An Evaluation of Microbial Communities and the Fate of Pathogens During Thermophilic Composting of Agricultural Land Waste

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Abstract

Thermophilic composts are exceptional dynamic ecosystems where organic waste is degraded and biologically stabilized. With the global importance of safe waste management, thermophilic composting has been a suggested point source method to eliminate agricultural waste from going to landfills and human waste from being transported by water-based sanitary systems. Surprisingly, considering the implications of composting practices on agricultural productivity, carbon-sequestration, and human health, little is known regarding the microbial dynamics of thermophilic composts and the fate of pathogens introduced. Using a custom 16S rRNA high-density DNA microarray (PhyloChip), bacterial community dynamics and related pathogenic bacteria in thermophilic composts of agricultural waste were investigated. Interestingly, almost 1600 subfamily taxa and 63 related pathogens were detected as present between raw manure and composting samples. It was shown that many spore-bearing bacteria such as *Bacilli* and *Streptosporanginea* increased directly with compost temperatures, while the overall presence and richness of potential pathogens declined. Our study concludes the first, high-density DNA microarray evaluation of microbial communities and pathogenic relatives in waste before and during thermophilic composting.

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Introduction

The use of clean drinking water to transport and treat waste has become an unsustainable and possibly economically non-viable means of sanitation control. In developed countries, the infrastructure of pipelines and septic tanks for water-based transport sanitary systems has become a significant economic burden. According to the U.S. Environmental Protection Agency's findings in their 2007 report, the costs of maintaining the current infrastructure for withdrawing clean drinking water is projected to be $334.8 billion dollars over the next 20 years in the US [1]. And more specifically, if they maintain their zero tolerance policy for sewage overflows because of public and environmental health concerns, it is projected to cost the US over $88 billion dollars in the next 20 years [2]. Consequently, both the direct and indirect costs for maintaining aging water pipelines and cleaning after sewage spills are a major expenditure for both the United States and other well-developed countries. As for many developing countries that have access to cleaning water sources and the financial capability to build the infrastructure for a water-based transport sanitary system, the economic costs to protect public and environmental health and to maintain the required infrastructure is unrealistic.

In addition to the high costs of water-transport sanitary systems, waste treatment plays a critical role to public health. In fact, half of all hospitalizations worldwide are from a lack of access to safe drinking water, inadequate sanitation, or poor hygiene. [3]. According to the World Water Council, more than 1.1 billion people live without access to safe drinking water in the world and more than 2.6 billion people do not have access to adequate sanitation. [4] Because of the global problem of adequate sanitation and clean drinking water, detrimental consequences are continually seen on every continent in the world. According to the World Health Organization's report in 2004, every year approximately 1.8 million people die from diarrheal diseases, and every day, 3900 children die from water borne diseases [4]. With this in mind, it is necessary to look towards more sustainable and economic practices of processing waste without using valuable drinking water and still maintain a high level of environmental and public health. One possibly crucial way to regain sustainability on processing waste is to use a source control method such as thermophilic composting, which eliminates the need for water to be used to process and transport waste.

Thermophilic composting involves the monitoring and maintaining of ideal conditions for aerobic, thermophilic bacterial growth in an organic compost pile. Often, waste is initially mixed with wood or straw and watered so that the C:N ratio is about 1:25 and the moisture is about 60% inside the pile. Typically during the second stage of composting (after mesophilic growth), thermophilic bacteria metabolize the organic waste in the pile at a fast rate and, in the process, heat the compost to high temperatures ranging between 40 °C and 71.1 °C [5]. Finally, at the end of the third stage known as curing, it is believed that as bacteria consume the organic material, the pile becomes

biologically stabilized. As a result, the compost cools and can be used as an excellent source of fertilizer to enrich agricultural land and aid in sequestration of carbon.

It has been hypothesized that during the thermophilic stages of bacterial growth and metabolism, the physical heat produced in the compost piles greatly reduces the number of pathogens [5]. However, it also has been shown that attempting to correlate the fate of pathogens such as *E. coli* and *Salmonella* with either temperature or time is difficult, which suggests that the fate of pathogens is more complicated and needs to be further investigated [6]. In addition, detecting the presence or absence of Bacteria using traditional culture-based assays has proved to be insufficient since most bacterial communities are viable but non-culturable [7]. Because culturing methods can't give a fair representation of true communities, molecular methods have replaced culture-based methods. Specifically, the PhyloChip developed at Lawrence Berkeley National Laboratory (LBNL) hybridizes targets of conserved sequences and variable regions of the universal 16S rRNA gene found in nearly all prokaryotes to simultaneously detect relative abundance and presence of microbial communities. PhyloChip technology has proven to be an invaluable way to detect Archaea and Bacteria without cultivation and was used to determine the microbes present in the thermophilic compost piles for this experiment.

In this experiment, our research group investigated the fate of pathogens and the microbial community dynamics in thermophilic agricultural (cow manure) composts at unique time intervals using the high-density 16S rRNA PhyloChip (LBNL, Andersen Lab). The results of this study will give scientists and researchers an idea of how these complex ecosystems interact and change, and what bacteria are present. More

importantly, the results of this experiment could provide insight to the safety of using composting as a point source on human waste in developing countries where water-based sanitary transport systems are a distant possibility.

