

# Report to the Manitoba Rural Adaptation Council

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## Use of on-farm composting to dispose of Johne's infected cattle

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## INTRODUCTION

Composting can be used as a means to inactivate pathogenic bacteria. Most of the work on inactivating bacteria has been on municipal waste, manure, and garden refuse and much less information is available for composting of livestock mortalities. Compared to other methods for disposal of livestock mortalities, composting has proven to be an inexpensive, safe and an environmentally sustainable way to eliminate disease causing organisms that may be present on-farm (13)(10). Most of the studies on bacterial pathogens has been done on *E. coli* and *Salmonella* but almost no work has been done with recalcitrant bacterial like *Mycobacterium avium* subspecies *paratuberculosis* (MAP).

MAP causes Johne's disease (JD) in cattle and has become more problematic in recent years because it is difficult to detect and can survive in the environment for long periods of time. The disease has a long incubation period and in its early stages, animals are asymptomatic with intermittent shedding of MAP in milk and feces. A recent study has shown that 4.5% of dairy and 1.7% of beef cattle in Manitoba test positive for MAP (11). But it is estimated that between 68% of dairy and 29% of beef herds have at least one animal testing positive (11). Presently there is no diagnostic test with specificities or sensitivities accurate enough to detect MAP shed at such low numbers or during intermittent shedding in early stages of the disease (8).

Some studies have suggested MAP may be a potential agent responsible for causing Crohn's disease in humans (12)(5). Although there is conflicting research about the etiology of the disease, studies have shown that MAP has survived pasteurization in milk products (3), which may be a possible route for human acquisition. Another possible cause of infection

sought out by researchers, is through meat products contaminated from infected animals entering the food chain (2)(1), although one study positively identified MAP in ground beef at the retail level (6). Most infected animals are culled on the basis of a loss in production levels, generally before the disease becomes symptomatic, and shipped to auction marts for slaughter rather than just disposed of on-farm. It is this cycle and prevalence of MAP in specific food products that has lead many to research its possible role in Crohn's disease establishment.

Johne's infected animals also have a significant impact at the economic level. A study of losses directly affecting milk yield, premature culling, mortality and reproductive losses from Johne's infected animals estimates that it would cost \$2,462 per annum for a 50 head herd (4). A similar study estimated that it costs the US Dairy Industry \$27 per animal and up to \$250 million per annum (9). Voluntary control programs have already been implemented in some countries (7) but more strict programs may become mandatory if a zoonotic connection is verified. This begs the question of how producers can eliminate this bacterium in a safe, environmentally friendly and cost-effective way.

Recently (13), an biosecure mortalities static composting system for large scale disposal cattle was developed. In this system it could be demonstrated that bacterial pathogens lost viability, but MAP was not tested. The purpose of this study is to determine whether static composting of cattle mortalities can inactivate MAP. Piles were constructed, and samples consisting of natural and lab-cultured MAP were allowed to compost for 250 days. Samples were removed at five separate intervals and the DNA analyzed with polymerase chain reaction (PCR) to determine survival. A secondary experiment was conducted *in vitro* to determine

whether MAP is inactivated solely by temperature or through the composting process. Tissues were sampled at nine separate intervals and again the DNA analyzed with PCR.

## **Experiment #1**

### **Biocontainment structures**

On March 6 and 7 2008, two biocontainment composting structures (Pile 1 and Pile 2) were constructed at the Glenlea Research Facility south of Winnipeg, Manitoba. Each structure dimensions were 2500 cm length by 440 cm width by 240 cm height (Figure 1A and 1B) built on top of a concrete pad. The outer walls were made of large barley straw bales, with the dimensions 260 cm length by 90 cm width by 120 cm height, into a rectangular shape. Smaller straw bales, with dimensions 90 cm length by 45 cm width by 45 cm height were used to create the floor of the piles. The inside of each structure was lined with heavy black silage plastic. Weeping tile was used to drain leachate into a stainless steel kitchen sink in the middle of the straw floor. Wire mesh was placed on top of the sink to prevent large particles from blocking the drain. A hose attached to the bottom of the sink ran underneath the pile to the outer length of the structure and used as a leachate sampling port (Figure 1A and 1B). Within the structure, loose straw was spread on the bottom of the plastic sheeting to form a layer about 60 cm thick. Three cull JD negative Holstein cows were purchased from the Glenlea Dairy Unit, euthanized on site and then laid on top of the straw layer, head to tail aligned along the length of the pile. Next, a layer of mixed warmed cattle manure 120 cm thick overlaid the carcasses. Flexible perforated air tubing was embedded in between each carcass in the straw with air vents at the ends of the tubes passing through the silage plastic to allow for passive aeration of the compost

(Figure 1A and 1B). The silage plastic was sealed and old tires were placed on top to prevent the plastic from opening and excess moisture seeping in.

