

BIOLOGICAL INDICATORS OF COMPOST-MEDIATED DISEASE SUPPRESSION  
AGAINST THE SOILBORNE PLANT PATHOGEN *RHIZOCTONIA SOLANI*

A Thesis Presented

by

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to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements  
for the Degree of Masters of Science  
Specializing in Plant and Soil Science

August, 2015

Defense Date: July 6, 2015  
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## ABSTRACT

Compost can suppress soilborne plant pathogens that cause significant damage on globally important food crops. However, reports of plant pathogen suppression are inconsistent likely because there are no established standards for feedstock material, application rate, and maturity age upon application. Excellent results can be achieved in greenhouse trials, but field applications are much less reliable. Disease suppression occurs through the activity of biocontrol organisms (direct antagonism), and general microbial competition. Biocontrol species are hypothesized to colonize the pile during the curing phase, but single species may not be as important as microbial consortia. Substrate composition during maturation may give rise to a suppressive microbial community. More research is needed to understand the relationships between feedstock, maturity, and production process on compost microbial ecology. The thesis had two main objectives: 1) identify biological indicators in compost that could (a) characterize maturity, process, and feedstock, and (b) predict disease suppression against *R. solani*, and 2) identify bacterial and fungal community composition and/or structure that is associated with suppression of soilborne disease.

*Rhizoctonia solani* is a facultative saprophytic fungus and soilborne plant pathogen that attacks many globally important food crops and turfgrass. Prior research suggests that managing carbon quality and compost maturity will alter relative competition between biological control microbes and the *R. solani* pathogen. The pathogen is responsible for economic losses to organic vegetable production in Vermont and there are no available methods to manage the disease that meet organic certification. *R. solani* on radish was chosen as a model system given its global importance, competitiveness affected by carbon quality, and lack of disease management options for organic production.

Compost samples were most abundant in the bacterial phyla Proteobacteria and Bacteroidetes, and known biocontrol species were not detected in abundance. Compost samples did not differ significantly in fungal community composition, suggesting a dominance effect from the native soil fungal community.

Overall, anaerobic digestate and vermicompost were most suppressive against *R. solani*. Thermophilic composts were not very suppressive overall, though a specially made hardwood bark compost was comparable to the suppressiveness of vermicompost application. Ecoenzyme analysis was able to integrate information on environmental substrate composition, microbial nutrient acquisition, and microbial community metabolism, offering the best view of current ecological conditions in compost. Ecoenzyme analysis showed that the most suppressive composts, anaerobic digestate and vermicompost, were most nutrient limited. All compost samples were severely nitrogen (N) limited, and anaerobic digestate and vermicompost were severely limited in both N and phosphorus (P). The additional P limitation may support non-pathogenic species to outcompete *R. solani*. The key to disease suppression may lie in matching up the ecology of the plant pathogen with the ecology of biocontrol, which may be engineered in compost.

## ACKNOWLEDGEMENTS

There are many people that supported me in finishing my graduate program. The saying often refers to other major undertakings in life, such as the raising of a child into a mature adult, or to the development of a solvent business, but I think it fits in the achievement of pursuing a graduate degree – *it takes a village* to earn a master's degree. Foremost I would like to thank my partner Aaron for enduring my long hours away at the lab or stuck on my computer screen, and offering his perpetual encouragement and belief in me. My personal friends and my parents have continually cheered me on through the good times and the hard.

I would like to thank my advisor Deb Neher for her unyielding positivity and inspired vision as a leader in the field of Soil Ecology, and for the rare opportunity to study compost ecology. My gratitude goes to Tom Weicht, our senior lab technician, for expressing his vast lore of science through his creative and skillful development of new laboratory methods, and his dedication to helping students succeed. I am grateful for my committee members, Josef Görres – for introducing me to soil analytical methods, and Jane Molofsky – for integrating the disparate aspects of my project under the purview of ecological theory.

I wish to thank the staff at Highfields Center for Composting, in particular Tom Gilbert and James McSweeney, for their technical guidance, custom compost blend, and leadership for a zero waste Vermont. Local organic farmers Andy Jones at the Intervale

Community Farm and Bruce Kaufman at Riverside Farm provided soil samples and the initial inspiration to study *Rhizoctonia solani* as a model disease system. I am grateful to the composters who freely provided samples for my study – Dan Goossen from Green Mountain Compost, Karl Hammer from Vermont Compost, Lisa Rankin from Grow Compost, Jim from Vermont Natural Ag Products, and Tom Herlihy from Worm Power Vermicompost based in Avon, NY. Terry Bradshaw and Andy Bessette of the UVM Horticultural Research Farm supplied me with field soil on multiple occasions, and trained me to operate a tractor/bucket loader so that I could manage a compost pile on my own.

Colleen Armstrong, Tom Doubleday, and Dave Heleba of the UVM greenhouse staff have been incredibly helpful and patient in coordinating greenhouse space for my research. Alan Howard has been tremendous help in statistical analyses. I am grateful for my intern and collaborator Flore Costumé, for her contribution in nematode identification and quantification. My intern Catherine Ferguson assisted me with my work in the laboratory and greenhouse. I am grateful to Jonathan Leff at the University of Colorado for assisting me with statistical analyses of sequencing data.

No graduate experience is complete without the camaraderie of fellow grad students. I am grateful for my fellow grad students at UVM, for they have been kind and clever, inspiring and knowledgeable.

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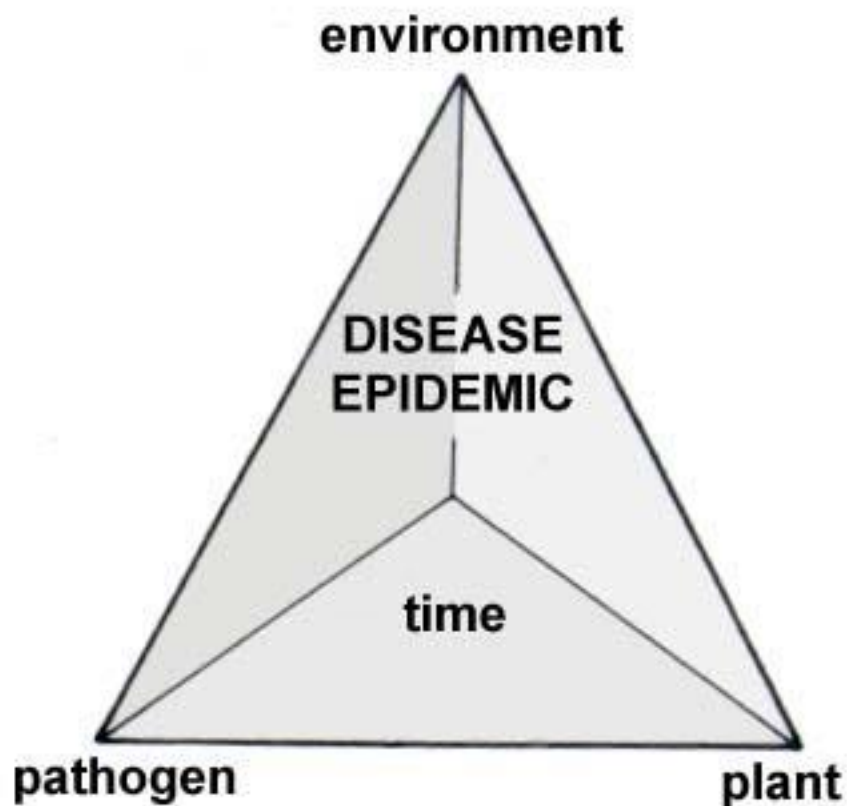
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## **CHAPTER 1. LITERATURE REVIEW**

### **1.1 Soilborne Plant Disease and Compost**

Persistent soilborne plant diseases cause considerable economic losses, and are a major barrier to growing food more sustainably. At least 10% of global food production is lost to plant disease, contributing to the issue of global hunger, where at least 800 million people are insufficiently fed (Strange and Scott, 2005). Conventional growers depend on fungicides and pesticides to control plant disease. In many cases, residual persistence from fungicide application leads to nonpoint-source pollution and groundwater contamination, and has resulted in banning effective chemical biocides (Neumann et al., 2002). Organic growers are further limited in their disease management opportunities: they are typically relegated to choosing resistant varieties, that may not exist, and crop rotation that cannot control facultative saprophytes or pathogens with extensive host ranges that includes common and abundant weeds (Baysal-Gurel et al., 2012).

Plant diseases establish based on the plant disease pyramid (Figure 1) – diseases theoretically occur when there is a perfect storm of a virulent pathogen and a susceptible plant host together in an environment conducive to disease development (Schumann and D'Arcy, 2006). Under this model, the role of compost in disease suppression is to decrease the conduciveness of the environment, and to enhance the health and immunity of the plant host to reduce the severity of infection and mitigate an epidemic.



**Figure 1.** Plant disease pyramid (Schumann and D'Arcy, 2006).

### **1.2. *Rhizoctonia solani* as a Model Disease System**

Vermont organic farmers expressed interest in examining suppression of a fungal pathogen, *Rhizoctonia solani* (teleomorph: *Thanatephorus cucumeris*), a facultative saprophyte with a wide host range including brassicas, lettuce, and potatoes. *R. solani* causes approximately \$10,000 in damage to lettuce and potatoes on small organic farms in Vermont (personal communication), and is listed as one of most important pathogens

on crop plants that provide the primary sources of human nutrition (Strange and Scott, 2005). Successful and reliable suppression of *R. solani* has been demonstrated in greenhouse trials with composted hardwood bark, municipal waste, and vermicompost (Nelson and Hoitink, 1983; Van Assche and Uyttendaele, 1981; Mathout, 1987; Ersahin et al., 2009). Suppression of *R. solani* damping off has also been shown in field trials using composted sewage sludge (Lewis et al., 1992) and green manure (Fuchs 1995). However, little to no suppression of *R. solani* on field-grown potatoes occurred using hardwood and bark compost (Larkin and Tavantzis 2013), suggesting that the native soil microbial community may alter compost disease suppressiveness in the field.

### **1.3. Biology and Chemistry of the Composting Process**

Composting is the controlled, aerobic decomposition of organic wastes into a stable end material that can be used in a variety of ways, such as a soil fertility amendment, a potting mix ingredient, for erosion control, and in disease suppression (Mehta et al., 2014). Mediated by the resident microbial community, composting occurs through three distinct successional phases, determined primarily by temperature changes: a mesophilic phase (temperatures rising to ~45°C), a thermophilic phase that kills weed seeds and pathogens (peaking at ~70°C), and then a curing phase (cooling to ambient temperature) which can last several months (Tuomela et al., 2000). Feedstock materials can also be fed to the compost worm, *Eisenia fetida*, whose castings become vermicompost (Ersahin et al., 2009). In another vermicompost process, compost that has just passed its thermophilic

phase is fed to the compost worm, ensuring that the compost meets EPA standards for pathogen safety, known as Process to Further Reduce Pathogens (PFRP) (Pathogen Treatment Processes, 2015). The PFRP requires composts produced using the aerated static pile (ASP) method to maintain a temperature of 55°C or higher for three days. Composts produced using the windrow method are required to maintain a temperature of 55°C or higher for 15 days or longer, during which time the pile is turned at least five times (Pathogen Treatment Processes, 2015).

The main components of the raw starting material (organic matter) are lignocellulose, proteins, and lipids. Lignocellulose consists of cellulose, hemicellulose, and lignin, which are strongly bonded by non-covalent forces and covalent cross-linkages (Pérez et al., 2002). During the early stage of composting, it is the soluble and easily degradable carbon sources (such as monosaccharides, starch, and lipids) that are consumed by micro-organisms. Organic acids are formed from degradation of these compounds, decreasing the pH. Proteins are broken down next, releasing ammonium and increasing pH. Finally, the more resistant lignocellulose compounds are degraded and partially transformed into humus (Tuomela et al., 2000).

In most composting environments, bacteria are about 100 times more abundant than fungi (Stofella and Kahn, p. 23). An estimated 80-90% of all the microbial activity in composting is attributed to bacteria (Golueke 1977, p. 9). Gram positive bacteria such as *Bacillus* spp. are dominant throughout the composting process (Klamer & Bååth, 1998;

Ryckeboer et al., 2003b). *Bacillus* spp. are the most dominant and abundant bacteria in compost. They produce a thick-walled endospore that is resistant to heat and chemical degradation. They can survive on a variety of food sources and tolerate both mesophilic and thermophilic temperatures.

In the first few days of composting, levels of lactate are high, as are populations of Gram-positive fermenting bacteria such as *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Staphylococcus* (Peters et al., 2000; Ishii et al., 2000). Yeasts are also abundant during the initial mesophilic stage. Gram-negative bacteria including *Pseudomonas*, *Arthrobacter*, and *Aliccaligenes* are present during this time. The predominant mesophilic fungus in the raw organic material is *Geotrichum* sp. (Nusbaumer et al., 1996). Fungal populations decrease as the temperature rises, with all thermophilic fungi undetected at 64°C (Tuomela et al., 2000).

Maximum temperatures are associated with peak population sizes of total aerobic heterotrophs, and as heterotroph populations decrease, so does temperature (Tiquia et al., 1996; Tiquia et al., 1997). Heterotrophic metabolic activities include cellular respiration, fermentation, and coenzyme activity. Increasing heterotroph populations releases increasing amounts of heat from their associated metabolic activities and coenzymatic breakdown of organic matter.

Actinobacteria co-dominate the thermophilic phase with other gram positive bacteria, particularly *Bacillus* spp (Ryckboer et al., 2003a; Ishii et al., 2000). Mesophilic organisms are inhibited during the thermophilic phase. Yeasts are not detected in the thermophilic phase, and re-appear in the cool-down and maturation phase when the temperature cools down to below 54°C. The thermotolerant fungus *Aspergillus fumigatus* is abundant during the initial mesophilic stage and its spores can withstand temperatures above 60°C. *Aspergillus fumigatus* specializes in degrading cellulose and hemicellulose (Tuomela et al., 2000; Stofella & Kahn, p. 25).

White-rot fungi are most efficient at degrading lignin, but because most of them do not survive the thermophilic phase, they cannot play a significant role in lignin decomposition. However, *Phanerochaete chrysosporium* is a white-rot Basidiomycete that can grow well in elevated temperatures (Tuomela et al., 2000). Because fungal populations are low during the thermophilic phase (Klamer & Bååth, 1998), it is the *Bacillus* spp. and actinobacteria such as *Streptomyces* that degrade most of the lignocellulose substrates during the thermophilic phase (Ryckboer et al., 2003b).

In the cooling phase, mesophilic and thermotolerant organisms re-colonize the pile. Most of the substrate now consists of partially decomposed organic matter and humus. The dominant fungi after peak heating are *Aspergillus* sp., *Thermomyces lanuginosus*, *Mucor* sp. (Tuomela et al., 2000). *Bacillus*, *Pseudomonas*, and *Rhodococcus* are abundant during the cooling phase (Ryckboer, 2003).



During maturation, lignin, hemicellulose, and cellulose may be the main substrates for micro-organisms. At this point, less complex carbon sources have already been consumed and transformed into humus (Danon et al., 2008). Microbial diversity increases at this time.

Humus is considered the end product of composting, as it is resistant to microbial degradation, and is the primary source of nutrients and conditioning when applied to soil. Humus binds to plant nutrients and sequesters heavy metals; it increases soil cation exchange capacity, retains moisture through microporosity, and accumulates in nature as soil organic matter, peats, coals, oils, and organic sediments. Humus is formed through the transformation of lignin decomposition products, quinones, proteins, and sugars (Stevenson 1994, p. 189).

#### **1.4. Effects of Compost Characteristics on Disease Suppression against *R. solani***

Compost has been shown to suppress symptoms of plant disease, but reliable and efficient disease suppression has been difficult to replicate in the field (Hoitink and Boehm, 1999; Stone et al., 2001; Noble and Coventry, 2005; Noble 2011). The maturity age, feedstock materials, and application rate (% v/v) of compost all contribute to its potential and ability to suppress disease (Noble and Coventry, 2005).

#### **1.4.1. Compost Maturity and Suppression against *R. solani***

Compost maturity is typically measured by age, or curing time post-thermophilic phase, which typically lasts three to six months. For the purpose of this study, composting just past the thermophilic phase is considered immature compost, and curing for three to six months is considered mature compost. Immature composts are warmer than mature composts, as they have just finished the thermophilic phase and are just beginning to cool down to ambient temperature. Greenhouse trials show that mature compost provides significant suppression against *R. solani*, whereas immature compost is conducive to disease development (Tuitert et al., 1998; Kuter et al., 1988; Hoitink et al., 1996).

Compost that had been cured for five months showed better suppression than compost that had been cured for three or seven months (Tuitert et al., 1998). Similarly, compost samples taken from the high-temperature center of the pile are conducive to *R. solani* infection, but samples taken from the low-temperature edge of the pile are suppressive (Chen et al., 1987; Chung and Hoitink, 1990). Immature composted hardwood bark and any compost that is heat-treated does not provide any disease suppression (Nelson and Hoitink, 1983; Hoitink et al., 1996).

#### **1.4.2. Compost Feedstocks and Suppression against *R. solani***

*R. solani* has been suppressed in greenhouse trials using compost made from hardwood bark (Daft et al., 1979; Stephens et al., 1981; Nelson and Hoitink, 1982, 1983; Kuter et

al., 1983; Stephens and Stebbins, 1985), organic household waste (Tuitert et al., 1998), viticulture and enological factory residues (Pane et al., 2011), cow manure (Pane et al., 2011; Gorodecki and Hadar, 1990), municipal waste (Van Assche and Uyttebroeck, 1981; Mathout, 1987), and grape marc (Gorodecki and Hadar, 1990). However, grape marc compost also showed no suppression against *R. solani* (Santos et al., 2008). Municipal waste compost stored near piles of composted hardwood bark suppressed *R. solani*, while those that were not stored near composted hardwood bark were not suppressive (Kuter et al., 1988). These conflicting findings stress the importance of consistent feedstock chemistry and microbial community composition in *R. solani* suppression.

#### **1.4.3. Compost Application Rate and Suppression against *R. solani***

Several studies report significant disease suppression against *R. solani* from compost amendment in greenhouse trials using application rates of 20% (v/v) or less in soil (Lumsden et al., 1983; Gorodecki and Hadar, 1990; Tuitert et al., 1998; Tuitert and Bollen 1996; Ryckeboer 2001; Diab et al., 2003; Daft et al., 1979; Kuter et al., 1988). These suppressive composts were made from a variety of materials such as cattle manure and grape marc (Gorodecki and Hadar, 1990), vegetable and fruit waste (Tuitert et al., 1998; Tuitert and Bollen 1996; Ryckeboer, 2001), or hardwood bark (Nelson and Hoitink, 1983; Daft et al., 1979). Low compost application rates are less likely to cause negative effects such as phytotoxicity and high pH and electrical conductivity (Sullivan and Miller, 2001).

#### 1.4.4. Compost Microbial Communities and Disease Suppression

Compost microbial community composition differs across feedstock recipe, curing method, and maturity age (Neher et al., 2013). Assessment of bacterial communities in compost have shown clear successional transitions during compost curing.

*Proteobacteria* are the most abundant phylum in all cases, and *Bacteroidetes* and *Gammaproteobacteria* were ubiquitous. *Actinobacteria* dominate the midcuring stage, and varying members of nitrifying bacteria and cellulose-degrading bacteria are found during the curing process (Danon et al., 2008).

Succession of microbial communities during composting is directly related to the establishment of the phenomenon of disease suppression (Hadar and Papadopoulou, 2012). At the start of the maturation phase, labile substrates have already been oxidized by the microbial community, and the remaining substrates consist of semihumified materials, lignins, recalcitrant microbial metabolites, and partly decomposed cellulosic substances. These substrates appear to favor the rise of a competitive microbial community.

Hardwood bark was hypothesized to be more conducive to colonization of *Trichoderma* (teleomorph: *Hypocrea*) biocontrol species, allowing it to outcompete and/or antagonize fungal pathogens (Hoitink et al., 1996). *Trichoderma* is a prolific sporulator and is able to

use various mechanisms for biocontrol, such as antibiosis, parasitism, and competition. However, *Trichoderma* spp. are not detected in high-throughput sequencing analysis of mature hardwood bark compost (Neher et al., 2013), though its order Hypocreales Helotales was present. Additionally, high cellulose content colonizes abundant *Trichoderma* spp., but because it characterizes immature composts, creates an environment that is conducive to *R. solani* proliferation and infection (Hoitink and Fahy, 1986). These conflicting results indicate that a high population of antagonist does not necessarily equate to suppression, and microbial consortia may be more responsible for biocontrol activity than single species (Hadar and Papadopoulou, 2012). Chung and Hoitink (1990) found that suppression against *R. solani* using *Trichoderma hamatum*-inoculated compost was reduced in compost at 40-50°C, and improved in compost at 55°C or hotter, but there was no significant difference in population densities of *T. hamatum* among compost at temperatures ranging from 40-60°C and greater, indicating that growth and abundance of biocontrol species is not the only variable in disease suppression.

Microbial succession that occurs during the production of compost offers an opportunity to engineer microbial communities with biological control activity to cosmopolitan and persistent soilborne plant pathogens. Successful biological control requires understanding the impacts that both carbon source and successional stage have on microbial community and ecological niche. However, most studies have treated all composts equally when considering biological control activity.

## **1.5. Biological Indicators of Disease Suppressive Compost**

### **1.5.1 Indicators of Compost Maturity**

One principal factor limiting widespread use of compost in disease suppression is variation in compost maturity. Compost maturity has traditionally been measured by pile temperature, age, and humification (humus is the end product of composting, and is measured through the amount of humic and fulvic acids). Parameters such as ecoenzyme activity (Castaldi et al., 2008), respiration (Hoitink et al., 1996), and C:N ratio (Goyal et al., 2005), are correlated most closely with maturity. Although respiration is affected by a number of parameters including temperature, humidity, and incubation conditions, it is still the most common of maturity tests (Gómez et al., 2006; Wichuk and McCartney, 2010), and constitutes the main component of a popular commercial maturity test kit produced by Solvita. The Solvita Compost Maturity kit measures carbon dioxide and ammonia emissions (Woods End Laboratories, Maine), based on the premise that more stable composts release low amounts of carbon dioxide and ammonia. Maturity indicators are useful for making decisions about how, and for what purpose, the compost should best be applied.

### **1.5.2. Eoenzyme Activity as Indicators of Microbial Community Metabolism**

Several terms have been used to describe extracellular enzymes secreted by soil microbes; this paper will use the term eoenzyme to include all enzymes located outside of cell membranes, and to refer to the correlation between environmental enzyme activity and organic matter decomposition (Sinsabaugh and Shah, 2012). Eoenzymes are the primary means by which soil organic matter is decomposed. Eoenzyme activity is an indicator of microbial nutrient demand in relation to environmental nutrient availability (Sinsabaugh et al., 2008). Transcription of eoenzyme expression is ultimately linked to environmental signals, such as substrate concentration, indicators of toxicity, or quorum sensing molecules (Sinsabaugh and Shah, 2012).

Because enzymes that hydrolyze related groups of compounds, such as cellulose and hemicellulose, correlate with each other, a single indicator enzyme can be used as a representative of the combined activities of a suite of enzymes that degrade a particular substrate. For example,  $\beta$ -glucosidase (BG) can serve as an indicator enzyme for hydrolyzation of hemicellulose and cellulose,  $\beta$ -N-acetyl-glucosaminidase (NAG) for chitin, L-leucine aminopeptidase (LAP) for protein degradation and general microbial activity, and phosphatase (PP) for organic phosphorus (phosphoesters) and microbial turnover (Moorhead et al., 2013). These are some of the most studied eoenzymes, and they catalyze the degradation of the largest environmental sources of organic carbon, nitrogen, and phosphorus. Humus is formed by the oxidative degradation of lignin, which

is catalyzed by phenol oxidases and peroxidases, requiring the use of molecular oxygen and peroxide, respectively, as electron acceptors (Sinsabaugh and Shah, 2012).

The ratio of ecoenzymatic carbon (C) acquisition to ecoenzymatic nitrogen (N) and phosphorus (P) is represented in the following ratios of ecoenzyme activity (Sinsabaugh and Shah, 2012):

$$EEA_{C/N} = BG/(LAP+NAG)$$

$$EEA_{C/P} = BG/PP$$

These ratios are correlated with the C:N and C:P ratios of labile organic matter in the environment.

Assessing ecoenzyme activity in compost captures the current state of microbial community metabolism, and serves as an indicator of which substrates and decomposition functions are most abundant. Ecoenzyme activity may be affected by the substrate composition of the original feedstock materials. Few studies have quantified ecoenzyme activity in compost (Neher et al., 2015), and none have been done to predict disease suppression.