Materials and Methods

Sampling Sites

On June 22, 2011, 11 samples were collected from a thermophilic compost pile and manure pile at a grassland research ranch (Marin Carbon Project, Nicasio, Ca). The compost pile was constructed as stacked layers vertically with the oldest layer at one end and the freshest at the other end. The starting materials of the compost pile were raw cow manure, food waste, and wood chips. In order to collect the samples from the core of each pile, a bulldozer was used to plow and later reveal each cross-section of the pile for sampling. After testing each sample area with a temperature gauge, duplicate physical samples were collected with a sterile spatula into 50 mL Falcon tubes. The spatula was sterilized between sampling 10% bleach and rinsed with water. Clean gloves were used for each sampling. After collecting duplicate samples from each of the four layers, the temperature and stage of composting were recorded. Lastly, triplicate time-zero samples were collected from a raw cow manure pile used to build the compost layers. Samples were immediately placed in a dry ice cooler and transported to Lawrence Berkeley National Lab (Andersen) where they were stored at -80 °C.

DNA Extractions

To isolate DNA, each sample was physically homogenized in a mortar and pestle. Following homogenization of each sample, samples were stored overnight at -80 °C. For DNA extractions, roughly 0.15 gram of each sample was placed in separate sterile ZR BashingBead Lysis Tubes with 750 µL of Lysis Solution (Zymo Research, Irvine, Ca), and the samples were physically lysed in a Disruptor Genie for 5 minutes. The samples were centrifuged for 1 minute at 10,000 *x* g. Four-hundred micro liters of supernatant was then filtered at 7,000 x *g* for 1 minute in Zymo-Spin IV Spin Filters into collection tubes and treated with 1200 µL of Fecal DNA Binding Buffer. The samples were washed with 200µL of Pre-Wash Buffer, and 500µL of Wash Buffer. Following washing, the IIC columns were treated with 100µL of Elution Buffer and centrifuged at 10,000 x *g* for 30 seconds to elute the DNA. Finally, the extracted DNA was quantified with the Qubit 2.0 Florometer (Life Technologies, Carlsbad, Ca) and stored at -20°C.

16S PCR Amplification for Microarray Hybridization

Polymerase Chain Reaction amplification for microarray hybridization was carried out on each sample using universal bacterial 16S rRNA 27F (5' - AGAGTTTGATCCTGGCTCAG- 3') and 1492R (5' –GGTTACCTTGTTACGACTT - 3') gene primers in a Gradient Thermocycler for 25 cycles at 48 °C to 58 °C. Following amplification of the 16S rRNA gene, the bacterial PCR products were purified and run on

a 2% Ethidium Bromide Agarose gel by gel-electrophoresis for quantification. Finally, the amplified gene products were compared and quantified using a UV gel-dock (Bio-Rad, Hercules, Ca) against a calibrated DNA ladder on each gel.

PhyloChip Hybridization and Image Processing

For each sample, replicate samples were run independently and their results were compared. The amplified bacterial 16S rRNA gene products were eventually hybridized onto custom PhyloChip G3 microarrays. The gene products were first fragmented to 50-200 base pairs using DNAseI and incubated at 25 °C for 25 minutes and 98°C for 10 minutes. Following fragmentation, the samples and spikes were labeled with biotin as reported in [11]. The labeled fragments were then loaded into the hybridization chambers of the PhyloChips (Affymetrix GeneChips, Santa Clara, Ca) and hybridized at 48 °C and 60 rpm for 16 hours. The PhyloChips were then washed stringently on an Affimetrix 450 Fluidic Automated System according to the protocol described in [11] in order to remove unbound targets and to label bound targets with streptavidin phycoerytherin.

Each chip was scanned and saved as a fluorescent image using a GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, Ca). After the images were taken, data normalization, background reduction, and probe pair scoring was preformed by a computer software program developed by Todd DeSantis at LBNL known as PhyCA. To determine the presence of certain taxa, sets of criteria had to be passed in each of two stages. In stage one, the response score to measure the potential that the probe pair was responding to a target and not background was calculated for three quartiles: rQ_1, rQ_2,

and rQ_3 using the following equation as described in [11]. In order for an OTU to pass stage one, all of the quartiles had to pass $rQ_1 \geq 0.70$, $rQ_2 \geq 0.95$, and $rQ_3 \geq 0.98$, based on the specificity of each probe set for the given OTU. Following the stage one criteria, stage two used the OTU that passed stage one adjust for cross-hybridization. After assigning penalties to probes as described in [11] for potential cross-hybridizations, if the $r_xQ_3 \geq 0.48$, then the subfamily was considered present in the compost sample. Finally, three data tables were created with the image data, one of probe intensities $\log_2$ transformed, one of subfamily presence/absence (binary), and one with binary values for every OTU for statistical analysis of potential pathogens and microbial communities.

Statistical Analysis

The R software environment [12] was used for all statistical calculations and operations on the PhyloChip data after PhyCA. For each sample, the intensity values were first compared amongst each other using a Bray-Curtis-based distance matrix by inputting the function "metaMDS" within the Cran Vegan package [13]. The Bray-Curtis measure of dissimilarity is considered a standard way of measuring ecological distances amongst communities because it allows for non-linear responses to environmental factors [8]. Next, the results of the Bray-Curtis distance matrix were plotted as an ordination plot using NMDS.