### **Bacterial inoculation**

The design of the composting experiment is outlined in Table 2, with the use of four treatments. The first treatment included as part of this experiment, was a control using extra lean ground beef. Ground beef was purchased from Safeway in Winnipeg, MB and used as the control as well as the substrate for our lab-cultured bacteria. About 15 grams was then placed into either a heat sealed nylon bag, to determine if the biochemical factors in compost were responsible for inactivation, or into a plastic Whirlpak bag, to assess if heat was the principle factor.

The second and third treatments were lab-cultured MAP (ATCC strain) and lab-cultured *Mycobacterium smegmatis* (ATCC strain) both inoculated into ground beef. Three strains of MAP were each grown in 500 ml of Middlebrook 7H9 broth with OADC (oleic acid, albumin, dextrose and catalase powder) enrichment and mycobactin J (growth factor for MAP). All three strains were then mixed together and dispersed into 50 ml centrifuge tubes. One strain of *M. smegmatis* was grown in plates on Middlebrook 7H10 agar with ADC (albumin, dextrose and catalase powder) and mycobactin J supplement. One well-separated colony was then grown in an eight ml centrifuge tube in Middlebrook 7H9 broth with OADC enrichment and mycobactin J supplement, for a total of 40 tubes. *M. smegmatis* was included because it is a fast-growing, non-pathogenic mycobacteria thought to parallel the survival and growth of MAP. Both strains were allowed to grow for four to six weeks and resulting growth was recorded and verified with Mycobacteria Growth Indicator Tubes (MGIT). MGIT tubes positive for MAP fluoresce under

UV light. At the time of inoculation, the broth was centrifuged for 10 min. at 3,000 x g and the supernatant decanted leaving 1-2 ml at the bottom of the tube to make a slurry with the pellet. The cell slurry was then inoculated into lean ground beef, hand massaging the mixture for approximately five minutes to ensure thorough mixing. Once mixed 15 grams of inoculated ground beef was added to either a nylon bag or a plastic bag.

The final treatment that we included was naturally infected MAP positive tissues from Johne's infected cattle. Six animals that tested positive for MAP through a fecal ELISA test were slaughtered and the terminal ileum and mesenteric lymph nodes were couriered from the University of Saskatchewan. The tissue was ground together in a food processor and 15 grams placed into either nylon or plastic bag for a total of 40 bags.

Degradation and inactivation of the samples in the nylon and plastic bags were investigated using special pyramid-shaped retrievable cages called Baker Retrieval Pyramids (BRP) (Figure 1C). Dimensions of each cage had a base of 210 mm<sup>2</sup> and a height of 310 mm. Manure was used to pack eight samples (each treatment within each bag type) into a BRP in a manner so that there was no contact between the bags. Two BRP's within the vicinity of each cow were suspended at varying depths from a stainless steel cable anchored to wooden poles at 1.5 m intervals along the length of the pile (Figure 1A and 1B). A total of five pyramids in Pile 1 and six pyramids in Pile 2 were embedded as each structure was filled with manure.

### **Extraction of samples and disinfection of equipment**

Removal of the BRP's from each compost structure occurred at five different time intervals. For extraction, the stainless steel cable holding the suspended BRP was unclamped

from the 4x4 pole and reattached to the front-end loader of a tractor while leaving the plastic covering in tact. One BRP per structure was extracted at days 35, 67, 96, 131 and 250.

Safety precautions were established during structure construction, sample extraction and deconstruction to ensure that MAP did not proliferate throughout the research facility. Any persons working with materials around and within each of the composting structures had to wear protective attire that included biohazard suits, latex gloves, goggles, dust masks and boot covers. Any equipment, such as tractors, backhoes and extendable highhoe, used on site needed to be pressure washed then disinfected with a 10% bleach solution. During structure deconstruction, all plastic products (silage plastic, weeping tile, perforated air tubes, etc.) were disposed of at the Brady Landfill and the remaining compost was taken to the prearranged disposal location (field location at Glenlea).

Leachate disposal also had precautionary measures taken to avoid any additional contamination. It was not know if the leachate contained MAP so when the liquid was drained out of the sinks from the hose (Figure 1A) and into 10 L biohazard jugs, a 10% bleach solution was mixed with the leachate. After one hour of exposure to the bleach solution, the mixture was poured into a cistern at the Glenlea Research Facility and treated as septic waste.

