MICROBIAL ANALYSIS OF ON-SITE COMPOST MADE FROM MEAT BY-PRODUCTS AT SUNNYSIDE MEATS IN DURANGO, COLORADO

by,

Susan Philip Paret

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Golden, Colorado

Date ________________

Signed: ____________________________

Susan Philip Paret

Signed: ____________________________

Dr. John R. Spear

Thesis Advisor

Golden, Colorado

Date _________________

Signed: ____________________________

Dr. John McCray

Professor and Department Head

Department of Civil & Environmental Engineering

ABSTRACT

Composting of different substrates from different environments has been studied in great detail. Composting is the biodegradation of organic material into matured, stable humus in which microorganisms play an important role. The process is important for the cycling of elements, the removal and treatment of unwanted wastes and the prevention of disease. The microbial community responsible for the decomposition of any organic material is a complex mix of organisms from all three domains of life—Bacteria, Archaea and Eucarya. Microbiota found in a compost mix are a blend of organisms that can thrive under different conditions; wet to dry; cold to hot; low pH to high pH, etc. Thermophiles that can thrive in high temperature zones in a compost pile can drive the composting process. This research investigated an on-site composting of meat by-products at Sunnyside Meats, a local meat processing facility in Durango, Colorado. This experimental, pilot-scale composting system was initiated with slaughterhouse wastes, wood chips and Sudan grass, ground together, mixed, aerated and given time for microbiota to digest the organic material to composted humus. The simple design and construction of this compost addressed the safety regulations put forward by the U.S. Department of Agriculture (USDA) whose on-site inspectors aided in the oversight of the experiment and its compliance with slaughterhouse operation. The primary objective of the study was to analyze the microbial risks associated with composting of animal wastes. Samples for microbial identification were taken from both pre-composting units and aerated compost bin. With a DNA sequence-based, molecular microbial community analysis approach, a wide variety of possible pathogens were identified at the different locations of the composting unit, but the research confirmed that this method of composting helps in the reduction of survival and occurrence of pathogens. Some of the pathogens identified belong to the Phylum Actinobacteria, Bacteroidetes, Proteobacteria and Firmciutes. The results also indicate that the on-site composting of slaughterhouse wastes is a viable option for the treatment of slaughterhouse waste and prevents the deposition of this organic material into landfills, while returning nutrients to the Earth in the form of rich humus that can be deposited on to agricultural fields as an organic fertilizer.

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LIST OF ACRONYMS

C...…………………………………………………………………………………….............. Carbon
N……………………………………………………………………………………………... Nitrogen
USDA…………………………………………………….......... U.S. Department of Agriculture
SRM ………………………………………………………………………… Specified Risk Material
BSE………………………………………………………… Bovine Spongiform Encephalopathy
OTU …………………………………………………………………….. Operational Taxonomic Unit

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CHAPTER 1

INTRODUCTION

Improper storage of waste can result in serious health, safety & environmental issues. Therefore, proper waste management is important for the sustainability of the surrounding ecosystem as well as to ensure the quality of life for Earth's inhabitants. Composting is a sustainable waste management practice where almost all the organic waste material is degraded into organic materials through the activities of successive groups of microorganisms (Dees and Ghiorse, 2001). An efficient and satisfactory composting process is dependent on the presence of a high microbial diversity (Beffa et al., 1996). In the process of composting, nutrients are added back to the soil and thereby provide many essential nutrients for plant growth as well as a more nutrient-rich and healthy soil matrix. Compost will improve the structure of the soil and thereby improve the ability to hold nutrients and water. Compost has long term benefits as it will make the soil healthy over time whereas chemical fertilizers will decrease the fertility of soil over time.

Conventional composting can be performed using a variety of methods. These methods include static windrows, turned windrows, aerated static piles and a range of in-vessel systems. Each method has been studied to analyze its advantages and disadvantages, in great detail. Despite the method used, substrate quality is probably the most important parameter that determines the quality of a compost. Any compost pile is a nutrient-rich treasure that consists of both living and dead microorganisms. Therefore, the choice of substrate and the physical and chemical characteristics of the organic material used can greatly determine both the richness and abundance of particular microorganisms in the compost. As the substrate becomes the only source of food to the microorganisms in a composting matrix, the nature of the substrates is the primary controlling factor in any composting process (Gajalakshmi and Abbasi 2008). In this study, a pilot-scale on-site composting process was constructed using meat by-products from a slaughterhouse as the substrate, adjacent to Sunnyside Meats in Durango, Colorado. The overall approach chosen for this system was a hybrid of in-vessel and openly aerated system.

A traditional method for dealing with slaughterhouse waste is landfilling the waste, which is ultimately a waste of a nutrient-rich waste stream and is not economically viable. The driving factor behind the work was to minimize what goes to a landfill and to return the nutrient rich biomass of meat and slaughterhouse by-products to the Earth. Therefore, we worked with the owners of Sunnyside Meats to recycle the waste stream by mixing the waste with straw, grinding the masses together, turning the mixed waste in a large drum followed by aerobic incubation in an open pit to create a nutrient-rich compost. The recycled

slaughterhouse wastes are rich in nitrogen, phosphorous, calcium, amino acids, small peptides, and fatty acids and all are excellent growth nutrients for microorganisms to feed on and cycle the elements (Franke-Whittle et al, 2013).

In addition, slaughterhouse wastes are considered to be a potential reservoir of bacterial, viral, prion and parasitic pathogens, capable of infecting both animals and humans. Not much research has been carried out to determine the extent to which true human and animal pathogens, including viruses and parasites, persist in animal waste management systems and enter the non-farm environment to contaminate water, land or air (Sobsey et al, 2006). Animal waste treatment processes like composting and thermophilic biological processes have been designed to operate at certain conditions capable of inactivating or removing pathogens but the extent to which they actually reduce pathogens has not been adequately studied (Sobsey et al, 2006). Many pathogens found in animal waste compost are difficult to detect and quantify because of their infectivity (Berge et al, 2009). Dead or inactivated pathogens can still be present in animal waste and they may be detectable by some analytical methods but detection of these dead pathogens will give us an incorrect result because they no longer pose threat to humans or other animals. Accurate detection of infectious pathogens requires highly sophisticated techniques, which are both time and money consuming. The primary aim of this project was to identify the microbial diversity in the compost prepared from the slaughter house waste and see if the compost process is a safe and viable option for treating the waste out of a slaughterhouse. A detailed study by molecular microbial community analysis to better understand the microbial ecology of the composting process, as well as to determine the presence of pathogens, was carried out to see if this pilot-scale composting process could provide a safe and effective method for treating slaughterhouse waste.

1.1 Background Information

The intended purpose of the pilot-scale composting process was to create usable compost as an end product of livestock harvest waste. During the operations at the meat processing plant, U.S. Department of Agriculture (USDA) regulations are considered priority at all times. The pilot-scale composting process begins by using a trailer parked adjacent to the slaughterhouse that was equipped with augers fed by a loading hopper containing grinding teeth. Material is left for a few days grinding and mixing with both wood chips and Sudan grass to create a homogenous mixture of organic plant / wood material with slaughterhouse by-products. Once enough composting material has been created, it is transferred into a rotation drum with preloaded hay bales that are broken up. The temperature of the compost is measured while in the rotating drum and is recorded by sensors that are in capsules, tumbling within the drum. The drum is emptied after several

days, and the compost is moved to an aeration bin for finishing. Once finished, the compost may need to be separated, but if expectations are met, it can be used as soil fertilizer, ideally by tilling the compost in with the topsoil of an agricultural field. For the purpose of this study, the following flowchart was created (See Figure 1.1).

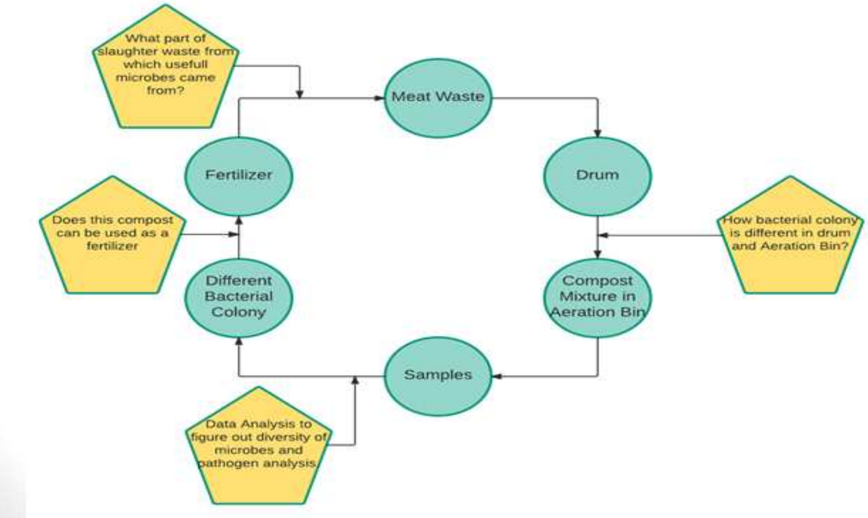

Figure 1.1: Design of the study.

1.2 Trial Setup

The compost team followed USDA safety regulations throughout the design and operation of the trial setup. Material that comes from the kill floor of the meat processing facility has to be screened before it can be ground up into the compost mixture. No specified risk materials (SRMs) were taken from the animals, especially cattle harvested after 30 months of age (30 mo.) which include: spinal cord, distal ileum, tonsils, brain, and vertebral column. These parts of the cattle pose a threat of Bovine Spongiform Encephalopathy (BSE), but no other body parts were restricted. All parts used for compost were expected to be denatured, and marked as not being food for consumption. The plant used a blue detergent dye as a marker for composting, but black carbon ash could also be used on the by-products if available.

There can be no offensive and persistent odors in or near the plant as it is considered adulterant to the process, and if serious enough may cause the meat processing facility to be shut down by the on-site USDA inspectors. For example, no pieces of flesh can be left anywhere on-site in, or around the pilot-scale composting operation. Any vessels that enter the facility must be clean and inspection-ready for USDA inspectors. Containers that enter with any material inside them must be pre-inspected before use in the process. In Figure 1.2, the process layout designed in the beginning of this study is shown.

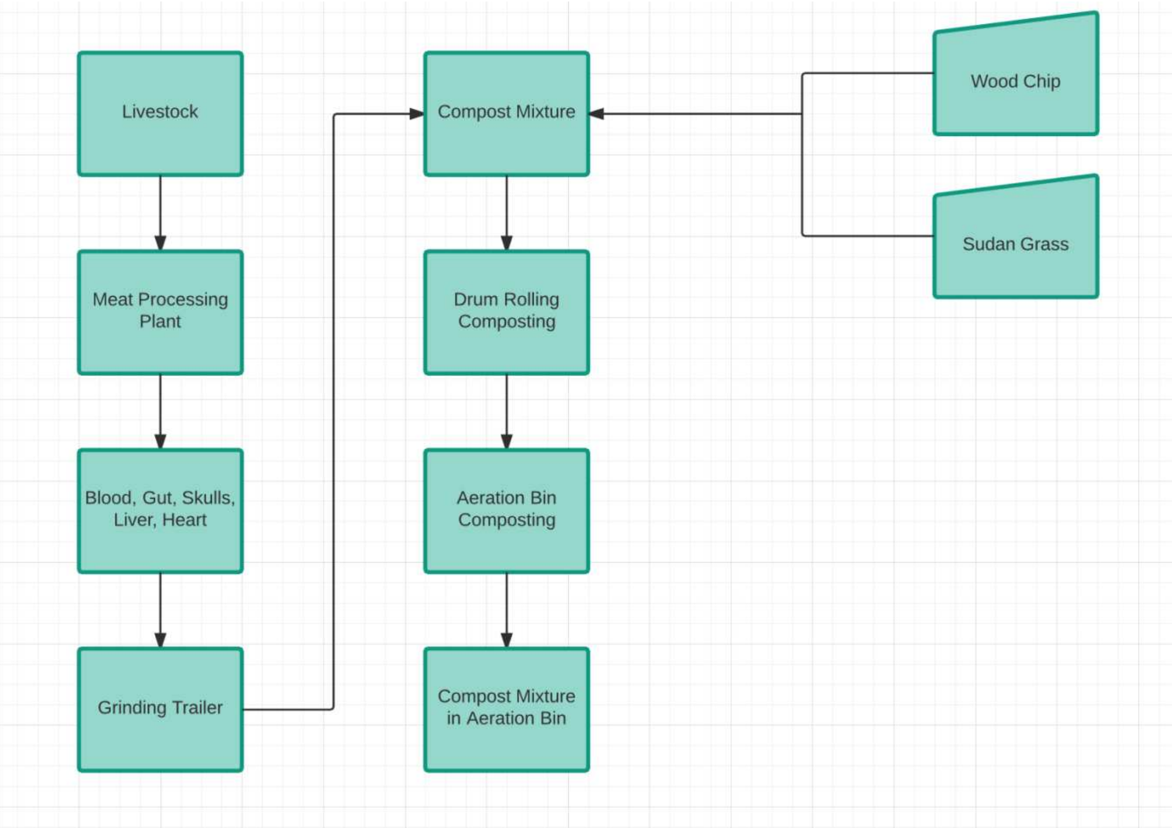

Figure 1.2: Process layout of the total compost system.