Compost temperature, age, and bacterial phase were recorded for environmental parameters. The environmental parameters were assessed for influence in variance of OTU intensities using "Adonis()" in the Vegan package [14]. From this test, the $P_r(>F)$

values showed what environmental factor most significantly influenced the distance matrix. Next, student t-tests were preformed between the manure samples and the thermophilic samples, between manure samples and end-stage curing samples, and lastly between thermophilic samples and curing samples. P values were corrected using the Benjamini-Hochberg correction. The Benjamini-Hochberg correction is considered a good correction for PhyloChip data because it is less stringent for large numbers of tests compared to the Bonferroni correction. From the significant OTU (corrected P-values <0.05) a heat map was constructed using heat map(stats, R) to show the major microbial shifts using hierarchal-cluster analysis. The OTU were then grouped into their respective Subphyla and Classes. Their binary (presence/absence) values were used to graph taxa richness between the sample points.

To investigate potential pathogens in each sample, 47 pathogens commonly found in agriculture and sanitation waste were compiled and their corresponding OTU were determined from the greengenes G3 OTU sequences database using "grep" in R. From there, the OTU intensity and binary values were filtered for the pathogenic OTU. Family affiliations were graphed as a function of time, and OTU intensities were graphed with a color gradient on a phylogenetic tree using ITOL [13]. Lastly, a heat map of significant pathogens was constructed after performing another student T-test between pre-treatment and treatment samples.

Results

Microbial Community Shifts

For all samples, abundance values for every OTU were compared using a Bray-Curtis-based distance matrix in R. The results were analyzed using Nonmetric Multidimensional Scaling ordination (Figure 1). The two dimensional representation of the Bray-Curtis-based distance matrix was plotted using a Shephard plot, and the stress was 13.8 with two convergent solutions after 17 attempts.

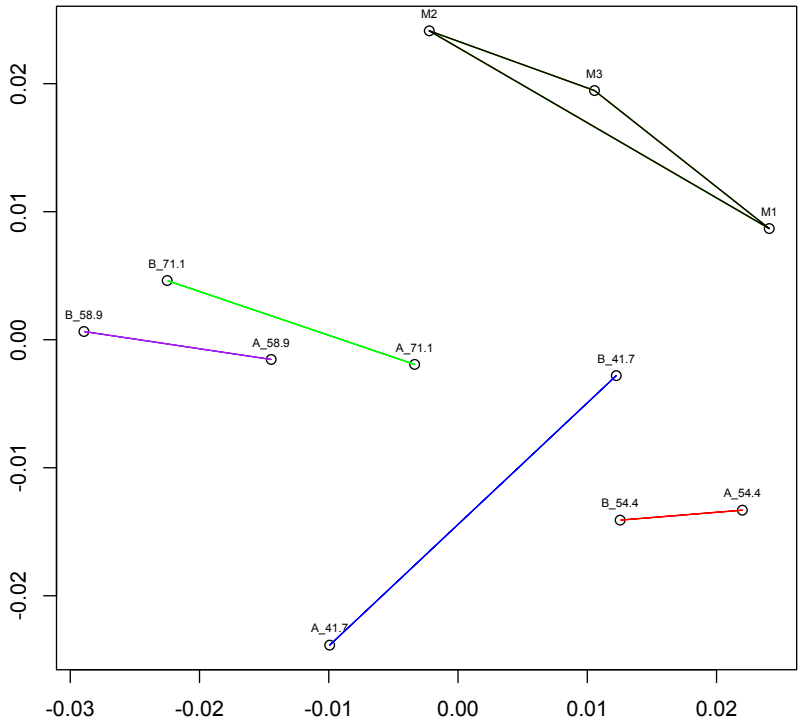


Figure 1. NMDS Plot using Bray-Curtis Dissimilarity Matrix. Replicates of each treatment were connected by colored lines for clarity

The NMDS ordination shows that the manure microbial communities differed significantly from the composting samples. In addition, the compost samples clustered based mainly on their temperatures rather than time intervals. Next, to detect which organisms significantly changed between the manure samples and the thermophilic composting samples, the compost samples and manure samples were compared using a

student's t-test with Benjamini-Hochberg corrected P-values. The 242 significant OTU ($P<0.05$) were plotted in a heat map using a hierarchal-cluster analysis of their log_2 transformed intensity values (Figure 2). While the *Bacteroidales* and *Flavobateriales* Classes declined between the manure and compost samples, the *Streptosporangineae*, *Bacillus*, and *Paenibacillaceae* dramatically increased in intensities.

