phylum *Proteobacteria*, all genera including *Pseudomonas* (1.87%), *Psychrobacter* (4.68%), *Serpens* (15.98%), UF (*Alcaligenaceae*) (1.15%), UO (*Chromatiales*) (2.60%), UC (*Gammaproteobacteria*) (7.43%) and UP (*Proteobacteria*) (4.19%), were more ($p < 0.05$) abundant in < 50°C samples, except UO (*Chromatiales*) and UP (*Proteobacteria*), which were more abundant ($p < 0.05$) in > 55°C samples (Figures 4.4 and 4.5).

4.4.5 Effect of compost temperature on community structure

The NMDS ordination plot (Fig. 4.6) demonstrated a significant difference in the community structure (similarity and abundance of OTU's) between samples exposed to each temperature (Stress = 0.24; $R^2 = 0.55$; AMOVA: $p < 0.001$). Genera (>

Figure 4.4 Heatmap of the relative abundance (%) of genus groups > 1% in samples after exposure to mesophilic (< 50°C) temperatures and after exposure to thermophilic (> 55°C) temperatures following a return to mesophilic temperatures (pooled SEM = 2.67).

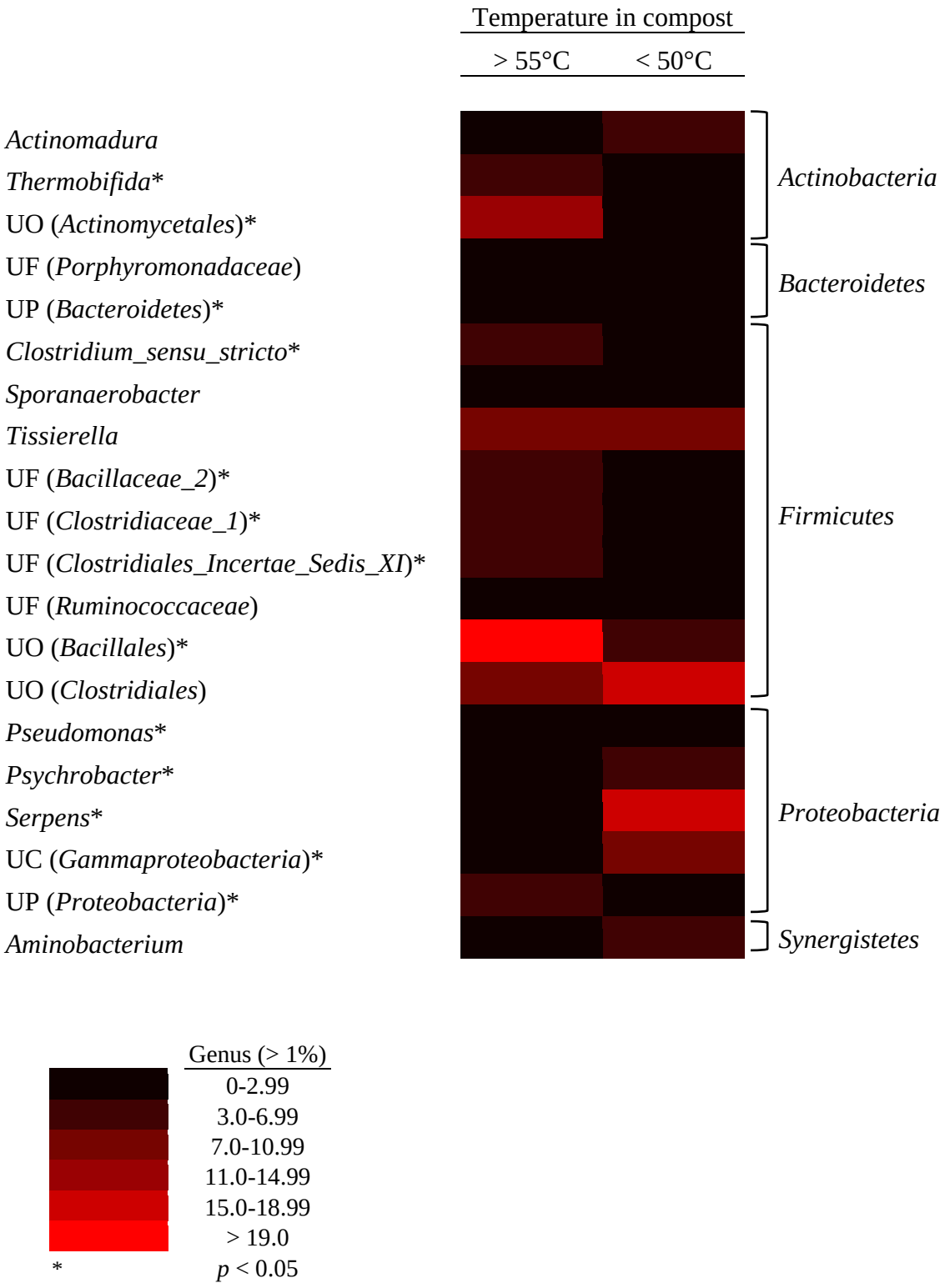
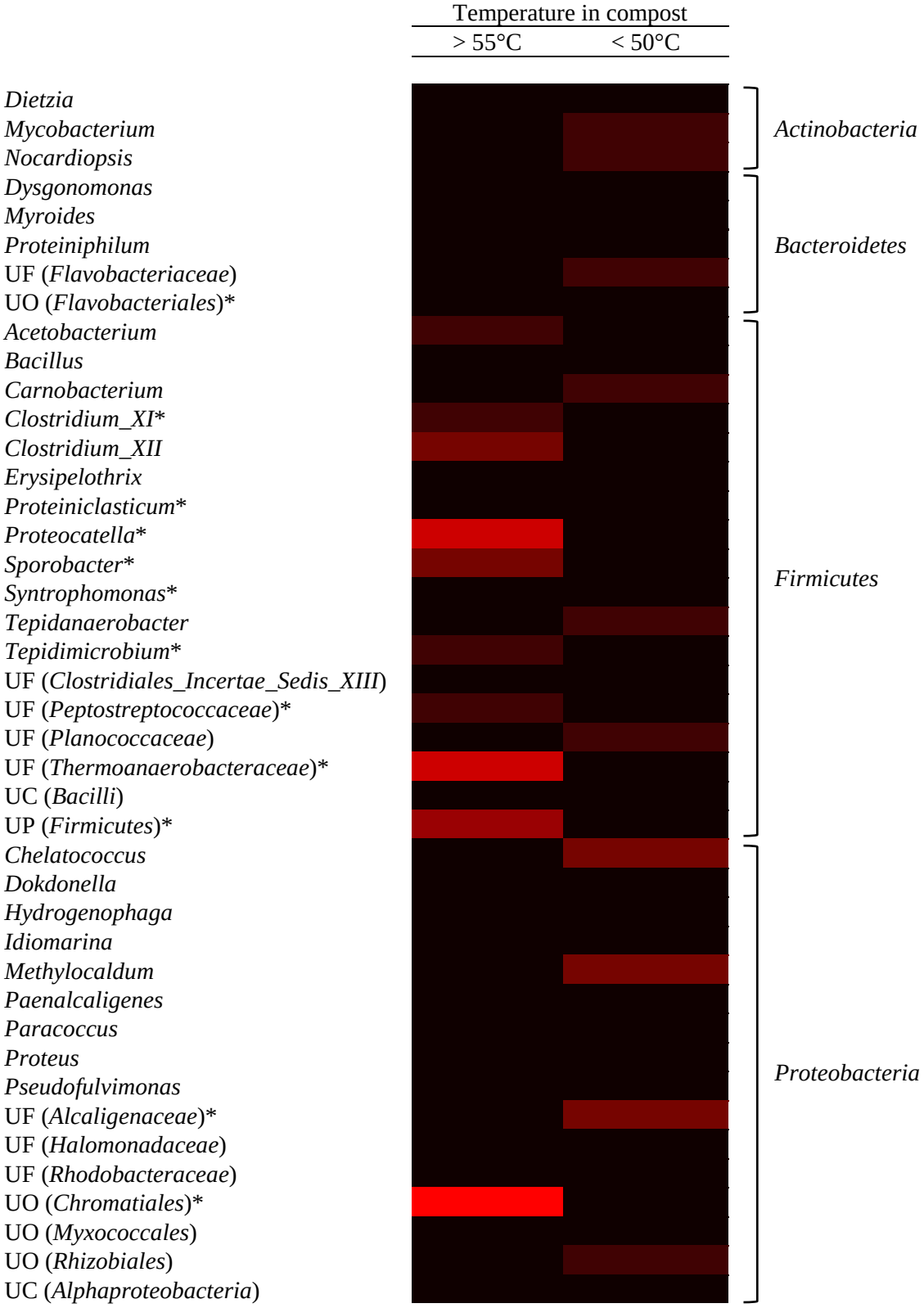
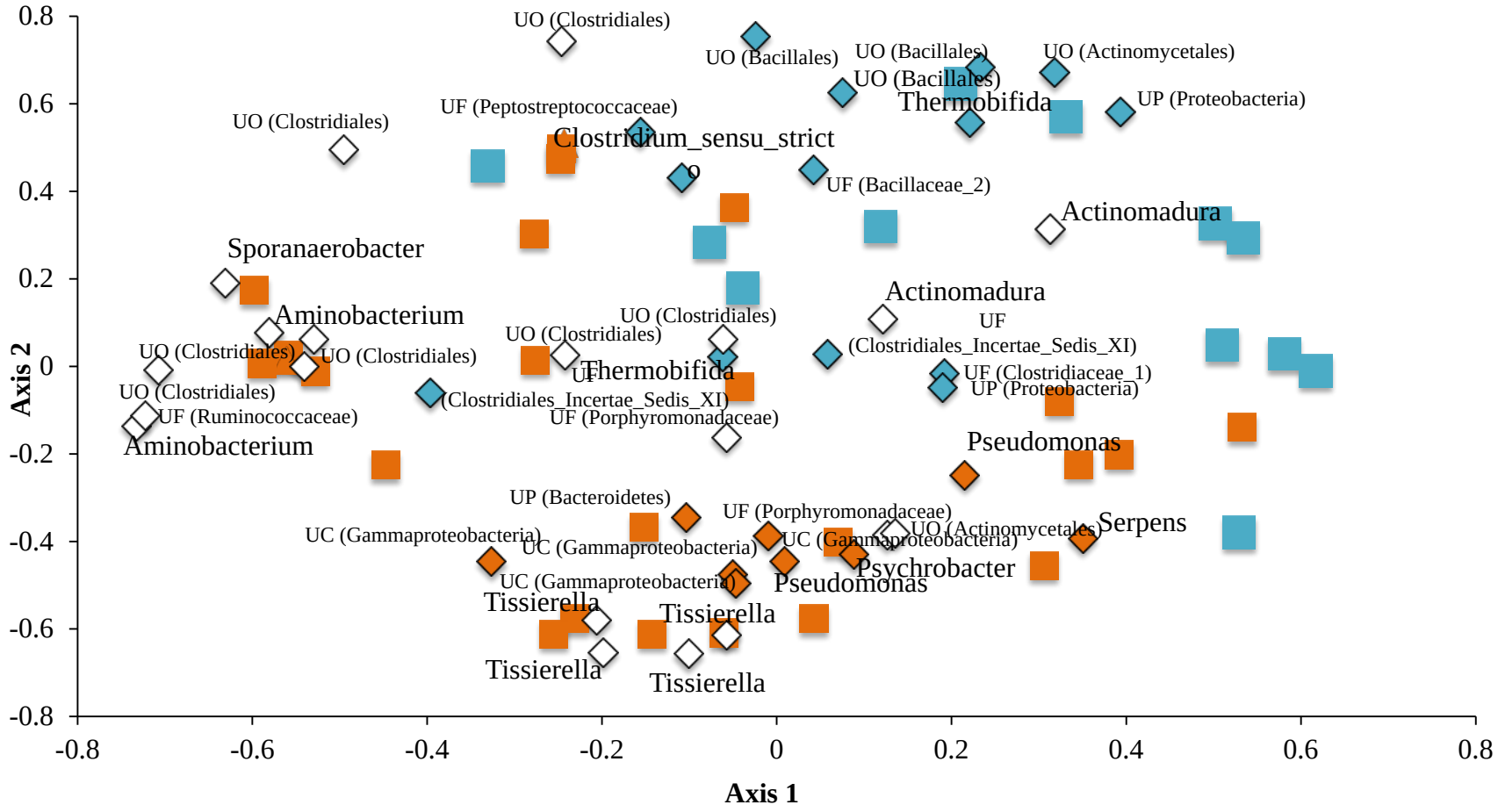