### **1.5.3. Use of Nematodes as Indicators of Compost Maturity**

Nematodes are well known as indicators of soil quality and ecosystem health, but they are less well understood in composting (Steel et al., in press). They are favored as biological indicators because of their distribution among multiple trophic groups, occupation of central positions in the soil food web, and their ubiquitous presence in all soil types, climates, and environments (Bongers and Ferris, 1999; Neher 2001). They are responsive to disturbance, enrichment, and pollution, which makes them good candidates as indicators of compost maturity.

Opportunistic nematodes typically occupy rapidly changing environments with an abundance of food – enrichment opportunists (cp-1) are gradually replaced by general opportunists (cp-2) (Bongers, 1999). Typically, a maturity index is computed from colonizer-persister (c-p) values. To avoid confusion with compost maturity, this paper will refer to the nematode maturity index as an ecological succession index (ESI). There appear to be clear shifts in nematode ecological successional index and F:B ratio during composting, so nematodes can potentially be used as an indicator of compost maturity. The ecological successional index increases from  $0.81 \pm 0.59$  during the thermophilic phase to  $1.24 \pm 0.12$  during cooling and finally to  $1.34 \pm 0.34$  during maturation ( $P \leq 0.001$ ) (Steel et al., 2010).

The maturity and stability of the compost ecosystem may play a role in its ability to outcompete or antagonize disease pathogens. Nematodes have never been assessed in compost to determine effects on disease suppression against *R. solani*.

## **1.6. Conclusion**

As the movement for more sustainable food production grows, so does the need for alternative methods of managing soilborne plant pathogens on globally important food crops. Compost is a promising method of suppressing soilborne plant pathogens that complies with organic standards of food production, but its use in disease suppression is currently met with varying efficacy, depending on feedstock materials, production process, application rate, and maturity age at the time of application. The best methods for assessing compost maturity and stability are still unclear, though the most popular methods favor respiration and C:N ratio. Ecoenzyme activity and nematode community composition are potentially reliable indicators of compost maturity and stability.

The microbial community composition and associated substrate composition are important to establishing disease suppression. More research is needed to determine the microbial metabolic profile that is best suited for disease suppression against a specific soilborne plant pathogen. This study aims to identify the indicators that most contribute to disease suppressive activity of compost against the soilborne fungal pathogen *R. solani*.

## **CHAPTER 2. EFFECTS OF MICROBIAL COMMUNITY COMPOSITION, COMPOST TYPE, AND FIELD APPLICATION RATE ON DISEASE INCIDENCE**

### **2.1. INTRODUCTION**

Compost has been shown to suppress soilborne plant diseases including the fungal pathogen *Rhizoctonia solani* (teleomorph: *Thanatephorus cucumeris*), a facultative saprophytic fungus with a wide host range including brassicas, lettuce, and potatoes (Hoitink et al., 1996). Several different types of compost have been shown to suppress *R. solani* in greenhouse trials, including composted hardwood bark and vermicompost, suggesting that managing carbon quality and compost maturity will alter relative competition between biocontrol microbes and the pathogen (Nelson and Hoitink, 1983; Ersahin et al., 2009). *R. solani* is responsible for economic losses to organic vegetable production in Vermont and there are no available methods to manage the disease that meet organic certification. It is also listed as one of most important pathogens on crop plants that provide the primary sources of human nutrition (Strange and Scott, 2005). Because of these reasons, *R. solani* on radish was chosen as the model pathogen for this study.

Compost microbial community composition has been shown to differ across feedstock recipe, production process, and maturity age (Neher et al., 2013). High-throughput

genetic sequencing has enhanced the ability of researchers to identify prevalent microbial groups in soil and compost. Culture-dependent techniques of microbiota identification are limited as not all soil organisms can be easily cultured in the laboratory. Genetic sequencing of the highly conserved 16S ribosomal subunit in bacteria and archaea provides a more accurate picture of the taxonomic composition of soil and compost (Fierer et al., 2005). In fungi, it is the internal transcribed spacer (ITS) sequence that is used (Bates et al., 2013). Field applications of compost are typically measured in tons per acre.

## **Objective**

This study aims to identify the effects of application rate and compost type (hardwood bark compost or vermicompost) on 1) disease incidence of *R. solani* on radish, and 2) bacterial and fungal community composition associated with disease suppression.

## **2.2. METHODS**

### **2.2.1. Compost Selection**

Commercial products were chosen because they have met the temperature requirements set by the EPA, known as Process to Further Reduce Pathogens (PFRP). A thermophilic compost and vermicompost were chosen for comparison. The thermophilic compost was made by Highfields Center for Composting (Hardwick, VT) using the aerated static pile

(ASP) method, from a 5:5:3 ratio of manure/silage : hardwood bark : softwood shavings, resulting in a C:N ratio of 34:1 (Neher et al., 2013). The vermicompost was made by Worm Power (Avon, NY) from ASP-composted manure/silage.

### **2.2.2. Model Pathogen System**

Local field isolates were collected from Vermont grown potatoes and radishes infected with *R. solani*; the infected pieces were excised, grown in water agar, and incubated at room temperature. Inoculum was grown in a sterile mix of 96g sandy soil, 4g corn meal, and 20ml water. The culture medium was autoclaved for one hour over three consecutive days to ensure that endospores of *Bacillus* spp. were killed. Plugs of *R. solani* on PDA were transferred to the culture medium, covered with foil, and let sit at room temperature for 2-3 weeks until the culture medium was overgrown with white fuzzy mycelium. At this point the inoculum was considered ready for infestation in soil. Pathogenicity tests were performed on radish seedling populations, detailed in the next section, 2.2.3. The most virulent isolates were kept in long-term storage on a minimal media of corn meal agar slants (at 5°C), and used to infest soils for greenhouse bioassays.

Radish (*Raphanus sativus*), Ping Pong variety from Johnny's Seeds, was chosen for its quick growth and ease of detecting *R. solani* infection at the root crown. Field soil was used in greenhouse bioassays to mimic field conditions while controlling for temperature and minimizing weather variability.

### 2.2.3. Greenhouse Bioassay

Field soil was provided by the UVM Horticultural Research and Education Center (Adams and Windsor loamy sands); it was steam pasteurized at 70°C for four hours to destroy native pathogens, then re-inoculated with its endemic microbial community by adding 4L of 10µm filtered soil extract, and sat for three weeks to allow the microbial community to re-establish itself. Half the soil was inoculated with mixed isolates of *R. solani* at a rate of one inoculum (approximately 100ml volume) per liter of soil, and was allowed one week to equilibrate with the soil before compost was applied. After compost application, another equilibration period of one week was given before 25 radish seeds were planted into each pot using a customized dibble-stick to ensure a distance of 2.54 cm (1 inch) between each seed. Four replicate pots were ascribed to each treatment sample. Pots were grown in the greenhouse under natural day lengths, watered daily. Seedlings were harvested after two weeks, with roots in tact. Each seedling was assessed for disease incidence, standardized as a proportion of diseased seedlings to healthy seedlings. A treatment of infested soil with no compost applied was used as a control.

#### *Pathogenicity Test*

Each isolate of *R. solani* was tested for pathogenicity in four replicate pots. Prepared field soil was bulk infested with a particular isolate of *R. solani* and distributed amongst four

pots. Twenty-five radish seeds were planted per pot and watered daily in the greenhouse. Seedlings were harvested after two weeks and assessed for disease incidence. The most virulent isolates of *R. solani* expressing severe disease symptoms on at least 75% of seedlings were kept and used to inoculate soils for disease severity trials.

#### *Field Application Rate Assays*

Low and high field application rates of 2.75 and 27.5 metric tons per hectare (MT/ha) (one and 10 tons per acre) of vermicompost (VMO), and 27.5 and 55 MT/ha (10 and 20 tons/acre) of hardwood bark compost (HM) were used for this greenhouse bioassay. These values were scaled down to a greenhouse pot size in four replicates. Controls of no compost in both infested and uninfested soil, and rice hulls in infested soil were used for comparison. Twenty-five radish seeds were planted per pot, and seedlings were harvested after two weeks of growth in the greenhouse to be assessed for disease incidence. The treatment soils were assayed for bacterial and fungal community composition using 16S and ITS high-throughput sequencing.

#### *16S and ITS High-Throughput Sequencing*

Genomic DNA was extracted using the MoBio PowerSoil™ kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions with the following exceptions: 0.1 gram of compost (instead of 0.25 g) was added to each tube of PowerBeads. Sample C1

was incubated at 65° C for 10 minutes and then secured and vortexed horizontally with beads for 2 minutes. PCR amplification of the 16S rRNA gene (for bacteria and archaea) or the internal transcribed spacer region (ITS1) of the nuclear ribosomal RNA gene (for fungi) followed the approach described in Lauber et al. (2009). Briefly, each sample was amplified in triplicate, and amplicons were composited together in equimolar concentrations prior to sequencing. PCR reactions contained 13 µL PCR-grade water, 10 µL 5 Prime Hot Master Mix, 0.5 µL each of the forward and reverse primers (10 µM final concentration), and 1.0 µL genomic DNA (diluted 1:10 with PCR-grade water). Reactions were held at 94°C for 3 min to denature the DNA, with amplification proceeding for 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s; a final extension of 10 min at 72 °C was added to ensure complete amplification. For the bacterial and archaeal analyses, the 200-bp PCR primers (515f/806r) targeted the V4 region of the 16S rRNA gene (Fierer et al., 2012). For the fungal analyses, we used PCR primers (ITS1-F/ITS2) to amplify the ITS1 spacer (Caporaso et al., 2012). Both primer pairs contained 12-bp barcodes unique to each sample and the appropriate adapters to permit sequencing on the Illumina MiSeq platform (Fierer et al., 2012; Gardes and Bruns, 1993).

#### **2.2.4. Statistical Analyses**

Analysis of variance (ANOVA) with three main effects (production process, maturity, and feedstock) was performed using the MIXED procedure in SAS 9.3, with Tukey post-



hoc tests groupings generated by a SAS macro called PDMIX800, created by Arnold M. Saxton of the University of Tennessee, Knoxville. No other interactions were possible given their unavailability on the market. Bray-Curtis dissimilarity and PERMANOVA (permutational multivariate analysis of variance) were performed on 16S and ITS sequences to determine differences in microbial community composition among compost treatments, using Primer 6/PERMANOVA+. Principal coordinates analysis (PCO) was performed on Bray-Curtis results. Multiple Kruskal-Wallis tests using false discovery rate p-values were performed on 16S and ITS sequences to determine taxonomic differences between compost treatments, using R code provided by Jonathan Leff at the University of Colorado (Appendix D.1).

## **2.3. RESULTS**

Disease incidence appeared lowest with high vermicompost application of 27.5 MT/ha (10 tons/acre), and all compost applications suppressed *R. solani* more than the control of no compost (Figure 2), though no significant differences were found with compost ( $P \leq 0.22$ ) or application rate ( $P \leq 0.7$ ).

Principal coordinates analysis of 16S sequences shows clustering of infested vermicompost samples and uninfested hardwood bark compost controls, and pure compost samples tend to have unique community composition from each as well as when

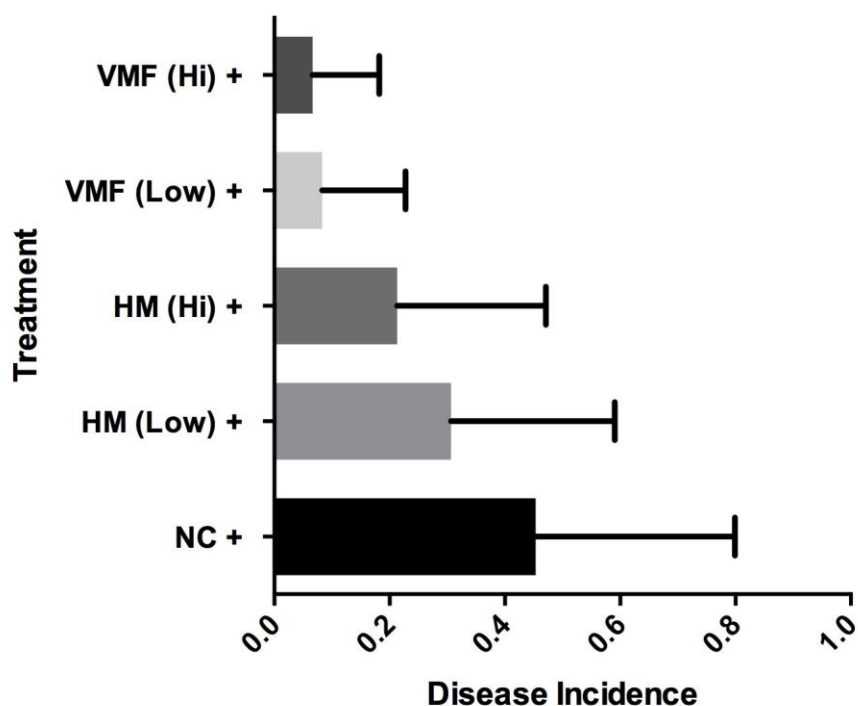
mixed with field soil samples (Figure 3). There is less evident clustering or consistency among fungal ITS sequences (Figure 4).

Compost type and application rate had significant effects on 16S community composition ( $P \leq 0.001$ ), including significant interaction effects ( $P \leq 0.001$ ) (Table 1). However, no significant effects were observed on ITS fungal community composition for compost type, application, or their interaction ( $P \leq 0.703$ ,  $P \leq 0.792$ ,  $P \leq 0.491$ ) (Table 1), indicating highly variable fungal communities even within the same compost type.

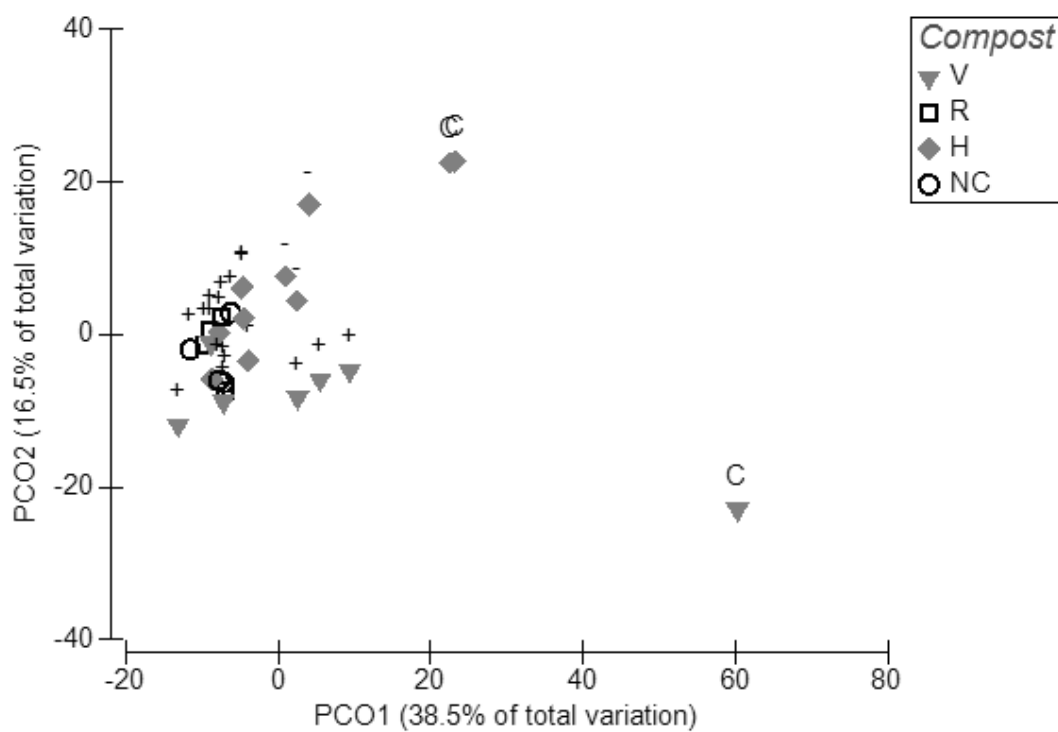
Just comparing relative abundances within each treatment sample, hardwood bark compost had greater median abundance of Proteobacteria, followed by Bacteroidetes, and smaller abundances of Verrucomicrobia, Firmicutes, Acidobacteria, Actinobacteria, Planctomycetes, and Gemmatimonadetes, and there was minimal presence of Armatimonadetes (Table 2). Similarly, vermicompost contained relatively abundant Proteobacteria, with Bacteroidetes as second most abundant, and a minimal presence of Armatimonadetes. The rice hulls treatment was highest in Proteobacteria, followed by Bacteroidetes, and there was somewhat greater abundance of Armatimonadetes. The no compost treatment was highest in Proteobacteria, followed by Bacteroidetes, and had the greatest abundance of Armatimonadetes compared to all other treatments (Table 2). The trends here indicate that Proteobacteria and Bacteroidetes are ubiquitous in both soil and compost.

There appears to be a trend of increasing abundance of Acidobacteria with increasing rates of compost application (Table 3), and hardwood bark compost was significantly more abundant in Acidobacteria ( $P \leq 0.0238$ ). Additionally, Acidobacteria were more abundant in uninfested treatments ( $P \leq 0.073$ ) (Table 4), indicating there is some relationship between Acidobacteria abundance and *R. solani* growth suppression.

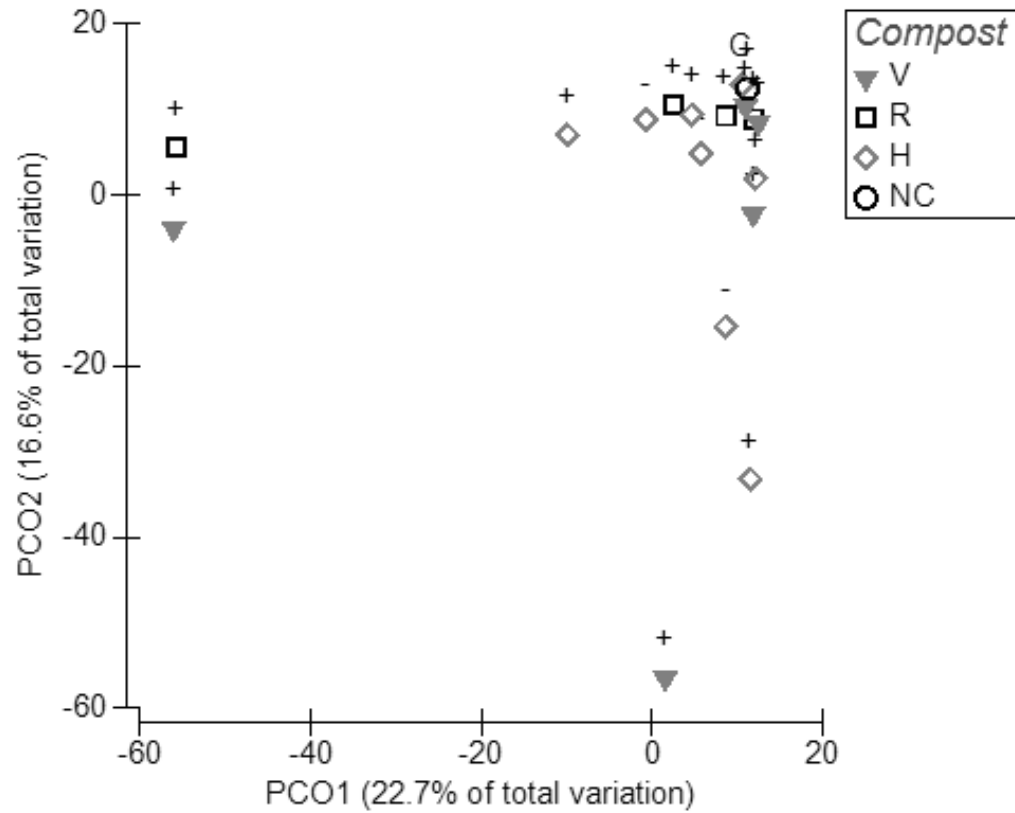
Acidobacteria is more abundant when *R. solani* is more suppressed. Acidobacteria are found in many soil types as well as freshwater habitats, hot springs, sewage sludge, and wastewater treatment plants (Quaiser et al., 2003). The application rate of 10 tons per acre yielded more abundant *FBP* ( $P \leq 0.0387$ ) (Table 3). Chloroflexi, Firmicutes, and Proteobacteria were all more abundant in infested treatments ( $P \leq 0.066$ ,  $P \leq 0.075$ ,  $P \leq 0.08$ ) (Table 4). No differences were found in ITS fungal phyla for compost type, application rate, or pathogen presence. No interaction effects between compost type and application rate were found (Table 1).



**Figure 2.** Disease incidence of hardwood bark compost and vermicompost. + indicates soil infested with *R. solani*; - indicates uninfested soil. VMF = Vermicompost/Mature/Fresh; HM = Hardwood bark/Mature; NC = No Compost. VMF (Hi) = 27.5 MT/ha (10 tons/acre); VMF (Low) = 2.75 MT/ha (1 ton/acre); HM (Hi) = 55 MT/ha (20 tons/acre); HM (Low) = 27.5 MT/ha (10 tons/acre)



**Figure 3.** Principal coordinates analysis of 16S sequences, bacteria and archaea. V=Vermicompost, R=Rice Hulls Control, H=Hardwood Bark Compost, NC=No Compost. On the graph + indicates soil infested with *R. solani*, - indicates soil not infested with *R. solani*, and C indicates it is a pure compost sample.



**Figure 4.** Principal coordinates analysis of fungal ITS sequences. V=Vermicompost, R=Rice Hulls Control, H=Hardwood Bark Compost, NC=No Compost. On the graph + indicates soil infested with *R. solani*, - indicates soil not infested with *R. solani*, and C indicates it is a pure compost sample.

**Table 1.** PERMANOVA effects of compost and application rate on 16S and ITS sequencing microbial community composition. Pseudo-F and Monte Carlo (MC) P-value are shown. Both are permutational versions of the traditional F statistic and P-value.

	16S Bacteria & Archaea		ITS Fungi	
	<i>Pseudo-F</i>	P (MC)	<i>Pseudo-F</i>	P (MC)
<b>Compost</b>	4.89	<b>0.001</b>	0.747	0.703
<b>Rate</b>	9.5	<b>0.001</b>	0.695	0.792
<b>Compost*Rate</b>	4.775	<b>0.001</b>	0.526	0.491

**Table 2.** Multiple Kruskal-Wallis test of 16S bacterial phylum differences among compost types with false discovery rate P-values and median abundances (expressed as percentage of sequences) for each compost type.  $P \leq 0.05$  is highlighted in bold. NC = No Compost; HM = Hardwood Bark Compost/Mature; VMF = Vermicompost/Mature/Fresh; R = Rice Hulls

Phylum	P (FDR)	NC	HM	VMF	R
Acidobacteria	<b>0.0238</b>	0.0138	0.0321	0.014	0.019
Armatimonadetes	0.1545	0.0079	0.009	0.004	0.011
Firmicutes	0.1514	0.029	0.02045	0.0394	0.0255
Verrucomicrobia	0.2202	0.0319	0.035	0.027	0.0369
Bacteroidetes	0.1791	0.2619	0.2819	0.3107	0.217
Planctomycetes	0.3785	0.0281	0.028	0.0227	0.034
Proteobacteria	0.518	0.5351	0.4746	0.4534	0.5422
Actinobacteria	0.597	0.04	0.0325	0.0408	0.0422
Gemmatimonadetes	0.589	0.0315	0.0264	0.035	0.031

**Table 3.** Multiple Kruskal-Wallis test of 16S bacterial phylum differences among application rates with false discovery rate P-values and median abundances (expressed as percentage of sequences) for each application rate.  $P \leq 0.05$  is highlighted in bold. Application rates are listed in MT/ha with tons/acre in parentheses.

Phylum	P (FDR)	0	2.75 (1)	27.5 (10)	55 (20)
FBP	<b>0.0387</b>	0.003	0.004	0.0111	0.003
Acidobacteria	0.118	0.017	0.012	0.0214	0.0367
Proteobacteria	0.432	0.511	0.529	0.4478	0.4854
Bacteroidetes	0.342	0.271	0.273	0.2953	0.2622
Planctomycetes	0.3113	0.028	0.022	0.0281	0.0298
Fibrobacteres	0.3408	0.001	0	0.0105	0.0113
Firmicutes	0.411	0.0266	0.0373	0.0334	0.0225
Gemmatimonadetes	0.74	0.0301	0.035	0.0342	0.027
Verrucomicrobia	0.676	0.0342	0.0269	0.0327	0.0375
Actinobacteria	0.6303	0.0396	0.0366	0.041	0.036

**Table 4.** Multiple Kruskal-Wallis test of 16S bacterial phylum differences among treatments uninfested and infested with *R. solani*, with false discovery rate P-values and median abundances (expressed as percentage of sequences) for each treatment group.  $P \leq 0.08$  is highlighted in bold.

