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EVALUATION OF TWO SUSTAINABLE PRACTICES FOR PLANT PATHOGEN ERADICATION

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Abstract

Boxwood blight is a disease impacting much of the United States, at least 26 states as of the time of this writing. Caused by the pathogens *Calonectria pseudonaviculata* and *C. henricotiae*, the disease is found worldwide. Currently, only *C. pseudonaviculata* is found in the United States, while both species are found in Europe. Like other species in the genus, both *C. pseudonaviculata* and *C. henricotiae* are prolific microsclerotia producers. Due to the resistant nature of these survival structures, extended survival under extreme environmental conditions is possible, which could then lead to subsequent dissemination of the pathogen. During the past 10-20 years, much attention has been given to diverting organic matter from our landfills, with composting often being the proposed method for the disposal of organic matter. However, if infected plant material containing microsclerotia are present in the organic waste stream, it is possible that the pathogen could survive the composting process, therefore the use of finished compost could inadvertently serve as a vector for the pathogen. Another area of concern potentially leading to the spread of the pathogen is through water runoff from nurseries. This wastewater can also be high in nutrients, like nitrate and ammonium, spurring eutrophication processes in bodies of water in which they discharge. Capturing and reusing this wastewater can help reduce fertilizer costs, and prevent pollution, however it may also lead to pathogen levels building up due to water recirculation in these systems. Treatment solutions like constructed wetlands could possibly serve to mitigate this problem.

To address these challenges, two systems were constructed, one consisting of nine composting bioreactors, and one consisting of nine constructed wetlands. To evaluate

compost survival, microsclerotia of *C. pseudonaviculata* were grown on cellophane and inoculated into compost. Survival was compared at 24, 48, and 72 h at temperatures of 40, 50, and 60 °C. Based on the results of these experiments, it was conclusively demonstrated that microsclerotia survival in compost ≥ 50 °C for 24 h or longer is not expected. Survival in compost was less than that of heat alone, leading to an experiment evaluating the impacts of ammonia. Both species of *Calonectria* were evaluated for ammonia sensitivity at the USDA BSL-3 facility housed at Ft. Detrick (Frederick, MD). Based on these results no significant differences in ammonia sensitivity between the two species were observed. Exposing the *C. pseudonaviculata* microsclerotia to gaseous ammonia at 40 °C for 24 h reduced survival rates compared to heat alone, but still not as efficient as compost. Similar work was done to evaluate *C. pseudonaviculata* survival in a wetland, with a few key differences. Due to the regulatory nature of *C. pseudonaviculata* research, *Verticillium dahliae* was used as a proxy. Microsclerotia were grown on cellophane and inoculated into the wetlands. Nine wetlands were constructed using one of three different substrates: unplanted gravel, planted gravel, and planted gravel/spent mushroom compost mix, with three cells per treatment. None of the three treatments caused complete eradication of the microsclerotia through 7 days. This indicates that while constructed wetlands could serve as a filter reducing the number of pathogens in the irrigation water system, survival of the pathogens can be expected, leading to future reintroduction. While not ideal for plant pathogen eradication, both planted treatments showed promise in nitrate removal. All findings and results discussed do not necessarily reflect the views of any of the funding agencies.

Table of Contents

List of Figures

List of Tables

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Chapter 1 Introduction

Boxwood Blight: An Overview

Boxwoods (*Buxus* spp.) have been a staple ornamental in both Europe and the United States for hundreds of years (Bir et al. 1997; Henricot & Culham 2002; Varela et al. 2009). Controversy exists surrounding the current naming of the pathogen responsible for boxwood blight. This stems from the pathogen being isolated and proposed as a new species independently by two different lab groups in 2002. The first of these reports (Crous et al. 2002), documented a new species of fungus infecting boxwoods in New Zealand and described it as *Cylindrocladium pseudonaviculatum*. Shortly thereafter, Henricot and Culham (2002) published a paper documenting their findings and named the fungus *Cylindrocladium buxicola*. Although the teleomorph has yet to be observed, the name *Calonectria pseudonaviculata* has been proposed for the sexual stage by Lombard et al. (2010). As per the rules set forth by the Botanical Code of Nomenclature, *Calonectria pseudonaviculata* is now the official name.

Due to the polycyclic nature of the disease, boxwood blight poses a significant threat to the boxwood industry. The adhesive nature of C. *pseudonaviculata* spores also contributes to the rapid spread and infection of new hosts (Henricot 2006). Infested tools and clothing, if not properly sanitized, can inadvertently vector pathogen propagules to healthy plants and non-threatened areas. The life cycle for the pathogen is rather straightforward. Germination takes place approximately 3 hours after inoculation, with penetration occurring approximately 5 hours post-inoculation under ideal weather conditions (Henricot 2006). Penetration occurs directly through the cuticle, or through a stoma. The presence of an appressorium has not been reported for this pathogen. Once

the fungus enters the host, the mycelium grows intercellularly within the mesophyll; the fungus re-emerges through the stomata 2 to 3 days after initial infection. After 1 week, conidiophores can be observed on the abaxial leaf surface (Henricot 2006). The presence of microsclerotia, which represents a method of survival during adverse environmental conditions, has also been noted (Ivors et al. 2012).