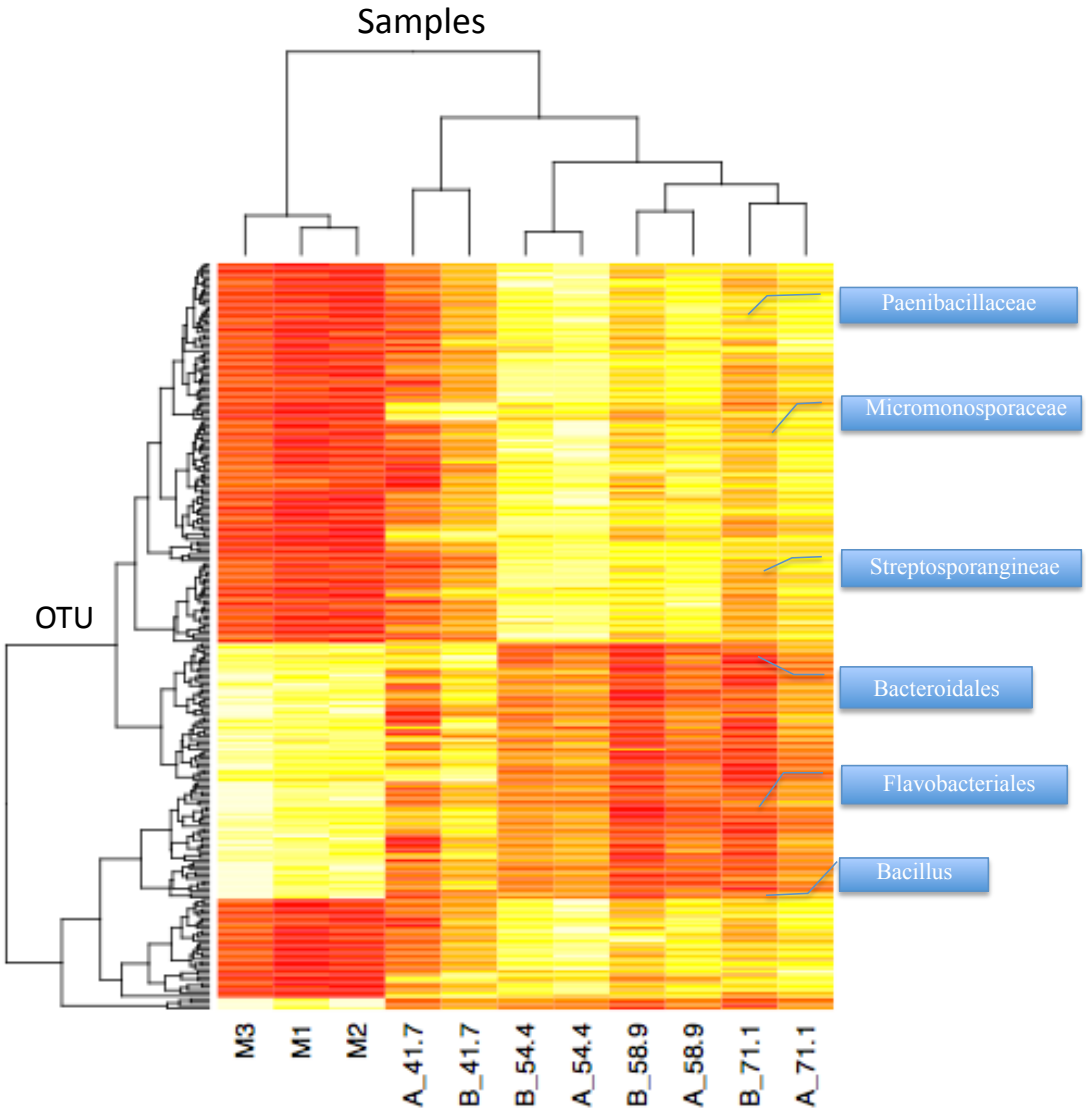


Figure 2. Bacterial community heat map based on intensities. Red: low intensity, Yellow: high intensity

An Adonis test was preformed on all 11 samples with environmental factors and the Bray-Curtis-based distance matrix as input to determine the variance due to

environmental factors. It was shown that the temperature had a greater influence on the variance between samples. Even more importantly, the bacterial phase had the most influence on the microbial communities.

Table 1. Adonis Test Results

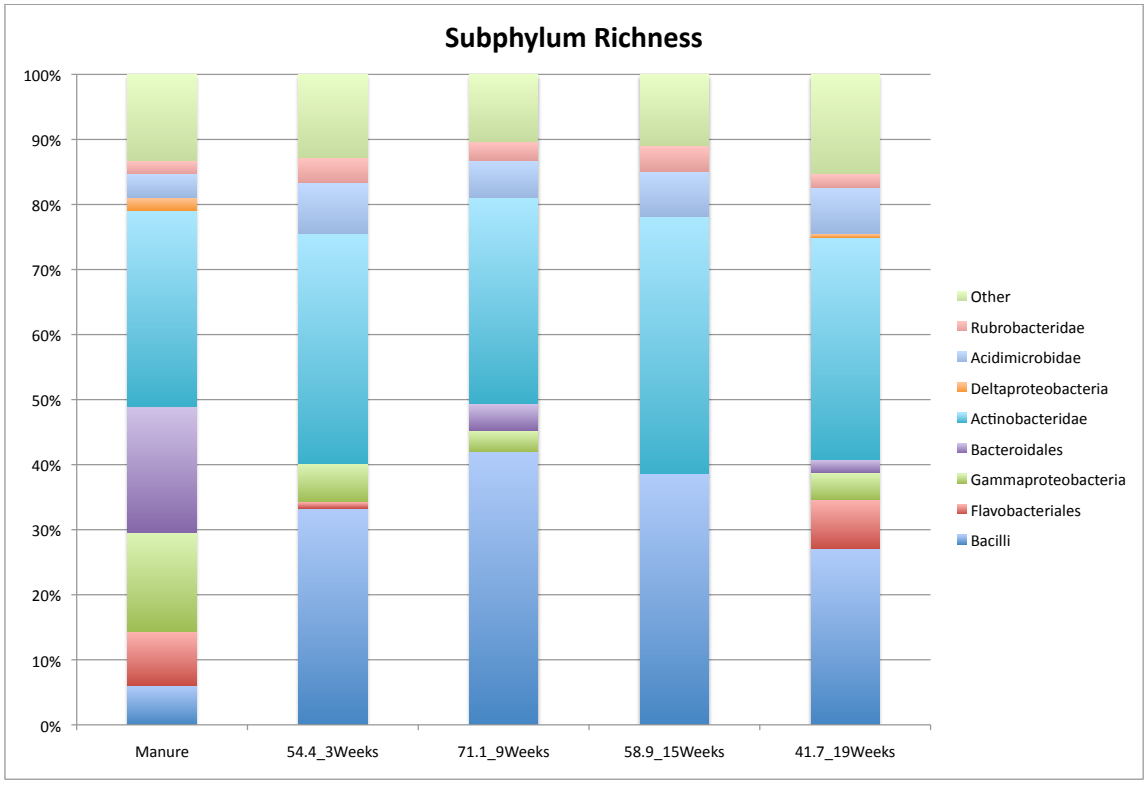
Environmental Factor	R2 Pr(>F)
Temperature	0.005 **
Time	0.047 *
Bacteria Phase	0.004 **

