Biodegradation (2011) 22:1029–1043
DOI 10.1007/s10532-011-9461-5

ORIGINAL PAPER

Biodegradation of specified risk material and characterization of actinobacterial communities in laboratory-scale composters

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Received: 28 October 2010 / Accepted: 12 February 2011 / Published online: 1 March 2011
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Abstract As a result of bovine spongiform encephalopathy in Canada, specific tissues at risk of harbouring prions are not allowed to enter the food chain. Composting may be a viable alternative to rendering and land filling for the disposal of specified risk material (SRM). Two types of laboratory-scale composters, actively-heated and ambient systems were constructed to assess the biodegradation of SRM over 30 days. A second heating cycle was generated by mixing the compost after 15 days. Compared to ambient composters, temperature profiles in actively-heated composters were above 50°C for 5 and 4 days longer in the first and second composting cycles, respectively. Degradation of SRM was similar between two composter types during two composting cycles, averaging 52.2% in the first cycle and 43.9% in second cycle. Denaturing gradient gel electrophoresis (DGGE) revealed that changes in the actinobacteria populations in the first composting cycle were of a temporal nature, whereas alterations in populations in the second composting cycle were more related to active heating of compost. Sequencing of the dominant DGGE bands showed the predominance of *Corynebacterium*, *Promicromonospora*, *Pseudonocardia*, and *Thermobifida* in the first composting cycle and *Corynebacterium*, *Mycobacterium, Nocardia, Saccharomonospora*, and *Streptomyces* in the second composting cycle. Active heating can alter the nature of actinobacteria populations in compost, but does not appear to have a major impact on the extent of degradation of SRM.

Keywords Specified risk material (SRM) · Laboratory-scale composter · Biodegradation · Actinobacteria · Denaturing gradient gel electrophoresis (DGGE)

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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^{Sc}), 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).

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 Milke 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 (Berge et al. 2009; Xu et al. 2010). 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, 2003) Our research group has also previously isolated a novel keratinolytic actinobacteria from a composting environment (Puhl et al. 2009). In addition, Hui et al. (2004) isolated a PrP^{Sc}-degrading enzyme from culture medium of *Streptomyces* sp. and an alkaliphilic actinobacterial isolate capable of degrading PrP^{Sc} 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. We also examined the nature of actinobacterial communities using DGGE due to the potential important role that these bacteria may play in the degradation of recalcitrant proteins. Our 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.

Materials and methods

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. Composters were constructed of polyethylene (Fig. 1a; 110-l cylinders; 0.7 m × 0.45 m, 1601 M; Eagle, Wellsburg, WV). The surface of the vessels was insulated with 50 mm of polyurethane foam with a