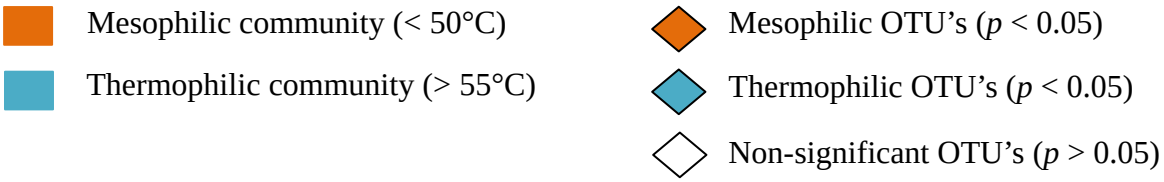
Figure 4.5 Heatmap of the relative abundance (%) of genus groups > 0.1% in samples after exposure to mesophilic (< 50°C) temperatures and after exposure to thermophilic (> 55°C) temperatures following a return to mesophilic temperatures (pooled SEM = 0.24).



Genus (> 0.1%)
0-0.49
0.5-0.99
1.0-1.49
1.5-1.99
2.0-2.49
> 2.5
* $p < 0.05$

Figure 4.6 Non-metric multidimensional scaling (NMDS) ordination plot describing the relationship of community structure based on exposure to only mesophilic (< 50°C) temperatures and after exposure to thermophilic (> 55°C) temperatures following a return to mesophilic temperatures during composting (Stress = 0.24; R^2 = 0.55; AMOVA: $p < 0.001$). OTU groups (> 1%) at the most specific classification were considered thermophilic (blue) or mesophilic (orange) if a significant difference ($p < 0.05$) was observed by temperature (Fig. 4.4).




Mesophilic community (< 50°C)
Thermophilic community (> 55°C)
Mesophilic OTU's (p < 0.05)
Thermophilic OTU's (p < 0.05)
Non-significant OTU's (p > 0.05)

1%) in compost that differed ($p < 0.05$) in samples exposed to > 55°C or < 50°C (Fig. 4.4) were depicted in this scatterplot (Fig. 4.6). Clustering was observed for genera that were more abundant in samples exposed to > 55°C (*Clostridium sensu stricto*, *Thermobifida*, UF (*Bacillaceae* 2), UF (*Clostridiaceae* 1), UF (*Clostridiaceae Incertae Sedis* XI), UO (*Actinomycetales*), UO (*Bacillales*), UP (*Proteobacteria*)) and < 50°C (*Pseudomonas*, *Psychrobacter*, *Serpens*, UC (*Gammaproteobacteria*), UP (*Bacteroidetes*) (Fig. 4.6).

4.5 Discussion

In compost, bacteria can be categorized as either thermophilic (optimum growth at temperatures above 55°C) or mesophilic (optimum growth between 20-45°C) depending on their temperature tolerance. During the initial phase of composting, mesophilic organisms predominate, as OM is degraded resulting in an increase in the temperature of compost to above 40°C (Kalbasi et al. 2005; Xu et al. 2009). As the temperature increases further (52-60°C), thermophilic organisms proliferate, resulting in continued decomposition of OM, as well as inactivation of many bacterial species including pathogens at temperatures greater than 55°C (Kalbasi et al. 2005; Xu et al. 2009). As remaining available OM is degraded, the temperature decreases (10-40°C) due to reduced microbial activity, and remains in this range until the compost matures (Bernal et al. 2009).