The disease and symptom progression of boxwood blight is as follows. Circular lesions appear initially on the leaf, forming concentric rings which appear due to the outward growth of the fungus (Akilli et al. 2012; Cech et al. 2010; Crepel et al. 2003; Elmhirst et al. 2013; Gorgiladze et al. 2011; Henricot & Culham 2002; Henricot 2006; Ivors et al. 2012; LaMondia et al. 2012; Mirabolfathy et al. 2013; Saurat et al. 2012; Varela et al. 2009). Over time, the lesions expand, eventually coalescing and leading to leaf death. Symptoms are not limited to the leaves. Large black cankers and streaks appear on the stems, eventually leading to total defoliation and plant death (Akilli et al. 2012; Cech et al. 2010; Crepel et al. 2003; Elmhirst et al. 2013; Gorgiladze et al. 2011; Henricot & Culham 2002; Henricot 2006; Ivors et al. 2012; LaMondia et al. 2012; Mirabolfathy et al. 2013; Saurat et al. 2012; Varela et al. 2009).

The host range of *C. pseudonaviculata* is not fully understood; however, *in vitro* experiments have yet to uncover a completely resistant species of *Buxus*. *Buxus baleaica* appears to be most resistant to the pathogen. This putative resistance is attributed to its thick leaves, leading to the postulate that the pathogen experiences difficulties penetrating the leaf. Unfortunately, *Buxus sempervirens* represents one of the most popular boxwood varieties and shows the highest level of susceptibility towards the pathogen (Henricot 2006; Henricot et al. 2008). Other member species of ornamental importance in the

Buxaceae family include *Sarcococca* sp. and *Pachysandra* sp., both of which have been evaluated for susceptibility. *Sarcococca* has illustrated some susceptibility to the pathogen, but not to the same extent as in *Buxus* (Henricot 2006; Henricot 2008). Also, *Pachysandra terminalis* (LaMonida et al. 2012) and *P. procumbens* (LaMondia and Li 2013) have been confirmed as being susceptible.

Severe damage and economic losses have occurred due to the rapid rate of boxwood blight spread in the United States. Ten thousand plants were confirmed to have boxwood blight in North Carolina alone, with the number of infected plants found in Connecticut being 15-fold higher. Within two nurseries, 150,000 boxwood plants were confirmed to have boxwood blight (Ivors et al. 2012). The estimated monetary loss in Connecticut alone amounted to $3,000,000 (LaMondia 2014). Boxwood blight is a major concern for the nursery industry, as the boxwood market is valued at $103 million annually. Currently, 26 states in the US have had confirmed cases of boxwood blight as well as multiple provinces in Canada.

Compost as a Method for Control

Composting is a complex process involving multiple physical and biological factors. Generally, composting involves microbial decomposition processes that transform heterogeneous organic waste to a homogenous soil-like material. Decomposition processes produce heat, leading to internal temperatures that vastly exceed ambient (Hassen et al. 2001). Overall, the composting process can be divided into three separate phases: mesophilic, thermophilic, and cooling (Hassen et al. 2001; Hoitink et al. 1997).

Temperature appears to be the key factor involved with pathogen eradication by composting (Fayolle et al. 2006; Harnik et al. 2004; Hassen et al. 2001; Hoitink et al. 1997; Noble and Roberts 2004; Noble et al. 2009). Heat can be an effective killer, even when not with-in the composting system. Harnik et al. (2004) reported that chlamydospores of the pathogen *Phytophthora ramorum* were killed in 3 minutes when exposed to temperatures of 53 °C. Indirect evidence of pathogen eradication was reported by Hassen et al. (2001), when they observed that fungal populations declined during the thermophilic stage of the composting process, indicating that many fungi cannot tolerate the high temperatures. Many fungi can be eliminated under composting conditions at 52 °C for 7 consecutive days (Hoitink et al. 1976, Noble and Roberts 2004; Noble et al. 2009). However, not all fungi are eradicated under these conditions. Windrow composting produces a temperature cross sectional profile, with uneven heating, due to air flow and insulation properties of the substrate. Therefore, windrows must be turned on a regular basis to ensure that all material is exposed to high temperatures (Hoitink et al. 1997).

Aeration is another factor that influences pathogen eradication. Fayolle et al. (2006) noted that under aerated conditions, pathogen eradication was successful in compost; however, no aeration led to incomplete pathogen eradication. When compost is not aerated or turned properly, the system can become anaerobic, which results in lower temperatures in the compost pile (Fayolle et al. 2006).

High moisture leads to eradication temperatures that are lower than in drier composts (Noble et al. 2004). Fayolle et al. (2006) demonstrated that *Plasmodium brassicae* eradication was not as efficient in drier composts as compared to composts that

had higher moisture contents. There was one exception, however, the level of moisture in wood-chip-compost did not influence eradication by heating (Fayolle et al. 2006).

Constructed Wetland Use

Constructed wetlands (CW) originated in Germany, being first designed by Käthe Seidel (Vymazal 2008). The original goal was to treat wastewater originating from livestock operations, as well as human origins (Vymazal 2008). There are three basic types, free water surface (FWS), horizontal subsurface flow (HSSF) and vertical flow (VF) wetlands. From this point on, HSSF wetlands will be discussed exclusively unless otherwise noted. Generally, HSSF wetlands are rectangular and lined with an impermeable liner that has enough flexibility to allow for ease of installation. Wastewater is introduced in one end and collected horizontally at the opposite end (Vymazal 2008). Gravel is most commonly used as the substrate, which serves as the medium for plants. Plants can be beneficial to HSSF wetlands by providing insulation in colder weather (Vymazal 2008) as well as increased surface area for microbial biofilm production. Plants also can supply carbon to microbes (Berghage et al. 1999; Gruyer et al. 2011b), as well as uptake contaminants directly (Berghage et al. 1999). An advantage of utilizing HSSF wetlands is that they are inexpensive, and require little maintenance compared to other strategies (Davison et al. 2005; Vymazal 2008). However, they have several disadvantages including large demand for space, and the increased risk of clogging (Davison et al. 2005; Vymazal 2008). Interestingly, Davison et al. (2005) discovered that earthworms in their systems helped to alleviate clogging and suggested that it could extend the life of a system.