1.2.1 Harvest Floor Material

During animal harvest, compostable material was placed into green colored barrels, and was then emptied into a 'gut truck' for further separation. The distal ileum was hard to eliminate and therefore, the entire intestinal tract of all cattle had to be removed prior to use in compost. The following materials were used from the kill floor for composting: eviscerations, heads, blood, internal organs, rumen sacks and bones. Femurs, horns, cattle hooves, non-organic material, and SRMs were avoided to ensure compliance with USDA regulations to ensure best operation of the grinding equipment, and thereby to reduce non-compostable material. Conservative techniques were used and precautions were taken to avoid anything that was near an SRM. Once placed into the grinding hopper of the trailer, the material was sprayed with colored detergent to designate it as inedible as shown in Figure 1.3.

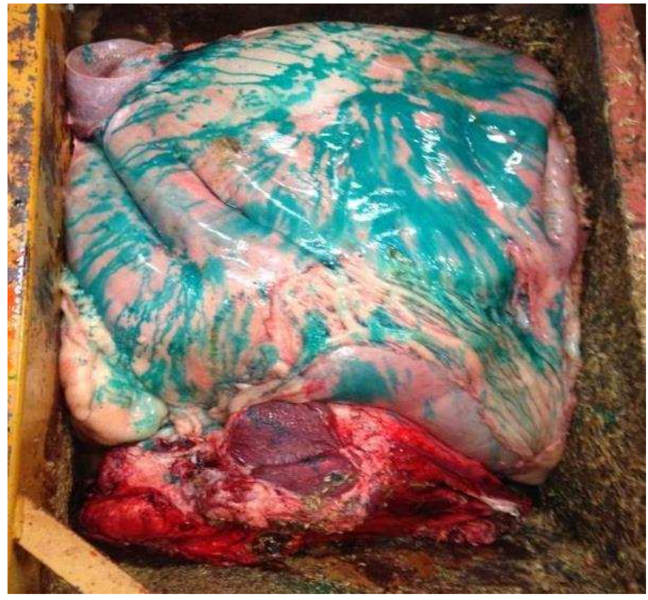

Figure 1.3: Denatured material from the kill floor, purposely dyed blue, in the grinding hopper.

The protocol laid out in the plant was Hazard Analysis Critical Control Point (HACCP) was always maintained while working on the harvest room floor. Any material spilled during transport off of the kill floor through the garage to where the grinding trailer was parked, or from the trailer itself, were cleaned up quickly to avoid any non-compliance issues.

1.3 Carbon – Nitrogen Ratio

Figure 2 shows a worksheet that was designed to be a tally mark count of the different materials that come from the animals for compost. The tally marks can be used as an input into an Excel spreadsheet to facilitate easier Carbon to Nitrogen ratio (C: N) calculations. The C: N ratio should be in the ballpark of 25:1 or 35:1, as this allows for a more successful composting of meat processing waste, based on measures known for compositing processes in general (Sadek,2013). If the ratio is too low, the compost will become anaerobic, and if it is too high it will not heat up enough to meet regulation requirements. Carbon sources were wood chips, Sudan grass, and hay bales. Nitrogen sources were all the animal products that came from the slaughterhouse free of SRMs. Figure 1.4 also shows how the quantity of wood chips, Sudan grass and hay bales were quantified in the compost mixtures to balance out the C: N ratio.

Tally Mark Worksheet for On-Floor Use										
Animal Parts Used		Cattle	Hogs	Sheep	Goats	Lambs	Bison		Processing Waste (# Buckets)	
These parts are always sent to compost	Blood									
	Lungs									
	Stomach or Paunch									
These parts are not always sent to the compost	Heads									
	Livers								Total # Buckets	
	Hearts									
	Tails								X weight/vol. chips	X bales of grass
Total Weights										
For X Blood Waste...... weight/vol. chips & grass For Y Kill Floor Waste...... weight/vol. chips & grass									Total Chip Weight	Sudan Grass Weight

Figure 1.4: Worksheet for C: N ratio calculation

1.4 Grinder Operation

A $\frac{1}{8}$ hp (horsepower) motor was installed on the side of trailer, and a ½ hp motor that was attached to the tongue of the trailer for connection to turn the auger. The $\frac{1}{8}$ hp motor on the side was sufficient for grinding during days with very little harvesting occurring or on days with small animals only. This motor was always used for overnight mixing. The ½ hp motor was used when cattle were being harvested, or during very busy harvests. The electric motors were never connected to the trailer while the trailer was being moved. Wet materials were observed to leak out from the front of the grinder near the exit door and the auger shaft. Pans or buckets were used to be compliant with the HAACP plan, and the liquid caught was thrown back into the mix. If needed, wood chips were used for temporary spill cleanup and then placed back into the mix. While in the garage of the meat processing facility, the grinder was covered by a ventilation hood to reduce unwanted odors from the grinding trailer. Figure 1.5 represents the grinder operation.

Figure 1.5: From left to right: grinding trailer, ventilation hood suspended by rope above the grinder, and the 1/8 hp electric motor attached to the grinder.

1.5 Grinder to Drum Transfer

Once a batch of sufficient compostable material was created in the grinder, it was transferred to the drum for rolling. The drum had several bales of hay broken up and spread down like a bed inside the drum before transfer. The trailer was pulled out of the garage with a tractor after all electric motors were disconnected, and the Power take-off (PTO) driveshaft was connected to the tractor and trailer for further mixing and subsequent dispensing. A metal connection housing was placed fully into the drum and then was hooked up to the side of the trailer as shown in Figure 1.6. Once the housing was in place, and the trailer was in line with the housing, it was connected by rope and the operator made sure there were no gaps between the housing and trailer, and also between the housing and drum. This was to avoid any unnecessary spills that can happen from spraying of the compost materials from the trailer to the drum.

Figure 1.6: Left - Transfer housing is placed into the rolling drum. Right - Trailer is connected to the housing and able to transfer material.

A hydraulic system as shown in Figure 1.7 connected to the tractor opened and closed the exit hatch of the trailer, and the hatch was about 1/3 open during transfer. The PTO drive was then switched on, the transfer was commenced, and the entire trailer contents emptied into the drum in minutes. While transferring a very large load, there was the potential for the transfer housing to back up and become clogged if the compost material piled up enough in front of the housing exit. This caused the tractor to cease running.

Figure 1.7: Interior of the rolling drum with and without any compost material inside.

Two or three sealed steel containers were placed into the compost with data-logging sensors as shown in Figure 1.8 to track temperature shifts while composting, mixing and turning in the drum. Sensors were placed within 3-5 feet of each end, and one placed in the center. Smart Buttons (ACR, Inc., Contoocook, NH) were reset using the trend reader software provided, and then inserted into the sealed steel containers before insertion into the compost. A long colorful cord or string was attached to the vessel so it could be identified and retrieved when the drum was unloaded.

Figure 1.8: Temperature Vessel for Smart Buttons, and a Smart Button temperature sensor.

1.6 Grinder Re-Entry Cleaning

Once transfer and sensor placement were completed, the housing and grinder were immediately washed to allow for easier cleaning. The sooner they were washed, the less likely they were to have a fly infestation that leads to maggots in the grinder. The grinder was washed into the septic system as shown in Figure 1.98 that had a screen cover, and the materials caught on the screen were put into the drum for composting.

Figure 1.9: Septic tank system and screen cover

All materials were removed from the grinder, and the grinding teeth were found to be somewhat clean, but most importantly free of maggots. It was very difficult to completely clean the grinding teeth, and it was not necessary to clean unless the teeth were creating a foul smell or carrying vectors of disease (i.e. flies, insects, mold, etc.). The grinder was sprayed on the inside with a bleach solution, for disinfection, and then was inspected by the plant operations manager before being brought back into the facility.

1.7 Rolling Drum Operation

The mixtures that were produced by the grinder tended to be very wet, and this moisture content aided the mix to heat up very slowly. If there was dripping from the exit end of the drum, wood chips were placed beneath the drip to catch the liquid. Once dripping ceased, the wood chips were put into the composting drum, or on the aeration pile.

The drum was powered on by a 110-volt electric motor on an outlet that had a timer attached. The timer had the ability to set 20 different times, and the drum was rotated for a set amount of time in a start and stop rotation range. Each rotation required approximately 9 minutes and 15 seconds. The material inside did not need a complete rotation to aerate, and was turned as infrequently as possible to discourage the formation of “balled up” material in the mix, but turned enough to supply oxygen for decomposition. During the initial testing, 5 minute periods, 20 times a day were created, but this could change as operation continues to create better results. Colorado composting regulations requires the mixture within a closed vessel to achieve 130 degrees F and maintain this temperature for three days. Once that was met, the compost was transferred into the aeration bin for finishing.

1.8 Drum to Bin Transfer

Once the mixture was ready to move to the aeration bin, the hatch at the end of the drum was fully opened, and the drum was turned to allow the material to fall out. The material was pushed from the drum entrance, and raked out the drum exit to completely empty. Cycles of pushing, raking, and turning were helpful. The pile of material that accumulated at the exit was moved by a Bobcat tractor. Though odiferous, the material was observed to be loamy and fluffy, with a small amount of moisture absorbed in it as shown in Figure 1.10. The mixture had some balls of material in it as well, and though this was not desired this will often occur as grease and fats separate and coagulate together. If the material has too many balls, it will not compost aerobically very well, and will end up producing ammonia gas. Rolling the material too much will create balls, so it was limited as much as possible.

Figure 1.10: Material that exits the drum should look like loamy, and have a visible structure of grass/hay.

Layers of drum material were placed in the aeration bin in layers, with grass/hay layers in between successive batches of compost. The bottom of the aeration bin had permeated steel pipes attached to a blower motor on the outside of the bin to dispense air into the bin. As the material builds up in the aeration bin, it was retained by wooden boards placed at the front of the bin. The pile was then covered, and the aeration fan turned on. The fan pulls heated air from the rolling drum and forced it through the pile in an effort to capture gasses and moisture coming off the material in the drum. This process can be seen in Figure 1.11.

Figure 1.11: Left: Aeration tubes in the bins which must be seated to provide aeration path. Right: Drum ventilation and fan supplying moist air and gases to the aeration tubes.

Figure 1.12: From left to right, top to bottom: Bobcat moves material into the aeration bin from the drum exit. Grass/hay is spread over the composting material in the bin. Wooden slats placed in front of the pile retain the material. A cover is placed over the top of the pile.