In this study, we have characterized the phylogenetic, richness, diversity and taxonomic changes that occurred in this bacterial community via pyrosequencing of the16S rRNA gene using 454 technologies.

Phylogenetic relatedness of bacteria is defined in terms of how recently species, or OTU's, last shared a common ancestor, assuming that traits change less frequently than the rate of branching among species (Baum 2008). Figure 4.1 depicts the relatedness of the bacteria after exposure to thermophilic (> 55°C) temperatures, which had declined to mesophilic temperatures, or only mesophilic temperatures (< 50°C). The impact of temperature on the microbial community (Schloss et al. 2005; Partanen et al. 2010; Martins et al. 2013) is reflected in the clustering of thermophilic and mesophilic samples (Fig. 4.1).

In this study, piles were considered replicates, as richness and diversity were not significantly different between temperature classifications (Table 4.1). A comparison of this study to those reported in the literature is difficult as a consequence of differences in composting method (active or static aeration), scale (full or pilot), substrate matrix and the methodology used to characterize the composition of microbial communities. Tiquia (2005) studied the microbial diversity of composted swine and cattle manure using terminal restriction fragment length polymorphisms (T-RFLP) and observed higher bacterial diversity at thermophilic-mesophilic temperatures (> 55°C) as compared to only mesophilic temperatures. Similarly, a 454-sequencing study of coffee hulls, rice straw and sugar cane bagasse compost revealed a steady increase in richness (Chao1) in all compost types as composting progressed through mesophilic, thermophilic and maturation phases (de Gannes et al. 2013). However, in the current study, thermophilic-mesophilic and mesophilic samples did not differ with respect to richness and diversity but instead clustered OTU's separately by temperature in the phylogenetic tree.

Therefore, OTU's were classified taxonomically to provide further insight into the functionality of individual OTU's, within each sample, exposed to different temperatures.

Firmicutes was the most abundant phylum at thermophilic-mesophilic and mesophilic temperatures, following exposure to temperatures between 50-65°C (Tkachuk et al. 2013) for approximately 25-35 d without active mixing of the compost. Other studies have reported a higher abundance of *Firmicutes* after exposure to thermophilic temperatures, irrespective of exposure time, ranging over a period of 96 h to 60 d. (Schloss et al. 2005; Yamamoto et al. 2009; Partanen et al. 2010; Martins et al. 2013). Yamamoto et al. (2009) used denaturing gradient gel electrophoresis (DGGE) to identify the microbial composition in three full-scale cattle and swine manure composting facilities, two of which were passively aerated. An abundance of *Firmicutes*-like sequences, compared to *Proteobacteria* and *Bacteroidetes*, were observed in one of the passively aerated facilities after temperatures decreased from 67°C to 38°C (Yamamoto et al. 2009). In the second passively aerated facility, which used cattle manure as the composting substrate, when temperatures did not exceed 40°C, all sequences grouped with either *Bacteroidetes* or the more predominant *Firmicutes* (Yamamoto et al. 2009). In this study, as *Firmicutes* was the predominant phylum in both thermophilic-mesophilic and mesophilic samples, Yamamoto et al. (2009) speculated that members of this group play an important role in degrading organic materials throughout the entire composting process, regardless of substrate composition, mixing activities or temperature exposure. In the current study, it was also demonstrated that the abundance of UF (*Bacillaceae* 2), UF (*Clostridiaceae* 1), UF (*Clostridiales Incertae Sedis* XI), UO (*Bacillales*) and UO

(*Clostridiales*), are groups of organisms known to degrade organic materials at both thermophilic and mesophilic temperatures.

A predominance of *Proteobacteria,* followed by the phylum *Bacteriodetes* at mesophilic temperatures reported in the current study, is consistent with that reported elsewhere in both static (Schloss et al. 2005) and mixed (Yamamoto et al. 2009; Partenan et al. 2010) compost systems. Schloss et al. (2005) reported a high abundance of *Pseudomonas* spp., which is within the phylum *Proteobacteria,* until temperatures exceeded 50°C, thereafter the abundance of those organisms declined. Presence of *Bacteroidetes* in this study was consistent with that reported by Yamamoto et al. (2009), by demonstrating a greater abundance at mesophilic temperatures and a lower relative abundance compared to *Firmicutes* and *Proteobacteria* (Fig. 4.3). It is speculated that *Bacteroidetes,* like *Firmicutes,* play an important role in the degradation of organic matter in compost (Yamamoto et al. 2009).