Although not initially developed for use in nurseries, constructed wetlands show promise in mitigating wastewater generated in nurseries and greenhouses (Arnold et al. 2003; Berghage et al. 1999; Fernandez et al. 1999; Gruyer et al. 2011a; Gruyer et al. 2011b). Nurseries represent an industry that utilize large amounts of both water and chemicals (Berghage et al. 1999). In fact, upwards of 19,000 L of water can be used per hectare per day in nurseries depending on the irrigation system, with large amounts of fertilizer also used each year (Berghage et al. 1999). Gilliam et al. (1992) conducted trials evaluating herbicide runoff based on spacing between pots. Even with pots directly adjacent to one another, 30% of the herbicide application misses the pots. When spacing is increased to 30 cm, this value almost tripled to 80%. These results demonstrate the ease at which chemicals and fertilizer can mix with runoff water. This fact is not lost on some authorities, with several states as well as Australia passing laws stating that nurseries need to manage their run off (Berghage et al. 1999; Lea-Cox et al. 2001; Huett et al. 2005). Many nurseries are switching to a system where they catch and recycle their irrigation water (Berghage et al. 1999; Huett et al. 2005 to minimize environmental impacts, reducing the threat to surrounding ecosystems (Arnold et al. 2003). However, reusing the water could lead to the buildup of harmful contaminants, including plant pathogens (Berghage et al. 1999; Lévesque et al. 2011; Stewart-Wade 2011). Other types of active treatments such as UV lamps can be costly (Éniel et al. 2006) solutions to address buildup of pathogen levels within the water. Results suggest that constructed wetlands perform well removing many of the nutrients and other contaminants commonly found in nursery runoff water (Arnold et al. 2003; Berghage et al. 1999; Davison et al. 2005; Fernandez et al. 1999; Huett et al. 2005; Lévesque et al. 2011)

Literature investigating plant pathogen removal via constructed wetlands is not as plentiful as that pertaining to human pathogens, although information from these studies can be useful, especially regarding bacterial pathogens as well as general processes of pathogen treatment in wetlands. An example is Diaz et al.'s (2010) work regarding the treatment of agricultural runoff with constructed wetlands. Diaz et al. (2010) determined that the runoff could pose a risk due to the presence of human pathogens, mainly in the form of *E.* coli. Overall, reduction by 66 – 91% of *E.* coli and 86-94% of general enterococcus bacteria was documented from the water effluent in the wetland system. Until more research is done evaluating bacterial plant pathogens, these numbers can be used as proxies for rough estimations. Gruyer et al. (2013) evaluated the spore reduction for *Pythium ultimum* and *Fusarium oxysporum* through constructed wetlands and found a 99.62-99.99% efficiency in treatment of these pathogens spores. This study demonstrated that treatment of fungal plant pathogens with constructed wetlands was feasible, however, there seems to be a lack of information regarding the possibility of pathogen survival inside the wetland. Vymazal (2008) described three main processes for the treatment of biological organisms in constructed wetlands, also corroborated by Kadlec and Wallace (2009), physical, biological, and chemical. Physical mechanisms include filtration and sedimentation (Kadlec and Wallace 2009; Vymazal 2008). These physical mechanisms do not directly cause the death of the organism, and the possibility of survival of resting structure such as microsclerotia should be examined in future studies. In contract, chemical and biological mechanisms usually result in direct death of the organism. Chemical processes include solar disinfection (UV rays), oxidation, biocides

and adsorption, while biological mechanisms consist predominately of antibiotic and predation (Kadlec and Wallace 2009; Vymazal 2008).

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Chapter 2 Survival of lab grown Calonectria pseudonaviculata microsclerotia during small scale composting

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ABSTRACT

Boxwood blight, caused by *Calonectria pseudonaviculata,* is a devastating fungal disease of *Buxus* spp., first observed in the United States in 2011. Due to the persistent nature of the produced microsclerotia, concern arose over the potential for compost to serve as a disease vector. Previous work demonstrated that *C. pseudonaviculata* is very stable at mesophilic temperatures, however no previous work evaluated *C. pseudonaviculata* during composting. Our objective was to evaluate the survival of *C. pseudonaviculata* after being composted for 24, 48, and 72 h at temperatures of 40, 50, and 60 °C. Composting was performed using a newly created bioreactor system, allowing for precise control of the composting process. In conjunction with the composting evaluations, the same temperature/time combinations were evaluated in incubators. While the pathogen survived 40 °C through 72 h without issue, compost survival was minimal, with only some survival seen at 24 h for the same temperature. We were able to determine that heat ≥ 50 °C for 24 h or longer would kill the microsclerotia, as well as composting for 48 h or longer at 40 °C.