1.9 Aeration Bin Finishing Stage

Once the compost is in the aeration bin, it needs to sit there for at least a few weeks to allow all of the microbial activity to complete a further digestion of the substrate. Ideally, the compost pile should aerate for a month to ensure that this has occurred. If unfinished, i.e. partially digested compost is placed on soil, it could be capable of killing the plant life due to increased oxygen demand. A quick test to see if the compost is finished is to place the compost in a sealed plastic bag. After a few days, if the samples smell rotten, it indicates the presence of unfinished compost. After the compost is finished, it needs to be screened to remove any large unwanted particles such as bone that did not fully break down. Before removing the compost from the aeration bin, the cover, wooden slats, and aeration tubes must be removed. The tubes have a square bracket welded to their ends to allow them to be rotated and detached, and then pulled out and are shown in Figure 1.13.

Figure 1.13: Aeration tube brackets.

CHAPTER 2

MATERIALS AND METHODS

Sample Collection

Sampling was conducted during the months of July and September of 2015, from both the initial and final stages of composting. Samples were mainly collected from outside of the aerated bin and also from within the aerated bin. All the units outside of the aerated bin were collectively termed as pre-composting system and this comprised of all the equipment from grinder to drum. The samples from the compost pit were assumed to have a higher content of microbiota than the pre-composting units. Sampling was collected as aseptically as possible to avoid any contamination and samples were stored in plastic containers upon collection. Samples were all stored in the freezer at -20 ˚C for subsequent DNA extractions in the laboratory. Table 2.1 lists all the 28 samples collected from the composting unit.

Table 2.1: Sample name and locations

Name of Sample	Specific locations	In/Out
SP-1	White colored sample from the compost pit	Compost Inside
SP-2	Mid Pile, 2 ft down of the compost pit	Compost Inside
SP-3	Surface from middle, 1 ft down	Compost Inside
SP-4	Next to wall, 1 ft down	Compost Inside
SP-5	Septic Tank (Lipid Tank)	Pre compost
SP-6	Bottom most while middle turned over	Compost Inside
SP-7	Lipid tank system top	Pre compost
SP-8	Mid Pile, 3 ft down	Pre compost
SP-9	Lipid tank system bottom	Compost Inside
SP-10	Wet top corner of compost bin	Compost Inside
SP-11	Compost drum	Pre compost

Table 2.1: cont.

SP-12	Septic filter dirt	Pre compost
SP-13	From the roller	Pre compost
SP-14	Mix from measure side of drum	Pre compost
SP-15	Post roller grass	Pre compost
SP-16	Aerated ball from compost pit	Compost Inside
SP-17	Random sample from the compost pit	Compost Inside
SP-18	Top ball from compost pit	Compost Inside
SP-19	White colored from center of the pit	Compost Inside
SP-20	Bottom chunk from the compost pit	Pre compost
SP-21	1st Mix Pre-barrel	Compost Inside
SP-22	Drip From roller	Pre compost
SP-23	Batch 1 of drum	Pre compost
SP-24	Batch 2 of drum	Pre compost
SP-25	Batch 3 of drum	Pre compost
SP-26	Batch 2 drum after 8-9 days	Pre compost
SP-27	Mixture 3 pre drum	Pre compost
SP-28	Batch 4 of drum	Pre compost

Following sample collection in the field, samples are brought back to the laboratory frozen and molecular microbial community analysis begins16S ribosomal RNA (16S rRNA) sequencing is the most commonly performed method in the identification of microbes present within the samples for metagenomic studies (Janda, et al, 2007). The 16S rRNA gene is approximately 1,500 bp (base pair) long and contains nine variable regions between conserved regions. This gene codes for the manufacture of RNA, by a cell, to be used in the cell's own ribosomes, where proteins are made. Since all cells need proteins to carry out the instructions of the DNA, metabolize and maintain cellular function, the 16S rRNA gene is said to be a central housekeeping gene. A cell cannot survive without this gene, and to

date, every known kind of life possesses at least one copy of this gene as part of its genetic makeup. Because of its importance, the 16S rRNA gene is highly conserved in DNA basepair sequence, and when occasional mutations or deletions of a base pair happens within the gene sequence, a comparison of the 16S rRNA gene sequences between organisms, allows for the construction of an evolutionary distance map—between the organisms. Maps of relatedness can be built between organisms, much like the maps of relatedness between towns along the Front Range of Colorado, or anywhere else in the world. A typical workflow for this sequencing method includes protocols such as DNA extraction, library preparation (Polymerase Chain Reaction or PCR and DNA Quantification), DNA sequencing and analysis of the data with bioinformatics tools such as the Quantitative Insights into Microbial Ecology (QIIME) (Caporaso, et al, 2010).

2.1 DNA Extraction Technique

DNA extraction is arguably the most important stage in a molecular microbial research. For the analysis of the microbial community in the compost, nucleic acids must be recovered first through DNA extraction. DNA was extracted using the MoBio PowerSoil (MO BIO, Carlsbad, CA) kit following the manufacturer's protocol. The kit provides PowerBead tubes to which 0.25 mg of each sample were added and the tubes were then mixed with a vortex machine. In order to validate the data and to check for the consistency of the samples, it was advised to extract DNA from the same sample twice. Following this, 60 uL of solution C1 was added to the tubes which was then vortexed for 10 seconds. The PowerBead tubes were then centrifuged at a room temperature at 10000 x g for 30 seconds, from which the supernatant was transferred to a clean 2 mL collection tube. Then 250 mL of solution C2 was added which was also vortexed for mixing. These tubes were then incubated at 4 ℃ for 5 minutes and were then centrifuged again at 10000 x g for 30 seconds. 600 mL of supernatant was then transferred to another 2 mL tube into which solution C3 was added. Vortexing, incubating and centrifuging were repeated again for this collection tube. Then, up to 750 mL of the supernatant was carefully transferred to a clean 2 mL collection tube. To this, 1200 uL of solution C4 was added and was also vortexed for 5 seconds. Approximately 675 uL of this mixture was loaded onto a spin filter to bind extracted DNA and centrifuged at 10000 x g for 1 minute at room temperature for 1 minute. The flow through was discarded and this was followed by adding another 675 uL of the mixture to the spin filter. Centrifuging was repeated as in the previous step. The same was done with the remaining mixture. Then 500 uL of the solution C5 was added, but this was centrifuged only for 30 seconds. The flow was then discarded through and the samples were centrifuged. The spin filters were then placed in a clean 2 mL collection tube. Finally, 100 mL of the solution C6 was added to the center of the spin filter and a last run of centrifugation was performed again at room

temperature but only for 30 seconds at 10000 x g. The spin filter was discarded and the DNA from the samples were ready for the next procedure, i.e. DNA quantification to check for the DNA concentration and purity of the samples collected.

2.2 DNA Quantification

Quantification, PCR and normalization of amplified DNA from samples for 16S rRNA gene sequencing were performed in accordance with published methods, using primers for the V4 region, a variable region of the 16S rRNA gene with minor modifications which exclude the Bioanalyzer steps and include an additional concentration step (Kozich et al., 2013). The Qubit dsDNA high sensitivity quantification kit for extracted double stranded DNA was used with the Qubit fluorometer. The kit also provides concentrated assay reagent, dilution buffer, and pre-diluted DNA standards.

The following is a step by step guide to the Qubit quantification protocol.

- Use Qubit spreadsheet to determine the volume necessary for the Qubit buffer (QB) and Qubit reagent (QR).
- Bring the following items into the hood:
    - Vial for working solution (WS)
    - Holder for WS vial
    - Extracted DNA samples
    - Holder for extracted DNA samples
    - Sharpie
    - Qubit kit: reagent, buffer, and standards
- Locate following objects within the hood:
    - Small vials
    - Holder for small vials
    - Micropipettes
    - Pipetting tips
- Vortex extracted DNA, then centrifuge for approximately 15 seconds
- Make working solution in WS vial (add small volume into large volume)
- Vortex working solution to mix
- Procure enough small vials for every sample and both standards
    - Shake vial bag in order to grab one vial at a time
- Close vials; label for each sample and both standards
- Add 198 µL WS to each sample vial; 190 µL WS to each standard vial
    - Feel free to use same pipette tip for all WS additions
    - Cap vials in between fluid additions to maintain clean practices
    - Keep pipette tip case closed between uses

- Add 2 µL of each sample to corresponding labeled vial
- Add 10 µL of each standard to corresponding labeled vial
- Vortex each vial, then centrifuge ~15 seconds
    - Use black collar inserts
- Set quantification level (e.g. Broad Range DNA)
- Calibrate with Standard 1, then Standard 2
- Change stock calculation to 2 µL and then to ng/µL
    - Save calculation

2.3 Sequencing (Illumina Sequencing)

PCR amplicons of the V4 region from each sample (2 µl) was prepared for DNA sequencing by a second PCR amplified with Illumina dual indexed primers for 30 cycles (Phusion Master Mix New England BioLabs, Inc). Amplicons were normalized and purified with the SequalPrep Normalization Plate Kit. Normalized amplicons were pooled and concentrated with Amicon Ultra-0.5mL 30K Centrifugal Filter Devices. Pooled concentrates were quantified and sequenced by the Biofrontiers Institute at the University of Colorado, Boulder using the Illumina MiSeq Platform with a v2 2x250 cycle reagent kit.

2.4 Analysis using QIIME

QIIME (Quantitative Insights into Microbial Ecology) is an open-source bioinformatics software package designed for microbial community analysis based on DNA sequence data. Resultant 250 base-pair sequences were processed in QIIME 1.9 (Caporaso et al., 2010). Individual sequences are typically clustered into phylotypes, or operational taxonomic units (OTUs), which are arbitrarily defined groups of sequences that share a certain level of similarity. OTUs were picked using 'pick_open_reference_otus.py' with Usearch 6.1 (Edgar, 2010) and were filtered out using the Greengenes gold database (DeSantis et al., 2006). The next step was to assign and define taxonomies to the representative sequences in the seqs_rep_set.fasta file for which sequences were compared with established databases. Representative sequences were aligned using PyNAST (Caporaso et al., 2009). DNA sequence reads were clustered against a reference sequence collection and any reads which do not hit a sequence in the reference sequence collection were excluded. Alpha diversity was assessed for species richness and evenness within the samples, whereas beta diversity was analyzed for comparison between the samples using weighted UniFrac distance matrices generated in QIIME and visualized using the Phyloseq package (McMurdie & Holmes, 2013). In addition, the principal coordinates diagram was also generated in QIIME using the program Emperor (Vazquez-Baeza et al., 2013) to group samples based on their diversity. Microbial sequencing data was primarily analyzed using an OTU table of abundant sequences in each sample providing meaningful taxonomic

identifiers for each OTU, hence, building an OTU table was the most relevant step followed in this study. OTU tables gave provide the number of unique OTUs in each sample. Relative abundance bar charts were also created where all OTUs >1% were grouped together.

CHAPTER 3

RESULTS

During the sampling period, the full-scale composting system was operating under sub-optimal conditions; the temperature and pH rose slowly to the levels typical for thermophilic composting and then the composting process progressed well.

3.1 Microbial Abundance of the Total Composting Unit

Data analysis was carried out using QIIME and taxa summary plots were generated to characterize the biodiversity in the total compost system that includes both the aerated bin and pre-composting unit. These plots helped to visualize the taxonomic composition of the samples at a phylum, class and order level. The biodiversity of microbes based on different samples were identified along with their percentage of abundance. Results are summarized in charts below. Phylum representation in a given sample above 1% is only taken into consideration while analyzing the results. Euryarchaeota, Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria constitute 2.1%, 11.3%, 7.6%, 47.1%, 23.7% respectively in the total compost. The Figure 3.1 represents the microbial phylum composition of all the 28 samples in their consecutive order.