The marked decrease in *Proteobacteria* from 33.7% to 8.6% following exposure to thermophilic-mesophilic temperatures is inconsistent with previous reports that *Proteobacteria* are abundant during early (Ryckeboer et al. 2003a; Schloss et al. 2005) and later stages of mesophilic composting (Yamamoto et al. 2009). However, all studies consistently report a decline in *Proteobacteria* abundance during thermophilic composting (Ryckeboer et al. 2003a; Schloss et al. 2003; Yamamoto et al. 2009). It is possible that after exposure to > 55°C, some species within *Proteobacteria* became dormant while others declined, leading to an overall decrease in relative abundance, which would not be detected with current sequencing technologies. Nonetheless, an observed increase in abundance of *Proteobacteria* upon return to mesophilic conditions

suggests that some members of this phylum could resume growth during the maturation phase of composting. Some researchers have rejected this theory of recolonization following a period of dormancy, associated with exposure to thermophilic temperatures. Research by de Gannes et al. (2013) observed that changes in the microbial community in coffee hulls, rice straw and sugar cane bagasse compost, assessed with 454-pyrosequencing with in-vessel rotary type drums at early mesophilic, thermophilic and mature phases of composting, were distinct between all phases and many of the species from the thermophilic phase were supplanted in the mature compost (de Gannes et al. 2013). Further, the results from de Gannes et al. (2013) were consistent with the findings in this study, observing a higher abundance of *Proteobacteria* in mesophilic compost that did not increase in abundance after thermophilic temperature exposure following a return to mesophilic temperatures, further disputing the dormancy theory (de Gannes et al. 2013).

Within each phylum the composition of microorganisms demonstrating significance was different between thermophilic-mesophilic and mesophilic temperatures. Members that were significantly different at mesophilic temperatures provide a reasonable estimation of the composition of the initial microbial community. However, because the compost at those sites were never exposed to thermophilic temperatures, it was assumed that the composting process had never initiated and changes in the community were reported but not considered in further detail.

At > 55°C, UO (*Bacillales*) and UO (*Actinomycetales*) were the two most abundant orders at 19.5% and 12.3%, respectively, although *Bacillus*, at the genus level, did not differ in abundance between thermophilic and mesophilic temperatures (Fig. 4.5).

The presence of both orders in compost has been associated with a tolerance to thermophilic temperatures, the ability to degrade cellulose and solubilize lignin and ability to colonize substrates under conditions of low nutrient availability (Ryckeboer et al. 2003a; Ryckeboer et al. 2003b; Martins et al. 2013). These results are consistent with other culture-independent findings demonstrating that *Bacillus*, within the order *Bacillales*, is among the most abundant taxa recovered from compost in the thermophilic phase (Schloss et al. 2003; Schloss et al. 2005; Partanen et al. 2010), Conversely, low abundance of *Bacillus* has been reported in coffee hulls, rice straw and sugar cane bagasse compost, although, genera, such as *Thermobacillus*, within the order *Bacillales*, were a major component in composted coffee hulls during the thermophilic phase (de Gannes et al. 2013).

As depicted in Figure 4.4, *Thermobifida* also increased in abundance ($p < 0.05$) after exposure to temperatures > 55°C. Several species of *Thermobifida*, which possess cellulose and lignin degrading capabilities, have been isolated from compost and exhibit optimal growth between 45-65°C (Kukolya et al. 2002; Lykidis et al. 2007). This suggests that the impact of thermophilic conditions on the communities in composting was long lasting, as *Thermobifida* was isolated after the compost returned to mesophilic conditions.

Several *Clostridia* members increased in abundance following exposure to thermophilic temperatures. These include, *Clostridium sensu stricto*, *Clostridium* XI, UF (*Clostridiaceae* 1) and UF (*Clostridiales Incertae Sedis* XI) (Figures 4.4 and 4.5), which consist of many thermophiles that are obiligately anaerobic and possess pathogenic properties (Maeda et al. 2010; Martins et al. 2013). Maeda et al. (2010) observed a

disparity in spatial distribution as *Clostridia*, despite mixing the compost every two weeks, was observed in greatest abundance in the core and bottom of the composting structure. As temperatures > 55°C were achieved, anaerobic conditions were likely a consequence of lack of oxygen associated with compaction, as moisture content was within an optimal range (Maeda et al. 2010). The spatial distribution and temporal changes observed in Figures 4.4 and 4.5 are not unique to the current study but rather, are a known occurrence in passively aerated composting systems (Xu et al. 2010; Wang et al. 2011). Within a biosecure system, spatial and temporal distribution of organisms such as *Clostridia* suggest that temperatures of > 55°C may not be uniformly achieved throughout the pile for a sufficient period of time (three days) to inactivate potential pathogens (Kalbasi et al. 2005; Xu et al. 2010). Recently, Xu et al. (2010) improved the design of a biosecure system through burial of carcasses 60 cm higher than previously reported (Xu et al. 2009), and addition of manure, with less moisture (60%; Xu et al. 2010) thereby achieving temperatures > 55°C for 79 d. In the current study, it is speculated that low ambient temperatures and high moisture (-25°C; Tkachuk et al. 2013) impeded initial heating making it extremely difficult to achieve optimal, homogeneous thermophilic conditions throughout the pile.

The importance of the role of temperature in the composting process is illustrated in Figure 4.6. When individual OTU's are plotted concurrently with community samples exposed to either thermophilic or mesophilic temperatures, there was a strong correlation of individual thermophilic and mesophilic OTU's ($p < 0.05$) to the community samples exposed to the same temperatures (Fig. 4.6). With the use of an OTU-based approach, it is possible to assign all sequences to clusters on the same basis (3% genetic distance)

(Schloss and Westcott 2011). The likelihood that two sequences will be found in the same OTU depends on the presence of other sequences in the data set (Schloss and Westcott 2011). Therefore, OTU's with the same taxonomic classification may be observed based on the percent similarity between sequences when they are binned together (Fig. 4.6). Further, it is apparent that many bacterial sequences lack a defined taxonomy at the genus or species level necessary for a more accurate OTU identification.