Phylum	P (FDR)	Infested	Uninfested
Chloroflexi	<b>0.066</b>	0.0024	0.011
Gemmatimonadetes	0.086	0.0316	0.0207
Acidobacteria	<b>0.073</b>	0.0203	0.0347
Firmicutes	<b>0.075</b>	0.0299	0.017
Proteobacteria	<b>0.08</b>	0.5019	0.4546
Bacteroidetes	0.102	0.272	0.3166
Actinobacteria	0.425	0.0399	0.0325
Planctomycetes	0.566	0.0281	0.025
Verrucomicrobia	1	0.0343	0.032



## 2.4. DISCUSSION

This study is novel in its comparison of the effects of bacterial and fungal community composition on disease suppression against *R. solani*, as well as its examination of the bacterial and fungal community composition of compost after amendment to soil.

Additionally, few studies have compared application rates of vermicompost and hardwood bark compost for suppression of *R. solani*.

While compost type and application rate had significant effects on bacterial community composition, these effects did not translate to differences in disease incidence. The differences in bacterial community composition may not have been large enough to affect disease incidence, and it is also likely that the native soil microbial community dominated the compost microbial community, rendering any biocontrol activity ineffective.

Additionally, the sample size may have been too small for the large amount of natural variation in microbial community analyses.

Similar to the findings of Neher et al. (2013), Proteobacteria and Bacteroidetes were the most abundant bacterial phyla in compost. Hardwood bark compost also contained more Acidobacteria than other compost types made from manure/silage only or hay as the primary carbon component. The hardwood bark compost used in this study was the same as that used in the study by Neher et al. (2013), but it had continued to mature for another year. The change in bacterial community composition and reduced abundances of

Chloroflexi, Acidobacteria, and Actinobacteria reflect the additional year of maturation time and overall reduction in microbial activity and abundance.

Similarly, in a mature thermophilic compost made from a mixture of sewage sludge and yard waste, aerated by biweekly windrow turning, Gammaproteobacteria, Proteobacteria, and Actinobacteria were reported to be the most abundant bacterial phyla (Danon et al., 2008). In this case, Bacteroidetes was dominant during the initial phases of composting, but the population was reduced during maturation. However, in a thermophilic compost made from organic wastes and yard trimmings that matured for over a year, only Actinobacteria and Firmicutes were reported to be most abundant (Fracchia et al., 2006). Firmicutes was barely detected by Danon et al. (2008), and was present but not abundant in this study as well as the composts used in Neher et al. (2013).

These studies all used 16S rRNA PCR amplification and sequencing to determine bacterial community composition in compost, making it possible to compare the results from different studies using different compost samples. Using other methods such as culture isolation or phospholipid fatty acid analysis (PLFA) would vary the results, and comparison would be more accurately focused on methods comparison rather than comparison of community composition.

Vermicompost has been reported to be most abundant in Bacteroidetes followed by Gammaproteobacteria, and somewhat abundant in Verrucomicrobia and Chloroflexi

(Neher et al., 2013), and in another study vermicompost was dominated by Chloroflexi, Acidobacteria, Bacteroidetes, and Gemmatimonadetes (Fracchia et al., 2006). In this study, vermicompost was most abundant in Proteobacteria and Bacteroidetes. Gemmatimonadetes and Verrucomicrobia were present but not abundant.

Similar phyla continue to show up in high-throughput sequencing studies of mature compost microbial communities, though their relative abundances and dominance vary, indicating a potentially strong influence from compost feedstock, production process, and maturity age at the time of analysis. Compost used in the study by Fracchia et al. (2006) had matured for at least one year and up to 12 years, which reflects the reduced abundance of bacterial phyla detected.

In contrast to the findings of Neher et al. (2013), our results show vermicompost had levels of abundance of Bacteroidetes and Verrucomicrobia similar to hardwood bark compost. Overall bacterial taxonomic composition differed by compost type, application rate, and their interaction, but differences in individual taxa were not found.

Because no significant differences were found in the fungal taxonomic composition of vermicompost and hardwood bark compost, they may have similar diversity and abundance of fungi. Diverse and abundant fungal populations may be important to the suppression of *R. solani*. Additionally, the soil fungal community may be dominating the compost community, thus the fungal communities become less distinguishable.

Certain biocontrol species such as *Bacillus* spp., *Enterobacter* spp., *Flavobacterium balustinum*, *Pseudomonas* spp., *Streptomyces* spp., *Penicillium* spp., *Trichoderma* spp., and *Gliocladium virens* have been identified in compost-amended substrates (Chung and Hoitink, 1990; Hadar and Gorodecki, 1991; Hardy and Sivasithamparm, 1991; Hoitink and Fahy, 1986; Nelson et al., 1983; Phae et al., 1990). Abundances of these biocontrol organisms was minimal in all treatments, and *Trichoderma* and *Gliocladium* were not detected at all. In Neher et al. (2013) *Trichoderma* spp. were also not detected. *Trichoderma* spp. are the predominant parasites recovered using cultural isolation methods from composts made from lignocellulosic wastes (Kuter et al., 1983; Nelson et al., 1983), and are known to be effective against *Rhizoctonia* damping-off, especially when added as an isolated microbial inoculant (Kwok et al., 1987). High-throughput genetic sequencing is a more accurate method, compared to cultural isolation, of determining microbial community composition in soil and compost. Culture-dependent techniques are limited by and biased towards the organisms that favor laboratory media, whereas sequencing amplifies the genes that are already present in the soil, minimizing these limitations and biases.

Because the composts used in this study and in Neher et al. (2013) were suppressive, abundance of specific biocontrol species may not necessarily determine significance, lending support to the idea that microbial consortia are more important in disease suppression than specific species of biocontrol organisms.

This study utilizes state of the art high-throughput sequencing to determine bacterial and fungal community composition in compost and soil, offering a culture-independent view of the compost microbial life. Taxonomic abundance is interesting to compare among compost samples made from different feedstocks and processes, and the differences are most prominent in different production processes. However, abundance of specific biocontrol species may not be the most significant factor in disease suppression. Certain taxonomic groups such as Acidobacteria increase with increasing rates of compost application, but this is not true for all other taxonomic groups. Ecological attributes of many of these taxa are not well known at this time. This study contributes to another view of compost microbial community composition via high-throughput genetic sequencing, and relates it to disease suppression against the model pathogen *R. solani*. While a handful of studies have looked at microbial community composition during the composting process using high-throughput genetic sequencing of 16S rRNA (Danon et al., 2008, Fracchia et al., 2006; Neher et al., 2013), none have related these changes and differences to disease suppression.

This study could be strengthened with the use of a disease severity assessment, as the two compost types may express significantly different degrees of disease severity, which would not be shown when recording the more general assessment of disease incidence. Using an application rate in terms of % v/v may also strengthen the comparison between the two different types of compost.

## CHAPTER 3. INDICATORS OF DISEASE SUPPRESSION IN COMPOST

### 3.1. INTRODUCTION

Compost has been shown to suppress soilborne plant diseases including the fungal pathogen *Rhizoctonia solani* (teleomorph: *Thanatephorus cucumeris*), a facultative saprophyte with a wide host range including brassicas, lettuce, and potatoes (Hoitink et al., 1996). However, compost is a heterogeneous material with varying efficiency in disease suppression, depending on feedstock chemistry, production process, and maturity upon application (Noble and Coventry, 2005). Several different types of compost have been shown to suppress *R. solani* in greenhouse trials, including composted hardwood bark and vermicompost, suggesting that managing carbon quality and compost maturity will alter relative competition between biocontrol microbes and the pathogen (Nelson and Hoitink, 1983; Ersahin et al., 2009). *R. solani* is responsible for economic losses to organic vegetable production in Vermont and there are no available methods to manage the disease that meet organic certification. It is also listed as one of most important pathogens on crop plants that provide the primary sources of human nutrition (Strange and Scott, 2005). Because of these reasons, *R. solani* on radish was chosen as the model pathogen for this study.

Successful and reliable suppression of *R. solani* has been shown in greenhouse trials with vermicompost (Ersahin et al., 2009), and thermophilic composts made from hardwood

bark (Nelson and Hoitink, 1983), organic household waste (Tuitert et al., 1998), viticulture and enological factory residues (Pane et al., 2010), cow manure (Pane et al., 2010; Gorodecki and Hadar, 1990), municipal waste (Van Assche and Uyttebroeck, 1981; Mathout, 1987), and grape marc (Gorodecki and Hadar, 1990). However, grape marc compost also showed conduciveness towards *R. solani* (Santos et al., 2008), showing the inconsistency of compost use in disease suppression. Municipal waste compost stored near piles of composted hardwood bark suppressed *R. solani*, while those that were not stored near composted hardwood bark were not suppressive (Kuter et al., 1988). These conflicting findings stress the importance of consistent feedstock chemistry and microbial community composition in *R. solani* suppression.

Disease suppression occurs through mechanisms of general competition for nutrients and resources and specific antagonism through toxicity (microbial production and release of antibiotics, antifungals that target pathogenic organisms), parasitism, and predation. The use of more recalcitrant carbon sources, such as lignocellulosic woody materials, in composting is hypothesized to favor the colonization of biocontrol organisms during maturation (Hoitink et al., 1996), supporting the view that it is the substrate composition during maturation that gives rise to a suppressive microbial community (Hadar and Papadopoulou, 2012). Maturity age is an indicator of substrate composition and microbial community. Greenhouse trials show that mature compost provides significant suppression against *R. solani*, whereas immature compost is conducive to disease development (Tuitert et al., 1998; Kuter et al., 1988; Hoitink et al., 1996). Compost that had been

cured for five months showed better suppression than compost that had been cured for three or seven months (Tuitert et al., 1998). Similarly, compost samples taken from the high-temperature center of the pile are conducive to *R. solani* infection, but samples taken from the low-temperature edge of the pile are suppressive (Chen et al., 1987; Chung and Hoitink, 1990). Immature composted hardwood bark and any compost that is heat-treated does not provide any disease suppression (Nelson and Hoitink, 1983; Hoitink et al., 1996), which suggests that suppression is largely due to microbial activity.

Compost maturity has traditionally been measured by pile temperature, age, and humification (humus is the end product of composting, and is measured through the amount of humic and fulvic acids). Parameters such as ecoenzyme activity (Castaldi et al., 2008), respiration (Hoitink et al., 1996), and C:N ratio (Goyal et al., 2005), are correlated most closely with maturity. Respiration rate is the basis for the industry standard maturity test produced by Solvita, Woods End Laboratories, which measures CO<sub>2</sub> and NH<sub>3</sub> evolution and computes a maturity index based on the combined evolution rates. It remains the most popular method of maturity assessment among commercial compost producers. However, because respiration rate is influenced by a number of parameters including temperature, humidity, and incubation conditions (Gómez et al., 2006; Wichuk and McCartney, 2010), it may not be the most accurate method in determining ecological maturity and stability, and may not support prediction of disease suppressive qualities in compost.



Ecoenzyme activity is an indicator of microbial nutrient demand in relation to environmental nutrient availability (Sinsabaugh et al., 2008). Because enzymes that hydrolyze related groups of compounds, such as cellulose and hemicellulose, correlate with each other, a single indicator enzyme can be used as a representative of the combined activities of a suite of enzymes that degrade a particular substrate. For example,  $\beta$ -glucosidase (BG) can serve as an indicator enzyme for hydrolyzation of hemicellulose and cellulose,  $\beta$ -N-acetyl-glucosaminidase (NAG) for chitin and peptidoglycan, L-leucine aminopeptidase (LAP) for proteins and general microbial activity, and phosphatase (PP) for organic phosphorus and microbial turnover (Moorhead et al., 2013).

Assessing ecoenzyme activity in compost captures the current state of microbial community metabolism, and serves as an indicator of which substrates and decomposition functions are most abundant, or which nutrients are most limited. Ecoenzyme activity may be affected by the substrate composition of the original feedstock materials. An abundance of labile carbon substrates such as cellulose can be more conducive to disease establishment than the use of more recalcitrant carbon substrates such as lignin (Hoitink et al., 1996).

The ecological stability of compost is important in disease suppression. Nematode quantification and identification is commonly used to assess ecosystem health, stability, and successional maturity in soils, though it has not commonly been used in compost.

Nematodes are favorable as biological indicators for a number of reasons (Bongers and Ferris, 1999; Neher 2001). As they are one or two steps higher on the food chain, they occupy multiple trophic groups and as such they can serve as integrators of physical, biological, and chemical properties within the soil ecosystem. Their occupation in multiple trophic groups and key positions in the soil food web allow them to be responsive to disturbance, enrichment, and pollution. Their generation time is longer (days to years) than metabolically active microbes (hours to days), making them more stable temporally, and buffered against ephemeral nutrient flushes (Neher 2001). Finally, they can be identified inexpensively and easily without the need for dissection. Recently, the nematode maturity index (MI) and fungivore to bacterivore (F:B) ratio showed potential to serve as an indicator of compost maturity and stability (Steel et al., 2010). Thus, nematode MI and F:B may be relevant in disease suppression as well. To avoid confusion with compost maturity, the MI will be referred to as a ecological successional index (ESI) in this paper.

The overarching aim of this project was to identify biological indicators that could be used to assess whether a particular type of compost is capable of suppressing the soilborne plant disease *R. solani*, as a model pathogen system of significant importance to the global food supply. Since compost is a heterogeneous material, and all compost is made differently, the indicators would serve to not only assess the microbial community composition and function, but also to determine how well they can characterize ecological properties of compost based on its feedstock materials, production process,

and age. The framework of this research study was designed for potential future applications to other soilborne plant pathogens and compost types.

### **Objective**

A suite of biological indicators was chosen for this study to determine their 1) ability to represent compost feedstock, process, and age, and 2) ability to predict disease severity (DS) on *R. solani* in several different types of compost. Additionally, the study aims to assess the ability of a *Rhizoctonia* plate assay to predict disease suppressiveness in greenhouse bioassays. The biological indicators include microbial biomass carbon, CO<sub>2</sub> respiration, pH, electrical conductivity (EC), C:N ratio, a *Rhizoctonia* plate competition assay, ecoenzyme activity, nematode ecological successional index (ESI), and nematode F:B ratio.

## **3.2. METHODS**

### **3.2.1. Compost Sample Selection**

#### *Criteria for Selection of Composts*

Composts from a variety of different commercial facilities were chosen based on maturity and pile type. Pile types included standard windrows aerated by a bucket loader or excavator, static piles aerated by a positive pressure automated fan system, and vermicompost. Vermicomposts were produced by feeding the compost worm, *Eisenia fetida*, material that had been composted past the thermophilic phase, having already met PFRP. Compost samples came from Green Mountain Compost, Vermont Compost, Grow Compost, Vermont Natural Ag Products, and Highfields Center for Composting. Two vermicompost samples and two vermicompost liquid extracts came from Worm Power in Avon, NY.

*Profile of Compost Facilities and Compost Samples*

**Table 5.** Profile of each compost facility. Compost samples were obtained from six commercial facilities, one anaerobic digester, and one farmer's field.

<b>Facility</b>	<b>Location</b>	<b>Process</b>	<b>General Description</b>
Highfields Center for Composting	Hardwick, VT	ASP/Windrow & ASP/Vermicompost	Highfields Center for Composting makes compost from local agricultural wastes including dairy and horse manure, apple, brewery, and coffee processing waste, food scraps, wood chips, straw, hay, sawdust, yard waste, etc. They compost in an aerated static pile for 90 days before moving to windrow piles for finishing the thermophilic phase and curing. They also have a small vermicompost digester where they feed material that has composted and met PFRP to the compost worms.
Green Mountain Compost	Williston, VT	ASP/Windrow	Green Mountain Compost is run by the Chittenden County Solid Waste District, collecting food scraps, yard waste, and agricultural waste from residents and businesses within Chittenden County. They also incorporate dairy manure/silage, straw, wood chips, and charcoal into their compost piles.
Vermont Compost	Montpelier, VT	Windrow	Vermont Compost receives food scraps and local agricultural wastes including manure, sawdust, wood chips, hay, etc. They incorporate chickens into their process. Fresh materials are blended into a new pile. Chickens dig into the fresh pile for food scraps, insects, and other food, leaving behind their manure, and contributing to the aeration process through their scratching, digging, and pecking.
Grow Compost	Moretown, VT	Windrow	Grow Compost receives food scraps and local agricultural waste including brewery processing, spoiled dairy, coffee chaff, manure, hay, sawdust, wood chips, etc. They use an excavator to turn windrow piles and their process takes 12 months to produce finished compost for sale.
Vermont Natural Ag Products	Middlebury, VT	Windrow	Vermont Natural Ag Products receives in dairy manure/silage and sawdust only, and composts using a turned windrow system.
Worm Power	Avon, NY	ASP/Vermicompost	Worm Power pioneered a vermicompost digester system (adopted by Highfields Center for Composting) where material that has been

			composted and met PRFP is fed to the compost worms. They partner with a dairy farm in Avon, NY, which uses a nutritionally consistent feed to its animals every day. A sawdust bedding is used. Manure and bedding are composted in aerated static piles before being fed to compost worms.
Riverside Organic Farm	Hardwick, VT	Windrow	Riverside Farm produces its own compost on-farm using poultry manure and softwood cedar shavings.
Green Mountain Power	Burlington, VT	Anaerobic Digestate	Manure/silage is fed into an anaerobic digester. The resulting liquid is separated from the solids and applied as fertilizer onto farmers' fields. The solids are dehydrated and used in our disease severity trials.

**Table 6.** Key to compost sample abbreviations. All samples that were assayed in the disease severity greenhouse trials were abbreviated and identified based on process and maturity.

Abbreviation	Description
-	soil that is not infested with <i>R. solani</i>
+	soil that is infested with <i>R. solani</i>
TI	Thermophilic (mixed feedstocks) Immature
MI	Manure/silage Immature
TM	Thermophilic (mixed feedstocks) Mature
MM	Manure/silage Mature
HM	Hardwood bark Mature
AD	Anaerobic Digestate
TMF	Thermophilic Mature Farmer's Compost
VMO	Vermicompost Mature 1 Year Old
VMF	Vermicompost Mature Fresh
VEO	Vermicompost Liquid Extract 1 Year Old
VEF	Vermicompost Liquid Extract Fresh
NC	No Compost (control)
R	Rice Hulls (control)

**Table 7.** Compost feedstocks

<i>Feedstock</i> <i>Compost</i>	<b>Manure/ Silage</b>	<b>Food Waste</b>	<b>Sawdust</b>	<b>Yard Waste</b>	<b>Poultry Manure</b>	<b>Hardwood</b>	<b>Softwood</b>
<b>TI1</b>	X	X	X	X	X		X
<b>TI2</b>	X	X	X	X			
<b>TI3</b>	X	X	X	X			
<b>TI4</b>	X	X	X	X			
<b>MI</b>	X		X				
<b>TM1</b>	X	X	X	X	X		X
<b>TM2</b>	X	X	X	X			
<b>TM3</b>	X	X	X	X			
<b>TM4</b>	X	X	X	X			
<b>TM5</b>	X	X	X	X			
<b>MM</b>	X		X				
<b>HM</b>	X					X	X
<b>AD</b>	X		X				
<b>TMF</b>				X	X		X
<b>VM0</b>	X		X				
<b>VMF1</b>	X	X	X	X			
<b>VMF2</b>	X		X				
<b>VE0</b>	X		X				
<b>VEF</b>	X		X				

### 3.2.2. Disease Severity Bioassays

#### *Rhizoctonia Plate Competition Bioassay*

A half gram of compost was added to 50ml sterile water and shaken overnight (adapted from Alfano et al., 2011). The next day, 1.5g agar was added to 50ml deionized water and autoclaved for 30 minutes. It was cooled to 55°C, mixed in with the compost water extract, swirled gently to mix, and poured into plates. The next day, plugs of *R. solani* growing on potato-dextrose agar were transferred onto the compost water extract plates, and pure water agar plates were used as a control. They were incubated for 24 hours at room temperature. The mycelium radius was then measured to the nearest 1 mm under the microscope. Three of the longest radii were recorded, and the mean was used as a representative measure to compare suppressive potential among different compost samples. Five replicates of each compost sample were assessed in this way. All measurements were standardized against the control of mean mycelium radial growth on water agar.

#### *Greenhouse Bioassay*

Greenhouse bioassays are set up as described in Chapter 2, Section 2.2.3. Each seedling was examined under a stereoscope and rated for disease severity according to the scale described in Table 8. Control treatments included an uninfested soil with no compost



applied, infested soil with no compost applied, infested soil with rice hulls as an inert treatment, uninfested soil with hardwood bark compost, and uninfested soil with vermicompost. Treatments were done in quadruplicate. Based on the application rate assay (described below), thermophilic composts were applied at 10% (v/v) and vermicompost samples were applied at 1.25% (v/v).

Disease severity was rated on scale of 1-5. A percentage disease severity was ascribed each rating, based on the Horsfall-Barratt (H-B) scale. The H-B scale is based on two assumptions: 1) there is a logarithmic relationship between the reflected light from disease and the estimated area of disease; and 2) the human eye perceives diseased area below 50% and healthy area above 50% (Horsfall and Cowling, 1978b). The second assumption explains why the severity intervals decrease above 50% in the H-B scale. A midpoint was taken from the center of the percentage range, to be used as an estimate of plant disease severity in each rating category (Madden et al., 2007):

**Table 8.** Disease severity scale

<b>Rating</b>	<b>Description</b>	<b>Midpoint %</b>
0	0% infection; healthy plant	0
1	1-10% infection; light symptoms of disease – lesions are small, light, and rare	5.5
2	11-69% infection; moderate symptoms of disease – lesions are dark, large, common. There may be root constriction and stunted growth.	40
3	70-94% infection; severe symptoms of disease – lesions are black, covering large areas of the root and stem, roots are highly constricted, and growth is severely stunted	82

4	95-100% infection; death soon after germination – a small seedling germinated but was so infected that it did not grow further	97.5
5	100% infection; no germination of seed	100

The mean disease severity was computed from the midpoint percentages:

$$\frac{\sum[(\text{number of plants in rating category}) * (\text{midpoint \% of rating category})]}{25}$$

$$= (25 \text{ seeds/pot}) * 100\% \text{ disease}$$

Each sample was then standardized against the negative control treatment of no compost in uninfested soil, as percent change in disease severity from the negative control.

### 3.2.3. Biological Indicators & Compost Characteristics

The biological indicators used in this study are summarized in Table 9, followed by a detailed protocol provided below.

**Table 9.** Summary table of biological indicators & compost characteristics

Category	Assay	Function	Units
Microbial Function	Chloroform Fumigation Extraction	Microbial Biomass Carbon	µg C/g dry weight compost
Microbial Function	Sodium Hydroxide Titration	Microbial Activity via CO <sub>2</sub> Respiration	mg CO <sub>2</sub> /hr/g dry weight compost

Microbial Function	Extracellular Enzyme Analysis	Decomposition Activity; Nutrient Availability	nmol/h/g dry weight compost or $\mu\text{mol/h/g}$ dry weight compost; ratios of ecoenzymatic C:N:P acquisition
Microbial Function	<i>R. solani</i> Plate Bioassay	Competition Against Pathogen	millimeters (mm)
Microbial Function, Ecosystem health	Nematode Quantification & Identification	Nematode Ecological Successional Index, N Mineralization	count
Microbial Community Composition	16S & ITS Sequencing	Prokaryotic & Fungal Community Composition	abundance
Chemical Properties	CHN Analysis	C:N Ratio	ratio
Chemical Properties	pH and EC	Salt Concentration	EC measured in mS/cm

### *Chloroform Fumigation Extraction*

The chloroform fumigation extraction was performed on 10g fresh weight of each compost sample in triplicate (Allison 2008). Non-fumigated samples were immediately extracted with 50ml 0.5M  $\text{K}_2\text{SO}_4$  for 1 hr on a shaker. Fumigated samples were placed in a vacuum desiccator with a beaker containing 20 ml ethanol-free chloroform (Sigma-Aldrich Co. St. Louis, MO). The desiccator was evacuated until the chloroform had boiled for 1 min. The desiccator was then sealed and incubated overnight in the dark. The next day, the beaker of chloroform was removed and the desiccator evacuated 10–12 times to remove all traces of chloroform from the compost. The fumigated compost

samples were extracted with 0.5M K<sub>2</sub>SO<sub>4</sub> under the same conditions as the non-fumigated samples. The C content of the K<sub>2</sub>SO<sub>4</sub> extracts was measured on a Shimadzu TOC-5000A soluble C analyzer. Dissolved organic carbon (DOC) of non-fumigated samples was subtracted from DOC of fumigated samples, and a k<sub>EC</sub> value of 2.64 was applied (Vance et al., 1987).

#### *Microbial Activity via CO<sub>2</sub> Respiration*

CO<sub>2</sub> respiration was measured using a sodium hydroxide incubation and titration, as adapted from Coleman et al. (2004: pp 301-303). An open plastic cup (100ml volume) filled with 10ml of 1M sodium hydroxide was set into a sealed jar of compost and incubated for 24-36 hours. After incubation, 10ml of 1M barium chloride was added to each cup to stop the reaction of CO<sub>2</sub> and NaOH. A drop of thymolphthalein was added as a blue color indicator, and the solution was titrated with 1M hydrochloric acid until the color indicator turned clear.