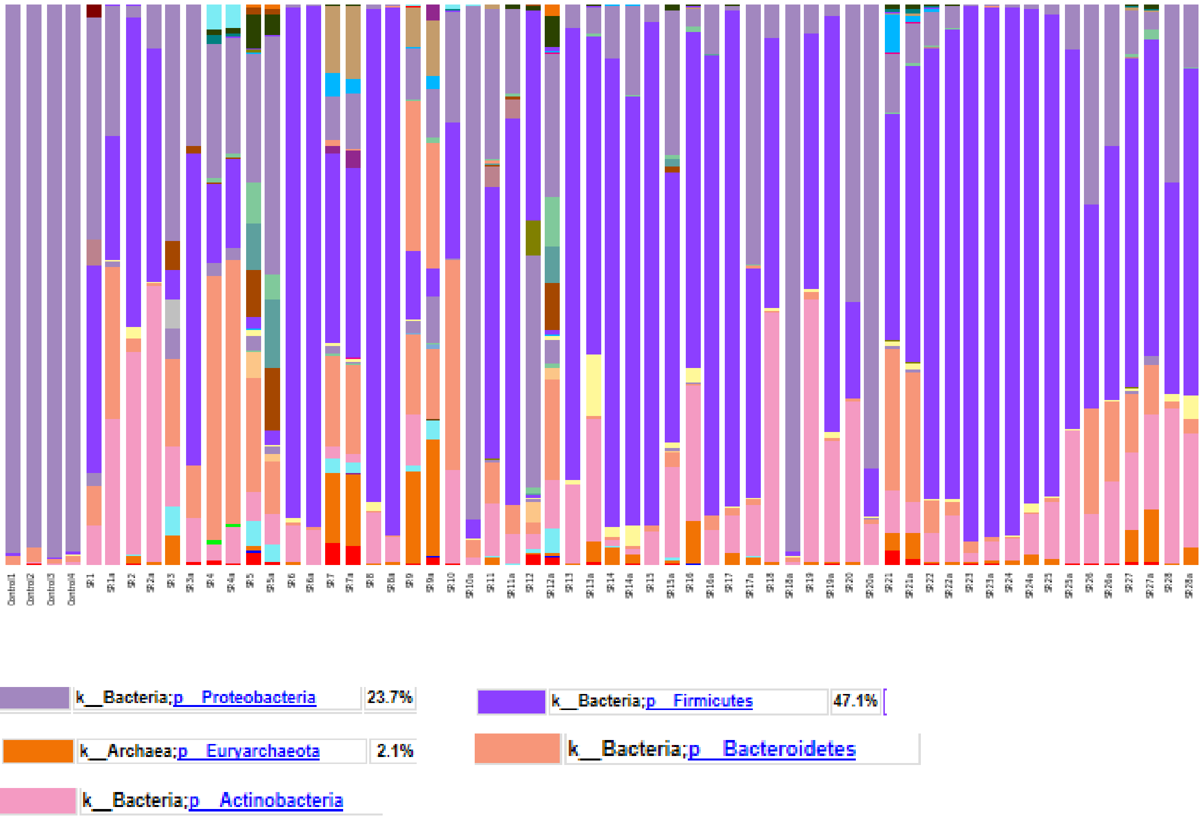

Figure 3.1: Microbial abundance at the phylum level of the total compost system.

Class analysis was carried out and the results are summarized in the Figure 3.2.

Figure 3.2: Microbial abundance at the class level of the total compost system

Order analysis was carried out and the results are summarized in the Figure 3.3.

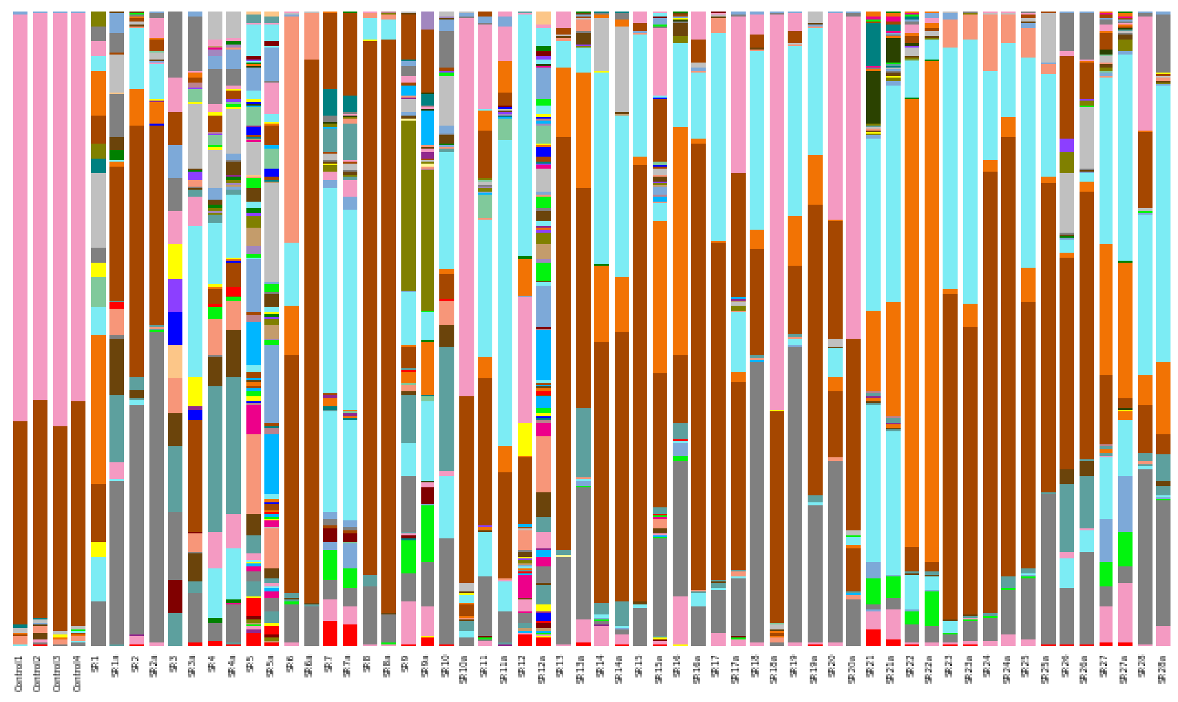

Figure 3.3: Microbial abundance at the order level of the total compost system.

3.2 Beta Diversity

Beta diversity was analyzed to measure the similarity and dissimilarity of the samples. The higher the beta diversity, the less similar the samples are. An OTU (similarity threshold)

graphs were generated and is shown in the Figure 3.4.

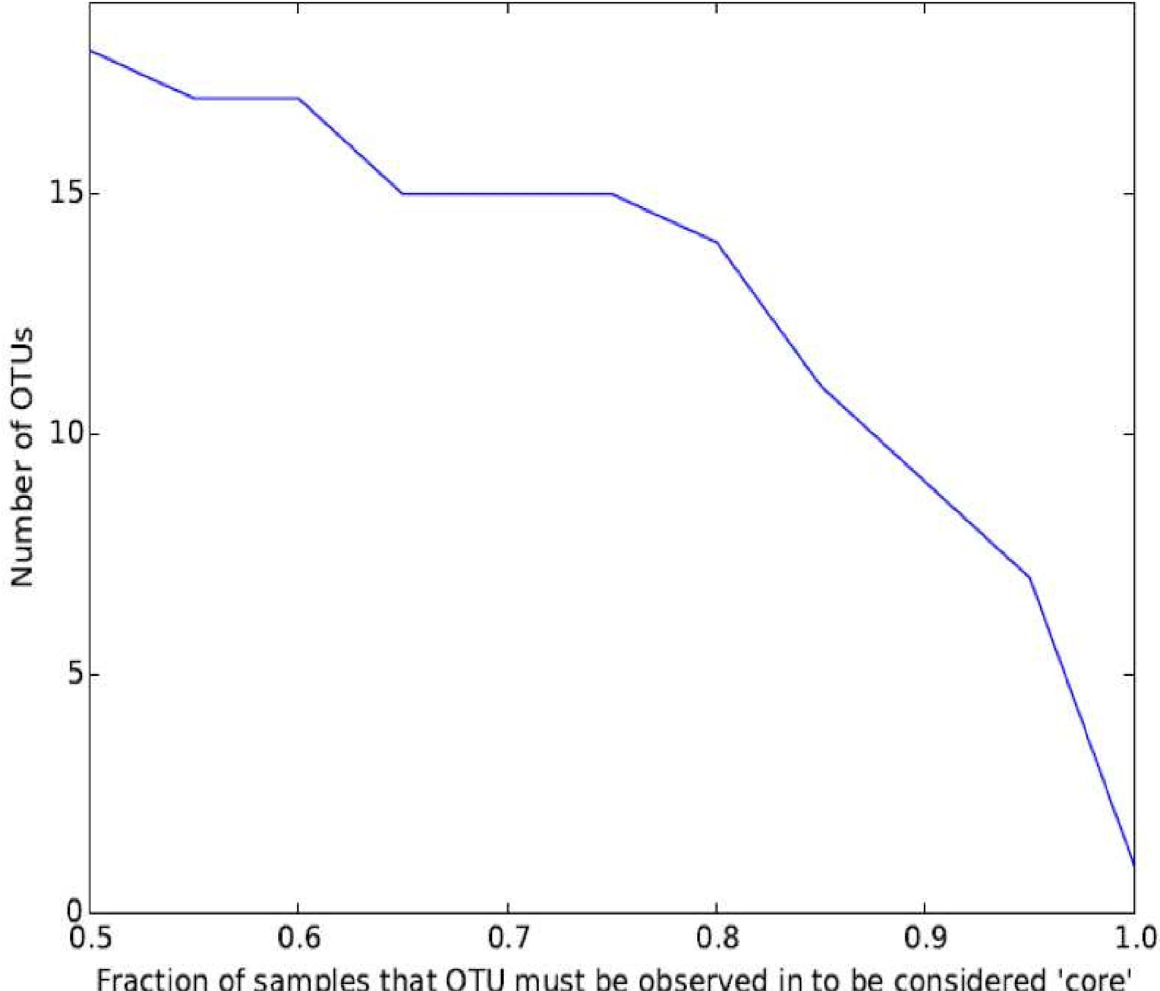

Figure 3.4: OTU chart of the total compost

The number of OTUs indicates the similarity threshold of the compost. Following is a representation of how the samples show variation in sequencing depth using PCoA (principal coordinate analysis). A principal coordinates analysis attempts to group things based on their diversity. Each coordinate (the axes) shows how much of that diversity is being explained by that coordinate. In the Figure 3.5, clusters of different colors at different locations are a representation of shift in the microbial communities.