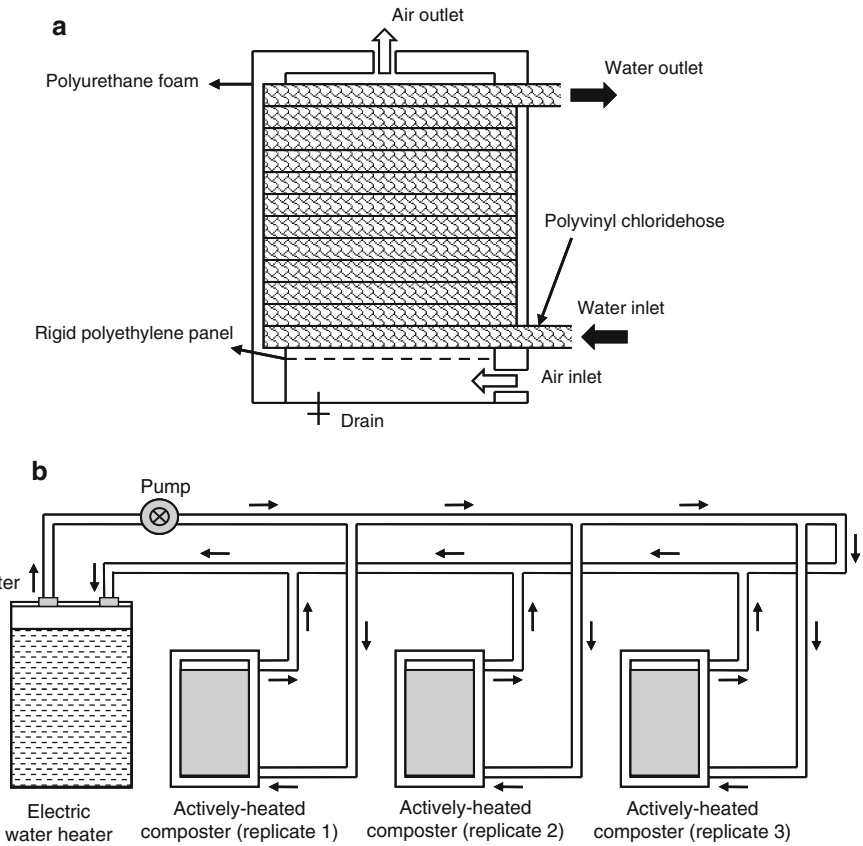

Fig. 1 **a** Schematics of a 110-l actively-heated laboratory-scale composter and **b** the water circulation system

density of 32 kg m^{-3} and a thermal resistance value of 1.2 km^2/W. For the purpose of passive aeration, an air plenum (0.1 m height) was created at the bottom of the composters using rigid polyethylene panels of 5 mm thickness (Model 2615; Sissiboo Farm Supplies, Weymouth, NS) perforated with 10 mm diameter holes at a spacing of 25 mm. Inlet and outlet air holes (25 mm diameter) were drilled at the bottom and the top of the composters to enable passive ventilation. Any leachate formed during composting was removed periodically through drains (15 mm diameter) at the bottom of each composter.

For actively-heated composters, the vessels were surrounded with 15 m of polyvinyl chloride (PVC) hoses (20 mm diameter; K3150; Kuriyama, Schaumburg, IL). The hoses were connected to a water circulation system (Fig. 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).

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 1. The remaining mixed matrix were freeze-dried and frozen at −40°C for later extraction of DNA for use in denaturing gradient gel electrophoresis (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;

Table 1 Characteristics of initial materials used for compost experiment

Materials	Moisture (%)	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 moisture, which is on a wet weight basis

[a] Values are cited from Xu et al. (2010). *NM* not measured

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.

Biodegradation of SRM and compost properties

The degradation of SRM was estimated on the basis of dry matter disappearance from the nylon bags during the composting process. Before composting, dry matter of fresh brain tissue was determined via drying triplicate samples at 105°C for 3 days. Upon removal from the mesh bags, nylon bags containing SRM were immediately rinsed with cold tap water, placed in a glass beaker and reweighed after 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, AB). Temperature was measured at the same three depths as the mesh bags were implanted. Oxygen concentration at each depth was measured twice daily using an oxygen monitor (Model OT-21; Demista Instruments, Arlington Heights, IL).

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. Moisture contents of 100 g samples were determined after drying for 5 days at 60°C (Larney and Olson 2006). Dried samples were ground through a 2 mm screen with additional subsamples (5 g) being ground to <150 μm for analysis of TC and TN using an automated CNS analyzer (NA2100; Carlo Erba Strumentazione, Rodano, Milan, Italy).

Compost pH was determined after mixing 25 g of compost with 50 ml of distilled water and shaking for 1 h. Samples were centrifuged at 10,000×*g* for 15 min and the extract was used to measure electrical conductivity (Model 125; Orion, Beverly, MA). Mineral N (NH_4^+ and $NO_2^- + NO_3^-$) was measured using an auto analyzer (AutoAnalyzer III, Bran + Luebbe GmbH, Norderstedt, Germany). Wood shavings samples were analyzed in a manner similar to the manure and compost, except that a 1:10 ratio of wood shavings to distilled water was used.

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 (Phillips 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. DNA from control isolates was isolated using a DNeasy tissue kit (Qiagen, Mississauga, ON) following the manufacturer's protocol for Gram positive bacteria.