CO<sub>2</sub> evolution was calculated using the following equation:

$$\text{CO}_2 - \text{C (mg)} = (\text{B} - \text{X})\text{ME}$$
where B = HCl (ml) needed to titrate NaOH solution from the blank, X = HCl (ml) needed to titrate NaOH solution in the experimental jars, M = 1.0 (HCl molarity), and E = equivalent weight (22 for CO<sub>2</sub> and 6 for C). The data is expressed as milligrams of CO<sub>2</sub> per hour per gram dry weight compost.

## Ecoenzyme Analysis

The following enzymes were assessed for their activity in compost:

**Table 10.** Ecoenzymes assayed and their associated functions in soil microbiota

Ecoenzyme Class	Ecoenzyme	Substrate	Functional Indicator	Abbreviation
Hydrolase	$\beta$ -1,4-N-acetylglucosaminidase (chitinase)	MUB <sup>1</sup> - $\beta$ -1,4-N-acetylglucosamine	Microbial cell wall turnover	NAG
	$\beta$ -glucosidase	MUB- $\beta$ -glucoside	Cellulose degradation	BG
Oxidase	peroxidase	hydrogen peroxide	Lignin degradation	NETPEROX
	phenol oxidase	L-DOPA <sup>2</sup>	Lignin degradation	PHENOX
Amino peptidase	L-leucine aminopeptidase	MC <sup>3</sup> -L-leucine aminopeptide	General microbial activity; Protein degradation; N availability	LAP
Esterase	phosphatase	MUB-phosphate	Microbial turnover; P availability	PP
1. MUB = 4-methylumbelliferone 2. MC = methylcoumarin 3. L-DOPA = 3,4-dihydroxyphenylalanine				

Six ecoenzymes were assayed for functional activity in compost treatments. The activities of hydrolase enzymes and phosphatase were quantified fluorometrically using a methylumbelliferone (MUB) linked substrate, L-leucine aminopeptidase was quantified fluorometrically using a methylcoumarin (MC) linked substrate, and the oxidase enzymes

were quantified using a 3,4-dihydroxyphenylalanine (L-DOPA) linked substrate. Enzyme activities were quantified using the modified protocol of Saiya-Cork et al. (2002) with the following modifications in methodology. Oxidative enzyme activity was quantified spectrophotometrically in clear polystyrene 96-well microplates. All other enzymes were quantified in all black polystyrene 96-well microplates.

Hydrolase substrates, L-leucine aminopeptide, and phosphate were assayed in four concentrations: 20, 40, 80, 120  $\mu$ M. Sample suspensions were prepared by adding a half gram of compost to 100ml of 50mM sodium bicarbonate buffer (pH 7) and homogenizing for 90 seconds with a Polytron (Brinkman PT 3100). Because enzyme activity is pH sensitive, the pH was adjusted to 7 to match the mean pH of compost samples. The microplates were designed to assay three samples per plate, with two columns of 8 wells each, for 16 replicates for each sample, along with controls (250  $\mu$ l buffer alone, 200  $\mu$ l buffer with 50  $\mu$ l reference MUB/MC, and 200  $\mu$ l buffer with 50  $\mu$ l substrate). Each MUB/MC linked substrate was prepared as a 200 $\mu$ M solution in nanopure water and stored at 4°C. The reference standard MUB/MC was a 50 $\mu$ M solution. Substrates were prepared as 200 $\mu$ M solutions in nanopure water. Microplates are covered and incubated at 20°C for 2 hours. After incubation, they are quantified using a microplate fluorimeter (FLx800, Bio-Tek Instruments) with 360nm excitation and 460nm emission filters.

Oxidative enzyme substrates consisted of 50mM L-DOPA for the phenol oxidase assay and 50mM L-DOPA with 0.3% hydrogen peroxide for the peroxidase assay. The plates

were covered and incubated for 1.5 hours at 20°C. Absorbance was read on a microplate spectrophotometer (Bio-Tek  $\mu$ Quant microplate reader) with a 520nm filter. All enzyme activities were calculated as  $\text{nmol h}^{-1} \text{g}^{-1}$  of dry compost.

### *Nematode Quantification and Identification*

Mixed subsamples of compost were packed into a small mesh-screened PVC pipe 5cm in diameter, 4cm tall, for a total volume of 78.5ml, set atop a glass stem funnel into a large test tube in an automated mist chamber. The mister sprays water for one minute every five minutes, releasing 1.5L water per hour. Nematodes swim out into a continuous column of water and settle at the bottom of the tube by gravity. After 48 hours, the contents of the test tube are poured into a beaker. The top of the water column is siphoned off to just less than 100ml and transferred into a bottle. The bottle is homogenized prior to counting – 10ml of water is withdrawn using a pipette and transferred into a counting chamber. All nematodes are counted, representing 10% of the sample.

After counting, 20ml of the top of the sample suspension is siphoned off and transferred into a Petri dish. The remaining sample is transferred to a 100ml bottle, allowed to settle (one hour per inch of water column), and then the top of the water is transferred into a 15ml centrifuge tube. Glass microslides with a thin wax ring in the middle are prepared by dipping a heated copper ring into wax and onto the middle of the slide. The contents

of the centrifuge tube are allowed to settle and 20-25  $\mu$ l is pipetted into the wax ring of each glass microslide preparation. Slides are created as necessary to achieve desired identification. A square cover slip is placed over the wax ring, held gently over a heating plate to melt the wax, and cooled to solidify the wax and create a partial seal with the cover slip. Clear nail polish is applied in a ring around the cover slip.

Nematodes were identified and classified according to the following families and trophic groups (Table 11) (Bongers 1990; Bongers and Bongers, 1998):

**Table 11.** Nematode families, c-p values, and trophic groups

<b>Family</b>	<b>c-p Value</b>	<b>Trophic Group</b>
Rhabditidae	1	bacterivore
Tylenchidae	2	plant-parasitic
Aphelenchidae	3	fungivore
Prismatolaimidae	3	bacterivore
Leptolaimidae	2	bacterivore
Monhysteridae	1	bacterivore
Plectidae	2	bacterivore
Paraphelenchidae	2	fugivore
Seinuridae	2	predatory
Meloidogyne	3	plant-parasitic
Alirhabditidae	1	bacterivore
Aphelenchidae	3	fungivore
Pseudodiplogasteroididae	1	bacterivore
Diplosapter	1	bacterivore
Hypodontolaimidae	3	plant-parasitic
Qudsianenatidae	4	fungivore
Diplogasteridae	1	bacterivore
Neodiplogasteridae	1	bacterivore
Pseudogasteridae	1	bacterivore



The nematode Ecological Successional Index (ESI) is calculated as follows (Bongers 1990):

$$ESI = \sum [(v_i f_i) / n]$$

where  $v_i$  equals the  $c-p$  value of the  $i$ th family,  $f_i$  equals the frequency of the  $i$ th family in the sample, and  $n$  equals the total number of individual nematodes in a sample.

The ratio of fungivorous to bacterivorous nematodes was calculated as follows (Yeates et al., 1993):

$$F:B = \text{fungivores} / (\text{fungivores} + \text{bacterivores})$$

### *Physical Properties*

pH, electrical conductivity (EC), and C:N ratio were assessed for all compost samples.

Ten grams of compost was extracted with 10ml 1M potassium chloride for pH assessment, using an Accumet AB15 glass pH electrode filled with saturated potassium chloride. Ten grams of compost was mixed in with 10ml of deionized water for EC assessment, using an Orion conductivity meter. C:N ratio was measured by drying compost samples overnight at 90°C, grinding into a fine powder, and then sent through a CHN analyzer.

### 3.2.4. Statistical Analyses

All variables were assessed for normality using the UNIVARIATE procedure in SAS 9.3, and adjusted as necessary to best fit a normal distribution. Electrical conductivity and C:N ratio were the least normally distributed variables, and so were adjusted with a natural log transformation of  $\ln(\text{EC}+1)$  and  $\ln(\text{C:N} + 1)$ . All statistically analyses were done with these transformed variables.

Pearson's correlation was used to determine independence between variables, performed in SAS using the CORR procedure. Multiple stepwise regression (forward selection) was performed in SAS using the REG procedure, with disease severity of all compost samples as the dependent variable, and, based on the Pearson's correlation test (Table A3.2.), the independent variables used were microbial biomass carbon, respiration, plate competition, pH, electrical conductivity, C:N, nematode ecological successional index, and nematode F:B ratio.

Analysis of covariance (ANCOVA) were performed using the MIXED procedure in SAS, with Tukey post-hoc tests groupings generated by a SAS macro called PDMIX800, created by Arnold M. Saxton of the University of Tennessee, Knoxville. Disease severity, microbial biomass carbon, respiration, plate competition, pH, electrical conductivity, and C:N ratio were analyzed as dependent variables in separate tests. Maturity, process, and feedstock were analyzed as the independent variables, with facility as a random effect.

Chi square analyses were performed to determine differences in nematode community composition, using JMP Pro 11. GraphPad Prism 6 was used for enzyme kinetics analysis and to generate all graphs.

### 3.3. RESULTS

#### 3.3.1. Biological Indicators and Compost Feedstock, Production Process, and Maturity Age

**Table 12.** Key to compost sample abbreviations

Abbreviation	Description
-	soil that is not infested with <i>R. solani</i>
+	soil that is infested with <i>R. solani</i>
TI	Thermophilic (mixed feedstocks) Immature
MI	Manure/silage Immature
TM	Thermophilic (mixed feedstocks) Mature
MM	Manure/silage Mature
HM	Hardwood bark Mature
AD	Anaerobic Digestate
TMF	Thermophilic Mature Farmer's Compost
VMO	Vermicompost Mature 1 Year Old
VMF	Vermicompost Mature Fresh
VEO	Vermicompost Liquid Extract 1 Year Old
VEF	Vermicompost Liquid Extract Fresh
NC	No Compost (control)
R	Rice Hulls (control)

The most suppressive compost sample was the vermicompost VMF1 (Table 12, Table C.1., Figure 5), followed by the vermicompost liquid extracts (VEO, VEF), anaerobic digestate (AD), and the hardwood bark compost (HM). A one-year-old vermicompost sample (VMO) and a mature manure/silage compost (MM) had little effect on disease suppression, comparable to the positive control of no compost with infested soil (NC+). Compost sample TM5 was the most conducive to disease, followed by TM4, TM1, TI2, TI1, and the thermophilic mature farmer's compost TMF. Cluster analysis of disease severity shows that the effects of VMF1 application to infested soil is most similar to uninfested control treatments (Figure 6). These treatments are most different from all

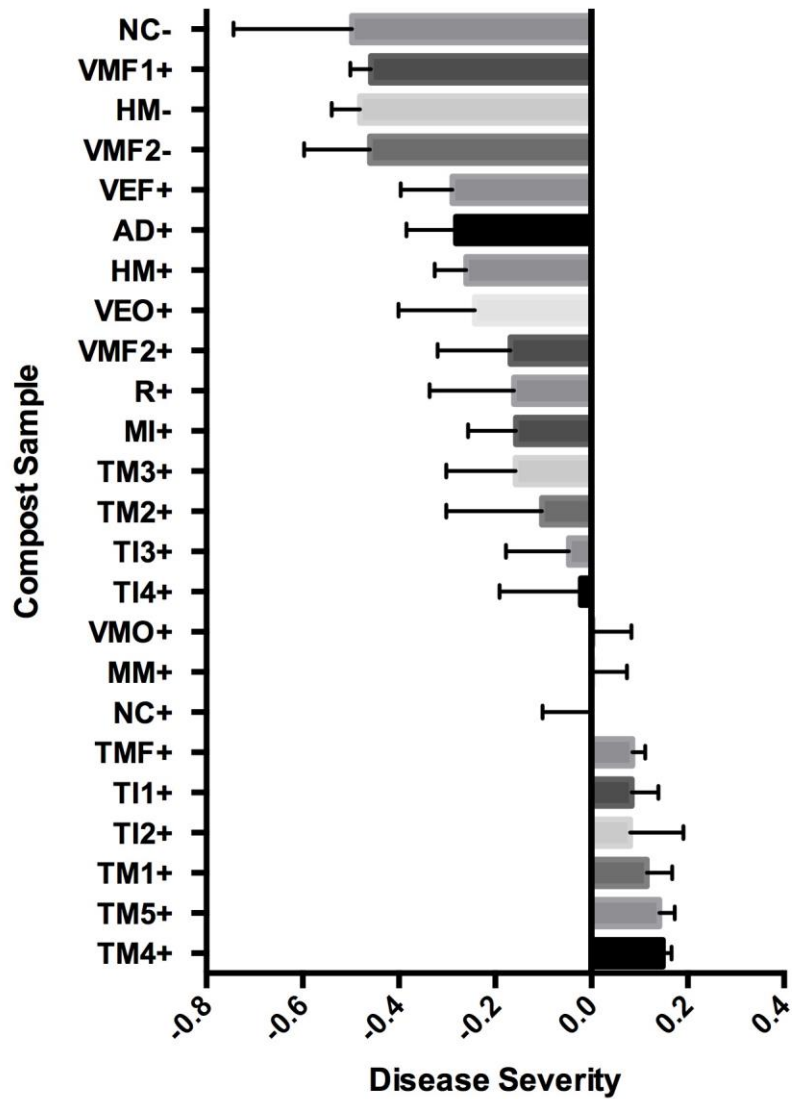
other treatments, distinguished by uninfested vs. infested soils. Most of the thermophilic composts cluster together, and the most suppressive treatments (AD, HM, VEN, VEF) also cluster together. The vermicomposts do not cluster together at all. Cluster analysis of indicators show that compost samples cluster differently than they do when only comparing disease severity values (Figure 7). Not all of the indicators predict disease suppression.

Vermicompost and anaerobic digestate were more suppressive than thermophilic (windrow and ASP) composts ( $P \leq 0.0014$ ), indicating that while they may use the same feedstock materials (manure/silage only), the different processes of decomposition produce materials with very different biological properties and effects than thermophilic composting alone. Mature composts were more suppressive than immature ( $P \leq 0.041$ ), confirming the need for a more stable microbial ecosystem in disease suppression. Both process and feedstock had significant effects on respiration ( $P \leq 0.0001$ ,  $P \leq 0.0001$ ), pH ( $P \leq 0.011$ ,  $P \leq 0.0003$ ), and C:N ratio ( $P \leq 0.0014$ ,  $P \leq 0.0001$ ). Respiration rate for vermicompost and anaerobic digestate were different than for thermophilic (windrow and ASP) composts ( $P \leq 0.0001$ ) (Table 14). Process, maturity, and feedstock all contributed to significant effects on electrical conductivity ( $P \leq 0.001$ ,  $P \leq 0.012$ ,  $P \leq 0.0028$ ).

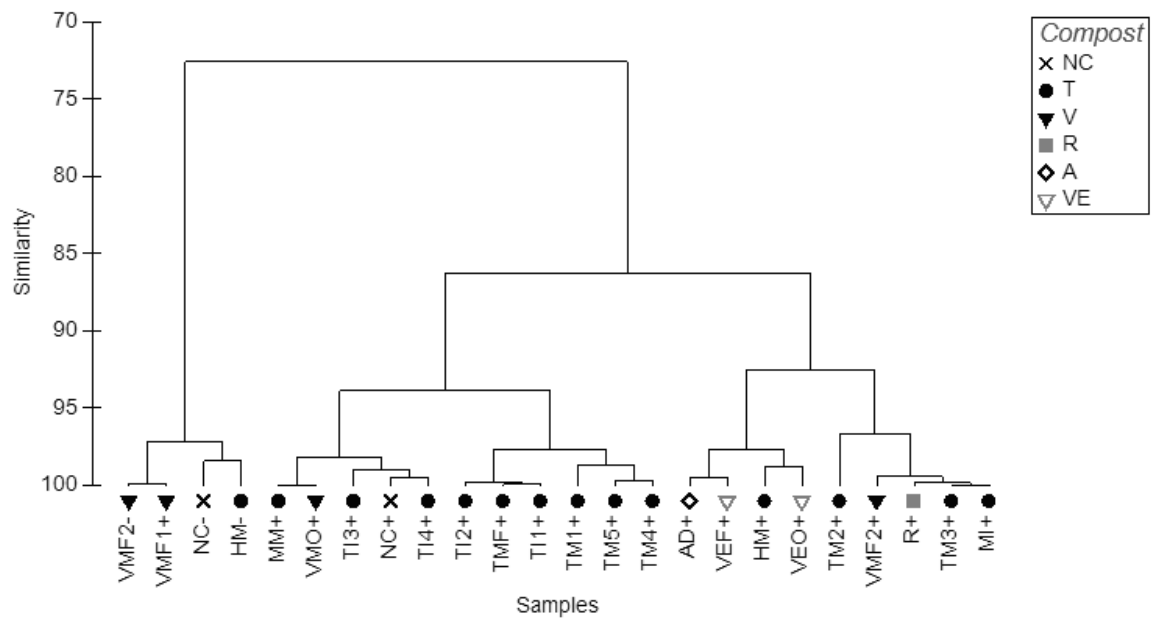
No linear relationships were found between disease severity and any of the indicators. Multiple stepwise regression showed disease severity was best predicted by five variables ( $R^2=0.6861$ ):

$$DS=0.4787+(-0.3189)*(ESI)+(0.3796)*(plate)+(0.0092)*(PP)+(0.06435)*(BG)+(-0.1338)*(NAG)$$

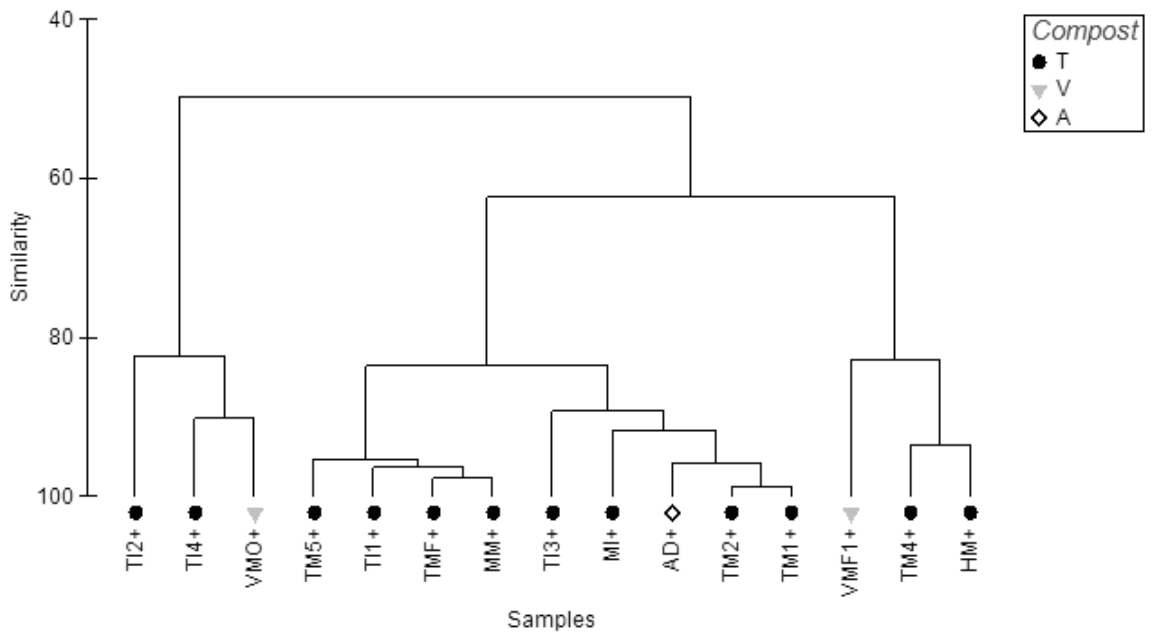
Where DS = disease severity; ESI = nematode ecological successional index; plate = *R. solani* growth *in vitro*; PP = phosphatase activity (nmol/h/g dry compost); BG =  $\beta$ -glucosidase (nmol/h/g dry compost); NAG =  $\beta$ -1,4-N-acetylglucosaminidase (nmol/h/g dry compost).



**Figure 5.** Disease severity of all compost samples. All samples are standardized against the positive control of no compost with infested soil (NC+). Negative disease severity values indicate more suppressive treatments; positive disease severity values indicate more conducive treatments.

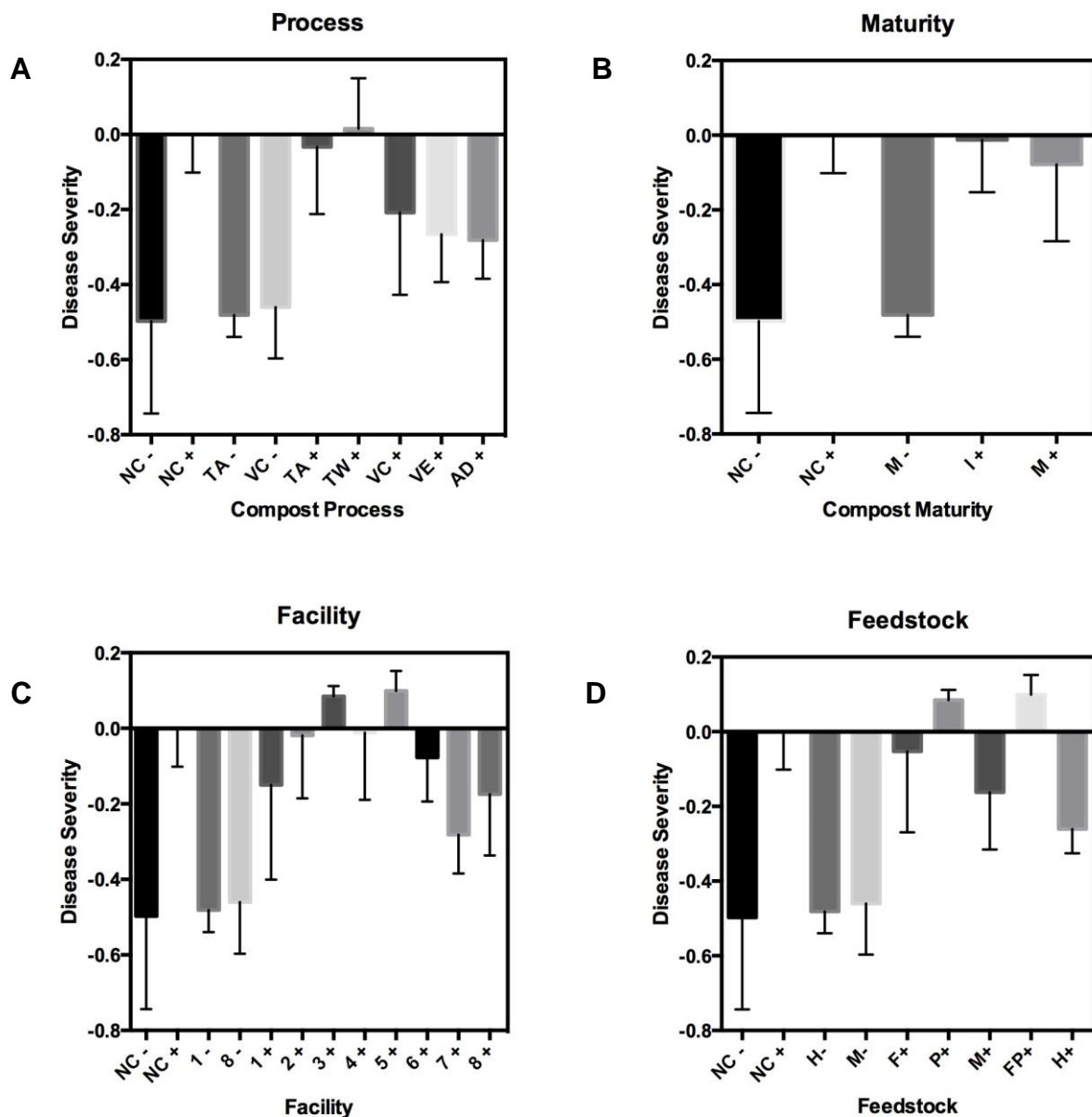


**Figure 6.** Cluster analysis of disease severity of all treatments.



**Figure 7.** Cluster analysis of indicators for all treatments except VEO, VEN, VMF2, TM3. Does not include Disease Severity values. Includes indicators Microbial Biomass Carbon, Respiration, Plate Competition, pH, EC, C:N, Nematode Maturity Index, and Nematode F:B Ratio

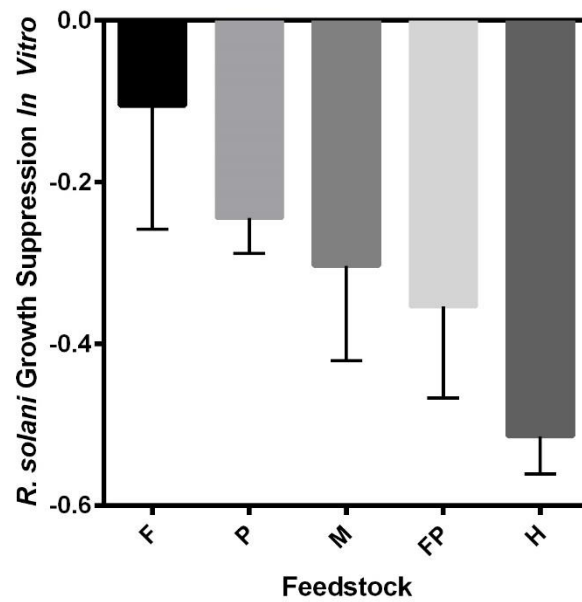




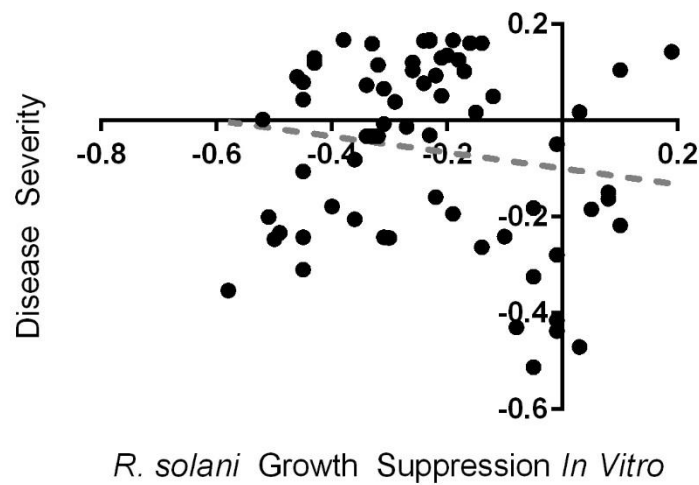
**Figure 8.** Disease severity of composts by A) process, B) maturity, C) facility, and D) feedstock. Key: TA=Thermophilic ASP; TW=Thermophilic Windrow; VC=Vermicompost, VE=Vermicompost Liquid Extract; AD=Anaerobic Digestate; M=Mature; I=Immature; H=Hardwood Bark, M=Manure, F=Food Waste, P=Poultry Manure, FP=Food Waste and Poultry Manure.