High-throughput sequencing provided a more comprehensive picture of the microbial biodiversity during composting of livestock mortalities, however, there is no differentiation between metabolically active and dead bacteria with the use of this technique. Composting is a complex process with many variables including, temperature, pH, oxygen availability, nutrient availability and competition from other microorganisms, which all have an affect on the persistence and survival of constituents within the microbial community. Compost temperature, induces rapid cell lysis and DNA degradation in some microorganisms, limiting the ability to isolate DNA of dead bacteria and explain observed differences of the microbial communities exposed to different temperatures. Xu et al. (2011) explored the relationship between bacterial 16S rDNA copy numbers (quantified with real-time PCR) and genomic DNA yield from carcass tissues throughout mortality composting. Within the first seven days of composting, bacterial 16S copy numbers increased by 300% as temperatures exceeded 55°C and tissues were rapidly degraded (Xu et al. 2011). However after day seven, copy number declined continuously for the remainder of the experiment (below the initial copy number on day zero) as temperatures exceeded 55°C for about 100 days. Although both active and dead bacteria were quantified in that study, the decline in total bacterial DNA below

initial levels would suggest that temperature largely affected the degradation of bacterial DNA, as moisture and pH were within normal levels (Xu et al. 2011).

Although this study focused on changes in the bacterial community, it is important to acknowledge other microorganisms such as archaea and fungi, which also play an important role during composting. Similar to bacteria, fungi and archaea are diverse groups of microorganisms, with certain species known to be facultatively anaerobic, demonstrating thermophilic properties and both groups are important in organic matter degradation during composting (Hultman et al. 2010; Lee et al. 2010). However, fungal and archaeal diversity identified with high-throughput sequencing, associated with various composts and their succession patterns, are limited (Hultman et al. 2010; de Gannes et al. 2013; Martins et al. 2013). Despite consisting of a significantly lower proportion of the total microbial community compared to bacteria (Lee et al. 2010; de Gannes et al. 2013), changes in the archaeal and fungal communities would likely be valuable indicators of the overall composting process and provide further insight into the occurance of spatial variation.

In conclusion, the microbial community in a biosecure, static composting system for cattle mortalities was distinctly different at thermophilic (> 55°C) and mesophilic (< 50°C) temperatures. The phylogenetic tree approximated differences in habitat adaptation with distinct clustering in community membership after exposure to temperatures > 55°C or < 50°C. However, richness and diversity estimates were not significantly different between temperature profiles, indicating that community membership was responsible for the clustering in the tree following exposure to those temperatures. At the phylum level, *Firmicutes* was the most abundant group at both thermophilic and mesophilic

temperatures, with greater abundance at thermophilic temperatures. *Proteobacteria*, however, were significantly higher at mesophilic temperatures. Members of the groups UO (*Bacillales*), UO (*Actinomycetales*), *Thermobifida* and the class *Clostridia* were all significantly more abundant after exposure to thermophilic temperatures. Spatial distribution, indicated by the presence of anaerobic, potentially pathogenic species of *Clostridia*, and the lack of heating at several sites within the composting pile, is a challenge that requires further consideration to ensure pathogen reduction throughout the pile. As high-throughput sequencing technologies develop, differential identification of bacterial organisms may be extended to the genus or species levels.

5.0 GENERAL DISCUSSION

Carcass disposal is an issue for livestock producers as it presents many challenges including, biosecurity risks depending on cause of death, possible contamination of feed and depending on the method of disposal, expense. Composting is one of four approved methods, including burial, incineration and rendering, for on-farm disposal of mortalities in Canada. Compared with the other methods of disposal, composting has been demonstrated as an effective way to eliminate pathogens (Senne et al. 1994; Grewal et al. 2005; Xu et al. 2009) and is an inexpensive, environmentally sound process of turning waste products, such as livestock mortalities or manure, into a source of nutrients for plants.

The process of composting can be divided into two phases, the biooxidative phase and the maturation phase (Kalbasi et al. 2005; Bernal et al. 2009). Throughout either phase, temperatures changes are associated with the aerobic metabolic activity of composting microorganisms. The bioxidative phase typically begins within the first few days of composting when primarily mesophilic organisms (optimum growth at temperatures between 20-45°C; Misra et al. 2003; Kalbasi et al. 2005; Xu et al. 2009) decompose OM, increasing the temperature to greater than 40°C. As the temperature increases, thermophilic organisms (optimum growth at temperatures between 50-70°C; Misra et al. 2003; Kalbasi et al. 2005; Xu et al. 2009) continue to decompose OM and inactivate many pathogens (Kalbasi et al. 2005; Bernal et al. 2009). Optimal composting during this phase should result in temperatures between 40-65˚C (Rynk 1992); temperatures between 52-60˚C are optimal for OM decomposition while temperatures >

55˚C are optimal for pathogen inactivation (Miller 1993; Kalbasi et al. 2005). During the maturation phase, the compost temperature remains constant between 10-40˚C while the remaining OM, which includes cellulose and lignin, undergoes humification (Bernal et al. 2009).

