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Composting as a method for disposal of specified risk material and degradation of prions

by

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Abstract

Provided that infectious prions (PrP^{TSE}) are inactivated, composting of specified risk material (SRM) may be a viable alternative to rendering and land filling. The overall objective of this research was to utilize laboratory-scale composters to assess the degradation of SRM and PrP^{TSE} during composting. Under non-containment conditions, co-composting of SRM with cattle manure revealed that SRM was degraded rapidly in compost, with approximately 60% and 80% dry matter loss after 14 and 28 days, respectively. Composter depth, types of bulking agent, or prolongation of thermophilic temperature using a water jacket did not influence SRM degradation. However, mixing of feathers with manure increased the extent of SRM degradation. Phospholipid fatty acid (PLFA) profiles demonstrated that both mesophilic and thermophilic microbial communities were responsible for SRM degradation. Furthermore, PCR-denaturing gradient gel electrophoresis (DGGE) analysis suggested that bacterial genera of *Thermoactinomycetaceae*, *Thiohalospira*, *Pseudomonas*, *Enterobacter*, *Corynebacterium*, *Promicromonospora*, *Pseudonocardia*, *Thermobifida*, *Mycobacterium*, *Nocardia*, *Saccharomonospora*, *Streptomyces*, *Actinomadura*, and fungal genera of *Dothideomycetes*, *Cladosporium*, *Chaetomium*, and *Trichaptum* may play a role in SRM degradation in compost. Prior to and after 14 or 28 days of composting, PrP^{TSE} was detected by Western blotting (WB) after extraction using sodium dodecyl sulphate (SDS) and sodium phosphotungstic acid (PTA) precipitation. The WB findings suggested at least 1-2 $\log_{10}$ reduction of PrP^{TSE} signals after 14 to 28 days of composting. Although scrapie prions (PrP^{Sc})

degradation can not be definitively concluded, the disappearance of chronic wasting disease prions (PrP^{CWD}) and bovine spongiform encephalopathy prions (PrP^{BSE}) may reflect biological degradation in compost. This is the first study to investigate possible biological degradation of PrP^{CWD} and PrP^{BSE} during composting, suggesting that it has merit as a means of SRM disposal.

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List of Abbreviations

ANOVA – analysis of variance

BH – brain homogenate

BSE – bovine spongiform encephalopathy

CCME – Canadian Council of Ministers of the Environment

CFIA – Canadian Food Inspection Agency

CJD – Creutzfeldt-Jackob disease

CWD – chronic wasting disease

DGGE – denaturing gradient gel electrophoresis

DNA – deoxyribonucleic acid

EC – electrical conductivity

ELISA – enzyme-linked immunosorbent assay

FAME – fatty acid methyl esters

Hamster PrP^{TME} – a hamster-adapted prion infected from transmissible mink encephalopathy

iQ-RT-PCR – immuno-quantitative real-time polymerase chain reaction

mcrA – methyl-coenzyme M reductase

PC analysis – principal component analysis

PCR – polymerase chain reaction

PK – proteinase K

PLFA – phospholipid fatty acid

PMCA – protein misfolding cyclic amplification

pmoA – particulate methane monooxygenase

PrP^{BSE} – an infectious prion that causes bovine spongiform encephalopathy in cattle

PrP^{C} – a normal cellular prion that is susceptible to proteinase K digestion

PrP^{CJD} – an infectious prion that causes Creutzfeldt-Jackob disease in human

PrP^{CWD} – an infectious prion that causes chronic wasting disease in deer and elk

PrP^{Sc} – an infectious prion that causes scrapie in sheep and goats

PrP^{TSE} – an infectious prion that causes transmissible spongiform encephalopathy and is resistant to PK digestion

PrP^{263K} – a hamster-adapted prion infected from scrapie (strain 263K)

PTA – sodium phosphotungstic acid

qPCR – quantitative polymerase chain reaction

RNA – ribonucleic acid

SDS – sodium dodecyl sulphate

SRM – specified risk material

TC – total carbon

TN – total nitrogen

TSE – transmissible spongiform encephalopathy

UPGMA – unweighted pair group method with arithmetic average

USEPA – United States Environmental Protection Agency

VFA – volatile fatty acid

WB – Western blotting

Chapter 1 Introduction

1.1 Composting

1.1.1 Concept of composting and typical composting process

Composting is a natural biological process of decomposition and stabilization of organic matter under conditions that allow development of thermophilic temperatures in a predominantly aerobic environment (Haug 1993; Keener et al. 1993). It is a common technology believed to have been used for centuries to recycle agricultural and horticultural residuals, such as animal manure and plant residues, and manage diverse organic waste produced by urban life (Rynk 1992; Kutzner 2000). Generally, the product of composting is sufficiently stable and free of pathogens and plant seeds for storage and land application (Haug 1993). Research has indicated that the composting process provides an inexpensive and technologically straightforward solution for management of some hazardous industrial wastes, inactivation of pathogens, and remediation of contaminated soils (Civilini et al. 1996; Diaz et al. 1996; Turner et al. 2004).

The composting process can be described in many ways. Figure 1-1 shows a typical temperature curve as a function of composting time. The shape of the curve varies with the substrates being composted and the composting method. There are two major composting stages, active composting and curing. The temperature zones can be divided into mesophilic and thermophilic stages. There is no precise definition of mesophilic and thermophilic, but mesophilic generally refers to temperatures up to approximately 40°C, and thermophilic to

temperatures from 40°C up to 70°C (Miller 1996). The active composting stage accelerates the decomposition of organic matter while at the same time creating an environment that is unsuitable for the survival of most pathogens (Xu et al. 2009a). High temperatures (i.e., > 50°C) can persist for days or weeks depending on the characteristics and quantity of the substrate and availability of oxygen (Haug 1993). When easily degradable organic substrates are depleted, the microbial activity and compost temperature decline. As a result, the composting process enters the curing phase in which the compost turns to relatively stable nutrient content and alters microbial communities. The duration of the curing process varies from weeks to months. During curing, the carbon compounds are less susceptible to mineralization so that it is difficult to detect changes over short periods of time (Epstein 1997). Contents of mineral N and humic acids continue to increase, improving the value of compost for agricultural or horticultural use (Goyal et al. 2005; Chen et al. 1996; Fauci et al. 1999).

After the curing phase, compost gradually comes to mature. To be considered mature, CCME (Canadian Council of Ministers of the Environment) (2005) suggest that the compost shall be cured for a minimum of 21 days and meet one of the following three requirements: (1) the respiration rate is ≤ 400 mg of oxygen per kilogram of organic matter per hour; (2) the CO_2 evolution rate is ≤ 4 mg of carbon in the form of CO_2 per gram of organic matter per day; (3) the temperature rise of the compost above ambient temperature is less than 8°C.

1.1.2 Parameters affecting composting process

1.1.2.1 Temperature

Heat production during composting is almost completely derived from microbial metabolism. Thus, compost temperature is a measurable indicator of microbial activity, because it changes in direct response to heat production (Kutzner 2000). The time-temperature relationships affect the rate of decomposition of organic matter. Temperature can therefore be used to assess the progress of decomposition and thus the performance of a composting system (Yu et al. 2008). Temperature, in any system, is rarely uniform throughout the composting mass with the center of the mass tending to be hotter than the peripheral edge.

There have been some controversial discussions as to the optimal compost temperature. Previous literature indicate that the optimum temperature for composting is about 50-60°C (Epstein 1997). However, it may be that there is no optimum temperature for all compost substrates as the available substrates are diverse (e.g., agricultural wastes, municipal garbage, sewage sludge) and exhibit varying optimal temperatures for decomposition (Epstein 1997). A certain level of temperature for a period of time is required to render pathogens in wastes non-viable. Guidelines from CCME (Canadian Council of Ministers of the Environment) (2005) and USEPA (United States Environmental Protection Agency) (1995) both suggest that the temperatures for all compost feedstock sources should reach or exceed 55°C for at least 15 consecutive days in windrows that are turned three times, and 3 consecutive days in confined or in-vessel composters.

1.1.2.2 Oxygen and aeration

Oxygen is essential for aerobic microbial activity during composting. Typically, oxygen consumption increases early in the composting process and declines as the composting process proceeds. For optimal composting, oxygen concentrations should not be below 5% (Strom et al. 1980) with levels greater than 10% being ideal in order to avoid anaerobic conditions and the generation of undesirable odours (Kutzner 2000). De Bertoldi et al. (1983) recommended concentrations of oxygen as high as 18%, whereas Suler and Finstein (1977) found no difference in composting efficiency between oxygen concentrations of 10% and 18%. Regardless of aeration methods used in composting system, it has been suggested that anaerobic microenvironments can develop, enabling anaerobic processes to occur (Atkinson et al. 1996). Thus, in practice, composting is not entirely an aerobic process (Kutzner 2000). Atkinson et al. (1996) estimated that almost 1% of bacteria found in aerobic municipal solid waste compost are anaerobic. An adequate supply of oxygen gives the more efficient aerobes a competitive advantage over the anaerobes and avoids the generation of offensive odours that are produced primarily by the anaerobic microbial populations (e.g., hydrogen sulphide).

In general, three primary methods are employed to provide oxygen during composting: physical turning of the substrate, convective air flow and mechanical aeration (Epstein 1997). Oxygen reaches microbial cells, either by convection and/or diffusion within the free air space, as well as through dissolution in the liquid phase (Kutzner 2000). Oxygen concentrations in compost vary with aeration method, the porosity and the moisture content of the substrate. These

factors can influence the movement of air within compost and thus the availability of oxygen (Rynk 1992).

1.1.2.3 Moisture

Moisture plays an essential role in supporting the metabolism of compost microbes as they can utilize only those organic molecules that are dissolved in water (Kutzner 2000). In addition, water provides a medium for chemical reaction, nutrient transportation, and microbial motility. Thus, moisture can affect microbial activity and rate of decomposition in compost (Epstein 1997). The moisture content of compost is a balance of water production as a result of the biological oxidation of organic matter and water loss through evaporation. However, the heat and air flow generated during composting evaporate significantly more water than is produced and as a result the compost matrix becomes drier as composting proceeds (Nakasaki et al. 1987). The changes of moisture during composting are rather complex, varying with composting methods, bulking agents and composting substrates (De Bertoldi et al. 1982; Oppenheimer et al. 1997). Optimum moisture content is also essential to achieve high temperatures during composting. Wiley and Pierce (1955) determined that moisture content in municipal solid waste of 55 to 69% (wet basis) produces the highest temperature during composting with temperatures being lower either below or above this moisture range. In practice, moisture contents of compost materials ranging between 40% and 65% generally result in adequate composting of most substrates (Rynk 1992). Below 40%, microbial activity declines, a condition that can be overcome through the addition of water. When moisture

exceeds 65%, air flow in compost is limited and anaerobic conditions can develop. However, composting may be feasible even if the moisture content exceeds the recommended limit. Leonard et al. (1997) observed that a sludge-straw-sawdust mixture could be successfully composted even though it had a moisture content of 80%, but subsequent disturbance of this mixture resulted in loss of porosity. Thus, moisture management is required to provide sufficient water to encourage microbial activity while still permitting adequate oxygen supply and sufficient structure (Richard et al. 2002).

1.1.2.4 Porosity

Porosity, bulk density, particle density, air filled porosity, and free air space are common terms cited in the composting literature to describe the physical nature of the composting matrix (Alburquerque et al. 2008). All of these factors influence the efficiency of composting by altering aeration. Adequate porosity in compost is necessary to prevent oxygen depletion and the maintenance of aerobic conditions. Since microbes grow primarily on the surface of compost particles (Nakasaki et al. 1986), the availability of substrate for microbial growth is dependent on the surface area of particles. Consequently, the larger surface area of small particles usually accelerates the composting process. However, smaller particle size can reduce the degree of porosity, leading to anaerobic conditions and failure of the composting process (Agnew and Leonard 2003). For optimum performance of the composting process, the free air space should account for 20-30% of the total volume of compost (Kutzner 2000) and particle size should range

from 3 to 50 mm (Rynk 1992). In practice, compost porosity can be controlled by adding raw materials or by grinding.

Materials added to adjust the porosity are referred to as bulking agents. Bulking agents can correct deficient physical properties, enhance aeration and improve composting performance (Haug 1993; Chang and Chen 2010; Gea et al. 2007; Adhikari et al. 2008). This is because most waste materials such as sewage sludge, food waste and animal manure, have moisture contents that are too high or C/N ratios that are too low for optimal composting, and bulking agent can also serve to add carbon.

Compost porosity varies with location within a pile or windrow. Generally, samples from the bottom of the pile are more compressed and have higher bulk densities (Schaub-Szabo and Leonard 1999; Mu and Leonard 1999). During the course of composting, porosity is influenced by the compressibility of initial materials and the loss of organic matter (Randle and Flegg 1985; Van Ginkle et al. 1999). Van Ginkle et al. (1999) suggested that over time, compaction has a greater influence on bulk density than organic matter degradation.

1.1.2.5 Nutrients and C/N ratio

Carbon and nitrogen are the primary nutrients required by the microbes involved in composting. Carbon is provided to the microbes from decomposing organic matter. Generally, the microbes digest carbon as an energy source and utilize carbon for cellular growth via aerobic respiration (Epstein 1997). Most animal manure has high levels of nitrogen, but low levels of carbon depending on the amount and type of bedding material used. Softwood shavings, sawdust and

straw are good sources of carbon (Larney et al. 2008). Other inexpensive sources of carbon include municipal waste and shredded newsprint or cardboard (Rynk 1992).

Microbes need nitrogen for protein synthesis. Bacteria may contain 7% to 11% nitrogen (dry weight basis) and fungi from 4% to 6% (Anderson 1956). As cells die, their stored nitrogen becomes available to living cells (Bishop and Godfrey 1983). Composting materials blended to provide a C/N ratio of 25:1 to 30:1 are ideal for active composting as microbes required 20 to 25 times more carbon than nitrogen to sustain active growth. If the C/N ratio is too high, nitrogen limits microbial cellular growth, reducing the decomposition of plant carbohydrates such as hemicellulose and cellulose (Eiland et al. 2001).

Low C/N ratios in the compost under alkaline conditions can result in the excess nitrogen volatilization in the form of ammonia (Epstein 1997). The loss of nitrogen reduces the value of compost as a fertilizer for land application. Meanwhile, the excess ammonium may produce alkaline conditions that inhibit the production of ligninolytic enzymes and further diminish the amount of carbon available for utilization by microbes (Eiland et al. 2001).

1.1.2.6 pH

The preferred pH for composting is in the range of 6.5-8.0 (Rynk 1992). However, composting may proceed effectively over a range of pH without limiting the process because of its natural buffering capacity. Compost pH affects the growth of microorganisms with bacterial growth often being optimal near neutral pH. De Bertoldi et al. (1983) showed that fungi typically grow better in a

mildly acidic environment, although they can often tolerate a wide range of pH (Tuomela et al. 2000). Most actinobacteria tolerate a higher pH than fungi with their optimal growth occurring at a pH between 7 and 8 (Fermor et al. 1979).

The pH does not remain constant during the composting process. The pH is influenced by the decomposition of substrates as well as other factors such as aeration. Anaerobic conditions can produce fermentation products, such as volatile fatty acids (VFA) that can lower compost pH (Beck-Friis et al. 2000; Eklind et al. 1997). The formation and decomposition of VFA strongly affects the pH in the compost and slurries (Beck-Friis et al. 2003; Paul and Beauchamp 1989). At the beginning of the mesophilic composting stage, pH may decrease slightly due to the formation of VFA even in a well aerated condition (Kutzner 2000). This may be followed by an increase in pH caused by the decomposition of proteins and the liberation of ammonia as a result of deamination of amino acids or caused by the decomposition of VFA due to the high temperature. During the latter stage of composting, the volatilization of NH_3 and nitrification may again result in a decline in compost pH.

1.1.2.7 Electrical conductivity

Electrical conductivity (EC) gives an indication of the total salt (or electrolyte) content of the compost and, consequently, is of interest to end users from a plant nutrient perspective (Wilson 1983; Epstein 1997). Ringer (1997) showed that EC of the water extract from manure compost was in the range of 0.7 to 1.5 mS cm^{-1} and had no phytotoxic effects on plants grown in a compost-soil

mixture. Epstein (1997) suggests that EC levels in excess of 5 mS cm^{-1} may lead to phytotoxicity.

Raviv et al. (1987) and Inbar et al. (1993) observed a drop in EC early in the composting process, but a steady increase as composting proceeded. They explained that the initial drop was due to the fixing of ammonia and other nutrient ions by the microbial population, while the subsequent increase in EC was attributed to the increase of mineral concentration as organic matter continued to be decomposed. Increase of compost EC value is widely reported in the literature and was found to be highly correlated with time of composting (Larney and Olson 2006; Grebus et al. 1994). Therefore, EC could be one of the parameters potentially used as an indicator of compost maturity.

1.1.3 Dynamics of organic matter degradation

Organic matter has been defined as seven major elements, including carbohydrates and sugar, protein, fats, hemicellulose, cellulose, lignin and mineral matter (MacCarthy et al. 1990). The rate of microbial decomposition of organic matters varies among these sources (Stentiford 1993). Usually, sugars, starch, lipids, amino acids and nucleic acids are readily biodegraded. Hemicellulose, cellulose, and chitin degrade at a slower rate. The third group is much more resistant to biodegradation, consisting of lignin and lignocellulose.

The composition of organic matter in the compost depends considerably on the source of substrate selected for the composting process. For example, composition of livestock manure depends on the type of livestock and the diet they are fed (Gray et al. 1971). Similarly, composition of degradable carbon in

plant materials depends on the source (e.g., straw vs. wood) or age (fresh vs. old) (Larney et al. 2008). Knowledge of the composition of these materials to be composted is important for the composting design. For example, mixing high protein materials with cellulolytic materials adjusts the C/N ratio to a value that is more favorable for the microbial growth.

1.1.4 Gas emissions during composting

Aerobic decomposition from well managed composting results in the emission of CO_2 and H_2O. The emission of CO_2 from the compost is a result of the microbial respiration. Measuring either CO_2 evolution or O_2 depletion has been used to estimate the degree of microbial activity in compost (Manios and Balis 1983; Nakasaki et al. 1985) and are positively correlated with organic matter decomposition (Ntougias et al. 2006). However, measurement of the fluxes of these gases does not provide the information on specific enzyme activities or the taxonomic nature of microbial communities in compost.

Another carbon compound gas emitted from compost is CH_4. The net CH_4 emission is determined by the activities of methanogen and methanotroph communities in the compost (Thummes et al. 2007; Halet et al. 2006). Due to the heterogeneous nature of a compost pile, CH_4 can be generated by methanogens in anaerobic microniches within the compost pile (Brown and Subler 2007). However, studies have shown that the majority of the CH_4 is oxidized to CO_2 by methanotrophs in aerobic regions near the surface of the compost pile, resulting in negligible CH_4 emissions (Zeman et al. 2002; Brown and Leonard 2004). However, this claim is refuted by Amlinger et al. (2008), who reported

considerable CH_4 emission in a laboratory-scale composter. In windrow composting systems, Hellmann et al. (1997) and Hao et al. (2001) also observed high CH_4 emission in the first 20 days with negligible emissions occurring after 30 days.

Ammonia emission is the main form of nitrogen loss during composting (Hellebrand and Kalk 2001). During composting, organic nitrogen is mineralized and released as soluble NH_4^+. The soluble NH_4^+ is volatile as NH_3 during the first few days or weeks of composting, when compost pH and temperature are high (Hao and Benke 2008). Bishop and Godfrey (1983) observed that about 50 to 90% of all NH_3 losses occurred during the first few weeks of sludge composting. Under aerobic conditions, NH_4^+ can be oxidized to NO_2^- and subsequently to NO_3^- by a nitrification process. Some evidence indicated that ammonia-oxidizing microbial communities are responsible for this process in compost (Jarvis et al. 2009). However, high temperature and elevated NH_3 level inhibit the growth of ammonia-oxidizing microbial communities during the early composting, leading to a low concentration of NO_3^- (Bustamante et al. 2008). When temperature drops at the latter composting, the increase of nitrification enhances NO_3^- and lowers NH_4^+ (Tiquia et al. 2002). Under aerobic conditions, NO_3^- is stable, but under anaerobic conditions, NO_3^- is denitrified by denitrifier community to N_2O and N_2 (Maeda et al. 2010). However, N loss in the form of N_2O is much lower than NH_3 (Parkinson et al. 2004).

1.2 Microbiology of composting

1.2.1 Dynamics of microbial communities during composting

Microbial communities in compost evolve during the composting process as determined by the selective pressures of temperature, moisture, nutrient supply and substrate characteristics. Efficiency of the composting process depends on the activity of diverse microbial communities, such as bacteria and fungi (Kutzner 2000; Haug 1993). Microbial diversity and the succession of populations is a prerequisite to ensure adequate degradation of organic matter (Ryckeboer et al. 2003). Generally, at the start of mesophilic composting stage, diverse populations of mesophilic and/or thermotolerant bacteria and fungi are predominant and primarily responsible for the degradation of soluble and easily degradable carbon sources. Subsequently, elevated temperature and increase of pH caused by ammonification are favourable for bacteria that out-compete fungi within a few hours or days. During this composting stage, bacteria have a competitive advantage over fungi to generate a board range of enzymes to degrade a variety of organic matter (Ryckeboer et al. 2003). Consequently, mesophilic bacteria are responsible for most of initial metabolic activity that contributes to initial temperature increase. When the temperature increases above 40°C, thermophilic bacteria begin to dominate in the compost. The higher temperature accelerates the decomposition of organic matter and creates an environment that is unsuitable for the survival of most human and plant pathogens (Kutzner 2000). However, overall bacterial diversity decreases during the thermophilic stage (Fogarty and Tuovinen 1991). As the composting process nears completion, microbial activity is reduced

and less heat is generated. Mesophilic microbes start to re-colonise remaining substrates and continue the composting process until curing. Some diverse bacteria and fungi favour the lower water content and are responsible for the degradation of recalcitrate substrates, such as lignocellulose (Ryckeboer et al. 2003).

1.2.2 Current techniques for studying microbial ecology in compost

Research on compost microbiology has employed traditional approaches of microbial cultivation including quantitative estimation and isolation of the microbes using selective media and growing conditions. Isolates obtained have been subject to both morphological and biochemical characterization in an effort to understand their role within the microbial ecosystem responsible for composting (Ryckeboer et al. 2003). However, traditional microbiological techniques are limited in that the identification and classification of microbes using this procedure can be expensive and time consuming. Furthermore, a large proportion of the microbes in compost are unculturable in the laboratory due to lack information on the culture conditions required to facilitate their isolation (Amann et al. 1995). Therefore, alternative culture-independent approaches have been used to gain a better understanding of the diversity of microbes involved in the composting process.

1.2.2.1 Phospholipid fatty acid analysis

One method of investigating the changes of the microbial community during composting is phospholipid fatty acid (PLFA) analysis, and it is regarded as a sensitive and reliable method to qualitatively and quantitatively assess the

changes of biomass and microbial diversity (Zelles 1999; Ramsey et al. 2006). The rationale of this method is that phospholipids within the membranes of most microorganisms rapidly turn over and different microbial communities differ in their phospholipid fatty acid composition (Petersen and Klug 1994; Lei and VanderGheynst 2000). Therefore, total PLFA in environmental samples can be used to estimate the quantity of microbial biomass, and to gain an understanding of the major groups of microorganisms (i.e., fungi, actinobacteria, Gram positive and Gram negative bacteria) present in compost. However, the technique has a limited ability to differentiate microbes at the species level (Klamer and Bååth 1998; Bolta et al. 2003). In addition, PLFA analysis also provides a statistically powerful method to demonstrate the impact of various composting treatments on the changes in the microbial community (Ramsey et al. 2006).

Phospholipid fatty acid analysis has been used to monitor shifts in microbial communities during composting. For example, a study by Amir et al. (2008) utilized PLFA analysis as an indicator to characterize microbial communities (i.e., Gram positive bacteria, Gram negative bacteria, and fungi) during composting of sewage sludge and straw. They found that Gram negative bacteria decreased during the thermophilic composting phase, and that fungi as opposed to bacteria were responsible for the majority of organic matter decomposition during the maturation mesophilic composting phase. Kato and Miura (2008) employed PLFA analysis to observe the succession of bacterial communities in a windrow composting system was accelerated after inoculation of 4-moth matured cattle

manure compost, and indicated that PLFA analysis can be an indicator for evaluating the maturity of cattle manure compost.

1.2.2.2 Denaturing gradient gel electrophoresis

The development of molecular fingerprinting techniques provides a pattern or profile of the genetic diversity in a microbial community on the basis of the physical separation of unique nucleic acid sequences (Muyzer and Smalla 1998). The general strategy for this technique consists of (a) the extraction of nucleic acids, (b) the amplification of target genes by polymerase chain reaction (PCR), and (c) the analysis of PCR products by molecular fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE).

Denaturing gradient gel electrophoresis (DGGE) separates DNA fragments of identical length, but with different nucleotide sequences (Pedro et al. 2001). Separation of DNA fragments in DGGE is based on the decreased electrophoresis mobility of partially melted double-stranded DNA molecules in polyacrylamide gels containing a linear gradient of DNA denaturants. The DNA fragments with different sequences may have different melting behaviour and therefore stop migrating at different positions in a gradient gel. Excision and sequencing of the DGGE bands further provides information on the microbial species present in the community of interest (Muyzer 1999). Muyzer et al. (1993) revealed that the DGGE process can detect microbial members that account for as little as 1% of the total community. Despite the high sensitive detection for DGGE, Buchholz-Cleven et al. (1997) demonstrated that DGGE is not always possible to separate the DNA sequences differing in two to three nucleotides under the electrophoretic

conditions they used, probably leading to multiple sequences in a single DGGE band. Furthermore, short DNA fragments (up to 500 bp) may lack the sequence variability needed to differentiate members of the microbial community at the species level (Muyzer and Smalla 1998). Other factors such as the presence of multiple *rrN* operons within a single species may contribute to multiple bands in the DGGE display, overestimating community diversity (Nübel et al. 1996).

In practice, DGGE is being employed increasingly in the field of compost microbiology to provide rapid and reliable results, despite the above limitations. The succession of the main microbial communities (i.e., bacteria and fungi) by targeting ribosomal genes during composting have been revealed and identified by DGGE (Steger et al. 2007; Novinscak et al. 2009). Also, DGGE can be used for targeting non-ribosomal genes in compost and detecting the presence of microbial populations of biochemical interest. For instance, Maeda et al. (2010) monitored the emissions of N_2O in composted cattle manure and linked the dynamics of denitrifying microbial communities using the gene coding for N_2O reductase. Cahyani et al. (2009) employed DGGE to examine T4-type bacteriophage communities by targeting the capsid gene (*g23*) in a rice straw compost pile and evaluated the effect of bacterial succession on bacteriophage communities during composting.

1.2.2.3 Quantitative PCR

The application of PCR in combination with the extraction of nucleic acids (DNA or RNA) from environmental matrices has been central to the development of culture-independent approaches in microbial ecology. Quantitative PCR (qPCR

or real-time PCR) combines the detection of target genes in the environment with quantification by using fluorescence to estimate the amplification rate of a PCR product and relating it to a standard curve constructed from known amounts of the target gene (Smith 2005). Quantitative PCR has been shown to be a robust, highly reproducible and sensitive method to quantitatively track the changes of the phylogenetic and functional gene or the levels of gene expression across temporal and spatial scales within the environment (Smith and Osborn 2009).

However, some biological and methodological factors can affect the quantification of genes from environmental samples. The method used for nucleic acid extraction and extraction efficiencies can be a major determinant on the final quantification of the target (Martin-Laurent et al. 2001). Various extraction protocols used for different environmental samples and within different laboratories make direct comparison of absolute gene numbers between studies extremely problematic. Therefore, the generation and comparison of absolute gene numbers can only be considered within a single reaction and using the same standard curve (Smith et al. 2006). Also, the numbers from environmental samples can not be converted to cell numbers as the copy number of genes may vary among microbial species (Klappenbach et al. 2000). Furthermore, the presence of PCR inhibitors in extracted DNA may affect subsequent qPCR as the gene isolated from the environment and the target gene used to generate the standard curve may not have equivalent amplification efficiencies (Stults et al. 2001).

Most applications of qPCR in compost have been for the quantification of pathogens, such as *Campylobacter jejuni*, *Clostridium perfringens*, and *Salmonella spp.*, during the composting process (Inglis et al. 2010; Karpowicz et al. 2010; De Clercq et al. 2007). In addition, qPCR has been employed to detect the abundance and/or the activity of the microbial communities in the compost. For example, Halet et al. (2006) used qPCR to observe that the activity of the methanotroph population declined during the thermophilic stage, subsequently increased to the maximum and finally declined during the mature stage in a 200 L lab-scale composter, by quantifying the copy numbers of reverse transcribed methanotroph 16S rRNA. Although qPCR has shown great promise in evaluating the microbial ecology of complex environmental samples (Smith and Osborn 2009), this technique has not been widely employed to study composting microbiology.

1.3 Specified risk material and prions

1.3.1 Transmissible spongiform encephalopathy

Transmissible spongiform encephalopathy (TSE) is a fatal neurodegenerative disease that includes scrapie in sheep and goats, chronic wasting disease (CWD) in deer and elk, bovine spongiform encephalopathy (BSE) in cattle, and Creutzfeldt-Jackob disease (CJD) in humans. The cause of TSE is a protein conformational change in which a normal prion protein found in the body (PrP^{C}) transforms into an infectious prion protein (PrP^{TSE}) (Prusiner 1998).

1.3.2 PrP^{C} and PrP^{TSE}

Normal prion protein (PrP^{C}) is attached to the outer surface of the plasma membrane by a glycosylphosphatidyl inositol anchor. It is readily released from the cell surface by cleavage with phosphatidyl inositol-specific phospholipase C and is highly susceptible to proteinase K (PK) digestion (Weissmann 2004). Infectiou prion protein (PrP^{TSE}) was originally defined as a form of prion protein that was largely resistant to PK digestion under conditions in which PrP^{C} and most other proteins were readily degraded (Meyer et al. 1986). Importantly, digestion of PrP^{TSE} by PK causes cleavage at residues 87 to 91 (the exact position depends on the prion strain) of the mature prion protein sequence, leading to a characteristic electrophoretic mobility shift of the three bands that correspond to di-, mono- and unglycosylated species (Weissmann 2004). Normal prion protein, PrP^{C}, can be converted into PrP^{TSE} through a process whereby a portion of its α-helical and coil structure is refolded into a β-sheet (Prusiner 1998). Pan et al. (1993) showed that PrP^{C} contains about 40% α-helix and little β-sheet, whereas PrP^{TSE} is composed of about 30% α-helix and 45% β-sheet. Nevertheless, these two prion proteins have the same amino acid sequence (Prusiner 1998). This structural transition is accompanied by profound changes in the physicochemical properties. All the prion proteins discussed in this thesis are designated in List of Abbreviations (Prefatory pages).

Two models have been hypothesized for the conversion of PrP^{C} to PrP^{TSE} as reviewed by Weissmann (2004). One is known as a virus model, postulating that the infectious agent consists of a TSE-specific nucleic acid associated with or

packaged in PrP^{TSE}. The other one is a protein model, which postulates that exogenous PrP^{TSE} causes catalytic conversion of PrP^{C} to PrP^{TSE}, either at the cell surface or after internalization. More evidence has provided the support for the protein model, as PrP^{TSE} is devoid of nucleic acid and resistant to the conventional virus inactivation (Prusiner 1998). However, the precise structure of PrP^{TSE} is not yet known, nor are the mechanisms of infection, conformational conversion and pathogenesis understood.

1.3.3 Bovine spongiform encephalopathy in Canada

As of January 2012, 19 cases of BSE had been confirmed in Canada (Dudas et al. 2010; CFIA 2012). Considering the findings in the UK and the rest of Europe, the consumption of BSE contaminated meat and bone meal was the most likely cause of the first case of BSE in Canada. Other possible causes of BSE have been proposed for a number of years including: spontaneous mutation of PrP^{C} to PrP^{BSE}, or exposure to another PrP^{TSE}, such as PrP^{Sc} and PrP^{CWD}. However, there is little scientific evidence or data to support these theories.

Classical or typical BSE cases (C- type) that occurred as part of outbreaks in the UK, Europe and Japan were believed to be caused by the consumption of contaminated feeds (Nicholson et al. 2008). Atypical BSE (H- and L-type) refer to cases of BSE that do not fit the typical C type pattern of the disease as documented by differences in histology, site of accumulation and molecular weight (Yamakawa et al. 2003; Béringue et al. 2006; Capobianco et al. 2007). Up to date, atypical types of BSE have been identified in Europe, Japan, the United States and Canada (Terry et al. 2007; Hagiwara et al. 2007; Richt et al. 2007;

Dudas et al. 2010). The origins of atypical BSE are unknown. However, it has been speculated that atypical BSE may be sporadic or genetically caused, and indeed, strong evidence for a causative role of genetic mutations has been found in at least one case (Richt and Hall 2008).

In Canada, only two BSE cases exhibited molecular weight and glycoform profiles similar to those of previously reported for atypical cases described from Europe, Japan and the USA, one corresponding to H-type BSE and the other to L-type BSE. All other cases were classified as C-type (Dudas et al. 2010). Thus, BSE prevalence in Canada follows the trend with low occurrence of atypical BSE cases, reflecting that most cases of BSE in Canada have arisen from the consumption of contaminated feed (Dudas et al. 2010).

1.3.4 Specified risk material

As a consequence of the occurrence of BSE, the Canadian Food Inspection Agency (CFIA) introduced a feed ban in 1997 that outlawed feeding of meat and bone meal derived from ruminants back to ruminants. Subsequent to this feed ban, CFIA introduced regulations in 2003 for the removal of specified risk material (SRM) from entering the food chain. Specified risk material refers to specific body tissues known to be at risk of harbouring PrP^{BSE}, including the skull, brain, trigeminal ganglia, eyes, palatine tonsils, spinal cord and dorsal root ganglia of cattle aged 30 months or older, as well as the distal ileum of cattle of all ages (CFIA 2010). On July 12, 2007, CFIA implemented an enhanced feed ban that bans SRM from all animal feeds, pet foods and fertilizers.

1.3.5 Current methods for specified risk material disposal

It is estimated that there are approximately 250,000 tonnes of SRM generated in Canada annually (Gilroyed et al. 2010). Alberta is estimated to produce 74,000 tonnes per year of SRM, including dead stock (Alberta Agricultural Research Institute 2005). Proper disposal of carcasses and SRM is important both to prevent livestock disease transmission, and to protect air and water quality. Currently, the majority of SRM are rendered and then disposed of in landfills, which is both uneconomical and a poor environmental practice (Ayalon et al. 2001; Ma et al. 2007). Consequently, alternative and safe methods of SRM disposal are needed in an attempt to derive value from SRM generated within Canada.

1.3.6 Current and potential methods for PrP^{TSE} inactivation

However, the disposal of SRM infected with PrP^{TSE} are challenging, as the infectivity of PrP^{TSE} exhibits resistance to inactivation by a wide range of physicochemical methods, commonly used for microorganisms, nucleic acids, and proteins, including strong alkali solutions, chaotrophic salts, alcohols, oxidizing agents, radiation, and heat (Ernst and Race 1993; Taylor 2000). This may be because PrP^{TSE} typically exist as large aggregates, a self-protecting arrangement that may be particularly resistant to chemical inactivation (Hörnlimann et al. 2007).

Currently, there are several effective methods for the inactivation of PrP^{TSE} in the laboratories (Table 1-1). Among the chemical disinfectants that are effective at inactivating PrP^{TSE}, a 1-hour disinfection with 2 N sodium hydroxide is best suited for surfaces and materials that can not be autoclaved, but can resist alkaline

treatment in the lab. Another current practice for PrP^{TSE} inactivation is autoclaving. Although the thermostability of PrP^{TSE} has been recognized (Hunter and Millson 1964), autoclaving of PrP^{TSE}-contaminated material in saturated steam at 132°C for a period of 60 minutes is sufficient to inactivate PrP^{TSE} (Hörnlimann et al. 2007). Incineration of PrP^{TSE}-contaminated material is considered the most effective method, as combustion at 1,000°C can destroy the infectivity of PrP^{TSE}. However, low infectivity remains after treatment at 600°C (Brown et al. 2004).

In Canada, several methods have been approved by CFIA for the disposal of SRM infected with PrP^{TSE}, including incineration, gasification, combustion in a cement kiln, thermal hydrolysis, and alkaline hydrolysis (Table 1-2). Incineration is the primary method of SRM disposal in Europe (Paisley and Hostrup-Pedersen 2005). Gasification is a process through which solid and liquid carbonaceous materials are converted to a combustible product gas. These gases can be combusted to provide energy or can be used for a variety of industrial applications, as SRM contains 60% to 75% of the energy content of coal (Fedorowicz et al. 2007). Thermal hydrolysis refers to a process in which biological materials are treated with high temperature (150-180°C) and high pressure (3-12 atmospheres) steam (Schieder et al. 2000). Similar to thermal hydrolysis, alkaline hydrolysis combines sodium hydroxide or potassium hydroxide with temperature (150°C) and pressure (5 atmospheres) to catalyze the hydrolysis of biological material into a sterile aqueous solution consisting of small peptides, amino acids and sugars (Murphy et al. 2009). Currently, alkaline

hydrolysis has been employed for laboratory-scale disposal of SRM in Alberta, Canada.

Recently, some evidence suggests that PrP^{TSE} may be degraded by a variety of other potential methods, such as ultraviolet-ozone and hydrogen peroxide (Table 1-3). In addition, several purified biological enzymes (Table 1-3) also have exhibited the ability to degrade recalcitrant PrP^{TSE} (Dickinson et al. 2009; Yoshioka et al. 2007; McLeod et al. 2004). However, only studies from McLeod et al. (2004) and Dickinson et al. (2009) examined the infectivity of PrP^{TSE} after enzyme digestion in mouse bioassays.

1.4 Composting of specified risk material and PrP^{TSE}

1.4.1 Application of composting for specified risk material disposal

The use of on-farm composting for the disposal of SRM is of interest because it is a relatively simple procedure, environmentally sound, and economical. Composting uses materials and equipment that are often readily available on farms. Moreover, composting is particular attractive as the temperature obtained during the process (> 55°C) is sufficient to inactive the pathogens, such as bacterial species of *Salmonella* (De Clercq et al. 2007), *Campylobacter* (Inglis et al. 2010), *Listeria* (Erickson et al. 2009), *Escherichia coli* (Xu et al. 2009a), *Clostridium* (Karpowicz et al. 2010), and zoonotic parasites, such as *Giardia* cysts and *Cryptosporidium* oocysts (Van Herk et al. 2004), and viruses such as those responsible for foot and mouth disease (Guan et al. 2010), avian influenza and Newcastle disease (Guan et al. 2009).

Under proper management, composting appears to be a viable method of carcass disposal. Composting of carcasses using windrows, static piles, and bins or vessels has been investigated for poultry, swine, sheep and cattle (Lawson and Keeling 1999; Murray et al. 2007; Stanford et al. 2000; Xu et al. 2009a). Stanford et al. (2000) reported that sheep carcasses including keratinized wool were completely degraded in composting systems in which temperature of over 60°C was maintained for a period of 41 days. Using a ratio of five parts manure to one part cattle mortalities in a windrow composting trial, Stanford et al. (2009) demonstrated that < 1% of residual bone from cattle remained in the cured compost. Xu et al. (2009a) reported that more than 90% dry matter of bovine brain disappeared after 7 days and 80 % dry matter of bovine hoof decomposed after 56 days in a biosecure composting system. Also, in the same composting system, Xu et al. (2009b) demonstrated a 99% reduction in genomic DNA of composted bovine tissue and > 93% reduction of bovine mitochondrial DNA to prove almost complete decomposition of carcass soft tissue after 147 days of composting.

Therefore, composting, when done correctly with proper attention to the design, layout, monitoring, maintenance and environmental impacts of the system, may be considered as potentially efficient and safe method of disposing of SRM in Canada.

1.4.2 Projected fate of PrP^{TSE} during composting

Composting might be an attractive alternative for the disposal of SRM or animal mortalities infected with PrP^{TSE}, as methods currently used are impractical

on farm. Recent evidence has indicated that several microbial proteinases exhibit the ability to degrade recalcitrant PrP^{TSE} (Table 1-3). The source for those enzymes has been previously found in compost microbial species (Ryckeboer et al. 2003). The optimal conditions (alkaline pH and 50-60°C) for enzymes with the ability of high degradation of PrP^{TSE} are feasible in the composting process. Also, the period of time that PrP^{TSE} would be exposed to enzymatic activity during composting was far more than the duration of exposure of PrP^{TSE} to these proteases required for degradation (i.e., weeks to months vs. minutes to hours). Generally, the more complex the substrate, the more extensive and comprehensive is the enzyme system required to achieve degradation (Tuomela et al. 2000; Golueke 1992). Therefore, compost could be an effective ecosystem for the inactivation of PrP^{TSE} as a wide range of enzymes are produced by complex microbial consortia. Up to date, only Huang et al. (2007) reported that PrP^{Sc} was near or below the Western blotting detection limit by directly testing scrapie-infected tissues left after 108-148 days of composting.

1.4.3 Characteristics of PrP^{TSE} in the environment

Infectious prion protein (PrP^{TSE}) may enter the environment through a number of routes. First, PrP^{TSE} may enter through shedding from infected hosts. It has been shown that PrP^{Sc} and PrP^{CWD} can be shed in urine (Seeger et al. 2005), feces (Safar et al. 2008), saliva and blood (Mathiason et al. 2006). A second route of entry is through animal mortalities, including those of farmed sheep, goats and cervids as well as of free-ranging cervids. For example, during the early years of the BSE outbreak in the United Kingdom (1988–1991), it is estimated that 6,000

carcasses that were suspected of having BSE were disposed of in 59 landfill sites (Saunders et al. 2008a). Another potential route of entry may be via solid or liquid waste generated in rendering plants and slaughterhouses that are unknowingly processing infected carcasses (Hinckley et al. 2008).

Generally, various proteins have been observed to be strong, fast and irreversible adsorption to a wide range of particle surfaces (Brash and Horbett. 1995). As with other proteins, PrP^{TSE} adsorption is most likely a function of electrostatic attractions and repulsions and hydrophobic interactions. Because the N-terminal domain is known to be flexibly-disordered and contain a high number of positively-charged amino acids, it may play a significant role in electrostatic attraction to negatively-charged mineral surfaces (Revault et al. 2005). It is known that PrP^{TSE} is highly insoluble and aggregated. The hydrophobic interactions could therefore also play a larger role in PrP^{TSE} adsorption (Saunders et al. 2008a). However, since the three-dimensional structure of PrP^{TSE} remains unknown, it is a challenge to model the specific mechanisms that are responsible for PrP^{TSE} adsorption.