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Introduction

Boxwood blight is a new and emerging disease affecting some members of the Buxaceae family (LaMondia and Li 2013; Malapi-Wight et al. 2016), caused by *Calonectria pseudonaviculata* (syn. *Cylindrocladium pseudonaviculata, Cylindrocladium buxicola*). Boxwood blight was reported by both Crous et al. (2002) and Henricot and Culham (2002) almost simultaneously in New Zealand and the United Kingdom, respectively. Although the reports of the disease were officially published in 2002, Henricot and Culham (2002) indicated that disease symptoms were first observed in 1994 in the United Kingdom. The pathogen has spread through Europe and North America (Akilli et al. 2012; Cech et al. 2013; Crepel et al. 2003; Elmhirst and Auxier 2013; Gorgiladze et al. 2011; Ivors et al. 2012; Mirabolrathy et al. 2013; Saurat et al. 2012; Varela et al. 2009). Originally confirmed in several states on both coasts of the United States, *C. pseudonaviculata* has since spread up and down both coasts, as well as most of the eastern United States (Fig. 2-1).

When originally evaluating long term survival of *C. pseudonaviculata,* Henricot and Culham (2002) reported survival of 5+ years within infected leaf material. The authors did not observe microsclerotia and concluded that the pathogen was surviving as mycelium. Weeda and Dart (2012), aware that various other species in the genus can produce microsclerotia (Crous 2002), examined infected leaf material from Virginia. By clearing, leaves infected with *C. pseudonaviculata* clearly showed microsclerotia present within the infected tissue. Weeda and Dart (2012) then examined boxwood leaves infected with other pathogens (*Macrophoma candollei* and *Volutella buxi*) but did not observe microsclerotia. Therefore, Weeda and Dart (2012) reported the presence of microsclerotia in *C. pseudonaviculata* for the first time. This observation had serious implications for management and control. (Weeda and Dart 2012).

Multiple studies have been performed evaluating survival attributes of *C. pseudonaviculata.* Shishkoff (2016) discovered that exposure to 10% bleach (0.6% sodium hypochlorite) for 15 min had little impact on germination of *C. pseudonaviculata* microsclerotia. Sixty minutes of exposure were required to reduce germination levels to less than 20%. However, 5 min of exposure to 70% ethanol was enough to completely inhibit microsclerotia germination. Evaluations of conidia and microsclerotia survival and infectivity were performed by Dart et al. (2015). Using boxwood leaf discs, they demonstrated microsclerotia survival at 40 weeks post inoculation, and conidial survival out to 3 weeks, as well as infectivity as levels as low as approximately 25 microsclerotia per gram of soil (Dart et al. 2015). Also, based on leaf evaluations, Dart et al. (2015) estimate that one boxwood leaf could contain upwards of 3600 microsclerotia. Shishkoff and Camp (2016) took microsclerotia survival a step further, evaluating survival at psychrophilic and mesophilic temperatures, specifically-10, 0, 10, 20 and 30 °C. Both lab-grown (colonized cellophane) and natural (within infected plant tissue) microsclerotia were utilized. Samples were incubated in sand at two moisture levels: 36% and 5% (v:v). Mortality of microsclerotia was observed at the more extreme temperatures (up to 5 months at 30 °C and 7 months at -10 °C for leaf tissue; up to 2 months at 30 °C and 7 months at -10 °C for culture-derived microsclerotia). However, microsclerotia in other treatments survived at least 30 months, suggesting that the pathogen might be able to survive extended periods in infested plant material at less extreme temperatures.

Composting is a complex microbiological process, involving the transformation of organic compounds to a more stable humus-like product, and CO_2 that generates heat (Agnew and Leonard 2003; López-González et al. 2015; Tuomela et al. 2000). Composting is

usually divided into three stages: (i) the mesophilic (temperatures below 40 °C); (ii) thermophilic (temperatures usually between 40-70 °C); and (iii) the cooling/maturation stage, high temperature compost cooling to ambient (Agnew and Leonard 2003; Li et al. 2013; Tuomela et al. 2000). Liang et al. (2003) studying the effects of temperature on microbial populations determined that temperatures < 20 °C and > 60 °C slowed the composting process, and temperatures > 83 °C severely impacted the microbial population. Overall, due to the complex nature of composting microbiology, many of these processes are still not fully understood, requiring more research (Kvok et al. 2012).

Composting can serve as a viable way to eradicate plant pathogens (Bollen et al. 1989; Downer et al. 2008; Fayolle et al. 2006; Hoitink et al. 1976; Lodha et al. 2002; Noble et al. 2009; Noble and Roberts 2004; Ryckeboer et al. 2002; Swain et al. 2006; Wichuk et al. 2011). Compost temperature is considered to be the most important aspect influencing pathogen eradication (Bollen et al. 1989; Hoitink et al. 1976; Lodha et al. 2002; Noble et al. 2009; Noble and Roberts 2004, Ryckeboer et al. 2002; Swain et al. 2006; Wichuk et al. 2011).