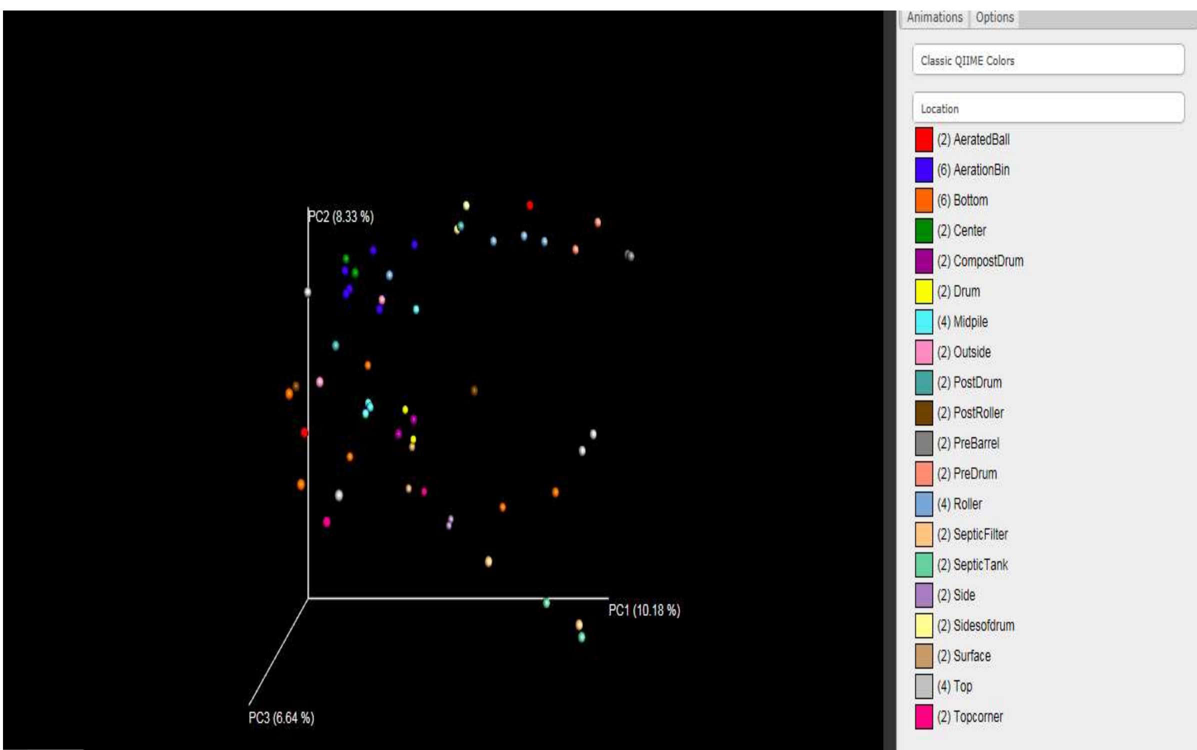

Figure 3.5: Principal coordinates analysis of the total compost w.r.t to the locations

3.3 Alpha Diversity

Species richness of individual sample curves is a plot of the number of species as a function of number of samples that were analyzed and the results are summarized in charts (see Figure 3.6). The number of species is expected to increase until a point where producing more sequences does not significantly increase the number of observed species.

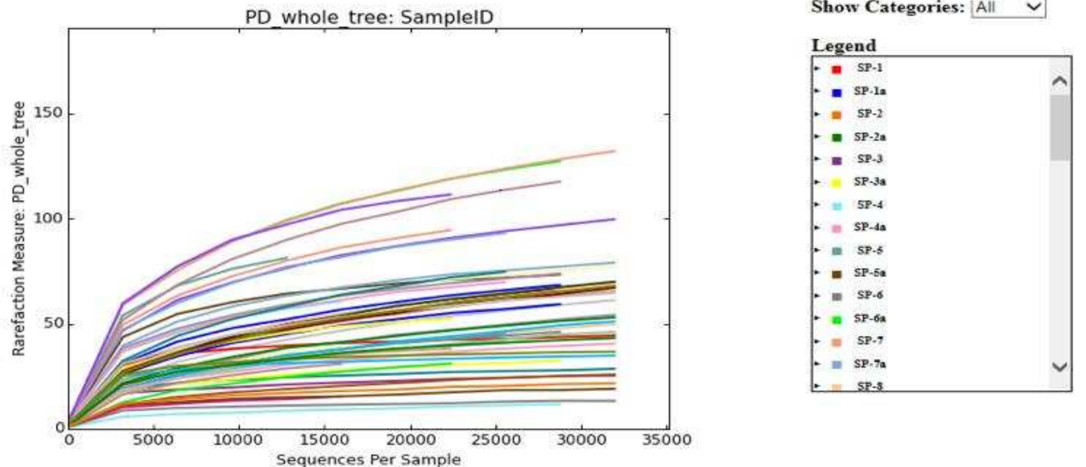

Figure 3.6: Alpha diversity chart

The results show that within a single sample, the species richness increases as more sequences are analyzed but they level off around 7500 sequences per sample indicating

that a wide diversity and species richness is captured in and around that sequence number point. Importantly, with the exception of only a few samples (the top 6 lines in Figure 3.6), the leveling off of the rarefaction curves indicates that we had adequate sequence coverage, that is an appropriate number of sequences per sample, which provides enough information to make inferences about the kinds of microorganisms present per sample.

3.4 Chemical Characterization of the Compost

The pH of the samples collected from the compost pit remained neutral around the range of 6-8 during the time of sample collection. Other studies have recorded change in the pH between different phases varying from neutral, to alkaline and slightly acidic (Sundberg, 2005). The samples in the composting unit were observed to be hot and slightly steaming indicating a higher temperature than the environment. This would imply the evolution and presence of an active microbial population in the compost unit. The high temperature regime in the compost heap indicated that the microbiota in the organic materials passed through different phases, e.g., from mesophilic to a thermophilic phase. As expected, no trace of the original organic material was found in the compost and the finished compost became dark brown to black in color by the second round of sample collection that took place in the month of September. As far as the moisture content was concerned, the compost was not too wet or dry, instead it was moist and loamy.

3.5 Microbial abundance of the Pre-Compost

Pre-compost samples include all of the samples collected outside of the aerated compost pit and consist of samples from septic tank, septic tank filter, roller, post roller mix, compost drum, pre-drum and post-drum. Most of the sequences observed in the pre-compost were similar to the bacterial species identified in other composts and mainly included archaea from the phylum Euryarchaeota (methanogens), and bacteria from the phyla Actinobacteria, Bacteroidetes, Proteobacteria and Firmicutes. All the phyla of microbiota are given in Table 3.2. All the family of microbiota with an average percentage above 1% is given in Table 3.1. These families are considered as the prominent families in the pre-compost samples. Of all the Firmicutes, the families Bacillaceae and Tissierellaceae dominated the pre-compost. Members of Bacillaceae, which belongs to thermophilic genera, is considered to be the most robust bacteria on Earth due to its ability to form resistant endospores. This indicates their high resistance to temperature and other adverse conditions. Most of the bacteria from the phylum Firmicutes were found to belong to the class Clostridia, another Order dominated by spore formers, which tends to exist in anaerobic habitats such as soils, aquatic sediments and in the intestinal tracts of animals **(**Suihko et al, 2008**)**. In addition to Firmicutes, the phylum Proteobacteria made up almost 13% of the pre-compost microbial population. Among the phyla, Halomonadaceae is the

dominant family. These bacteria are identified as a type of extremophiles that can thrive in high salt concentrations. In addition, the phyla Actinobacteria, Bacteroidetes and Euryarchaeota constitute 9.7%, 8.6% and 3.7 % respectively in the pre-compost samples. Microbial abundance in the pre-compost samples at the phylum level is represented with a pie-chart as shown in Figure 3.7. Figure 3.8 shows the microbial abundance at the family level.

Table 3.1: Family of microbes above 1% abundance in pre-compost

Prominent Family	Average Percentage
Methanobacteriaceae	2.0259%
Corynebacteriaceae	6.7185%
Bacteroidaceae	1.4296%
Prevotellaceae	1.7778%
Chitinophagaceae	1.1593%
Bacillaceae	11.6593%
Lactobacillaceae	9.2741%
Leuconostocaceae	1.4889%
Clostridiaceae	1.4370%
Lachnospiraceae	1.2037%
Ruminococcaceae	2.4074%
Veillonellaceae	2.8963%
Tissierellaceae	6.4259%
Nitrospiraceae	1.0704%
Synergistaceae	1.0296%
Halomonadaceae	3.1185%
Shewanellaceae	1.8963%
Alcaligenaceae	1.5111%
Comamonadaceae	1.2481%

The phylum Actinobacteria, which are a group of Gram-positive bacteria has members that usually adopt to different lifestyles in its association with other microorganisms. They are known to play a huge role in the organic matter degradation and humification process Actinobacteria are known to occur not only in most kinds of environments, but also in extreme environments, which are characterized by acidic/alkaline pH, low or high temperatures, salinity, high radiation, low levels of available moisture, and nutrients (Shivlata and Satyanarayana, 2015). It also includes pathogens that are known to cause disease in animals and plants. The phylum Bacteroidetes was significant among the samples collected from the septic tank and from the septic filter solids on top, but was not found abundantly among samples collected from roller, post roller, pre-drum, drum, or post drum. These were further divided into orders of Bacteroidales, Cytophagales, Flavobacteriales, Sphingobacteriales and Saprospirales. The phylum Bacteroidetes is phenotypically a diverse group of Gram-negative rods that do not form endospores (Bergey et al, 2009**)**. From the pre-compost samples, phylum Euryarchaeota was the only microorganism that belonged to

the kingdom Archaea and the observed sequences shows the presence of Methanobacteria, Methanomicrobia and Thermoplasmata.

Table 3.2: Phyla of microbes in the pre-compost

Phylum	Average Percentage
Other	0.841%
Euryarchaeota	3.796%
Acidobacteria	0.596%
Actinobacteria	9.774%
Bacteroidetes	8.600%
Chlamydiae	0.356%
Chloroflexi	0.689%
Cyanobacteria	1.033%
Firmicutes	45.307%
Fusobacteria	0.278%
Gemmatimonad	1.037%
Lentisphaerae	0.152%
Nitrospirae	1.070%
OP8	1.881%
Planctomycetes	0.870%
Proteobacteria	13.630%
Spirochaetes	0.659%
Synergistetes	1.544%
TM6	0.233%
TM7	1.422%
Verrucomicrobi	0.385%
WPS-2	0.096%
WS3	0.126%
WWE1	0.115%

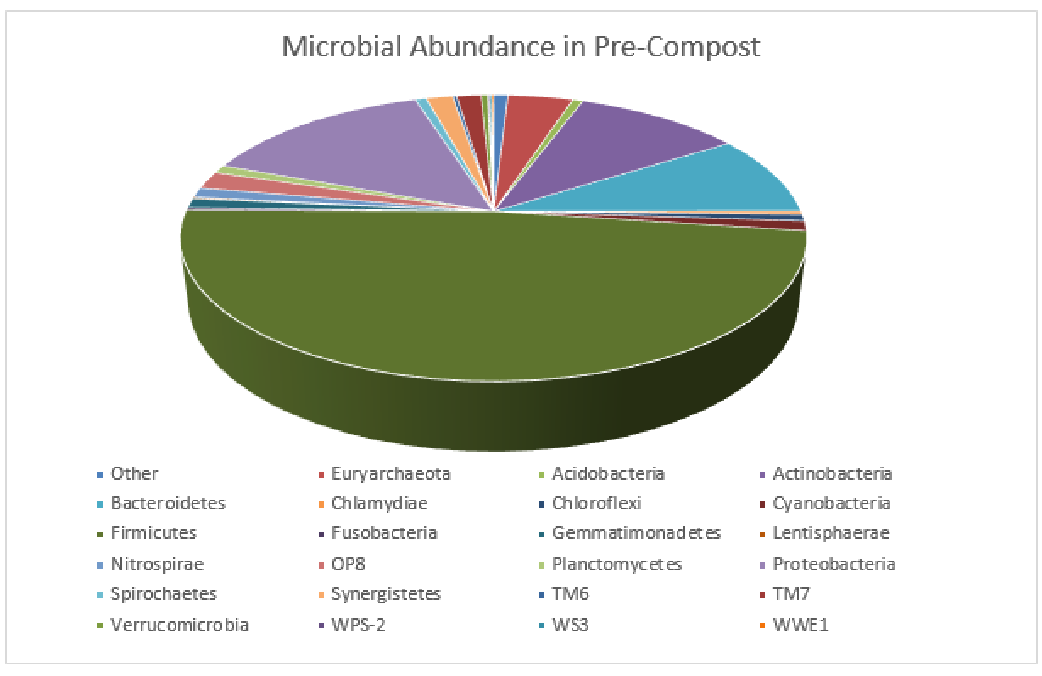

Figure 3.7: Microbial abundance in the pre-compost at the phylum level.