Significant codes: 0 '***' 0.001 '**' 0.01 '*'

Subphylum and Class Richness

Subphylum richness was compared between the manure samples and compost samples. The binary values for each OTU were averaged between duplicate samples of each time point and summed for each subphylum. While the *Acidimicrobidae*, *Actinobacteridae*, and *Rubrobacteridae* differed the least across treatments, the *Bacilli*, *Flavobacteriales*, *Gammaproteobacteria*, and *Bacteroidales* changed dramatically over the course of composting (Figure 3). Because the Subphylum *Bacilli* showed a dramatic increase as the compost temperature increased, it was investigated in detail by plotting a richness graph for the Class-level. Although all of the classes increased in presence between the manure and compost samples, the *Bacillus* and *Paenilbacillaceae* increased the most dramatically (Figure 4).

Figure 3. Subphylum Richness



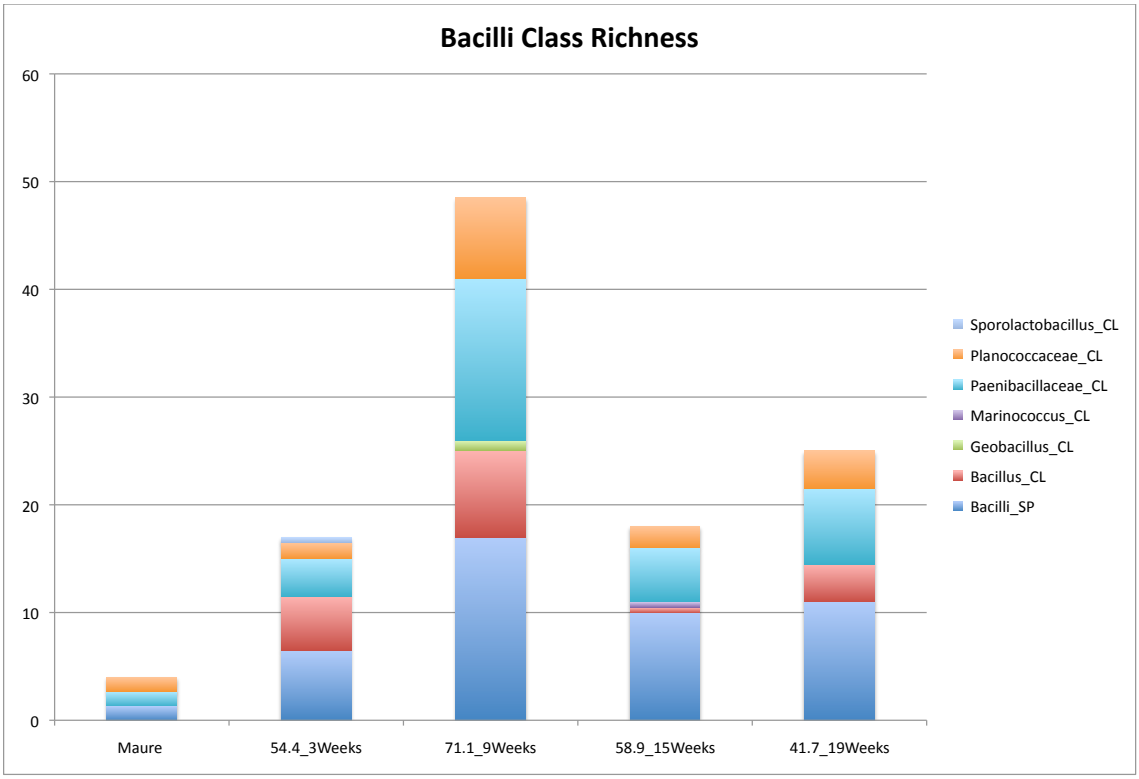
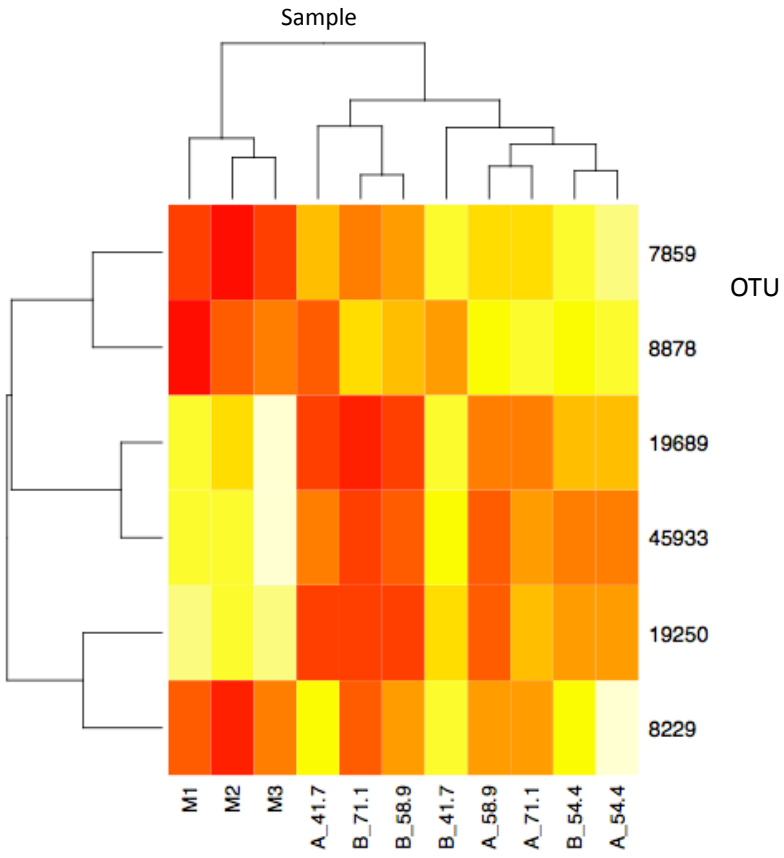