Downer et al. (2008) evaluated the survival of the fungal plant pathogens *Armillaria mellea, Phytophthora cinnamomi, Sclerotinia sclerotiorum,* and the nematode pathogen *Tylenchulus semipemetrans* for 8 weeks in static piles of green waste. Static composting differs from actively turned composting in that it is not managed by scheduled turning, therefore leading to either lower temperatures or temperatures similar to turned compost that are typically not evenly distributed within the pile. All the pathogens were killed during the course of the experiments except for *S. sclerotiorum,* a sclerotium producer, which survived the entire 8 weeks of the trial. The authors recommend turning such waste piles to eliminate

S. sclerotiorum, which may help eradication of this and other sclerotium forming pathogens. More evidence for heat playing an important factor in pathogen eradication in compost was presented by Bollen et al. (1989), who used an innovative experimental design to decouple the thermophilic and maturation phases of compost adding the pathogen to compost at different times. Pathogens which were introduced during the thermophilic period perished, while those that were introduced later and experienced only the maturation phase survived. Based on the results of Bollen et al., avoiding the period of exposure to heat permitted continued pathogen survival.

Ammonia production during composting plays an important role in pathogen eradication. Therefore, it is important to accurately quantify ammonia produced by the compost. Previous methods have utilized a boric acid titration using stoichiometry to determine ammonia (Elwell et al. 1994; Grewal et al. 2006; Ndegwa et al. 2009; Tubail et al. 2008). As ammonia in the exhaust bubbles through the boric acid, ammonium and $H_2BO_3^-$ is formed. Addition of a colorimetric pH indicator allows the solution to be titrated back to the original color shade. Using stoichiometry, the mass of ammonia in the flask can be calculated based on the amount of acid, typically 0.7 M HCl is used, needed to fully titrate the solution.

Other studies evaluating the pathogens *Phytophthora* spp. (Hoitink et al. 1976; Swain et al. 2006), *Rhizoctonia* spp. (Hoitink et al. 1976), *Botrytis* spp. (Hoitink et al. 1976), *Erwinia* spp. (Hoitink et al. 1976), *Plasmodiophora* spp. (Fayolle et al. 2006), and *Macrophomina* spp. (Lodha et al. 2002; Rycokeboer et al. 2002) showed that compost was effective in either eradicating or reducing these pathogens.

The objective of this study was to evaluate the survival of the new and emerging pathogen *C. pseudonaviculata* under various combinations of composting time and

temperature. As a microsclerotium producer, *C. pseudonaviculata* has the potential to survive extended periods of time under adverse environmental conditions. Although multiple factors can play a role in eradication of pathogens in compost, this research focused on the influence of time and temperature combinations in reducing or eliminating *C. pseudonaviculata* by composting lab grown microsclerotia. A secondary objective was to create a composting bioreactor system, as well as to set a standardized protocol for evaluation of future compost/plant pathogen studies.

Materials and Methods

Bioreactor construction. Composting under field conditions does not allow for accurate and precise control over composting conditions. Therefore, a composting bioreactor system was constructed for this research (Fig. 2-2). In addition, due to the emerging nature and regulatory constraints of working with this pathogen, it was necessary to work in a contained system to prevent pathogen escape. All described work was performed in accordance to APHIS regulations (permit number: P526P-17-02656). The bioreactor consisted of a section of 6-in diameter PVC pipe with a flat cap on the bottom, and a removable rubber cap on the top. Caps were fitted with air-tight couplings to allow insertion of a temperature probe in each reactor. Connection fittings for ¼ in tubing were attached on the bottom and top side of the bioreactor (2.5 cm from the bottom, 6.35 cm from the top) to allow for air inlet and exhaust. To maintain temperature, three bioreactors were housed in one high temperature incubator, for a total of nine reactors in three incubators (Binder Inc., Bohemia, NY). To prevent compost desiccation, inlet air was bubbled though distilled water before entry into the vessel to humidify the air. A system of flow values (Omega Eng. Inc,

Norwalk, CT) allowed for precise control of air flow rate. Outflow air was bubbled through 100 ml of 0.67 M boric acid flasks maintained at 4 °C to serve as an ammonia trap and condensation point (Elwell et al., 1994). After the ammonia traps, exhaust air entered carbon dioxide and oxygen detectors. To allow for optimal air flow through the compost, a stainless-steel mesh was used as a floor and placed in every reactor 3.8 cm from the bottom, placing it above the air inlet point. Various combinations of temperature and aeration were achieved by adjusting both incubator temperature and air flow rates. Compost temperature was measured and recorded in real time using Mushroom Master software

Ammonia determination. An experiment was designed to produce a regression model and evaluate if it could be used to replace the previously described titration. The nine boric acid flasks were pulled every day over a 6-day period, their pH recorded, then titrated to the original pH of the boric acid. Fifty-two data pairs were collected for the analyses, but several pairs were discarded due to titration errors. Regression analyses were performed in R using the RStudio interface with command lm(). Several regressions were performed, a regression with untransformed data (Ammonia ~ pH), a polynomial-natural log regression (ln(Ammonia) ~ pH + pH^2), and a polynomial-log base 10 regression (log(Ammonia) ~ pH + pH^2). R^2 and residuals were compared amongst the models to select the best model. The units determined using this assay were in mass. To convert to ppm ammonia the following equation was used, where m is the mass of ammonia (g), R is the ideal gas constant 0.08206 $\frac{L*atm}{mol*K}$, T is the temperature (K), M is the molecular mass of ammonia (g), P the pressure (atm), and V_s the total volume of air passing through the flask (m^3).

$$ppm = \frac{1000mRT}{MPV_S}$$

Microsclerotia production. Production of microsclerotia was performed based on methods described by Shishkoff (2016), in which cellophane squares (Sigma Aldrich LLC.) were autoclaved and aseptically placed on glucose yeast extract tyrosine (GYET) agar (15g glucose, 10g agar, 100mg yeast extract, 40mg tyrosine, 500 mL water). A plug of colonized agar was placed on the middle of the square and allowed to grow for 10 weeks in the dark at 20 °C before use.