Xu et al. (2009) successfully achieved rapid heating of compost (temperatures exceeded 55°C for > 35 d) using a comparable system to that described herein. An initial temperature spike was not observed in the current study and average internal temperatures of compost were 20°C. It was speculated that the higher ambient temperatures of 20°C in the study by Xu et al. (2009), compared to -25°C in the current study, were responsible for rapid initial heating of compost. Upon closer evaluation of the internal temperatures, each BRP was subject to a unique temperature profile as a consequence of the spatial heterogeneity associated with carcass composting (Xu et al. 2010). As all other biochemical parameters (pH, moisture, C/N ratio) were within recommended ranges for effective composting, it was concluded that low ambient temperature was likely the limiting factor for uniform heating, as only one location in Pile 1 and two locations in Pile 2 achieved temperatures > 55°C.

Under the described conditions, MAP remained viable after 250 d of composting in large scale composters designed for the disposal of cattle mortalities, despite achieving internal temperatures for several weeks that inactivate many types of pathogens (Kalbasi et al. 2005; Bernal et al. 2009; Xu et al. 2009). Furthermore, MAP was still recovered in an *in vitro* study after sustained exposure to 80°C for 90 d, suggesting that even effective composting conditions are an unlikely to inactivate MAP. Firstly, it is unlikely that temperatures during carcass composting will be homogeneous throughout the pile,

making it likely that some regions will not achieve 80°C (Kalbasi et al. 2005). Secondly, if these temperatures were reached, many beneficial organisms that contribute to the composting process would be inactivated (Rynk 1992; Bernal et al. 2009).

Several characteristics and adaptive functions have been suggested to explain why MAP can survive for extended periods of time under extreme temperature conditions. The thick, lipid-rich cell wall is responsible for its extreme tolerance to heat, surviving pasteurization (Cerf et al. 2007; Rademaker et al. 2007; Whittington et al. 2010), and exhibiting resistance to desiccation (Lovell et al. 1944; Whittington et al. 2004) and UV radiation (Donaghy et al. 2009). Although all studies demonstrated a reduction in numbers of MAP, viable cells could still be isolated after these treatments. Dormancy has also been suggested to extend the survival of MAP in the environment however the exact physiological mechanism triggering dormancy remains unclear (Whittington et al. 2004). Recently, a MAP spore-like morphotype was produced that remained viable after exposure to 70°C for 30 min (Lamont et al. 2012). In this study, the authors speculated that temperature-induced sporulation may be the mechanism by which MAP survived at > 55°C in the compost piles and at 80°C *in vitro*.

Mycobacterium smegmatis possesses desirable characteristics as a surrogate for MAP. It is a fast-growing species of mycobacteria (Chaturvedi et al. 2007) with no linkage to human disease and possesses dormancy genes similar to those in MAP (Whittington et al. 2004). However, our data suggests that *M. smegmatis* is not a suitable surrogate for MAP, as it could not be detected after 67 d of composting. Further, MAP may have a resistance mechanism, such as sporulation, that is not active or present in *M. smegmatis* under composting conditions.

As a consequence of the lack of pathogen inactivation in the first study, the second study characterized the microbial community after exposure to thermophilic temperatures in order to characterize that portion of the microbial community contributing to adequate pathogen inactivation. Although the composition and dynamics of microbial communities differs among substrates, the use of 454-pyrosequencing provided an indication of the microbial community structure after thermophilic composting of cattle mortalities.

Studies of microbial habitat adaptation using high-throughput sequencing techniques suggest that phylogenetic information provides a good first approximation of habitat range, as microbial preferences have remained fairly stable over evolutionary time (Zaneveld et al. 2011). The relatively stable properties of the 16S rRNA gene (Janda et al. 2007), targeted for high-throughput sequencing in this study, provided a clear indication of phylogenetic relatedness of the samples in terms of how recently the OTU's (within each sample) last shared a common ancestor. It was observed that samples clustered according to whether they were exposed to thermophilic (BRP's achieving temperatures > 55°C) or mesophilic (BRP's achieving temperatures < 50°C) temperatures.

Richness (Chao1 and ACE) and diversity (Shannon and Simpson) were measured however, no statistical differences were observed between the indices and the temperature groups. Given the clear clustering of the samples based on temperature exposure, sequences were taxonomically classified to provide further insight into the organisms present within the community.

At the phylum level, several groups exhibited significant differences between samples exposed to mesophilic or thermophilic temperatures. Firstly, *Firmicutes* was the

most abundant phylum at both thermophilic and mesophilic temperatures, although significantly higher when temperatures exceeded 55°C. These observations were similar to other studies (Schloss et al. 2005; Partenan et al. 2010; Martins et al. 2013) suggesting that members of this phylum play an important role in degrading OM throughout the entire composting process irregardless of temperature, substrate composition, scale or management procedures used for composting (Yamamoto et al. 2009). Secondly, *Proteobacteria* was significantly higher when temperatures remained < 50°C. Several studies have also found similar results in early mesophilic composting (Schloss et al. 2005; Ryckeboer et al. 2003; Yamamoto et al. 2009; Partanen et al. 2010). However, it is not known if these early inhabitants are inactivated at these temperatures or whether they enter a state of dormancy until temperatures return to mesophilic levels (Yamamoto et al. 2009; de Gannes et al. 2013). A recent high-throughput sequencing study of coffee, rice and bagasse compost has rejected the theory of dormancy, observing distinct microbial communities in different phases of composting, suggesting that *Proteobacteria* did not recolonize or increase in abundance post-exposure to thermophilic temperatures (de Gannes et al. 2013).