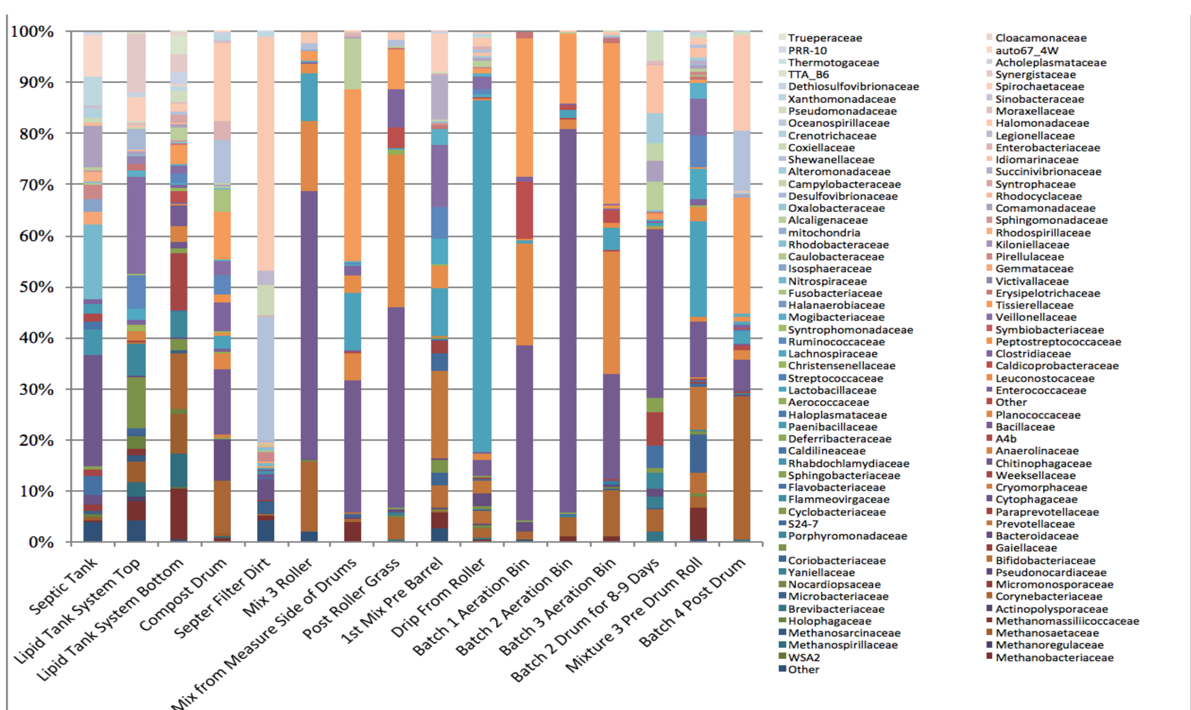

Figure 3.8: Microbial abundance at different locations of pre-compost at family level

3.6 Microbial Abundance in the Aerated Compost Bin

Microbial diversity in the aerated compost bin was studied for which the samples were collected from top, bottom and corners of the compost pile. Most of the microbes identified were categorized into 4 different phyla as shown in Table 3.4. The Firmcutes, Proteobacteria, Actinobacteria and Bacteroidetes constitute 37%, 23.39%, 13.5% and 10.2% respectively. The families of these microbes were also identified and recorded as shown in Table 3.3.

Table 3.3: Family of microbes above 1% abundance in the aerated compost bin

Prominent Family	Average Percentage
Corynebacteriaceae	10.725%
Porphyromonadaceae	1.561%
Weeksellaceae	2.857%
Sphingobacteriaceae	1.146%
Chitinophagaceae	1.596%
Bacillaceae	17.636%
Lactobacillaceae	1.182%
Veillonellaceae	1.250%
Tissierellaceae	5.393%
Alcaligenaceae	1.536%
Comamonadaceae	1.254%
Shewanellaceae	5.200%
Halomonadaceae	8.893%
Pseudomonadaceae	1.568%

Table 3.4: Phyla of microbes in the aerated compost bin

Phylum	Average Percentage
Other	0.51%
Euryarchaeota	2.55%
Actinobacteria	13.50%
Bacteroidetes	10.22%
Chlamydiae	0.19%
Chloroflexi	0.82%
Cyanobacteria	0.26%
Deferribacteres	0.22%
Firmicutes	37.07%
Fusobacteria	0.19%
Gemmatimonad	0.93%
Lentisphaerae	0.15%
Nitrospirae	0.73%
OP8	1.83%
Planctomycetes	0.38%
Proteobacteria	23.39%
Spirochaetes	0.31%
Synergistetes	1.41%
Tenericutes	0.07%
Thermotogae	0.10%
Verrucomicrobi	0.25%
WPS-2	0.09%
WWE1	0.11%
Thermi	0.357%

The majority of bacteria in the aeration pit compost belonged to the families of Corynebacteriaceae (10.7%), Weeksellaceae (2.85%), Bacillaceae (17%), Trissierellaceae (5.3%), Shewanellaceae (5.2%), and Halomonadaceae (8.89%). 0.5% of the microbes are unidentified, which is not a surprise due to the use of shorter-read Illumina sequencing of the 16S rRNA gene. Full-length sequencing of the ~1500 base pair gene would help to minimize this number and could also likely identify candidate and unknown phyla. The phylum Actinobacteria plays an important role in the degradation of complex, recalcitrant compounds. (Steger et al,2006). The microbial abundance at the phylum level was plotted as show in Figure 3.10.

The phylum Bacteroidetes is mainly found in the gastrointestinal tract of animals. Firmicutes was the largest phylum in the compost by average percent abundance. Among Firmicutes, Bacillaceae were the most prominent microbes in the composting unit. The family Bacillaceae is mostly aerobic and they form endospores which gives them resistance to heat radiation, chemicals etc. (Mandic-Mulec et al, 2016). Proteobacteria is the second most abundant phylum of microbes in the compost by percent abundance. Halomonadaceae was the most prominent among all Proteobacteria.

Microbial abundance in each of the samples at the family level was determined and is plotted in Figure 3.9. Bacillaceae was more concentrated in the lower region rather than the surface with an exception of found on the surface where a white fungus was visibly present. Trissierellaceae was not found on the surface and it was more concentrated at 1-2 ft below the surface. Corynebacteriaceae was evenly distributed throughout the aeration bin and was found in almost all samples. Shewanellaceae and Halomonadaceae were present in a bottom sample, but was also distributed in small percentages in different samples collected from the surface. Weeksellaceae was mainly identified in 1-2 ft below the surface and it was literally not present anywhere near the surface.

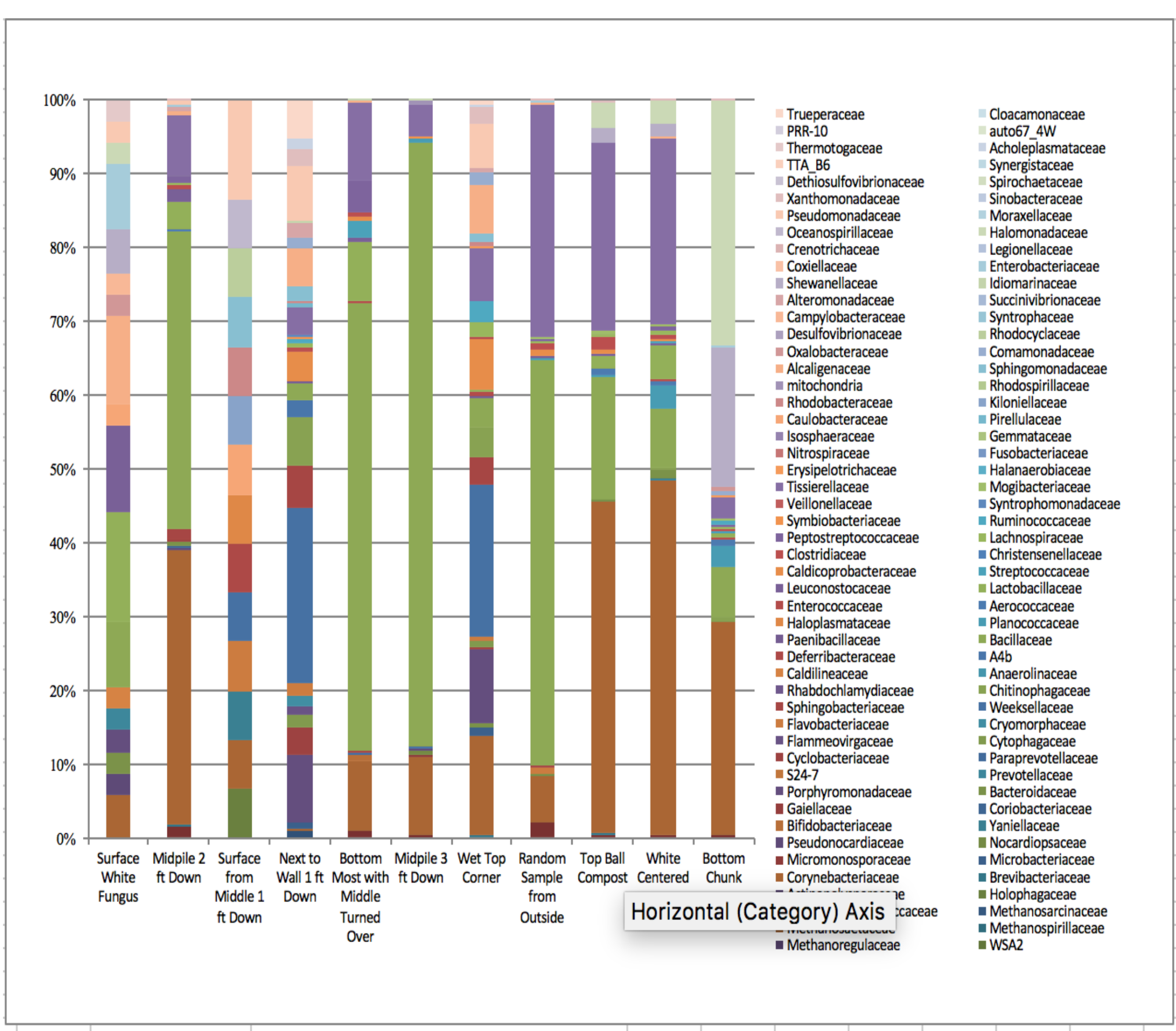

Figure 3.9: Microbial abundance at different locations of compost pit at family level.

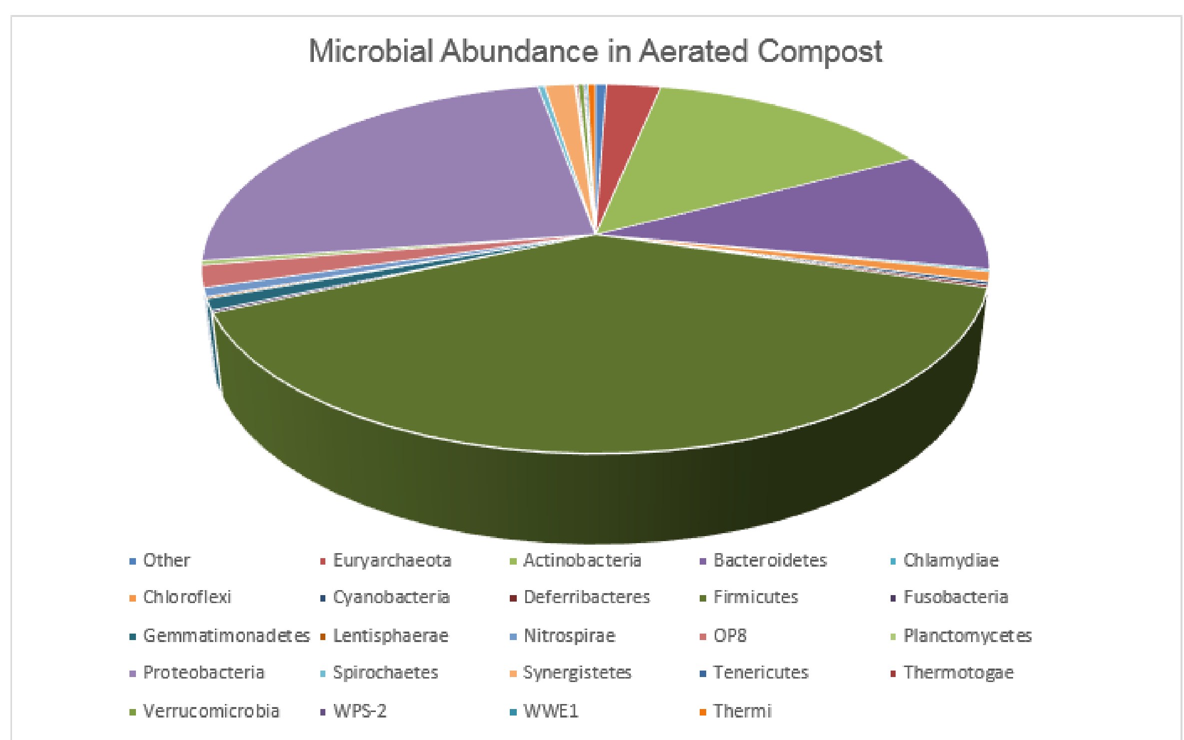

Figure 3.10: Microbial abundance in the compost pit at the phylum level.