The isolate of *C. pseudonaviculata* utilized, PA1, was isolated from an infected *Buxus sempervirens* 'Suffruticosa' plant collected from a Philadelphia County nursery on 8 July 2015. The newly isolated microsclerotia were treated with sodium hypochlorite (0.08%), followed by single spore isolation. Conidia morphology matched that of the *Calonectria* genus, as well as confirmed with a 100% match of the ITS region of previously deposited sequences on NCBI Blast (KF815098.1). After confirmation, the isolated was labeled PA1 and used in all subsequent experiments.

Compost heat experiments. Composting experiments were performed in the bioreactors described previously. Compost formulation was based on typical compost C:N ratio of 30:1. Fresh compost was created at the beginning of each experiment, rather than importing it from municipal operations. Although municipal compost may be vulnerable for *C. pseudonaviculata* contamination from homeowners depositing infected boxwoods, its use has drawbacks. Using municipal compost adds another layer of complexity to an already variable process, our creation of compost using the same formulation in every experiment reduced this variability. Therefore, compost was created using wheat straw (1750 g), dried distiller's grain (440 g), and dried, pelletized poultry manure (440 g). Water was added to obtain 60-70% moisture. Ingredients were mixed, and 700 g of the mixture was added to

each reactor vessel. Airflow was set to 100 mL/min to simulate aerobic composting, based on results of preliminary airflow experiments (data not shown).

Composting followed a 16-day profile (Figure 2-3), with 13 days of composting prior to inoculation, and 3 subsequent days of composting post inoculation. For the first week of composting the temperature was set to 60 °C, and turned twice, days 3 and 7. Days 7 through 10 were held at 55 °C, then turned on day 10. Following the turn on day 10, each incubator was set to the goal temperature for the assay (40, 50, or 60 °C), and inoculated on day 13. One reactor was pulled per temperature per day for survival analysis (24, 48, and 72 h). Stainless steel mesh pouches (38 micron) were used to inoculate the compost with the microsclerotia. Each pouch received one square of colonized cellophane, with each reactor receiving 5 pouches. The temperature was recorded in each incubator every 6 hours. Control pouches were held at 20 °C, not in compost, and sampled at the same time points as the compost treatment. On day 13, before inoculation, 50 g of compost was removed from each bioreactor, consolidated by temperature treatment, and taken to the Ag Analytical service at The Pennsylvania State University for analysis.

All squares were removed from the pouches to undergo surface sterilization. Squares were rocked in 50 mL conical tubes for 5 min in 0.08% sodium hypochlorite, followed by 30 min rocking in sterile Milli-Q water in fresh 50 mL conical tubes. After the final wash, each square was transferred individually to GYET plates and evaluated 20 days later. Each square was rated plus or minus for *C. pseudonaviculata* survival using three indicators: spore production, pigment production, and/or microsclerotia production. *C. pseudonaviculata* produces a distinctive chestnut coloration on GYET agar, a reliable indicator.

Non-compost heat experiments. To access the impact of temperature alone on *C. pseudonaviculata*, a non-compost experiment was designed to complement the composting experiment. In the composting experiments, heat was provided via the compost (stabilized and controlled with incubators), whereas in the non-compost experiment the heat was produced by and within incubators (Quincy Lab Inc. and VWR International). We also could evaluate if there were any interactions between temperature and the bleach wash utilized in the composting experiments.

The colonized cellophane was removed from the media surface of 10-week old PA1 cultures and cut into 0.75 cm^2 squares. However, unlike in the composting experiments, each square was placed in a Petri dish of GYET agar. To evaluate the impacts of time and temperature on microsclerotia survival, four temperature treatments were established 20 °C (positive control), 40 °C, 50 °C, and 60 °C, to be sampled at 24, 48, and 72 h. Samples taken at each time point were further divided into two subgroups, wash and no-wash. Samples in the no-wash group were directly placed into the control incubator, whereas the wash group was removed from respective petri plates and washed in the method utilized in the compost experiments. After the wash procedure, these plates were placed on fresh GYET agar and placed in the control incubator. Plates were rated +/- for survival after 20 days based on attributes as described in the following composting section. Results from all three treatments were analyzed in RStudio (Ver. 1.0.153) using a logistic regression followed by an ANOVA Chi Square of the model. There were 5 replicate squares per temperature/time/wash, and the entire experiment was repeated 3 times.

Results

Model validation. The results from the untransformed linear regression were significant, but the R^2 and adjusted R^2 values were not large enough to warrant use as a model (0.769 and 0.7644 respectively). However, both regression models utilizing a polynomial and ln() and log() transformation had similar R^2 and adjusted R^2 values, 0.9902 and 0.9898 respectively, as well as similar residual plots. With no distinguishing separation between the two models, the log() based model was selected arbitrarily, in which the equation was $\log(NH_3) = -6.81170 + 1.99691pH - 0.10297pH^2$. When rewritten as $NH_3 = 10\text{^}(-6.81170 + 1.99691pH - 0.10297pH^2)$, the amount of ammonia released from composting could be determined by inserting the recorded pH value for each sample.