### 3.3.2. Relationship between *Rhizoctonia* Plate Assay and Greenhouse Bioassay

The plate competition was most significantly effected by feedstock ( $P \leq 0.0085$ , Figure 9). Food waste and/or poultry manure compost generated more mycelial growth than manure/silage or hardwood bark compost ( $P \leq 0.006$ ) (Table 14). Microbial biomass carbon was the only indicator that contributed to differences in the *Rhizoctonia* plate competition ( $P \leq 0.048$ ) (Table 13). This contrasts the findings from the greenhouse bioassay showing no effects from feedstock differences. There is minimal linear relationship between *R. solani* growth *in vitro* and disease severity in the greenhouse ( $R^2 = 0.025$ ) (Figure 10).



**Figure 9.** Effects of compost feedstock on *R. solani* growth inhibition *in vitro*. F = Food waste, P = Poultry manure, M = Manure/silage only, FP = Food waste and Poultry manure, H = Hardwood bark.



**Figure 10.** Linear regression of *R. solani* growth suppression *in vitro* vs. disease severity in the greenhouse.  $Y = (-0.1664) * X - 0.09931$ ;  $R^2 = 0.025$

**Table 13.** Summary of analyses of covariance: Effects of facility as random effect, process, maturity, and feedstock on disease severity, microbial biomass carbon, respiration, pH, electrical conductivity, C:N ratio, and *Rhizoctonia* plate competition. *F*-values and levels of significance are shown.  $P \leq 0.05$  are highlighted in bold.

Dependent Variable	Process		Maturity		Feedstock		Covariables											
							MBC		Resp		pH		EC		C:N		Plate	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Disease Severity	8.82	<b>0.0003</b>	4.59	<b>0.041</b>	0.59	0.674	1.47	0.24	4.43	<b>0.045</b>	1.57	0.22	1.73	0.199	0.99	0.328	0.05	0.82
Microbial Biomass Carbon	1.14	0.349	0.08	0.783	0.35	0.843	-	-	0	0.996	0.21	0.65	1.19	0.284	0.6	0.444	2.37	0.135
Respiration	15.88	<b>0.0001</b>	0.45	0.509	6.84	<b>0.0001</b>	15.9	0.8	-	-	8.99	<b>0.0055</b>	2.18	0.151	12.28	<b>0.0015</b>	0.3	0.59
pH	4.42	<b>0.011</b>	2.87	0.1007	7.57	<b>0.0003</b>	0.32	0.57	5.32	<b>0.029</b>	-	-	0.56	0.459	2.02	0.166	1.11	0.3
ln(Electrical Conductivity+1)	16.04	<b>0.0001</b>	7.18	<b>0.012</b>	5.21	<b>0.0028</b>	3.71	0.06	2.18	0.15	0.68	0.418	-	-	1.79	0.1908	0.31	0.58
ln(C:N Ratio)	6.7	<b>0.0014</b>	0.83	0.3694	8.23	<b>0.0001</b>	0.62	0.44	12.28	<b>0.0015</b>	2.56	0.12	1.79	0.19	-	-	0.29	0.59
Plate Competition	2.1	0.1217	0.02	0.8993	4.19	<b>0.0085</b>	4.25	<b>0.048</b>	0.47	0.5004	0.91	0.3483	0.32	0.5787	0.28	0.6	-	-

**Table 14.** Means with standard error, letter superscripts indicate Tukey post-hoc differences at  $P \leq 0.05$ . Feedstock Key: P = Poultry Manure, M = Manure/Silage Only, FP = Food Waste and Poultry Manure; H = Hardwood Bark; F = Food Waste

Dependent Variable	Process				Maturity		Feedstock				
	ASP	W	V	AD	I	M	P	M	FP	H	F
Disease Severity	0.0124 ( $\pm 0.1585$ ) <sup>a</sup>	-0.0192 ( $\pm 0.157$ ) <sup>ab</sup>	-0.1907 ( $\pm 0.2443$ ) <sup>b</sup>	-0.2819 ( $\pm 0.1025$ ) <sup>b</sup>	-0.0125 ( $\pm 0.1399$ ) <sup>a</sup>	-0.0923 ( $\pm 0.2046$ ) <sup>b</sup>	0.0848 ( $\pm 0.0271$ ) <sup>a</sup>	-0.169 ( $\pm 0.1565$ ) <sup>a</sup>	0.0994 ( $\pm 0.0528$ ) <sup>a</sup>	-0.2605 ( $\pm 0.0652$ ) <sup>a</sup>	-0.0275 ( $\pm 0.2$ ) <sup>a</sup>
Microbial Biomass Carbon	421.05 ( $\pm 304.8$ ) <sup>a</sup>	366.14 ( $\pm 219.25$ ) <sup>a</sup>	581.705 ( $\pm 492.2$ ) <sup>a</sup>	299.493 ( $\pm 308.99$ ) <sup>a</sup>	535.3221 ( $\pm 306.4284$ ) <sup>a</sup>	334.893 ( $\pm 247.319$ ) <sup>a</sup>	384.8 ( $\pm 80.6376$ ) <sup>a</sup>	463.3955 ( $\pm 317.7215$ ) <sup>a</sup>	335.174 ( $\pm 74.342$ ) <sup>a</sup>	130.2533 ( $\pm 137.4$ ) <sup>a</sup>	419.223 ( $\pm 308.87$ ) <sup>a</sup>
Respiration	0.03 ( $\pm 0.012$ ) <sup>c</sup>	0.0375 ( $\pm 0.0323$ ) <sup>b</sup>	0.0217 ( $\pm 0.0117$ ) <sup>a</sup>	0.14 ( $\pm 0.02$ ) <sup>ab</sup>	0.0493 ( $\pm 0.0337$ ) <sup>a</sup>	0.0352 ( $\pm 0.0368$ ) <sup>a</sup>	0.03 ( $\pm 0.01$ ) <sup>ab</sup>	0.0431 ( $\pm 0.0562$ ) <sup>ab</sup>	0.0065 ( $\pm 0.0138$ ) <sup>a</sup>	0.0033 ( $\pm 0.0058$ ) <sup>c</sup>	0.037 ( $\pm 0.0249$ ) <sup>a</sup>
pH	7.245 ( $\pm 0.2524$ ) <sup>a</sup>	6.7954 ( $\pm 0.78$ ) <sup>b</sup>	6.795 ( $\pm 0.2461$ ) <sup>b</sup>	8.09 ( $\pm 0.466$ ) <sup>a</sup>	7.3358 ( $\pm 0.4041$ ) <sup>a</sup>	6.862 ( $\pm 0.7178$ ) <sup>a</sup>	5.4467 ( $\pm 0.08$ ) <sup>d</sup>	6.964 ( $\pm 0.7363$ ) <sup>cd</sup>	7.577 ( $\pm 0.0903$ ) <sup>a</sup>	6.72 ( $\pm 0.446$ ) <sup>ab</sup>	7.144 ( $\pm 0.4474$ ) <sup>bc</sup>
EC	3.462 ( $\pm 1.3595$ ) <sup>c</sup>	3.7752 ( $\pm 1.497$ ) <sup>b</sup>	12.235 ( $\pm 4.258$ ) <sup>a</sup>	1.497 ( $\pm 0.1955$ ) <sup>c</sup>	4.47 ( $\pm 1.1002$ ) <sup>a</sup>	4.257 ( $\pm 3.975$ ) <sup>b</sup>	3.3233 ( $\pm 1.0162$ ) <sup>abc</sup>	5.8342 ( $\pm 5.1522$ ) <sup>a</sup>	2.643 ( $\pm 0.76$ ) <sup>c</sup>	2.1467 ( $\pm 0.35$ ) <sup>bc</sup>	3.8859 ( $\pm 1.57$ ) <sup>b</sup>
C:N	16.5726 ( $\pm 1.967$ ) <sup>ab</sup>	18.8028 ( $\pm 5.251$ ) <sup>b</sup>	11.5852 ( $\pm 0.4045$ ) <sup>c</sup>	27.208 ( $\pm 0.3453$ ) <sup>a</sup>	19.6216 ( $\pm 6.005$ ) <sup>a</sup>	17.0338 ( $\pm 4.477$ ) <sup>a</sup>	19.43 ( $\pm 0.1073$ ) <sup>bc</sup>	16.756 ( $\pm 6.58$ ) <sup>d</sup>	23.778 ( $\pm 6.81$ ) <sup>ab</sup>	20.6357 ( $\pm 3.3488$ ) <sup>a</sup>	16.3244 ( $\pm 2.6437$ ) <sup>cd</sup>
Plate Competition	-0.104 ( $\pm 0.1384$ ) <sup>b</sup>	-0.307 ( $\pm 0.1642$ ) <sup>b</sup>	-0.2833 ( $\pm 0.175$ ) <sup>ab</sup>	-0.134 ( $\pm 0.0445$ ) <sup>a</sup>	-0.2428 ( $\pm 0.195$ ) <sup>a</sup>	-0.2178 ( $\pm 0.1702$ ) <sup>a</sup>	-0.244 ( $\pm 0.0439$ ) <sup>ab</sup>	-0.297 ( $\pm 0.1213$ ) <sup>b</sup>	-0.3533 ( $\pm 0.11$ ) <sup>ab</sup>	-0.514 ( $\pm 0.0472$ ) <sup>b</sup>	-0.1154 ( $\pm 0.1541$ ) <sup>a</sup>

### 3.3.3. Ecoenzyme Analysis

Overall, there is greater potential protein degradation (LAP) than cellulose degradation (BG) or chitin degradation (NAG) in all the compost samples (Figure 11A-C). Looking at  $EEA_{C/N}$  ( $BG/(LAP+NAG)$ ) vs.  $EEA_{C/P}$  ( $BG/PP$ ), samples above the 1:1 line are more N - limited, while samples below the 1:1 line are more P-limited (Figure 12A). All of the compost samples are severely N-limited. Vermicompost and anaerobic digestate samples appear to be more limited in both P and N (Figure 12B). The ecoenzyme analysis indicates that most of the nitrogen has been hydrolyzed, and the recalcitrant carbon materials constitute the remaining substrates in mature compost samples.

#### *Effects of Compost Process, Maturity, and Feedstock on Ecoenzyme Activity*

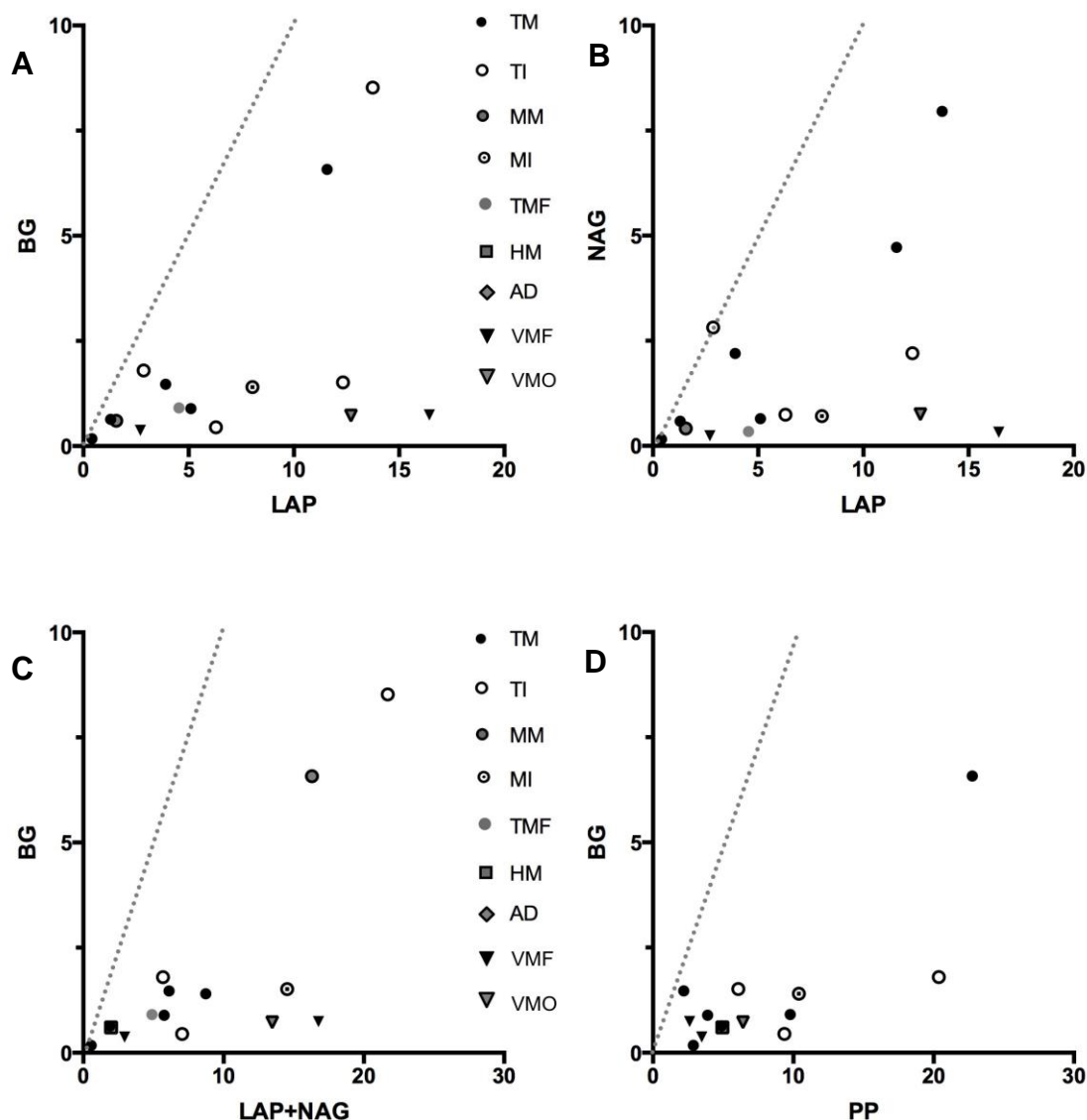
Compost process contributed to differences in PP, LAP, and NAG activity at 40 $\mu$ M substrate concentration ( $P \leq 0.0001$  for all three), as well as differences in  $EEA_{C/N}$  ( $P \leq 0.0396$ ),  $EEA_{C/P}$  ( $P \leq 0.0019$ ), and BG/OX (BG/total oxidative activity) ( $P \leq 0.0001$ ) (Table 15). Thermophilic composts (ASP and W) had different effects than anaerobic digestate (AD) and vermicompost (V) on  $EEA_{C/N}$  ( $P \leq 0.022$ ),  $EEA_{C/P}$  ( $P \leq 0.0042$ ), BG/OX ( $P \leq 0.0001$ ), PP ( $P \leq 0.0001$ ), BG ( $P \leq 0.028$ ), LAP ( $P \leq 0.0001$ ), NAG ( $P \leq 0.0045$ ), though no significant differences were found with OX (total oxidative activity) ( $P \leq 0.246$ ). Overall, anaerobic digestate had the highest rates of ecoenzyme activity in all hydrolytic and oxidative enzymes except NAG (Table 16). Anaerobic digestate had

low  $EEA_{C/N}$  similar to vermicompost,  $EEA_{C/P}$  similar to ASP, and low BG/OX similar to W. It is less P-limited than vermicompost, but more P-limited than W; and it is slightly less N-limited than vermicompost, but much less C-limited than N-limited compared to ASP or W. AD had the highest rate of oxidative activity, but the ratio of BG/OX is low and similar to W. Overall, AD is abundant in native substrate, particularly cellulose, indicated by its high BG activity.

Vermicompost process had the highest rate of NAG activity, lowest  $EEA_{C/P}$ , and lowest  $EEA_{C/N}$ , making it more C-limited than N-limited or P-limited compared to all other compost processes. Vermicompost had the lowest rate of oxidative activity compared to all other compost processes, which explains it having the highest ratio of BG/OX (Table 16). Vermicompost appears to be more abundant in nitrogen and phosphorus than cellulosic carbon or lignin.

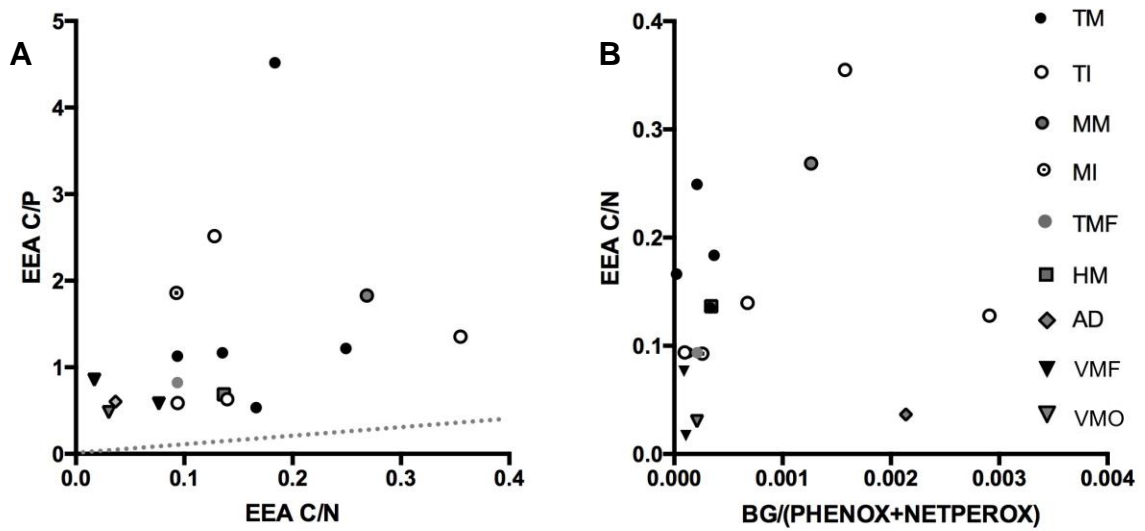
In thermophilic composts, windrow process (W) contributed to higher activity levels in PP and BG ( $P \leq 0.05$ ) (Table 16), as well as higher  $EEA_{C/P}$  ratios, compared to aerated static pile (ASP), indicating greater overall phosphorus turnover, but still more P-limited than ASP. W had the highest  $EEA_{C/P}$  and  $EEA_{C/N}$  ratios compared to all other processes, indicating that it is more P-limited and N-limited than all other processes. ASP had lowest activity rates for BG, LAP, NAG, and lowest ratio of BG/OX, indicating a low overall substrate concentration of carbon and nitrogen.

Compost maturity contributed to differences in LAP ( $P \leq 0.0175$ ) and  $EEA_{C/P}$  ( $P \leq 0.0279$ ), and feedstock had significant effects on PP ( $P \leq 0.0001$ ), BG ( $P \leq 0.0009$ ),  $EEA_{C/N}$  ( $P \leq 0.0213$ ),  $EEA_{C/P}$  ( $P \leq 0.0042$ ), and BG/OX ( $P \leq 0.0079$ ) (Table 15). Mature composts have higher LAP ( $P \leq 0.05$ ) and lower  $EEA_{C/P}$  ( $P \leq 0.05$ ), indicating that they are generally less C-limited than immature composts. Composts made with manure/silage only, mixed food wastes and poultry manure, or mixed food wastes without poultry manure, are much higher in BG than composts made with poultry manure as the primary nitrogen source, or those made with hardwood bark as the primary carbon source ( $P \leq 0.05$ ) (Table 16).



**Figure 11.** (A) LAP vs. BG; (B) LAP vs. NAG; (C) LAP+NAG vs. BG; (D) PP vs. BG. All hydrolase activities are expressed as nmol/h/g dry weight compost, from the 40 $\mu$ M substrate concentration. LAP represents protein degradation, nitrogen and microbial turnover; BG represents cellulose degradation; NAG represents chitin degradation; PP represents phosphorus and microbial turnover; PHENOX and NETPEROX represent lignin degradation (Table 11). The 1:1 line is shown for reference in all graphs. TM = Thermophilic mixed feedstocks/Mature; TI = Thermophilic mixed feedstocks/Immature; MM = Manure/silage feedstock/Mature; MI = Manure/silage feedstock/Immature; TMF = Thermophilic mixed feedstocks/Mature/Farmer's; HM = Hardwood bark feedstock/Mature; AD = Anaerobic Digestate; VMF = Vermicompost/Mature/Fresh; VMO = Vermicompost/Mature/One Year Old.





**Figure 12.** (A)  $EEA_{C/N}$  ( $BG/(LAP+NAG)$ ) vs.  $EEA_{C/P}$  ( $BG/PP$ ) (Sinsabaugh and Shah, 2012); (B)  $BG/(PHENOX+NETPEROX)$  vs.  $EEA_{C/N}$ . All hydrolase activities are expressed as nmol/h/g dry weight compost, from the 40 $\mu$ M substrate concentration. LAP represents protein degradation, nitrogen and microbial turnover; BG represents cellulose degradation; NAG represents chitin degradation; PP represents phosphorus and microbial turnover; PHENOX and NETPEROX represent lignin degradation (Table 11). The 1:1 line is shown for reference.

**Table 15.** Summary of analyses of covariance: Effects of facility as random effect, process, maturity, and feedstock on ecoenzyme activities,  $EEA_{C/N}$  (BG/(LAP+NAG)),  $EEA_{C/P}$  (BG/PP), and BG/OX, with microbial biomass carbon (MBC), respiration (Resp), pH, electrical conductivity (EC), C:N Ratio, and *Rhizoctonia* plate competition (Plate) as covariables. OX represents total oxidative activity (PHENOX+NETPEROX) and lignin degradation (enzyme abbreviations and general functions are explained in Table 11). *F*-values and levels of significance are shown.  $P \leq 0.05$  are highlighted in bold. Dpdt = Dependent

Dpdt Variable	Process		Maturity		Feedstock		Covariables											
							MBC		Resp		pH		EC		C:N		Plate	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
PP	18.98	<b>&lt;0.0001</b>	0.17	0.688	8.91	<b>0.0001</b>	0.00	0.959	16.15	<b>0.0004</b>	2.21	0.1495	1.38	0.251	12.5	<b>0.0016</b>	11.6	<b>0.002</b>
BG	2.79	0.0606	0.14	0.7144	6.54	<b>0.0009</b>	1.72	0.201	5.56	<b>0.0261</b>	3.06	0.092	0.22	0.6394	12.58	<b>0.0015</b>	6.26	<b>0.019</b>
LAP	42.54	<b>&lt;0.0001</b>	6.44	<b>0.0175</b>	2.45	0.0714	0.53	0.473	0.32	0.575	2.89	0.1009	0.00	0.9886	1.66	0.2088	2.9	0.1
NAG	453.63	<b>&lt;0.0001</b>	1.32	0.262	1.21	0.3302	0.00	0.99	4.39	<b>0.0461</b>	1.33	0.2588	0.39	0.5399	13.74	<b>0.001</b>	8.49	<b>0.007</b>
OX	1.12	0.3613	0.98	0.3314	1.34	0.2843	0.82	0.37	1.49	0.234	16.6	<b>0.0004</b>	20.4	<b>0.0001</b>	3.03	0.094	2.5	0.126
$EEA_{C/N}$	6.13	<b>0.0396</b>	4.91	0.0775	7.99	<b>0.0213</b>	6.82	<b>0.048</b>	1.41	0.289	0.09	0.782	2.46	0.1776	1.21	0.321	2.93	0.15
$EEA_{C/P}$	25.41	<b>0.0019</b>	9.4	<b>0.0279</b>	16.86	<b>0.0042</b>	0.4	0.55	2.9	0.1492	31.9	<b>0.0024</b>	3.12	0.1378	0.1	0.7663	0.00	0.985
BG/OX	590476	<b>&lt;0.0001</b>	0.55	0.464	4.4	<b>0.0079</b>	0.11	0.741	1.57	0.2221	3.91	0.0591	0.16	0.6896	9.3	<b>0.0053</b>	3.52	0.072

**Table 16.** Activity means with standard error, letter superscripts indicate Tukey post-hoc differences at  $P \leq 0.05$ . Feedstock Key: P = Poultry Manure, M = Manure/Silage Only, FP = Food Waste and Poultry Manure; H = Hardwood Bark; F = Food Waste. PP, BG, LAP, NAG activities are expressed in nmol/h/g, OX represents total oxidative activity (PHENOX+NETPEROX), and is expressed in  $\mu\text{mol/h/g}$ , except in BG/OX where both activities are calculated in nmol/h/g.