Precautionary measures with excess leachate seepage from the compost structures were also addressed. Any seepage areas were covered with wood shavings to allow excess moisture to be soaked up and then the area was disinfected with a 10% bleach solution. Wood shavings were disposed of at the same location as the compost.

## **Temperature monitoring**

Ambient and inner composting temperatures were monitored for each compost structure. At each cow within each structure, three type-T thermocouples were embedded at 40 cm, 140 cm and 190 cm depths (Figure 1A) for a total of nine temperature reads per pile. Temperature was measured with a digital thermometer (Digi-Sense® Thermometer) daily in the beginning to weekly at the end of the composting experiment, and averaged each measurement day throughout the 523 days of composting. Stainless steel Hobo Temp Data loggers U12-015 were also attached to each BRP and to the outer north wall of each structure measuring the temperature hourly.

## **Chemical analysis of compost**

Initial cattle manure retrieved from the BRP and composting material were analyzed for moisture, pH, electrical conductivity, volatile fatty acids, total nitrogen, total carbon, total sulfur, nitrate and ammonia nitrogen and water activity. Compositated subsamples of manure and compost were removed from each pile on days 1, 96, 131, 194, 203, 250, 303, 498 and 523 stored at -20°C until further analysis. All analyses were conducted according to AOAC ()

## **DNA analysis of bacteria**

After BRP's were extracted they were transported from the Glenlea Research Facility to the Level 2 Large Animal Microbiology lab in the Animal Science department at the University of Manitoba. Sample bags were recovered from the manure in the BRP and one half of the remaining tissues in each bag were stored at -20°C and the other half was used for culturing purposes.

In order to identify MAP from composting tissues, a decontamination step was performed with the BBL® MycoPrep™ specimen digestion/decontamination kit. Due to the slow growth of MAP, and rapid overgrowth and contamination from other organisms, the decontamination step is used to increase mycobacterial yield. Samples are digested and decontaminated with the reagent, N-acetyl-L-cysteine (NALC) combined with 2% sodium hydroxide (NaOH) in trisodium citrate solution and then washed with phosphate buffer. When combining equal amounts of sample and reagent, a final concentration of 1% NaOH is an effective decontaminating agent, NALC is a mucolytic agent less toxic to mycobacteria, sodium citrate binds heavy metals that could inactivate the NALC and then washed with phosphate buffer that lowers the activity of the reagent solution and lowers the specific gravity of the sample. Samples were then centrifuged at 3000 x g for 20 min and the pellet was re-suspended with phosphate buffer. Next, 200 µl of the resuspension was inoculated onto BBL® Herrold's Egg Yolk Agar slants with mycobactin J, amphotericin, naladixic acid and vancomycin and allowed to incubate at 37°C for 12 weeks. Growth was recorded weekly and at the end of 12 weeks, all growth from the surface of the slants were removed and stored at -20°C in 1 ml peptone water before further analysis.

The DNA of the harvested samples was extracted using the ZR Fungal/Bacterial DNA Kit™ following the protocol outlined in the kit. Each DNA sample had 50 µl eluted into two 1.5 ml microcentrifuge which were then ready for PCR analysis, and stored at -20°C. Survival of MAP and *M. smegmatis* was verified with PCR. Primers used for verification are outlined in Table 2. MAP primers were taken from Vansnike et al. (2004) and amplified a region 569 base pairs long and primers for *M. smegmatis* were designed using NCBI BLAST (2008) and

amplified a region 624 base pairs in length. PCR was performed in a final volume of 25  $\mu$ l containing 12.5  $\mu$ l of PCR Master Mix (Taq DNA polymerase, deoxyribonucleotide triphosphates (dNTPs), and magnesium chloride (MgCl<sub>2</sub>), Promega Corporation), 10.5  $\mu$ l of nuclease free water, 0.5  $\mu$ l of the forward primer, 0.5  $\mu$ l of the reverse primer and 1  $\mu$ l of DNA template. Tubes were placed in a thermocycler (Bio-Rad C1000™ Thermal Cycler; Bio-Rad Laboratories) and amplification, adapted from Vansnike et al. (2004), was as follows: one cycle of denaturation at 94°C for 4 minutes followed by 40 cycles of denaturation at 94°C for 45 seconds, annealing at 61°C for 45 seconds and extension at 72°C for 45 seconds and a final extension at 72°C for 10 minutes. Bacterial DNA of both MAP and *M. smegmatis* were used as positive controls for differentiation between fragment sizes of such closely related species, along with water as template as the negative control. The final PCR products were analyzed by electrophoresis on a 1% (w/v) agarose gel stained with ethidium bromide and visualized under UV light.