Infectious prion protein (PrP^{TSE}) enters the environment concurrently with the adsorption to the organic matter from the host. For example, in the wastewater system, PrP^{TSE} are unlikely to be transported long distances in surface water as it has been shown that both hamster PrP^{TME} and PrP^{BSE} tend to strongly associate with the sludge solids (Hinckley et al. 2008; Kirchmayr et al. 2006). In the soil, PrP^{Sc} and PrP^{263K} have been known to remain infectious for years (Seidel et al. 2007; Brown and Gajdusek 1991). Similarly, the strong adsorption of PrP^{TSE} to

soil particles may also result in the limited PrP^{TSE} mobility in soil (Cooke and Shaw 2007). To date, the persistence of various types of PrP^{TSE} is being explored. By incubating infectious brain homogenate at 37°C for up to 1 month, PrP^{CWD} was shown to be greater resistant to degradation than hamster PrP^{TME} as observed by Western blotting (Saunders et al. 2008b). After the exposure to SDS (sodium dodecyl sulphate), PrP^{BSE} was 10- and 10^6-fold more resistant to inactivation than PrP^{CJD} and hamster-adapted PrP^{Sc} via an infectivity titer in transgenic mouse (Giles et al. 2008). In anaerobic sludge, the stability of PrP^{BSE} was observed to be higher than mouse-adapted PrP^{Sc} under 55°C for 12 days as indicated by Western blotting (Kirchmayr et al. 2006). All these stable characteristics of PrP^{CWD} and PrP^{BSE} in the environment may directly impact or promote TSE transmissibility and pathogenesis. However, the consequences of PrP^{TSE} adsorption in the environment, including the conformational changes that may impact TSE infectivity, altered bioavailability or uptake by animal, and protection from enzymatic, chemical or physical degradation, are poorly understood (Saunders et al. 2008a).

1.4.4 Potential methods for PrP^{TSE} detection in compost

Highly-sensitive and accurate detection of PrP^{TSE} quantification and infectivity in compost environments present a significant challenge. This is because the compost environment is an exceedingly complex system, owning to higher temperatures, complex enzymes activities and dynamic changes in microbial communities. Once PrP^{TSE} enter the compost environment, they are exposed to or involved in a wide variety of biological, chemical and physical

mechanisms that can influence PrP^{TSE} existence. For example, solution pH, ionic concentration and humic acids have been reported to influence PrP^{TSE} adsorption (Ma et al. 2007; Polano et al. 2008). The factors can be largely altered during composting and possibly impede the detection of PrP^{TSE} in compost. Moreover, PrP^{TSE} adsorption in the environment is irreversible and not readily mobilized. Sarkosyl (sodium-laurylsarcosinate) and SDS have the ability to release PrP^{TSE} bound to soil (Cooke et al. 2007; Seidel et al. 2007). Therefore, most common methods for the measurement of PrP^{TSE} associated with infected tissues may not be directly suitable for detection of PrP^{TSE} in complex matrices such as compost.

Several methods have been applied to detect PrP^{TSE} in complex environments, such as soil. These methods could be potentially used for detection of PrP^{TSE} in compost. Methods for PrP^{TSE} extraction from soil have been focused on using detergents and boiling to dissociate PrP^{TSE} into solution where they are detected by immunoblotting techniques, such as Western blotting and ELISA (enzyme-linked immunosorbent assay) (Cooke et al. 2007; Johnson et al. 2006). Soil bound PrP^{TSE} are usually first separated from dissociated PrP^{TSE} by low-speed centrifugation (Johnson et al. 2006; Genovesi et al. 2007; Leita et al. 2006). Dissociated PrP^{TSE} can be then be detected by standard immunoblotting procedures. Although immunoblotting techniques are rapid and specific for PrP^{TSE}, detergent and boiling extraction methods typically have very low recoveries of bound PrP^{TSE}, presumably due to the strong and near-irreversible binding of PrP^{TSE} to soil particles. Recoveries have been reported to be 61 – 67%

in wastewater sludge (Hinckley et al. 2008) and 5 – 40% for sandy and clay soils (Cooke et al. 2007; Cooke and Shaw 2007).

Reuter et al. (2009a) describe a simple and robust assay for the quantitative detection of PrP^{Sc} using immuno-quantitative real-time PCR (iQ-RT-PCR) made possible by a direct conjugate of a PrP^{Sc}-specific antibody (ICSM35) and a synthetic 99-bp DNA tail. The DNA tail was engineered to include a single ScrFI restriction site, which enabled subsequent quantification of restricted DNA tails using real-time PCR. This iQ-RT-PCR assay had a detection limit corresponding to 2.32×10^2 PrP^{Sc} epitopes, which represented a 1000-fold increase in detection sensitivity over the commercial assay and the same sensitivity as the bioassay in transgenic mice. However, a number of substances with humic acids being the most predominant within compost are known to inhibit subsequent enzyme-based analysis of DNA, such as PCR (Reuter et al. 2009b). The high concentrations of humic acids in the compost may impede this iQ-RT-PCR analysis of PrP^{TSE} throughout composting.

Protein misfolding cyclic amplification (PMCA), in which small amounts of PrP^{TSE} can be amplified by incubating and sonicating PrP^{TSE}-containing brain homogenate diluted in normal brain homogenate (Saá et al. 2006), has generated much interest for use as a promising environmental detection method. Up to date, PMCA has been used with PrP^{CWD} and PrP^{263K} extracted from soil with SDS (Seidel et al. 2007; Kurt et al. 2007), but not with PrP^{BSE}. Recently, Nagaoka et al. (2010) developed a direct detection of PrP^{Sc} in soil to avoid the effects of inefficient extraction, which used soil-bound PrP^{Sc} to seed PMCA and provided

10^3-10^4 higher sensitivity than the extraction methods followed by Western blotting. However, further development of PMCA is needed for the detection of PrP^{TSE} in the compost.

Infectivity of PrP^{TSE} may be still present in the absence of detectable PrP^{TSE} (Barron et al. 2007). Thus, animal bioassay represents the golden standard for assessing TSE infectivity and involves injecting PrP^{TSE} materials into the brain of a host animal and observing for visual signs of infection. Although ruminants were used in early animal bioassays, such as sheep and goats (Cuille and Chelle 1938), animal bioassays for research were subsequently improved with the introduction of rodent-adapted PrP^{Sc} and their titration by intracranial inoculation of mice and hamsters (Marsh and Kimberlin 1975). A further breakthrough of animal bioassays was achieved by the genetic engineering of transgenic mice (Raeber et al. 1998), sensitively expressing high level of heterologous PrP^{C} (e.g., human or bovine). The results of animal bioassays are expressed in terms of infectious units (IU_{50}). One IU_{50} represent the amount of PrP^{TSE} required to infect half the populations of test animals used in a study. Quantification is gained by either end-point titration or on the basis of the incubation period time (Prusiner et al. 1980). Animal bioassays have been applied in soil samples to test the infectivity of soil-bound PrP^{TSE}. For example, Seidel et al. (2007) used either the mixtures of soil and PrP^{263K} or the aqueous extract from those mixtures for the oral application into the hamsters to demonstrate PrP^{263K} can remain infectious in soil at least over 29 months. Furthermore, Johnson et al. (2007) observed that the hamster PrP^{TME} bound to soils increased oral transmission relative to that unbound

agent, by orally dosing the mixture of hamster PrP^{TME} and soil into hamster. Although the practical limitations of animal bioassay include the long incubation periods and the high expense of maintaining large numbers of animals (Klohn et al. 2003), those applications of animal bioassay in soil hold promise for the use in the future studies of PrP^{TSE} in compost as any PrP^{TSE} inactivation procedures must be validated by animal bioassay.

1.5 Conclusions

Composting may be an attractive alternative for disposal of carcases or SRM infected with PrP^{TSE}, because it combines multiple advantages, such as high temperature (> 55°C), fluctuation of pH (5.5-10), and wide range of proteolytic enzymes produced by dynamic microbial communities. The previous fundamental knowledge about the understanding of composting process, composting microbiology and composting of animal carcasses leads to a hypothesis that composting may be effective to inactive recalcitrant PrP^{TSE}.

However, the study of PrP^{TSE} in compost is highly challenging, as no other environmental pathogens have the complex structure and also the complex and dynamic characteristics owned by composting itself. Moreover, the fact that PrP^{TSE} structure is not well known and that many PrP^{TSE} analytical methods could not be well-developed adds to these challenges (Saunders et al. 2008a). To answer the questions about composting as an alternative method for disposal of SRM infected with PrP^{TSE}, it is necessary to first assess the degradation of SRM and PrP^{TSE} in compost at the laboratory-scale, which helps to determine whether it can be employed at full-scale. In practice, more critical importance is the ability to

study PrP^{TSE} in complex composting process as determined in three aspects: (1) developing a suitable composting model enabling this approach to compost PrP^{TSE} under laboratory biocontainment conditions; (2) building a practical and reliable method to analyze the compost-associated PrP^{TSE} in compost; (3) quantifying any degree of PrP^{TSE} degradation (i.e., logarithmic reduction), and ultimately the reduction in infectivity.

1.6 Objectives of thesis

The research work discussed in this thesis aimed to address the aforementioned issues. The objectives set for this thesis were:

(1) To assess the degradation of SRM and characterize the microbial communities responsible for SRM degradation in laboratory-scale composters.

(2) To utilize the laboratory-scale composters from Objective (1) to investigate the degradation of PrP^{Sc}, PrP^{CWD} and PrP^{BSE} in compost under biocontainment conditions.

1.7 Outline of thesis

This thesis is presented in a paper format. The figures and tables are grouped at the end of each chapter before the bibliography. Chapter 1 provides a general literature review for this work. Chapter 2 assesses the degradation of SRM and investigates the microbial communities during composting of SRM in the laboratory-scale composters; Chapters 3 and 5 further characterize the bacterial, actinobacterial, and fungal species that could be responsible for SRM degradation in compost; In Chapter 4, a method for the detection of compost-bound PrP^{TSE} is

described and applied to assess the fate of PrP^{Sc} in compost; The possible degradation of PrP^{263K}, PrP^{CWD} and PrP^{BSE} in compost is further explored in Chapter 6. Results are summarized and discussed in Chapter 7, together with directions for future work.

Table 1-1 Currently recommended methods for PrP^{TSE} inactivation

Destruction methods	Required conditions	References
Sodium hydroxide	2 N concentration at least for 1 hour	Hörnlimann et al. (2007)
Autoclaving	134°C for 60 minutes	Hörnlimann et al. (2007)
Incineration	1,000°C	Brown et al. (2004)

Table 1-2 Currently approved methods for the disposal of specified risk material infected with PrP^{TSE} in Canada

Destruction methods	Required conditions	References
Incineration, gasification, combustion	1,000°C	Brown et al. (2004)
Thermal hydrolysis	180°C and 12 atmospheres	Schieder et al. (2000)
Alkaline hydrolysis	pH 14, 150°C and up to 5 atmospheres	Murphy et al. (2009)

Table 1-3 Potentially effective methods for PrP^{TSE} inactivation

Methods	Source enzymes	Source microbes	Reaction conditions	Infectivity reduction	References
Ultraviolet-ozone	N/A	N/A	8 weeks	≥ 5 log	Johnson et al. (2009)
Ozone	N/A	N/A	20°C, pH 4	≥ 4 log	Ding et al. (2012)
Hydrogen peroxide	N/A	N/A	90%, 53°C	≥ 5-6 log	Rogez-Kreuz et al. (2009)
Biological enzymes	Alkaline serine proteinase	*Streptomyces* sp.	60°C, pH 11	N/A	Hui et al. (2004)
	MSK 103 serine proteinase	*Bacillus licheniformis*	50°C, pH 9	N/A	Yoshioka et al. (2007)
	Properase, protease M, Purafect Ox, Purafect	*Bacillus* sp.	60°C, pH 12	1-3 log	McLeod et al. (2004)
	Protease E, Protease F	*Thermus* sp.	80°C, pH 7	N/A	McLeod et al. (2004)
	Keratinolytic protease	*Thermoanaer-obacter* subsp.	60°C, pH 7	N/A	Tsiroulnikov et al. (2004)
	Keratinolytic enzyme	*Thermosipho* subsp.	80°C, pH 7	N/A	Tsiroulnikov et al. (2004)
	MC3 alkaline protease	*Bacillus lentus*	60°C, pH 10	> 7 log	Dickinson et al. (2009)
	Proteinase K	*Tritirachium album*	50°C, pH 7	N/A	Langeveld et al. (2003)
	PWD-1 keratinase	*Bacillus licheniformis*	50°C, pH 7-8	N/A	Langeveld et al. (2003)
	Keratinolytic protease	*Nocardiopsis* sp.	60°C, pH>10	N/A	Mitsuiki et al. (2006)

N/A, not applicable

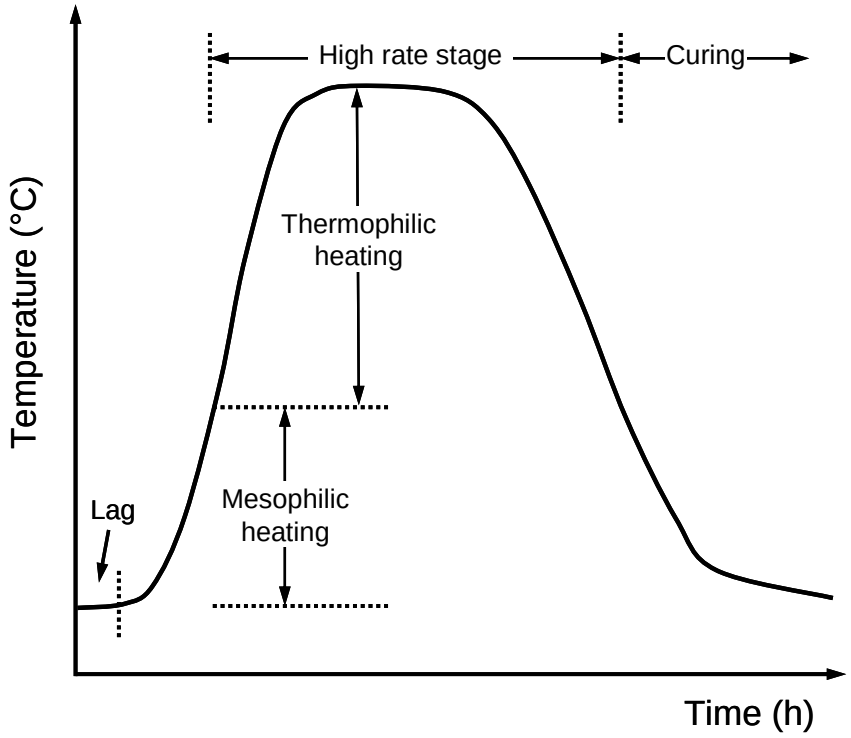

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Figure 1-1 Schematic of typical compost temperature vs. time curve.

1.8 References

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Chapter 2 Assessment of microbial communities in decomposition of specified risk material using a passively aerated laboratory-scale composter*

2.1 Introduction

Transmissible spongiform encephalopathies (TSE) are fatal neurodegenerative diseases that include scrapie in sheep and goats, chronic wasting disease (CWD) in deer and elk, bovine spongiform encephalopathy (BSE) in cattle, and Creutzfeldt-Jackob disease (CJD) in humans. Transmissible spongiform encephalopathy's result when the normal prion protein (PrP^{C}) undergoes a conformational change to form a misfolded infectious prion protein (PrP^{TSE}) that leads to neurodegenerative disease (Prusiner 1998). Interaction of PrP^{TSE} with PrP^{C} leads to the generation of more misfolded prion proteins. As a consequence of the occurrence of BSE in Canada in 2003, the Canadian Food Inspection Agency (CFIA) introduced regulations in 2007 to prevent the introduction of specified risk material (SRM) into the food chain (CFIA 2008). Specified risk material refers to specific body tissues known to be at risk of harbouring infectious prion protein, including the skull, brain, trigeminal ganglia, eyes, palatine tonsils, spinal cord and the dorsal root ganglia of cattle aged 30 months or older, as well as the distal ileum of cattle of all ages (CFIA 2008). A similar regulation is presently being considered in the United States (Dewell et al. 2008).

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A variety of approaches are being used for the disposal of SRM from abattoirs and on-farm cattle mortalities. In Canada, rendering followed by deposition of the resultant meat and bone meal in landfills is the main method of SRM disposal. Thermal hydrolysis and gasification technologies are also being assessed for the disposal of SRM (Kalbasi-Ashtari et al. 2008; Fedorowicz et al. 2007). However, both of these approaches require considerable capital investment and energy expenditure for SRM disposal. Currently, incineration is the primary method of SRM disposal in Europe (Paisley and Hostrup-Pedersen 2005), but this process derives little to no value from SRM, is energetically costly and raises concerns over air quality. Furthermore, the geographically vast nature of Canadian beef production makes incineration impractical as a disposal method throughout Canada. Burial of SRM is another option, but Canadian winters and ground water conditions often limit the utility of this disposal method in many regions of Canada.

Composting has been long considered as an environmentally acceptable method for treating industrial and agriculture wastes. Because temperatures within the compost typically exceed 60°C, this high temperature accelerates the decomposition of organic matter and creates an environment that is unsuitable for the survival of microbial pathogens (Van Herk et al. 2004; Ceustermans et al. 2007). Composting of cattle carcasses under proper management appears to be a potentially attractive option and a well-established pathogen reduction technology (Stanford et al. 2007; Xu et al. 2009). However, little information exists about the utility of composting as a disposal method of SRM or about the microbial

populations that may be involved in the decomposition of this material during composting.

The objective of the current study was to develop a passively aerated laboratory-scale composting system to investigate the decomposition of SRM and to use phospholipid fatty acid (PLFA) profiling to assess changes in the microbial community. The model was developed with the intention of eventually assessing the potential degradation of PrP^{TSE} under laboratory containment conditions.

2.2 Materials and methods

2.2.1 Laboratory-scale composter design

Six 110-L cylindrical polyethylene vessels (1601M, Eagle, Wellsburg, West Virginia, USA), 0.7 m high and 0.45 m diameter were used to construct laboratory-scale composters (Figure 2-1). To reduce the dissipation of heat through the composter wall, the surface of the composter was sealed with a 50-mm layer of polyurethane foam with a density of 32 kg m^{-3} and a thermal resistance value of 1.2 K m^2 W^{-1}. For the purpose of passive aeration, an air plenum (0.1 m height) was created at the bottom of the composter using a supporting rigid polyethylene panel (5 mm thickness, Model 2615, Sissiboo Farm Supplies, Weymouth, Nova Scotia, Canada) perforated with 10-mm diameter holes. An inlet air hole and outlet air hole of the same diameter (25 mm) were drilled at the bottom and the top of the composter to enable passive ventilation. Any leachate formed during composting was collected through a 15 mm drain in the bottom of each composter.

2.2.2 Composting setup and sampling

Fresh feedlot beef manure and two different bulking agents (i.e., barley straw or wood shavings) were used as the composting matrix for SRM. Fresh cattle brain tissue from non-infectious cattle mortalities under 30 months of age were obtained from a nearby slaughterhouse and used as a representative component of SRM. Each composter contained a mixture of 35 ± 0.1 kg fresh feedlot beef manure (74.1% water content) and 3.5 ± 0.1 kg of full length barley straw or wood shavings with an average particle size of 10 mm × 5 mm. The manure and the bulking agents were thoroughly mixed by hand and placed in three replicate composters per treatment. In their respective treatments, 20 mm of straw or wood shavings were placed in the bottom of each composter prior to introduction of the mixed matrix. During filling, triplicate samples of SRM, fresh manure, barley straw, wood shavings and the complete mixture were collected for later analysis.

Specified risk material was placed in each composter using the procedure illustrated in Figure 2-2. Fresh cattle brain tissues (150 ± 0.1 g) were weighed and sealed in nylon bags (140 × 90 mm; 25 µm pore size). The nylon bag, along with 400 g of mixed compost, was then placed into a larger polyester mesh bag (200 mm × 200 mm; 5-mm pore size) with attached polyester twine to enable recovery during composting. When the compost vessels were filled, six mesh bags were placed at each of three depths, 0.45 m (bottom) 0.3 m (middle) and 0.15 m (top) resulting in a total of eighteen mesh bags in each composter. Sample bags were collected on days 5, 9 and 15. At each sampling time, two mesh bags were removed from each sampling depth resulting in a total of six bags being removed

from each composter. Composting was terminated on day 15. After sampling, subsamples of compost (150-200 g) were collected from the mesh bags and stored at 4°C for up to 12 h before chemical and physical analysis, while the remaining compost in the mesh bags was freeze-dried in a freeze dryer (Series 24D × 48, Virtis, Gardiner, New York, USA) and vacuum-packed in a plastic bag using a vacuum sealer (Model T000-3502-300, FoodSaver, Ontario, Canada), and frozen at -80°C until PLFA analysis.

2.2.3 Specified risk material decomposition and compost properties

Decomposition of SRM was estimated on the basis of dry matter disappearance from the nylon bags during the composting process. Upon removal from the mesh bags, nylon bags containing cattle brain tissue were immediately rinsed with cold tap water, then placed in a glass beaker and gravimetrically measured after oven drying at 105°C for 3 days.

Compost and ambient temperatures were measured every hour using thermocouples attached to a data logger (CR10X, Campbell Scientific, Edmonton, Alberta, Canada). Temperature was measured at the same three depths that SRM were implanted in each composter. Oxygen concentrations at each depth were also measured twice daily using a compost oxygen monitor (Model OT-21, Demista Instruments, Arlington Heights, Illinois, USA).

Water content of a 100-g sample of each compost matrix was determined by difference after drying for 5 days at 60°C. Dried samples were then coarsely ground through a 2-mm screen with additional subsamples (5 g) being ground to size <150 µm for analysis of total carbon (TC) and total nitrogen (TN) using an

automated CNS analyzer (NA2100, Carlo Erba Strumentazione, Rodano, Milan, Italy). For SRM, TC and TN contents were measured in similar procedures using freeze dried samples after being finely ground using a ball mill (MM200, Retsch GmbH, Haan, Germany).

Compost pH was determined after mixing 25 g of compost with 50 ml of distilled water and shaking for 1 h. After this, the bottles were centrifuged at 10,000 × *g* for 15 min and the extract was used to measure electrical conductivity (EC) using an EC meter (Model 125, Orion, Beverly, Massachusetts, USA). Mineral N (NH_4^+ and NO_X^- [NO_2^- & NO_3^-]) was measured using a Bran + Luebbe AutoAnalyzer III (Bran + Luebbe GmbH, Norderstedt, Germany). Barley straw and wood shavings samples were analyzed in a manner similar to the manure and compost, except that a 1:10 ratio of bulking agent to distilled water was used. Bulk density was measured under unloaded conditions without compressive forces in a 10 L cylindrical bucket as described by Raichura and McCartney (2006).

2.2.4 Phospholipid fatty acid analysis

The procedure for PLFA analysis was modified from Lei and VanderGheynst (2000). Briefly, the whole freeze-dried compost (4 g) collected from within the vicinity of the SRM was weighed into a centrifuge tube and 33 ml of a one-phase mixture of chloroform, methanol and citric buffer (pH 4.0) in a ratio of 1:2:0.8 were added. After two hours of shaking for fatty acid extraction, the mixture was centrifuged at 2100 × *g* for 20 min and the supernatant was decanted into a test tube. Compost samples were re-extracted with 23 ml of one-phase mixture for

another 30 min and the supernatant from the second extraction was also added to the same tube with citric buffer and chloroform resulting in a final chloroform, citric buffer and methanol mixture of 1:0.9:1. After shaking for 2 min, the mixture was allowed to stand overnight for phase separation. The lower organic phase containing the lipids was collected into another test tube and evaporated to dryness under N_2 at 32°C in a water bath. At this point, dried extracts were kept at -20°C until further analysis. Phospholipids were separated from neutral and glycolipids using a solid phase extraction silica gel column. The columns were conditioned with chloroform and lipids were then transferred to the column in chloroform. Neutral lipids, glycolipids and phospholipids (polar lipids) were fractionated and eluted with a series of organic solvents of 5 ml chloroform, 10 ml acetone, and 5 ml methanol respectively. Subsequently, the phospholipids in methanol were evaporated under N_2 at 32°C in a water bath and stored dry at -20°C until methylated. Fatty acid methyl esters (FAME) were formed by methylation in a 1 ml 1:1 mixture of methanol and toluene along with 1 ml of 0.2 M KOH at 37°C for 10 min. The FAME was extracted with 2 ml hexane, 2 ml distilled water and 0.3 ml of 1.0 M acetic acid, and the hexane fraction was collected. Another 2 ml of hexane was used for further extraction of the sample. The hexane samples containing the FAME were combined and washed with 4 ml of 0.03 M NaOH and then dried under N_2 at room temperature. The dried FAME were dissolved into 500 µl hexane with C19:0 as an internal standard and analyzed using a Hewlett Packard 5890 gas chromatograph with a 25-m Ultra 2 (5% phenyl) methylpolysiloxane column (J&W Scientific, Folsom, California,

USA) and a flame ionization detector. Peaks were identified using fatty acid standards and associated identification software (MIDI Inc., Newark, Delaware, USA).

In fatty acid nomenclature, the basic form was used in 'A:BωC', where A is the total number of carbon, B is the number of double bonds, and C is the position of the double bond from the methyl end of the molecule. The suffixes 'c' and 't' stand for *cis* and *trans* configurations of double bonds. The prefixes 'i' and 'a' refer to *iso* and *anteiso* branching fatty acids. The number followed by 'Me' indicates the position of the methyl group from the carboxyl end of the chain. The prefix 'cy' refers to cyclopropyl rings (Zelles 1999).

The concentration of total PLFAs was determined by summing all PLFAs in the sample and expressed as nmol of total PLFAs per gram (dry weight) of compost, a value that was used as an indicator of total microbial biomass as PLFA are present in the membranes of most microorganisms (Klamer and Bååth 1998; Zelles et al. 1995).

The sum of the following fatty acids was considered to represent bacteria: i14:0, i15:0, a15:0, 15:0, i16:0, 16:0, 16:1ω7c, 16:1ω9c, i17:0, a17:0, cy17:0, 17:0, 10Me18:0, 18:1ω7 and cy19:0 (Bååth 2003; Bolta et al. 2003). Gram-positive bacteria were represented by the sum of i15:0, a15:0, i17:0, a17:0 and i16:0 (Klamer and Bååth 1998) and Gram-negative bacteria by cy17:0, cy19:0, 16:1ω7c, 16:1ω5c and 18:1ω7c (Klamer and Bååth 1998). Fungi were represented by 18:2ω6, 9c and 18:1ω9c (Bååth 2003) and actinomycetes by 10Me 18:0 (Klamer and Bååth 1998). All branched fatty acids were considered as

representative of thermophiles, and anteiso-branched and unsaturated fatty acids of nonthermophiles (Bolta et al. 2003).

The microbial community in compost was presented in the particular microbial groups as described above and determined as a molar percentage (mol%) against total identified PLFAs. In this context, the development of different groups of microorganisms during composting was inferred from the changes in PLFAs (mol%). Although this approach could not be used to determine absolute amounts of the biomass associated with specific communities, it has been used as an indicator of possible shifts in the composition of soil microbial communities (Hill et al. 2000; Ramsey et al. 2006).

2.2.5 Statistical analysis

The initial compost properties were analyzed as a factorial design using Proc GLM and Tukey's significant difference test (< 0.05 probability level) in SAS (SAS Institute 2001). The analysis of variances on the decomposition of SRM and the compost chemical properties was performed using the Mixed procedure of SAS with time and compost depth treated as repeated measures in the model (< 0.05 probability level). Principal component (PC) analysis was used to analyze the changes in the composition of PLFA over the composting period. For PC analysis, the PLFA data set was transformed by removing the fatty acids that accounted for less than 1% of the profile (Herrmann and Shann 1997; Steger et al. 2003) leaving 31 individual PLFAs for PC analysis.

2.3 Results

2.3.1 Characteristics of initial compost materials

The chemical and physical properties of the materials used to formulate the composting matrix are shown in Table 2-1. With regard to the characteristics of the two bulking agents, barley straw had higher ($P < 0.05$) water content, TN, pH, EC, NH_4^+, and NO_x^- (NO_2^- and NO_3^-), and lower ($P < 0.05$) TC, C/N ratio, and bulk density as compared to wood shavings. Although the two bulking agents were chemically different, once mixed with manure the mixture exhibited similar TN, C/N ratio, pH, EC and mineral N (NH_4^+, NO_2^- and NO_3^-). The wood shavings compost did exhibit higher ($P < 0.05$) water content, TC and bulk density, and lower ($P < 0.05$) EC than the straw compost. Specified risk material had higher ($P < 0.05$) water content, TC and TN content as compared to barley straw and wood shavings.

2.3.2 Specified risk material decomposition

The effect of the compost matrix or depth on SRM decomposition was not significantly different ($P > 0.05$), but SRM decomposition exhibited a three-way interaction among compost matrix, depth and time. Decomposition of SRM was notably lower in the top layer of the straw compost on day 5 (9.2% vs. 25.0% and 37.2% at the middle and bottom depths), but was similar to that of other depths after day 9 (Table 2-2). Composting matrix exerted no major effect on rate of decomposition of SRM except that at day 5, degradation was more ($P < 0.05$) extensive with wood shavings than with straw (37.1% vs. 9.2%) at the top depths in the composters. SRM decomposition increased with composting time ($P <$

0.05), but reached a plateau after 9 days at the middle and bottom compost depths. At the top compost depth, however, decomposition of SRM continued beyond 9 days of composting and degradation was more extensive ($P < 0.05$) after 15 days than after 9 days, irrespective of the type of compost matrix (Table 2-2).

2.3.3 Temperature and oxygen profiles

At day 3, temperature peaked in both straw and wood shavings compost at 59°C and 67°C, respectively (Figure 2-3a). Temperature increased more rapidly in the straw compost than the wood shavings compost. After day 3, the temperature in all composters began to steadily decline, but remained above 50°C until day 10. Temperatures were generally higher in the wood shavings compost than the straw compost at all compost depths. Temperature also exhibited a gradient from low to high between the bottom and top depths. Oxygen concentrations were initially 18% in all composters on day 0 and after two days of composting decreased to 16% in the straw compost and to 5% in the wood shavings compost (Figure 2-3b). Oxygen concentration remained at 5% for two days in the wood shavings compost at which point it subsequently returned to 18% and remained at this level for the remainder of the composting period. Oxygen concentration in the straw compost remained relatively constant throughout the composting period.

2.3.4 Changes in chemical properties

Both the straw and wood shavings compost had high water content (~70%) at the start of the experiment (Figure 2-4). With subsequent composting, water contents of both compost types declined, with this decline occurring more rapidly

in the straw compost than the wood shavings compost after day 9. After 15 days of composting, the water contents in the straw compost was lower ($P < 0.05$) than that in the wood shavings compost at all depths. The wood shavings compost initially had a higher ($P < 0.05$) TC content (42.0%) than the straw compost (40.4%). During composting, TC content declined gradually in both compost types. Although TC content in the wood shavings compost remained higher ($P < 0.05$) than in the straw compost, the straw compost exhibited a more rapid TC loss than the wood shavings compost during the initial composting period. Prior to composting, the TN content was 1.9% in the straw compost and 2.0% in the wood shavings compost, with TN increasing in both the straw and wood shavings compost over the composting period. Although initial C/N ratios were similar between the matrices prepared with straw and wood shavings, the C/N ratio was lower ($P < 0.05$) with straw than with wood shavings during composting. The C/N ratios in both compost types declined as the experiment progressed, from 22 initially to a range of 13 to 18 at day 15.

Both the straw and wood shavings compost had the similar initial pH (i.e., 7.7). Subsequently, the pH in the both composts increased and tended to be higher in wood shavings compost than in straw compost. By day 15, the compost pH in both compost types had declined slightly and was lower ($P < 0.05$) at the bottom than at other depths. Compost EC declined during the first 5 days of composting and thereafter increased in both types of compost at all depths. The straw compost exhibited a higher ($P < 0.05$) EC than the wood shavings compost throughout the composting period. Over the composting period, NH_4^+-N declined from 2275

mg kg^{-1} to 470 mg kg^{-1} in the straw compost and from 2975 mg kg^{-1} to 587 mg kg^{-1} in the wood shavings compost. Levels of (NO_3^- + NO_2^-)-N were low in both compost types until day 5, but peaked after 9 days, reaching a maximum of 363 mg kg^{-1} at the bottom depth in the straw compost and 4 mg kg^{-1} at the top depth in the wood shavings compost. At day 15, concentrations of (NO_3^- + NO_2^-)-N had declined back to pre-composting levels, with the exception of the bottom depth in the straw compost.

2.3.5 Phospholipid fatty acid analysis

The concentration of total PLFAs mirrored changes in temperature, increasing at most locations until day 5 and declining thereafter (Figure 2-5). Similarly, the proportions of PLFA associated with thermophilic and Gram-positive bacteria also tended to increase from day 0 to day 5 and declined as the temperature dropped below 40°C. Levels of PLFA associated with Gram negative bacteria decreased at day 5 and showed a small increase on the final sampling day. The proportions of PLFAs associated with fungi declined as temperature exceeded 55°C at day 5, and with the exception of straw compost on day 15, did not appear to recover during the remainder of the composting period. For actinomycetes on day 5, the proportions of PLFAs indicative of this group microorganism slightly increased in the straw compost, but declined in the wood shavings compost. However, after 15 days of composting, the proportion of actinomycetes increased in both compost types at most depths. In contrast to the thermophiles, the proportions of PLFAs associated with non-thermophiles

declined sharply after 5 days of composting and continued to be low until the end of the experiment on day 15.

Principal component analysis of the PLFA concentrations measured in the samples collected on days 0, 5 and 15 revealed localizations on the left, middle and right of the score plot, respectively (Figure 2-6), indicating a shift in PLFA composition with time that corresponded to the changing temperature phases associated with composting. However, PC analysis of the PLFA data did not show any spatial separation with compost type or sampling depth (Figure 2-6).

2.4 Discussion

2.4.1 Temperature and oxygen profiles

Most composting models reported to date employ forced aeration during composting. However, forced aeration is not desirable in laboratory experiments involving human pathogens because of concern over generation of aerosols. The 110-L passively aerated, laboratory-scale composter used in this trial reached temperatures above 55°C after 3 days and remained above 40°C for a period of 15 days, indicating successful establishment of the composing process. Relatively higher temperatures in the upper layers of compost are consistent with results reported by Yu et al. (2005) for a 200-L composter. The lower initial bulk density in the straw compost may have allowed for greater air flow through the compost, leading to a higher oxygen concentration during the early stages of composting. However, oxygen levels above 16% in both the straw and wood shavings composts in the later stages of composting indicated that oxygen was not limiting the composting process.

2.4.2 Changes in chemical properties

Water content remaining higher in the wood shavings compost than in the straw compost may reflect the greater porosity of wood shavings compared with straw, as well as the greater decomposition and loss of physical integrity during composting exhibited by straw in comparison with wood (Michel et al. 2004). Water contents in both compost types were within a range considered adequate for efficient composting (Richard et al. 2002; Ahn et al. 2008).

The higher TC content in wood shavings than in barley straw contributed to the consistently higher TC during composting of wood shavings compared with straw. Although cellulose and hemicellulose contents are similar between barley straw and wood shavings, the wood shavings contain more carbon in the form of lignin (Larney et al. 2008). The more rapid and extensive TC loss in the straw compost than in the wood shavings compost during the thermophilic composting stage was likely attributable to the lignification having impaired the degradability of the carbohydrates in wood shavings (Hao et al. 2004; Ward et al. 2000).

The observed increase of TN content in both compost types over the 15 days contrasts with the decreased TN typically reported (Peigné and Girardin 2004). This may be attributable to the release of a large amount of protein N into the surrounding compost from SRM as it decomposed. This conclusion is supported by the observation that at day 15, TN content was greater in compost collected from inside of the mesh bags than in compost from outside of the bags (Table 2-3). Xu et al. (2007) also recorded a higher TN content in a compost pile

containing cattle mortalities, after 310 days of composting, as compared to a compost pile that did not contain bovine carcasses.

Volatile fatty acids (VFA) are present in fresh manure waste and are produced during the initial mesophilic stages of composting even with adequate aeration (Beck-Friis et al. 2003) and can potentially affect compost pH. The increase in compost pH over the initial 5 days of this study was likely due to rapid decomposition of accumulated VFA during the transition to thermophilic conditions. The elevated pH and high temperature during early composting also likely promoted volatilization of NH_3 (Curtis et al. 2005; Larney and Olson 2006), which may have contributed to the decline in pH between days 9 and 15. The nitrification evident at day 9 (i.e., increased NO_x-N) may also have reduced pH during later stages of composting (Tiquia et al. 1998). The tendency of pH to be higher in the top than in the bottom sampling depths at day 15 may reflect the upward movement and sequestration of volatilized NH_3 in the upper regions of the composters. The increase in EC as composting progressed is consistent with other reports (Wang et al. 2004; Rasapoor et al. 2009). Consistently greater EC in the straw compost than in wood shavings compost reflected the lower soluble salts content in wood chips compared with straw (Allison and Anderson, 1951).

2.4.3 Specified risk material decomposition and phospholipid fatty acid analysis

The SRM decomposition pattern in this study (50% gone within 15 days; majority of that within 5 days; Table 2-2) is consistent with a report by Smårs et al. (2002), that 60% of initial compost substrate was degraded within 14 days in a

200-L composter when the temperature (controllable) was maintained at 55°C. Xu et al. (2009) also found that SRM was rapidly decomposed (> 90%) during the first 7 days of passive windrow composting. This present period of most rapid SRM decomposition corresponded with the early increase in microbial biomass revealed by PLFA analysis.

Gram-positive bacteria dominated the microbial community involved in SRM decomposition during the period of elevated temperature, consistent with reports from earlier studies (Strom 1985; Herrmann and Shann 1997; Bolta et al. 2003; Takaku et al. 2006; Amir et al. 2008). Gram-negative bacteria tended to decline as temperature increased during the active phase of composting, as has been indicated both by PLFA analysis (Klamer and Bååth 1998; Bolta et al. 2003) and by traditional culture techniques (Lafond et al. 2002). It has been confirmed that pathogenic Gram-negative bacteria associated with animal feces, such as *Escherichia coli*, *Salmonella* and *Shigella* spp., are killed by thermophilic composting (Hassen et al. 2001; Ceustermans et al. 2007).

Similar to other studies (Klamer and Bååth 1998; Steger et al. 2003), my data suggest that the relative proportion of fungi declined as temperatures increased. A slight recovery of fungal populations appeared to take place in the straw compost during the later stages of composting. This recovery may have been due to the lower compost temperature in the straw compost (35°C) as compared to the wood shavings compost (40°C) at this time.

Some thermotolerant actinomycetes, including *Nocardia*, *Streptomyces*, *Thermoactinomyces* and *Micromonospora*, are commonly isolated from compost

during periods of elevated temperature (Strom 1985). In this study, however, actinomycetes declined in the wood shavings compost over the first 5 days, probably because temperatures were above 60°C and few species of actinomycetes are tolerant of this temperature. The marked recovery of actinomycete populations during the later stages of composting is consistent with reports by others that actinoycetes are frequently visible on the surface of mature compost (Herrmann and Shann 1997; Tuomela et al. 2000; Bolta et al. 2003). Although actinomycetes lack the cellulose- and lignin-degrading activities exhibited by fungi (Crawford 1983; Godden et al. 1992), they do play an important role in organic matter degradation (Lacey 1997). Previous work has shown that actinomycetes can be highly proteolytic (Puhl et al. 2009) and consequently may play an important role in the decomposition of protein rich substrates such as SRM. Therefore, further identification of these species in the compost is merited although PLFA analysis suggests that multiple microbial communities may be involved in SRM decomposition.

2.5 Conclusions

Passively aerated laboratory-scale composters were constructed to enable study of manure composting under containment conditions. The composters were loaded with non-infectious specified risk material (SRM) to test whether this high-moisture substrate could be disposed of by composting in conjunction with typical livestock production substrates (manure and bedding). Physicochemical analyses conducted over 15 days confirmed that composting was proceeding typically, i.e., that the composter design was appropriate for representative test-

composting. Decomposition of SRM was relatively rapid (50% within 15 days), and PLFA profiling revealed time/temperature-dependent shifts in the populations within the compost microbial ecosystem. Further research will include specific study of prion protein degradation within these test composters.

Table 2-1 Characteristics of the materials and initial mixtures used for composting

Property*	Starting materials				Mixture at day 0	
	Beef manure	Barley straw	Wood shavings	Specified risk material	Straw	Wood shavings
Water content (%)	74.1 ± 0.6	12.1 ± 0.1b	7.7 ± 0.0c	81.0 ± 0.7a	68.2 ± 0.5B	69.9 ± 0.3A
Total C (%)	40.2 ± 0.3	45.4 ± 0.1c	51.1 ± 1.1b	56.7 ± 1.2a	40.4 ± 0.6B	42.0 ± 0.2A
Total N (%)	2.5 ± 0.0	1.1 ± 0.0b	0.1 ± 0.0c	7.4 ± 0.4a	1.9 ± 0.1A	2.0 ± 0.1A
C/N	16.1 ± 0.1	40.2 ± 0.8b	492.1 ± 31.5a	7.7 ± 0.6c	21.1 ± 0.5A	21.6 ± 0.8A
pH	7.6 ± 0.1	6.0 ± 0.0a	4.2 ± 0.0b	NM	7.6 ± 0.0A	7.7 ± 0.1A
EC (ds m^{-1})	8.8 ± 0.4	7.0 ± 0.4a	0.1 ± 0.0b	NM	9.3 ± 0.1A	8.1 ± 0.2B
NH_4–N (mg kg^{-1})	3095 ± 1032	158 ± 2a	12 ± 2b	NM	2275 ± 278A	2975 ± 458A
NO_x–N (mg kg^{-1})	< 0.5	3746 ± 67a	5 ± 0b	NM	< 0.5A	< 0.5A
Bulk density (kg m^{-3})	871 ± 13	39 ± 0b	117 ± 1a	NM	330 ± 3B	394 ± 1A

*All characteristics except water content are expressed on a dry weight basis (wt wt^{-1}). Water content is expressed on a wet weight basis. EC: Electrical Conductivity.
a-c: Within a row, values followed by different letters differ ($P < 0.05$) among bulking materials.
A,B: Within a row values followed by different letters differ ($P < 0.05$) between mixture types.
NM: not measured.

Table 2-2 Decomposition of specified risk material (% dry matter loss) during composting in laboratory-scale composters

Sampling time	Compost types and depths					
	Straw compost			Wood shavings compost		
	Top	Middle	Bottom	Top	Middle	Bottom
Day 5	9.2aAX	25.0aBX	37.2aCX	37.1bABX	26.6aAX	35.7aBX
Day 9	37.6aAY	37.6aAY	33.6aAX	43.2aAY	39.0aAY	45.4aAY
Day 15	47.8aBZ	36.6aABY	37.3aAX	49.2aAZ	40.9aAY	48.3aAY

a,b: Within a row and compost depth, values followed by different letters differ ($P < 0.05$) between compost types.
A-C: Within a row and compost type, values followed by different letters differ ($P < 0.05$) among compost depths.
X-Z: Within a column, values followed by different letters differ ($P < 0.05$) among sampling days.

Table 2-3 Total nitrogen content (%) of compost materials collected inside and outside of mesh bags at day 15 in laboratory-scale composters

Sampling location	Compost types and depths					
	Straw compost			Wood shavings compost		
	Top	Middle	Bottom	Top	Middle	Bottom
Inside	2.77a	2.92a	3.15a	2.33a	2.26a	2.73a
Outside	2.59a	2.40b	2.66b	2.18b	2.17a	2.20b

a,b: Within a column and compost depth, values followed by different letters differ ($P < 0.05$) between sampling location.