PCR-DGGE and sequencing analysis

For all extracted DNA, fragments of actinobacterial 16S rRNA gene were amplified using a nested PCR approach. In the first-round PCR, a template DNA (40 ng) was amplified with the specific forward primer F243 and reverse primer R1378 (Heuer et al. 1997). The total reaction volume was 50 µl with each reaction containing 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) 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 using primers F984GC and R1378 (Heuer et al. 1997). The second-round PCR was conducted in the same manner as described above.

DGGE using the DCode™ Universal Mutation System (Bio-Rad, Hercules, CA) was performed by loading PCR products (500 ng) onto a polyacrylamide gel containing 6% (v/v) acrylamide-bisacrylamide, 0.09% tetramethylenediamine (v/v), and 0.09% ammonium persulfate (w/v). 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) 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.

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.

Results

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 (Fig. 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 (Fig. 2a). During the second cycle, peak temperatures occurred on day 19 (47°C)

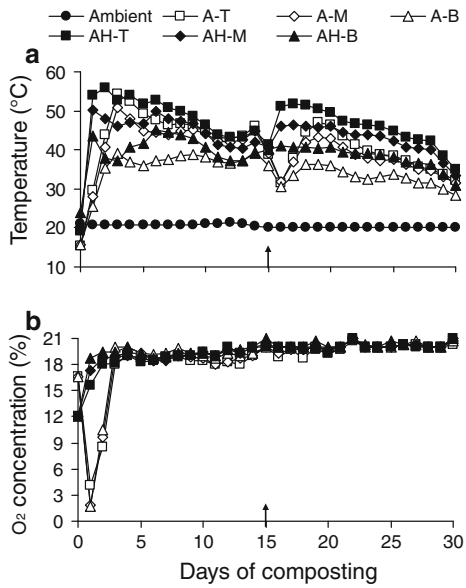

Fig. 2 **a** Temperature and **b** O_2 concentration during composting of specified risk material in ambient (A) and actively-heated (AH) laboratory-scale composters. *T* top depth, *M* middle depth, *B* bottom depth. *Arrows* indicate the date compost was mixed

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 (Fig. 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.

Changes of physicochemical properties

Moisture contents remained relatively constant during the initial composting cycle, but declined in both