Dependent Variable	Process				Maturity		Feedstock				
	ASP	W	V	AD	I	M	P	M	FP	H	F
PP	8.2761 ( $\pm 6.5757$ ) <sup>c</sup>	19.2928 ( $\pm 20.54$ ) <sup>b</sup>	3.1772 ( $\pm 3.065$ ) <sup>bc</sup>	138.548 <sup>a</sup>	26.584 ( $\pm 21.86$ ) <sup>a</sup>	19.1685 ( $\pm 39.7049$ ) <sup>a</sup>	9.7866 <sup>bc</sup>	40.0953 ( $\pm 56.8498$ ) <sup>ab</sup>	22.1777 ( $\pm 11.178$ ) <sup>d</sup>	4.9488 <sup>cd</sup>	14.759 ( $\pm 21.1881$ ) <sup>a</sup>
BG	0.7893 ( $\pm 0.5683$ ) <sup>b</sup>	4.0357 ( $\pm 3.99$ ) <sup>a</sup>	6.404 ( $\pm 7.57$ ) <sup>ab</sup>	15.46 <sup>a</sup>	4.711 ( $\pm 4.436$ ) <sup>a</sup>	3.6588 ( $\pm 5.478$ ) <sup>a</sup>	0.9065 <sup>b</sup>	7.7815 ( $\pm 6.786$ ) <sup>a</sup>	6.8853 ( $\pm 5.2008$ ) <sup>a</sup>	0.6006 <sup>b</sup>	2.03 ( $\pm 2.685$ ) <sup>a</sup>
LAP	3.197 ( $\pm 2.2734$ ) <sup>b</sup>	15.728 ( $\pm 20.954$ ) <sup>b</sup>	5.81 ( $\pm 6.543$ ) <sup>b</sup>	226.11 <sup>a</sup>	20.6 ( $\pm 24.4007$ ) <sup>a</sup>	25.8736 ( $\pm 66.0645$ ) <sup>b</sup>	4.5433 <sup>b</sup>	61.4456 ( $\pm 94.4154$ ) <sup>ab</sup>	41.2115 ( $\pm 31.478$ ) <sup>a</sup>	1.5599 <sup>b</sup>	4.5586 ( $\pm 4.1891$ ) <sup>ab</sup>
NAG	0.9901 ( $\pm 0.9528$ ) <sup>b</sup>	3.1939 ( $\pm 02.86$ ) <sup>ab</sup>	25.76 ( $\pm 23.772$ ) <sup>a</sup>	18.485 <sup>ab</sup>	4.1056 ( $\pm 2.84$ ) <sup>a</sup>	7.0721 ( $\pm 13.8068$ ) <sup>a</sup>	0.3446 <sup>a</sup>	12.2824 ( $\pm 15.0258$ ) <sup>a</sup>	4.0985 ( $\pm 3.2185$ ) <sup>a</sup>	0.4115 <sup>a</sup>	4.643 ( $\pm 10.83$ ) <sup>a</sup>
OX	73.0599 ( $\pm 26.573$ ) <sup>a</sup>	64.042 ( $\pm 19.18$ ) <sup>a</sup>	30.4179 ( $\pm 0.00$ ) <sup>a</sup>	91.7267 <sup>a</sup>	70.007 ( $\pm 18.6343$ ) <sup>a</sup>	65.7496 ( $\pm 26.204$ ) <sup>a</sup>	79.09 <sup>a</sup>	67.4583 ( $\pm 22.3907$ ) <sup>a</sup>	63.562 ( $\pm 6.8675$ ) <sup>a</sup>	23.1177 <sup>a</sup>	72.659 ( $\pm 24.0887$ ) <sup>a</sup>
$EEA_{C/N}$	0.236 ( $\pm 0.1101$ ) <sup>a</sup>	0.2408 ( $\pm 0.106$ ) <sup>a</sup>	0.0539 ( $\pm 0.00$ ) <sup>ab</sup>	0.0632 <sup>b</sup>	0.2056 ( $\pm 0.1298$ ) <sup>a</sup>	0.222 ( $\pm 0.1097$ ) <sup>a</sup>	0.1855 <sup>bc</sup>	0.1481 ( $\pm 0.1418$ ) <sup>a</sup>	0.1554 ( $\pm 0.005$ ) <sup>ab</sup>	0.3047 <sup>a</sup>	0.2589 ( $\pm 0.1079$ ) <sup>cd</sup>
$EEA_{C/P}$	0.1112 ( $\pm 0.0669$ ) <sup>c</sup>	0.258 ( $\pm 0.1856$ ) <sup>b</sup>	0.024 ( $\pm 0.028$ ) <sup>bc</sup>	0.1116 <sup>a</sup>	0.1744 ( $\pm 0.1157$ ) <sup>a</sup>	0.1729 ( $\pm 0.1801$ ) <sup>b</sup>	0.0926 <sup>c</sup>	0.1512 ( $\pm 0.1092$ ) <sup>b</sup>	0.2581 ( $\pm 0.1168$ ) <sup>a</sup>	0.1214 <sup>bc</sup>	0.1833 ( $\pm 0.2044$ ) <sup>a</sup>
BG/OX	0.000 <sup>b</sup>	0.0001 ( $\pm 0.0001$ ) <sup>b</sup>	1.6643 ( $\pm 1.564$ ) <sup>a</sup>	0.0002 <sup>b</sup>	0.0001 ( $\pm 0.0001$ ) <sup>a</sup>	0.2881 ( $\pm 0.8877$ ) <sup>a</sup>	0.0000 <sup>c</sup>	0.3826 ( $\pm 0.9303$ ) <sup>ab</sup>	0.0001 ( $\pm 0.0001$ ) <sup>a</sup>	0.0000 <sup>bc</sup>	0.1878 ( $\pm 0.796$ ) <sup>ab</sup>

### 3.3.4. Nematode Quantification & Identification

Nematode community composition differs among compost samples ( $P \leq 0.0001$ ). Feedstock contributes to differences in ESI ( $P \leq 0.0001$ ), and maturity contributes to F:B ratio ( $P \leq 0.005$ ) (Table 17). Hardwood bark feedstock has much higher ESI than all other feedstocks ( $P \leq 0.0001$ ) (Table 18), which reflects its longer aging time. The covariables pH, C:N, and *Rhizoctonia* plate assay contribute to differences in ESI ( $P \leq 0.0164$ ,  $P \leq 0.0002$ ,  $P \leq 0.0001$ ), while microbial biomass C, respiration rate, pH, and C:N ratio contribute to differences in F:B ( $P \leq 0.008$ ,  $P \leq 0.002$ ,  $P \leq 0.04$ ,  $P \leq 0.0032$ ) (Table 17).

**Table 17.** Analysis of covariance: Effects of facility as random effect, process, maturity, and feedstock on Ecological Successional Index (ESI) and F:B ratio with microbial biomass carbon (MBC), respiration (Resp), pH, electrical conductivity (EC), C:N ratio, and *Rhizoctonia* plate competition (Plate) as covariables. *F*-values are shown,  $P \leq 0.05$  are highlighted in bold.

Dependent Variable	Process		Maturity		Feedstock		Covariables											
							MBC		Resp		pH		EC		C:N		Plate	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
ESI	0.64	0.537	2.54	0.127	12.19	<b>&lt;0.0001</b>	2.6	0.126	0.1	0.751	6.9	<b>0.0164</b>	0.34	0.565	20.52	<b>0.0002</b>	34.8	<b>&lt;0.0001</b>
F:B Ratio	1.67	0.213	10.01	<b>0.005</b>	2.22	0.1031	8.7	<b>0.008</b>	13.3	<b>0.002</b>	4.8	<b>0.04</b>	0.3	0.59	11.2	<b>0.0032</b>	2.93	0.1024

**Table 18.** Means with standard error, letter superscripts indicate Tukey post-hoc differences at  $P \leq 0.05$ . Feedstock Key: P = Poultry Manure, M = Manure/Silage Only, FP = Food Waste and Poultry Manure; H = Hardwood Bark; F = Food Waste

Dependent Variable	Process			Maturity		Feedstock				
	ASP	W	V	I	M	P	M	FP	H	F
ESI	1.207 ( $\pm 0.1886$ ) <sup>a</sup>	1.1798 ( $\pm 0.3199$ ) <sup>a</sup>	1.0526 ( $\pm 0.0815$ ) <sup>a</sup>	1.16 ( $\pm 0.2204$ ) <sup>a</sup>	1.184 ( $\pm 0.2885$ ) <sup>a</sup>	1.1 ( $\pm 0.00$ ) <sup>b</sup>	1.0611 ( $\pm 0.0955$ ) <sup>b</sup>	1.0068 ( $\pm 0.00$ ) <sup>b</sup>	1.9091 ( $\pm 0.00$ ) <sup>a</sup>	1.1413 ( $\pm 0.1745$ ) <sup>b</sup>
F:B Ratio	0.0068 ( $\pm 0.0085$ ) <sup>a</sup>	0.0033 ( $\pm 0.0056$ ) <sup>a</sup>	0.00 ( $\pm 0.00$ ) <sup>a</sup>	0.0051 ( $\pm 0.0091$ ) <sup>a</sup>	0.0037 ( $\pm 0.0053$ ) <sup>b</sup>	0.0149 ( $\pm 0.00$ ) <sup>a</sup>	0.00 ( $\pm 0.00$ ) <sup>b</sup>	0.00 ( $\pm 0.00$ ) <sup>ab</sup>	0.00 ( $\pm 0.00$ ) <sup>ab</sup>	0.0053 ( $\pm 0.0073$ ) <sup>ab</sup>

### 3.4. DISCUSSION

The potential of compost to suppress *R. solani* has been demonstrated in many studies, with particular success in greenhouse media (Ersahin et al., 2009; Nelson and Hoitink, 1983; Tuitert et al., 1998; Pane et al., 2010; Gorodecki and Hadar, 1990; Van Assche and Uytendaele, 1981; Mathout, 1987), though less consistent success has been shown in field trials (Lewis et al., 1992; Fuchs 1995; Larkin and Tavantzis, 2013). The disease suppressive activity of compost has been associated with microbial activity (Hoitink and Fahy, 1986; Hoitink and Boehm, 1999; Noble and Coventry, 2005), utilizing both general (competition for nutrients and resources) and specific (toxicity, parasitism, predation, etc.) mechanisms. Many studies on disease suppression focus on inoculation with biocontrol organisms such as *Trichoderma* spp. (Trillas et al., 2006; Chung and Hoitink, 1990; Postma et al., 2003), or only consider single biocontrol species as the primary mechanism of disease suppression. However, recent views have proposed that microbial consortia and ecological stoichiometry may be more responsible for suppressive phenomenon, rather than single biocontrol species. Substrate and nutrient composition correlating to specific states of compost maturity may be important to consider as they culture and give rise to suppressive microbial communities (Hadar and Papadopoulou, 2012).

Additionally, the ecology of the plant pathogen may be important in engineering and choosing which type of compost would be best suited for suppression. Plant pathogens

are spread across the *r-K* strategist continuum. *Rhizoctonia* is considered an opportunistic species that can attack young, predisposed plants, but is a poor competitor (Fisher et al., 1999). *Botrytis* and *Pythium* are similar in this way, while other pathogens such as *Penicillium* spp. produce antibiotics that inhibit competitors (Fisher et al., 1999). The key to consistent disease suppression may be in matching up the ecology of the plant pathogen with the ecology of the biocontrol mechanism, which may be engineered in compost.

Among all of the indicators assessed, ecoenzymes seem to be the best potential indicator of disease suppressive compost, as they integrate information about environmental substrate composition, microbial nutrient acquisition, and microbial community metabolic function. Additionally, LAP activity has the potential to serve as an indicator of compost maturity. Nematode community analysis did not offer a clear indication of disease suppression, though nematode ESI has the potential to serve as an indicator of compost maturity. Production process had the strongest influence on disease suppressive potential, followed by maturity age. Most of the other indicators did not correlate well with disease suppression.

### **3.4.1. Ecoenzyme Activity and Disease Suppression**

Ecoenzymes can be used for inferring microbial nutrient needs in relation to environmental supply. Transcription of ecoenzymes is ultimately linked to environmental

signals, such as indicators of toxicity or quorum-sensing molecules (Sinsabaugh and Shah, 2012). The most studied case of ecoenzymatic stoichiometry is the generally inverse relationship between phosphatase activity and environmental P availability (Reichardt et al., 1967; Berman 1970; Jones 1972; Speir and Ross, 1978; Wetzel 1981; Chróst and Overbeck, 1987). Changes in substrate availability affect resource allocation and multiple resource limitation, altering the functional organization of microbial communities, and ultimately, altering microbial metabolism (Allison et al., 2007).

Thus, the original feedstocks used in composting may be important in engineering the substrate composition and nutrient supply of the compost ecosystem as it matures.

However, since no differences in disease severity were found between different feedstocks, the production process may be more important in determining the substrate composition and microbial metabolism during maturation. Nutrient limitation may be important to compost-mediated disease suppression, as the most suppressive samples were severely limited in N and P. Because anaerobic digestate had very high rates of ecoenzyme activity, while vermicompost and hardwood bark compost were generally low (Table 17), rates of ecoenzymatic activity for single enzymes do not seem to be as important as the ratios of enzyme classes representing ratios of nutrient acquisition. Additionally, because microbial metabolism depends on nutrient ratios to continue, the microbial community composition is more accurately inferred from ecoenzymatic nutrient acquisition ratios.

A nutrient limited environment will favor oligotrophs over copiotrophs, and can be indicative of a late-successional ecosystem with tighter nutrient cycles. Recently disturbed environments are expected to have higher nutrient availability in the soil, and possibly a soil microbial community of reduced diversity, which may favor biological invasions (van der Putten et al., 2007). Additionally, because competition for nutrients is one of the mechanisms important to compost-mediated disease suppression, a nutrient limited environment may support non-pathogenic species to outcompete pathogenic species. This may explain why a nutrient-limited compost is more successful in suppressing *R. solani*.

Oxidative activity was expected to be significant in disease suppression, but no significant differences were found between process, maturity, or feedstock. These results contrast the findings of Van Beneden et al. (2010), which showed that incorporation of kraft lignin into soil reduced the viability of *R. solani* sclerotia. They hypothesized that abundance of lignin-degrading basidiomycetes might play an important role in control of *R. solani* sclerotia. Although statistical difference was not detected, anaerobic digestate had the highest rate of oxidative activity overall, compared to all other compost samples. By incorporating indicators of ecological stoichiometry and ecosystem health and stability, this study takes a novel approach in examining the nature of compost-mediated disease suppression against *R. solani*. Little work has been done on the ecological nature of compost-mediated disease suppression. The use of ecoenzyme and nematode community analyses allowed this study to further examine the relationship between

microbial consortia and ecological stoichiometry.

### **3.4.2. Nematode Community Analysis**

As fungi have been suggested to be important in suppression against *R. solani* (Hoitink et al., 1996), fungivorous nematodes were expected to be abundant, but few were found in all compost samples. In contrast, Steel et al. (2010) detected abundant fungivorous nematodes in mature compost with F:B values of  $11.90 \pm 8.15$ . Fungivorous nematodes *Aphelenchus avenae* and *Aphelenchoides* spp. have been found to be successful in suppressing *Rhizoctonia* damping off on cauliflower (Lagerlöf et al., 2011). However, since few fungivorous nematodes were found in compost samples that were suppressive, they may not play a crucial role in suppression of *R. solani*.

Nematode ESI for the compost samples in this study were in the range of that found by Steel et al. (2010), however ESI did not differ significantly between immature and mature compost samples in this study.

### **3.4.3. Maturity, Production Process, and Feedstock**

Respiration rate is most commonly used in determining compost maturity (Gómez et al., 2006; Wichuk and McCartney, 2010), but there were no correlations found between compost maturity and respiration rate in this study. However, respiration rate did



contribute to differences in ecoenzyme activity of phosphatase (PP) ( $P \leq 0.0004$ ),  $\beta$ -glucosidase (BG) ( $P \leq 0.0261$ ), and  $\beta$ -1,4-N-acetylglucosaminidase (chitinase) (NAG) ( $P \leq 0.0461$ ). Mature composts (composts that have aged for 3-6 months) are more suppressive than immature composts, confirming findings from previous research (Tuitert et al., 1998; Kuter et al., 1988; Hoitink et al., 1996).

Compost maturity contributed to differences in LAP activity and  $EEA_{C/P}$ . Mature composts have greater LAP activity and lower  $EEA_{C/P}$ , indicating that they are less C-limited than immature composts. LAP activity has potential to serve as an indicator of compost maturity. Greater LAP activity is an indication of increased microbial N acquisition from protein sources, rather than cell wall components such as chitin. Regardless of maturity, overall LAP activity is much greater than NAG activity. Microbial N acquisition is more focused on peptidase activity, rather than chitinase activity. Greater overall LAP activity than NAG activity is consistent with findings from Neher et al. (2015) in three different types of compost (manure/silage only, hay as primary C, and hardwood bark as primary C).

Vermicompost and anaerobic digestate behave very differently from thermophilic composts – they are more suppressive and more limited in N and P than thermophilic composts. Anaerobic digestate (AD) had much greater ecoenzyme activity overall than any of the other composts, indicating an abundance of microbial metabolic activity and an abundant supply of nutrients. However, the microbial biomass carbon of AD was

generally lower than all other compost samples, and vermicompost (VC) had the highest concentration of microbial biomass carbon. AD had the highest rate of respiration, while VC had the lowest.

This study showed inconsistent effects from feedstock chemistry, similar to previous research (Santos et al., 2008; Kuter et al., 1988). Feedstock had significant effects on the *Rhizoctonia* plate bioassay, but none on the greenhouse bioassay. This may be due to unique effects of the feedstock microbiota on *R. solani* growth *in vitro*, which are different in the native soil ecosystem. The hardwood bark feedstock was most suppressive *in vitro*. This may be due to a larger fungal community competing against or antagonizing *R. solani*, although no fungivorous nematodes were found in the hardwood bark compost. Feedstock may ultimately be less important than production process in compost-mediated disease suppression

#### **3.4.4. Indicators**

The primary indicators of significance to disease suppression are ecoenzymes, nematodes, and the *Rhizoctonia* plate competition bioassay. The *Rhizoctonia* plate competition bioassay can serve as a preliminary assessment of disease suppression, but is not strong or reliable enough as a standalone assay. Maturity and production process are most important to consider in disease suppression. Feedstock chemistry is less important, though hardwood bark compost seems to provide the best suppression among

thermophilic composts.

Other indicators that looked solely at microbial activity or physical properties, without integrating information about ecosystem maturity and stability, did not exhibit potential to predict disease suppression in compost, confirming the view that both substrate composition and microbial community composition are important in compost-mediated disease suppression.

In some ways, such as with feedstocks food waste, manure/silage, and hardwood bark, the *R. solani* assay *in vitro* reflected the results of disease severity in the greenhouse bioassay. However, it was not consistent with poultry manure or food waste mixed with poultry manure. There is more happening in the soil and compost ecosystem that could not be reflected in the laboratory assay. Microbial communities play a significant role, as does the presence of a plant. These results confirm the theory behind the plant disease triangle (Madden et al., 2007) – not only is the presence of a pathogen important in developing infection and pathology, but so is the presence of a conducive soil environment and susceptible plant host. Similar to the conclusions and recommendations of Alfano et al. (2011), the plate assay could be used as a quick preliminary assessment of disease suppression, but would need to be strengthened and confirmed by a greenhouse bioassay, and is not reliable as a standalone assay.

Microbial biomass carbon, pH, EC, and C:N ratio did not have significant effects on

disease severity. Respiration rate was greater for anaerobic digestate and vermicompost, reflecting their greater suppressive ability compared to thermophilic composts.

Additionally, respiration rate had significant effects on PP, BG, and NAG, which were the ecoenzymes that most contributed to disease severity in the multiple stepwise regression. However, while respiration was also the covariable that contributed to significant differences in disease severity ( $P \leq 0.045$ ), there was no significant linear correlation between respiration rate and disease severity ( $R^2=0.005$ ). Based on the relationships between respiration rate, production process, and ecoenzyme activity, it may have a different relationship with disease severity that is not linear in nature. This confirms the finding by Scheuerell et al. (2004) that respiration potential did not have a significant linear relationship with compost-mediated disease suppression of *R. solani*.

Ecoenzyme activity and nematode community analyses may serve as potential indicators of compost maturity and disease suppression. Both integrate information about the ecological conditions of the compost environment. Nutrient ratios, metabolic activity, and presence of microorganisms based on metabolic function may be inferred from analyses of ecoenzymes and nematodes. Since suppressive ability depends on a specific ecological environment (Hadar and Papadopoulou, 2012), these parameters may be most pertinent in choosing a compost best suited for disease suppression.

### **3.4.5. Recommendations**

Overall, the recommended composts to manage *R. solani* would be vermicompost, anaerobic digestate, and hardwood bark compost. The combination of ecoenzyme activity, nematode MI, and *Rhizoctonia* plate bioassay would serve well to predict disease suppression against *R. solani*. The plate bioassay is effective as a preliminary screen, but would need to be followed up with a greenhouse bioassay to make reliable conclusions about disease severity.

### **3.4.6. Future Applications**

This study created a framework that will allow further research to be done in similar fashion, to examine the characteristics of compost, and to identify indicators that can predict suppression of other soilborne plant pathogens. It was one of the first to explore ecoenzyme activity and analysis in compost, for the purpose of disease suppression. Future work can focus on the relationship between ecoenzyme activity and biocontrol, as well as the relationships between ecoenzyme activity, feedstock, and maturity.

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## **APPENDIX A. EFFECTS OF APPLICATION RATE ON DISEASE SEVERITY IN HARDWOOD BARK COMPOST AND VERMICOMPOST**

### **A.1. Objective**

Because the compost microbial community is important in disease suppression, application of higher rates of compost may increase competition for nutrients and resources and antagonism against disease pathogens. The objective of this study was to assess the effects of increasing application rate of hardwood bark compost and vermicompost on disease suppression, measured as disease severity on radish seedlings, against *R. solani*.

### **A.2. Methods**

Four concentrations (% v/v) of HM and VMO (Worm Power, Avon, NY) were assayed for the effects of application rate on suppressiveness against *R. solani*. Concentrations of 5, 10, 15, and 25% were assayed for hardwood bark compost, while concentrations of 0.25, 1.25, 2.5, and 5% were assayed for vermicompost in quadruplicate. These values were chosen based on the results from the field application rate assay detailed in Chapter 2. The field application rate was converted to % v/v application rate, and a range of concentrations was developed around that. Infested soil and compost applications were prepared as described in the greenhouse bioassay in Chapter 2, Section 2.2.3. Twenty-

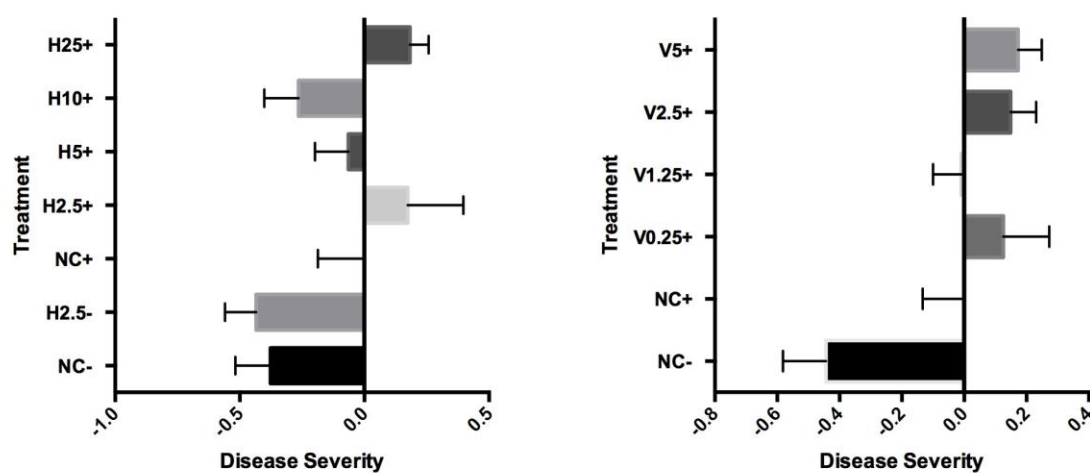
five radish seeds were planted per pot and seedlings were harvested and assessed for disease severity after two weeks of growth in the greenhouse, as described in Chapter 3, Section 3.2.2. Chi square analysis was done in JMP Pro 11 to determine effects of application rate on disease severity.