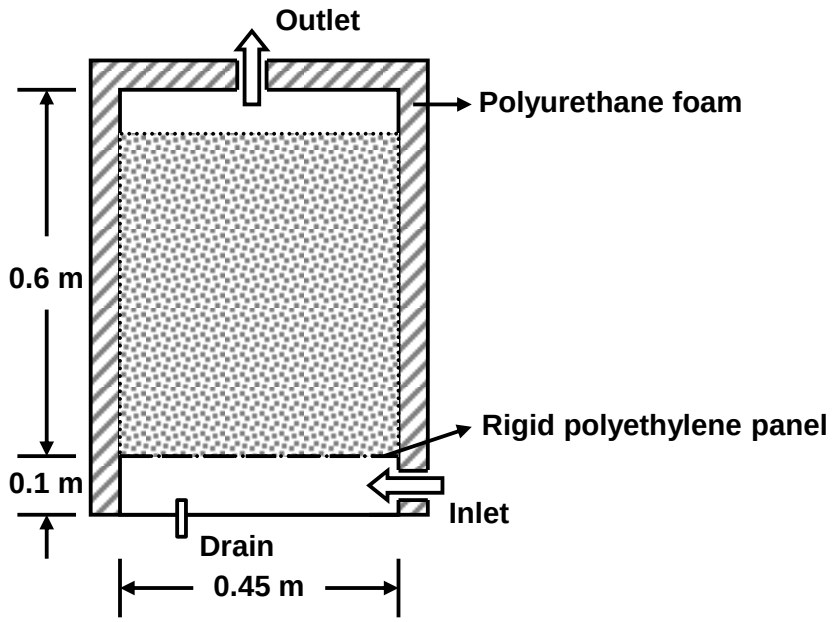

Figure 2-1 Schematic of the 110 L passively aerated laboratory-scale composter developed for work in containment.

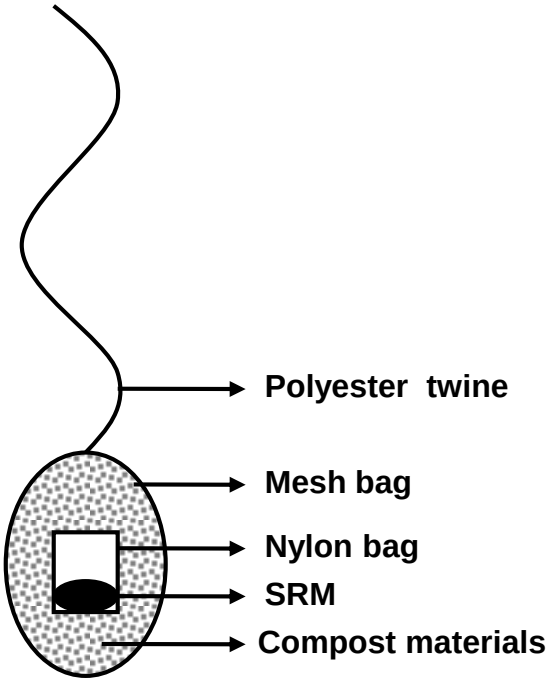

Figure 2-2 Schematic of the technique used to implant specified risk material (SRM) into the laboratory composter. SRM was placed into nylon bags with a pore size of 25 µm, which was subsequently surrounded by a mesh bag containing either a straw or wood shavings mixed with beef manure. The entire structure was removed from the composter at each sampling time via the polyester twine.

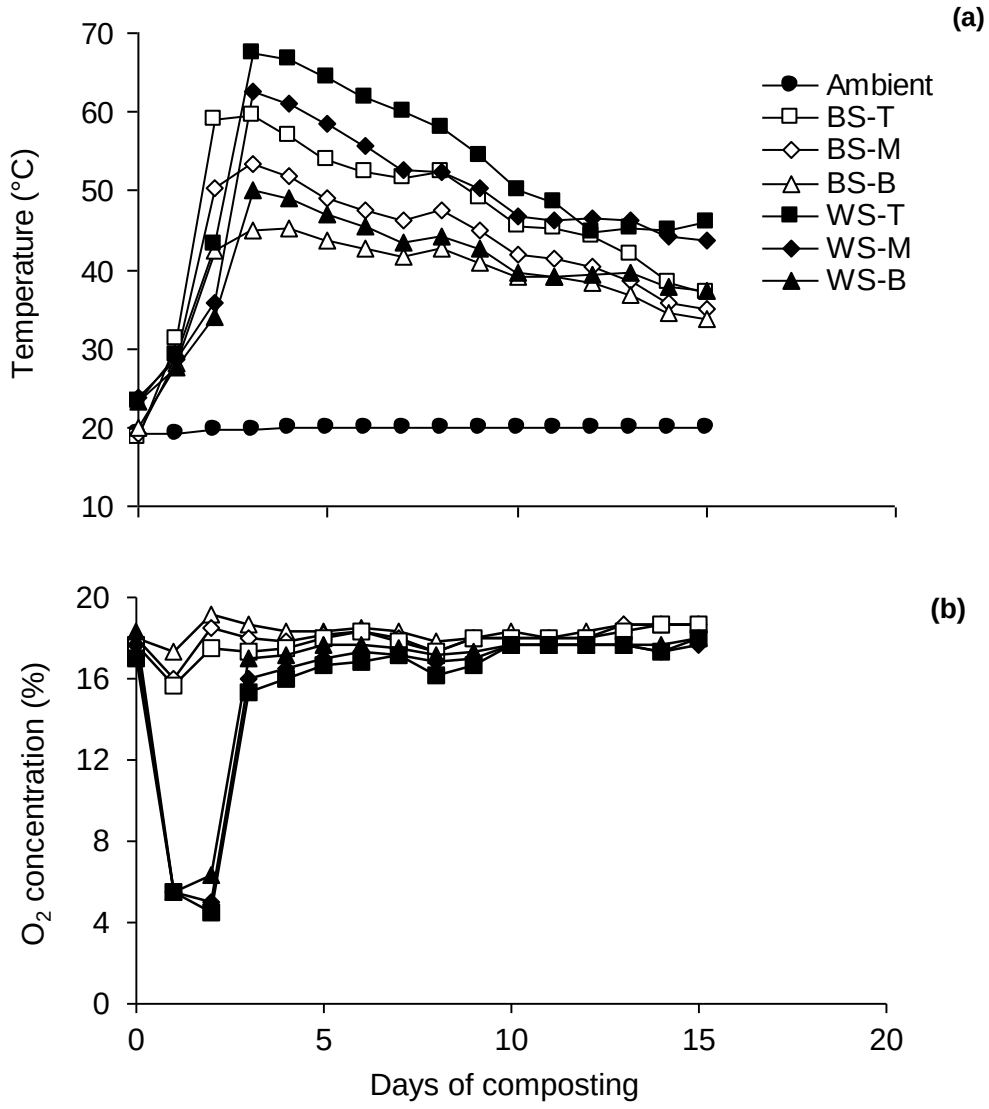

Figure 2-3 Temperature (a) and O_2 concentration (b) changes during composting of specified risk material in a laboratory-scale composter. BS (-T, -M and –B) and WS (-T, -M, and –B) indicate barley straw or wood shavings compost, sampled at top, middle or bottom depths, respectively.

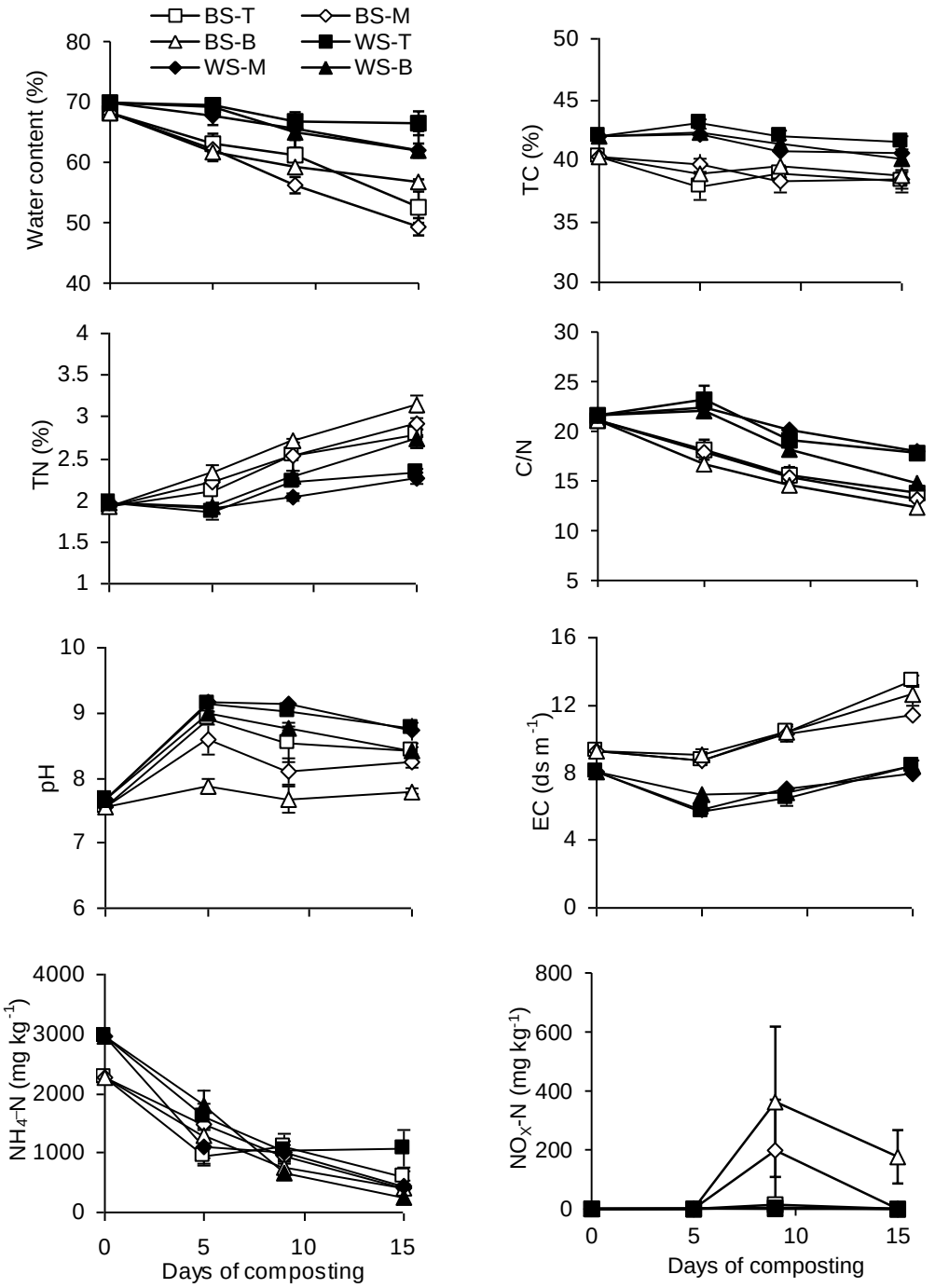

Figure 2-4 Chemical properties during composting of specified risk material in a laboratory-scale composter. BS (-T, -M and –B) and WS (-T, -M, and –B) indicate barley straw or wood shavings compost, sampled at top, middle or bottom depths, respectively.

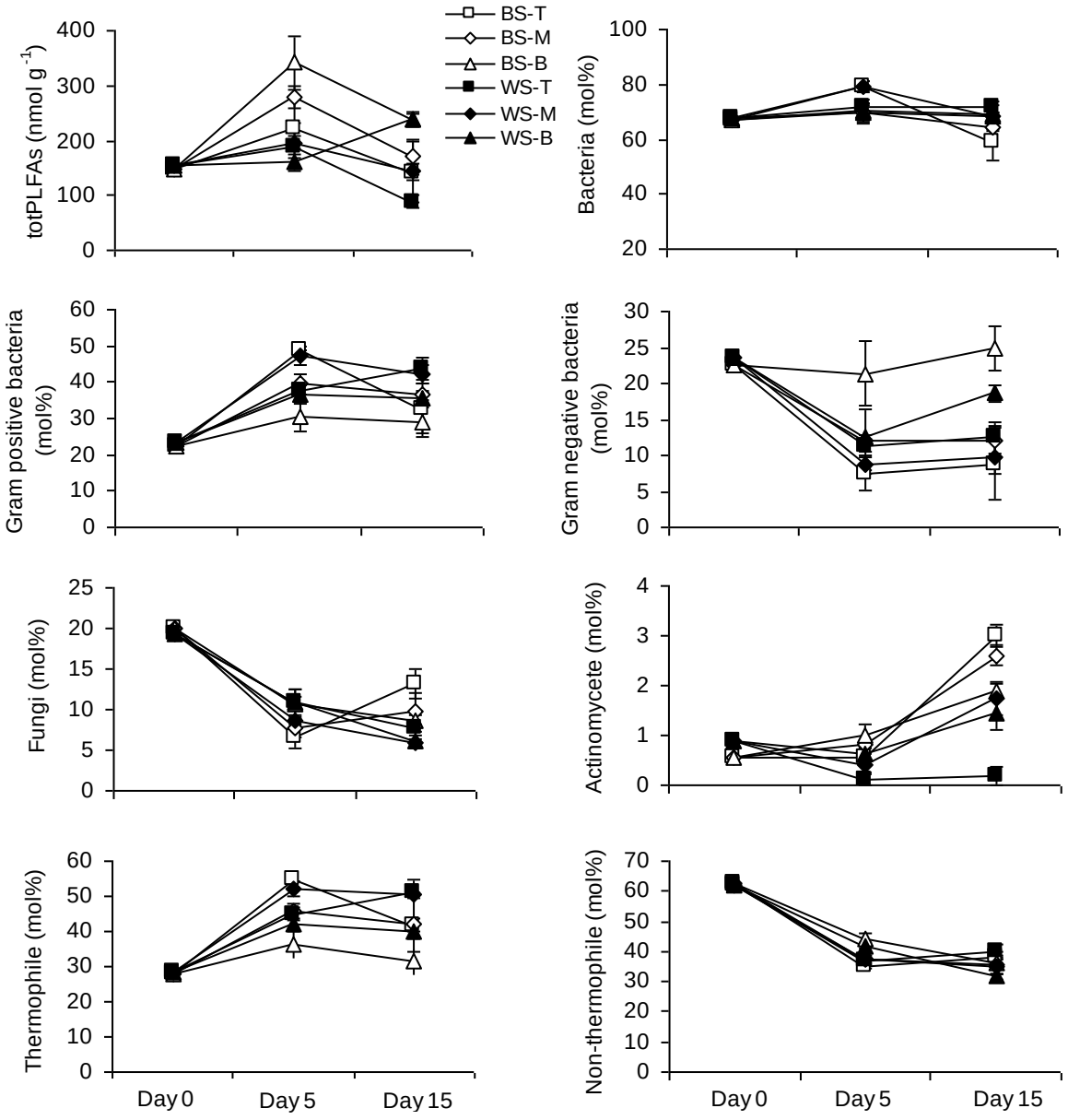

Figure 2-5 Relative proportions of microbes during composting of specified risk material in a laboratory-scale composter. Populations are defined based on phospholipid fatty acid profiles as described in the materials and methods. BS (-T, -M and –B) and WS (-T, -M, and –B) indicate barley straw or wood shavings compost, sampled at top, middle or bottom depths, respectively.

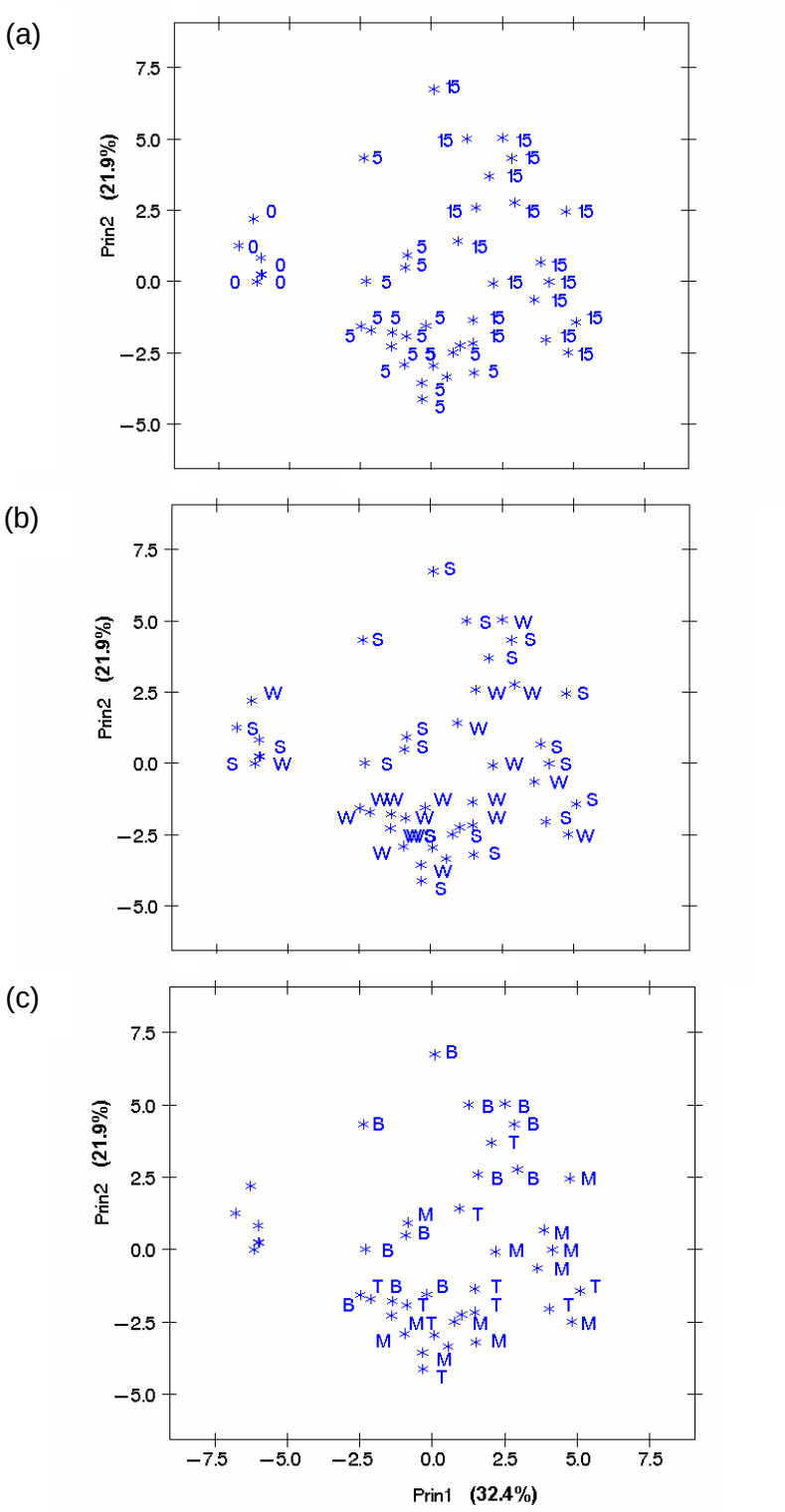

Figure 2-6 Principal component analysis of phospholipid fatty acids during composting of specified risk material in a laboratory-scale composter. (a) Score plot by sampling time (0, 5, 15: days 0, 5 and 15); (b) Score plot by compost matrix (S: straw compost; W: wood shavings compost); (c) Score plot by compost depth (T: top; M: middle; B: bottom).

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Chapter 3 Biodegradation of specified risk material and characterization of actinobacterial communities in laboratory-scale composters[†]

3.1 Introduction

As a consequence of the occurrence of bovine spongiform encephalopathy (BSE) in Canada in 2003, the Canadian Food Inspection Agency (CFIA) imposed an enhanced feed ban in July of 2007 to prevent the introduction of specified risk material (SRM) into the food chain (CFIA 2008). Specified risk material refers to specific body tissues known to be at risk of harbouring infectious prion proteins (PrP^{TSE}), including the skull, brain, trigeminal ganglia, eyes, palatine tonsils, spinal cord and the dorsal root ganglia of cattle aged 30 months or older, as well as the distal ileum of cattle of all ages (CFIA 2008). Currently, rendering followed by deposition of the resultant meat and bone meal in landfills is the main method of SRM disposal in Canada. The enhanced feed ban has prompted renderers to impose fees for SRM disposal and consequently there is a desire to develop economical on-farm disposal methods for SRM. One economically feasible option may be composting as this has been shown to reduce pathogens (Van Herk et al. 2004; Xu et al. 2009) while producing a valuable fertilizer for agriculture crops (Ros et al. 2006; Ceustermans et al. 2007).

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Effective composting of SRM requires that animal tissues are fully biodegraded and that precautions are taken to minimize the risk of pathogen transmission or the generation of odours or effluent (Berge et al. 2009; Xu et al. 2009). To inactivate human pathogens, guidelines from CCME (Canadian Council of Ministers of the Environment) and USEPA (United States Environmental Protection Agency) both suggest that all materials are exposed to temperatures of at least 55°C for at least 3 consecutive days. During the composting process, degradation of organic matter occurs more rapidly under thermophilic than under mesophilic conditions (Ekinci et al. 2004; Qdais and Hamoda 2004). Achieving elevated temperatures for a prolonged duration might be the key to establishing composting conditions that kill pathogens and biodegrade SRM (Kalbasi et al. 2005; Wilkinson 2007; Stanford et al. 2009).

Self-heating laboratory-scale composters rely solely on microbial heat production to obtain process temperatures, as well as on heat retention by external insulation (Campbell et al. 1990a). However, self-heating composters can have large heat losses resulting in a short thermophilic phase, even when well-insulated (Mason and Mike 2005). In contrast, actively-heated laboratory-scale composters sustain temperatures through active aeration with moist hot air (Smårs et al. 2001), placement in a heated water bath (Qdais and Hamoda 2004) or circulation of warm water through a jacket (Campbell et al. 1990b; Huang et al. 2000). Actively-heated laboratory-scale systems may increase the degree of SRM degradation.

The biodegradation of SRM in compost depends on the complex microbial consortia that are naturally present in this environment (Chapter 2; Berge et al. 2009). Although composting of cattle carcasses on-farm has been evaluated (Stanford et al. 2007; Xu et al. 2009), information on the microbes participating in this process is lacking. Actinobacteria are a group of Gram-positive bacteria (Class *Actinobacteria*) characterized by a high G + C content (Strom 1985). Actinobacteria play an important role in degradation of organic matter in compost, such as cellulose, hemicellulose, lignin and chitin (Lacey 1997). Some actinobacteria isolated from soil, in particular *Amycolatopsis* and *Saccharomonospora*, have exhibited activity against recalcitrant proteins, such as keratin (Al-Zarban et al. 2002; Al-Musallam et al. 2003). A novel keratinolytic actinobacteria has also previously isolated from a composting environment (Puhl et al. 2009). In addition, Hui et al. (2004) isolatedas a PrP^{TSE}-degrading enzyme from culture medium of *Streptomyces* sp. and an alkaliphilic actinobacterial isolate capable of degrading PrP^{TSE} has recently been described (Mitsuiki et al. 2010).

In the current study, two types of composters, ambient and actively heated, were constructed to assess the extent of SRM biodegradation during composting. The nature of actinobacterial communities were also examined using denaturing gradient gel electrophoresis (DGGE) due to the potential important role that these bacteria may play in the degradation of recalcitrant proteins. The overall objective was to develop a laboratory scale composting system that would be suitable for examining the biodegradation of PrP^{BSE} under level III containment conditions.

3.2 Materials and methods

3.2.1 Laboratory-scale composting apparatus design

Six laboratory-composters were used in the present experiment with three of these composters being actively heated via a circulating water jacket. Ambient composters were constructed in the same manner as described in Chapter 2 (Section 2.2.1). For actively-heated composters (Figure 3-1a), the vessels were surrounded with 15 m of polyvinyl chloride hoses (20 mm diameter; K3150; Kuriyama, Schaumburg, IL). The hoses were connected to a water circulation system (Figure 3-1b) in which heated water (60 ± 2°C) was continuously circulated from an electric water heater (JW50SDE130; GSW Water Heating, Fergus, ON) using a nonsubmersible pump (UPS15-35SFC; GRUNDFOS, Oakville, ON).

3.2.2 Composting setup and sampling procedure

Fresh feedlot beef manure (35 ± 0.1 kg) and white spruce (*Picea glauca*) wood shavings (3.5 ± 0.1 kg) were thoroughly mixed by hand to form a matrix with a moisture content of 76.0 ± 0.3%. Before filling, 20 mm layers of wood shavings were placed in the bottom of each composter. Duplicate samples of fresh manure, wood shavings and the mixed matrix were collected from each replicate composter and stored at 4°C for physical and chemical analysis. The basic properties of the compost ingredients are provided in Table 3-1. The remaining mixed matrix were freeze-dried in a freeze dryer (Series 24D × 48; Virtis, Gardiner, NY) and frozen at -40°C for later extraction of DNA for use in DGGE.

Fresh bovine brain tissue (i.e. cerebrum, cerebellum and brain stem) from mortalities under 30 months of age were obtained from a nearby slaughterhouse and used as a model for SRM. Brain tissue (100 ± 0.1 g; wet basis) was weighed and sealed in 140 × 90 mm nylon bags (25 µm pore size; Sefar BDH Inc., Chicoutimi, QC) and the bags along with 400 g of freshly mixed compost matrix were placed in larger polyester mesh bags (200 × 200 mm; 5 mm pore size). Polyester twine was attached to each bag to enable easy recovery of the material during composting.

As each compost vessel was filled, six mesh bags were placed at three depths, 0.45 m (bottom) 0.3 m (middle) and 0.15 m (top) resulting in a total of eighteen mesh bags in each composter. Mesh bags were collected after 5, 10 and 15 days of composting. A total of six mesh bags were removed at each sampling time with two bags being collected from each of the three depths per replicate composter. After collecting the mesh bags on day 15, compost materials in each composter were dumped and mixed using a shovel and returned to the composter from which they originated for a second heating cycle. As the composters were filled, mesh bags containing a mixture of fresh brain tissue (100 ± 0.1 g; wet basis) and compost were placed in the composter in the same manner as for the first composting cycle. In the second composting cycle, mesh bags were collected after 20, 25 and 30 days. After each sampling, compost samples (150-200 g) were collected from the mesh bags and stored at 4°C for further physicochemical analysis, while the remaining compost in the mesh bags was freeze-dried and frozen at -40°C for later DNA extraction.

3.2.3 Biodegradation of specified risk material and compost properties

The degradation of SRM was estimated on the basis of dry matter disappearance from the nylon bags during the composting process in the same manner as detailed in Chapter 2 (Section 2.2.3).

Compost temperatures and oxygen concentration (%) were measured at the same three depths as the mesh bags were implanted using the methods from Chapter 2 (Section 2.2.3). Fresh manure, wood shavings and the mixed matrix used for composting setup and compost samples retrieved from mesh bags were analyzed for moisture, total carbon (TC), total nitrogen (TN), pH, electrical conductivity (EC), and mineral N (NH_4^+ and $NO_2^- + NO_3^-$) as previously described in Chapter 2 (Section 2.2.3).

3.2.4 DNA extraction

Prior to DNA extraction, all freeze-dried compost samples were ground using a Ball Mill (MM200; Retsch GmbH, Haan, Germany) and pooled by replicates to decrease the variability of replicates within the given treatments (Philips et al. 2006). For the samples collected prior to composting, an equal amount of compost matrix collected in duplicate from the six composters was pooled. Duplicate samples collected after composting originated from mesh bags implanted at the middle depth with equal amounts being pooled from the three replicate composters within each treatment. Samples were thoroughly mixed prior to DNA extraction. Subsequently, DNA was extracted from 100 mg of pooled sample by QIAamp DNA stool mini kit (Qiagen, Mississauga, ON). Extracted DNA was

quantified using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA). Extracted DNA was stored at -20°C prior to further analysis.

As positive controls for PCR and DGGE analysis, *Thermobifida fusca*, *Streptomyces thermovulgaris*, *Saccharomonospora viridis*, *Actinomadura hallensis*, *Streptomyces thermophilis* and *Nocardiopsis sp.* were isolated from a cattle mortality composting system developed by Xu et al. (2009). Isolates were identified on the basis of morphological characteristics and 16S rRNA gene sequence. The DNA from control isolates was isolated using a DNeasy tissue kit (Qiagen, Mississauga, ON) following the manufacturer's protocol for Gram positive bacteria.

3.2.5 PCR-DGGE and sequencing analysis

For all extracted DNA, fragments of actinobacterial 16S rRNA gene were amplified using a nested PCR approach from Heuer et al. (1997). In the first-round PCR, the primers F243 and R1378 (Heuer et al. 1997) were used to specifically amplify actinobacterial 16S rRNA gene. The products from the first-round PCR were then used as a template for the second-round PCR with the primers F984GC (Nübel et al. 1996) and R1378 (Heuer et al. 1997) to amplify bacterial 16S rRNA gene, which obtained a suitable length fragment (~400 bp) for the analysis of DGGE. In the first-round PCR, the total reaction volume was 50 µl with each reaction containing 40 ng template DNA, 1 × HotStarTaq *Plus* DNA Master Mix (Qiagen, Mississauga, ON), 0.2 µM of each primer as well as 0.1 µg/µL bovine serum albumin (New England Biolabs, Pickering, ON). Amplification was conducted using a thermal cycler (Mastercycler ep*gradient*;

Eppendorf, Hamberg, Germany), with conditions of 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 62°C, 2 min at 72°C, and finally 10 min at 72 °C. PCR products were visualized on a 1.5% (w v^{-1}) agarose gel and purified using a QIAquick gel extraction kit (Qiagen, Mississauga, ON). The purified PCR product was used as templates in the second-round PCR, which was conducted in the same manner as described above.

Denaturing gradient gel electrophoresis using the DCode^{TM} Universal Mutation System (Bio-Rad, Hercules, CA) was performed by loading PCR products (500 ng) onto a polyacrylamide gel containing 6% (v v^{-1}) acrylamide-bisacrylamide, 0.09% tetramethylenediamine (v v^{-1}), and 0.09% ammonium persulfate (w v^{-1}). A linear gradient of denaturant from the top (30%) to the bottom (60%) of the gel was applied with the 100% denaturant containing 7 M urea and 40% (v v^{-1}) formamide. Gels were run at a constant voltage of 150 V and a temperature of 60°C in 1 × TAE buffer for 6 h. Gels were stained with SYBR Gold, 10^{-4} dilution (Invitrogen, Eugene, OR) for at least 30 min and the migration patterns were visualized using a UV transilluminator (Biospectrum 800 imaging system; UVP, LLC, Upland, CA).

All dominant DGGE bands and emergence appearance or disappearance DGGE bands were excised for sequencing and placed in 50 µl of elution TE buffer (pH=7.4) overnight. The eluted DNA fragments were re-amplified with F984 and R1378 primers as described above for the second-round PCR. The PCR products were purified with EXOSAP-IT enzyme (Affymetrix, Santa Clara, CA)

and sequenced at a commercial sequencing centre (Macrogen Inc., Rockville, MD).

For phylogenetic analyses, nucleotide sequences were aligned using Sequence Scanner Software v1.0 (Applied Biosystems 2005). The trimmed clean and high quality 16S rDNA sequences were compared with actinobacterial sequences from the GeneBank database (NCBI, http://www.ncbi.nlm.nih.gov/) using BLASTN. The phylogenetic tree was constructed by the Neighbour joining method. TREEVIEW (Page 2001) was used to generate a rooted phylogenetic tree. The reliability of the phylogenetic estimates was evaluated with the DNADIST, NEIGHBOR, SEQBOOT and CONSENSE programs in the PHYLIP package (Felsenstein 2005) based on 1000 replications. *Rubrobacter radiotolerans* (GenBank accession no. U65647) used as an outgroup from the NCBI database was included. The nucleotide sequences reported in this study were deposited in NCBI nucleotide sequence databases with the accession numbers HQ911331 to HQ911358.

3.2.6 Statistical analysis

Statistical analysis of the DGGE bands was conducted with BioNumerics software (Applied Maths Inc. 2007) according to the provider's instructions. Calculation of the pair-wise similarities of relative abundance-based densitometric profile was performed using Pearson's correlation coefficients. Cluster analysis based on this similarity matrix was done by the unweighted pair group method with arithmetic average (UPGMA) to form a complete linkage dendrogram.

For each time point, duplicate data from two mesh bags collected at each depth per composter were averaged as the mean of each replicate within treatment before statistical analysis. Physicochemical parameters of the compost as well as SRM degradation were analyzed using the Mixed procedure of SAS (SAS Institute 2001) with time and compost depth treated as repeated measures in the model. Differences between ambient and actively heated compost were reported at the < 0.05 probability level. The comparisons between two composting cycles for SRM degradation at the middle depth and compost temperatures at three depths were respectively analysed using the Mixed procedure of SAS with time as the only repeated measure in the model.

3.3 Results

3.3.1 Temperature and oxygen profiles

In the first composting cycle, temperature peaked at 54°C on day 3 in ambient compost and at 56°C on day 2 in actively-heated compost (Figure 3-2a). Subsequently, temperatures steadily declined, but remained above 50°C for 2 days in ambient compost and for 7 days in actively-heated compost. Temperatures in actively-heated compost remained higher ($P < 0.05$) than that in ambient compost for 7 days (days 0, 1, 6, 7, 8, 9, and 15) at the top depth, for 2 days (days 1 and 7) at the middle depth, and for 6 days (days 0, 1, 5, 6, 7, and 8) at the bottom depth.

Upon mixing, temperatures rapidly increased in actively-heated compost at day 15, but continued to decline in ambient compost before it once again increased on day 16 (Figure 3-2a). During the second cycle, peak temperatures occurred on day 19 (47°C) in ambient compost and on day 17 (52°C) in actively-

heated compost. A greater divergence in temperatures was observed between the two types of composters. Temperatures did not exceed 50°C in ambient compost, but did remain above this level for 4 days in actively-heated compost. In contrast to the first composting cycle, temperatures in the second cycle were lower ($P < 0.05$) for 7 and 4 days of the 15 days at all three depths in ambient and actively-heated compost, respectively.

Oxygen concentrations were 17% in ambient compost and 12% in actively-heated compost at day 0 (Figure 3-2b). In the early composting period, oxygen concentration in ambient compost declined to 2% after 1 day, but had returned to 19% by day 3. A decline in oxygen concentration was not observed for actively-heated compost as it steadily increased to 19% by day 3. Oxygen concentration remained at this level in both ambient and actively-heated compost for the remainder of composting period.

3.3.2 Changes of physicochemical properties

Moisture contents remained relatively constant during the initial composting cycle, but declined in both compost types during the second composting cycle, with the decline being more rapid in actively-heated compost (Figure 3-3). By day 30, moisture contents in actively-heated compost ranged from 17.6% to 31.6% at all compost depths and were lower ($P < 0.05$) at all depths than those in ambient compost. Levels of TC gradually declined ($P < 0.05$) over the entire composting period and did not differ between ambient or actively-heated compost. Similarly, composting method did not influence TN content which gradually increased over

the composting period. Compost C/N ratio declined from an initial ratio of around 22 to a range between 15 and 18 upon completion of the experiment.

Initial compost pH was 8.2, a level that increased over the first 5 days of composting and thereafter declined over the remainder of composting period in both compost types. Compost EC increased over the initial composting cycle, but declined in ambient compost and increased in actively-heated compost after mixing in the second cycle. By day 30, actively-heated compost exhibited a higher ($P < 0.05$) EC than ambient compost.

The concentrations of NH_4^+-N in the lower and upper depths peaked at days 5 and 10, respectively, and thereafter declined until day 30. During the decline, ambient compost had a higher NH_4^+-N ($P < 0.05$) than actively-heated compost, but no differences in NH_4^+-N were observed between two compost types at the end of composting. Low levels of (NO_2^- + NO_3^-)-N (< 3 mg kg^{-1}) were observed over the majority of the composting period with a greater ($P < 0.05$) value in ambient than in actively-heated compost at the final day of composting.

3.3.3 Biodegradation of specified risk material

In the first composting cycle, the loss of SRM dry matter depended on three factors (i.e. the exposure time, depth of placement, and ambient or active heating), as a three way interaction ($P < 0.05$) among these factors occurred (Table 3-2). The extent of SRM degradation was almost completed after 10 days at three depths in both composters with only about 50% of SRM dry matter remaining in the implanted bags. The exception was at bottom depth in actively-heated compost, with this level of SRM being degraded after 5 days (Figure 3-4a).

Although the overall degradation of SRM did not differ between ambient and actively- heated compost, there was a greater ($P < 0.05$) dry matter loss in ambient than in actively-heated compost at top depth on day 5.

In the second composting cycle, 46.6% and 41.1% of SRM were degraded in ambient and actively-heated compost but still did not differ as a result of an additional 15 days of composting (Figure 3-4b). The extent of SRM degradation also did not statistically differ between the first and second composting cycle.

3.3.4 PCR-DGGE and sequencing analysis

Preliminary experiments using three replicate PCR products from the compost samples showed the consistent band patterns of DGGE profile among the replicate samples (Figures 3-5 and 3-6). Thus, it demonstrated the low variability of the PCR amplification and the good repeatability of DGGE results.

Considerably different actinobacterial DNA banding profiles were produced as a result of DGGE depending on sampling time and if the compost was actively heated or not (Figure 3-7a). In general, the average number of DGGE bands tended to increase from 9 at day 0 to 19 at day 15 during the first composting cycle and decrease from 23 at day 20 to 13 at day 30 during the second composting cycle. The UPGMA analysis revealed that the DGGE profiles clustered on the basis of time of sampling in first composting cycle (Figure 3-7b) and on if they originated from ambient or actively-heated compost during the second cycle.

In general, the average numbers of bands observed were 14 in ambient compost and 18 in actively-heated compost in the first cycle. However, the band

number became more numerous in ambient compost in the second cycle with 19 and 16 in ambient and actively-heated compost, respectively. In the second cycle, the pooled samples from ambient and actively-heated compost clustered separately at each sampling day. A divergence was especially obvious in actively-heated compost samples from day 30, which exhibited only a 65% similarity with other samples collected in the second composting cycle (Figure 3-7b).

Sequencing all of the dominant bands generated the Neighbor joining tree in Figure 3-8. Sequences from bands 1, 2, 3, and 4 at day 0 were closely affiliated to *Corynebacterium*. Similarly, sequence from bands 5, 6, and 7 in ambient compost and from band 8 in actively-heated compost at day 5 formed a robust clade with *Corynebacterium*. On day 5, a faint band (band 9) derived from actively-heated compost at day 5 was distantly associated with *Promicromonospora sp*. Subsequently, a pronounced band (band 10), closely associated with band 9, appeared in ambient compost on day 10. At day 15, sequencing bands formed a non-robust clade with *Pseudonocardia* (band 15) and a robust clad with *Thermobifida* (band 16). After mixing the compost, band sequences (bands 17, 18, and 19), derived from day 20 in both compost types, were associated with *Corynebacterium*, whereas sequences associated with *Mycobacterium* (bands 21, 24, 27, and 28) and *Nocardia* (bands 20 and 23) were found at day 25 and with *Saccharomonospora* (bands 22 and 25) and *Streptomyces* (band 26) on day 30.

3.4 Discussion

3.4.1 Temperature and oxygen profiles

Production of heat in the compost is associated with the microbial degradation of organic matter. The temperature of compost is a reflection of the balance between heat-production and heat-dissipation, a factor that is influenced by the nature of the composting matrix, aeration and the insulative value of the compost container (Krogmann and Körner 2000; Berge et al. 2009). In this study, ambient compost experienced two days of temperature above 50°C in the primary composting cycle. However, use of a simple water jacket prolonged the period of thermophilic composting for 5 of 15 days in the primary cycle, with temperature above 50°C occurring 2 days earlier and continuing 3 days longer than in the non-heated system. This suggests that the high temperature (60°C) inside the water jacket decreased heat loss reducing the rate of temperature decline after peak values were obtained. Additionally, the system likely radiated heat to the composters, accelerating the rise in temperature during the early stages of composting. Before mixing, the average temperature in actively-heated compost were 3.8°C, 2.4°C and 4.3°C higher than ambient compost at the top, middle and bottom depths, respectively.

Mixing of the compost promoted a renewed heating cycle with both types of composters developing a lower temperature profile, but a pattern that was similar to that observed the first composting cycle. Periodic turning of compost is used in windrows and in-vessel systems, as it breaks up aggregates, redistributes moisture and exposes substrate surfaces, promoting microbial activity and prolonging the period of thermophilic biodegradation during composting (Manios et al. 2006; Stanford et al. 2009). Ambient compost exhibited a typical decline in compost

oxygen concentration at the start of composting, indicating that oxygen was being consumed by the microbes via aerobic respiration. A similar phenomenon has been observed in a previous study using a same composting system (Chapter 2; Figure 2-3b). However, active-heating resulted in oxygen concentrations similar to atmospheric concentrations, a result that has been confirmed by others using actively heated composter (Hogan et al. 1989). Active heating may have initially enhanced passive airflow in the composter, improving aeration and oxygen diffusion during the early stages of composting. After five days of composting oxygen levels remained above 19% indicating that oxygen availability did not limit the composting process.

3.4.2 Changes of physicochemical properties

Initial moisture levels of the compost (70% wet basis) were within a range considered optimal for effective composting (Rynk 1992). Active-heating resulted in a greater loss of moisture than ambient compost, a response that was accelerated after the compost was mixed after the first cycle. Upon completion of the experiment, moisture content was reduced to approximate 30%, a level that is not optimal for organic matter degradation (Ahn et al. 2008). The average TC loss (2.4%) in the first composting cycle was slightly higher than the 1.3% loss observed in a previous trial using the same laboratory-scale composters (Chapter 2; Figure 2-4). This slight difference likely arises from differences in the composition of the feedlot manure between the two studies. The increase in TN content observed in this study supports the hypothesis put forward by Xu et al.

(2007) that the degradation of SRM releases a large amount of free nitrogen into compost that surround the nylon bags.

Compost EC is generally measured as an indicator of the sum of soluble ions in compost extract (Inbar et al. 1993). An increase of compost EC in my results was similar to Larney and Olson (2006), a result that has been attributed to the loss of organic matter and a resultant increase in the relative concentration of soluble salts in the remaining substrate. The lower water content in actively-heated compost, likely concentrated the soluble salts in the compost resulting in a higher EC at day 30 as compared to ambient compost.

Patterns in compost pH were consistent with the changes in NH_4^+-N concentration during composting. Compost pH increased at day 5 likely due to NH_4^+-N arising from the deamination of amino acids released from the hydrolysis of protein in SRM. The decline in pH in the second composting period likely reflects the volatilization of NH_3 (McCrory and Hobbs 2002). Loss of NH_3 appeared to be accelerated in actively-heated compost and in some instances development of conditions conducive for nitrification have accounted for N losses during the latter stages of composting (Hao et al. 2001).

3.4.3 Biodegradation of specified risk material

Close to 50% of SRM dry matter disappeared within 15 days of composting, similar to that observed in Chapter 2. Schwarzlose et al. (2008) reported that insulation of composters improved heat retention and accelerated the biodegradation of poultry carcasses during a 30 day composting period. In this study, actively-heated compost prolonged thermophilic composting (50-60°C),

but only for a relatively short period of 5 days. This extent of heat transfer may not have been sufficient to significantly increase the activity of thermophilic microbes involved in the degradation of SRM at all depths. Degradation of SRM was accelerated at the bottom depth in actively-heated compost, but tended to be decreased at the top depth in the first 5 days. This suggests that active-heating may have accelerated the development of mesophilic microbial communities leading to increased degradation of SRM, but that a longer thermophilic phase actually reduced SRM decomposition. Beck-Friis et al. (2001) observed a similar phenomenon where extension of the thermophilc composting period using an external heat source inhibited the degradation of organic matter in a 200 L actively-aerated composter.