CHAPTER 4

DISCUSSION

Microbial Abundance at Different Stages in the Aerated Composting Unit

In studies regarding compost, it has been determined that microbial diversity is rapidly high in the initial stages (Pasi Partanen et. al, 2010). Different microbial communities predominate during the various composting phases, each of which being adapted to a particular environment (Bagstam, 1978). The initial degradation and breakdown of organic materials are initiated predominantly by mesophilic bacteria that prefer to live at temperatures between 12 – 40 ℃ . At the beginning of the decomposition process, another group of bacteria, psychrophilic bacteria, start the process because they are active in lower temperatures up to 12 ℃ (but can function from betweenbelow zero up to 20 ° C), and generate heat in the process. When the temperature inside the decomposing layer reaches 12 – 37 ℃, it attracts mesophilic bacteria to continue the biodegradation and furthe r increases the temperature in the compost environment (Fuller and Norman, 1943). The mesophilic microbes that initiated the decomposition process belonged mostly to the phylum Actinobacteria, Bacteroidetes and Firmicutes. When the temperature reaches 40 – 65℃ (Chen, 2011), mesophilic microbes die off and are replaced by heat loving thermophilic microbes that prefer to live between 60 – 85 ℃ . Approximately 87% of randomly selected colonies during the thermophilic phase of the composting belong to the genus Bacillus (Strom, 1985). The samples were collected from both the pre-composting and composting units as recorded in the tables and figures. The succession of different microbial communities observed explains how specific microbial communities dominate and adapt to the different stages of composting. Some microbial communities were identified to exist in both the units as they can survive in both mesophilic and thermophilic stages of composting. For example, the appearance of Firmicutes in both the pre-composting and composting units suggested adaptation to wider temperature ranges. From the compost unit samples collected in the second round of sample collection, the presence of the Phylum Actinobacteria was identified and this supports the idea that the compost has reached the thermophilic stage of the composting.

4.1 Pathogens identified in the whole compost

A matured compost is primarily a source of nutrients, and can lead to the contribution of a diverse set of beneficial microorganisms once applied to a soil but the presence of pathogens in the compost can be a threat to animals, plants and humans. Generally, the survival of bacterial pathogens in manure can be influenced by the characteristics of the manure, which depends on boththe management and on-farm practices which in turn

determine the method of treatment as well as the microbial makeup in terms of diversity and load (Manyi-Loh et al, 2016). The occurrence and survival of pathogens in this compost has been studied. Table 4.1 includes commonly identified pathogens in a compost made from meat by-products but this has this has limited use for the present study due to the fact that QIIME-generated OTU table didn't provide reliable information of microbiota present at the genus level. To identify specific pathogens, microbes are to be identified at a species level which was not available in the OTU table, therefore this table is broad and common.

The phylum Actinobacteria is responsible for many human and animal diseases and are also is responsible for agricultural losses (Gao et al, 2012). Major pathogens in thephylum Actinobacteria belong to the genus *Mycobacterium, Actinomyces, Renibacterium, Atopobium, Gordonia, Gardnerella, Leifsonia,* and *Clavibacter.* Mycobacterium is responsible for diseases like tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*Mycobacterium leprae*) (Smith, 2003). However, these specific genera were not identified in the compost but the family of microbes that includes those genera were identified in compost. Hence, these pathogens may or may not be present in the system. Many genera of Mycobacterium that are prevalent in the environment can sometimes affect people and sometimes not. An example is *Mycobacterium avium*, a pathogen to immune-compromised people, but not too healthy people. *M. avium* is found in almost every shower head and people are exposed to it upon every bathing shower (Whiley et al, 2015). Other genera found in the compost from the phylum Actinobacteria included Leifsonia and Clavibacter, belonging to the family Microbacteriaceae. Although no plant pathogens have been identified in the family Microbacteriaceae, it is closely related to Clavibacter that consists of plant pathogens. For example, *Clavibacter michiganesis* is a seedborne tomato pathogen (Savidor et al, 2012). Another family found was Bifidobacteriaceae, often found in yogurt, though present in small abundance.

The phylum Bacteroidetes are often classified as opportunistic pathogens meaning they will cause infections if the integrity of the intestinal mucosal barrier is broken (Thomas et al, 2011). Notable human pathogens in the phylum Bacteroidetes are Bacteroides, Porphyromonas, Prevotella and Flavobacterium. Families that contain those pathogens were identified in the compost however these pathogens may require time-intensive tests for an accurate interpretation of their survival in the compost. Flavobacterium attacks hosts with poor immune system and cause diseases (Liu et al, 2011). Bacteroidetes not only affects humans and animals but also affects plants and algae. Bacteroidetes produce polymer degrading enzymes which target host cellular components (Thomas et al, 2011).

The phylum Proteobacteria mainly contains four pathogenic class of microbes which are Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria, and Epsilonproteobacteria among which Gammaproteobacteria contains several species that are highly pathogenic.

Pathogenic Gammaproteobacteria are *Aeromonas, Klebsiella, Pseudomonas, Salmonella, Vibrio, and Yersinia*. Pathogenic Alphaproteobacteria includes members of *Brucella spp.*, and *Bartonella spp*. Pathogenic Betaproteobacteria are *Bordetella, Burkholderia*, and *Neisseria.* Pathogenic Epsilonproteobacteria are *Arcobacter, Campylobacter*, and *Helicobacter* (Liu et al, 2011). Bartonellaceae, Burkholderiales, Neisseriales, and Helicobacteraceae are the only family of microbes that contain at least some of the pathogenic genus mentioned above but all these families are identified to have less than 1 % of abundance in the compost. Because of the low abundance in DNA sequences analyzed, the phylum Proteobacteria members present in the compost should not be considered pathogenic.

The phylum Firmicutes mainly contains two pathogenic classes of microbes which are Bacilli, and Clostridia. A number of bacteria, particularly from the genera Bacillus and Clostridium produce resistant endospores which enable them to survive for long periods in the environment" (Nicholson et al, 2000). They have the ability to form spores that are resistant to the dormant state even when growth conditions are unfavorable, but can revert to vegetative cells through germination as growth conditions becomes favorable in the soil (Manyi-Loh et al, 2016), which typically already contains a host of spore forming microbes. Therefore, these identified pathogens in the aerated compost may not increase the risk of disease transmission. Pathogenic Bacilli includes members of *Bacillus, Enterococcus, Listeria, Staphylococcus, Streptococcus* and pathogenic Clostridia includes members of *Clostridium, Eubacterium* and *Peptostreptococcus*. Among the pathogenic genus mentioned above, *Enterococcus, Staphylococcus, Streptococcus,* and *Eubacterium* have abundances less than 1%in our DNA sequence analysis. On the other hand, *Bacillus, Clostridium*, and *Peptostreptococcus* has abundance greater than 1%, and this is likely due to the nature and in-growth of anoxic zones in the compost process, which favors the growth and development of these anaerobic species.

Typically, temperatures reached in a well-managed compost operation are in a range of 50 to 65 ℃. Such temperatures are well above the thermal death points of mesophili c pathogens (Goluke, 1977). Epstein et al. (2002) reported that high temperatures were extremely effective in the destruction of pathogens. It has been proposed that the attainment and maintenance of a temperature higher than 55 ℃ over a 3-day period should have been sufficient to have eliminated all pathogens (Anon, 1981). In a study conducted by Kudva et al. the findings highlighted that the survival period of pathogenic bacteria was lower in laboratory studies as opposed to observations in field studies, which were influenced by fluctuations in ambient temperature. As a consequence, laboratory results are difficult to apply in field conditions (Manyi-Loh et al, 2016). On the other hand, in scenarios where solid manure is stockpiled outside, i.e., exposed to the environment and ambient temperature, the

survivability of pathogens will be influenced by the seasons or time of the year as the environmental temperature varies and fluctuates from winter (cold) to summer (hot) months and from wet to dry seasons. Owing to the lower temperatures in winter, pathogens have been reported to demonstrate longer survival times compared to summer season (Manyi-Loh et al, 2016)

In addition, a recent paper “Dead or Alive: Molecular Assessment of Microbial Viability the limitations of traditional microbiological methods on analyzing the physiological states of microorganisms present in the samples was addressed. It is simply too difficult to replicate an organism’s natural environment, out of that environment, in the laboratory. Molecular microbiological studies, as herein, have several caveats of its own. Traditional PCR fails at differentiating DNA of a viable microbial cell from that of an inactivated or dead microbial cell. The observation of a viable microorganism in a sample means something entirely different from the observation of a dead one (Cangelosi- et al, 2014), leading to the assumption that the pathogens identified in the system probably consists of both the viable and inactivated microbial cells, hence this study may not be an accurate representation of all the existing pathogens in the compost system. Table 4.1 is a representation of common pathogens identified and their average percentage abundance at the family level.

Table 4.1: Common pathogens in compost unit

Phylum	Family/Class	Presence of pathogenic family	Average Percentage Abundance of Family	Genus	Notable pathogens	Disease caused
Actinobacteria	Mycobacteriaceae	Yes	0%	Mycobacterium	Mycobacterium tuberculosis	Tuberculosis
					Mycobacterium leprae	Leprosy
	Actinomycetaceae	Yes	0%	Actinomyces	Actinomyces israelii	Actinomycosis
	Micrococcaceae	Yes	0%	Renibacterium	Renibacterium salmoninarum	Bacterial Kidney Disease
	Microbacteriaceae	Yes	0.143%	Leifsonia	Leifsonia aquatica	Infections
				Clavibacter	Clavibacter michiganensis subsp. sepedonicus	Ring Rot
Bacteroidetes	Bacteroidaceae	Yes	0%	Bacteroides	Bacteroides fragilis	Wound Infections
	Porphyromonadaceae	Yes	1.561%	Porphyromonas	Porphyromonas gingivalis	Chronic periodontitis
	Prevotellaceae	Yes	0%	Prevotella	Prevotella melaninogenica	Footrot and foot abscesses of cattle
	Flavobacteriaceae	Yes	0.775%	Flavobacterium	Flavobacterium psychrophilum	Bacterial Coldwater Disease
Proteobacteria	Gammaproteobacteria	Yes	17.393%	Aeromonas	Aeromonas hydrophila	gastrointestinal or non-gastrointestinal
				Klebsiella	Klebsiella pneumoniae	pneumonia, bloodstream infections
				Pseudomonas	Pseudomonas aeruginosa	Infections
				Salmonella	Salmonella typhi	Typhoid
				Vibrio	Vibrio vulnificus	Acute gastroenteritis
				Yersinia	Yersinia enterocolitica	Yersiniosis
	Alphaproteobacteria	Yes	1.693%	brucella	Brucella melitensis	Brucellosis
	Betaproteobacteria	Yes	1.511%	bordetella	Bordetella bronchiseptica	Kennel Cough
	Epsilonproteobacteria	Yes	0%	Arcobacter	Arcobacter butzleri	diarrhea
Firmicutes	Bacilli	Yes	0%	Bacillus	Bacillus anthracis	Anthrax
	Enterococcaceae		0%	Enterococcus	Enterococcus faecalis	Endocarditis
	Listeriaceae		0%	Listeria	Listeria monocytogenes	Listeriosis
	Staphylococcaceae		0%	staphylococcus	Staphylococcus aureu	Skin infections
	Streptococcaceae		0%	streptococcus	Streptococcus pyogenes	Rheumatic fever