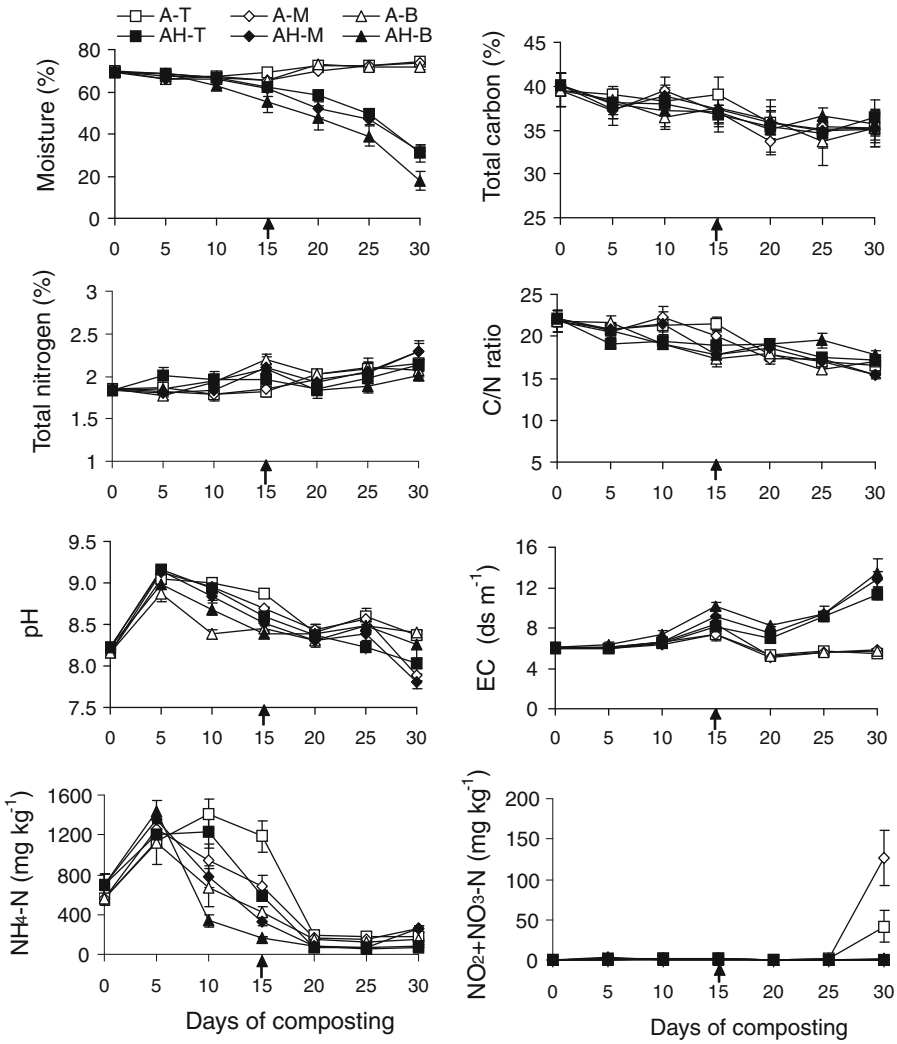

Fig. 3 Physicochemical changes during composting of specified risk material in ambient (A) and actively-heated (AH) laboratory-scale composters. *T* top depth, *M* middle depth, *B* bottom depth. *Arrows* indicate the date compost was mixed

compost types during the second composting cycle, with the decline being more rapid in actively-heated compost (Fig. 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.

Biodegradation of SRM

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 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 (Fig. 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 (Fig. 4b). The extent of SRM degradation also did not statistically differ between the first and second composting cycle.

Table 2 ANOVA (analysis of variance) table for effects of compost types, compost depths and compost time on the biodegradation of SRM

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 $P < 0.05$

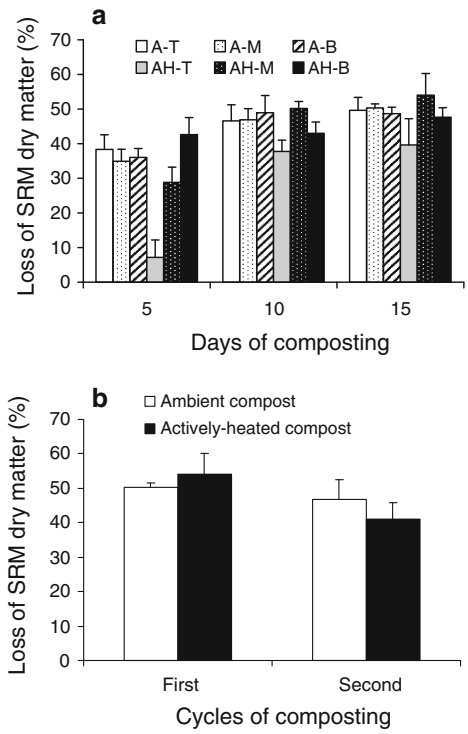

Fig. 4 **a** Degradation of specified risk materials (% dry matter loss) during the first composting cycle in laboratory-scale composters. *A-T* top depth of ambient compost, *A-M* middle depth of ambient compost, *A-B* bottom depth of ambient compost, *AH-T* top depth of actively-heated compost, *AH-M*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

PCR-DGGE and sequencing analysis

Preliminary experiments using three replicate PCR products from all the compost samples showed the consistent band patterns of DGGE profile among the replicate samples (data not shown). 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 (Fig. 5a). 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