Figure 4. Bacillus Class Richness

Pathogen Family Richness and Overall Shifts

Sixty-three OTU containing pathogenic members were compared using a T-test and six were found to differ significantly ($P<0.05$). The intensity values of the six significant pathogenic OTU, were then plotted in a heat map to show which pathogens declined or increased between the manure samples and composting samples as shown in Figure 5.

Figure 5. Significant pathogen heat map based on intensities. Red: low intensity, Yellow: high intensity



OTU #	Order	Family	P-Values	BH Corrected
45933	Bacteroidales	Unclassified	0.0010	0.0643
7859	Bacillus	Bacillus	0.0009	0.0689
8229	Bacillus	Bacillus	0.0083	0.1960
8878	Bacillus	Bacillus	0.0414	0.1707
19250	Aeromonadaceae	Aeromonadaceae	0.0001	0.0340
19689	Aeromonadaceae	Aeromonadaceae	0.0262	0.1482

Table 2. Pathogen P-Values

In Table 2, the six pathogenic OTU that had student t-test P-values <0.05 were compared to the Benjamini-Hochberg corrected P-values. The 63 pathogens were then grouped by Families and plotted (Figure 6) using their binary values averaged between duplicates and summed in the same way as Figure 3. While there was a noticeable increase in relative pathogenic families from the manure to the first composting time point, there was an overall decline in overall family richness and presence throughout the composting samples. More notably, the *Enterobacteriaceae, Bacillus,* and *Clostridiaceae* families showed a dramatic decline over the composting phases.