## **Experiment #2**

### **Bacterial inoculation**

The experimental design of the *in vitro* incubation of MAP is outlined in Table 3. Three treatments were used for the design of this experiment to determine what temperature can inactivate naturally infected tissues mixed with or without compost.

The first treatment was a control of compost not mixed with MAP infected tissues. Compost used for this experiment was collected just prior to the final composted material from Experiment #1 and transported to the University of Manitoba from the Glenlea Research

Facility. Approximately 100 g of material was placed into a total of eight plastic Whirlpak bags and hand massaged for ~5 min to thoroughly mix.

The second treatment was naturally infected MAP positive tissues from JD positive cattle. Three animals that tested positive for MAP through a fecal ELISA test were slaughtered and the terminal ileum and mesenteric lymph nodes were again couriered from the University of Saskatchewan. Tissues were ground in a food processor and again about 100 g of material was placed into eight plastic Whirlpak bags and hand massaged for about 5 min for thoroughly mixing.

The third treatment was a combination of MAP positive tissues and compost mixed in a 1:4 ratio, respectively. About 200 g of naturally infected tissues and 600 g of compost were mixed together in a food processor then 100 g of ground material was placed into 8 plastic Whirlpak bags and hand massaged for 5 min.

Duplicate bags of each treatment were then placed at the temperatures 4°C, 45°C, 60°C and 80°C. Sampling occurred at nine time intervals on 0, 1, 2, 4, 8, 16, 30, 60 and 90 days of incubation.

### **DNA analysis of bacteria**

Analysis of DNA for MAP was performed the exact same way as in Experiment #1. Half of the tissue on each sample day was used for culturing and then the other half was placed in -20°C. The samples were decontaminated using the BBL® MycoPrep™ specimen digestion/decontamination kit, allowed to grow for 12 weeks and then harvested into 1 ml of peptone water. DNA was extracted using the ZR Fungal/Bacterial DNA Kit™ and PCR was again performed and analyzed by electrophoresis for MAP survival.

## RESULTS

The construction of the two composting piles was undertaken in mid-winter under western Canadian conditions and temperatures were as low as  $-30^{\circ}\text{C}$ . Even under these extreme cold temperature the composting pile never froze and the internal temperature of the piles was always above ambient temperature. In general as the ambient temperature rose, so did the internal temperature of the piles. On days 194 and 250 the pile were mixed and in particular mixing on day 250 resulted in a spike in metabolic activity and a rise in the internal pile temperature to as much as  $50^{\circ}\text{C}$ . The high temperature peak on day 250 can be ascribed to the fact that we added dry straw to the pile.

Moisture and water activity (Fig. 3A) was relatively constant except for days 101 to 201. This period coincided with the summer months and the pile dried out. The pH remained relatively constant except for days 101 to 201. The major VFA produced in the piles was acetic acid (Fig. 3B). Much greater amount of nitrate was produced in the early part of the composting but this eventually declined (Fig 3C). There was generally a decline in Na, Ca, Mg, K, and electrical conductivity but P increased (Fig. 4A). Total nitrogen, S, Cl (Fig. B and C) and ratios of C:N (Fig. 5B) and C:P (Fig. 5C) were stable or declined.

When survival of MAP and Smeg was evaluated in the composting piles, almost no Smeg could be detected after 35 d (Table 4). On the other hand MAP, irrespective of the source survived for up to 250 d of composting. To further evaluate the ability of MAP to survive composting we set up an in vitro incubation experiment in which MAP cultures or MAP in lymph nodes was mixed with compost and incubated at 4, 45, 60, and  $80^{\circ}\text{C}$  for 1, 2, 4, 8, 16, 30,

60, or 90 days (Table 5). There was not MAP in the compost alone, indicating that there was no contamination of the experiment. MAP could survive 80°C incubation for up to 90 days.

## CONCLUSIONS

1. The biosecure composting system used is laborious to set up and is probably not practical for producers.
2. In a mass mortality situation it may be of use providing enough resources are available for the set up of a composting pile, but it is not of value to destroy MAP.
3. Using this composting system the carcass is almost completely degraded and only some of the long bones and skull remain intact to some extent.
4. There is no destruction of the MAP during field scale composting.
5. *M. smegmatis* is not a good proxy for MAP in composting studies.
6. Even under laboratory conditions it is not possible to destroy MAP and these bacteria survive at high temperatures for long periods of time.
7. If there is ever a Public Health need to destroy JD infected animals composting will not be suitable. This is because composting, whichever method is used, does not reach the temperature attained in our laboratory study, and even if it did the MAP would not be destroyed.
8. MAP is an extremely robust bacterium that is difficult to destroy and survives for long periods of time under harsh conditions.