At the genus level several members demonstrated increases in abundance ($p < 0.05$) after exposure to mesophilic v.s. thermophilic temperatures. The two most abundant groups after thermophilic composting were UO (*Bacillales*) and UO (*Actinomycetales*) at 19.5% and 12.3% of the total community, respectively. Both orders have shown tolerance to thermophilic temperatures, an ability to degrade cellulose and solubilize lignin and colonize complex C-sources (Ryckeboer et al. 2003; Martins et al. 2013). *Thermobifida* also accounted for 3.5% of the total community with known

cellulose and lignin degrading properties at temperatures between 45-65°C (Kukolya et al. 2002; Lykidis et al. 2007). Several members of the class *Clostridia*, including *Clostridium sensu stricto, Clostridium* XI, UF (*Clostridiaceae* 1) and UF (*Clostridiales Incertae Sedis* XI) at 3.6%, 0.6%, 5.3% and 6.2% of the total community, respectively, were also more abundant ($p < 0.05$) after exposure to thermophilic temperatures. As many members within *Clostridia* are known obligately anaerobic thermophiles, it was speculated that spatial variation resulted in regions within the piles that were both anaerobic and > 55°C (Tkachuk et al. 2013).

Uneven distribution of oxygen, moisture and nutrients is a known challenge in passively aerated composting systems. To overcome issues associated with inappropriate aeration, inconsistent initial heating and lack of product uniformity, compost is typically mixed to actively aerate the matrix prior to composting. In the event of a disease outbreak, if a biosecure, static system for composting for cattle mortalities was used to control the disease, and did not exceed 55°C, it would be deemed unsuitable for use as a fertilizer. In this study, initial heating to > 55°C was limited to three sites in both piles due to construction at sub-zero temperatures. Strategies to reduce heterogeneity and increase heating efficacy in the compost structure include, construction at a shallower pile depth, reduced moisture in manure added to initial compost matrix, limiting excess moisture seepage throughout composting process from precipitation, adequate mixing of the matrix prior to pile construction and during the composting process and avoiding pile construction during periods of extreme cold.

Several conclusions can be derived from both of these studies. In the first experiment, MAP was not inactivated in a biosecure, static composting system for cattle

mortalities after 250 d. Neither temperatures of 60°C in several sites nor biochemical conditions, which prevailed within the compost, rendered MAP non-viable. Further, an *in vitro* incubation experiment demonstrated MAP survival after 90 d at 80°C in the presence of a compost matrix, suggesting that MAP cannot be completely inactivated through composting. It may also be concluded that *M. smegmatis* is not a suitable surrogate for MAP. Dormancy and sporulation were speculated as possible mechanisms for MAP survival, making it necessary to develop an alternative means of disposing of JD infected cattle and improve prevention techniques on-farm, to avoid spread of the disease. To further explore the lack of pathogen inactivation in the first study, the second study characterized changes in the microbial community after exposure to thermophilic temperatures in an attempt to understand the system and optimize product quality for effective pathogen reduction during mortality composting. As heating to temperatures > 55°C was limited to one site in Pile 1 and two sites in Pile 2, the phylogenetic tree first approximated differences in habitat adaptation with distinct clustering of the samples according to exposure to thermophilic or mesophilic temperature. Further considerations, such as adequate mixing, limiting excess moisture seepage and avoiding construction during periods of extreme cold, need to be considered to ensure that passively aerated systems can consistently and safely inactivate pathogens in the event of a disease outbreak.

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APPENDIX

Appendix 1 Sequence and OTU counts from both Pile 1 and Pile 2 with means between the piles throughout data processing over 250 days of composting.

	Days of composting					
	35	67	96	131	250	Total reads[f]
All reads[a]	--	--	--	--	--	199,947
Post-quality removal[b]	(5846/11,257)* 8552**	(7586/7037) 7312	(11,771 /9431) 10,601	(11,020/16,427) 13,746	(9426/8343) 8885	98,189
Post-sequence error removal[c]	(5327/11,068) 8198	(7411/5218) 6315	(11,232/8893) 10,063	(10,375/16,133) 13,254	(9160/7862) 8511	92,679
Post-chimera and contaminant removal[d]	(5270/9659) 7465	(7256/5109) 6183	(11,052/8755) 9904	(10,222/15,736) 12,979	(9064/7699) 8382	89,822
3% OTU distance[e]	(373/548) 461	(723/553) 638	(732/393) 462	(714/756) 735	(1154/1238) 1196	7184

[a] Total read counts of unprocessed 454-pyrosequencing reads in standard flowgram format (sff) file for each pile over 250 days of composting.
[b] Redundant sequence counts after removal of barcode and primer mismatches, homopolymers and ambiguous bases in Mothur.
[c] Redundant sequence counts after screen, filter and precluster steps in Mothur.
[d] Redundant sequence counts after removal of chimeras and contaminants not classified at the Kingdom level in Mothur.
[e] Unique (non-redundant) sequences at each time interval clustered into OTU's at 3% (species) genetic distance.
[f] Total redundant read counts for both piles.
* First value in parentheses indicates the estimate for Pile 1 and the second value indicates the estimate for Pile 2.
** Average of Pile 1 and Pile 2 sequences after each processing step.