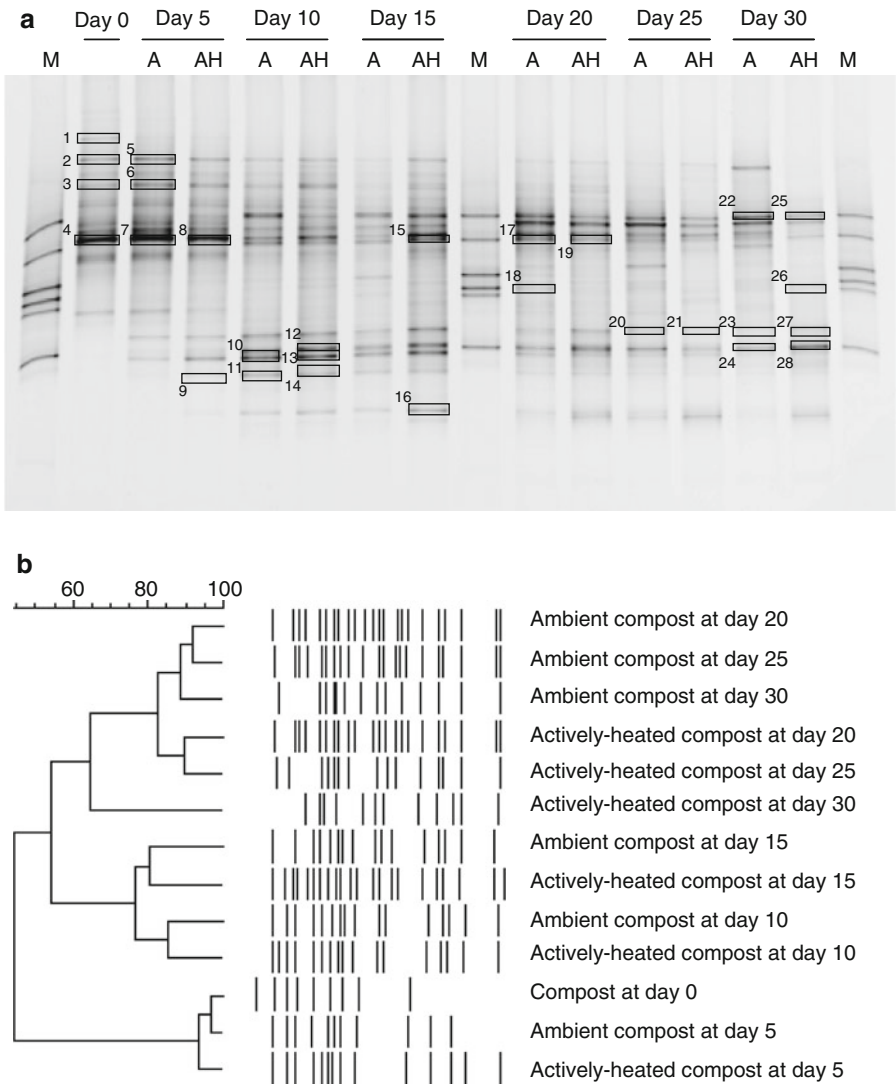

Fig. 5 **a** Denaturing gradient gel electrophoresis separation of 16S rRNA gene fragments after PCR with actinobacteria-specific primers F243-R1378 and F984GC-R1378 from different types of composter and composting times and **b** corresponding dendrogram using Pearson's correlation index and unweighted pair group method with arithmetic average. Marked bands were excised and sequenced. *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*

revealed that the DGGE profiles clustered on the basis of time of sampling in first composting cycle (Fig. 5b) 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 (Fig. 5b).