After mixing, addition of fresh SRM revealed that extent of degradation of SRM was similar to that achieved during the first composting cycle. Similarly, the decline in TC was off a comparable magnitude. Optimum moisture level is crucial for biodegradation of animal carcasses during composting (Ahn et al. 2008). The lower moisture content of actively-heated compost during the second composting cycle may have numerically reduced the decomposition of SRM as compared to ambient compost during this period. Turning of compost has been shown to enhance the breakdown of bones from cattle carcasses during windrow composting (Stanford et al. 2009). Therefore, mixing of compost in order to generate multiple heating cycles is likely to further increase the extent of SRM degradation, providing moisture levels remain optimal for composting.

3.4.5 PCR-DGGE and sequencing analysis

Denaturing gradient gel electrophoresis can provide detailed insight into temporal and environmentally-mediated changes in bacterial community structure (Boon et al. 2002; Siddique et al. 2005). The analysis of UPGMA on DGGE band patterns revealed a temporal increase in the diversity of actinobacteria as the composting process proceeded in the first composting cycle. As temperature increases during composting, actinobacteria play an active role in the degradation of organic matter after bacteria and fungi have consumed easily degradable substrates available at initiation of composting (Ryckeboer et al. 2003). A similar result was also confirmed in Chapter 2 in which phospholipids fatty acid profiling was used to assess the development of actinobacteria populations. However, a temporal decrease in the level of actinobacterial diversity was recorded in the second composting cycle. This may be because of the more recalcitrant nutrient components left in the compost at this stage, requiring the development of other bacterial populations that were more specific to the degradation of these substrates. Steger et al. (2007a) found that temperature was an important factor in the development of actinobacteria populations. In the second composting cycle, DGGE band patterns in ambient and actively-heated compost clustered separately at each time point, a result that may be attributed to the greater temperature differences associated with actively-heated compost. Actinobacteria develop poorly in compost that is either too wet or too dry (Ryckeboer et al. 2003). In the second composting cycle, moisture content of the actively-heated compost declined to less than 35% at all depths, a level that may have altered both the diversity and activity of actinobacteria at days 25 and 30.

In this study, the sequences of the DGGE bands were closely related to *Corynebacterium,* both at the initiation of composting as well as during the early thermophilic stage of composting. Steger et al. (2007b) observed that *Corynebacterium* was largely eliminated when thermophilic temperatures were achieved during field-scale composting of organic household waste. The relatively short duration of thermophilic composting in the laboratory-scale composters may not have been sufficient to completely suppress *Corynebacterium* during composting. The sequences of thermotolerant species, like *Promicromonospora* sp., *Pseudonocardia* sp., and *Thermobifida* sp. were largely obtained on days 10 and 15 in the first composting cycle. However, one band (band 9) associated with *Promicromonospora* sp. was only found at day 5 in actively-heated compost, a result that may have reflected the rapid increase in temperature that occurred in this system. Species of *Corynebacterium* were detected at day 20, suggesting that mixing created conditions that were conducive for this genus. Mesophilic *Mycobacterium* and moderately thermophilic *Saccharomonospora*, *Nocardia* and *Streptomyces* were founded on days 25 and 30 in the second composting cycle. Vasileva-Tonkova et al. (2009) reported thermophilic actinobacteria possessed highly active proteases that were capable of rapid hydrolysis of recalcitrant proteins such as the keratin contained in feathers. In the present study, although predominant bands related to thermophilic actinobacteria appeared earlier in actively-heated compost, this did not result in an increase in the extent of degradation of SRM.

3.5 Conclusions

It can be concluded that active heating of laboratory-scale composters constructed in this study did not substantially increase the degradation of SRM. However, mixing of compost can be used as a means of increasing the duration of thermophilic composting and multiple composting cycles may increase the extent of SRM degradation. Temporal changes of actinobacterial communities present in the compost were revealed by PCR-DGGE analysis. Actively-heated composters largely influenced the development of actinobacterial communities in the second composting cycle. Although actinobacteria characterized in this study were not directly shown to be involved in the degradation of SRM, some of actinobacteria identified were closely related to genera that may possess this capacity. The characterization of bacterial communities involved in SRM degradation is currently being conducted. The present laboratory-scale composting system is being employed under level III containment conditions to explore the possible role of actinobacteria in the degradation prions including the biodegradation of PrP^{BSE}.

Table 3-1 Characteristics of initial materials used for composting

Materials	Water content (%)	Total carbon (%)	Total nitrogen (%)	C/N ratio	pH	EC ($ds\ m^{-1}$)	NH_4-N ($mg\ kg^{-1}$)	$NO_2 + NO_3$-N ($mg\ kg^{-1}$)
Beef manure	76.0	35.3	2.3	15	8.2	6.1	1036	1
Wood shavings	7.1	51.3	0.1	666	4.3	0.1	1	1
SRM[a]	81.0	56.7	7.4	8	NM	NM	NM	NM

All values are expressed on a dry weight basis except water content, which is on a wet weight basis

[a] Values are cited from Chapter 2 (Table 2-1). NM, not measured

Table 3-2 ANOVA (analysis of variance) table for effects of compost type, compost depth and compost time on the biodegradation of specified risk material

Effects	Numerator degree of freedom	Denominator degree of freedom	*F* value	*P* value[a]
Compost type	1	5.77	6.07	0.0505
Compost depth	2	5.34	1.82	0.2498
Compost time	2	10.5	67.88	< 0.0001
Compost type*depth	2	5.34	1.97	0.2286
Compost type*time	2	10.5	3.54	0.0669
Compost depth*time	4	9.41	3.83	0.0416
Compost type*depth*time	4	9.41	4.34	0.0295

[a]Significant at $P < 0.05$

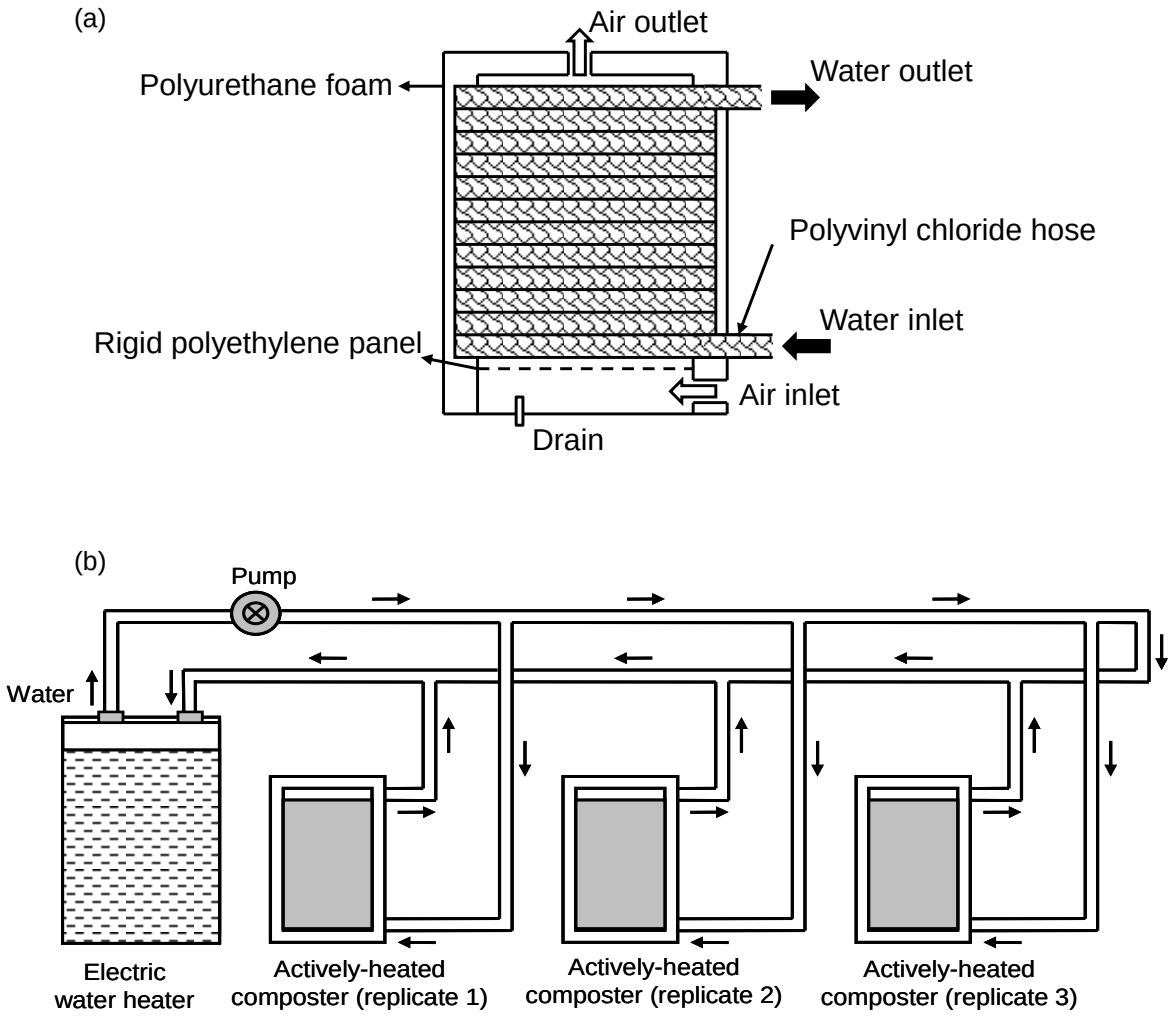

Figure 3-1 (a) Schematics of a 110-L actively-heated laboratory-scale composter and (b) the water circulation system.

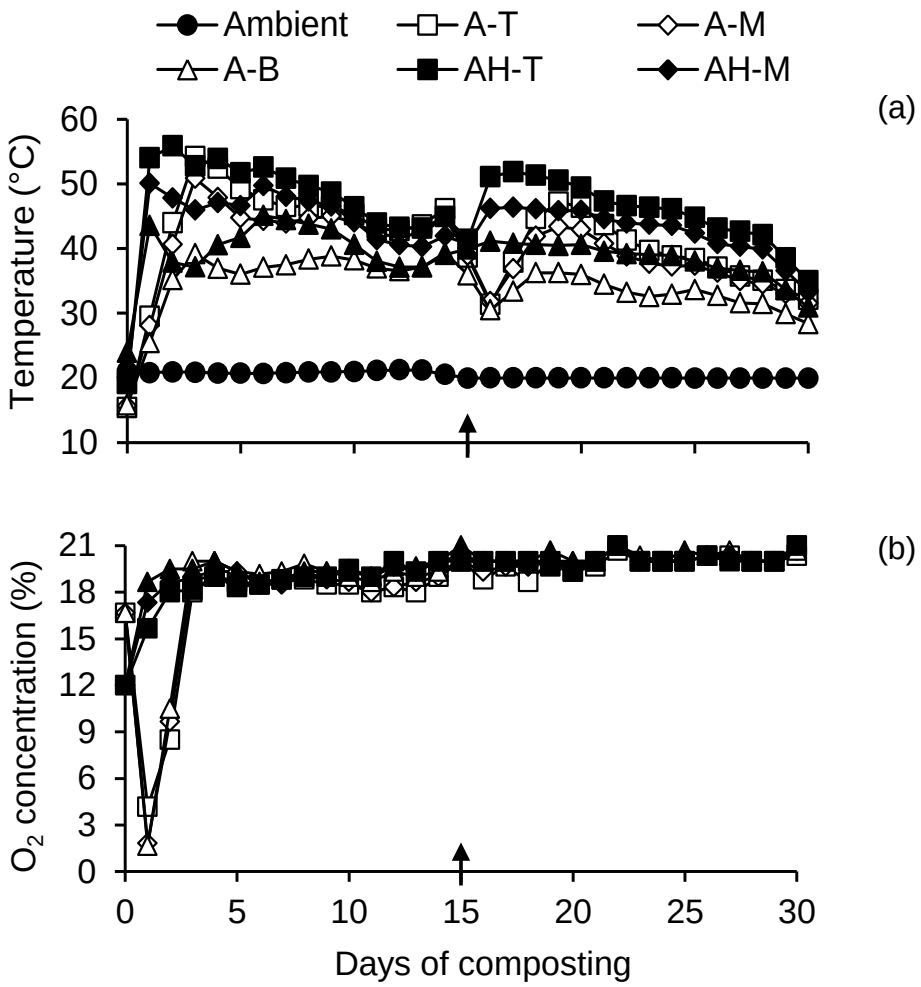

Figure 3-2 (a) Temperature and (b) O_2 concentration during composting of specified risk material in ambient (A) and actively-heated (AH) laboratory-scale composters. T = top depth; M = middle depth; B = bottom depth. Arrows indicate the date compost was mixed.

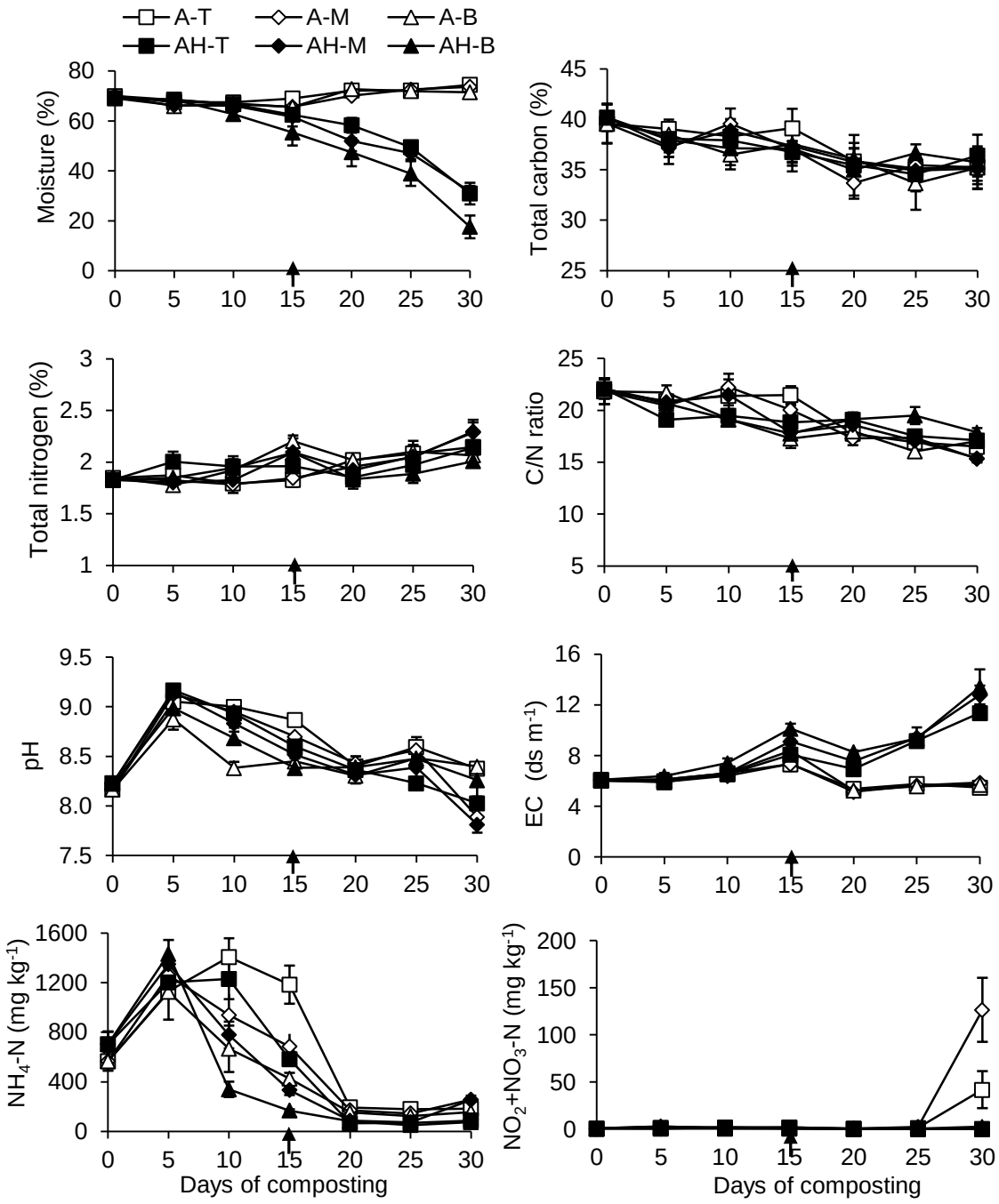

Figure 3-3 Physicochemical changes during composting of specified risk material in ambient (A) and actively-heated (AH) laboratory-scale composters. T = top depth; M = middle depth; B = bottom depth. Arrows indicate the date compost was mixed.

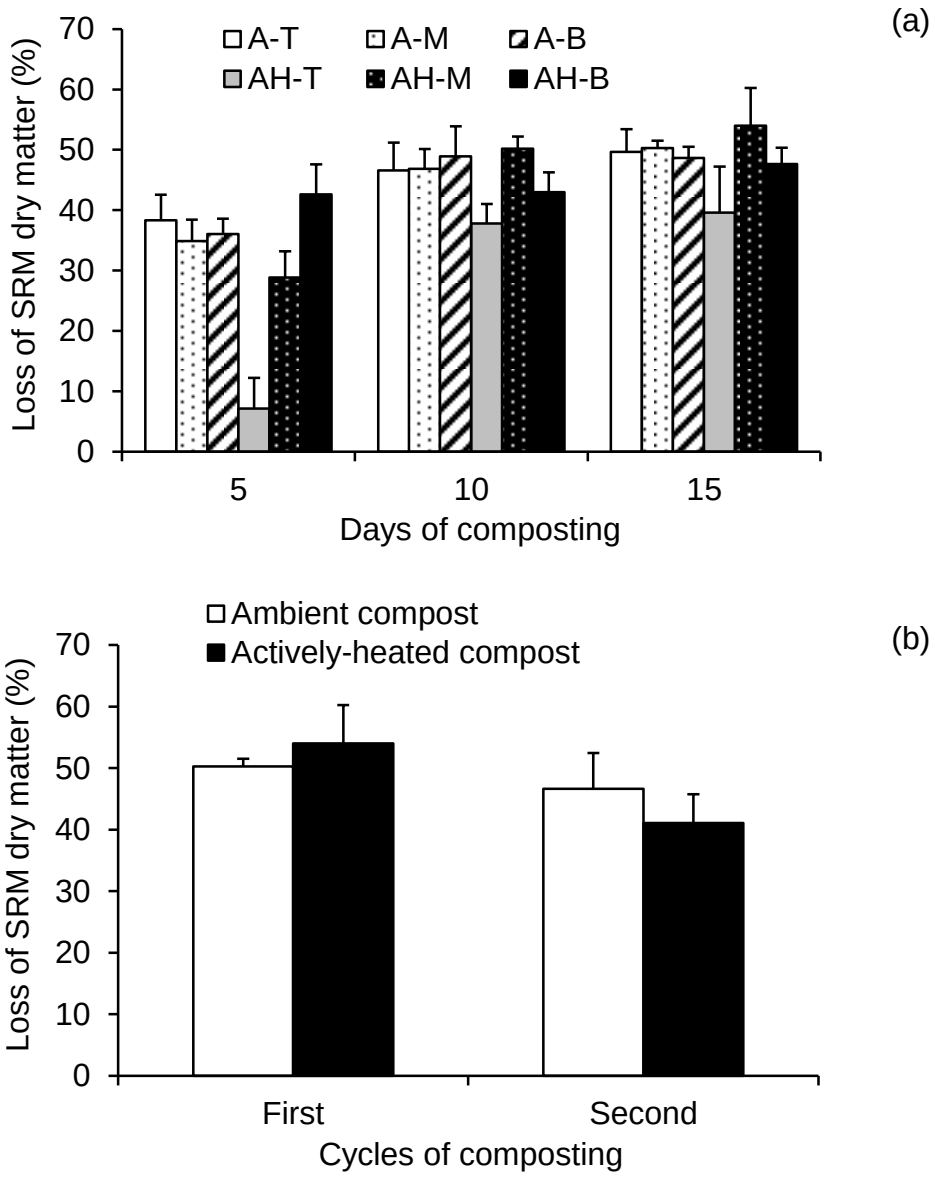

Figure 3-4 (a) Degradation of specified risk material (% dry matter loss) during the first composting cycle in laboratory-scale composters. A-T=top depth of ambient compost; A-M=middle depth of ambient compost; A-B=bottom depth of ambient compost; AH-T=top depth of actively-heated compost; AH-M=middle depth of actively-heated compost; AH-B=bottom depth of actively-heated compost. (b) Degradation of specified risk material (% dry matter loss) in the first and second composting cycles, respectively, at the middle depth in laboratory-scale composters (fresh specified risk material was added to the composters after mixing on day 15).

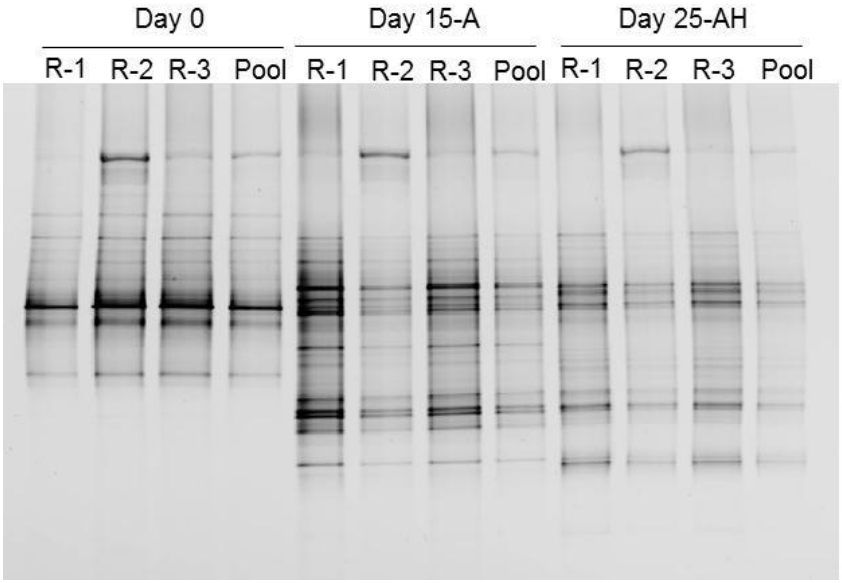

Figure 3-5 Denaturing gradient gel electrophoresis separation of 16S rRNA gene fragments after three replicate PCR amplifications on the same DNA template from different compost types and time using actinobacteria-specific primers F243-R1378 and F984GC-R1378. A=ambient compost; AH=actively-heated compost; R1-R3: three replicate PCR products; Pool: pooled PCR products from R1-R3.

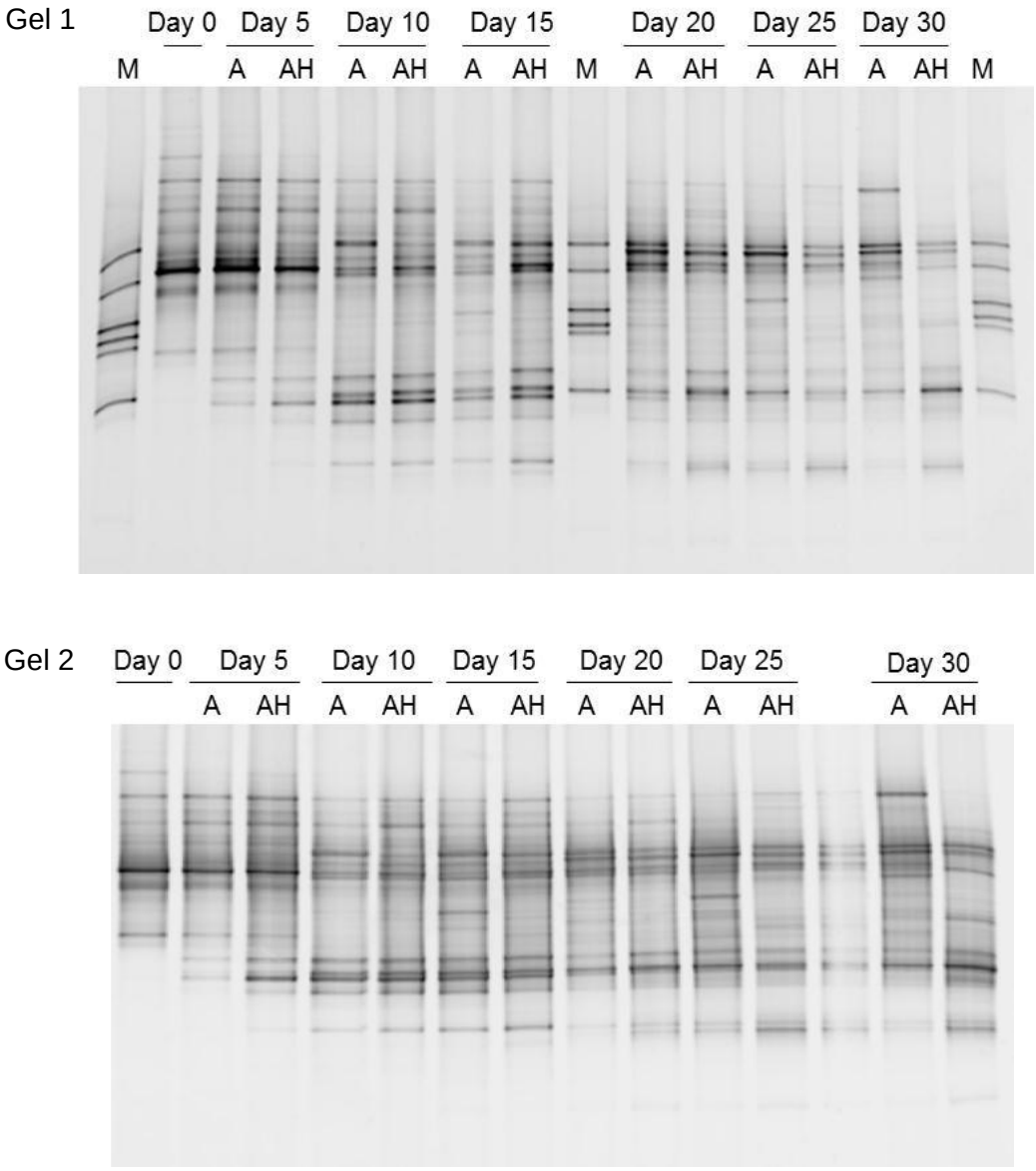

Figure 3-6 Analyses of denaturing gradient gel electrophoresis on the same PCR products amplified by actinobacteria-specific primers F243-R1378 and F984GC-R1378 from different compost types and time using duplicate gels (gels 1 and 2). A=ambient compost; AH=actively-heated compost; M=marker.

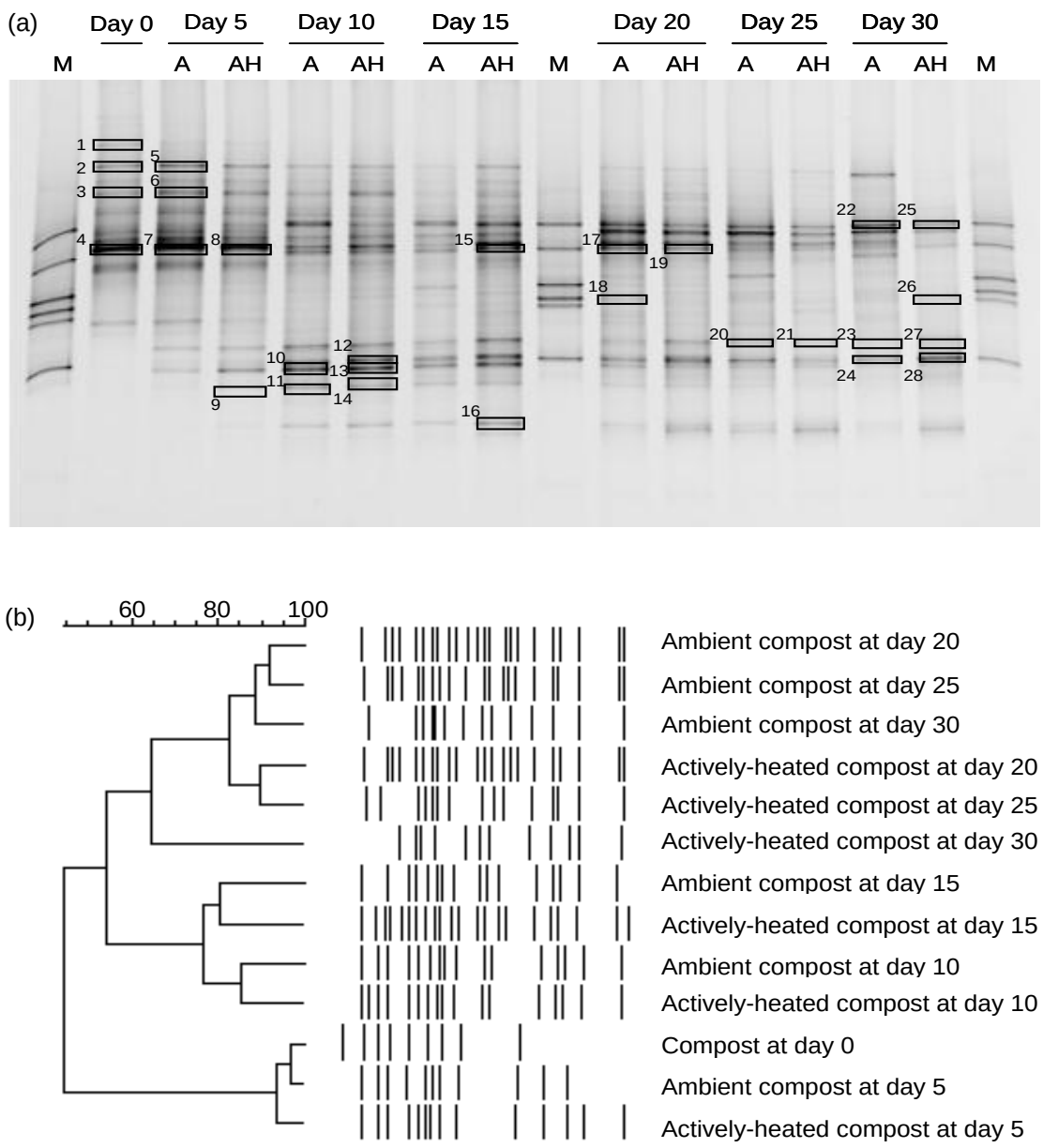

Figure 3-7 (a) Denaturing gradient gel electrophoresis separation of 16S rRNA gene fragments after PCR with actinobacteria-specific primers F243-R1378 and F984GC-R1378 from different types of composter and composting time and (b) corresponding dendrogram using Pearson's correlation index and unweighted pair group method with arithmetic average. Marked bands were excised and sequenced with sequence results being listed in Figure 3-8. A=ambient compost; AH=actively-heated compost; M=marker. Sequences of bands in the marker from top to the bottom of the gel were affiliated with the following actinobacteria species: *Saccharomonospora viridis, Actinomadura hallensis, Streptomyces thermophilus, Streptomyces thermovulgaris, Nocardiopsis* sp., and *Thermobifida fusca*.

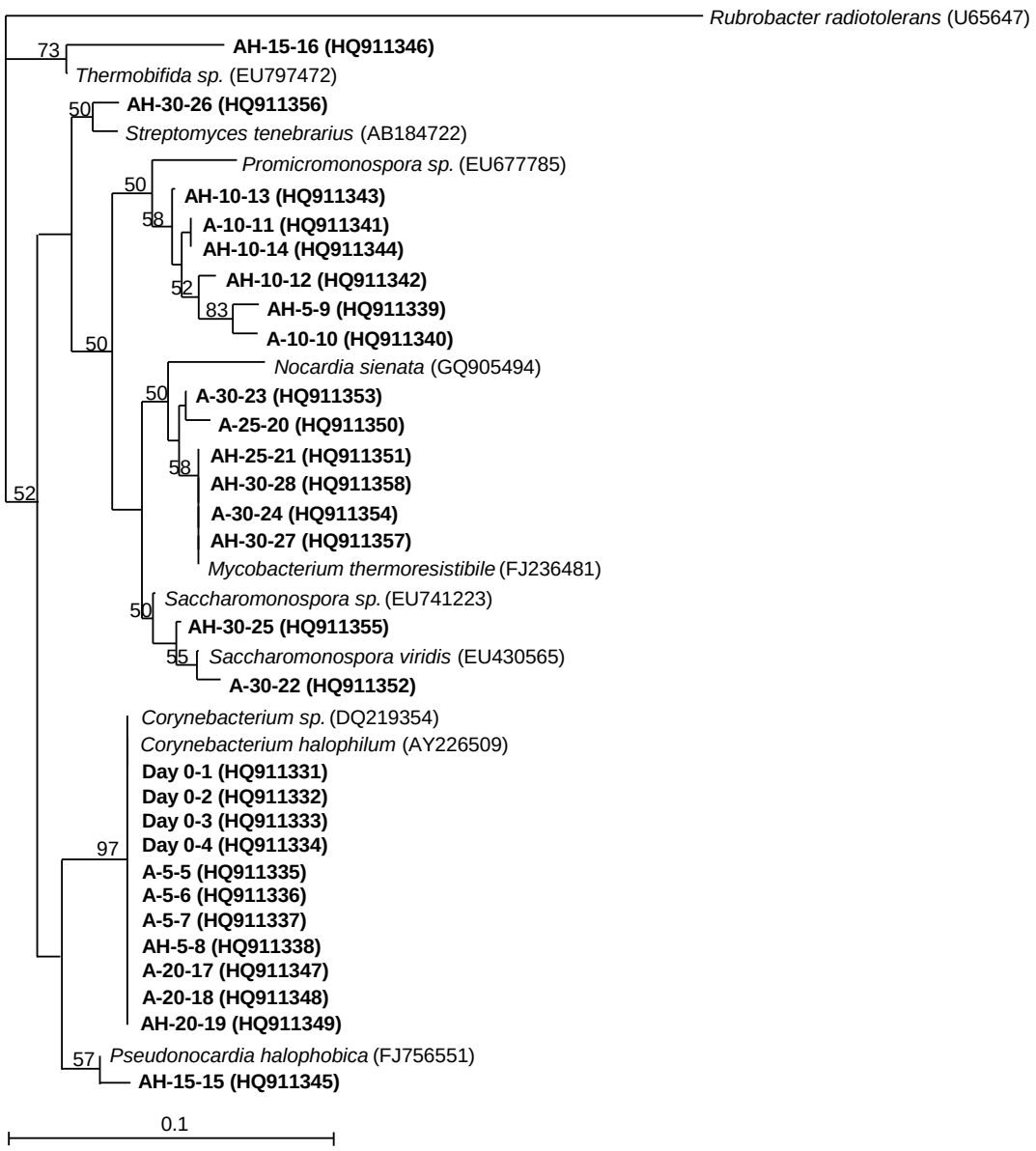

Figure 3-8 A Neighbour joining tree of phylogenetic relationships between DNA sequences obtained (see Figure 3-7a) during composting of specified risk material and species within the class *Actinobacteria* (accession numbers of the sequences retrieved from the database are enclosed in parentheses). The scale bar indicates 10% nucleotide substitutions and bootstrap values at 50-100% are displayed at the nodes. The sequences obtained in this study are bold-typed and names indicated Composter Type (A=ambient compost; AH=actively-heated compost)-Sampling Day (Days 0, 5, 10, 15, 20, 25 and 30)-Band Number (1-30). *Rubrobacter radiotolerans* served as an outgroup.

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Chapter 4 Biodegradation of specified risk material and fate of scrapie prions in compost[‡]

4.1 Introduction

Transmissible spongiform encephalopathies (TSE) are fatal neurodegenerative diseases including scrapie in sheep and goats, chronic wasting disease (CWD) in deer and elk, bovine spongiform encephalopathy (BSE) in cattle, and Creutzfeldt-Jackob disease (CJD) in humans. The diseases of TSE are a result of sequential conformational changes of normal prion protein (PrP^{C}) into misfolded and infectious prion protein (PrP^{TSE}) (Prusiner 1998).

As a consequence of the occurrence of BSE in Canada in 2003, the Canadian Food Inspection Agency (CFIA) introduced regulations to prevent specified risk material (SRM) from entering the food chain. The term "SRM" refers to body tissues with an increased likelihood to accumulate BSE prions (PrP^{BSE}), including the skull, brain, trigeminal ganglia, eyes, palatine tonsils, spinal cord and the dorsal root ganglia of cattle aged 30 months or older, as well as the distal ileum of cattle of all ages (CFIA 2010).

A variety of approaches are being used for the disposal of SRM from abattoirs and on-farm cattle mortalities. Currently, the majority of SRM are rendered, dehydrated and disposed of in landfills, a practice that is uneconomical and environmentally questionable (Ayalon et al. 2001). Due to its conformation,

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PrP^{TSE} exhibits resistance to inactivation by many methods, including chemical agents, radiation and heat that are commonly used to kill microorganisms or denature proteins (Taylor 2000). The practices currently recommended by CFIA (2009) for PrP^{TSE} inactivation in Canada include two stages of incineration at 850°C and 1000°C for approximately 16 h, 1-h disinfection with 2 N sodium hydroxide, autoclaving in saturated steam at 134°C for a period of 60 minutes, or alkaline hydrylysis at 150°C under 5 atmospheric pressure for 3-6 hours. These methods, however, are economically impractical to ensure the inactivation of PrP^{TSE} while disposing of the large volumes of SRM arising from meat processing plants or on farm mortalities.

Composting has long been considered to be an environmentally acceptable method for treating industrial and agriculture wastes (Rynk 1992). Thermophilic temperatures (>55°C) during composting accelerate the biodegradation of organic matter and create an environment that kills most microbial pathogens (Van Herk et al. 2004; Ceustermans et al. 2007). Composting of cattle carcasses under proper management has been shown to be an attractive option for the disposal of livestock carcasses and is a well-established technology (Stanford et al. 2007; Xu et al. 2009). Recent evidence has indicated that some bacterial proteinases exhibit the ability to degrade PrP^{TSE} (Langeveld et al. 2003; Hui et al. 2004; McLeod et al. 2004). The bacterial species capable of this activity have been shown to be associated with compost (Ryckeboer et al. 2003). Moreover, Puhl et al. (2009) have previously isolated a novel keratinolytic actinobacteria involved in the degradation of hoof keratin in compost.The microbial consortia in compost could

carry out the biodegradation of recalcitrant proteins such as keratin or possibly PrP^{TSE}, owing to the wide range of proteolytic enzymes produced by these complex microbial communities.

Poultry feathers are a nitrogen rich (15%) waste predominantly composed of β-keratin protein (90% dry matter) (Tiquia et al. 2005), which has some structural properties that are similar to PrP^{TSE} (Tsuboi et al. 1991; Suzuki et al. 2006). Keratinases that have the capacity to degrade feathers may also exhibit activity against PrP^{TSE} (Suzuki et al. 2006). Recent studies not only indicated that enrichment of a composting matrix with feathers produced effective non-specific proteolytic activity early in the composting process, but also promoted the growth of specialized keratinolytic fungi that degraded keratin in feathers during the latter stages of composting (Korniłłowicz-Kowalska and Bohacz 2010; Bohacz and Korniłłowicz-Kowalska 2009). Therefore, inclusion of feathers in the compost matrix may also promote the degradation of SRM or possibly PrP^{TSE} in compost. The objectives of the current study were to utilize laboratory-scale composters to investigate the degradation of SRM and the fate of scrapie prions (PrP^{Sc}) in compost with and without feathers.

4.2 Materials and methods

4.2.1 Tissue source and preparation

Fresh bovine brain tissue from mortalities under 30 months of age was obtained from a nearby slaughterhouse and used as SRM. Brain tissues from sheep confirmed to be negative or positive for scrapie using immunochemical and histopathological techniques were obtained from the Animal Diseases Research

Institute at the Canadian Food Inspection Agency in Ottawa, ON. Sheep brain tissues were homogenized (1 g + 9 ml) using the MediFASTH homogenizer (Consul AR, Villeneuve, Switzerland) in phosphate buffered saline to yield a 10% brain homogenate (BH).

4.2.2 Compost experiment design

Bovine SRM and scrapie BH were composted in two matrices. Control compost contained solely cattle manure and wood shavings. Feather compost contained cattle manure, wood shavings, and chicken feathers (*Gallus gallus*) obtained from a local poultry abattoir. Composting of SRM was conducted under non-containment conditions at the Agriculture and Agri-Food Canada Research Centre in Lethbridge, Alberta. Composting of PrP^{Sc} was conducted under level 3 containment conditions at the Canadian Food Inspection Agency in Lethbridge, Alberta. Both experiments were conducted simultaneously, using the same initial compost matrix and procedures. Six composters were used to compost SRM with triplicate composters per treatment. Four composters were used for composting of PrP^{Sc} with duplicate per treatment, due to the space restrictions in the level 3 containment laboratory. Laboratory-scale composters were used as detailed in Chapter 2 (Section 2.2.1).

4.2.3 Composting setup

The compost matrix was prepared by mixing the initial matrix in a mortar mixer (12S, Crown construction equipment, Winnipeg, Canada) at the feedlot at the Agriculture and Agri-Food Canada Research Centre in Lethbridge. For control

compost, a mixture of 35 ± 0.1 kg fresh cattle manure (74.8% moisture) and 3.5 ± 0.1 kg white spruce (*Picea glauca*) wood shavings was placed in each composter. For feather enriched compost, chicken feathers (5% wt wt^{-1}; dry basis) were substituted for a portion of the manure, with the final composition consisting of 34.2 ± 0.1 kg cattle manure, 3.5 ± 0.1 kg wood shavings and 0.8 ± 0.1 kg of feathers. The compost matrix was identical for both SRM and PrP^{Sc} experiments. Triplicate samples of fresh manure, wood shavings, chicken feathers and mixed matrix were collected before composting. All the samples were stored at 4°C for subsequent physicochemical analysis.

4.2.4 Sampling procedures

For composting of SRM, bovine brain tissue (50 ± 0.1 g; wet basis) was weighed and sealed in 140 × 90 mm nylon bags (53 µm pore size; ANKOM Technology, Macedon, USA). The nylon bags were then placed within larger polyester mesh bags (200 × 200 mm; 5 mm pore size) with 400 g of freshly mixed compost matrix. Polyester twine was attached to each bag to enable recovery of the material during composting. As each compost vessel was filled, four mesh bags were placed 0.3 m under the surface in each composter. One of the mesh bags was randomly collected at days 7 and 14. After collecting the mesh bags on day 14, composters were emptied and the matrix was mixed using a separate shovel for each composter. During the mixing process, water was added to the matrial to return it to original moisture level measured prior to initiation of composting. The moistened mixture was returned to each original composter for a second heating cycle. As the composters were refilled, the remaining two mesh

bags containing a mixture of SRM and composted manure from the first heating cycle were returned and placed in each composter in the same manner as for the first cycle. During the second cycle, one of the remaining two mesh bags was collected at days 21 and 28, respectively. Compost samples were collected from each composter after mixing at day 14, and from each mesh bag at days 7, 14, 21, and 28 days. All compost samples were stored at 4°C prior to physicochemical analysis.