## OTHER ACTIVITIES

To promote the research activities of the Animal Science Department at the University of Manitoba, as well as the funding support from MRAC, our group attended several meetings and conferences. These included:

- Poster presentation titled “ Inactivation of *Mycobacterium avium* spp. *paratuberculosis* (causative agent of Johne’s Disease) by dead-stock composting” at the Traceability Research and Development conference in June 2009
- Hosted a tour of the National Centre for Livestock and the Environment (NCLE) at the Glenlea Research Station for Manitoba Cattle Producers Association (MCPA) members in October 2009
- Invited talk titled “Dead-stock composting of cattle mortalities to inactivate MAP” at the 2<sup>nd</sup> Annual Canadian Johne’s Disease Meeting in October 2009
- Poster presentation titled “Dead-stock composting of cattle to inactivate Johne’s Disease causing organism” at the Manitoba Grazing School in December 2009
- The authors of this report also intend to publish these findings in the form of a Master’s thesis, as well as two peer-reviewed manuscripts that will be sent to your agency upon completion. Tentative titles for these publications include:
  - ❖ Inactivation of *Mycobacterium avium* subspecies *paratuberculosis* by large-scale composting of cattle mortalities during a Manitoba winter compared with *in vitro* heat treatment
  - ❖ Microbial community analysis during composting of cattle mortalities

It is also important to note that although the funding for this research project is providing immediate results to current questions circulating in the industry, it also has an added long-term value for training at the graduate level. In this particular instance, the graduate student working on this project has obtained a highly specialized skill set that will prove to be valuable well into her future.

With the funding provided from MRAC for this project, we have been able to be leaders in research areas not previously explored. Since beginning this project in March of 2008, interest within the areas of Johne's disease, composting and microbial community analyses within different environments has significantly increased. One particular project, funded through MRAC and again in collaboration with the principle investigators on this project, will investigate how and when young calves become infected with MAP and identify other microorganisms that accompany MAP during infection and use this as a diagnostic tool. Implications of this project include reduction in Johne's disease related economic losses and increasing confidence in our Canadian agricultural products especially with increasing concern about the possibility that consumption of MAP infected products may cause Crohn's disease. A recent publication in the NCLE March/April 2010 newsletter under newly funded projects describes this proposal.

There have also been discussions on several occasions about collaborating with MAFRI on different MAP related projects.

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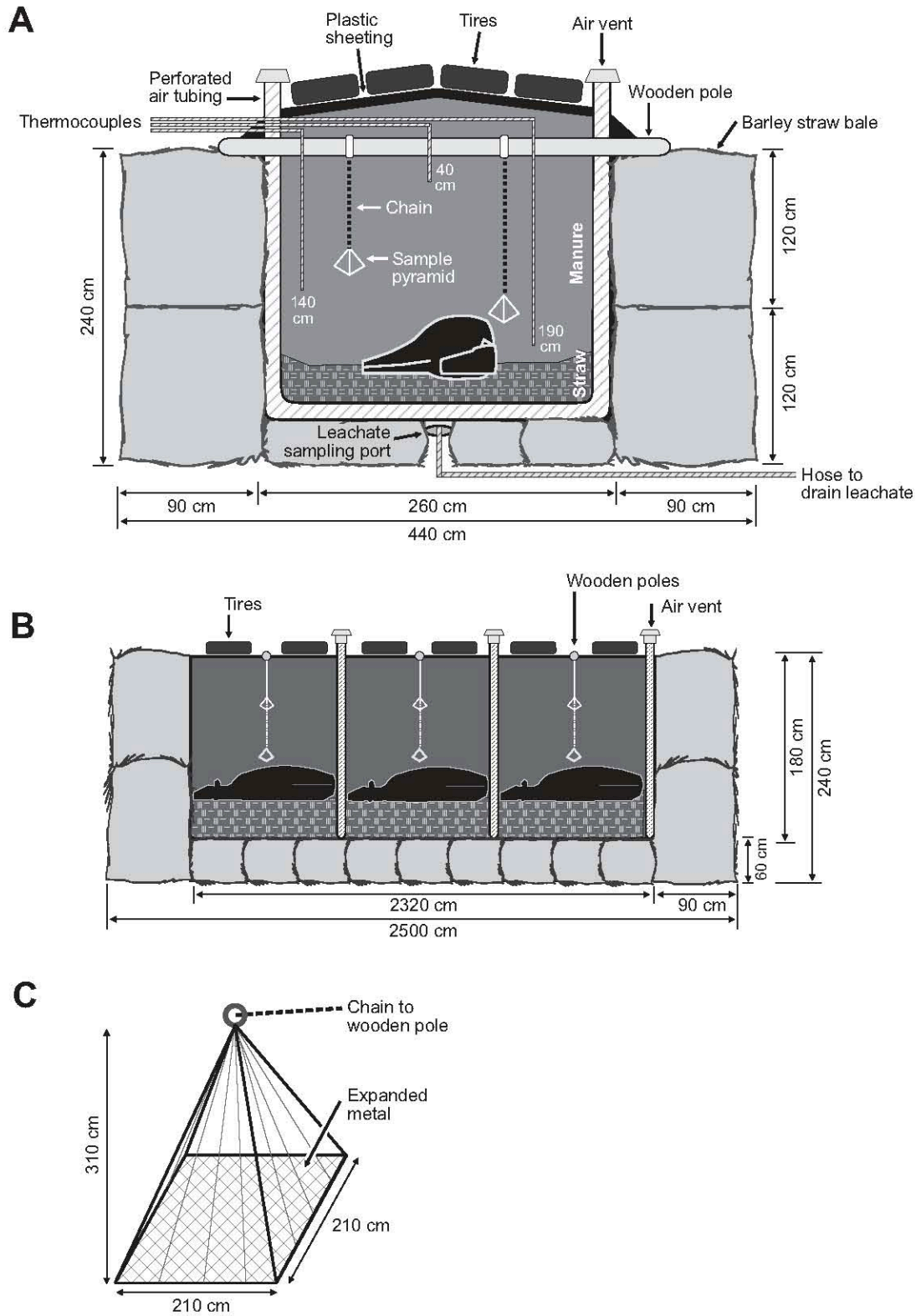