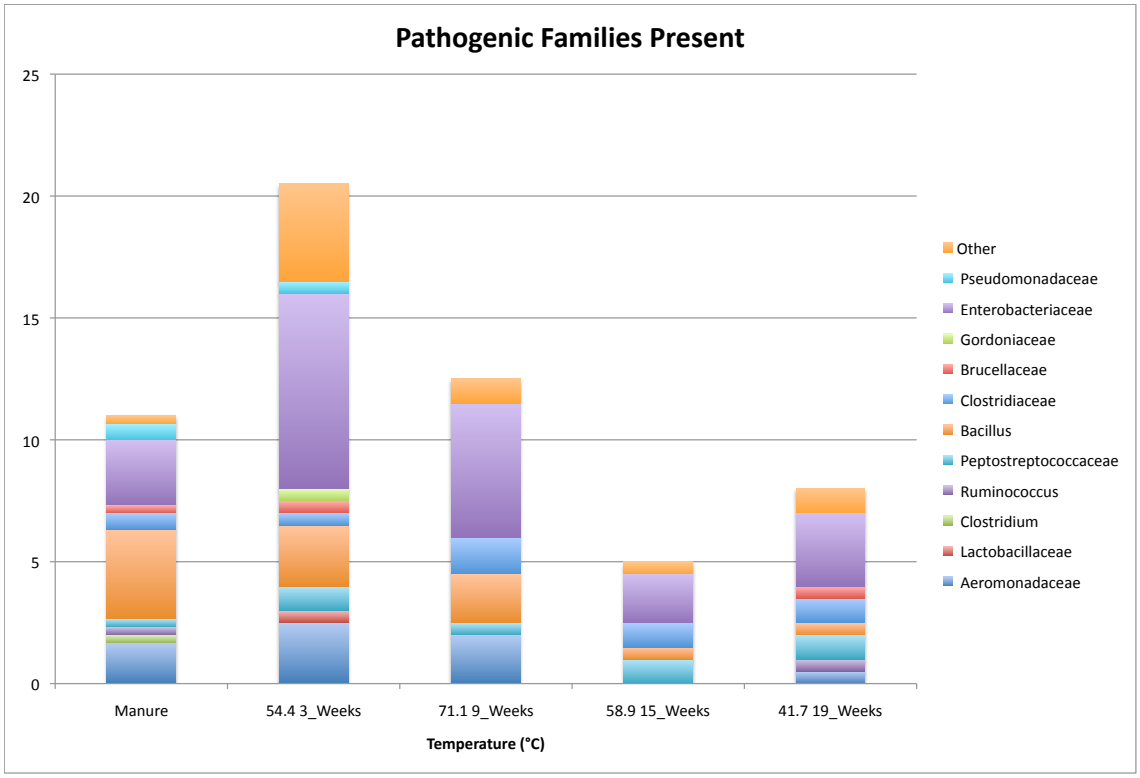


Figure 6. Pathogen Families Present

Next, the phylogenetic distance of all 237 target pathogenic OTU were compared using FastTree using their ProkMSA sequences. The results from FastTree were then uploaded to ITOL to create a phylogenetic tree with each leaf representing an OTU. Their families were colored at the node and tip of each leaf. In addition, a blue color gradient

was placed on the outside for each sample time point (internal: manure, exterior: 19 weeks) to track the intensity over time. While most of the intensity values did not decline over time, it was observed that certain OTU in the *Enterobacteriaceae* family declined during thermophilic composting (Figure 7).

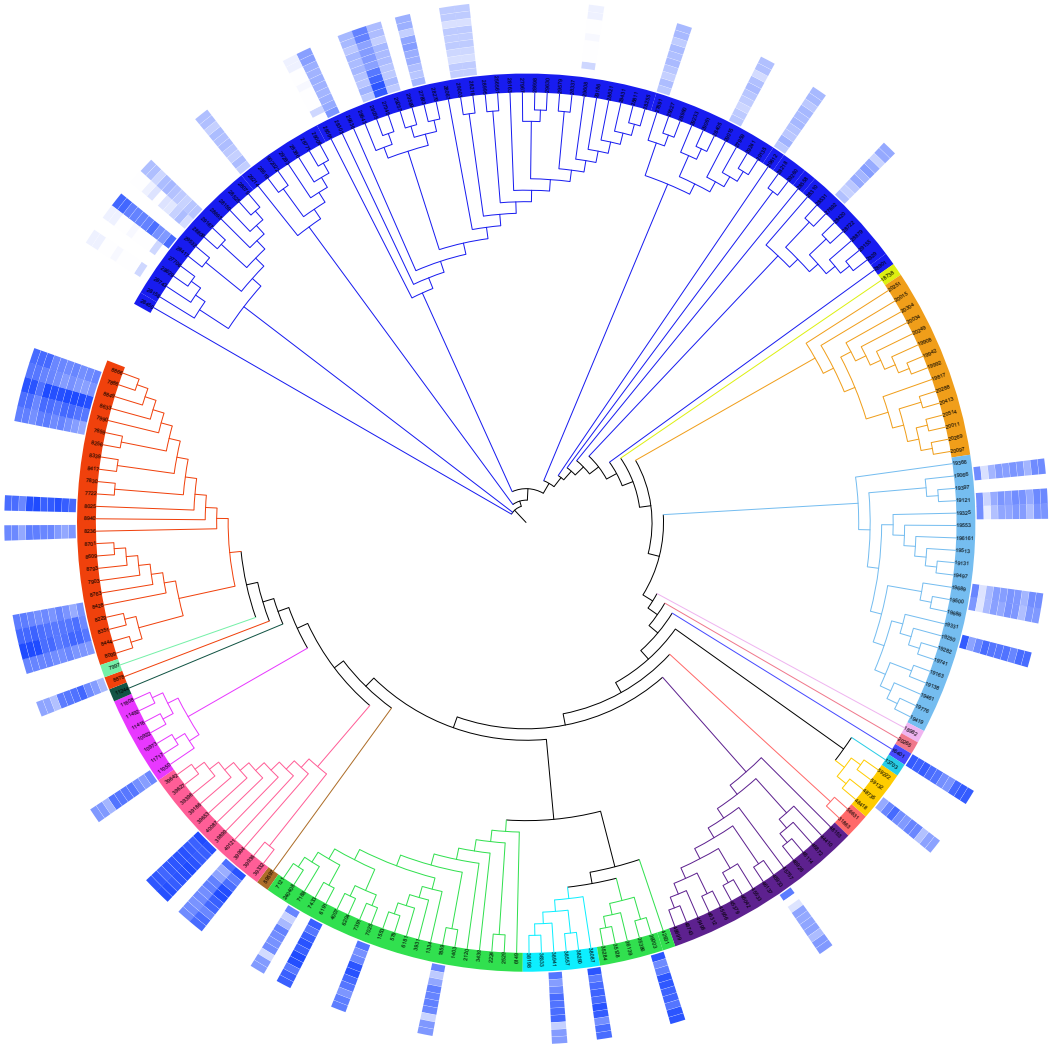
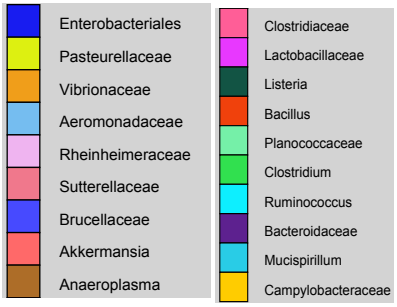