CHAPTER 5

CONCLUSION AND FUTURE RECOMMENDATION

Compared to 16S rRNA gene sequencing, the sequencing method used in this study, shotgun metagenomic analysis, the random sequencing of all of the DNA in a given sample to give a hint at the total kinds of genes present in that sample, is a more representative approach of the microbial communities present in the system. Although 16S rRNA gene sequencing will give necessary depth of most active players in the system, higher taxonomic resolution is made better possible through shotgun metagenomic analysis. Therefore, a truly accurate identification of pathogens in the compost system was probably not performed in this study. However, the work detailed here provides accurate information that better informs on the quality of the compost. The comparative analysis between these two approaches may actually provide a better insight into the complex microbial communities present, including the activities of pathogens present or not, in the compost system. Assessing the exact microbial content of biosolids, both for biosafety and operational reasons, has traditionally relied on the use of standard microbiological methods (Novinscak et al., 2008) that is traditional, determinative bacteriology with the culturing of microorganisms present in a sample, but for more specific monitoring of pathogens, the application of qPCR is recommended for the future studies. qPCR is Quantitative Polymerase Chain Reaction (qPCR) and is used to simultaneously detect a specific DNA sequence in a sample and determine the actual number of the sequences relative to a standard. Therefore, a qPCR approach can be applied in the future to reflect the real identification of pathogenic sequences in this study (Smith et al, 2009).

Microbial communities are diverse and abundant in slaughterhouse waste initiated compost. This study provides a solid understanding of the kinds of microbial communities present at two different locations of the composting unit. The variety of microbial communities observed also suggests how different types of microbes adapt according to their preferences of temperature zones. The detailed comparison of microbial communities at the pre-composting and composting unit revealed the presence and absence of some microbial communities, which indicates that the microbial succession is influenced by multiple factors such as composting technique, pH, C: N ratio, moisture content and temperature. This study confirms that the microbial activities are directly related to the initial substrate used, in this case, a combination of slaughterhouse wastes, wood chips and Sudan grass. Slaughterhouse wastes are observed to be a source of microbial pathogens that can infect both plants and animals, but a cost effective, safe disposal of wastes from the meat processing plant, as in this study, will reduce the risk of pathogens spreading diseases.

However, the possibility of a microbe spreading disease depends on the particular microbial species and the unique environment in which it thrives. Future research should therefore, be concentrated more on identifying the survival rate of these existing pathogens in the total compost system using time-intensive tests. In addition, it would be good to perform a detailed study on the minimization and inactivation of the pathogens present in the compost (Franke-Whittle et al, 2012).

Most importantly, the presence of an impressive variety of these microbial communities is a valuable source for the continued survey on the system to study on its potential production as an organic fertilizer. This is an ongoing process. Degradation of the organic materials in the compost was not complete when samples were collected but with the continued microbial succession, matured compost is expected to form and it is recommended to test for the nutrient value of the compost. Compost quality testing is necessary to determine the quality of the compost (Anon (a), 1998) and results of compost quality testing provide the basis for which recommendations can be made regarding suitable end uses for the product (Herity, 2003). In addition, a cost savings calculation and life-cycle assessment can be carried out for this on-site composting to compare it to other available feedstock.

REFERENCES CITED

Anon. (1998b). A Framework of Regional (Sub-National) Level Criteria and Indicators of Sustainable Forest Management in Australia. Montreal Process Implementation Group, Commonwealth of Australia, Canberra.

Bagstam, Gudrun. "Population changes in microorganisms during composting of spruce-bark:." *European Journal of Applied Microbiology and Biotechnology* 6.3 (1979): 279-88. Web.

Beffa T, Blanc M, Lyon PF, Vogt G, Marchiani M, Fischer JL, Aragno M. (1996). Isolation of Thermus strains from hot composts (60 to 80 degrees C). Appl Environ Microbiol, 62, 1723–1727.

Berge, Anna Catharina B., Thomas D. Glanville, Patricia D. Millner, and Donald J. Klingborg. "Methods and microbial risks associated with composting of animal carcasses in the United States." *Journal of the American Veterinary Medical Association*234.1 (2009): 47-56. Web.

Bergey, David H., David R. Boone, and George M. Garrity. Bergey's manual of systematic bacteriology. New York: Springer Science Business Media., n.d. Print.

Cangelosi, G. A., and J. S. Meschke. "Dead or Alive: Molecular Assessment of Microbial Viability." *Applied and Environmental Microbiology 80.*19 (2014): 5884-891. Web.

Caporaso, J. G., K. Bittinger, F. D. Bushman, T. Z. Desantis, G. L. Andersen, and R. Knight. "PyNAST: a flexible tool for aligning sequences to a template alignment." Bioinformatics 26.2 (2009): 266-67. Web.

Caporaso, J G. Kuczynski, Justin, Stombaugh, Jesse. William Anton Walters, Antonio González, and Rob Knight. "Using QIIME to Analyze 16S rRNA Gene Sequences from Microbial Communities." Current Protocols in Bioinformatics (2011): n. pag. Web.

Chen, L., A. Moore, C. Falen, and M. Haro Marti. *The Composting Process*(2011): n. pag. *www.cals.uidaho.edu*. Web.

Dees PM, Ghiorse WC. (2001). Microbial diversity in hot synthetic compost as revealed by PCR-amplified rRNA sequences from cultivated isolates and extracted DNA. FEMS Microbiol Ecol, 35, 207–216.

Desantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen. "Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB." Applied and Environmental Microbiology 72.7 (2006): 5069-072. Web.

Driks, Adam, and Patrick Eichenberger. The bacterial spore: from molecules to systems. Washington: ASM Press, 2016. Print.

Edgar, R. C. "Search and clustering orders of magnitude faster than BLAST." Bioinformatics 26.19 (2010): 2460-461. Web.

Epstein E. Human pathogens: hazards, controls and precautions in compost. Compost utilisation in horticultural cropping systems. Boca Raton, USA: Lewis Publishers; 2002. p. 361–80.

Fuller WH, Norman AG. Characteristics of Some Soil Cytophagas. J Bacteriol. 1943 Jun;45(6):565–572.

Franke-Whittle, Ingrid H., and Heribert Insam. "Treatment alternatives of slaughterhouse wastes, and their effect on the inactivation of different pathogens: A review." *Critical Reviews in Microbiology* 39.2 (2012): 139-51. Web.

Gao, B., and R. S. Gupta. "Phylogenetic Framework and Molecular Signatures for the Main Clades of the Phylum Actinobacteria." *Microbiology and Molecular Biology Reviews* 76.1 (2012): 66-112. Web.

Gajalakshmi, S and Abbasi, S (2008). “Solid waste management by composting: State of the art”, Critical Reviews in Environmental Science and Technology, Vol. 38, No. 5, 2008, pp. 311-400. DOI: abs/10.1080/10643380701413633.

Herity, Lorraine. *A Study of the Quality of Waste Derived Compost in Ireland*(2003): n. pag. Composting Association of Ireland. Web.

Janda, J. M., and S. L. Abbott. "16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls." Journal of Clinical Microbiology 45.9 (2007): 2761-764. Web.

Kozich, J. J., S. L. Westcott, N. T. Baxter, S. K. Highlander, and P. D. Schloss. "Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform." Applied and Environmental Microbiology 79.17 (2013): 5112-120. Web.

Liu, Dongyou. *Molecular detection of human bacterial pathogens*. Boca Raton, FL: Taylor & Francis/CRC Press, 2011. Print.

Mandic-Mulec I, Stefanic P, van Elsas J. 2016. Ecology of *Bacillaceae*, p 59-85. *In* Driks A, Eichenberger P (ed), *The Bacterial Spore: from Molecules to Systems*. ASM Press, Washington, DC. doi: 10.1128/microbiolspec.TBS-0017-2013.

Manyi-Loh, Christy, Sampson Mamphweli, Edson Meyer, Golden Makaka, Michael Simon, and Anthony Okoh. "An Overview of the Control of Bacterial Pathogens in Cattle Manure." *International Journal of Environmental Research and Public Health* 13.9 (2016): 843. Web.

Mcmurdie, Paul J., and Susan Holmes. "Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data." PLoS ONE 8.4 (2013): n. pag. Web.

Nicholson, W. L., N. Munakata, G. Horneck, H. J. Melosh, and P. Setlow. "Resistance of Bacillus Endospores to Extreme Terrestrial and Extraterrestrial Environments." *Microbiology and Molecular Biology Reviews 64*.3 (2000): 548-72. Web.

Novinscak, A., M. Filion, C. Surette, and C. Allain. "Application of molecular technologies to monitor the microbial content of biosolids and composted biosolids." *Water Science & Technology* 57.4 (2008): 471. Web.

Partanen, Pasi, Jenni Hultman, Lars Paulin, Petri Auvinen, and Martin Romantschuk. "Bacterial diversity at different stages of the composting process." BMC Microbiology 10.1 (2010): 94. Web.

Sadek, Omar . "Advanced Composting Discussion: Carbon to Nitrogen Ratio simplified Calculations." Lecture.

Savidor, Alon, Doron Teper, Karl-Heinz Gartemann, Rudolf Eichenlaub, Laura Chalupowicz, Shulamit Manulis-Sasson, Isaac Barash, Helena Tews, Kerstin Mayer, Richard J. Giannone, Robert L. Hettich, and Guido Sessa. "The Clavibacter michiganensissubsp.michiganensis–Tomato Interactome Reveals the Perception of Pathogen by the Host and Suggests Mechanisms of Infection." *Journal of Proteome Research*11.2 (2012): 736-50. Web.

Shivlata, L., and Tulasi Satyanarayana. "Thermophilic and alkaliphilic Actinobacteria: biology and potential applications." Frontiers in Microbiology 6 (2015): n. pag. Web.

Smith, Cindy J., and A. Mark Osborn. "Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology." *FEMS Microbiology Ecology* 67.1 (2009): 6-20. Web

Smith, I. "Mycobacterium tuberculosis Pathogenesis and Molecular Determinants of Virulence." Clinical Microbiology Reviews 16.3 (2003): 463-96. Web.

Sobsey, M. D., L. A. Khatib, and V. R. Hill. "Pathogens in Animal Wastes and the Impacts of the Waste Management Practices on their Survival, Transport and Fate." (2006): n. pag. Web.

Sundberg, Cecilia. Improving Compost Process Efficiency by Controlling Aeration, Temperature and pH (2005): n. pag. Web.

Suihko, Maija-Liisa, and Eija Skyttä. "Characterisation of aerobically grown non-spore-forming bacteria from paper mill pulps containing recycled fibres." Journal of Industrial Microbiology & Biotechnology 36.1 (2008): 53-64. Web.

Strom, P.E. 1985. Identification of thermophilic bacteria in solid waste composting. *Appl. Environ. Microbiol.,* 50(4): 906-913.

Thomas, François, Jan-Hendrik Hehemann, Etienne Rebuffet, Mirjam Czjzek, and Gurvan Michel. "Environmental and Gut Bacteroidetes: The Food Connection." *Frontiers in Microbiology* 2 (2011): n. pag. Web.

Vázquez-Baeza, Yoshiki, Meg Pirrung, Antonio Gonzalez, and Rob Knight. "EMPeror: a tool for visualizing high-throughput microbial community data." GigaScience 2.1 (2013): n. pag. Web.