### **A.3. Results**

Varying application rates of hardwood bark compost on soil infested with *R. solani* had a significant effect on disease severity ( $P \leq 0.0001$ ). Application rates of 2.5% and 25% hardwood bark compost were conducive to disease, whereas application rates of 5% and 10% were suppressive, compared with the positive control (Figure A1.1.). The 10% application rate was more suppressive than other rates, so this rate was used for all thermophilic compost samples in the greenhouse disease severity bioassay.

Varying application rates of vermicompost on soil infested with *R. solani* also had a significant effect on disease severity ( $P \leq 0.0018$ ). Vermicompost application was conducive to disease at all rates except 1.25%, where it was comparable to the positive control (Figure A1.1.). This particular sample had aged for several months since its initial use in the preliminary field application rate assay (Figure 1), and most likely began to lose its disease suppressive capability. The 1.25% application rate was used for all vermicompost samples.





**Figure A.1.** Disease severity of hardwood bark compost and vermicompost application at four concentrations (v/v). Hardwood bark compost: 2.5%, 5%, 10%, 25%. Vermicompost: 0.25%, 1.25%, 2.5%, 5%. NC = No Compost Applied; + indicates soil infested with *R. solani*; - indicates uninfested soil

**APPENDIX B: EFFECTS OF FILTRATION AND AUTOCLAVING ON  
*RHIZOCTONIA SOLANI* GROWTH ON COMPOST WATER EXTRACTS *IN*  
*VITRO***

**B.1. Objective**

Disease suppression occurs through general (competition for nutrients and resources) and specific (toxicity, parasitism, predation, etc.) activities of biocontrol antagonists.

Microbiota viability is killed through autoclaving, and filtration removes large particles from compost media, which may harbor microbiota important to disease suppression. The objective of this study was to determine the effects of filtration and autoclaving on *R. solani* growth *in vitro* on compost water extract media.

**B.2. Methods**

*Rhizoctonia solani* was isolated and cultures were maintained as described in Chapter 3, Section 3.2.2. Five plates each of eight treatments were prepared as follows:

vermicompost – autoclaved – filtered (VAF); vermicompost – autoclaved – unfiltered (VAU); vermicompost – raw – filtered (VRF); vermicompost – raw – unfiltered (VRU); hardwood bark compost – autoclaved – filtered (HAF); hardwood bark compost – autoclaved – unfiltered (HAU); hardwood bark compost – raw – filtered (HRF); hardwood bark compost – raw – unfiltered (HRU).

All treatments were shaken in deionized water overnight, as described in Chapter 3, Section 3.2.2. The next day, treatments destined for autoclaving were autoclaved along with eight flasks of water agar mixtures (1.5g agar in 50ml deionized water). After cooling to 55°C, treatments destined for filtration were vacuum filtered through Whatman No.1 paper. Each treatment was mixed in with a flask of autoclaved water agar, swirled gently to mix, and poured into plates. The non-autoclaved (raw) and unfiltered compost water media was prepared as mentioned above. *R. solani* is transferred, incubated, and the mycelium growth is recorded for all treatment plates as described above, using pure water agar as a control treatment.

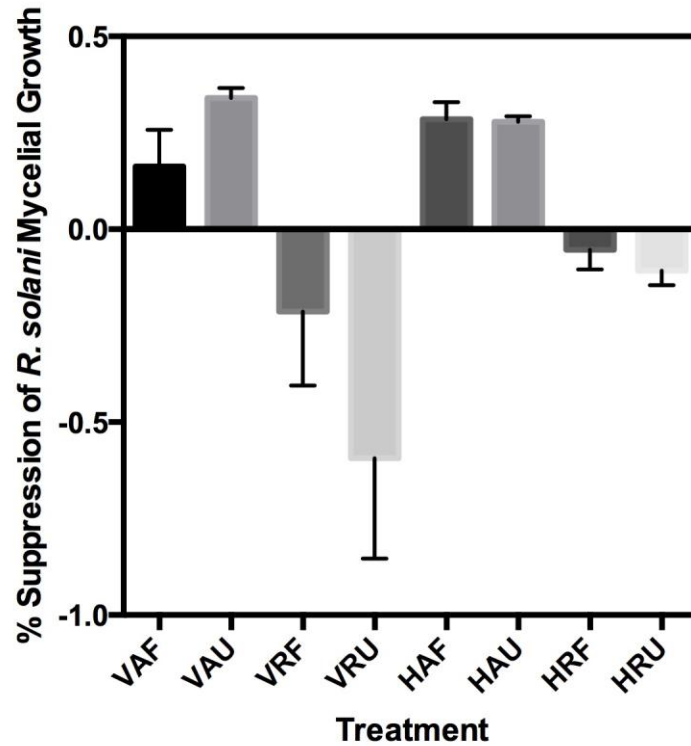
### **B.3. Results**

Raw compost water extracts suppressed *R. solani* growth *in vitro* much more than autoclaved samples ( $P \leq 0.0001$ ). Filtration appears to reduce the suppression effect, though not significantly ( $P \leq 0.1453$ ). Filtration reduced the overall conducive effect of autoclaved vermicompost, and slightly enhanced the overall conducive effect of autoclaved hardwood bark compost (Figure 5). Filtration of raw treatments in both vermicompost and hardwood bark compost appears to reduce the suppressive effect.

Autoclaving reverses the suppressive effect of raw treatments, and becomes conducive to *R. solani* growth *in vitro* ( $P \leq 0.0001$ ). Vermicompost suppresses *R. solani* growth *in*

*vitro* much more than hardwood bark compost ( $P \leq 0.0003$ ). An interaction effect was found between filtration and autoclaving ( $P \leq 0.0016$ ) and autoclaving and compost type ( $P \leq 0.0022$ ). Autoclaving treatments removed the effects of filtration, while treatments that were neither filtered nor autoclaved showed the greatest suppression of *R. solani* growth *in vitro* ( $P \leq 0.05$ ).

Since filtration of the compost water extract reduces *R. solani* suppression, there is something in the larger, solid particles that is important to disease suppression. Larger microorganisms such as microarthropods and nematodes may dwell in these aggregates, and may play a significant role in disease suppression against *R. solani*. Additionally, substrates holding the aggregates together may be important.



**Figure B.1.** Effects of filtering and autoclaving on *R. solani* growth *in vitro*, as measured by % change in mycelial growth from control. V=vermicompost; H=hardwood bark compost; A=autoclaved; R=raw; F=filtered; U=unfiltered.

## APPENDIX C: BIOLOGICAL INDICATORS DATA

**Table C.1.** Means and standard deviations for all samples and measurements. Refer to Table 6 for Sample IDs. DS = Disease Severity (% change from positive control NC+, MBC = Microbial Biomass Carbon ( $\mu\text{g C/g}$  dry weight compost), Resp = Respiration Rate ( $\text{mg CO}_2/\text{hr/g}$  dry weight compost), EC = Electrical Conductivity ( $\text{mS/cm}$ ), ESI = Nematode Ecological Successional Index, F:B = Nematode F:B Ratio ( $F/(F+B)$ ), Plate = *Rhizoctonia* Plate Assay (% change in mycelium radius (mm) from positive control water agar plate)

Sample	DS	MBC	Resp	pH	EC	C:N	ESI	F:B	Plate
NC-	-0.4974 ( $\pm 0.25$ )	-	-	-	-	-	-	-	-
NC+	-1.667E-08 ( $\pm 0.101$ )	-	-	-	-	-	-	-	-
R+	-0.1609 ( $\pm 0.175$ )	-	-	-	-	-	-	-	-
HM-	-0.481 ( $\pm 0.0586$ )	-	-	-	-	-	-	-	-
VMF2-	-0.46 ( $\pm 0.1366$ )	-	-	-	-	-	-	-	-
TI1+	0.0835 ( $\pm 0.056$ )	408.5 ( $\pm 1.078$ )	0.07724 ( $\pm 0.003$ )	7.55 ( $\pm 0.087$ )	3.177 ( $\pm 0.634$ )	28.5 ( $\pm 6.978$ )	0	0	-0.412 ( $\pm 0.097$ )
TI2+	0.0804 ( $\pm 0.1106$ )	713.52 ( $\pm 257.8$ )	0.0907 ( $\pm 0.004$ )	7.74 ( $\pm 0.181$ )	4.375 ( $\pm 0.431$ )	20.157 ( $\pm 1.189$ )	1	0	-0.322 ( $\pm 0.1$ )
TI3+	-0.047 ( $\pm 0.13$ )	244.25 ( $\pm 27.41$ )	0.04866 ( $\pm 0.0045$ )	7.51 ( $\pm 0.099$ )	4.287 ( $\pm 0.65$ )	18.0496 ( $\pm 0.21$ )	1.52	0	0.082 ( $\pm 0.033$ )
TI4+	-0.022 ( $\pm 0.1687$ )	932.753 ( $\pm 145.87$ )	0.023 ( $\pm 0.002$ )	7.32 ( $\pm 0.115$ )	4.693 ( $\pm 1.22$ )	19.034 ( $\pm 0.08$ )	1.12	0.02	-0.212 ( $\pm 0.07$ )
TM1+	0.115 ( $\pm 0.052$ )	286.29 ( $\pm 45.73$ )	0.05433 ( $\pm 0.0045$ )	7.6 ( $\pm 0.105$ )	2.11 ( $\pm 0.433$ )	19.047 ( $\pm 0.295$ )	1	0	-0.28 ( $\pm 0.094$ )
TM2+	-0.103 ( $\pm 0.199$ )	286.65 ( $\pm 324.02$ )	0.01697 ( $\pm 0.003$ )	6.45 ( $\pm 0.093$ )	3.623 ( $\pm 0.79$ )	14.125 ( $\pm 0.172$ )	1.01	0.007	-0.008 ( $\pm 0.12$ )
TM3+	-0.1578 ( $\pm 0.144$ )	276.89 ( $\pm 77.42$ )	0.0282 ( $\pm 0.003$ )	7.38 ( $\pm 0.186$ )	1.667 ( $\pm 0.33$ )	16.127 ( $\pm 0.7335$ )	-	-	0.014 ( $\pm 0.084$ )
TM4+	0.1482 ( $\pm 0.0178$ )	120.489 ( $\pm 73.07$ )	0.0174 ( $\pm 0.007$ )	6.91 ( $\pm 0.223$ )	3.963 ( $\pm 1.2$ )	13.795 ( $\pm 0.269$ )	1.12	0	-0.212 ( $\pm 0.037$ )
TM5+	0.1408 ( $\pm 0.0323$ )	430.6838 ( $\pm 99.38$ )	0.0351 ( $\pm 0.0067$ )	7.11 ( $\pm 0.072$ )	2.7 ( $\pm 0.07$ )	15.86 ( $\pm 1.198$ )	1.065	0.007	-0.192 ( $\pm 0.023$ )
TMF+	0.085 ( $\pm 0.0271$ )	384.806 ( $\pm 90.234$ )	0.02827 ( $\pm 0.0075$ )	5.44 ( $\pm 0.081$ )	3.32 ( $\pm 1.016$ )	19.43 ( $\pm 0.107$ )	1.1	0.015	-0.244 ( $\pm 0.044$ )

MI+	-0.157 (± 0.0989)	335.307 (± 179.1)	0.0075 (± 0.0057)	6.66 (± 0.197)	5.79 (± 0.451)	12.36 (± 0.093)	1	0	-0.35 (± 0.1)
MM+	0.0029 (± 0.0706)	397.929 (± 94.92)	0.0216 (0.0021)	6.18 (± 0.029)	5.857 (± 0.142)	16.157 (± 0.512)	1.2	0	-0.33 (± 0.01)
HM+	-0.2605 (± 0.0652)	130.2515 (± 137.39)	0.00376 (± 0.002)	6.72 (± 0.446)	2.147 (± 0.352)	20.636 (± 3.35)	1.9	0	-0.514 (± 0.047)
AD+	-0.2819 (± 0.1025)	299.494 (± 308.99)	0.13776 (± 0.018)	8.09 (± 0.466)	1.497 (± 0.1955)	27.208 (± 0.345)	0	0	-0.134 (± 0.044)
VMO+	0.00258 (± 0.0804)	1123.77 (± 235.86)	0.0152 (± 0.0046)	6.8 (± 0.04)	14.14 (± 0.54)	11.27 (± 0.116)	1	0	-0.4 (± 0.069)
VMF1+	-0.4586 (± 0.0424)	176.676 (± 72.845)	0.03539 (± 0.0057)	6.533 (± 0.02)	7.06 (± 1.06)	11.935 (± 0.162)	1.16	0	0.008 (± 0.049)
VMF2+	-0.1682 (± 0.1512)	-	0.0187 (± 0.005)	6.99 (± 0.14)	16.26 (± 0.96)	-	-	-	-0.314 (± 0.084)
VEO+	-0.2425 (± 0.1583)	-	-	-	1.683 (± 0.006)	-	-	-	-
VEF+	-0.2892 (± 0.107)	-	-	-	2.3 (± 0.01)	-	-	-	-

**Table C.2. Pearson Correlation Coefficients**

Pearson Correlation Coefficients														
Prob >  r  under H0: Rho=0														
Number of Observations														
	MBC	resp	pH	lnEC	lnCN	plate	eeaCN	eeaCP	bgox	phos	bg	lap	nag	ox
MB C	1	0.061	0.14816	0.48392	-0.04241	-0.2955	0.15949	-0.14191	-0.17771	0.10344	0.06782	-0.06815	-0.13747	-0.2315
		0.694	0.3371	0.001	0.7846	0.0515	0.313	0.3581	0.2485	0.504	0.6618	0.6603	0.3735	0.1402
	44	44	44	43	44	44	42	44	44	44	44	44	44	42
resp	0.061	1	0.63373	-0.40862	0.67003	0.10717	-0.20539	-0.1082	-0.11174	0.88314	0.63952	0.79811	0.18893	0.45534
	0.694		<.0001	0.0044	<.0001	0.4685	0.1811	0.4641	0.4496	<.0001	<.0001	<.0001	0.1984	0.0019
	44	48	48	47	46	48	44	48	48	48	48	48	48	44
pH	0.14816	0.63373	1	-0.27338	0.43452	0.09802	-0.15798	-0.20707	-0.11842	0.52379	0.39484	0.46656	0.07181	0.26229
	0.3371	<.0001		0.063	0.0025	0.5075	0.3057	0.1579	0.4228	0.0001	0.0055	0.0008	0.6277	0.0854
	44	48	48	47	46	48	44	48	48	48	48	48	48	44
lnEC	0.48392	-0.40862	-0.27338	1	-0.56516	-0.16231	-0.09645	-0.10831	0.49777	-0.33054	0.12355	-0.34915	0.41578	-0.33994
	0.001	0.0044	0.063		<.0001	0.2757	0.5384	0.4686	0.0004	0.0233	0.408	0.0162	0.0037	0.0257
	43	47	47	53	45	47	43	47	47	47	47	47	47	43
lnCN	-0.04241	0.67003	0.43452	-0.56516	1	-0.14511	0.042	-0.03621	-0.29517	0.61012	0.65217	0.56847	0.00063	0.19042
	0.7846	<.0001	0.0025	<.0001		0.3305	0.7841	0.809	0.044	<.0001	<.0001	<.0001	0.9966	0.2102
	44	46	46	45	47	47	45	47	47	47	47	47	47	45
plate	-0.2955	0.10717	0.09802	-0.16231	-0.14511	1	-0.11804	0.04012	0.06844	0.00096	-0.1904	0.03451	0.03744	0.55599
	0.0515	0.4685	0.5075	0.2757	0.3305		0.3269	0.7308	0.5569	0.9934	0.0995	0.7673	0.7482	<.0001
	44	48	48	47	47	76	71	76	76	76	76	76	76	71
eeaC N	0.15949	-0.20539	-0.15798	-0.09645	0.042	-0.11804	1	0.06753	-0.34137	-0.15062	-0.09495	-0.41051	-0.15707	-0.08132
	0.313	0.1811	0.3057	0.5384	0.7841	0.3269		0.573	0.0033	0.2066	0.4276	0.0003	0.1876	0.4971
	42	44	44	43	45	71	72	72	72	72	72	72	72	72
	-0.14191	-0.1082	-0.20707	-0.10831	-0.03621	0.04012	0.06753	1	-0.28515	-0.08468	-0.00988	-0.00738	-0.24666	-0.19235



<b>eeaC</b>	0.3581	0.4641	0.1579	0.4686	0.809	0.7308	0.573		0.0119	0.464	0.932	0.9492	0.0306	0.1055
	44	48	48	47	47	76	72	77	77	77	77	77	77	72
<b>bgox</b>	-0.17771	-0.11174	-0.11842	0.49777	-0.29517	0.06844	-0.34137	-0.28515	1	-0.15946	0.30206	-0.11291	0.90947	-0.37759
	0.2485	0.4496	0.4228	0.0004	0.044	0.5569	0.0033	0.0119		0.166	0.0076	0.3282	<.0001	0.0011
	44	48	48	47	47	76	72	77	77	77	77	77	77	72
<b>phos</b>	0.10344	0.88314	0.52379	-0.33054	0.61012	0.00096	-0.15062	-0.08468	-0.15946	1	0.70907	0.90841	0.24281	0.33289
	0.504	<.0001	0.0001	0.0233	<.0001	0.9934	0.2066	0.464	0.166		<.0001	<.0001	0.0334	0.0043
	44	48	48	47	47	76	72	77	77	77	77	77	77	72
<b>bg</b>	0.06782	0.63952	0.39484	0.12355	0.65217	-0.1904	-0.09495	-0.00988	0.30206	0.70907	1	0.68755	0.64487	0.24239
	0.6618	<.0001	0.0055	0.408	<.0001	0.0995	0.4276	0.932	0.0076	<.0001		<.0001	<.0001	0.0402
	44	48	48	47	47	76	72	77	77	77	77	77	77	72
<b>lap</b>	-0.06815	0.79811	0.46656	-0.34915	0.56847	0.03451	-0.41051	-0.00738	-0.11291	0.90841	0.68755	1	0.26616	0.25322
	0.6603	<.0001	0.0008	0.0162	<.0001	0.7673	0.0003	0.9492	0.3282	<.0001	<.0001		0.0193	0.0319
	44	48	48	47	47	76	72	77	77	77	77	77	77	72
<b>nag</b>	-0.13747	0.18893	0.07181	0.41578	0.00063	0.03744	-0.15707	-0.24666	0.90947	0.24281	0.64487	0.26616	1	0.28155
	0.3735	0.1984	0.6277	0.0037	0.9966	0.7482	0.1876	0.0306	<.0001	0.0334	<.0001	0.0193		0.0166
	44	48	48	47	47	76	72	77	77	77	77	77	77	72
<b>ox</b>	-0.2315	0.45534	0.26229	-0.33994	0.19042	0.55599	-0.08132	-0.19235	-0.37759	0.33289	0.24239	0.25322	0.28155	1
	0.1402	0.0019	0.0854	0.0257	0.2102	<.0001	0.4971	0.1055	0.0011	0.0043	0.0402	0.0319	0.0166	
	42	44	44	43	45	71	72	72	72	72	72	72	72	72

## APPENDIX D: CUSTOM STATISTICS CODES

### D.1. R Code for Multiple Kruskal Wallis Tests on 16S and ITS Sequencing Results,

Developed by Jonathan Leff, University of Colorado

```
#####  
###  
### R code to find the taxa driving differences between microbial communities ###  
### given a taxa summary table and mapping file from QIIME          ###  
### -- Jon Leff -- December 5, 2012 --                               ###  
#####  
###  
  
# This code will: (1) Filter the taxa summary to remove taxa that do not meet  
# an abundance threshold in any factor level. This is based on median abundance.  
# (2) Calculate which taxa have differences in relative abundance among factor  
# levels. This is based on either Mann-Whitney tests or Kruskal-Wallis (both  
# non-parametric tests). Use Mann-Whitney for 2 factor levels and K-W for more  
# than 2. (3) Output results including adjusted (Bonferroni and FDR) p-values  
# and medians.  
  
##### Functions needed for analysis: #####
```

```
#### Run all so that they will be usable ####
```

```
#### Example usage is at bottom of file ####
```

```
# get metadata values for a specific variable in the same order as the samples
```

```
# in the taxa table
```

```
get_metadata = function(t_table,map_file,variable)
```

```
map_file[match(names(t_table),row.names(map_file)),variable]
```

```
# function to filter taxa
```

```
filter_taxa = function(t_table,map_file,f_level,f_factor){
```

```
  # Check if the t_table only has one sample
```

```
  if(class(t_table)=="numeric"){
```

```
    "skip"
```

```
  } else {
```

```
    factorMeta = get_metadata(t_table,map_file,f_factor)
```

```
    rowsToKeep = c()
```

```
    for(i in 1:nrow(t_table)){
```

```
      # in the row, calculate medians for each factor level and keep if one is
```

```
      # greater than filter
```

```
      medianAbunds <- NULL
```

```
      medianAbunds <- aggregate(as.numeric(t(t_table[i,])),list(factorMeta),median)
```

```
      if(max(medianAbunds$x) >= f_level){
```

```

    rowsToKeepP ≤- c(rowsToKeep,i)
  }
}

t_table[rowsToKeep,]
}
}

# run Wilcoxon Rank-Sum test (Mann-Whitney U test) and return p-value
run_MW_test = function(dependent,factor){
  # check for only two factor levels
  if(length(unique(factor))!=2) print('Mann-Whitney test requires exacly two factor levels.')
  wilcox.test(formula=dependent~factor)$p.value
}

# run Kruskal-Wallis test
run_KW_test = function(dependent,factor){
  kruskal.test(formula=dependent~factor)$p.value
}

# run statistical test (ANOVA or Kruskal-Wallis) on each taxon
# in a provided taxa table
run_test = function(t_table,map_file,fctr,type){

```

```

fctrMeta = as.factor(as.vector(get_metadata(t_table,map_file,fctr)))

pvals = c()

for(i in 1:nrow(t_table)){

  if(type=='MW') pvals = c(pvals,run_MW_test(as.vector(t(t_table[i,])),fctrMeta))

  else if(type=='KW') pvals = c(pvals,run_KW_test(as.vector(t(t_table[i,])),fctrMeta))

  else print('Invalid test type specified')

  if(i==1){

    medianAbunds = aggregate(as.numeric(t(t_table[i,])),list(fctrMeta),median)

  } else{

    medians = aggregate(as.numeric(t(t_table[i,])),list(fctrMeta),median)[,2]

    medianAbunds = cbind(medianAbunds,medians)

  }

}

# generate bonferroni corrected pvals

pvalsBon = pvals*length(pvals)

# generate FDR corrected pvals (taken from otu_category_significance.py)

# Ranks p-values low to high and multiplies each p-value by the number of

# comparisons divided by the rank.

pvalsFDR = pvals*(length(pvals)/rank(pvals,ties.method="average"))

# prep medians to be added

factorLevels = as.character(medianAbunds[,1])

medianAbunds[,1] = NULL

```

```

# make result df

result = as.data.frame(cbind(pvals,pvalsBon,pvalsFDR,t(medianAbunds)))

row.names(result) = row.names(t_table)

colnames(result) = c("pvals","pvalsBon","pvalsFDR",factorLevels)

result

}

# filter out blanks code (not currently used)

# if(omitBlanks){

#   taxa_table <- taxa_table[,factorMeta!=""]

#   factorMeta <- as.factor(as.character(factorMeta[factorMeta!=""]))

# }

# function to show contributions of specific taxa to variation among communities

# using Mann-Whitney (2 factor levels) or Kruskal-Wallis (more than 2) tests

# PARAMETERS:

# ts_fp=taxa summary filepath

# map_fp=mapping file filepath

# out_fp=test results output filepath

# factor=mapping file header (in quotation marks) of factor for which you are testing for
differences