The same sampling procedures were conducted for composted PrP^{Sc} within a containment facility. Briefly, fresh cattle manure (1.0 ± 0.1 g; dry basis) was shaped into spheres and dried at 30°C for 1 day to reduce moisture levels. The dried manure spheres were inoculated with 1 ml of 10% scrapie BH containing PrP^{Sc} to return the manure to moisture content similar to that prior to drying. The inoculated spheres were placed in nylon bags which along with compost matrix were in turn placed in larger mesh bags. As each compost vessel was filled, six mesh bags were placed at 0.3 m under the compost matrix. Three of the mesh bags were randomly collected after 14 days with the remaining three being collected at day 28. On day 14, compost was mixed and moistened in the same manner as described above. Compost samples were collected from each composter after mixing at day 14, and also from each mesh bag at days 14 and 28 for analysis of moisture and pH. Manure spheres were collected from nylon bags before and after composting, and stored at -20°C for the detection of PrP^{Sc} by Western blotting (WB). In order to test for migration of PrP^{Sc} during the

composting process, compost samples in the vicinity of the nylon bags, as well as from the nylon bags itself were collected for WB analysis.

4.2.5 Composting conditions and properties

For composting of SRM, compost temperatures and oxygen concentration (%) were measured at the same depth as the mesh bags were implanted using the methods from Chapter 2 (Section 2.2.3). Samples of the initial compost matrix and those collected at each sampling date were analyzed for moisture, bulk density, total carbon (TC), total nitrogen (TN), pH, electrical conductivity (EC), and mineral N (NH_4^+ and $NO_2^- + NO_3^-$) as described in Chapter 2 (Section 2.2.3)

For composting of PrP^{Sc}, only temperature, moisture and pH were measured due to restrictions associated with removing material from the biocontainment facility.

4.2.6 Degradation of specified risk material in compost

The degradation of SRM in compost was estimated on the basis of dry matter disappearance from the nylon bags during the composting process in the same manner as described in Chapter 2 (Section 2.2.3).

4.2.7 PrP^{Sc} extraction

The infectious prion (PrP^{TSE}) is highly hydrophobic and aggregated, and exhibits a high affinity for soil minerals and sludge solids (Leita et al. 2006; Johnson et al. 2006; Kirchmayr et al. 2006). The adsorption of PrP^{TSE} to soils or soil minerals appears to be immediate, irreversible, and resistant to desorption

(Leita et al. 2006; Vasina et al. 2005). Thus, these characteristics may impose a challenge to highly sensitive and accurate quantification of PrP^{TSE} in compost.

Sodium dodecyl sulphate (SDS) treatment has been shown to be effective and practical among various detergents to release soil-bound PrP^{C} or PrP^{TSE} into solution for subsequent WB detection (Seidel et al. 2007; Johnson et al. 2006). A modification of the procedure used to extract PrP^{TSE}-contaminated soil (Seidel et al. 2007) was used to extract PrP^{Sc} from compost. Briefly, 10 ml of 1% aqueous solution of sodium dodecyl sulphate (SDS; Sigma-Aldrich, Oakville, Canada) was added to each manure sphere inoculated with 1 ml of 10% scrapie BH in a 50 ml Falcon conical centrifuge tube (Becton Dickinson, Mississauga, Canada). Subsequently, the tubes were vortexed using a vortex mixer (Fisher Scientific, Ottawa, Canada) at 3,000 rpm for 1 min and shaken in a Rocking Platform (Model 100; VWR, Edmonton, Canada) at 120 rpm for 1 h, followed by a centrifugation step at 3,200 g for 20 min to release PrP^{Sc} from manure into the SDS solution. The supernatant (7 ml) was then incubated with proteinase K (PK; 25 μg ml^{-1}) (Roche Diagnostics, Laval, Canada) at 37°C for 1 h. The PK digestion was terminated by the addition of Pefablock SC (Roche Diagnostics) to a final concentration of 2 mM.

A procedure of PTA (sodium phosphotungstic acid; Sigma-Aldrich) precipitation modified from Huang et al. (2005) was used to precipitate the extractable PrP^{Sc} from compost. After PK digestion, the supernatant was incubated with PTA (4%, w v^{-1}, in 170 mM $MgCl_2$, pH 7.4) at a final concentration of 0.3% (w v^{-1}), followed by centrifugation at 17,950 g for 30 min.

The pellet was resuspended in 200 µl of 1 × Laemmli's sampling buffer (Sigma-Aldrich) and heated to 100°C for 5 min for WB.

4.2.8 Western blotting

Each denatured sample (12 µl) was loaded onto pre-cast 17-well, 1 mm thick, 12% Bis-Tris NuPAGE gels (Invitrogen, Burlington, Canada). Magic Mark XP (Invitrogen) was used as a reference for estimation of molecular mass. Electrophoresis was performed for 1 h at 150 V in 3-(N-morpholino) propanesulfonic acid (MOPS) running buffer (Invitrogen) with antioxidant (Invitrogen) using XCell SureLock Mini-Cells (Invitrogen). Proteins were electrotransferred to polyvinyl difluoride membranes (Millipore, Bedford, USA) in a transfer tank equipped with plate electrodes (Criterion Blotter; Bio-Rad, Mississauga, Canada) at 150 V for 1 h. Subsequently, the membranes were immunoblotted with the first antibody of monoclonal antibody P4 (1:5,000 dilution; Rida, Germany) and the second antibody of goat anti-mouse IgG conjugated with alkaline phosphatase (1:5,000 dilution; Prionics, Zurich, Switzerland) in a Snap i.d. protein detection system (Millipore), as per manufacturer's instructions. After blotting, the membranes were incubated with chemiluminescence substrates of CDP-Star (Roche Diagnostics) for 5 min. Protein signals were visualized by exposing the membranes to BioMax Light Chemiluminescence film (Kodak, Herts, UK). Bands with molecular weights ranging from 20 to 30 kDa were used to show the glycoform profile of PK resistant core of PrP^{Sc}.

4.2.9 Evaluation of PrP^{Sc} extraction from manure

Initially, manure spheres were inoculated with 10% non-infectious sheep BH to determine if ovine PrP^{C} could be retrieved using the above mentioned extraction method. To evaluate the effect of PTA precipitation on the SDS-extracted PrP^{C}, one of two samples after SDS extraction was precipitated with and without PTA, respectively. In addition, a sample after SDS extraction applied with both PK digestion and PTA precipitation was included to evaluate the success of PK digestion.

Subsequently, the optimized extraction procedures were applied to the manure spheres inoculated with 10% scrapie BH to evaluate PrP^{Sc} retrieval. Similarly, to compare the effect of PTA precipitation, duplicate PrP^{Sc} extraction samples after PK digestion were treated with and without PTA, respectively. For the positive control, 10% scrapie BH without manure inoculation was treated with PK at a final concentration of 25 μg ml^{-1} at 37°C for 1 h. The PK activity was stopped by Pefablock SC (2 mM final concentration). For the negative control, the manure inoculated with water was extracted, PK digested and PTA precipitated.

To determine the sensitivity of WB, 10% scrapie BH was diluted in an equal volume of 10% non-infectious sheep brain homogenate to yield 5%, 2.5%, 1.25%, 0.625%, 0.313%, 0.156%, and 0.078% dilutions. The 10% to 0.078% dilutions were inoculated with manure and then extracted to test if PrP^{Sc} bands were obtained by WB.

4.2.10 Statistical analysis

The comparison of initial compost properties between control and feather compost were analyzed as a factorial design using the Mixed procedure (< 0.05 probability level) in SAS (2001). Changes in compost temperature and compost properties as well as SRM degradation during composting were analyzed using the Mixed procedure of SAS with time treated as repeated measures in the model. Main effects were considered to be statistically significant at a probability level of < 0.05.

4.3 Results and discussion

4.3.1 Temperature and oxygen profiles

Passively aerated laboratory-scale composters described in Chapter 2 were used to enable the composting of PrP^{TSE} to be studied under containment conditions. During the composting of SRM, temperature curves did not differ between control and feather compost ($P > 0.05$; Figure 4-1a). After 2 days of composting, temperatures peaked at 74°C in control compost and 75°C in feather compost. Subsequently, temperatures steadily declined, but remained above 50°C for 4 days. Upon mixing at day 14, temperatures rapidly increased again in both composts with peak temperatures of 49°C and 52°C occurring on day 16 in control and feather compost, respectively (Figure 4-1a). Mixing and moistening of compost successfully resulted in a second heating cycle and further extended the period of biodegradation of SRM and possible recalcitrant PrP^{Sc} (Ahn et al. 2008). Temperature profiles of the composters in containment containing PrP^{Sc} (Figure 4-1b) mirrored those obtained in the SRM compost (Figure 4-1a) used to assess the physicochemical parameters of the compost.

Oxygen concentrations in SRM compost were 7% in control compost and 6% in feather compost at day 0 (Figure 4-1c). After 1 day of composting, oxygen concentration decreased to 6% and 3% in control and feather compost, respectively. As composting proceeded, oxygen concentration increased to 18% in both compost types until day 5 and remained at a level between 18% and 20% from days 6 to 28 (Figure 4-1c). Oxygen profiles in the composters were indicative that aerobic microbial processes had sufficiently developed for degradation of organic matter in compost (Kutzner 2000).

4.3.2 Characteristics of initial materials

At the start of the experiment, fresh manure would not have been suitable for direct composting as it did not have the physicochemical properties necessary to optimize composting (Rynk 1992; Table 4-1). Wood shavings were added to the manure as a bulking agent to give an optimized mixture with a moisture level of 66.7% (wet basis), a bulk density of 498 kg m^{-3} and C/N ratio of 22.2 (Table 4-1). Inclusion of feathers in the compost had no effect on matrix properties, except increasing ($P < 0.05$) TN and decreasing C/N ratio (Table 4-1).

4.3.3 Changes in compost properties

During the composting of SRM, moisture content declined in both compost types during the first composting cycle (Figure 4-2). After 14 days of composting, the decline was more rapid in feather compost as the moisture content was reduced to 45.7%, while the control declined to 57.2%. In the second cycle, moisture content remained relatively constant throughout the cycle. Bulk density,

TC content, and C/N ratio steadily declined over each composting cycle (Figure 4-2). Compost EC gradually increased during the initial composting cycle, but decreased after the compost was mixed and water was added on day 14 (Figure 4-2). Concentrations of NH_4^+-N peaked at day 7 in all composters, and thereafter declined until day 28. Levels of (NO_2^- + NO_3^-)-N below 5 mg kg^{-1} were observed over the primary composting cycle, but increased up to 200 mg kg^{-1} at the end of the experiment (Figure 4-2). Physicochemical changes in compost over the experimental period demonstrated that laboratory-scale composters were appropriate as a model for examining the ability of this approach to degrade SRM and PrP^{TSE} (Larney and Olson 2006).

For each composting cycle, TN content in the compost in the vicinity of SRM steadily increased (Figure 4-2), probably due to a large amount of free nitrogen released into the surrounding compost from the degradation of SRM (Chapter 3; Xu et al. 2007). As feathers contain 16% nitrogen, their substitution for manure lowered ($P < 0.05$) the C/N ratio (Table 4-1), which may have negatively impacted the composting process (Barone and Arikan 2007). However, inclusion of feathers (5% dry weight) did not appear to alter the composting process as most parameters remained unchanged (Figure 4-2).

The pH of both control and feather compost were similar at the start of composting (i.e., 7.7), increasing to ~9.4 after 28 days of composting (Figure 4-2). Current evidence suggests that PrP^{TSE} or soil-bound PrP^{TSE} are most effectively degraded by enzymes at a neutral or alkaline pH (Suzuki et al. 2006; Saunders et

al. 2010). Alkaline conditions observed in the composters may have been favorable for the enzymatic degradation of PrP^{Sc}.

For composting of PrP^{Sc}, moisture content and pH (Table 4-2) were similar to the results from composting of SRM (Figure 4-2). However, moisture content declined to a lower ($P < 0.05$) level at the end of the first cycle as compared to SRM compost, with an average of 40.3% in control compost and 26.4% in feather compost (Table 4-2). Due to the maintenance of negative air pressure, an enhanced air flow was generated in the level 3 laboratories, probably resulting in a greater removal of heat and moisture from these passively aerated composters.

4.3.4 Degradation of specified risk material in compost

Within the first 7 days of composting, 60.9% of SRM dry matter disappeared (Figure 4-3) a value comparable to, or even higher than that observed in previous experiments in Chapters 2 and 3. Xu et al. (2009) also found that SRM was rapidly degraded (> 90%) after the first 7 days in a biosecure composting system. This period of rapid SRM degradation corresponded with an increase in microbial biomass as indicated by phospholipid fatty acid profiles within this passively aerated laboratory composter (Chapter 2; Figure 2-5). After the first composting cycle, 63.1% of SRM was degraded (Figure 4-3). However, an increase ($P < 0.05$) of SRM degradation occurred in the second cycle, with 76.9% of SRM being degraded by the completion of the experiment (Figure 4-3). This suggests that mixing of compost to generate repetitive heating cycles can be used to increase the duration of thermophilic composting, increasing the extent of SRM degradation and possibly keratin and PrP^{Sc}.

In the present study, inclusion of feathers increased ($P < 0.05$) the overall degradation of SRM. At the end of the experiment, 73.2% and 80.5% of SRM was degraded in control and feather compost, respectively (Figure 4-3). As SRM contains a large fraction of labile protein (Kolb et al. 1992), it is more easily degraded than recalcitrant proteins such as keratin, either in anaerobic digestion or aerobic composting (Xu et al. 2009; Gilroyed et al. 2010). Consequently, mineralization of protein nitrogen from feathers during composting may be limited in feather-enriched compost, resulting in an increase in the use of N that originates from SRM.

Alternatively, Fredrich and Kern (2003) reported that a keratinolytic protease from the fungus *Doratomyces microsporus* not only had the capacity to degrade keratin, but also hydrolysed a broad range of other protein types including casein, bovine serum albumin, and elastin. Therefore, inclusion of feathers in compost may have effectively stimulated the growth of keratinolytic microbes that also produced ancillary proteases with the ability to hydrolyze SRM proteins, leading to enhance SRM degradation.

4.3.5 Evaluation of PrP^{Sc} extraction

In this study, ovine PrP^{C} was successfully extracted by SDS solution after PTA precipitation from manure inoculated with non-infectious sheep BH (Figure 4-4a, lane 9). Protein bands corresponding to scrapie were observed between 20 to 30 kDa in the positive control (Figure 4-4a, lane 1) with no signals being observed in the negative control (Figure 4-4a, lane 6), confirming that SDS plus PTA did effectively extract PrP^{Sc} from manure (Figure 4-4a, lanes 4 and 5).

Previous studies of the extraction of soil-bound PrP^{TSE} indicated that low PrP^{TSE} recovery from SDS could affect the sensitivity of immunoblotting techniques (Saunders et al. 2008a). Protein enrichment by PTA has provided an effective method to enhance the ability of WB to detect PrP^{TSE} in tissue and soil (Huang et al. 2005; Cooke et al. 2007). In this study, PTA effectively precipitated the extractable ovine PrP^{C} from cattle manure, as evidenced by a faint PrP^{C} signal in the SDS extraction sample without PTA (Figure 4-4a, lane 8) becoming visible and more intense in the SDS extraction sample with PTA (Figure 4-4a, lane 9). More importantly, these results demonstrate that PTA concentrated the extractable PrP^{Sc} from cattle manure (Figure 4-4a, lanes 2 and 3 vs. lanes 4 and 5), with a detectable level by WB after 1-2 $\log_{10}$ dilution (Figure 4-4b).

4.3.6 Fate of PrP^{Sc} in compost

Scrapie prions (PrP^{Sc}) have been shown to remain stable and infectious after burial in soil for 3 years (Brown and Gajdusk 1991), and persist for 16 years in a sheep barn (Georgsson et al. 2006). In compost, the behaviour of PrP^{Sc} may be more complex, due to a wider variety of biological, chemical and physical degradation processes occurring during composting than in soil (Epstein 1997).

Little is known about the possibilities of PrP^{TSE} degradation in compost. To date, only Huang et al. (2007) have reported that PrP^{Sc} was near or below the WB detection limit in a compost heap after 108-148 days. However, they directly tested scrapie-infected tissues left in the compost, which did not consider the impact of the prion-compost interactions that may have had on the detection of PrP^{Sc}. Furthermore, composting conditions or the matrix was not formulated to

optimize protease activity during the composting process. In this study, a natural scenario was further simulated in which PrP^{Sc} were allowed to interact with the components in compost. By using WB, compost-associated PrP^{Sc} was analyzed before and after composting of cattle manure spiked with scrapie BH. Before composting, a positive WB signal of extracted PrP^{Sc} was detectable in all samples (Figure 4-5). However, PrP^{Sc} was not detected in the samples after 14 or 28 days of composting. Similar results were verified in each replicate composter for control and feather compost (Figures 4-5a and 4-5b). The sensitivity results of WB (Figure 4-4b) indicated that at least 1-2 $\log_{10}$ reduction in PrP^{Sc} signals was observed after composting for 14 days. The agent of PrP^{TSE} has been demonstrated to undergo limited migration in soil (Seidel et al. 2007; Cooke and Shaw 2007). In this study, the migration of PrP^{Sc} during the composting process was analyzed by WB, and no PrP^{Sc} specific signals were detected in compost samples collected from the vicinity of the nylon bag (Figure 4-6).

Several possible mechanisms could explain the decline in the detectable level of PrP^{Sc} in compost. First, the dynamic changes in manure properties during composting may reduce or inhibit the extractability of PrP^{Sc} from compost, resulting in signals at or below the detection limit of WB. Ionic concentration, pH and humic acids have been reported to influence PrP^{TSE} adsorption or promote PrP^{TSE} conformational changes in soil (Ma et al. 2007; Polano et al. 2008). Both pH and EC significantly increased during composting (Figure 4-2) and possibly influenced the recovery of PrP^{Sc} during SDS extraction. It was also observed that extracts from compost exhibited a darker brown colour as compared to extracts

from fresh manure, suggesting that concentration of humic acids or other phenolics during composting may have interfered with the extraction of PrP^{Sc} and subsequent WB detection.

Another possible explanation lies in the nature of the antibodies used for detection. The monoclonal N-terminus antibody P4 binds to residues 93 to 99 of the ovine prion protein and has been used for the diagnosis of both typical and atypical scrapie (Gretzschel et al. 2006). Several studies have suggested that the N-terminus of PrP^{TSE} is susceptible to proteolytic degradation (Saunders et al. 2008b), and it has been shown that PrP^{TSE} that comes in contact with soils frequently undergoes N-terminus cleavage (Johnson et al. 2006; Cooke et al. 2007). Therefore, N-terminus epitopes of PrP^{Sc} might be truncated by compost proteases after composting or structurally changed by SDS extraction, resulting in the failure of the P4 antibody to detect this altered structure.

The biological degradation of adsorbed PrP^{Sc} by compost microbes may also explain, at least in part, the observation of the disappearance of PrP^{Sc}. Over 28 days, there was considerable evidence of microbial activity in compost as indicated by peak temperature > 70°C, 5 days of thermophilic composting, significant reduction in TC content, and degradation of ~80% of SRM. In addition, some bacteria with the ability to degrade recalcitrant proteins such as keratin and PrP^{TSE}, including those of the genera *Actinomadura* (Puhl et al. 2009), *Streptomyces* (Hui et al. 2004), and *Saccharomonospora* (Al-Zarban et al. 2002), have been shown to be present in the composters (Chapter 3) and may play a role in the degradation of PrP^{Sc}.

4.4 Conclusions

In this chapter, the findings suggested that enhanced SRM degradation as a result of the addition of feathers also appears to have enriched for microorganisms with enhanced activity towards recalcitrant proteins. However, due to the challenges of detecting prions within the composting matrix, the present work does not definitively prove that PrP^{Sc} undergoes complete degradation during composting. Micro-climates within the composting matrix that do not achieve the same level of microbial activity may also limit the degradation of PrP^{Sc} in compost. However, the fact that PrP^{Sc} was clearly detectable in manure, but not detectable after 14 and 28 days clearly suggests that at least partial degradation of PrP^{Sc} occurs during composting. Our group is presently undertaking similar work on the composting of PrP^{BSE} and PrP^{CWD} where both the protein misfolding cyclic amplification procedure and laboratory bioassays will be employed to further document the extent that PrP^{TSE} are degraded during composting.

Table 4-1 Physicochemical characteristics of initial compost materials and mixtures

Property[a]	Starting materials				Mixture at day 0	
	Cattle manure	Wood shavings	Chicken feather	Specified risk material[b]	Control compost	Feather compost
Moisture (%)	74.8	10.5	10.0	81.0	66.7A	65.0A
Bulk density (kg m^{-3})	595	117	28	nm	498A	490A
Total carbon (%)	42.8	52.6	50.9	56.7	46.3A	46.6A
Total nitrogen (%)	3.17	0.06	15.56	7.35	2.09B	2.66A
C/N ratio	13.5	960.5	3.3	7.7	22.2A	17.5B
pH	7.63	5.81	6.69	nm	7.71A	7.75A
EC (ds m^{-1})	1.8	0.1	1.0	nm	1.3A	1.4A
NH_4–N (mg kg^{-1})	3264	509	575	nm	788A	899A
NO_x–N (mg kg^{-1})	6.6	1.4	1.6	nm	3.7A	3.1A

[a]All characteristics except moisture and bulk density are expressed on a dry weight basis (wt wt^{-1}). Moisture and bulk density are expressed on a wet weight basis. EC: electrical conductivity.
[b]Values are cited from Chapter 2 (Table 2-1). nm, not measured.
A, B: within a row values followed by different letters differ ($P < 0.05$) between mixture types.

Table 4-2 Moisture (%) and pH changes after composting of scrapie prions

Property	Day 14		Day 14 (after mixing)		Day 28	
	Control compost	Feather compost	Control compost	Feather compost	Control compost	Feather compost
Moisture[a] (%)	40.3A	26.4B	62.8A	60.8A	58.8A	57.4A
pH	9.35A	9.45A	9.06A	9.08A	9.63A	9.71A

[a]Moisture is expressed on a wet weight basis.
A, B: within a row values followed by different letters differ ($P < 0.05$) between compost types.

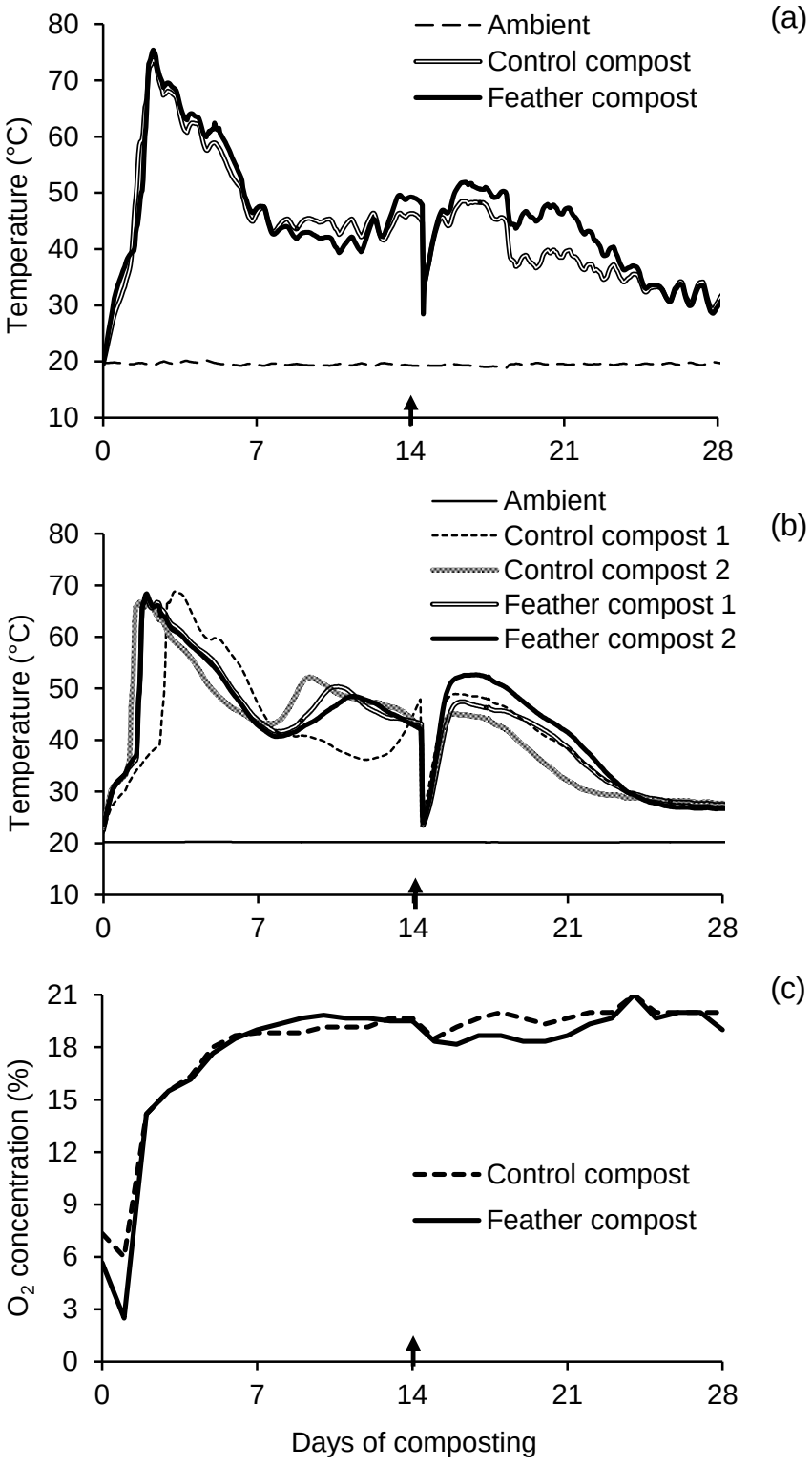

Figure 4-1 (a) Temperature during composting of specified risk material (SRM), (b) Temperature during composting of scrapie prions, and (c) O_2 concentration during composting of SRM. Arrows indicate the date compost was mixed.

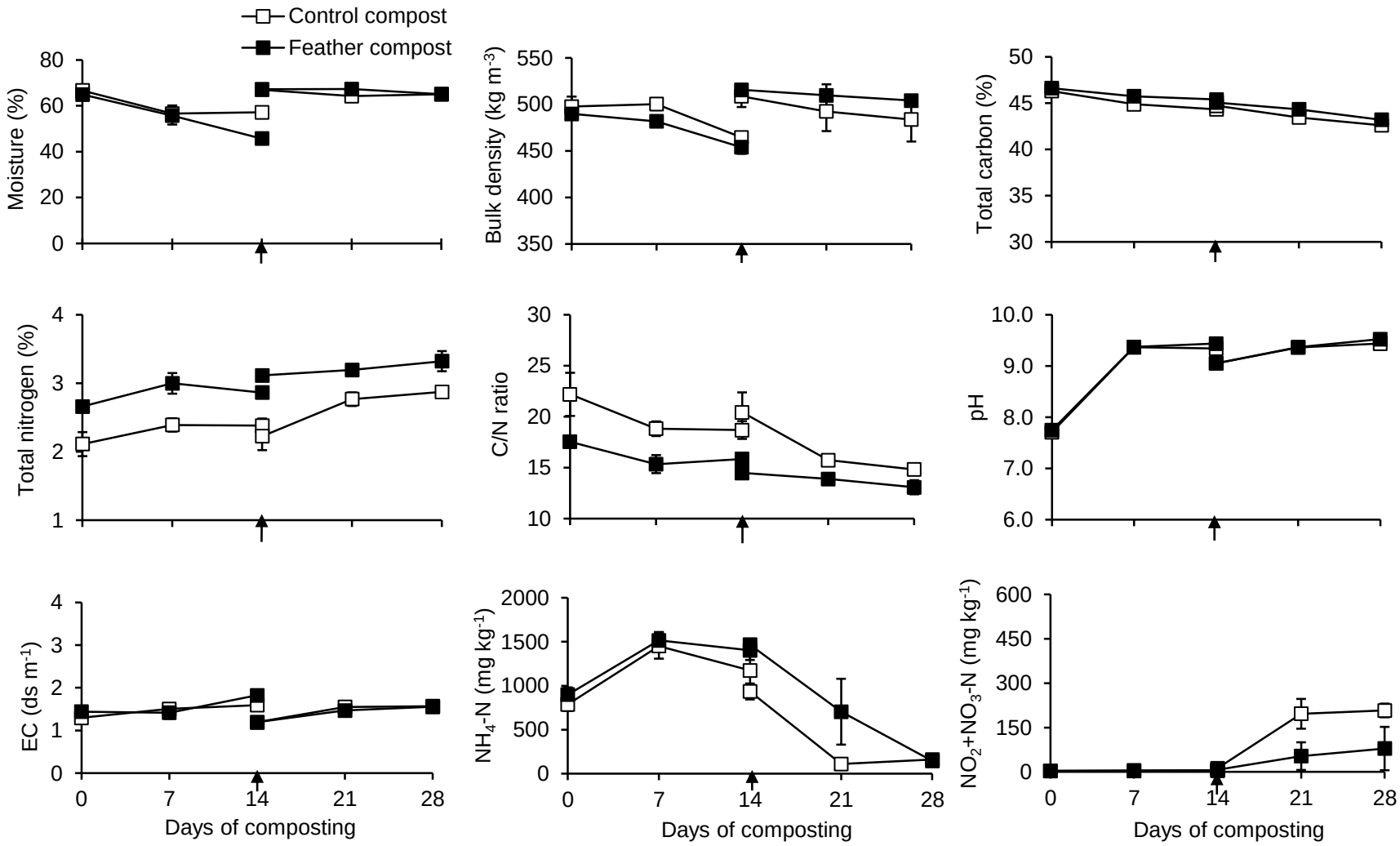

Figure 4-2 Physicochemical changes during composting of specified risk material. Arrows indicate the date compost was mixed.

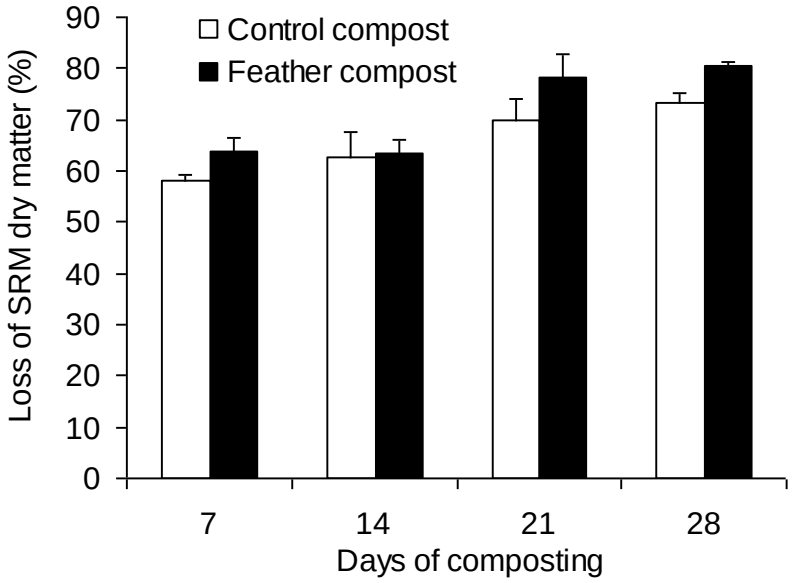

Figure 4-3 Degradation of specified risk material (% dry matter loss) at the middle depth in laboratory-scale composters.

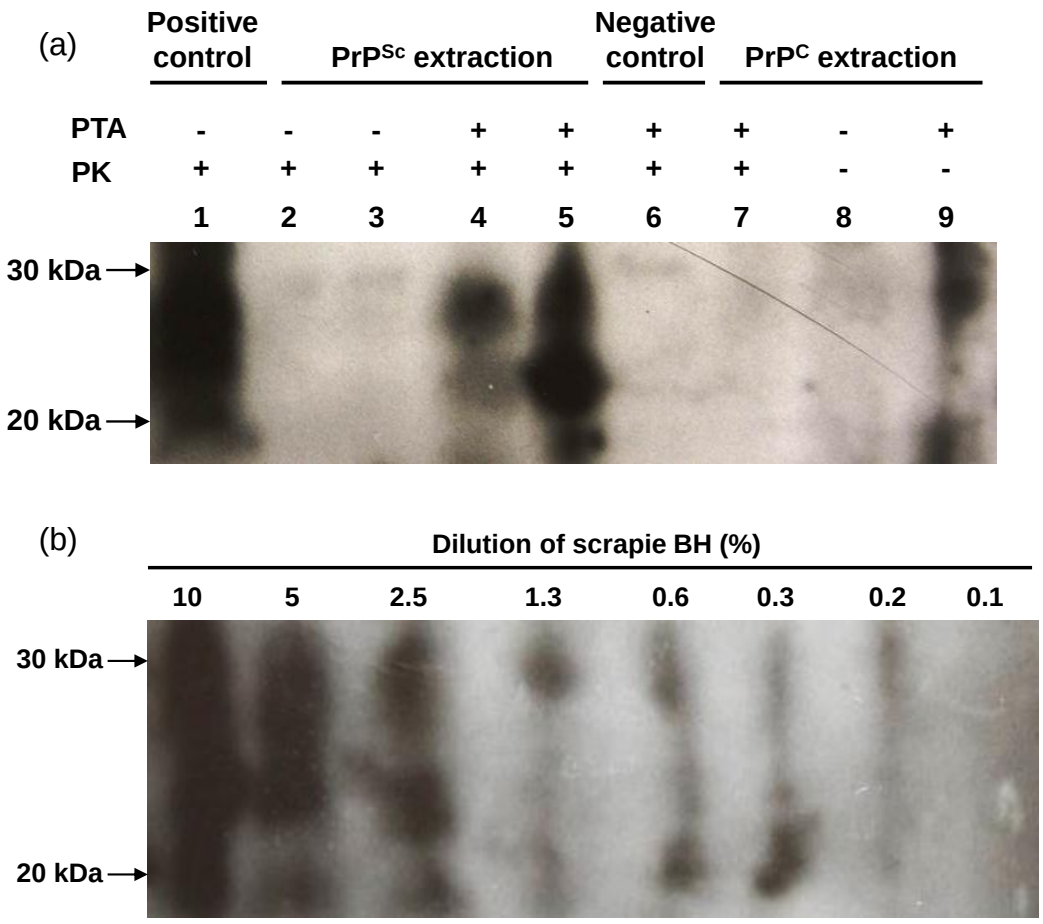

Figure 4-4 (a) Extraction of ovine normal prions (PrP^{C}) and scrapie prions (PrP^{Sc}) from fresh manure inoculated with non-infectious sheep and scrapie brain homogenate (BH), respectively. Lane 1: proteinase K (PK) digestion of scrapie BH (positive control); lane 6: extraction from manure inoculated with water with both PK and PTA (sodium phosphotungstic acid) precipitation (negative control); lanes 2 and 3: duplicate extracted PrP^{Sc} with PK but without PTA; lanes 4 and 5: duplicate extracted PrP^{Sc} with both PK and PTA; lane 7: extracted PrP^{C} with both PK and PTA; lane 8: extracted PrP^{C} without both PK and PTA; lane 9: extracted PrP^{C} with only PTA (b) Western blotting of PrP^{Sc} extracted from manure inoculated with decreasing concentrations of PrP^{Sc}. PrP^{Sc} extraction was PK digested and PTA precipitated prior to WB.

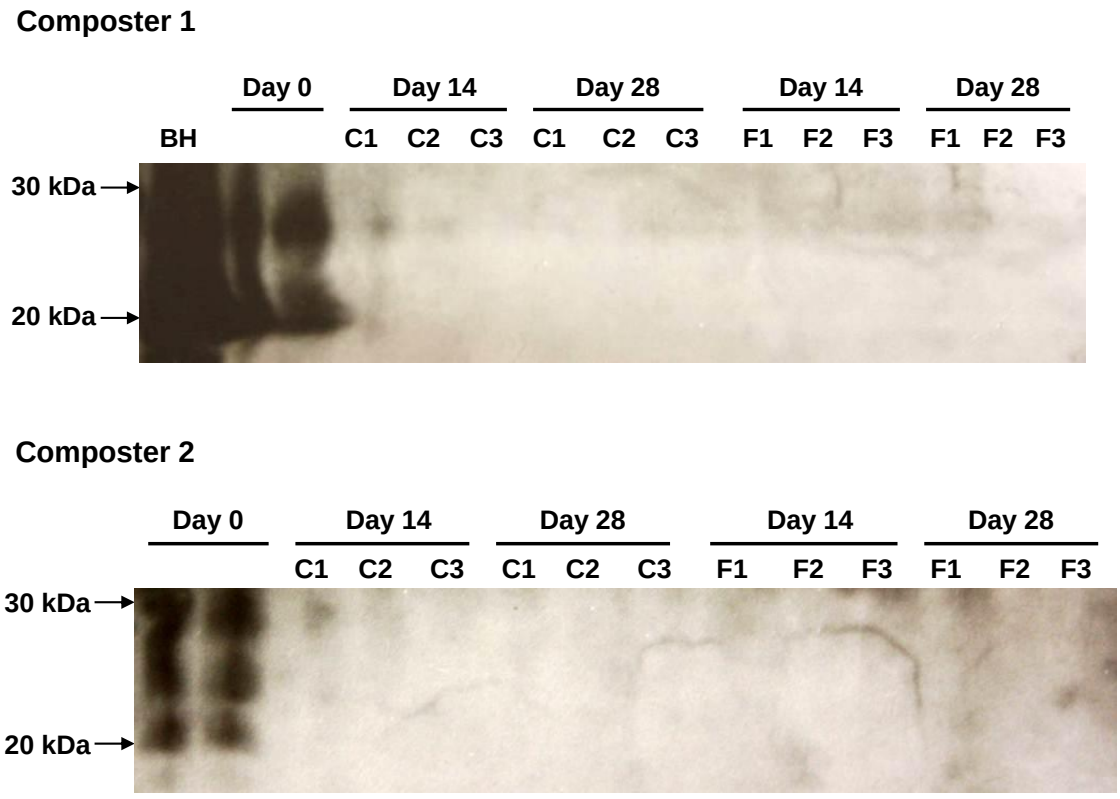

Figure 4-5 Western blotting (WB) of scrapie prions (PrP^{Sc}) extracted from samples (n=3) at days 0, 14 and 28 from replicate composters. C1-C3: Control compost; F1-F3: Feather compost. BH: (positive control) 10% scrapie brain homogenate. PrP^{Sc} extraction was proteinase K digested and phosphotungstic acid precipitated prior to WB.

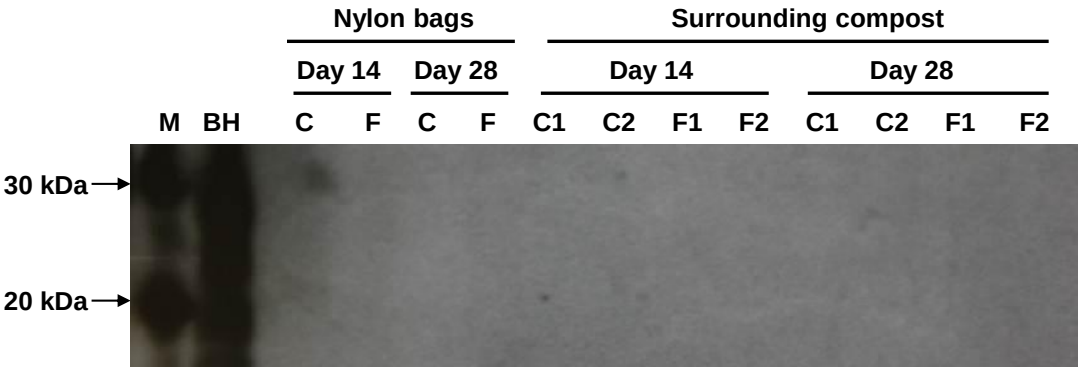

Figure 4-6 Western blotting (WB) of scrapie prions (PrP^{Sc}) extracted from nylon bags and surrounding compost materials at days 14 and 28. C: control compost; F: feather compost. C1-C2: duplicate control composters; F1-F2: duplicate feather composters. M: marker; BH: scrapie brain homogenate. PrP^{Sc} extraction was proteinase K digested and phosphotungstic acid precipitated prior to WB.

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Chapter 5 Microbial communities and greenhouse gas emissions associated with the biodegradation of specified risk material in compost[§]

5.1 Introduction

In response to the identification of bovine spongiform encephalopathy (BSE) in Canada in 2003, the Canadian Food Inspection Agency (CFIA) imposed an enhanced feed ban in July of 2007 to prevent the introduction of specified risk material (SRM) into the food chain. Specified risk material was designated to include the skull, brain, trigeminal ganglia, eyes, palatine tonsils, spinal cord and the dorsal root ganglia from cattle aged 30 months or older, as well as the distal ileum from cattle of all ages. These are tissues known to be at risk of accumulating infectious prion proteins (PrP^{TSE}). Currently, the majority of SRM in Canada are rendered and then disposed of in landfills. However, alternative on-farm disposal methods for SRM which are environmentally and economically feasible are desired. One option may be composting, which has been shown to inactivate pathogens while producing a valuable fertilizer for agricultural crops (Hao et al. 2009).

The microbial consortia in compost could potentially carry out the biodegradation of SRM infected with PrP^{TSE}, due to the wide range of proteolytic enzymes they produce. Previous research has revealed bacterial species that

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produce proteases that are capable of degrading recalcitrant PrP^{TSE} (Hui et al. 2004; McLeod et al. 2004), some of which reside in compost (Ryckeboer et al. 2003). In Chapter 3, research work has been focused on characterizing actinobacterial communities that may degrade SRM in compost. Puhl et al. (2009) also have previously isolated a novel actinobacterium with the ability to degrade recalcitrant proteins in compost. However, the nature of the various microbial communities associated with the degradation of SRM remains to be defined.

Despite the obvious advantages of using composting for SRM disposal, addition of carcasses to manure during composting has been shown to increase greenhouse gas emissions (Xu et al. 2007). Hao et al. (2009) observed that CH_4 emissions during co-composting of feedlot manure with cattle mortalities were higher than with slaughterhouse SRM wastes. Methanogens are involved in the production and methanotrophs in the oxidation of CH_4 during composting. Heterotrophic methanogens produce CH_4 utilizing acetate, formate or methanol as a carbon source, whereas autotrophic methanogens reduce CO_2 to CH_4. Methanotrophs utilize methane monooxygenase to catalyze the oxidation of CH_4 to methanol, which is further oxidized to formaldehyde (Xin et al. 2004). However, the relative abundance of methanogens and methanotrophs during the composting of SRM has not been investigated

Keratinases that have the capacity to degrade feathers may also exhibit activity against PrP^{TSE}, due to similarities in their structure (Suzuki et al. 2006). Recent studies indicated that enrichment of a composting matrix with feathers not only produced an effective non-specific proteolytic activity early in the

composting process, but also promoted the growth of keratinolytic fungi that degraded feathers in the later stages of composting (Korniłłowicz-Kowalska and Bohacz 2010; Bohacz and Korniłłowicz-Kowalska 2009). Specified risk material contains a large fraction of labile protein (8% fresh weight basis; Mcllwain and Bachelard 1985), so inclusion of feathers in compost may promote enzyme activity (i.e., non-specific proteolytic and keratinolytic) that could improve the degradation of SRM as well as PrP^{TSE}.

Chapter 4 has reported that mixing of feathers with cattle manure in a laboratory-scale composter increased total N content though enhanced SRM degradation, but did not alter the physicochemical properties of compost. Hence, it is hypothesized that inclusion of feathers may alter the composition of the microbial community in a manner that enhances SRM degradation in compost. The objectives in this study were to examine the composition of bacterial and fungal communities degrading SRM in compost with and without feathers. Furthermore, emissions of greenhouse gases (i.e., CH_4 and N_2O) and the related abundance of methanogens and methanotrophs were also investigated.