Compost heat survival. Continuous temperature confirmed all bioreactors were in an acceptable range around the set target temperature (Fig. 2-3), and there was limited variability among bioreactors within the same incubator. As anticipated, temperatures quickly returned to target levels after pathogen inoculation, and there was little lag time when the incubators were not at the desired temperature. Ammonia levels did vary between temperature treatments, with hotter treatments producing higher levels of ammonia (Fig. 2-4). These latter data could not be analyzed statistically since the destructive sampling reduced the sample size to one at the last time point. A marked difference was observed between run 1 and runs 2 and 3 (Fig 4. b, c), whereas the compost maintained at 40 °C in runs 2 and 3 had approximately half the ammonia produced in the first run. These values became more consistent for the 48 and 72 h time points. The differences observed at 50 °C were more substantial. At 24 hours, run 1 had an average ammonia production of 61.2 ppm, compared to

subsequent runs where the ammonia production was only13.7 ppm, and as low as 3.8 ppm. Physical properties were similar among all compost temperature treatments (Table 2-1).

Survival of *C. pseudonaviculata* microsclerotia was extremely limited in compost (Table 2-2). Of the three experiments performed, survival was only observed once at the 40 °C 24 h time point, with surviving microsclerotia on four of five squares. Other than that, survival was not noted any point past 48 h for 40 °C, or at any time point for 50 and 60 °C. Control treatments demonstrated 100% germination for all three experiments, at all three time points indicating that all samples were viable. Considering the variability often observed in compost, the fact that overall consistent results were observed in this study, indicates the reliability of the survival findings. Despite slight variations in ammonia production, compost at temperatures as low as 40 °C were still sufficient to cause complete eradication of microsclerotia after 48 h. Even at 24 h, there were substantial decreases in survival at 40 °C. Due to the large number of zeros in the data, no statistics could be performed.

Non-Compost heat survival. Survival was consistent across all three non-compost experiments (Table 2-3). Survival was not observed in any of the three experiments at temperatures ≥ 50 °C for any time interval, compared with the 20 °C and 40 °C heat treatments, in which survival was observed at every time point. Only at 72 hours in the 40 °C treatment was reduced survival observed. For the water-washed controls, only one square out of the 15 combined from all three experiments did not germinate, compared to 5 non-germinating sample from the 14 squares subjected to bleach. One square was compromised during processing and was not included in the results.

Results from the ANOVA indicated that both temperature and time significantly influenced survival ($p = 0.003$ and 0.0001 respectively), and that treatment was marginally

insignificant at 0.05 alpha (p=0.074). This latter result might indicate that another extraneous factor is involved. However, results from all other controls were as expected. While statistically insignificant, it appears that after 72 hours (and further) an interaction between bleach and temperature may start to influence survival results.

Discussion

Composting at temperatures ≥ 50 °C were adequate to cause pathogen inactivation. The dichotomy of results from the compost and temperature exposure experiments were interesting, particularly in the 40 °C temperature treatment. At 50 and 60 °C heat alone was sufficient to eradicate the microsclerotia. Similar results were obtained in the compost experiments. Our experiments confirmed that the compost did not protect the microsclerotia from thermal eradication. However, if temperature was the sole reason for microsclerotia eradication in compost, then survival would have been expected at 40 °C in the absence of compost. Almost the exact opposite was observed. Except for four of the 15 samples at 24 hours, all inoculated microsclerotia in compost died, with the expectation of complete survival through 72 hours based on temperature alone. This provides strong evidence that temperature, while important, is not the sole contributor to eradication in compost, at least at 40 °C. Other factors in the compost contributed to eradication of microsclerotia.

This work also corroborates the findings of Shishkoff (2016) indicating that low levels of bleach do not negatively impact viability of *Calonectria* microsclerotia. Thus, the described surface-sterilization procedure is a viable method for sanitation of *C. pseudonaviculata* microsclerotia without causing significant mortality at ambient temperatures. Without this step, other fungi could colonize the plate preventing detection.

Based on both the model statistics and graphical comparison, using this equation accurately determined ammonia values for this experiment, as well as subsequent composting experiments using this system. Ammonia detectors are expensive, and usually not optimized for low flow rates commonly used in these types of composting bioreactors, therefore boric acid remains one of the simplest and most consistent ammonia measuring tools available. However, the titration process in this method was a labor-intensive titration process, and the color perception was subjective. Creating a pH-ammonia correlation for a specific reactor system greatly reduced labor, and increased accuracy and precision, since human perception was no longer a determining factor. Composting is a variable process, as demonstrated in the varied amounts of ammonia evolved. While composting within a bioreactor system helps reduce variability, it is impossible to control all factors. Ammonia levels themselves can be extremely variable among samples, as Michel et al. (2004) reported from dairy manure composting after amendments. They observed variable ammonia levels of 7329 ± 4119 ppm, much higher than those observed in this study. The similarity in compost physical properties demonstrate the ability of the bioreactor system to create similar composting conditions among both the separate bioreactors and incubators. This is important because having consistent properties, other than those under study, is important for drawing valid conclusions regarding the results, whether it be survival, or unknown factors. This also addresses one of the main issues stated by Nobel and Roberts (2004), the lack of control of the system allowing for precise determination of the actual point of pathogen eradication. While we were not able to narrow the range down beyond the 24 h periods used, subsequent work with this system, or similar systems, can further narrow down the eradication temperature range for *C. pseudonaviculata*. Of specific interest would be identifying the

lower range of survival, perhaps in the range of 35 °C. By identifying the lower threshold needed for eradication, compost managers could make a better risk assessment for their product.