Figure 1

FIGURE 2. Ambient and pile 1 and 2 inner composting temperature profiles over 523 days of composting.

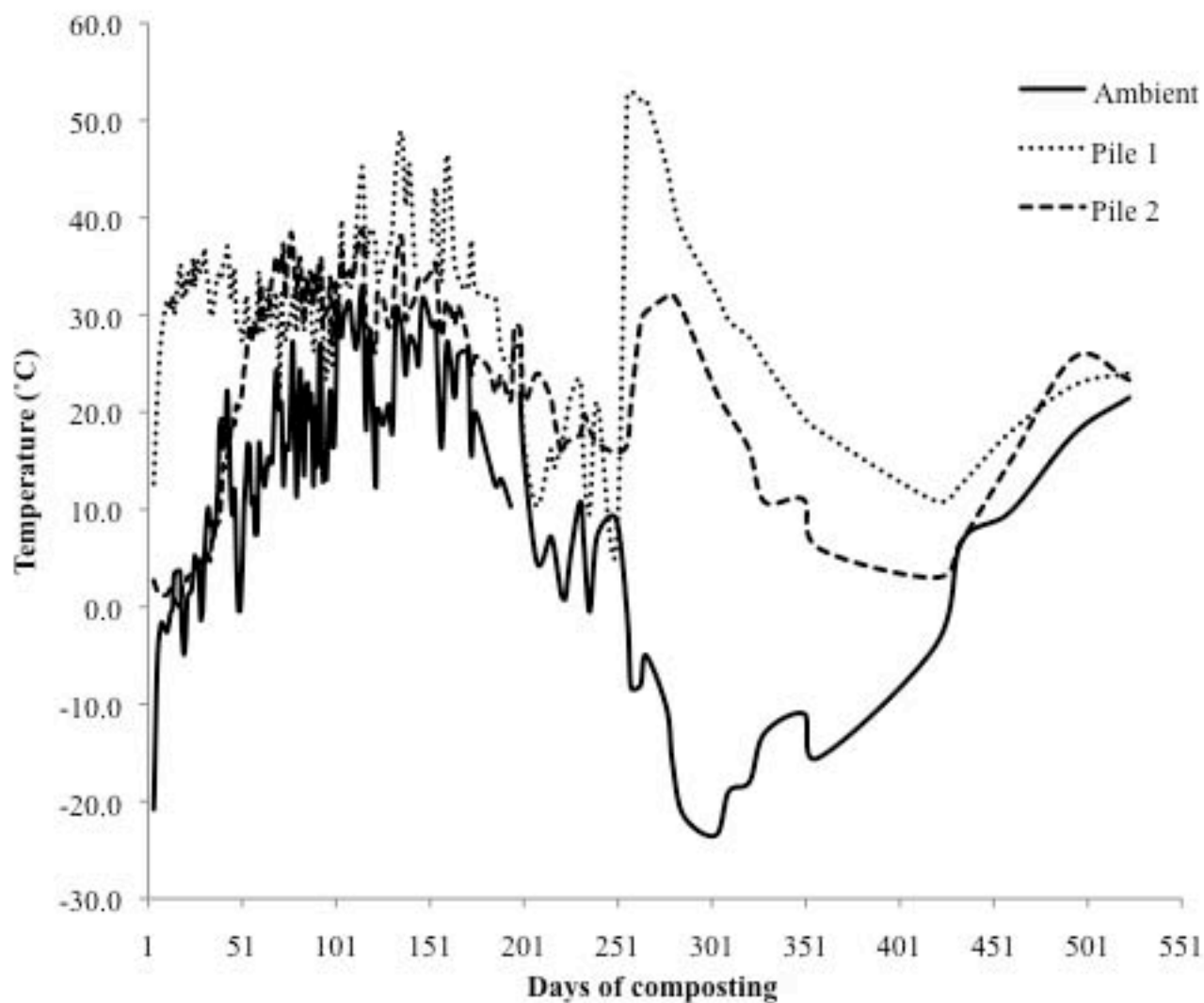


FIGURE 3. Moisture, compost pH, VFA's, nitrate and