```

```

# filterLevel=number from 0 to 1--the minimum median relative abundance needed in at least
one of the

#          factor levels for a taxon to be retained in the analysis

# testType=either 'MW' or 'KW' (i.e. Wilcoxon/Mann-Whitney U for 2 factor levels or Kruskal-
Wallis

#          for more than two factor levels)

differences_in_taxa = function(ts_fp,map_fp,out_fp,factor,filterLevel,testType){

  # import taxa summary and mapping file

  ts =

read.table(ts_fp,header=TRUE,sep="\t",row.names=1,comment.char="",check.names=FALSE)

  map =

read.table(map_fp,header=TRUE,sep="\t",row.names=1,comment.char="",check.names=FALSE
)

  # match up data from both

  samplesInBoth=intersect(row.names(map),names(ts))

  ts.use=ts[,match(samplesInBoth,names(ts))]

  map.use=map[match(samplesInBoth,row.names(map)),]

  # filter taxa summary table by abundance in any/either factor level

  taxa.use.filt <- filter_taxa(ts.use,map.use,filterLevel,factor)

  testResults <- run_test(taxa.use.filt,map.use,factor,testType)

  # Sort by pvalues

  testResults <- testResults[with(testResults,order(pvals)),]

```

```

# output data

write.table(x=testResults,file=out_fp,sep="\t",row.names=TRUE,col.names=NA)

}

#####

#### Example usage ####

#####

ts_fp=' '
map_fp=' '
out_fp=' '
factor=' '

filterLevel=0.01 # This is the minimum median relative abundance for taxa to be kept

testType='KW' # Either 'MW' or 'KW'

# This will produce results

differences_in_taxa(ts_fp,map_fp,out_fp,factor,filterLevel,testType)

```



**D.2. SAS Micro for Tukey Letter Groupings: PDMIX800, Developed by Arnold M. Saxton,  
University of Tennessee**

/\*\*\*\*\*

PDMIX800 08/08/2003 slice correction, handles groups with one mean;

03/26/2002 error in by processing;

10/18/2001 printing changed again, turned off log notes;

06/08/2001 bug in slice and printing modified;

/\*\*\*\*\*

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\* University of Tennessee, Knoxville TN 37996-4500 \*

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```

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*
*****/

```

\*\*\*\* PDMIX800, for SAS Version 8 \*\*\*\*;

/\*

#### ORIGINAL REFERENCE:

Saxton, A.M. 1998. A macro for converting mean separation output to letter groupings in Proc Mixed. In Proc. 23rd SAS Users Group Intl., SAS Institute, Cary, NC, pp1243-1246.

#### PURPOSE:

This macro takes two data sets from Proc MIXED (Version 8), created by the

DIFFS option on the LSMEANS statement. If an ADJUST= option is used, the pdiffs from this are used, not the unadjusted defaults.

The pdiffs are converted to groups, labeled by numbers, and this is merged onto the lsmeans data set.

The numbers are converted to letters, and for cases where more than 26 letters are needed, sections of letters are coded. For example, 3 means might have the letters A, (2)A, and (3)A. These 3 means are all different, because although all have the letter A, each A belongs to a different section, identified by (#).

#### CAUTIONS!!!!!!

Depends on computer using ASCII characters, with 32=blank and capital letters following this.

Requires temporary SAS datasets MSGRPZZ, LSDVALZZ, PDTEMPZZ, PDTEMPZZZ, PDTEMPMZZ,

so any existing SAS dataset with these names will be destroyed.

There may be an IML limit of 90 total characters in the group letter labels, but space for 200 are hardcoded.

Since SAS/IML is used, this must be installed on the computer, along with BASE and STAT.

#### Parameters.

-First required parameter must name a dataset created by

ODS OUTPUT DIFFS in proc mixed;

-Second required parameter must name a dataset created by

ODS OUTPUT LSMEANS in proc mixed;

-Optional parameters, given in any order, case insensitive.

**SORT=YES** - printing of means is in order of least squares mean

value. Any value other than YES leaves means in

the proc mixed sort order.

**ALPHA=.05** - critical probability value for deciding if means

differ or not. The default is .05, and values must

be between 0 and 1.

**WORKSIZE=1** - number of Kb of memory for IML to use. This should

only be needed in very extreme circumstances as IML

dynamically increases memory as needed.

**TEST0=YES** - this requests that 3 variables (df, t, p) be

included in the printing. Any value other than NO

prints all variables produced by the lsmeans.

**MIXFMT=NO** - this removes the formatting assigned by proc mixed,

which helps compress the page width of the output.

This also will result in the means and std. errors

being rounded, which usually is desirable. Any value

besides NO retains the proc mixed formatting.

**NUMLET=200** - This specifies maximum number of letters that will

be permitted. Many means may possibly require many letters, but memory requirements get excessive. The default of 200 should fail only in unusual cases. If failure occurs (error message in log), rerun with this option set higher.

`SLICE=variables` Effects containing all the slice variables will be subdivided, and mean separation reporting done within slice levels. Note that all comparisons are made, just reporting of comparisons across slice levels is suppressed. This is useful to reduce the complexity of letter groupings.

Example of use.

Assume the file `pdmix800.sas`, containing the macro code, is on the `a:` drive. Then the code below will run `MIXED`, and run `pdmix800` on the `lsmeans`. `MIXED` is told not to print the means and `pdiffs`, using the `ODS exclude` statement, as `pdmix800` does the printing in the more desirable format.

Also shown are two optional parameters.

```
proc mixed;  
class block a b;  
model y = a b a*b;
```

```

random block;

lsmeans a b a*b/pdiff;

ods output diffs=ppp lsmeans=mmm;

ods listing exclude diffs lsmeans;

run;

%include 'a:pdmix800.sas';

%pdmix800(ppp,mmm,alpha=.01,sort=yes);

*****/

*****;

%macro pdmix800(pname,lname,sort=NO,alpha=.05,worksize=1,test0=NO,
               mixfmt=YES,numlet=200,slice=);

/******

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```

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*****/

%let printdebug=0; **this does not turn on debug printing within IML;

*** check arguments;

%global bylistzz slicezz varlistzz; **put out for possible use by backtrans;

%let slicezz=&slice;

%local dsid chk3 error1 error neweffectlength lastslicevar var adjust bylist

printdebug;

%let error=0;

```

```

%if %length(&lname)=0 %then %let error=1;

%if %sysfunc(exist(&lname)) %then %do;

    %let dsid=%sysfunc(open(&lname,I));

    %let chk3=%sysfunc(varnum(&dsid,ESTIMATE));

    %if &chk3=0 %then %let error=2;

    %let chk3=%sysfunc(varnum(&dsid,EFFECT));

    %if &chk3=0 %then %let error=2;

    %let dsid=%sysfunc(close(&dsid));

%end;

%else %let error=1;

%if &error>0 %then %do;

    %if &error=1 %then %put WARNING: Dataset &lname does not exist.;

    %if &error=2 %then %put WARNING: Dataset &lname was not made by proc mixed.;

%end;

%let error1=&error;

%let error=0;

%if %length(&pname)=0 %then %let error=1;

%if %sysfunc(exist(&pname)) %then %do;

    %let dsid=%sysfunc(open(&pname,I));

    %let chk3=%sysfunc(varnum(&dsid,ESTIMATE));

```



```

%if &chk3=0 %then %let error=3;

%let chk3=%sysfunc(attrn(&dsid,nobs));

%if &chk3=0 %then %let error=2;

%let dsid=%sysfunc(close(&dsid));

%end;

%else %let error=1;

%if &error>0 %then %do;

%if &error=1 %then %put WARNING: Dataset &pname does not exist.;

%if &error=2 %then %put WARNING: There are no observations in dataset &pname.;

%if &error=3 %then %put WARNING: Dataset &pname was not made by proc mixed.;

%end;

%if (&error or &error1) %then %do;

%put NOTE: PDMIX800 terminated due to errors in input values.;

%goto skip;

%end;

%if &error %then %do;

%put PDMIX800 terminated due to errors in input values.;

%if &error=3 %then %put Alpha can only have values between 0 and 1.;

%if &error=4 %then %put ADJUST=Dunnett output not supported.;

%goto skip;

```

```

%end;

** save setting of notes option;

%let notesval=notes;

options nonotes;

%put PDMIX800 08.08.2003 processing;

****need list of variable names, either sliced or not;

data _null_;

*** First get unique list of all names used in BY statements;

*** these come before the variable EFFECT, but include EFFECT in list;

dsid=open("&lname",'i');

length namlist $ 512;

ii=1;

value=varname(dsid,ii);

do while (value ^= 'Effect') ;

    if ii=1 then namlist=value;

    else namlist=trim(namlist)||' '||value;

    ii=ii+1;

    value=varname(dsid,ii);

end;

call symput('bylistzz',compbl(namlist)); **list without effect;

if namlist="" then namlist=value;

```

```

else namlist=trim(namlist)||' '||value;

namlist=trim(namlist);

call symput('bylist',namlist);  **list with effect;

*****;

*** Now get list of all class variables (always between effect and estimate);

length list list1 list2 $ 3200;

start=varnum(dsid,"EFFECT")+1;

ii=1;jj=start;

slicein=upcase("&slice");

do while(ii);

name=varname(dsid,jj);

name1=upcase(name); **case sensitive names are returned by varname;

type=vartype(dsid,jj);

if name1 ^= 'ESTIMATE' then do;

kk=indexw(slicein,name1);

if kk=0 then do; list=compress(list||' '||name);

if type='N' then

list2= trim(list2)||' left('||trim(name)||left('= ' _ ' and" ) ;

else list2= trim(list2)||' left('||trim(name)||left('= " and" ) ;

end;

else do;

if type='N' then

```

```

list1= trim(list1)||' left("||trim(name)||left(")='_' or") ;

else list1= trim(list1)||' left("||trim(name)||left(")=" or") ;

end;

jj=jj+1;

end;

else ii=0;

end;

list=substr(list,2);

jj=length(list1); if jj>2 then list1=substr(list1,1,jj-2);

list2=substr(list2,1,length(list2)-3);

call symput('slice1',trim(list1));

call symput('varlist1',trim(list2));

list=translate(list,' ','=');

call symput ('varlistzz',trim(list));

run;

%if &printdebug=1 %then %do;

%put bylist    &bylist;

%put bylistzz  &bylistzz;

%put varlistzz &varlistzz;

%put varlist1  &varlist1;

%put slice1    &slice1;

%end;

```

```

***** add variables to datasets *****;

data pdtempzz; set &pname; by &bylist notsorted;

** if adjusted probs are not there, an LSD was used;

if ADJP=. then do; ADJP=PROBT; ADJUSTMENT='LSD  '; end;

length _mstech_ $ 30;

if ADJUSTMENT =" then _mstech_=compress('LSD(P<||"&alpha"||)');

else do;

    _mstech_=compress(ADJUSTMENT||'(P<||"&alpha"||)');

    if substr(ADJUSTMENT,1,7)='Dunnett' then call symput('error','4');

end;

*** numerical value check only possible in data step;

if &alpha < 0.0 or &alpha > 1.0 then call symput('error','3');

run;

data pdtempmzz; set &lname; by &bylist notsorted;

*** add bygroup variable to means dataset;

retain bygroup 0;

if first.effect then bygroup+1;

if first.EFFECT and last.EFFECT then  df0=1;

else df0=0;

dothiseffectzz=0;

run;

```

```

***means and diffs data may have different effects, due to 0 df,

    so copy bygroup over to diffs;

data pdtempzzz; set pdtempmzz; by bygroup notsorted;

if first.bypgroup;

keep &bypgroup bygroup effect;

run;

** use bylist for merging;

proc sort data=pdtempzzz; by &bypgroup ;

proc sort data=pdtempzzz; by &bypgroup ;

data pdtempzzz; merge pdtempzzz (in=have) pdtempzzz; by &bypgroup;

if have;

run;

***this sort is required to give IML data by slice;

proc sort data=pdtempzzz; by bygroup &slice; run;

%if %length(&slice) ne 0 %then %do;

*****;

*****;

*** sort, edit, relabel diff and mean data for the slice option ***;

*** this works by redefining effects that are being sliced ***;

*** Example: In a 2*2 factorial, slicing the A*B interaction by A

*** means only 2 comparisons are needed of the 4*3/2=6 possible.

```

```

*** These are A1B1-A1B2 and A2B1-A2B2;

%if %length(&varlistzz)=0 %then %put ERROR: No variables left after slicing.;

%else %do;

%let lastslicevar=%scan(&slice,-1);

*** identify sliced effects;

*** use pdtempzzz created above, with one record per effect;

proc sort data=pdtempmzz; by bygroup ;

data pdtempmzz ; set pdtempmzz;

dothiseffectzz=0;

*****test if effect should be sliced;

if not(&slice1) then do; **no slice vars missing;

    if not(&varlist1) then dothiseffectzz=1;

end;

run;

*** now fix up diffs dataset;

data pdtempzzz; set pdtempmzz; by bygroup;

if first.bygroup;

keep dothiseffectzz bygroup;

run;

proc sort data=pdtempzz ; by bygroup ;

```

```

data pdtempzz; merge pdtempzz (in=have) pdtempzzz;

  by bygroup ;

  if have;

  ***Delete any pdiffs information that compares across slices;

  ***compared factor levels must match on all slice variables;

  discardzz=0;

  if dothiseffectzz then do;

    %let ii=1;

    %let var=%scan(&slice,1);

    %do %while(%length(&var) ne 0);

      %let var2=_&var;

      %if %length(&var2)>32 %then %let var2=%substr(&var2,1,32);

      if &var ne &var2 then discardzz=1;

      %let ii=%eval (&ii+1);

      %let var=%scan(&slice,&ii);

    %end;

    if discardzz then delete;

  end;

drop discardzz ;

run;

%end;

```



```

**** if means data set has single means (eg 0 df)

    then sort these to the bottom so they do not

    merge with the msgrp letter output;

proc sort data=pdtempmzz; by &bylist &slice;

data pdtempmzz; set pdtempmzz; by &bylist &slice ;

**slicing is being done, so may have slice groups with just one level;

if dothiseffectzz >0 and first.&lastslicevar and last.&lastslicevar then df0=1;

run;

%end;

***sort single means to bottom, and get data back to original bygroup order;

proc sort data=pdtempmzz; by df0 bygroup ;


%if &printdebug=1 %then %do;

    proc print data=pdtempmzz; title3 'Means data set ready'; run;

    proc print data=pdtempzz; title3 'Diffs data set ready for IML'; run;

    title3 ;

%end;


*****;

*** ready to process for differences within each effect ***;

proc iml worksize=&worksize; reset nolog fw=7; printdebug=0;

```

```

alpha=&alpha;

use pdtempmzz; **for reading later;

**** create mean separation output dataset with length 200;

temp=j(1,&numlet,'0'); msgroup=rowcatc(temp);

ADJUSTMENT='          ';

create msgrpzz var{msgroup bygroup lsmrank ADJUSTMENT};

**** create indexes of effect and by group locations;

*** For all useful variable names, read in levels;

test='a'; ii=1;

use pdtempzz;

varlist= "&bylistzz &slice &varlistzz";

value='a'; ii=1;

do while (value ^= " " );

value=scan(varlist,ii);

if value ^= " " then do;

    *** the BY variables are not guaranteed to be character,

    *** so convert them if necessary;

    read all var value into hold;

    if type(hold)='N' then level=level||char(hold);

    else level=level||hold;

    free hold;

```

```

end;

ii=ii+1;

end;

if printdebug=1 then print varlist level;

if ncol(level)=0 then do;

    file log;

    put "NOTE: No variables found for use in &pname.";

    dataerr=1;

end;

else dataerr=0;

if dataerr ^= 1 then do;

    call change(level,"'-');

    level=rowcatc(level);

    idx=1;

    dim=nrow(level);

if printdebug=1 then print dim level;

***search down for number of comparisons in each section;

***read number of rows involving first mean to get number of means,

    then calculate number of comparisons;

byby=0;

do jj=1 to dim;

    first=level[jj,1];

```

```

byby=byby+1;

**go to end of comparisons with mean 1;

kk=jj; flag=1;

do while(flag=1);

    kk=kk+1;

    if(kk > dim) then flag=0;

    else if (level[kk,1] ^= first) then flag=0;

end;

num=kk-jj+1;

idx=idx || idx[1,byby] + num;

jj=jj-1+num*(num-1)/2; ** skip to next section;

end;

free level;

end;

if printdebug=1 then print idx byby;

** BIG BB loop through rows of prob data

** subsetting out block dealing with each effect;

pptr=1; **points to where probs start for current means;

do bygroup = 1 to byby;

    dim= idx[1,bygroup+1]-idx[1,bygroup];

    nn= dim*(dim-1)/2;

```

```

*****;

**for sorting letters need descending order, and antiranks;

setin pdtempmzz;

range=idx[1,bygroup] : idx[1,bygroup+1]-1 ;

read point range var {ESTIMATE} into lsmcur;


**stupid rank function fails on missing values;

**so must temporarily make them non missing;

test=lsmcur[><,]-1.e-30;

locmiss=loc(lsmcur=.); kk=ncol(locmiss);

if kk>0 then lsmcur[locmiss,]=test;

lsmrnk=dim+1-rank(lsmcur);

if kk>0 then lsmcur[locmiss,]=.;

lsmarnk=lsmrnk;

lsmarnk[lsmrnk,]=(1:(dim))`;

if printdebug=1 then print pptr nn;

*****;

**** get prob file data for these means.

_adj_ contains the probs, no matter what adjust method;

setin pdtempzz;

range=pptr:pptr+nn-1;

```

```

read point pptr var {_mstech_} into ADJUSTMENT;

read point range var {ADJP} into data;

pptr=pptr+nn;

if printdebug=1 then print data;

*** put p values into matrix;

p = j(dim,dim,0);

kk=1; do ii=1 to dim-1; do jj=ii+1 to dim;

  if data[kk,1]=. then p[jj,ii]=1;

  else p[jj,ii] = data[kk,1];

  p[ii,jj]=p[jj,ii]; **fill in upper triangle for next sort;

  kk=kk+1;

end;end;

*** sort matrix by lsm value, so high mean gets first letter;

temp=p;

p[,lsmrnk]=temp;

temp[lsmrnk,]=p;

p=temp; free temp;

if nn>&numlet then maxlet=&numlet; **memory use limit;

else maxlet=nn+1;

group = j(dim, maxlet, 0);

members=j(dim,1,0);

```

```

if printdebug=1 then print p dim data;

gcode=1; ngroup=1;

do ii=1 to dim;

    kk=0;

    flag=0;

    do jj=ii+1 to dim; * go down row, find group members ;

        if p[jj,ii] > alpha then do; * jj and ii are the same ;

            * check jj against members ;

            do mm=1 to kk ;

                ll=members[mm,1];

                if jj>ll then test1=p[jj,ll];

                else test1=p[ll,jj];

                if test1<0 then test1=-test1;

                if(test1 < alpha) then goto jmp0; * need new group ;

            end;

        jmp0:

        if mm=kk+1 then do;

            do mm=ii+1 to dim;

                if mm=jj then mm=mm+1; *skip jj (on diagonal);

                if mm>dim then go to jmp2;

                if jj>mm then test1=p[jj,mm];

                else test1=p[mm,jj];

```

```

if test1 > alpha && -p[mm,ii] > alpha then do;

* previous grouped mean mm may belong in this group ;

* so check if already in and current members;

* dont conflict ;

do ll=1 to kk;

    nn=members[ll,1];

    if nn=mm then goto jmp1;

    if nn<mm then test1=p[mm,nn];

    else    test1=p[nn,mm];

    if(test1<0.0) then test1=-test1;

    if(test1<alpha) then goto jmp1;

end;

jmp1: if(ll=kk+1)then do;

    group[mm,ngroup]=gcode;

    kk=kk+1; members[ll,1]=mm;

end;

end;

end;

jmp2: p[jj,ii]=-p[jj,ii]; * set so not put in next group ;

do mm=1 to kk;

    ll=members[mm,1];

    * set so not used again ;

```



```

    if ll<jj then do;

        if p[jj,ll]>0 then p[jj,ll]=-p[jj,ll]; end;

        else do;

            if p[ll,jj]>0 then p[ll,jj]=-p[ll,jj]; end;

        end;

        group[jj,ngroup]=gcode;

        kk=kk+1; members[kk,1]=jj;

    end;

    else flag=1;

end;

end;

if(kk=0) then do; * no members ;

    do jj=1 to ngroup until (group[ii,jj] ^= 0) ; end;

    * not in a group yet, so set flag ;

    if(jj=ngroup+1) then kk=kk+1;

end;

if(kk^=0) then do; * need to set current mean ;

    group[ii,ngroup]=gcode;

    ngroup=ngroup+1; gcode=gcode+1;

    if ngroup > &numlet then do;

        ** number of letters needed exceeded maximum;

        jj=dim; ii=dim; **stop loops this way to avoid warnings;

```

```

    bygroup=byby; dataerr=1;

    call symput('error','1');

end;

end;

if(flag^=0) then ii=ii-1; * need another group for this mean;

end;

if dataerr=0 then do; **skip below if error;

ngroup=ngroup-1;

group=group[,1:ngroup];

***** this section just takes the groups identified by numbers

above and converts numbers to letters. This depends on

the ASCII character definitions, eg. 64 value below is what

gets capital letters;

*** write out letters;

kk=nrow(group);

do ii=1 to kk;

    gc="";nsect=1;

    do jj=1 to ngroup;

        mm=group[ii,jj];

        if mm > 0 then do; ** blanks are 0, do not do them;

```

```

    sect=floor((mm-1)/26); *** 26 letters in alphabet;

    offset=mm-sect*26;

    sect=sect+1;

    if sect > nsect then do;

        nsect=sect;

        gc=gc||"("||char(sect)||")";

    end;

    gc=gc||byte(64+offset);

end;

end;

lsmrank=lsmarnk[ii,1];

msgroup=rowcatc(gc);

** save letters, by group and sort info;

append var {msgroup bygroup lsmrank ADJUSTMENT};

end;

end; **dataerr;


end; ** for the big bb loop over effect sections;

quit;


%if &error=1 %then %do;

    %put ERROR: PDMIX800 terminated due to exceeding NUMLET limit.;

```

```

%end;

**** put group letters back in original lsm order;

**** they were sorted so largest mean gets letter A;

proc sort data=msggrpzz; by bygroup lsmrank;

%if &printdebug=1 %then %do; proc print data=msggrpzz; run; %end;


**** merge letters with means and print ****;

data msggrpzz; merge pdtempmzz msggrpzz;

label msgroup='Letter Group';

if ESTIMATE=. then do;

    **do not print for missing means;

    msgroup=";

end;

%if %upcase(&mixfmt)=NO %then %do; format _all_; %end;

run;

proc sort; by &bylistzz bygroup effect; run;


*****;

**** before printing, add the lsdvalues;

```

```

proc means noprint data=pdtempzz; by &bylist &slice notsorted;

id df adjustment;

var STDERR ;

output out=lsdvalzz n=numcomp mean=meanse max=maxse min=minse;

run;

data lsdvalzz; set lsdvalzz;

if upcase(substr(adjustment,1,3))='LSD' then critt=tinv( (1-&alpha/2),DF);

if upcase(substr(adjustment,1,3))='BON' then critt=tinv( 1-&alpha/(2*numcomp), DF);

if upcase(adjustment)='SIDAK' then do;

    prob=exp( log(1-&alpha/2) /numcomp );

    critt=tinv( prob , DF);

end;

if upcase(adjustment)='SCHEFFE' then do;

    numdf=-1+(sqrt(1+8*numcomp)+1)/2;

    critt=sqrt(numdf*finv(1-&alpha,numdf,DF));

end;

if upcase(substr(adjustment,1,5))='TUKEY' then do;

    numdf=(sqrt(1+8*numcomp)+1)/2; ** number of treatments;

    critt=probmcc('RANGE', . , 1-&alpha,DF,numdf);

put critt;

    critt=critt/sqrt(2); **adjust for tukey needing sd of mean, not diff;

end;

```

```

AvgSigDiff=meanse*critt;

MaxSigDiff=maxse*critt;

MinSigDiff=minse*critt;

keep &bylist &slice avgsigdiff maxsigdiff minsigdiff;

format minsigdiff maxsigdiff avgsigdiff best7. ;

put adjustment ' values for ' &bylist &slice ' are ' avgsigdiff ' (avg) ' minsigdiff ' (min) '
maxsigdiff ' (max).';

run;

***** print mean separation *****;

proc sort data=msggrpzz; by &bylist &slice;

proc sort data=msggrpzz; by ADJUSTMENT bygroup EFFECT;

%if %upcase(&sort)=YES %then %do;

proc sort data=msggrpzz; by ADJUSTMENT bygroup EFFECT descending ESTIMATE;

%end;

%if %upcase(&test0)=NO %then %do;

data msggrpzz; set msggrpzz;

drop tvalue probt df;

run;

%end;

data msggrpzz; set msggrpzz;

** drop working variables before printing;

```

```
drop df0 dothiseffectzz lsmrank;

run;

proc print data=msggrpzz label ;

by effect adjustment bygroup notsorted;

label bygroup=' Set'

      adjustment=' Method';

run;

%skip:

*** restore notes option;

options &notesval;

%mend;
```