5.2 Materials and methods

5.2.1 Composting setup and sampling procedure

Passively aerated laboratory-scale composters were used as previously described in Chapter 2 (Section 2.2.1). Triplicate composters were filled with matrices of either control compost (35 ± 0.1 kg fresh feedlot cattle manure, 74.8% moisture; 3.5 ± 0.1 kg white spruce (*Picea glauca*) wood shavings) or feather compost (34.2 ± 0.1 kg cattle manure; 3.5 ± 0.1 kg wood shavings; 0.8 ± 0.1 kg

chicken (*Gallus gallus*) feathers). Compost substrates were prepared by mixing the initial materials in a mortar mixer (12S; Crown construction equipment, Winnipeg, Canada), resulting in 66.7% moisture in control compost (C/N ratio=22.2) and 65% moisture in feather compost (C/N ratio=17.5).

Fresh bovine brain tissue (SRM) from mortalities under 30 months of age were obtained from a nearby slaughterhouse. Bovine brain tissue (50 ± 0.1 g; wet basis) was weighed and sealed in 140 × 90 mm nylon bags (53 µm pore size; ANKOM Technology, Macedon, USA). The nylon bags were then placed within larger polyester mesh bags (200 × 200 mm; 5 mm pore size) with 400 g of freshly mixed compost substrate. Polyester twine was attached to each mesh bag to enable recovery of the material during composting. As each compost vessel was filled, four mesh bags were placed at 0.3 m below the top of each composter. One bag per treatment was randomly collected after 7 and 14 days. After collecting the bags on day 14, composters were emptied and the contents were mixed using a specific shovel for each treatment. During the mixing process, water was added to the material to return it to the moisture level measured prior to initiation of composting. The moistened mixture was returned to the original composter for a second heating cycle. As the composters were refilled, the remaining two mesh bags containing a mixture of SRM and composted manure from the first heating cycle were returned and placed in each composter in the same manner as for the first cycle. In the second cycle, one of the remaining two mesh bags was collected after 21 and 28 days, respectively.

Triplicate samples of the initial mixed matrix and fresh SRM were collected at day 0. Decomposed SRM and compost samples were collected from each mesh bag after sampling on days 7, 14, 21, and 28. All samples were freeze-dried in a freeze dryer (Series 24D × 48; Virtis, Gardiner, USA) after collection and used for subsequent DNA extraction.

5.2.2 Gas collection and analysis

Changes in CH_4 and N_2O concentrations (ppm) in the composter headspace were used for the qualitative estimation of CH_4 and N_2O emissions from the passively aerated composters. For gas collection, a flexible polyvinyl chloride tube (3.2 mm diameter; Nalgene, New York, USA) was inserted into each composter headspace through a hole drilled in the composter sidewall. One end of the tube was placed at the above centre of the compost matrix within the composter, while the other end, protruding out of the composter was sealed using an air-tight tube fitting (Swagelok, Medicine Hat, Canada). Gas samples (11 mL) were extracted from the tube fitting using a polypropylene syringe and then injected into 5.9-ml, pre-evacuated, septum-stoppered vials (Exetainer; Labco Limited, Buckinghamshire, UK). Gas samples were collected from each composter three times daily at 4 h intervals and analyzed for CH_4 and N_2O concentrations using a gas chromatograph (Varian 450; Varian Instruments, Walnut Creek, USA) equipped with flame ionization and electron capture detectors. Oxygen concentration (ppm) in the compost was measured at the same depth as the mesh bags were implanted using the method from Chapter 2 (Section 2.2.3).

5.2.3 DNA extraction

Prior to DNA extraction, freeze-dried SRM (n=27) and compost (n=30) samples collected at days 0, 7, 14, 21, and 28 were ground using a Ball Mill (MM200; Retsch GmbH, Haan, Germany). Subsequently, DNA was extracted and quantified using the same manner as described in Chapter 3 (Section 3.2.4). The extracted DNA was then stored at -20°C prior to further PCR-DGGE and qPCR analyses.

5.2.4 PCR-DGGE and sequencing analysis

To decrease the variability within a given treatment, an equal amount of DNA extracted from replicate samples at each sampling date was pooled as previously described in Chapter 3, resulting in a total of nine SRM and ten compost DNA samples used for the subsequent PCR-DGGE analysis. All PCR amplifications contained 1 × HotStarTaq *Plus* DNA Master Mix (Qiagen), 0.2 µM of each primer in a final volume of 50 µl. Amplification was conducted using a thermal cycler (Mastercycler ep*gradient*; Eppendorf, Hamburg, Germany). Partial fragments of the bacterial 16S rRNA gene were amplified with primers F984GC (Nübel et al. 1996; Table 5-1) and R1378 (Heuer et al. 1997; Table 5-1) as previously described in Novinscak et al. (2009). Each reaction contained 40 ng of template DNA and the cycling conditions consisted of 95°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 62°C, 2 min at 72°C, and final extension for 10 min at 72 °C. For fungi, partial fragments of the fungal 18S rRNA gene were amplified using nested PCR. In the first-round PCR, template DNA (100 ng) was amplified by primers EF4 and Fung5 (Marshall et al. 2003; Table 5-1) with

conditions of 95°C for 5 min, followed by 35 cycles of 30 sec at 94°C, 30 sec at 53°C, 1 min at 72°C, and final extension for 5 min at 72 °C. In the nested step, amplification was conducted with primers EF4 and NS2GC (Marshall et al. 2003; Table 5-1) in the same manner as described above in the first-round PCR. The PCR products were visualized on a 1.0% (w v^{-1}) agarose gel before DGGE analysis.

Bacterial and fungal PCR products were loaded onto 6% and 7.5% polyacrylamide gels in 1 × TAE buffer at 60°C using a DCode^{TM} Universal Mutation System (Bio-Rad, Hercules, USA). For bacteria, DGGE gels consisted of a linear gradient of denaturant (40% to 70% from top to bottom) and were run at a constant voltage of 150 V for 6 h. For fungal DGGE, the denaturant gradient was 18% to 38%, and the electrophoresis was performed at 50 V for 14 h. For bacteria, a previously developed DGGE marker in Chapter 3 (Section 3.2.4) consisting of bands representing *Thermobifida fusca*, *Streptomyces thermovulgaris*, *Saccharomonospora viridis*, *Actinomadura hallensis*, *Streptomyces thermophilis* and *Nocardiopsis sp.* was used. For fungal DGGE, a commercial DGGE marker (Marker IV, Nippon Gene, Toyama, Japan) was loaded between fungal samples. Gels were stained with SYBR Gold (Invitrogen, Eugene, USA) for 30 min, and the migration patterns were visualized using a UV transilluminator (Biospectrum 800 imaging system; UVP, LLC, Upland, USA).

Bacterial and fungal DGGE bands that were predominant in the SRM samples were excised and placed overnight in 50 µl of elution TE buffer (pH=7.4). The eluted bacterial 16S rRNA and fungal 18S rRNA gene fragments

were re-amplified using F984/R1378 primers and EF4/NS2 primers, respectively. The resulting PCR products were again electrophoresed in a DGGE gel and subject to a final purification using a QIAquick PCR kit (Qiagen) and sequenced (Eurofins MWG Operon, Huntsville, USA).

Phylogenetic analyses of nucleotide sequences were conducted in the same method as described in Chapter 3 (Section 3.2.5). Sequences from *Rubrobacter radiotolerans* (accession no. U65647) and *Mortierellales* sp. (accession no. EF126342) were obtained from the NCBI database and used as bacterial and fungal out groups, respectively. The bacterial 16S rRNA and fungal 18S rRNA gene sequences obtained in this study were deposited in the NCBI nucleotide sequence database with the accession numbers JN596829 to JN596841 and JN596842 to JN596857, respectively.

5.2.5 quantitative PCR analysis

The relative abundance of methanogens and methanotrophs in compost were quantified using qPCR targeting the copy numbers of *mcrA* (methyl-coenzyme M reductase) and *pmoA* (particulate methane monooxygenase) genes as previously reported by Steinberg and Regan (2009) and Kolb et al. (2003), respectively. The *mcrA* genes were amplified with primers mlas and mcrA-rev, while the *pmoA* genes were amplified using primers A189F and Mb661R (Table 5-1). To construct DNA quantification standards, *mcrA* and *pmoA* genes were cloned using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, USA), and sequenced at a commercial sequencing centre (Eurofins MWG Operon). The BLAST analysis of the cloned standard *mcrA* and *pmoA* genes indicated 99% similarity to the *mcrA*

gene of *Methanoculleus marisnigri* (accession no. CP000562; Anderson et al. 2009) and *pmoA* gene of *Methylomicrobium* sp. (accession no. AY195668; Bodrossy et al. 2003). The *mcrA* and *pmoA* sequences were deposited in the NCBI nucleotide sequence database with the accession numbers JN186796 and JN186797, respectively. To generate standard curves for qPCR, plasmid DNA was extracted, serially diluted over a range from 1 to10^7 copies μl^{-1}, and used as a template for PCR. All reactions were conducted in duplicate on a Mx 3005P qPCR system (Agilent Technologies, La Jolla, USA) and contained 1 × Brilliant II SYBR Green QPCR Master mix (Agilent Technologies), 20 ng of DNA and 0.1 µg/µl bovine serum albumin (New England Biolabs, Pickering, Canada) in a final volume of 50 µl. For methanogens, each reaction contained 0.2 µM primers and PCR conditions consisted of one cycle at 95°C for 10 min, followed by 40 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C. For methanotrophs, each reaction contained 0.5 µM primers and PCR conditions were similar to those for methanogens except that the annealing temperature was 63°C. Melt curve analysis was performed after the final extension step to test for the presence of primer dimmers and non-specific amplification. Florescence normalization and data analysis were conducted using MX3005P software (version 4.10; Stratagene).

5.2.6 Statistical analysis

Statistical analysis of the DGGE bands was conducted with BioNumerics software (version 5.1; Applied Maths Inc., Austin, USA) following the provider's instructions. Calculation of the pair-wise similarities based on the presence and absence of the bands was performed using Dice's correlation coefficients. Cluster

analysis based on the similarity matrix was performed using the unweighted pair group method with arithmetic average (UPGMA) to form a complete linkage dendrogram.

Changes in headspace CH_4 and N_2O concentrations, and copy numbers of *mcrA* and *pmoA* genes during composting were analyzed using the Mixed procedure of SAS (version 8; SAS Institute Inc., Cary, USA) with time treated as a repeated measure. Main effects of compost types were considered to be statistically significant at a probability level of < 0.05.

5.3 Results and discussion

5.3.1 Bacterial communities associated with specified risk material degradation

The DGGE analysis of bacterial communities throughout the composting process is shown in Figure 5-1. In general, the average numbers of bands observed in compost samples were 14 and 18 in the first and second composting cycles, respectively (Figure 5-1a). However, the band numbers were less numerous in SRM samples, with 8 in the first cycle and 9 in the second cycle (Figure 5-1b). This suggests that the bacterial community involved in SRM degradation may be less diverse than that involved in degrading other organic matter in compost. In Figure 5-2, the statistical analysis of bacterial DGGE bands revealed that all SRM samples clustered together and exhibited only 35% similarity to compost samples. Specified risk material contains a large fraction of labile protein and fat (Mcllwain and Bachelard 1985). However, manure contains a variety of proteins, lipids, starch, cellulose, hemicellulose, lignin and humic

acids (Ryckeboer et al. 2003). Therefore, these complex components in manure may have required more diverse bacterial population for decomposition.

Sequencing and phylogenetic analysis of the predominant bacterial DGGE bands from SRM (Figure 5-1b) generated the neighbor joining tree depicted in Figure 5-3. Bacteria belonging to the phyla of *Actinobacteria*, *Firmicutes* and *Proteobacteria* were detected over 28 days. In the first composting cycle at days 7 and 14, sequences corresponded to two robust clades with genera of *Saccharomonospora* (bands 1, 3, and 6) and *Thermobifida* (bands 2 and 4) identified in both compost types. Others have found the moderately thermophilic *Saccharomonospora viridis* and *Thermobifida fusca* to be associated with thermophilic microbial populations in laboratory-scale composters containing household food waste (Steger et al. 2007). This suggests that as temperature increases, thermophilic actinobacteria may dominate in compost and carry out SRM decomposition after the easily degradable substrates are utilized during the initial stages of composting. This pattern of decomposition was previously revealed in the investigation from Chapter 3 on the role of actinobacteria in SRM degradation during composting. Apart from those genera, additional sequences in feather compost (band 7), and control compost (band 5) were closely affiliated with *Thermoactinomycetaceae* and *Thiohalospira*. Species of *Thiohalospira alkaliphila* have been previously isolated from hypersaline habitats (Sorokin et al. 2008). The compost environment in this study was also highly saline as indicated by its high level of electrical conductivity (Chapter 4; Figure 4-2).

After mixing the compost at days 21 and 28, sequences in control compost (bands 8, 9, and 11; Figure 5-1b) were affiliated with *Pseudomonas*. *Pseudomonas* strains previously isolated from sewage sludge have been noted for their denitrification ability (Lai and Shao 2008). In this study, significant denitrification occurred in the second composting cycle. This is evident from Figure 5-7b, which shows a rapid increase in N_2O concentration in the composter headspace after mixing. In addition, sequences in feather compost associated with *Actinomadura* (bands 10 and 12) and *Enterobacter* (band 13) were identified (Figure 5-1b). The relative maturity of the compost could be indicative of the presence of these two genera, as *Actinomadura* and *Enterobacter* have been also found during the late stages of composting with sewage solids (Novinscak et al. 2009) and in mature manure compost-amended soil (Edrington et al. 2009). A novel species of *Actinomadura* has been isolated from compost with the ability to degrade keratin from bovine hooves (Puhl et al. 2009). The appearance of *Actinomadura* in feather compost suggested that this genus also likely played a role in the degradation of feather keratin and SRM in the present study.

5.3.2 Fungal communities associated with specified risk material degradation

In contrast to bacteria, the number of fungal DGGE bands associated with compost samples (Figure 5-4a) tended to be less than that in SRM samples (Figure 5-4b), suggesting that SRM as a substrate was colonized by a more diverse fungal community than compost. Ryckeboer et al. (2003) demonstrated that fungi can more easily attack organic residues in compost that are too dry for bacteria due to their hyphal network. The high fat content (11% fresh weight

basis; Mcllwain and Bachelard 1985) of SRM may have contributed to its hydrophobicity, making it a more suitable substrate for fungi than bacteria. Similar to bacteria, fungal DGGE bands from SRM samples clustered separately from the compost samples and exhibited a 20% similarity (Figure 5-5).

Sequencing and phylogenetic analysis of the dominant fungal DGGE bands from SRM (Figure 5-4b) generated the neighbor joining tree depicted in Figure 5-6. In the first composting cycle, fungi belonging to the *Ascomycota* were detected, while in the second cycle *Basidiomycota* predominated. On day 0, the major fungal taxa (bands 1 and 2) detected from freeze-dried SRM were associated with *Udeniomyces puniceus*. After 7 and 14 days of composting, sequences associated with *Dothideomycetes* (bands 3 and 5) and *Cladosporium* (band 4) were found in control compost, whereas other sequences (bands 6-10) from both control and feather compost formed a robust clade with *Chaetomium*. *Dothideomycetes* sp. have been characterized from the bone marrow of a bison carcass (Reeb et al. 2011), suggesting that they may be also capable of degrading substrates in SRM. Moreover, *Cladosporium cladosporioides* are highly proteolytic even at alkaline pH (Breuil and Huang 1994), a property that may make them particularly relevant to the degradation of protein in the alkali conditions encountered in compost (Chapter 4; Figure 4-2). C*haetomium* have been widely identified in soil and compost and produce serine protease that are thermally stable and exhibit activity over a broad range of pH (Li and Li 2009). The proteolytic nature of these genera suggests they may play an important role in the hydrolysis of SRM in compost.

After mixing of compost on day 14, the sequences of DGGE bands from SRM samples in control and feather compost (bands 11-16; Figure 5-4b) were closely related to *Trichaptum abietinum*. *Trichaptum* have been reported to have potent lipase activity (Goud et al. 2009), suggesting they may be involved in the degradation of lipids in SRM during composting. However, previous studies have focused primarily on the ability of this fungus to decompose wood (Worrall et al. 1997). The predominance of this microorganism in the later stages of composting may reflect its ability to utilize recalcitrant cell-wall carbohydrates that remained in compost after mobile carbon sources were consumed. As SRM were in contact with the compost matrix it is possible that some of these microorganisms originated from other organic components in compost.

5.3.3 Effect of feather addition on microbial communities associated with specified risk material degradation

For either bacterial or fungal DGGE, the band profiles from SRM and compost samples clustered based on the time of sampling (Figures 5-2 and 5-5). Thus, changes in the bacterial or fungal populations during composting were of a temporal nature. However, considerably different microbial communities associated with SRM degradation were also produced between control and feather compost. For bacteria, a divergence was especially obvious in feather compost at the later stage of the first composting cycle, which exhibited only a 50% similarity at day 14 with control compost (Figure 5-2). Similar to bacteria, inclusion of feathers also altered the composition of fungal populations in the first cycle, with similarities between composting types being 55% and 35% on days 7

and 14, respectively (Figure 5-5). However, differences in either bacterial or fungal populations induced by feather addition were relatively minor in the second cycle (Figures 5-2 and 5-5).

In this study, inclusion of feathers in compost enhanced ($P < 0.05$) SRM degradation in feather compost (Chapter 4). Bohacz and Korniłłowicz-Kowalska (2009) demonstrated that the presence of feathers in compost stimulated the growth of keratinolytic fungi during composting, enhancing the decomposition of feather keratin. Moreover, some keratinolytic fungi, like *Doratomyces microspores*, not only degrade keratin, but also hydrolyze other types of proteins including casein, bovine serum albumin, and elastin (Fredrich and Kern 2003). Therefore, in this study, it might be expected that these special keratinolytic microbes within feather compost had the capacity to degrade a wide range of proteins and significantly contribute to SRM degradation.

5.3.4 Greenhouse gas emissions and abundance of methanogens and methanotrophs in compost

Accurate measurement of low and variable gas airflow in laboratory-scale passively aerated composters is challenging (Yu et al. 2005). Although active aeration can produce a more consistent airflow, it also contributes to the drying of the compost and curtails microbial activity (Larney et al. 2000). Changes in gas concentrations of the composter headspace can be a suitable indicator for the qualitative estimation of gas emissions in passively aerated systems, where gases are dissipated through natural convective airflow.

At day 0, CH_4 concentrations in the composter headspace were 335 ppm in control compost and 454 ppm in feather compost (Figure 5-7a). After one day of composting, CH_4 concentrations peaked at 1108 ppm in control compost and 2338 ppm in feather compost ($P < 0.05$), declining thereafter to below 100 ppm in both compost types (Figure 5-7a). Similar to CH_4, the maximum N_2O concentrations (78 ppm for control compost and 90 ppm for feather compost; $P < 0.05$) occurred on day 1, thereafter declining to below 10 ppm (Figure 5-7b). Upon mixing at day 14, N_2O concentrations once again rapidly increased, peaking on days 16 and 17 in control (42 ppm) and feather (60 ppm) compost, respectively. Subsequently, N_2O concentrations steadily declined, but remained higher ($P < 0.05$) in feather compost than control compost for 4 days (Figure 5-7b).

The concentrations of O_2 were 7×10^4 ppm in control compost and 6×10^4 ppm in feather compost at day 0, and decreased to 6×10^4 ppm in control compost and 3×10^4 ppm in feather compost at day 1 (Figure 5-7c). After day 2, O_2 concentrations increased to 1.8×10^5 ppm in both compost types and remained constant (i.e., 1.8×10^5-2×10^5 ppm) thereafter (Figure 5-7c). As expected, maximum CH_4 and N_2O concentrations occurred in the early stages of composting when O_2 supplies were limited. However, mixing the compost resulted in an increase in N_2O concentrations, possibly due to redistribution of NO_3-N making it available for denitrification as proposed by others (Xu et al. 2007). In this study, after mixing, higher ($P < 0.05$) N_2O concentrations occurred in feather than control composters, partially due to an increase ($P < 0.05$) in total N (Chapter 4; Figure 4-2) and possible due to greater degradation of recalcitrant keratin during

the second composting cycle (Chapter 6; Figure 6-2). After turning, Xu et al. (2007) also reported higher N_2O emissions from compost windrows with increasing levels of total N.

During 28 days of composting, *mcrA* copy numbers, a reflection of methanogenic communities, ranged from 6.7 to 7.2 $\log_{10}$ copies g^{-1}dry weight at each sampling time (Figure 5-8a). No significant differences were observed in *mcrA* copy number between control and feather compost. In relation to CH_4 concentrations (Figure 5-7a), *mcrA* copy numbers were lower (6.8 $\log_{10}$ copies g^{-1}dry weight; Figure 5-8a) on days 7 and 28, a period associated with a decline in CH_4 concentrations (< 15 ppm). Meanwhile, the higher *mcrA* copy numbers (7.1 $\log_{10}$ copies g^{-1}dry weight; Figure 5-8a) in compost at days 14 and 21 was associated with an increase in headspace CH_4 concentration (Figure 5-7a). In contrast, *pmoA* copy numbers, an indicator of methanotrophic communities, remained relatively high (4.5 $\log_{10}$ copies g^{-1}dry weight; Figure 5-8b) at day 7 when CH_4 concentrations were low (Figure 5-7a). However, *pmoA* copy numbers declined in both compost types at day 14 (Figure 5-8b) after which headspace CH_4 concentrations increased (Figure 5-7a). These results suggested that the majority of composting CH_4 emissions occur when the density of methanogens increases and methanotrophs decreases.

By using qPCR, copy numbers of *mcrA* and *pmoA* genes have been proposed to reflect the abundance of methanogens and methanotrophs in environmental samples, such as soil, acidic peat and anaerobic sludge (Kolb et al. 2003; Steinberg and Regan 2009). However, little quantitative data on *mcrA* and *pmoA*

copy numbers have been reported in compost. Only Sharma et al. (2011) reported *mcrA* copy numbers ranged from 7.1 to 8.7 $\log_{10}$ copies g^{-1}wet weight during 15 weeks of windrow composting, while *pmoA* copy numbers were more variable and negatively correlated with CH_4 emissions. However, the results in this study showed that there was a lack of significant relationship between *pmoA* copy numbers and headspace CH_4 concentrations at day 21 (Figures 5-7a and 5-8b). This may be in part due to the extraction of DNA from non-viable microbial cells that would not have been actively oxidizing CH_4. In this study, relative low CH_4 concentrations in composter headspace were observed throughout the composting process (Figure 5-7a). It is important to emphasize that the qPCR procedure does not necessarily indicate activity as *mcrA* and *pmoA* copies would be detected in methanogens and methantrophs that were not metabolically active. Therefore, more studies are needed to specifically investigate enzymatic activities involved in CH_4 formation and oxidation.

Composting also has been proposed as a viable and effective option for the disposal of feather wastes (Bohacz and Korniłłowicz-Kowalska 2009). The findings from this experiment suggested that feather inclusion might enhance degradation of SRM (Chapter 4). Although addition of feathers in compost might increase greenhouse gas emissions, other methods of feather disposal, including rendering, landfilling and incineration, may also have similar greenhouse gas emissions.

5.4 Conclusions

In this chapter, results obtained by PCR-DGGE clearly underline the value of this molecular tool for characterization of bacteria and fungi that are fastidious to culture, but may possess the capacity to degrade SRM in compost. In agreement with the hypothesis, potential differences in microbial community composition were induced by the inclusion of feathers within the composting matrix, resulting in the establishment communities that were more adept at degrading SRM and possibly PrP^{TSE}. Inclusion of feathers in compost may increase greenhouse gas emissions, but composting still has merit for the disposal of this poultry by-product and enhancing the decomposition of SRM.

Table 5-1 Primers used in this study to target various microbial communities

DNA target	Primer[a]	Sequence (5'-3')	Reference
Bacteria	F984GC[b]	GC.-AACGCGAAGAACCTTAC	Nübel et al. (1996)
16S rDNA	R1378	CGGTGTGTACAAGGCCCGGGAACG	Heuer et al. (1997)
Fungi	EF4	GGAAGGGRTGTATTTATTAG	Marshall et al. (2003)
18S rDNA	Fung5	GTAAAAGTCCTGGTTCCCC	
	NS2GC[c]	GC.-TGCTGGCACCAGACTTGC	
Methanogen	mlas	GGTGGTGTMGGDTTCACMCARTA	Steinberg and Regan (2009)
mcrA gene	mcrA-rev	CGTTCATBGCGTAGTTVGGRTAGT	
Methanotroph	A189F	GGNGACTGGGACTTCTGG	Kolb et al. (2003)
pmoA gene	Mb661R	CCGGMGCAACGTCYTTACC	

[a] F, forward primer; R, reverse primer.
[b] GC. clamp, CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG, is attached to 5'-end of primer.
[c] GC. clamp, CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGC, is attached to 5'-end of primer.

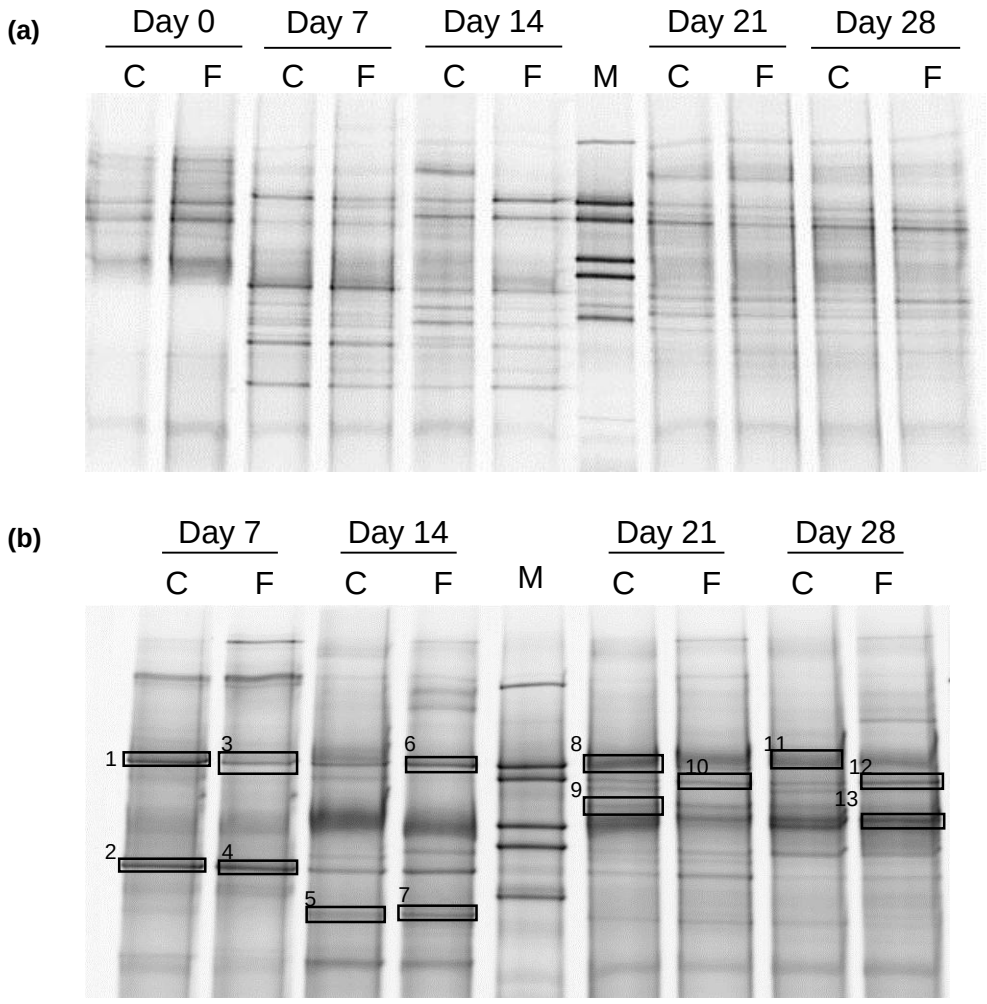

Figure 5-1 Denaturing gradient gel electrophoresis separation of 16S rRNA gene fragments after PCR amplification of extracted (a) compost DNA and (b) specified risk material DNA with bacteria-specific primers F984GC and R1378. Marked bands were excised and sequenced with sequence results being listed in Figure 5-3. C=control compost; F=feather compost; M=marker. Sequences of bands in the marker from top to the bottom of the gel were affiliated with the following bacteria species: *Saccharomonospora viridis*, *Actinomadura hallensis*, *Streptomyces thermophilus*, *Streptomyces thermovulgaris*, *Nocardiopsis* sp., and *Thermobifida fusca*. The SRM samples at day 0 were not included as it did not yield amplifiable DNA.

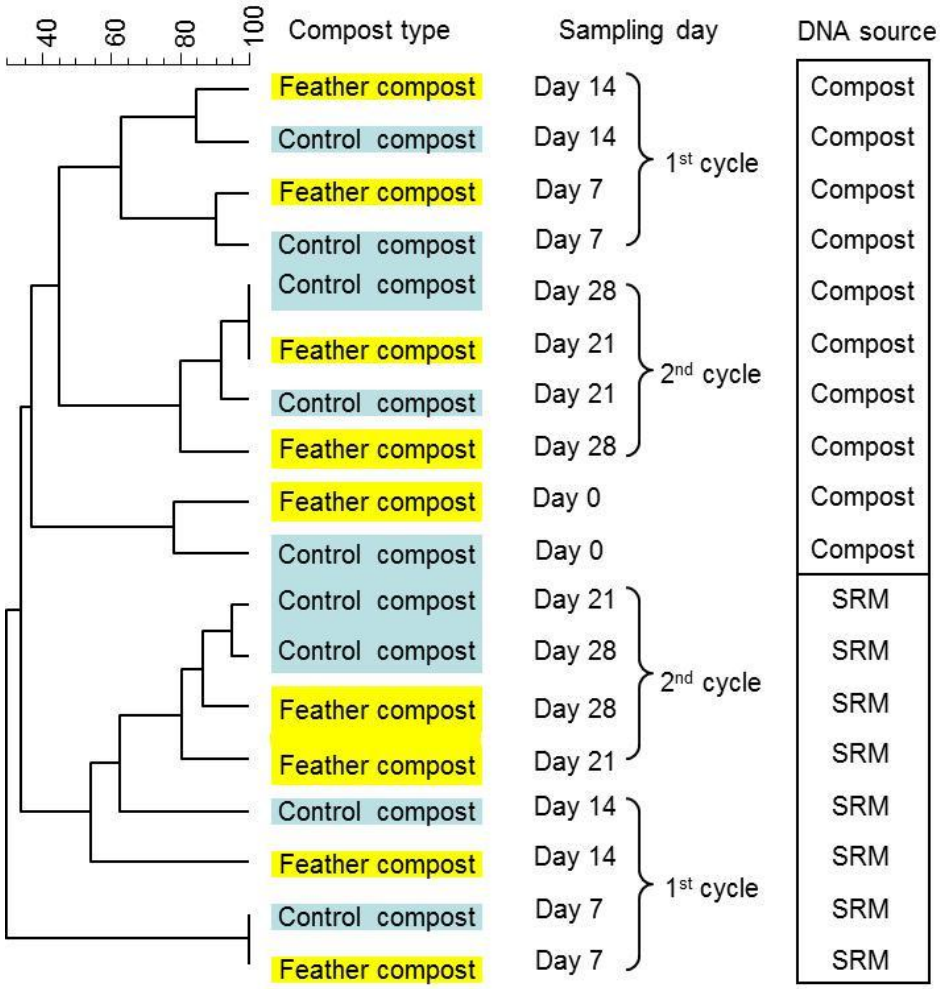

Figure 5-2 Dendrogram of bacterial denaturing gradient gel electrophoresis bands (Figure 5-1) using Dice's coefficient and unweighted pair group method with arithmetic average. The compost type (Control compost or Feather compost), sampling day (Days 0, 7, 14, 21, and 28), and DNA source (SRM-specified risk material DNA or Compost DNA) are given at the end of branches. The SRM samples at day 0 were not included as it did not yield amplifiable DNA.

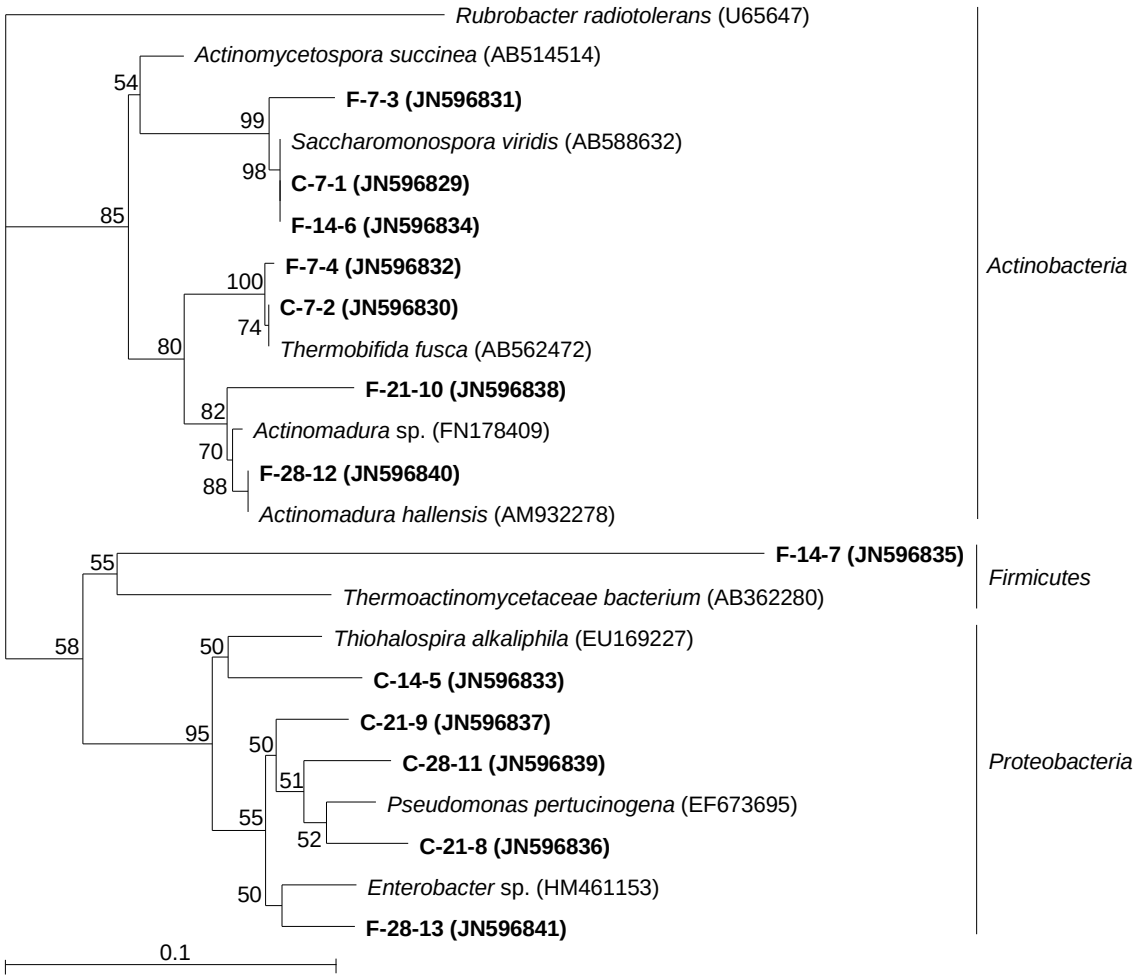

Figure 5-3 A neighbour joining tree of phylogenetic relationships among DNA sequences obtained from bacterial denaturing gradient gel electrophoresis gel (Figure 5-1b) after PCR amplification of extracted specified risk material DNA. Accession numbers of the sequences retrieved from the database are enclosed in parentheses. The scale bar indicates 10% nucleotide substitutions and bootstrap values at 50-100% are displayed at the nodes. The sequences obtained in this study are bold-typed and names indicated Compost Type (C=control compost; F=feather compost)-Sampling Day (Days 7, 14, 21 and 28)-Band Number (1-13). *Rubrobacter radiotolerans* served as an outgroup.

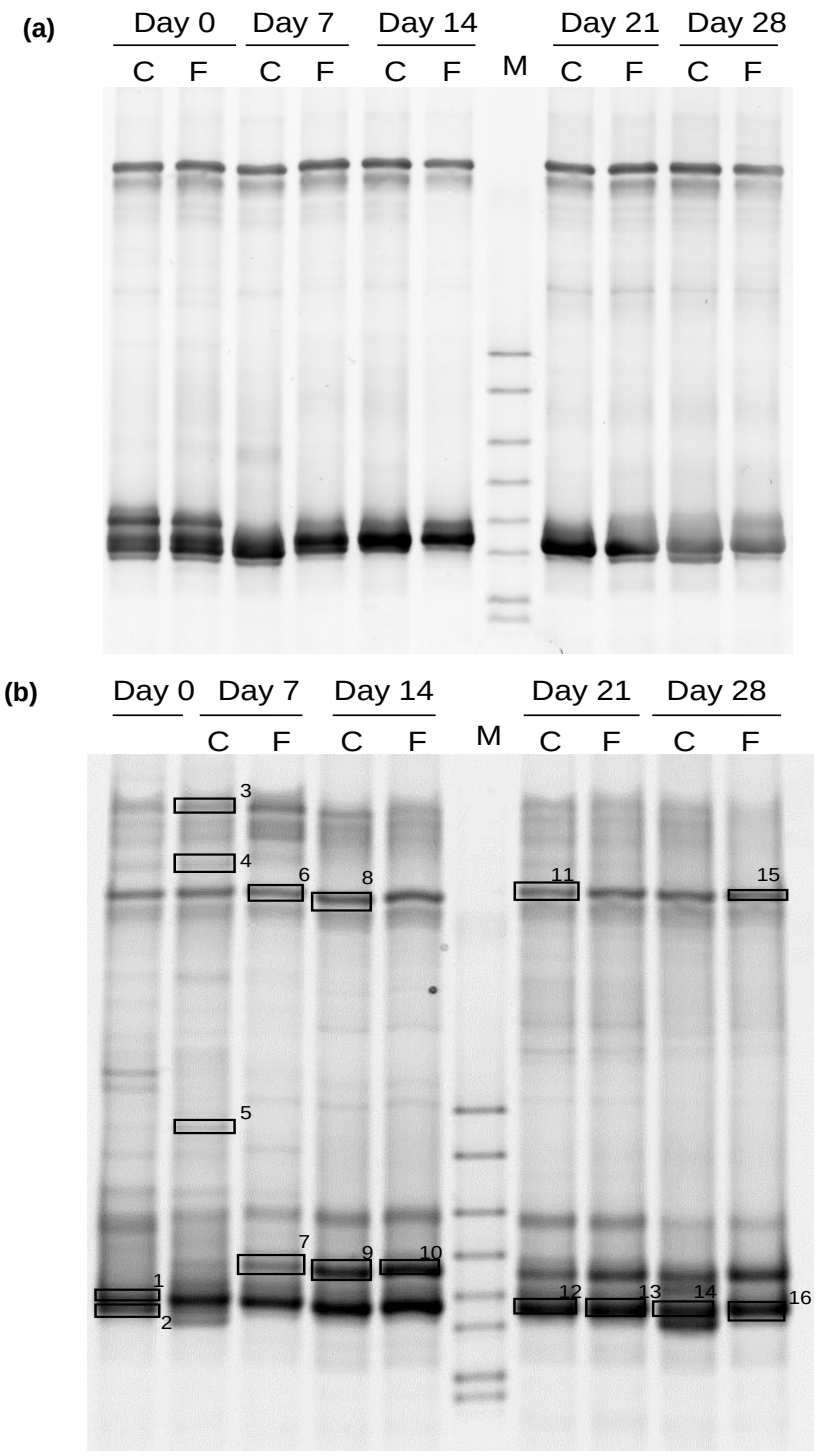

Figure 5-4 Denaturing gradient gel electrophoresis separation of 18S rRNA gene fragments after PCR amplification of extracted (a) compost DNA and (b) specified risk material DNA with fungi-specific primers EF4-Fung5 and EF4–NS2GC. Marked bands were excised and sequenced with sequence results being listed in Figure 5-6. C=control compost; F=feather compost; M=marker.

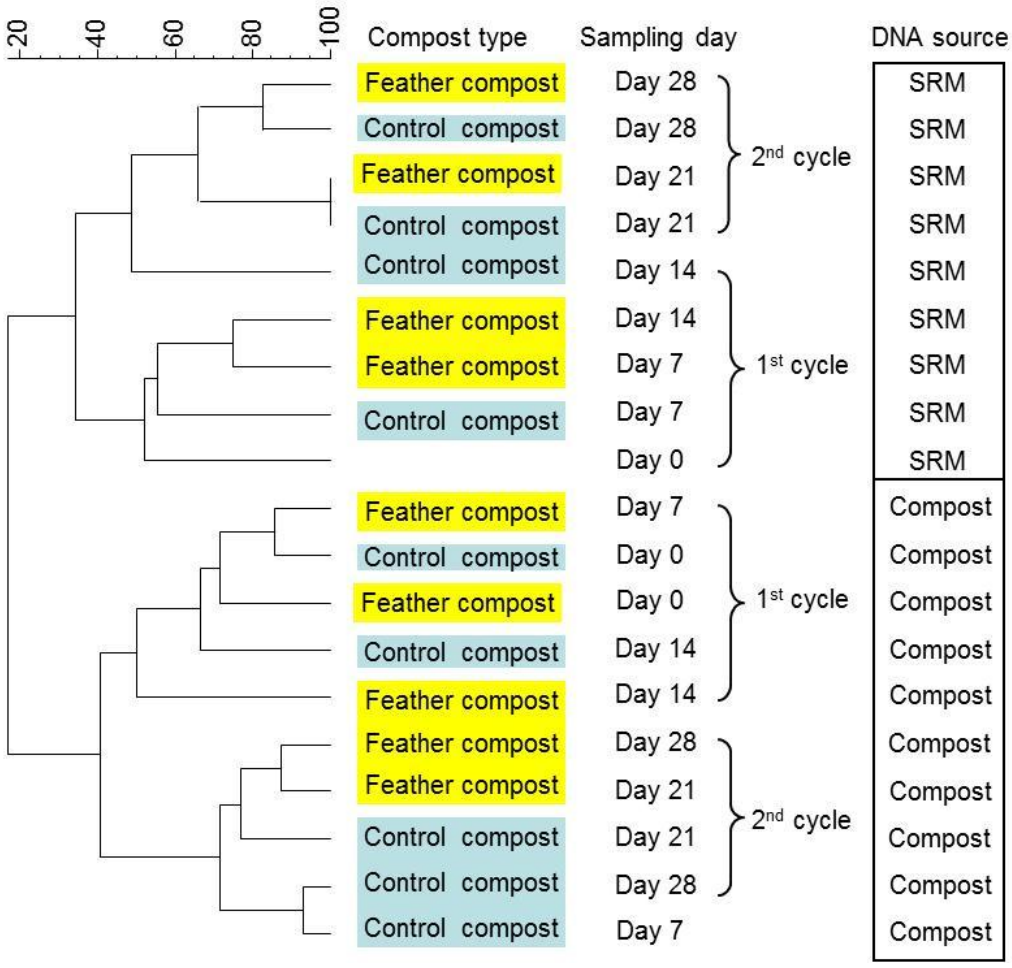

Figure 5-5 Dendrogram of fungal denaturing gradient gel electrophoresis bands (Figure 5-4) using Dice's coefficient and unweighted pair group method with arithmetic average. The compost type (Control compost or Feather compost), sampling day (Days 0, 7, 14, 21, and 28), and DNA source (SRM-specified risk material DNA or Compost DNA) are given at the end of branches.