ammonia nitrogen over the 523 days of composting.

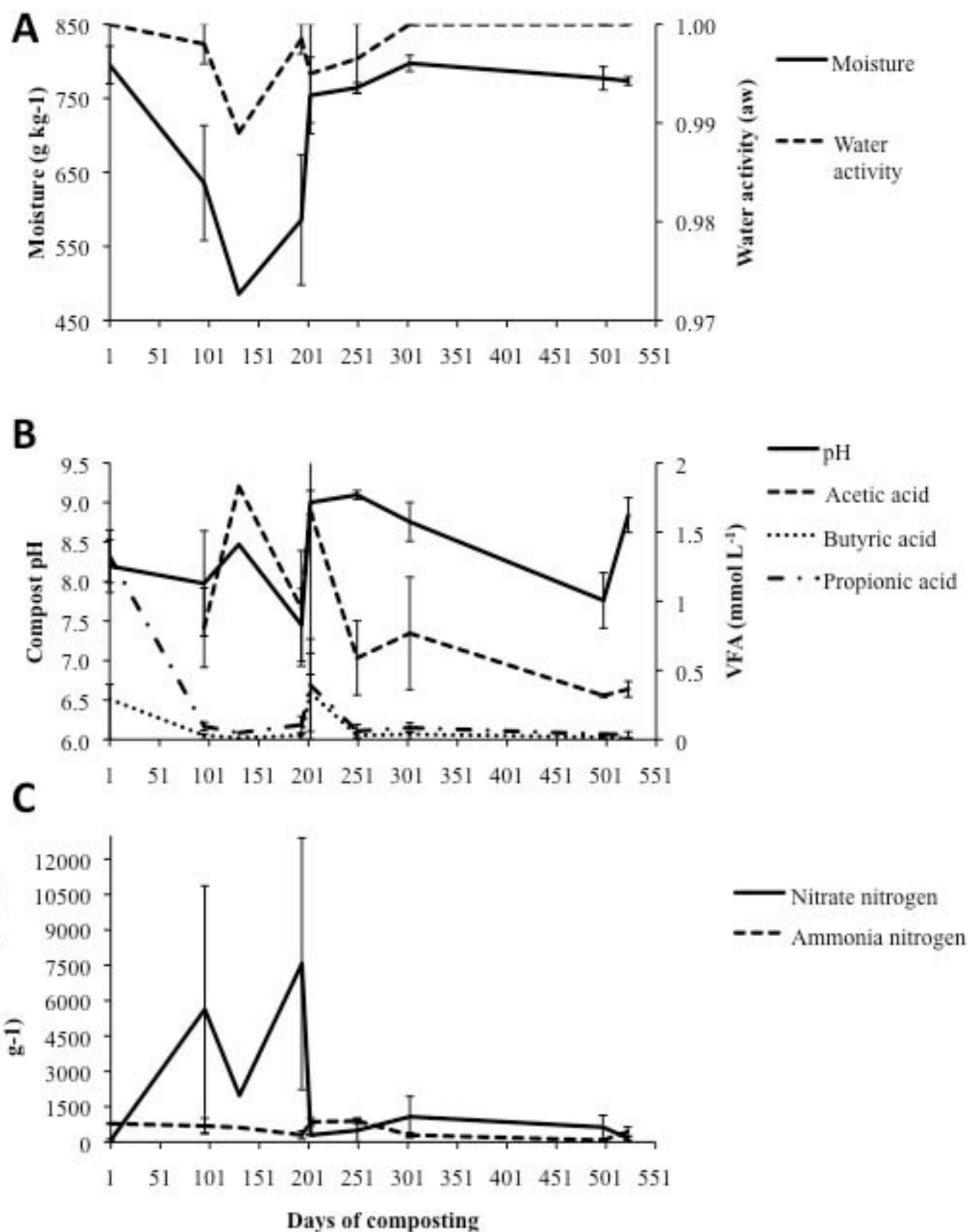


FIGURE 4. Electrical conductivity, total nitrogen, sulfur and chloride quantities over the 523 days of composting.