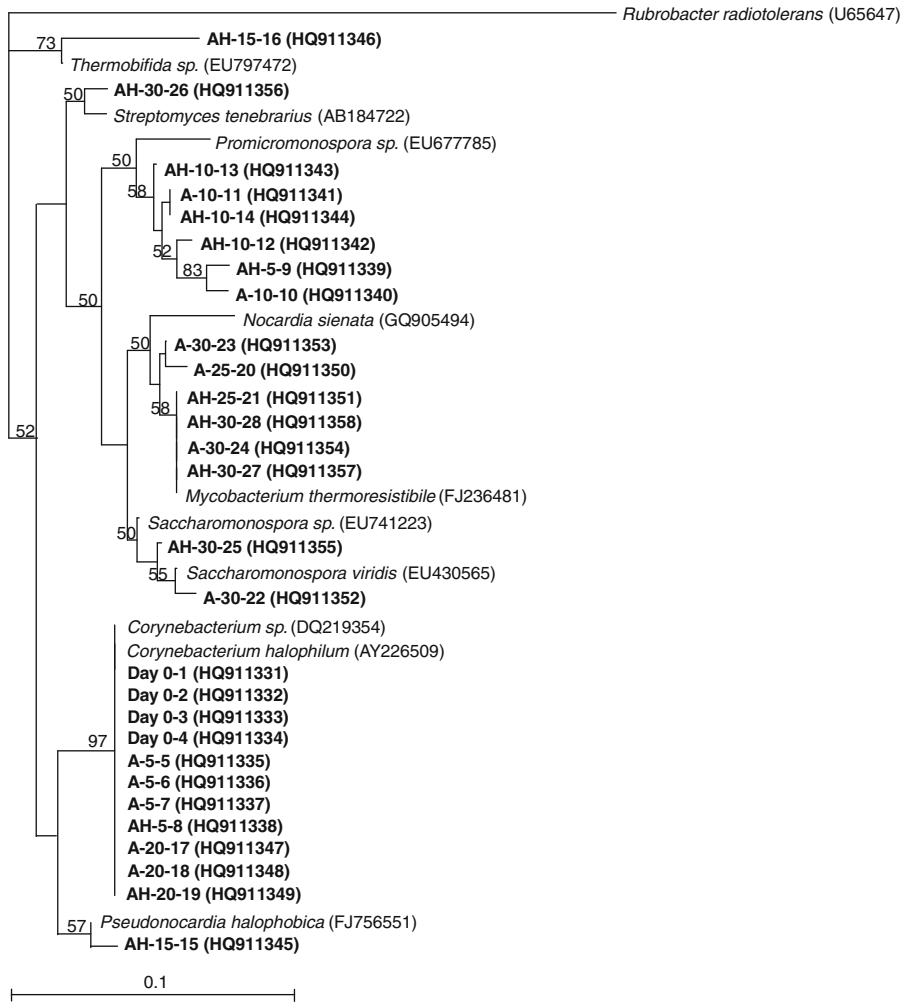

Fig. 6 A Neighbour joining tree of phylogenetic relationships between DNA sequences obtained during composting (see Fig. 4) of specified risk material and species within the class *Actinobacteria* (accession numbers of the sequences retrieved from the database are enclosed in *parentheses*). The *scale 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

Sequencing all of the dominant bands generated the Neighbor joining tree in Fig. 6. 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.

Discussion

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 our study, ambient compost experienced 2 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, 2.4 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 oxygen was being consumed by the microbes via aerobic respiration. A phenomena that we have observed in a previous study using a similar composting system (Xu et al. 2010). However, active-heating resulted in oxygen concentrations being 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 5 days of composting oxygen levels remained above 19% indicating that oxygen availability did not limit the composting process.

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 (Xu et al. 2010). 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, 2010) 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 our 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).

Biodegradation of SRM

Close to 50% of SRM dry matter disappeared within 15 days of composting, similar to that observed in a previous experiment in our laboratory (Xu et al. 2010). 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 our 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 phenomena 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.

PCR-DGGE and sequencing analysis

DGGE 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 a previous study in our lab in which phospholipids fatty acid profiling was used to assess the development of actinobacteria populations (Xu et al. 2010). 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 of our experiments, 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 our 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 our 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.

Conclusions

We can conclude that active heating of laboratory-scale composters constructed in our 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}.

Acknowledgments This project was conducted with funding from the Prion Inactivation and Environment project, from the Alberta Prion Research Institute and the Specified Risk Material Disposal Program of Agriculture and Agri-Food Canada. The technical assistance of F. Van Herk, B. Baker, S. Cook, L. Selinger, and W. Smart are much appreciated. Direction from Reive Pumping and Heating Ltd. in Lethbridge with regard to the design and construction of the water circulation piping system was also invaluable. The authors also acknowledge Ben's Meat in Lethbridge for the supply of bovine brain tissue from cattle under 30 months of age.

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