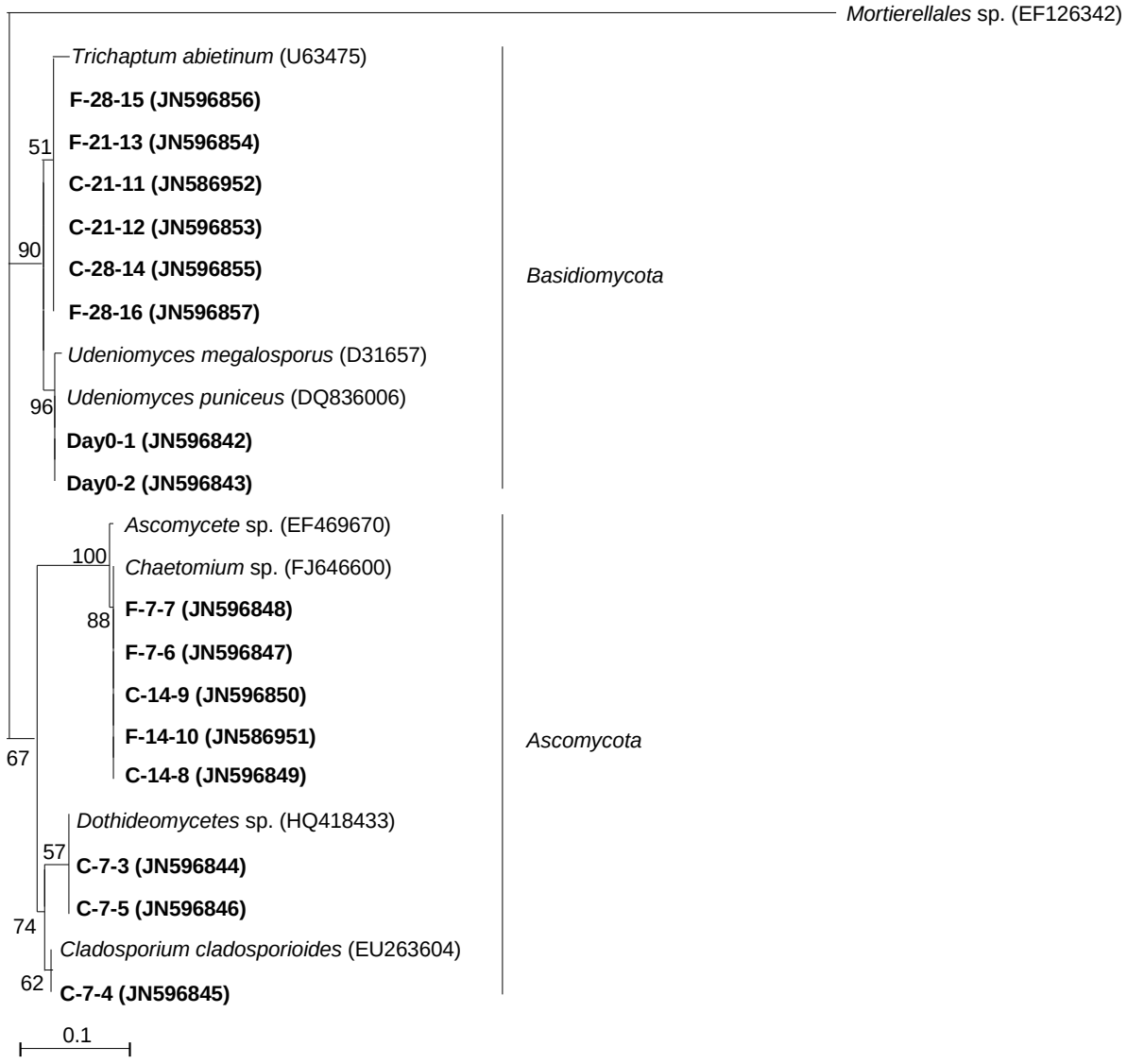

Figure 5-6 A neighbour joining tree of phylogenetic relationships among DNA sequences obtained from fungal denaturing gradient gel electrophoresis gel (Figure 5-4b) after PCR amplification of extracted specified risk material DNA. Accession numbers of the sequences retrieved from the database are enclosed in parentheses. The scale bar indicates 10% nucleotide substitutions and bootstrap values at 50-100% are displayed at the nodes. The sequences obtained in this study are bold-typed and names indicated Compost Type (C=control compost; F=feather compost)-Sampling Day (Days 0, 7, 14, 21 and 28)-Band Number (1-16). *Mortierellales* sp. served as an outgroup.

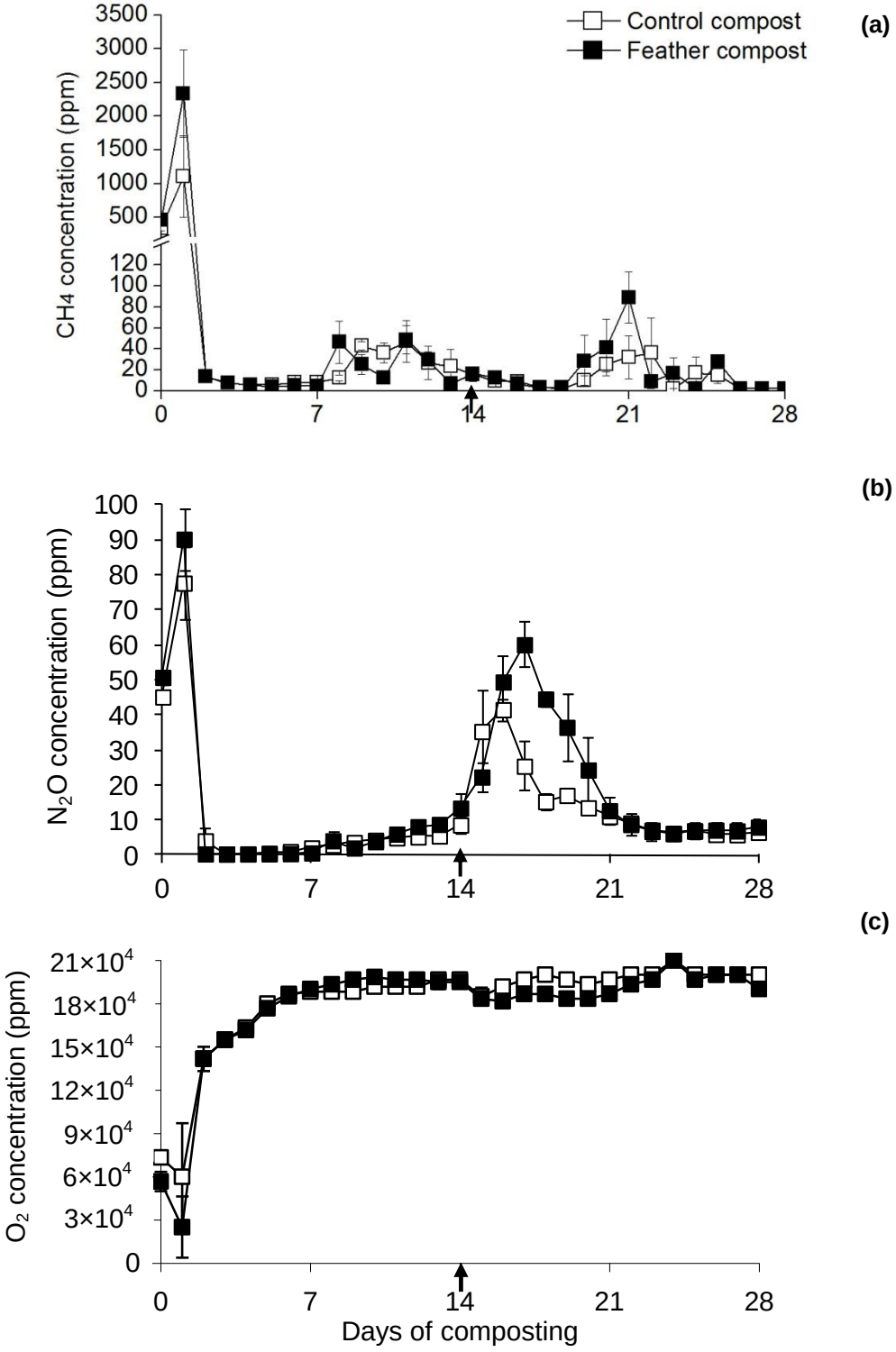

Figure 5-7 Changes of (a) CH_4 and (b) N_2O concentrations (ppm) in the headspace, and (c) O_2 concentration (ppm) in compost during composting of specified risk material. Arrows indicate the date compost was mixed. The data of O_2 concentration was cited from Chapter 4.

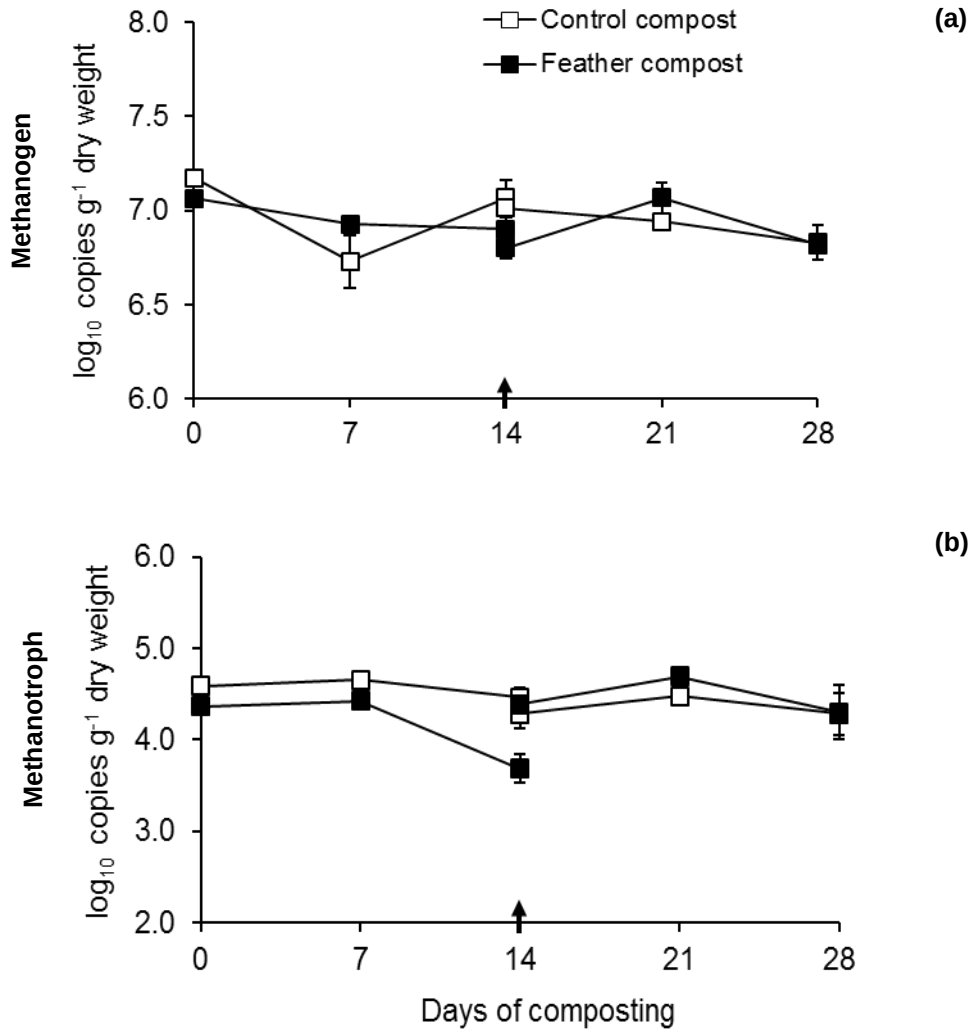

Figure 5-8 Quantitative PCR analyses on the changes of (a) *mcrA* (log_{10} copies g^{-1}dry weight) and (b) *pmoA* (log_{10} copies g^{-1}dry weight) genes during composting of specified risk material. Arrows indicate the date compost was mixed.

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Chapter 6 Potential biodegradation of prions in compost

6.1 Introduction

Transmissible spongiform encephalopathies (TSE) are a group of fatal neurodegenerative diseases including scrapie in sheep and goats, chronic wasting disease (CWD) in deer and elk, bovine spongiform encephalopathy (BSE) in cattle, and Creutzfeldt-Jackob disease (CJD) in humans. The cause of TSE is the sequential conformational changes of normal prion protein (PrP^{C}) into misfolded and infectious prion proteins (PrP^{TSE}) (Prusiner 1998).

As of January 2012, 19 cases of BSE and 66 cases of CWD were confirmed in Canada (CFIA 2012a and 2012b). The occurrence of these TSE diseases has resulted in huge ecomonic losses for cattle and cervid industries in Canada (Petigara et al. 2011). In response to the identification of BSE in Canada in 2003, the Canadian Food Inspection Agency (CFIA) imposed an enhanced feed ban in July of 2007 to prevent the introduction of specified risk material (SRM) which are tissues that include the skull, brain, trigeminal ganglia, eyes, palatine tonsils, spinal cord and the dorsal root ganglia from cattle aged 30 months or older, as well as the distal ileum from cattle of all ages, into the food chain. In Canada, it is estimated that there are 250,000 tonnes of SRM generated annually (Gilroyed et al. 2010). In contrast, the cervid industry in Canada is much smaller, but with 145,000 animals and an overall mortality rate of 5.7% (Canadian Cervid Alliance 2009; Haigh et al. 2005) considerable quantities of SRM are still generated. In addition, approximately 45,000 wild cervids are killed every year in Canada as a

result of vehicular collisions (Huijser et al. 2009). Therefore, proper disposal of these carcasses and SRM is important to prevent the transmission of TSE diseases and to protect soil and water quality.

Currently, the majority of SRM are rendered, dehydrated and disposed of in landfills, (Ayalon et al. 2001). However, disposal of SRM or carcasses infected with PrP^{TSE} in a manner that inactivates the infective agent is challenging due to the recalcitrant nature of PrP^{TSE} (Tylor 2000). Current approved disposal practices for PrP^{TSE} infected material in Canada include two stages of incineration at 850°C and 1000°C for approximately 16 h, 1-h disinfection with 2 N sodium hydroxide, autoclaving in saturated steam at 134°C for a period of 60 minutes, and alkaline hydrolysis at 150°C under 5 atmpospheric pressure for 3-6 hours (CFIA 2009). These methods, however, are economically impractical to ensure the inactivation of PrP^{TSE} while disposing of the large volumes of SRM or carcasses arising from farms, meat processing plants and road kills.

Composting can be an attractive alternative for the disposal of SRM or carcasses infected with PrP^{TSE}. Recent evidence has indicated that some bacterial proteinases exhibit the ability to degrade recalcitrant PrP^{TSE} in minutes or hours (Langeveld et al. 2003; Hui et al. 2004; McLeod et al. 2004). These bacterial species capable of this activity have been shown to be present in compost (Ryckeboer et al. 2003). Morever, some bacteria and fungi with the ability to degrade SRM and PrP^{TSE}, belonging to the genera of *Actinomadura*, *Streptomyces,* and *Dothideomycetes*, have been characterized from composting of SRM by PCR-DGGE (Chapters 3 and 5). The microbial consortia in compost may

biodegrade PrP^{TSE}, owing to the wide range of proteolytic enzymes produced by these complex microbial communities. Moreover, the period of time that PrP^{TSE} would be exposed to enzymatic activity during composting is far longer than the duration that PrP^{TSE} must be exposed to these proteases for degradation to occur (i.e., weeks to months vs. minutes to hours).

To date, only Huang et al. (2007) have reported that scrapie prion (PrP^{Sc}) was near or below the Western blotting (WB) detection limit by directly testing the scrapie-infected tissues left in a compost heap after 108-148 days. However, they did not quantify the reduction level of PrP^{Sc} during the composting process. In Chapter 4, research has been further advanced on composting of PrP^{Sc} and observed that at least 1-2 $\log_{10}$ reduction of PrP^{Sc} signals using WB in a laboratory-scale composter after 14-28 days may be due to biological degradation. However, the possible degradation of BSE and CWD in compost has not yet been reported. In soil, Rapp et al. (2006) demonstrated that buried animal carcasses enhanced the proteolytic activity in the surrounding soil, resulting in the degradation of a β-sheeted recombinant ovine prion protein that was used to mimic PrP^{Sc}. After 15 days of anaerobic digestion at 37°C in sludge solids, a 2.4 $\log_{10}$ infectivity reduction in mouse adapted PrP^{Sc} was observed in a cell-based culture assay (Miles et al. 2011), possibly as a result of biological degradation. Compost may represent a more promising matrix than soil and sludge for PrP^{TSE} degradation, as it achieves thermophilic temperatures (> 55°C), is typically highly alkaline, and possesses intense microbial activity (Epstein 1997).

Poultry feathers are predominantly composed of β-keratin protein (90% dry matter) (Tiquia et al. 2005). Feather β-keratin is structurally similar to PrP^{TSE}, as they both contain high contents of β-sheets (Tsuboi et al. 1991; Prusiner 1998). Keratinases that have the capacity to degrade feathers may also degrade PrP^{TSE} (Suzuki et al. 2006). Bohacz and Korniłłowicz-Kowalska (2007; 2009) reported that enrichment of a composting matrix with feathers not only increased the proteinase activity early in the composting process, but also promoted the growth of specialized keratinolytic fungi that degraded feathers later in the composting period. In Chapter 4, the results (Figure 4-3) have showed that mixing of feathers with cattle manure enhanced SRM degradation in compost. Therefore, inclusion of feathers may also be a means of promoting the degradation of SRM infected with PrP^{TSE} in compost. The objective of this study was to investigate the possible degradation of BSE and CWD prions (PrP^{BSE} and PrP^{CWD}) in compost enriched with and without feathers.

6.2 Materials and methods

6.2.1 Tissue source and preparation

Scrapie-infected hamster brain tissues (strain 263K) and non-infectious hamster brain tissues were provided by the Animal Diseases Research Institute at the Canadian Food Inspection Agency in Ottawa, ON. Brain tissues from elk and cattle confirmed to be negative or positive for CWD and BSE using immunochemical and histopathological techniques were obtained from the Canadian and OIE Reference Laboratories for BSE at the Canadian Food Inspection Agency in Lethbridge, AB. All brain tissues were homogenized to

yield a 10% brain homogenate (BH) as previously described in Chapter 4 (Section 4.2.1).

6.2.2 Compost experimental design

Three types of PrP^{TSE} (i.e., PrP^{263K}, PrP^{CWD} and PrP^{BSE}) were composted in two types of matrices. Control compost contained cattle manure and wood shavings. Feather compost contained cattle manure, wood shavings, and feathers (*Gallus gallus*) obtained from a local poultry abattoir. Composting of PrP^{TSE} was conducted in the level 3 containment laboratory at the Canadian Food Inspection Agency in Lethbridge, Alberta. Composting without PrP^{TSE} using an identical composting matrix was conducted in non-containment laboratories at the Agriculture and Agri-Food Canada Research Centre in Lethbridge, Alberta. The parellell composting experiment was conducted outside of containment and used to assess physicochemical compost parameters, due to restrictions associated with removing samples from level 3 biocontainment. Both experiments were initiated at the same time and conducted for the same duration. Passively aerated laboratory-scale composters were used in this study as described in Chapter 2 (Section 2.2.1). Six composters were used for the non-containment composting with triplicate composters per treatment. Four composters were used for the PrP^{TSE} composting with duplicate composters per treatment.

6.2.3 Composting setup

Compost substrates were prepared by mixing the initial components in a mortar mixer (12S, Crown construction equipment, Winnipeg, Canada) at the

feedlot of the Agriculture and Agri-Food Canada Research Centre in Lethbridge. For control compost, a mixture of 45 ± 0.1 kg fresh cattle manure (74.4% moisture) and 5 ± 0.1 kg white spruce (*Picea glauca*) wood shavings was placed in each composter. For feather enriched compost, chicken (*Gallus gallus*) feathers (5% wt wt^{-1}; dry basis) were substituted for a portion of the manure resulting in of 44 ± 0.1 kg cattle manure, 5 ± 0.1 kg wood shavings and 1 ± 0.1 kg feathers. The compost matrix was identical for both experiments under non-containment and containment conditions. Triplicate samples of fresh manure, wood shavings, chicken feathers and mixed matrix were collected before composting. All the samples were stored at 4°C for subsequent physicochemical analysis. The physicochemical properties of the compost ingredients used to generate the compost matrix are provided in Table 6-1.

6.2.4 Sampling procedures

For composting of PrP^{TSE}, fresh cattle manure (1.0 ± 0.1 g; dry basis) was shaped into spheres and dried at 30°C for 1 day to reduce moisture levels. Subsequently, 1 ml of 10% TSE BH was inoculated into the dried manure spheres, with each manure sphere containing one type of PrP^{TSE}. Manure spheres after inoculation with PrP^{TSE} were stored at -20°C for later detection of PrP^{TSE} by Western blotting (WB).

For sampling PrP^{TSE} during composting, inoculated spheres with PrP^{TSE} were sealed in 70 × 50 mm nylon bags (53 µm pore size; ANKOM Technology, Macedon, USA). The nylon bags along with 200 g of freshly mixed compost matrix were then placed within larger polyester mesh bags (100 × 100 mm; 5 mm

pore size), with each mesh bag containing one type of PrP^{TSE}. Polyester twine was attached to each mesh bag to enable recovery of the material during composting.

When each compost vessel was filled, triplicate PrP^{263K} mesh bags, and duplicate PrP^{CWD} and PrP^{BSE} mesh bags were placed at a depth of 0.3 m below the compost matrix surface, resulting in a total of seven bags in each composter. To determine if PrP^{TSE} can still be detected after short-duration exposure to compost, one PrP^{263K} bag was collected at day 2 prior to peak temperature. After 14 days of composting, three mesh bags with each bag containing PrP^{263K}, PrP^{CWD}, and PrP^{BSE} were randomly collected. After collecting the mesh bags on day 14, compost materials in each composter were emptied and the remaining matrix was mixed using a shovel. During the mixing process, sufficient water was added to the matrix to return it to the original moisture level measured on the first day of composting. Mixed and moistened compost was returned to the composter from which it originated for a second heating cycle. As the composters were refilled, the remaining PrP^{263K}, PrP^{CWD}, and PrP^{BSE} mesh bags originated from the first cycle were placed in each composter at the same depth as in the first composting cycle. After 28 day of composting, all the mesh bags were collected. Compost samples were also collected from each composter at day 14 after mixing, and also from each mesh bag after days 2, 14 and 28. All compost samples were stored at 4°C for later analyses of moisture and pH. Manure spheres were collected from nylon bags after composting, and stored at -20°C for analysis of WB.

Within the non-containment facility, the same sampling procedures were conducted for composting. To estimate the degradation of feathers in feather

enriched compost, chicken feathers (20 ± 0.1 g; wet basis) were weighed and sealed in the nylon bags. The nylon bags containing feathers along with compost matrix were in turn placed in larger mesh bags, which were placed in the feather composters. For control compost, freshly mixed compost matrix was placed in the mesh bags. Three bags were placed at a depth of 0.3 m below the matrix surface during the filling of each composter. One of the three mesh bags was randomly collected at days 2, 14 and 28. On day 14, compost was mixed and moistened in the same manner as described above. Compost samples were collected from each composter after mixing at day 14, and also from each mesh bag after days 2, 14 and 28. Compost samples were stored at 4°C prior to physicochemical analysis.

6.2.5 Compost properties

For composters outside of containment, compost temperatures and oxygen concentration (%) were measured at the same depth as the mesh bags were implanted using the methods from Chapter 2 (Section 2.2.3). Samples of the initial compost matrix and those collected from the mesh bags collected at days 2, 14 and 28, were analyzed for moisture, bulk density, total carbon (TC), total nitrogen (TN), pH, electrical conductivity (EC), and mineral N (NH_4^+ and NO_2^- + NO_3^-) as described in Chapter 2 (Section 2.2.3). For composting of PrP^{TSE}, only temperature, moisture, and pH of compost were measured due to restrictions associated with removing material from the biocontainment facility.

6.2.6 Degradation of feathers

The degradation of feathers in feather compost was estimated on the basis of dry matter disappearance from the nylon bags during the composting process. Dry matters of fresh chicken feathers were determined via drying triplicate samples (20 ± 0.1 g; wet basis) at 105°C for 3 days. Upon removal from the mesh bags, nylon bags containing degraded feathers collected at days 14 and 28 were rinsed with cold tap water until the water was clear and residual material was weighed after drying at 105°C for 3 days.

6.2.7 PrP^{TSE} extraction

The procedures used to extract PrP^{TSE}-contaminated manure were modified from the method described in Chapter 4 (Section 4.2.7). Briefly, 4.5 ml of 1% aqueous solution of sodium dodecyl sulphate (SDS; Sigma-Aldrich, Oakville, Canada) was added to each manure sphere inoculated with 1 ml of 10% PrP^{TSE} BH in a 50 ml Falcon conical centrifuge tube (Becton Dickinson). Subsequently, the tubes were vortexed (Fisher Scientific, Ottawa, Canada) at 3,000 rpm for 1 min and shaken on a Rocking Platform (Model 100; VWR, Edmonton, Canada) at 120 rpm for 1 h. Samples were centrifuged at 3,200 g for 20 min to release PrP^{TSE} from manure into SDS solution. To remove PrP^{C}, the supernatant (500 µl) was incubated with proteinase K (PK; 25 µg ml^{-1}) (Roche Diagnostics, Laval, Canada) at 37°C for 1 h. The PK digestion was terminated by the addition of Pefablock SC (Roche Diagnostics) to a final concentration of 2 mM. Sodium phosphotungstic acid (Sigma-Aldrich) precipitation was used to precipitate the extractable PrP^{TSE} from manure. After PK digestion, the supernatant was incubated with PTA (4%, w v^{-1}, in 170 mM $MgCl_2$, pH 7.4) at a final concentration of 0.3% (w v^{-1}),

followed by centrifugation at 17,950 g for 30 min. The pellet was resuspended in 200 µl of 1 × Laemmli's sampling buffer (Sigma-Aldrich) and heated to 100°C for 5 min for WB.

6.2.8 Western blotting

The procedures of WB were conducted in the same manner as previously detailed in Chapter 4 (Section 4.2.8). The mAb 3F4 (1:20,000 dilution; Human PrP109-112; Millipore) was used as the first antibody for the detection of PrP^{263K}, while mAb 6H4 (1:5,000 dilution; Ovine PrP148-157; Prionics, Zurich, Switzerland) was used for the detections of PrP^{CWD} and PrP^{BSE}. As an isotype control for the anti-PrP^{TSE} antibodies used, the first antibody was replaced with an antibody of the same isotype in the immunoblotting procedures. Thus, a commercial mouse monoclonal IgG2a antibody (Millipore) and mouse monoclonal IgG1 antibody (BD Biosciences, Mississauga, Canada) were selected for the substitutions of mAb 3F4 and mAb 6H4, respectively.

6.2.9 Sensitivity of Western blotting

To determine the sensitivity of WB on PrP^{TSE} extracted from manure, 10% hamster 263K, CWD and BSE BH were initially diluted in an equal volume of 10% non-infectious hamster, elk and cattle BH, respectively. A series of 5%, 2.5%, 1.25%, 0.625%, 0.32%, 0.16%, 0.08%, 0.04%, 0.02%, and 0.01% dilutions were generated for each type of PrP^{TSE}. The 10% to 0.01% dilutions of PrP^{TSE} BH were then inoculated onto manure spheres (1.0 ± 0.1 g; dry basis) and extracted for the analysis by WB. For the positive control, 10% PrP^{TSE} BH which was not

inoculated into manure spheres was subject to PK digestion at a final concentration of 25 μg ml^{-1} at 37°C for 1 h. The PK activity was stopped by Pefablock SC (2 mM final concentration). For the negative control, the manure inoculated with water was extracted, PK digested and PTA precipitated.

6.2.10 Detection of PrP^{TSE} before and after composting

To detect PrP^{TSE} before and after composting, manure spheres inoculated with PrP^{TSE} prepared before composting and manure speres collected after composting at days 14 and 28 were extracted by SDS for the subsequent analysis of WB.

Chapter 4 has suggested that the dynamic changes in manure properties over composting might reduce or inhibit the SDS extractability of PrP^{TSE} from compost. Therefore, as a control study, manure spheres without PrP^{TSE} inoculation were placed in composters prior to composting and collected after 14 and 28 days. Subsequently, these composted manure spheres were directly inoculated with 1 ml of 10% CWD or BSE BH and extracted by SDS to determine if PrP^{TSE} can be recoved and detected by WB.

6.2.11 Statistical analysis

The comparison of feather degradation between the first and second composting cycles were analyzed as a factorial design using the Mixed procedure (< 0.05 probability level) in SAS (2008). The changes of compost temperature and compost physicochemical parameters during composting were analyzed using the Mixed procedure of SAS with time treated as repeated measures, composters

treated as experiment units and mesh bags treated as replicates in the model. Main effects were considered to be statistically significant at a probability level of < 0.05.

6.3 Results

6.3.1 Temperature and oxygen profiles

During composting of PrP^{TSE}, temperature curves did not significantly differ between control and feather compost. Both types of compost heated rapidly in all replicate composters, with temperature peaking at 68°C in control compost and 66°C in feather compost after 3 days (Figure 6-1a). Subsequently, temperatures steadily declined but remained above 50°C for 3 days in control compost and for 2 days in feather compost. Upon mixing and moistening of compost on day 14, temperatures rapidly increased again in both composts (Figure 6-1a) with peak temperatures of 60°C and 56°C occurring on day 15 in control and feather compost, respectively. In the second composting cycle, temperatures remained above 50°C for 5 days in control compost and for 4 days in feather compost.

Temperature profiles of the composters used to assess physicochemical compost parameters (Figure 6-1b) were similar to those obtained in the composters in containment that were used to examine PrP^{TSE} (Figure 6-1a). Oxygen profiles (Figure 6-1c) reached levels as low as 1% at the beginning of composting, but had steadily increased to 17% in control and 18% in feather compost by day 3. As composting proceeded, oxygen concentration fluctuated remained between 18% and 20% in both compost types for the remainder of the composting period (Figure 6-1c).

6.3.2 Changes in compost properties

Compost moisture declined to a greater ($P < 0.05$) degree in the first heating cycle than in the second cycle, as the losses of moisture content were 25.8% and 7.3% within the first and second cycle, respectively (Table 6-2). Bulk density, TC content, and NH_4^+-N declined after each composting cycle. However, in the second cycle, the loss of TC in feather compost was greater ($P < 0.05$) than in control compost. Levels of TN declined ($P < 0.05$) in both compost types up to day 14, but subsequently remained constant in control compost and increased in feather compost over the remainder of the 28 day composting period. The pH of both control and feather compost tended to be similar, increasing ($P < 0.05$) over the first composting cycle and reaching ~9.2 after 28 days of composting (Table 6-2). Compost EC increased ($P < 0.05$) for each composting cycle, but declined after mixing and moistening of the compost. Concentrations of (NO_2^- + NO_3^-)-N were observed at a level below 5 mg kg^{-1} either at the start or the end of composting, but increased ($P < 0.05$) to ~10 mg kg^{-1} at the middle of composting on day 14.

Over 28 days of composting, inclusion of feathers in the matrix did not impact most physiochemical parameters with the exception that TN and NH_4^+-N were increased ($P < 0.05$) resulting in a reduction ($P < 0.05$) in C/N ratios (Table 6-2).

Under containment conditions, only the changes of moisture and pH were measured and shown in Table 6-3. Those results were comparable to the moisture

and pH observed in composters that were maintained outside of the containment facility (Table 6-2).

6.3.3 Degradation of feathers

Outside of containment, 12.6% of feathers were decomposed within the first composting cycle (Figure 6-2). However, more degradation of feathers ($P < 0.05$) occurred in the second cycle, with 27.9% of dry matter loss from the implanted nylon bags by the completion of the experiment.

6.3.4 Sensitivity of Western blotting

The sensitivities of WB on the detections of PrP^{263K}, PrP^{CWD}, and PrP^{BSE} extracted from manure are shown in Figures 6-3a, 6-3b, and 6-3c, respectively. These figures suggest that all types of PrP^{TSE} in manure substrate can be detected by WB after SDS extraction, allowing a detectable level after 2 $\log_{10}$ dilution for PrP^{263K}, 1-2 $\log_{10}$ dilution for PrP^{CWD}, and 1 $\log_{10}$ dilution for PrP^{BSE}.

6.3.5 Detection of PrP^{TSE} before and after composting

The reactivity of PrP^{TSE} detected by WB before and after composting was not related to the non-specific bindings of anti-PrP^{TSE} antibodies, as no signals were observed when isotype control antibodies were examined (Figure 6-4).

Prior to composting, positive WB signals for PrP^{263K}, PrP^{CWD}, and PrP^{BSE} were all detectable after extraction from manure spheres (Figures 6-5). After each composting cycle, decreasing levels of PrP^{TSE} were detected for all prion types. Signal intensity of PrP^{263K} was only reduced slightly after 2 days of composting (Figure 6-5a). However, by day 14, the signal PrP^{263K} was largely reduced in

control compost, but was absent in feather compost. After 28 days, PrP^{263K} reactivity was undetectable using mAb 3F4 in both compost types. Similarly, within 14 days, a reduction of PrP^{CWD} was observed with mAb 6H4, with no signal being detected in feather compost (Figure 6-5b). Upon completion of the experiment, PrP^{CWD} was not detected in either control or feather-enriched composter. After 14 days of composting, the signal intensities PrP^{BSE}were reduced slightly in control compost and completely absent in the other composters (Figure 6-5c). After 28 days, PrP^{BSE} was no longer detectable by WB in any of the composters.

6.4 Discussion

Infectious PrP^{TSE} are highly hydrophobic and aggregate into plaque-like complexes that exhibit a high affinity with soils, soil minerals, and sludge solids (Leita et al. 2006; Johnson et al. 2006; Kirchmayr et al. 2006). These adsorption characteristics may impose a challenge to highly sensitive and accurate quantification of PrP^{TSE} in soil and sludge. Previous studies (Seidel et al. 2007; Hinckley et al. 2008) have reported that a solution of SDS is effective and practical among various detergents to release soil- and sludge-bound PrP^{TSE} into solution for subsequent detection. However, composting owns a wide variety of biological, chemical and physical degradation processes that can more largely influence the adsorption and structure of PrP^{TSE} in compost than soil or sludge (Epstein 1997). The ability of SDS extraction to recover PrP^{TSE} in these complex substrates such as manure or compost has not been explored. Along with the results of a previous study (Chapter 4), I further confirmed that SDS can be

effectively used to extract scrapie, CWD and BSE from cattle manure and compost.

Prior to composting, this study showed the sensitivities of WB on extracted PrP^{TSE} from fresh manure ranked as $PrP^{263K} > PrP^{CWD} > PrP^{BSE}$. Three factors could have contributed to the different WB sensitivities among those prion strains: (1) varying concentrations of each type of PrP^{TSE} in the infectious brain tissues used; (2) strain-specific adsorption ability with manure that may affect the efficiency of SDS extraction; and (3) differing antibodies used and antibody affinities. After composting, all types of PrP^{TSE} exhibited a similar result that PrP^{TSE} was faintly or not detected in the extraction from composted manure spheres. The sensitivity results of WB (Figure 6-3) indicated that PrP^{TSE} signals were reduced after composting of 14 and 28 days, by at least 2 $\log_{10}$ for PrP^{263K}, 1-2 $\log_{10}$ for PrP^{CWD}, and 1 $\log_{10}$ for PrP^{BSE}.

Several possible mechanisms could explain the results of reduction or disappearance of PrP^{TSE} in compost. First, the dynamic changes in manure properties over composting might reduce or inhibit the extractability of PrP^{TSE} from compost, resulting in signals at or below the detection limit of WB despite the ongoing presence of PrP^{TSE}. Solution pH, ionic concentration and humic acids have been reported to influence PrP^{TSE} adsorption or promote PrP^{TSE} conformational changes in soil (Ma et al. 2007; Polano et al. 2008). In this study, physicochemical analysis suggested that the nature of manure properties were substantially altered after composting, including significant increases in compost pH and EC (Tables 6-2 and 6-3). Consequently, a control study was performed in

which manure spheres without PrP^{TSE} inoculation were placed in composters prior to composting. After 14 or 28 days, composted manure spheres were collected and directly inoculated with 10% CWD or BSE BH, followed by extraction procedures with analysis of WB. The results in Figures 6-5b and 6-5c showed that both PrP^{CWD} and PrP^{BSE} extracted from 14 or 28 days of composted manure in this control study were detectable at a similar level to the extraction from fresh manure. This offers evidence that the irreversible binding of PrP^{CWD} and PrP^{BSE} to humic acids or other organics in compost was not responsible for the temporal decline in the WB signal over the composting period.

Biological degradation of PrP^{TSE} by proteolytic enzymes in compost may have also been responsible for the decline in the WB signal with composting. Over 28 days, there was considerable evidence of microbial activity in compost as indicated by temperatures > 60°C in each composting cycle, 7 days of thermophilic composting, and significant declines in both TC and TN content. This suggests that the composters have developed an effective composting process, enabling this approach to sufficiently biodegrade a range of organic matter, possibly including recalcitrant PrP^{TSE}. At day 2, minor changes were observed in the physiochemical properties of manure (Tables 6-2 and 6-3) and the signal associated with PrP^{263K} remained prominant (Figure 6-5a), suggesting that microbial activity had not peaked by this point in time. However, manure properties as measured in all physicochemical parameters were substantially altered after 14 and 28 days of composting. In this study, I simulated a natural scenario in which PrP^{TSE} was inoculated into manure sphere to enable complete

contact with manure substrates during the composting process. Therefore, it may be concluded that the decline in PrP^{TSE} signal after composting was likely a result of biological degradation.

Infectious PrP^{CWD} and PrP^{BSE} have been shown to be more resistant to inactivation than other prions. For example, by incubating infectious BH in a PCR tube at 37°C, PrP^{CWD} was shown to have undergone less degradation after 35 days than hamster-adapted PrP^{TME} as indicated by WB (Saunders et al. 2008). After exposure to SDS, PrP^{BSE} was 10- and 10^6-fold more resistant to inactivation than CJD prions and hamster-adapted PrP^{Sc} via an infectivity titer in transgenic mouse, probably due more stable tertiary structure (Giles et al. 2008). In anaerobic sludge, PrP^{BSE} was also observed to be more stable than mouse-adapted PrP^{Sc} at 55°C after 12 days, by comparing the luminescence reduction levels from WB (Kirchmayr et al. 2006). Although different degrees of degradation among various prion types cannot be distinguished, the more recalcitrant nature of PrP^{CWD} and PrP^{BSE} observed from previous literatures adds the values of my results showing composting could be promising to inactivate CWD and BSE at least 1-2 $\log_{10}$ level.

In this experiment, higher ($P < 0.05$) TN and NH_4^+-N contents were observed in feather compost than control compost. However, feather inclusion did not lower the composting performance as reflected by temperature profiles. In agreement with the hypothesis, at day 14, PrP^{TSE} signals were detectable in control compost, while no signals were observed in feather compost. This suggested that feather addition might promote the proteolysis of PrP^{TSE}. More

feathers ($P < 0.05$) were degraded in the second cycle than the first cycle (Figure 6-2), a result that may be indicative of an increase in keratinase activity during the latter stages of composting. The increased keratinase activity in the second cycle could further enhance the degradation of PrP^{TSE} in feather compost, although this trend was not detected at day 28 as no WB signals were observed. To futher address my hypothesis, the level of keratinase activity and its effectiveness against PrP^{TSE} in feather compost needs to be examined.

Immunoblotting technique is a rapid and convenient way of determining the level of PrP^{TSE}, but it is always limited by a dynamic range of ~100-fold (Giles et al. 2008). Moreover, detergent of SDS has relatively low PrP^{TSE} recoveries in the extraction of PrP^{TSE} from environmental samples, with 61-67% recovery in wastewater sludge (Hinckley et al. 2008) and 5-40% in sandy and clay soils (Cook et al. 2007; Cook and Shaw et al. 2007). Therefore, these method limitations with dilution effect may result in an insuffieicnt sensitivity of WB (i.e., 1-2 $\log_{10}$) for the detection of PrP^{TSE} in compost. Possible improvements for the detectability of PrP^{TSE} in compost would be to use *in vitro* methods, such as PMCA and cell culture assay, which have improved the detectability of PrP^{Sc} and PrP^{263K} in soil and biosolids (Seidel et al. 2007; Nagaoka et al. 2010; Miles et al. 2011). Infectious PrP^{263K} were used in this experiment with the intention of being analyzed by PMCA. Currently, PrP^{263K} samples from this study are being analyzed by PMCA in the laboratories of Dr. Norman Neumann at the University of Alberta.

Animal bioassays should be further employed to determine the extent to which composting may reduce prion infectivity. However, bioassay of PrP^{TSE} from compost still presents a significant challenge, as animals may not be susceptible to infection as a result of structural changes or the binding of PrP^{TSE} to organic components in compost. Moreover, a wide range of toxic chemicals and pathogens produced in the compost also could affect the survival of the rodents during bioassay. Currently, our research group is examining a stainless steel bead method for bioassay, adopted from Flechsig et al. (2001). In this method, PrP^{TSE} are bound to a stainless steel bead and then exposed to the compost environment for biodegradation. The bead-bound method not only may reduce the possibilities of PrP^{TSE} binding to components in compost, but it also may be convenient for subsequent decontamination and inoculation into the animals.

6.5 Conclusions

In this chapter, at least 1-2 $\log_{10}$ reduction of PrP^{CWD} and PrP^{BSE} signals was observed by WB after composting, probably as a result of biological degradation. However, a high level (i.e., > 1-2 $\log_{10}$) of PrP^{TSE} degradation in compost is possible, since reduction levels as measured by WB were below the detectable limit associatated with this assay. Inclusion of feathers may have enriched the matrix with proteases capable of increasing the degradation of both PrP^{CWD} and PrP^{BSE} in compost. These fundamental but novel outcomes incease the confidence that the degradation of PrP^{TSE} maybe more extensive with full-scale composting as the duration of the thermophilic period is longer and microbial activity is more intense.

Currently, CFIA (2010) have published a regulation on the limited use of composting for the disposal of SRM by issuing a temporary permit. The contributions to knowledge in this area will open perspective avenues for CFIA to reconsider the current regulations and accelerate the adoption of this practice to be used for the disposal of SRM.