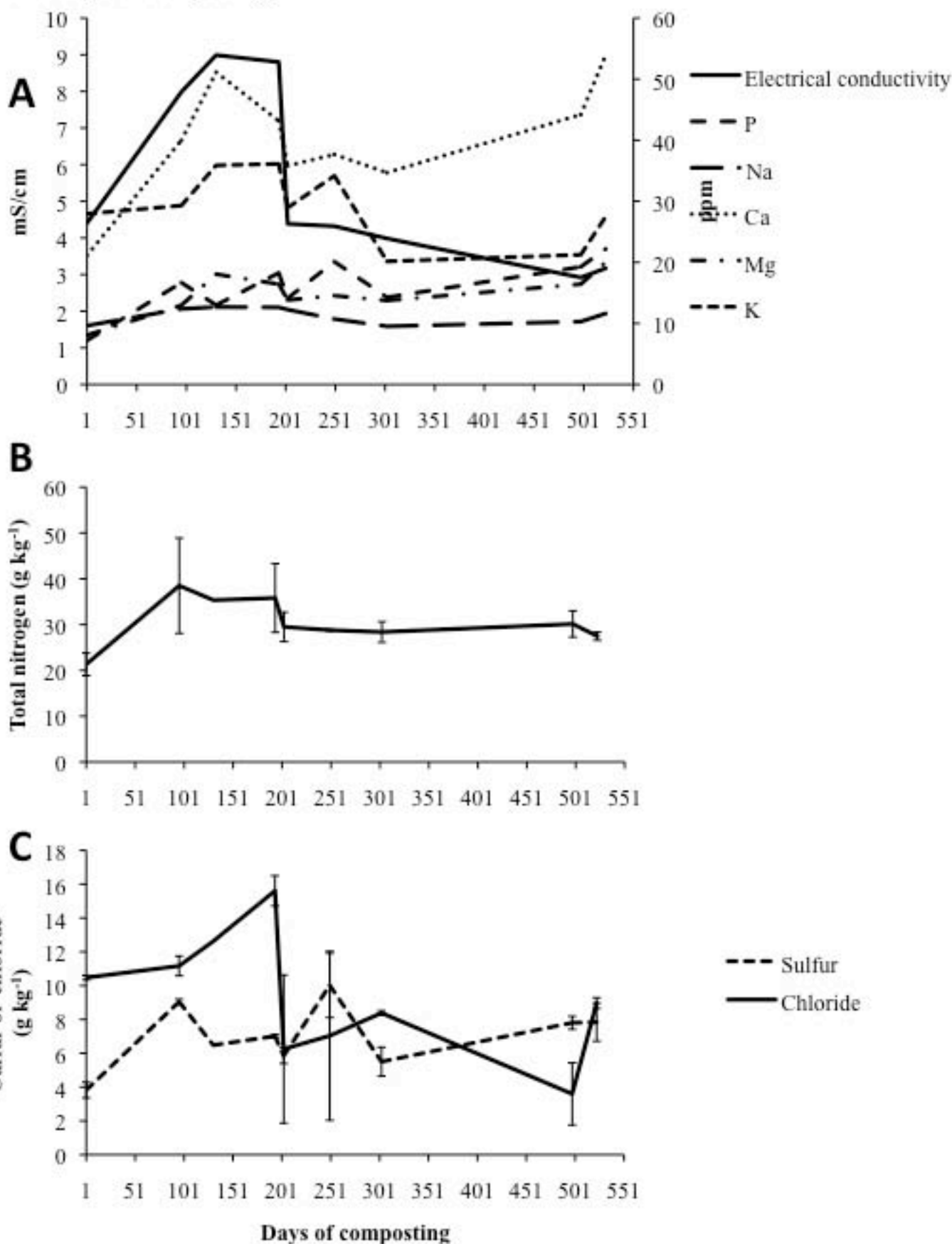


FIGURE 5. Total carbon, carbon: nitrogen and carbon: phosphorous ratios over the 523 days of composting.