Figure 7. Phylogenetic Tree of Pathogenic OTU



Discussion and Conclusion

Significant differences were observed between manure and compost samples. In Figure 1, it was shown that the samples clustered primarily based on whether they were in the compost or the manure pile, and secondarily based on their temperatures. It was expected that the samples would cluster based on treatment, however, it was unexpected from both the NMDS plot (Figure 1) and the Adonis Test (Table 1) that the samples would cluster by temperatures and not age. It has been shown that the physiochemical changes in compost piles affect the bacterial communities [14], and because of that, it was expected that as the composting process carried on, age would have a greater influence on the pile than temperature as nutrients are depleted. However, the Adonis Test showed that with the P-value of 0.005, temperature was a slightly larger factor than the age (weeks) since the P-value was 0.047.

In Figure 3, it was observed that significant changes to Subphylum richness between the samples took place. Most noticeably, the *Actinobacteridae* changed the least between the manure and composting samples. It is well known that many *Actinobacteridae* are soil bacteria with high GC-content [16], suggesting that they are well evolved to survive at high temperatures like hot compost piles. Interestingly, many cellulose-degrading bacteria are also known to fall in the same Subphylum, and therefore flourished in the wood-chip compost pile [16]. Noticeably, the Subphylum *Gammaproteobacteria* declined in richness immediately between the manure and compost samples.

It has been stated that pathogen reduction is reached when the compost reaches 55 °C [5], which was shown by the reduction the pathogen bearing Subphylum

Gammaproteobacteria. This pathogen destruction can be explained not only by the increase in temperature, but by the competition with thermophilic bacteria that are highly evolved and well-equip to live in hot compost piles [20].

In addition, we saw a significant increase in *Bacilli* between the compost and manure samples. Many of the known thermophiles in compost are known to be *Bacilli* because many are spore bearing and activate only when the compost reaches a suitable environment [21]. And since it has been shown that there are thousands of taxa of bacteria freely circulating the atmosphere [8], there is opportunity for bacteria to enter the compost and experience growth given the right environmental conditions. Because the Subphylum *Bacilli* showed a dramatic increase as the compost temperature increased, the classes were investigated in detail. The results showed that at the peak temperatures, a known set of thermophiles, *Geobacillus* [17], were present in greater numbers. In addition, although Class *Bacillus* increased with increasing temperature, it decreased dramatically during temperature reduction, possibly due to increased competition.

In Figure 6, we observed that the overall richness of pathogenic families showed a significant reduction between weeks three to fifteen, suggesting that the temperature and other selective pressures like competition with thermophiles caused for a reduction of pathogens. Most importantly, human and agricultural pathogens of concern with in the Families *Aeromonadaceae*, *Bacillus*, and *Enterobacteriaceae* showed the most significant reduction with time. In addition, after correcting the P-values of the pathogens, it was noted that the OTU (19250) belonging to the diarrheal causing bacterium *Aeromonas veronii* [18] showed the most significant reduction between manure and composting samples suggesting that composting is an effective method in reducing their abundance.

Because the 16S rRNA gene is highly conserved, it was chosen for probe analysis to identify entire microbial communities and their dynamics. However, because the 16S rRNA is so conserved over evolution, a resolution issue arises when looking at detailed levels of genera, species, or pathogenic strains like *Escherichia coli* O157:H7. This occurs because multiple species and strains can fall into a single OTU. Which means that while the PhyloChip may detect a certain OTU as present in our compost samples, it could be a number of related species containing the pathogenic species where the 16s rRNA gene sequence is too similar to differentiate it from non-pathogenic relatives with these probe sets. While it is likely that the species that fall into the same OTU as a pathogen will behave in the same or similarly across thermophilic composting, it has been shown that microbial mechanisms towards environmental changes can differ drastically between strains, species, and especially genera [15]. Despite such limitations in comprehensive pathogen analysis in this study, further analysis of improved set-ups and added analysis will answer the questions regarding pathogen destruction and public health.

Because of this, the research group is implementing a phase two and three continuation to this study. For phase two, the research group built three identical cow waste and straw compost piles further investigate the community dynamics and the fate of pathogens during composting for 90 days. Finally, in phase three of the Marin Carbon Project supported "Thermopile Project", we plan on investigating pathogens with quantitative PCR and primer specific methods in order to make strain specific identifications between three cow, three chicken, and three human waste compost piles. The results could differ drastically between the three phases of this project depending on the starting material. It has been suggested that many pathogens that do not release tough

spores, such as *E. coli* and *Salmonella*, are only able to survive in the environment for one to three weeks [19]. For this reason, the abundance of pathogens from this study and phase two of the "Thermopile Project" are likely to be lower than their initial concentrations since the manure pile was roughly a month old. To overcome this time zero difficulty, all starting materials for phase three of the project will be collected within five days.

This is the first comprehensive high-density DNA microarray analysis for the centuries-old practice of thermophilic composting. The results from this baseline analysis have led to more directed questions and ideas regarding the nature of composting, and we are already carrying out further studies to document how microbial communities change over time and exactly what pathogens are either reduced or destroyed.

Acknowledgements

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A group photo of a few of the dedicated individuals that made this research possible!

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