Table 6-1 Physicochemical characteristics of the materials included in the compost matrix

Parameters*	Cattle manure	Wood shavings	Chicken feathers
Moisture (%)	74.4 ± 3.0	9.1 ± 0.1	7.6 ± 0.1
Bulk density (kg m^{-3})	772 ± 26	89 ± 3	61 ± 3
Total carbon (%)	43.3 ± 0.2	51.7 ± 0.7	50.5 ± 1.3
Total nitrogen (%)	2.7 ± 0.1	0.2 ± 0.1	15.4 ± 0.4
C/N ratio	16.0 ± 0.2	311.9 ± 74.5	3.3 ± 0.1
pH	7.96 ± 0.03	4.23 ± 0.02	5.92 ± 0.05
EC (ds m^{-1})	1.31 ± 0.17	0.05 ± 0.02	1.09 ± 0.10
NH_4–N (mg kg^{-1})	2649 ± 953	9 ± 2	1525 ± 128
NO_x–N (mg kg^{-1})	3.8 ± 1.0	5.1 ± 0.2	1.9 ± 0.2

*All parameters except moisture and bulk density are expressed on a dry weight basis (wt wt^{-1}). Moisture and bulk density are expressed on a wet weight basis. EC: electrical conductivity.

Table 6-2 Physicochemical changes of compost mixtures during composting

Parameters*	Day 0		Day 2		Day 14		Day 14 (after mixing)		Day 28		Test of main effects and their interaction§
	C†	F‡	C	F	C	F	C	F	C	F	
Moisture (%)	60.7	62.3	60.7	61.5	32.3	39.1	62.2	64.4	53.9	58.2	Time
Bulk density (kg m^{-3})	468	468	494	488	371	397	405	431	428	425	Time
Total carbon (%)	45.2a	46.0a	45.5a	46.0a	44.6a	44.7a	44.3a	44.7a	44.2a	42.5b	Compost type×Time
Total nitrogen (%)	2.2	2.8	2.2	2.9	1.9	2.5	2.1	2.6	2.1	3.1	Time, Compost type
C/N ratio	21.1	16.4	21.3	15.9	23.2	17.7	21.0	17.2	20.9	13.6	Time, Compost type
pH	8.26	8.15	8.27	8.44	9.25	9.05	8.86	8.79	9.25	9.17	Time
EC (ds m^{-1})	1.66	1.61	1.64	1.59	2.13	1.98	1.31	1.36	1.61	1.56	Time
NH_4–N (mg kg^{-1})	1118	1719	2526	2398	329	834	547	959	97	110	Time, Compost type
NO_x–N (mg kg^{-1})	3.1	3.1	3.3	3.1	5.4	7.0	8.6	31.2	2.7	1.9	Time

*All parameters except moisture and bulk density are expressed on a dry weight basis (wt wt^{-1}). Moisture and bulk density are expressed on a wet weight basis. EC: electrical conductivity.
†C: control compost.
‡F: feather compost.
§Within a column, main effects of time, compost type and their interaction which are at $P < 0.05$ level are listed.
a, b: Within a row, values followed by different letters differ ($P < 0.05$) between compost types on the same sampling day.

Table 6-3 Moisture (%) and pH changes of compost mixtures during composting under biocontainment

Parameters[*]	Day 0		Day 2		Day 14		Day 14 (after mixing)		Day 28		Test of main effects and their interaction[§]
	C[†]	F[‡]	C	F	C	F	C	F	C	F	
Moisture (%)	60.7	62.3	59.7	56.3	40.1	35.7	64.3	63.2	54.0	49.5	Time
pH	8.26	8.15	8.96	9.14	8.90	9.65	9.17	9.47	9.61	9.65	Time

[*]Moisture is expressed on a wet weight basis.
[†]C: control compost.
[‡]F: feather compost.
[§]Within a column, main effects of time, compost type and their interaction which are at $P < 0.05$ level are listed.

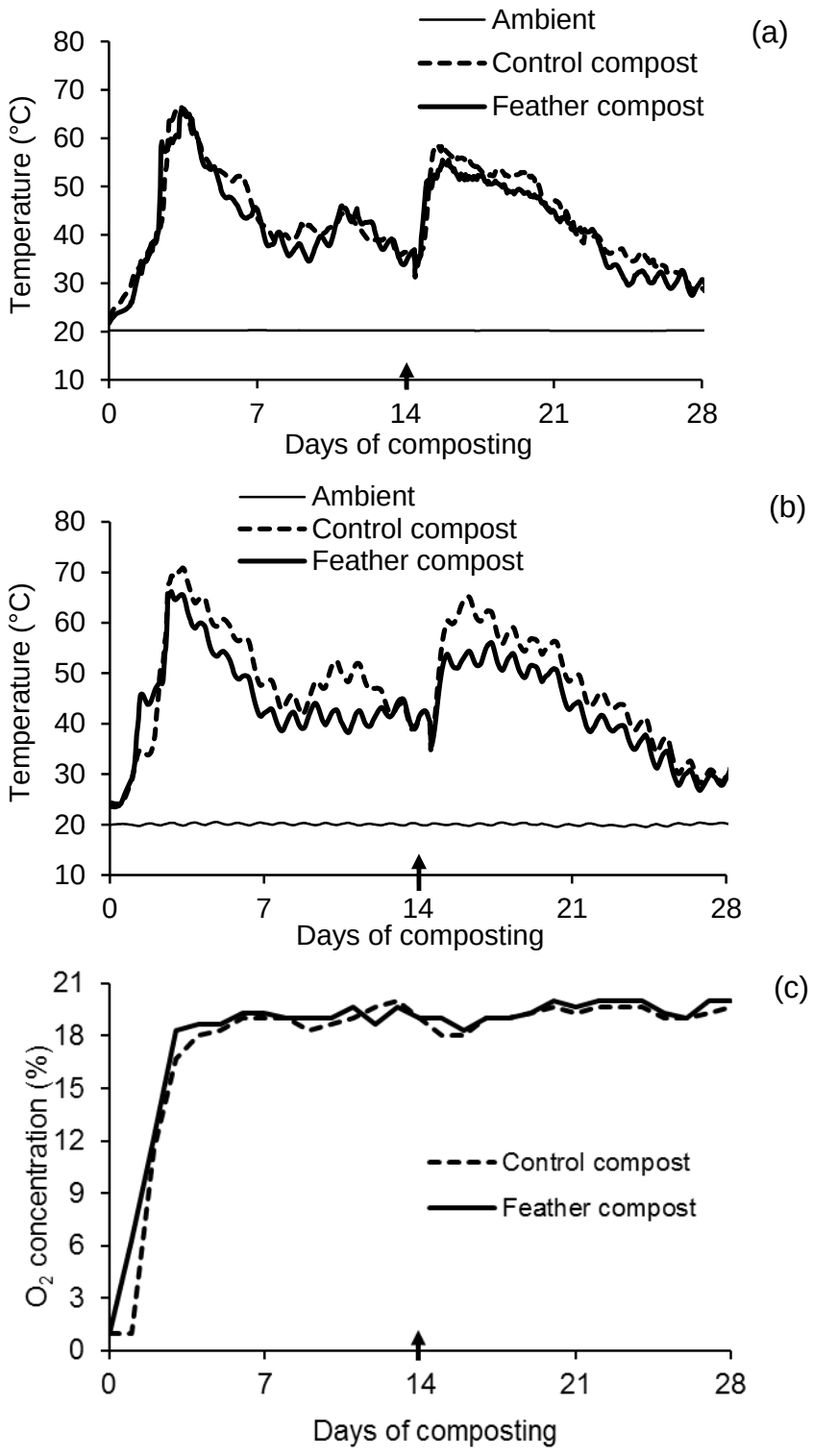

Figure 6-1 (a) Temperature during composting under containement (b) Temperature and (c) O_2 concentration during composting outside of containment. Arrows indicate the date compost was mixed.

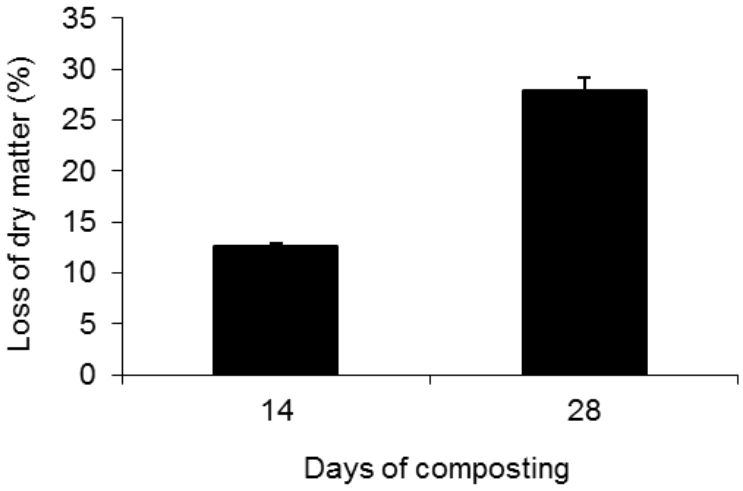

Figure 6-2 Degradations of feathers (% dry matter loss) in feather compost during composting.

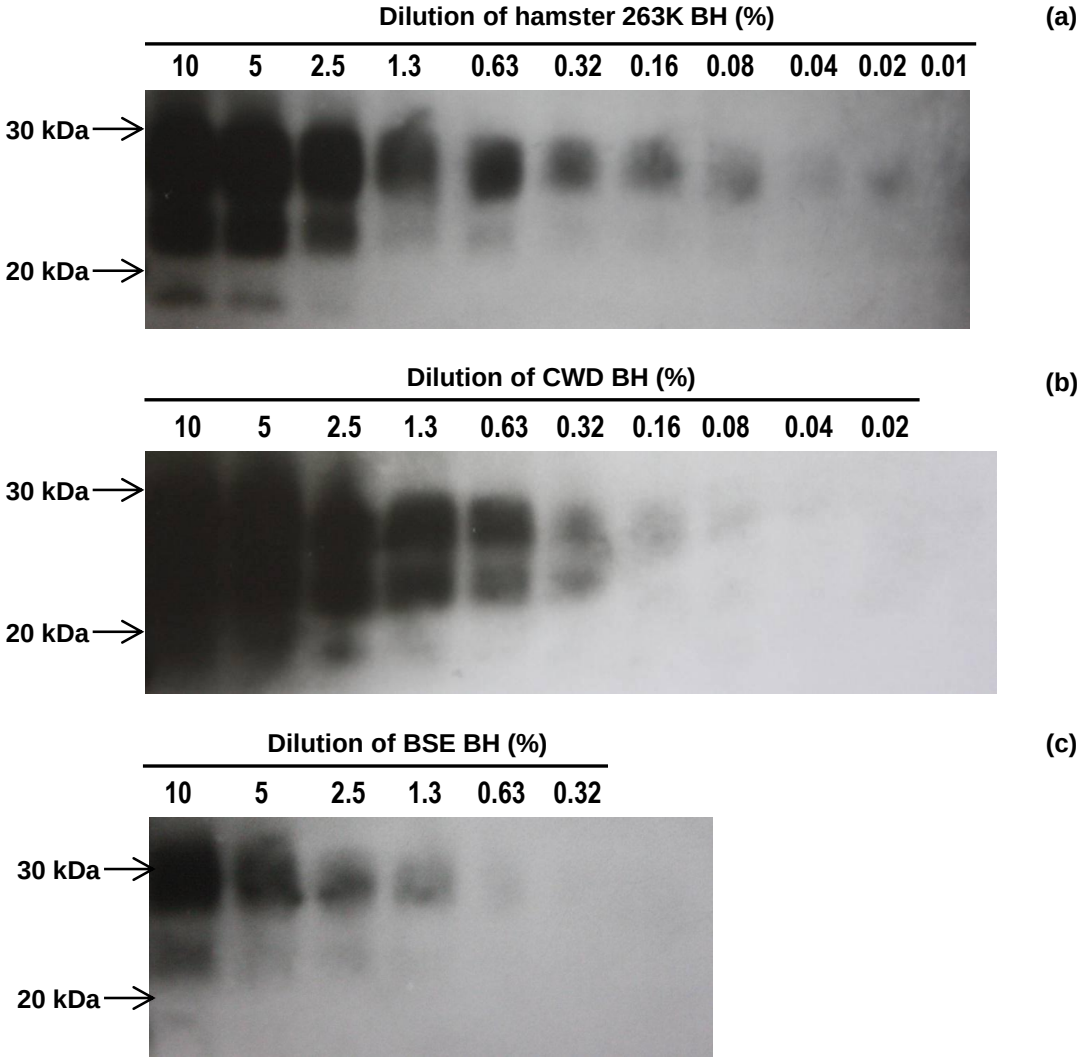

Figure 6-3 Western blotting (WB) of (a) PrP^{263K} with mAb 3F4, (b) PrP^{CWD} with mAb 6H4, and (c) PrP^{BSE} with mAb 6H4 extracted from fresh manure spheres inoculated with decreasing concentrations of hamster 263K, chronic wasting disease and bovine spongiform encephalopathy brain homogenates, respectively. The extractable PrP^{263K}, PrP^{CWD}, and PrP^{BSE} were proteinase K digested and precipitated with phosphotungstic acid prior to WB.

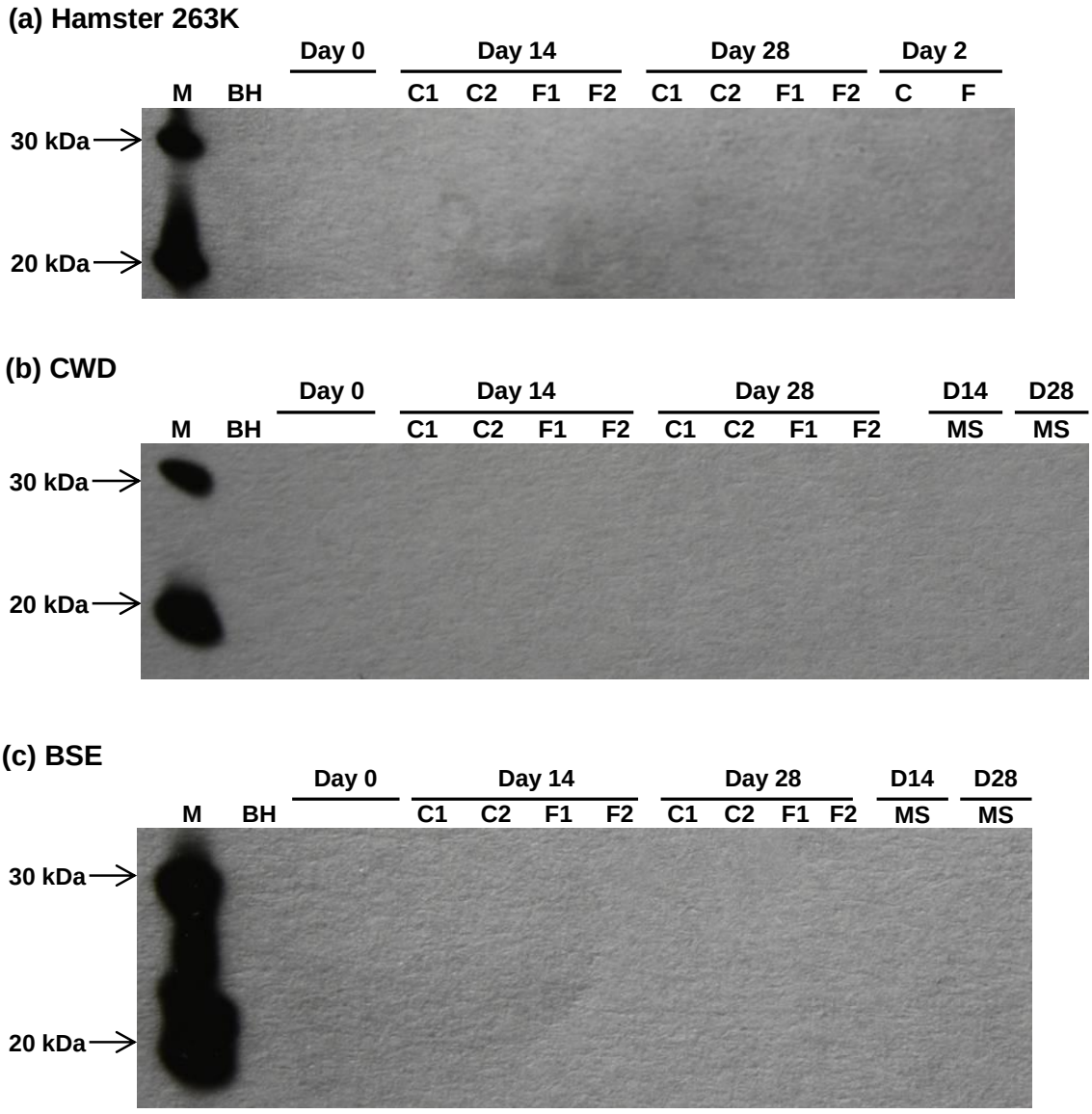

Figure 6-4 Western blotting (WB) of (a) PrP^{263K}, (b) PrP^{CWD}, and (c) PrP^{BSE} samples extracted from manure spheres collected at days 0, 2, 14, and 28 with isotype control antibodies instead of anti-PrP^{TSE} antibodies. M: Reference marker; BH: 10% infectious brain homogenate was PK digested; C: Control compost; F: Feather compost; C1-C2: duplicate control composters; F1-F2: duplicate feather composters; MS_D14 and MS_D28: manure spheres without PrP^{TSE} inoculation were composted for 14 and 28 days, and then collected and directly inoculated with PrP^{CWD} or PrP^{BSE}, followed by extraction and WB analysis. All extractable PrP^{263K}, PrP^{CWD}, and PrP^{BSE} samples were proteinase K digested and precipitated using phosphotungstic acid prior to WB.

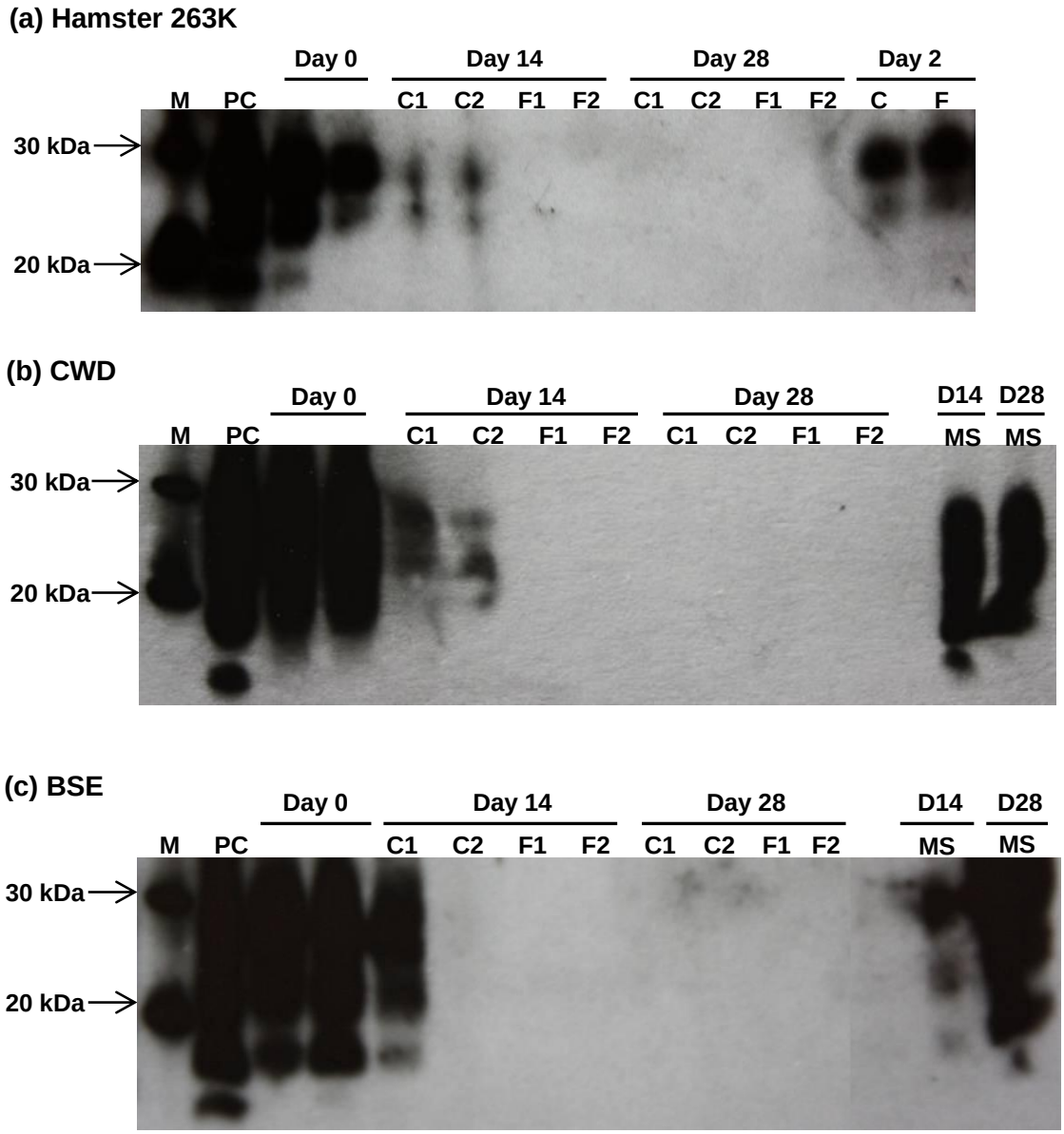

Figure 6-5 Western blotting (WB) of (a) PrP^{263K}, (b) PrP^{CWD}, and (c) PrP^{BSE} samples extracted from manure spheres collected at days 0, 2, 14, and 28 with anti-PrP^{TSE} antibodies. M: Reference marker; PC (positive control): 10% infectious brain homogenate was PK digested as a positive control; C: Control compost; F: Feather compost; C1-C2: duplicate control composters; F1-F2: duplicate feather composters; MS_D14 and MS_D28: manure spheres without PrP^{TSE} inoculation were composted for 14 and 28 days, and then collected and directly inoculated with PrP^{CWD} or PrP^{BSE}, followed by extraction and WB analysis. All extractable PrP^{263K}, PrP^{CWD}, and PrP^{BSE} samples were proteinase K digested and precipitated using phosphotungstic acid prior to WB.

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Chapter 7 General discussion and conclusions

7.1 Overview of results and their significance

Composting may be a viable alternative to rendering and land filling for the disposal of SRM, provided that PrP^{TSE} are inactivated. However, to test this hypothesis using field-scale composting of SRM containing PrP^{TSE} or carcasses confirmed to harbor TSE is challenging. Therefore, the present thesis undertook the development of a 110-L passively aerated laboratory-scale composting model that could be used to assess PrP^{TSE} degradation under containment conditions. The results obtained in this thesis open a promising perspective for composting as a disposal method of SRM and serve as a "stepping stone" to field-scale research on PrP^{TSE} degradation in compost.

7.1.1 Effective specified risk material degradation

In this thesis, fresh bovine brain tissues from mortalities under 30 months of age were used as representative samples of SRM. Studies on composting of SRM in Chapters 2, 3 and 4 suggested that the degradation of SRM during composting was rapid, with approximately 60% to 80% of dry matter being degraded after 14 and 28 days of composting, respectively. Physicochemical analyses conducted over 28 days confirmed that composting conditions were within optimal parameters for the effective decomposition of organic matter in compost (Rynk 1992). It is clear that compost depth, types of bulking agents, and attempts at prolonging thermophilic temperatures via a water jacket did not significantly impact SRM degradation (Chapters 2 and 3). However, Chapter 4 further

demonstrated that an increase in the extent of SRM degradation was induced by mixing feathers in the manure matrix. Xu et al. (2009) reported that SRM was degraded more rapidly (> 90% in first 7 days) in a field scale composting model as compared to the laboratory-scale composters used in the current study. Although the feasibility for composting of cattle carcasses or SRM on a field scale has been demonstrated (Stanford et al. 2007; Hao et al. 2009), the results from this study on SRM composting revealed that the laboratory-scale composters and composting methods developed were appropriate as a model for examining the degradation of PrP^{TSE} under containment conditions.

7.1.2 Degradation of PrP^{Sc}, PrP^{CWD} and PrP^{BSE}

To simulate a natural scenario in which PrP^{TSE} was completely exposed to the compost environment throughout the composting process, PrP^{TSE} brain homogenates were inoculated into manure spheres and then placed in the composters over 28 days within a level 3 biocontainment facility. Effective methods of SDS extraction plus PTA precipitation following the WB analysis were described in Chapter 4 for the detection of PrP^{Sc} and subsequently for PrP^{263K}, PrP^{CWD}, and PrP^{BSE} (Chapter 6) in compost. Prior to composting, positive WB signals were detectable for all four types of PrP^{TSE} in fresh manure. After 14 days, PrP^{263K}, PrP^{CWD} and PrP^{BSE} were faintly detected, but no signals were observed for PrP^{Sc}. By the completion of the experiment, all types of PrP^{TSE} were not detected by WB. The sensitivity of WB suggested that the reductions of PrP^{TSE} signals observed after composting were at logarithmic levels, with at least 2 $\log_{10}$ for PrP^{263K}, 1-2 $\log_{10}$ for PrP^{Sc} and PrP^{CWD}, and 1 $\log_{10}$ for PrP^{BSE}.

Over 28 days, composting temperature exceeded and remained thermophilic for 7 days and exhibited a decline in both TC and TN contents (Chapter 6). These results all suggest that the composters were active to biodegrade a range of organic matter, including possibly recalcitrant PrP^{TSE}. After 2 days of composting, minor changes were observed in physicochemical properties of manure, while the WB signal associated with PrP^{263K} remained prominent (Chapter 6). This suggests that microbial activity had not peaked by this point time. However, when manure physicochemical properties were substantially changed at days 14 and 28, the signals of PrP^{263K} were also faint or not detected. In this thesis, PrP^{TSE} were inoculated into manure spheres to enable complete contact with manure during composting. Therefore, the changes in manure properties may reflect that the decline of PrP^{TSE} signal was a result of biological degradation. Although the degradation of PrP^{Sc} cannot be definitively concluded (Chapter 4), the reduction of PrP^{CWD} and PrP^{BSE} after composting could reflect biological degradation (Chapter 6).

To date, no studies have been reported on the possible degradation of PrP^{CWD} and PrP^{BSE} in compost. Composting is an exceedingly complex ecosystem, involving a wide variety of biological, chemical and physical degradation processes that can influence the adsorption, structure and detection of PrP^{TSE} in compost (Polano et al. 2008; Saunders et al. 2008). Previous researchers have also described that PrP^{CWD} and PrP^{BSE} are more resistant to inactivation than other prions, including PrP^{CJD} and hamster-adapted PrP^{Sc} (Giles et al. 2008; Saunders et al. 2008). Therefore, the recalcitrant nature of PrP^{CWD} and PrP^{BSE} clearly

underline the significance of this work on the characterization of possible CWD and BSE degradation in compost.

7.1.3 Microbes involved in specified risk material degradation and possible degradation of PrP^{TSE}

The degradation of SRM in compost depends on complex microbial consortia that are naturally present within the composting environment (Berge et al. 2009). In this study, the period of rapid SRM degradation corresponded with an equally rapid increase in the microbial biomass at the thermophilic stage as indicated by PLFA profiles (Chapter 2). Analyses of PCR-DGGE in Chapters 3 and 5 revealed that a wide variety of bacteria and fungi were responsible for SRM degradation in compost. However, the statistical analyses of DGGE gels showed that the bacterial and fungal communities associated with the degradation of SRM differed from those involved in the degradation of manure. It was further observed that the bacterial community involved in manure degradation was more diverse than that involved in degrading SRM, while SRM was colonized by a more diverse fungal community than manure (Chapter 5). Specified risk material contains a large fraction of labile protein and fat (Mcllwain and Bachelard 1985). However, manure contains a variety of proteins, lipids, starch, cellulose, hemicellulose, lignin and humic acids (Epstein 1997). Therefore, these complex components in manure may have required more diverse bacteria to decompose. In addition, the high fat content (11% fresh weight basis; Mcllwain and Bachelard 1985) of SRM may have made it a more suitable substrate for fungi than bacteria,

as fungi can more easily attack organic residues in compost that are too dry for bacteria (Ryckeboer et al. 2003).

In this thesis, active heating and enrichment with feathers were assessed for their ability to increase SRM degradation during composting. Chapter 3 demonstrated that active heating did not appear to have a major impact on the extent of SRM degradation. However, inclusion of feathers in compost not only enhanced SRM degradation, but also appeared to alter bacterial or fungal populations (Chapter 5). Bohacz and Korniłłowicz-Kowalska (2007) demonstrated that the presence of feathers in compost stimulated the growth of keratinolytic fungi responsible for the degradation of feather keratin during composting. Moreover, some keratinolytic fungi, like *Doratomyces microspores*, not only degrade keratin, but also hydrolysze other types of proteins including casein, bovine serum albumin, and elastin (Fredrich and Kern, 2003). Therefore, as outlined in Chapter 5, it might be expected that some special keratinolytic microbes with the ability to degrade a wide range of proteins in feather compost could have enhanced SRM degradation.

Further sequencing analysis of DGGE bands indicated that bacteria related to the genera of *Corynebacterium*, *Saccharomonospora*, *Promicromonospora*, *Thermobifida*, *Thermoactinomycetaceae*, *Thiohalospira*, *Pseudomonas*, *Actinomadura*, *Mycobacterium*, *Nocardia*, *Streptomyces*, and *Enterobacter*, and fungi related to the genera of *Dothideomycetes*, *Cladosporium*, *Chaetomium*, and *Trichaptum*, appear to represent potential candidates that are involved in SRM degradation in compost.

Many studies have been performed to examine the susceptibility of PrP^{TSE} to proteolysis. Some serine proteinases have appeared to be among the most active in degrading PrP^{TSE}, and they are listed in Table 1-3 (Chapter 1). Moreover, some commercially available proteinases termed "prionases" are being considered as a decontaminant for for prions (Dickinson et al. 2009; Georgieva et al. 2004; Saunders et al. 2010). These proteinases with superior ability to inactivate PrP^{TSE} have been shown to be produced from bacteria belonging to the genera of *Bacillus*, *Streptomyces*, *Nocardiopsis, Thermus*, and *Thermosipho*, and from fungi belonging to the genera of *Tritirachium* (Table 1-3; Chapter 1). Theses taxa have been found in compost (Ryckeboer et al. 2003) a finding that I have confirmed for some of these microbes using PCR-DGGE in Chapter 3. In addition, Chapter 5 also revealed that some microbes associated with SRM degradation, such as the fungal genus of *Dothideomycetes* and bacterial genus of *Actinomadura*, have been `previously confirmed to be capable of degrading bison carcasses and bovine hooves (Reeb et al. 2011; Puhl et al. 2009). Therefore, the evidence above increases the likelihood that the reduction of PrP^{TSE} and the loss of SRM dry matter observed after composting were due to enzymatic degradation as a result of these complex microbial communities in compost.

The microbial studies on the composting of SRM described in this thesis add value for characterization of bacterial and fungal species that are fastidious to culture but may possess the capacity to degrade SRM in compost. Characterization of these microbes enhanced the understanding of the microbial

populations that may participate in the degradation of SRM and possible PrP^{TSE} during composting.

7.1.4 Advantages of feather addition

Feather keratin has also been previously used to mimic the degradation of prions (Suzuki et al. 2006; Gilroyed et al., 2010), as it has some structural similarities to PrP^{TSE}. Suzuki et al. (2006) also demonstrated that keratinases that have the capacity to degrade feathers may degrade PrP^{TSE}. Thus, it was hypothesized that inclusion of feathers in compost may promote the production of enzymes (i.e., non-specific proteolytic and keratinolytic) that would improve the degradation of SRM and PrP^{TSE} (Chapters 4 and 6).

In this thesis, 5% (wt wt^{-1}; dry basis) of chicken feathers were added into the control compost matrices along with cattle manure and wood shavings. Mixing of feathers with cattle manure did not alter the physicochemical properties of compost other than increasing total nitrogen content, but as hypothesized, tended to enhance SRM degradation (Chapter 4). After composting of $PrP^{TSE}$for 14 days (Chapter 6), faint WB signals were observed for PrP^{263K}, PrP^{CWD} and PrP^{BSE} in control compost, but no signals were observed from PrP^{TSE} in feather compost. This suggests that inclusion of feathers may have promoted the proteolysis of PrP^{TSE} in the first composting cycle. However, at day 28, PrP^{263K}, PrP^{CWD} and PrP^{BSE} were not detected by WB in either control or feather compost. In Chapter 6, the loss of dry matter from feathers showed that more feathers ($P < 0.05$) were degraded in feather-enriched compost in the second cycle than the first cycle, indicative of an increase in keratinase activity. This increased keratinase activity

in the second cycle may have further enhanced the degradation of PrP^{TSE} in feather compost, although this trend was not detected by WB at day 28 as no signals were evident.

Feathers are a significant waste by-product of the poultry industry (Ichida et al. 2001; Korniłłowicz-Kowalska and Bohacz 2010). Composting has been proposed as a viable and effective option for the disposal of feathers (Bohacz and Korniłłowicz-Kowalska 2007; Lloyd-Jones et al. 2010). Thus, my results suggested that inclusion of feathers in compost may have merit, not only from the perspective of enhancing the degradation of SRM and PrP^{TSE} but also through contributing to the disposal of this waste by-product. Although Chapter 5 suggested that inclusion of feathers in compost may increase greenhouse gas emissions, other feather disposal methods such as rendering, landfilling and incineration, may result in the release of similar or even higher levels of greenhouse gases. In addition, the agronomic value of compost may be increased as the mineral N content of compost was increased by 12.5% in compost fortified with feathers (Chapter 6).

7.2 Future directions

The work presented in this thesis was conducted to examine the degradation of SRM and PrP^{TSE} in laboratory-scale composters and characterize the microbial community and species involved in the process of SRM degradation. These experiments provide a fundamental but significant framework for examining the possibility that composting can be an alternative for the disposal of SRM infected

with PrP^{TSE}. However, to provide more convincing evidence for addressing this question, there are a number of future experiments that need to be performed.

7.2.1 Quantification of PrP^{TSE} in compost

The experiments from Chapters 4 and 6 showed that the WB signals for all types of PrP^{TSE} were not detectable after 28 days of composting. Due to the insufficient sensitivity of WB (i.e., 1-2 $\log_{10}$), I am unable to discern the degree of biological degradation of PrP^{TSE} as it may have still been present at levels below the detectable limit associated with this assay. Therefore, significant interest remains in developing methods that could precisely quantify PrP^{TSE} in compost. A possible improvement would be to use *in vitro* methods, such as PMCA and cell culture assay, which have improved the detectability of PrP^{Sc} and PrP^{263K} in soil and biosolids (Seidel et al. 2007; Nagaoka et al. 2010; Miles et al. 2011; Genovesi et al. 2007).

In Chapter 6, PrP^{263K} was used with the intention of being analyzed by PMCA. This work is currently being carried out in the laboratories of Dr. Norman Neumann at the University of Alberta. In addition, by testing the compost materials collected from the vicinity of PrP^{TSE} tissues and compost leachate, PMCA may be also used for examining the mobility of degraded PrP^{TSE} during composting.

7.2.2 PrP^{TSE} infectivity in compost

Animal bioassays remain the golden standard for testing TSE infectivity (Barron et al. 2007). Following the measurements from WB and PMCA, animal

bioassays should be further employed to determine the extent to which composting may reduce prion infectivity.

Seidel et al. (2007) have measured the infectivity of PrP^{263K} in soil using Syrian hamsters through oral application of soil materials containing PrP^{263K}, demonstrating that PrP^{263K} can persist in soil for over 29 months. This previous study might be used as a model for examining the reduction of PrP^{263K} infectivity in samples from experiment described in Chapter 6. Moreover, research should also be devoted to determine the infectivity of CWD and BSE in compost. Currently, bioassays of PrP^{CWD} and PrP^{BSE} from animal tissue are being developed. The infectivity of BSE in brain tissue can be determined by a bioassay using transgenic mice that are highly susceptible to PrP^{BSE}, such as T4092 mice from Giles et al. (2008) or Tg63 mice currently being used in our research team. Haley et al. (2009) also utilized 5037 transgenic mice in a bioassay to measure PrP^{CWD} infectivity in cervid nerve and lymph tissues. Therefore, these transgenic mouse models may have the potential candidates to be employed in future bioassays targeted at assessing the infectivity of PrP^{CWD} and PrP^{BSE} after composting.

Soil-bound PrP^{TSE} has been shown to remain infectious via the oral route of exposure, but PrP^{TSE} binding to soil can increase prion disease infectivity, enhancing the efficiency of oral transmission (Johnson et al. 2007). However, bioassay of PrP^{TSE} from compost still presents significant a challenge, as animals may not be susceptible to be infected by PrP^{TSE} in compost as a result of structural changes of PrP^{TSE} induced by biological degradation or the binding of PrP^{TSE} to

more complex compost components. In addition, toxic chemicals and pathogens in compost materials could affect the survival of the animals used in bioassay. A stainless steel bead method is being examined for bioassay in our research group, which is adopted from Flechsig et al. (2001). In this method, PrP^{TSE} are bound to a stainless steel bead and then placed in compost for biodegradation. The bead method may have several advantages, including reducing the likelihood of PrP^{TSE} binding to the components in compost and being convenient for subsequent decontamination and inoculation into the animals.

7.2.3 Characterization of microbes or enzymes effective to degrade PrP^{TSE}

In Chapter 6, possible degradation of CWD and BSE in the composters suggested that composting could be a potential reservoir of microbes or enzymes with the ability to inactivate PrP^{TSE}. Several methods could be used for characterizing these microbes and enzymes. To begin with, PCR-DGGE with sequencing might be used to further characterize the bacteria or fungi with the potential to degrade PrP^{TSE}, by extracting DNA from compost that was intentionally spiked with PrP^{TSE}.

Furthermore, Chapter 6 showed that feather-enrichment may enhance PrP^{TSE} degradation in compost. This finding deserves more research in order to characterize the keratinolytic bacteria and proteases that exhibit activity against PrP^{TSE}. Puhl et al. (2009) have isolated a novel actinobacterial species (i.e., *Actinomadura keratinilytica* sp. nov.) with the ability to degrade recalcitrant hoof keratin from cattle compost. Similar approaches might be employed to isolate novel keratinolytic bacterial species from feather-enriched compost. Further

purifying and characterizing the keratinases from these species are feasible, as similar studies have been previously done (Xie et al. 2010; Sharaf and Khalil 2011). Subsequently, these candidate keratinolytic bacteria and keratinases can be incubated with PrP^{TSE} infected tissue to further examine their ability to degrade PrP^{TSE}.

Another more advanced method may be derived from culture-independent metagenomic or metatranscriptomic approaches. A metatranscriptomic approach to investigate the functional diversity of the eukaryotic microorganisms within the rumen has been used by Qi et al. (2011) who characterized some lignocellulolytic enzymes with the ability to degrade plant cell wall. Therefore, by extracting and sequencing the compost RNA, metatranscriptomic methods may be used to examine the diversity of bacterial or fungal communities in feather-enriched compost and identify transcripts that potentially encode for proteases capable of degrading PrP^{TSE}.

7.2.4 Inoculation of microbes in compost

Inoculating microbes into the composting substrates has been used to increase the efficiency of the composting process. For example, Wei et al. (2007) reported that inoculation of a mixture of microorganisms (i.e., *Bacillus casei, Lactobacillus buchneri,* and *Candida rugopelliculosa*) into municipal solid waste compost resulted in a greater degree of aromatization of humic acids. Ichida et al. (2001) inoculated feather-degrading *Bacillus licheniformis* and *Streptomyces sp.* into a compost matrix containing poultry manure and feathers, and observed a higher degradation of feathers than the uninoculated treatment.

However, some studies also suggest that microbial inoculants do not affect the degradation of organic matter in compost. For example, Lloyd-Jones et al. (2010) reported that the degradation of poultry carcasses and feathers in compost, as indicated by dry matter loss, were not altered by inoculating feather-degrading bacteria belonging to diverse genera of *Chryseobacterium, Rhodococcus, Bacillus, Pseudomonas, Stenotrophomonas,* and *Achromobacter*.

The review presented in Chapter 1 indicates that some microbial species have been shown to have the capacity to degrade PrP^{TSE} (Table 1-3). Most of these species are culturable or commercially available. Inoculation of these microbes into the compost may hold promise for enhancing the degradation of SRM and PrP^{TSE}, but needs to be further investigated.

7.2.5 Inactivation of PrP^{TSE} in field-scale composting

It is likely that large-scale composting may result in more degradation of PrP^{TSE} than lab-scale composting. Along with the studies in the laboratory-scale composting models, characterization of PrP^{TSE} inactivation in field-scale composting is desired.

Xu et al. (2009) have developed an appropriate biosecure system for field-scale composting to dispose of cattle mortalities and inactivate environmental pathogens. A similar composting system could be considered as a model for examining the inactivation of PrP^{TSE} at field scale. In addition, the variation of PrP^{TSE} inactivation between replicated composting trials or compost piles should also be quantified.

7.3 Summary and conclusions

Composting may be a viable alternative for the disposal of the large volumes of SRM and animal carcasses arising from farms, meat processing plants and road kills as a control measure while still minimizing the risk of spreading TSE diseases. Currently, CFIA (2010) have published a regulation on the limited use of composting for the disposal of SRM by issuing a temporary permit. Therefore, to confirm the validity of this regulation and accelerate the adoption of this practice, demonstrating the inactivation of PrP^{TSE} during composting has become increasingly important.

This thesis describes utilization of a laboratory-scale composter to assess the degradation of SRM and PrP^{TSE} over 28 days. The microbial communities degrading SRM in compost were also investigated. A number of bacteria and fungi that may possess the capacity to degrade SRM or possible PrP^{TSE} were characterized. After these studies, significant outcomes that can be summarized from this thesis include:

1. Approximately 80% of SRM were effectively degraded after 28 days of composting.

2. At least 1-2 $\log_{10}$ reductions of PrP^{CWD} and PrP^{BSE} signals were indicated by WB analysis after 14 days of composting and possibly higher than this level after 28 days. These could be associated with biological degradation.

3. Feather inclusion enhanced both SRM and PrP^{TSE} degradation possibly by enriching proteolytic bacteria in the compost.

4. Analyses of PCR-DGGE revealed that a wide variety of bacteria and fungi were responsible for SRM and possible PrP^{TSE} degradation in compost.

5. Some bacteria and fungi with the ability to degrade SRM and PrP^{TSE}, belonging to the genera of *Actinomadura*, *Streptomyces*, and *Dothideomycetes*, were characterized from the composters by PCR-DGGE.

Due to the infectivity of prions, logarithmic reduction of PrP^{TSE} in contaminated materials is necessary to ensure that infectious prions do not enter the environment (Appel et al. 2001). The outcomes from this thesis increase confidence that the degradation of SRM infected PrP^{TSE} maybe more extensive with full-scale composting as the duration of the thermophilic period is longer and microbial activity is more intense. Moreover, contributions to the knowledge of SRM composting open a more promising perspective for CFIA to reconsider the current regulations that are in place with regard to the disposal of SRM.

However, the criteria for 100% inactivation of PrP^{TSE} by composting are difficult and unrealistic to achieve. The European Food Safety Agency (2005) recommended that an effective method can be approved to inactivation of pathogens if it meets minimum criteria, such as 5 $\log_{10}$ reductions in the number of non-spore-forming pathogenic bacteria, parasites, and non-thermoresistant viruses; 3 $\log_{10}$ reductions in infectivity titer of thermoresistant viruses; and 3 $\log_{10}$ reductions in the number of parasites. Therefore, for assessments of composting systems to inactivate PrP^{TSE}, a relative risk reduction or a risk limit might need to be defined. More important, further investigations on the inactivation of prion infectivity by composting are required.

7.4 References

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