Other future directions could evaluate if any differences would be seen if boxwood material was used in the compost mix, as well as evaluate infested plant materials. The scope of this work involved the use of lab grown microsclerotia on cellophane sheets, so the next logical step would be to assess infected plant material. Shishkoff and Camp (2016) made comparisons among cellophane sheets, infected leaves, and infected twigs. While overall there did not seem to be large discrepancies, there was more fluctuation in the cellophane sheet survival compared to both plant materials. Now that a streamlined methodology has be created, these studies can be performed quickly and efficiently.

The objectives of this paper were twofold: (i) first to evaluate the survival of *C. pseudonaviculata* microsclerotia in compost, and (ii) to evaluate the bioreactor system as a method for accurate and precise evaluation of pathogen survival. Noble and Roberts (2004) reviewed a multitude of compost eradication studies. However, few if any of these studies utilized a composting bioreactor system. Large scale windrows and compost piles, while realistic and important, may not be able to clarify the precise time and temperature eradication points that can be discovered utilizing a bioreactor system. Satisfactory performance of our bioreactor system allowed for accurate and precise evaluation of *C. pseudonaviculata* microsclerotia in a realistic setting. Based on our results, survival would not be expected in well managed compost systems, either windrow or static, provided 50 °C temperatures are sustained for at least 24 h. The dichotomy in the 40 °C survival data presents an interesting result that deserves further inquiry. Ammonia is an often-cited

contributor to pathogen eradication in compost (Noble and Roberts 2004). Therefore, ammonia could be an important factor, extraneous to heat, leading to microsclerotia eradication in compost. This is important since heat alone at 40 °C was not adequate to ensure eradication of *C. pseudonaviculata* microsclerotia after 24 h. with the goal of composting to include pathogen eradication, a minimum temperature setpoint of 50 °C should be used for *C. pseudonaviculata*.

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Table 2-1. Compost physical properties after 13 days

	pH	Moisture[1]	Org. Matter[1,2]	Total N[1,2]	Carbon[1,2]	C:N
Initial Mix[3]	-	75.5 ± 0.794	-	1.51	45.6	29.8
40 °C[4]	7.70 ± 0.346	77.0 ± 2.08	88.4 ± 1.21	2.30 ± 0.436	44.4 ± 4.35	19.3 ± 2.0
50 °C[4]	7.67 ± 0.513	76.2 ± 2.34	88.4 ± 0.853	2.60 ± 0.361	43.9 ± 1.58	17.0 ± 1.9
60 °C[4]	7.93 ± 0.0578	76.1 ± 2.50	88.8 ± 0.433	2.73 ± 0.709	47.2 ± 6.85	17.4 ± 2.0

1 – Units are percent (%)

2 – On a dry weight basis

3. Represents sampling of the initial mix of compost before being place in bioreactors. Moisture calculated using U.S. Compost Council test methods, total N, C, and C:N ratio were calculated mathematically based on the raw material proportions.

4. Values were calculated by samples sent for analysis at the Pennsylvania State University Ag Analytical service.

Table 2-2. Survival of *C. pseudonaviculata* microsclerotia in compost at 40, 50, and 60 °C, data combined from three replicate experiments.

	24 h[1]	48 h[1]	72 h[1]
Control[2]	100% ± 0	100% ± 0	100% ± 0
40 °C[3]	26.7% ± 42.2	0% ± 0	0% ± 0
50 °C[3]	0% ± 0	0% ± 0	0% ± 0
60 °C[3]	0% ± 0	0% ± 0	0% ± 0

1. Mean percent survival ± standard deviation for the three compost survival experiments
2. Control samples were housed in stainless steel pouches similar to compost samples but were held at 20 °C in an incubator.
3. 40, 50, and 60 °C samples were placed in stainless steel pouches, then inserted into the bioreactors in various locations in the compost.

Table 2-3. Survival of *C. pseudonaviculata* microsclerotia at 20 °C, 40 °C, 50 °C, and 60 °C in incubators, data combined from 3 replicate experiments.

	24 h		48 h		72 h	
	Wash[1,2]	No Wash[1,3]	Wash[1,2]	No Wash[1,3]	Wash[1,2]	No Wash[1,3]
20 °C[4]	100% ± 0	100% ± 0	100% ± 0	100% ± 0	100% ± 0	100% ± 0
40 °C[5]	100% ± 0	100% ± 0	100% ± 0	100% ± 0	60% ± 52.9	93.3% ± 11.5
50 °C[5]	0% ± 0	0% ± 0	0% ± 0	0% ± 0	0% ± 0	0% ± 0
60 °C[5]	0% ± 0	0% ± 0	0% ± 0	0% ± 0	0% ± 0	0% ± 0

[1] Mean ± standard deviation for the 3 replicate experiments
[2] The wash treatment included washing the cellophane squares with 0.08% sodium hypochlorite for 3 min, followed by water for 30 minutes
[3] The no wash treatment cellophane squares were transferred directly to a fresh Petri plate
[4] 20 °C samples were held in the same incubator that processed samples from other temperatures; these samples represented a positive control.
[5] Temperature ($p=0.003$) and time ($p<0.001$), but not treatment ($p=0.074$) were significant at this temperature

Figure Legends

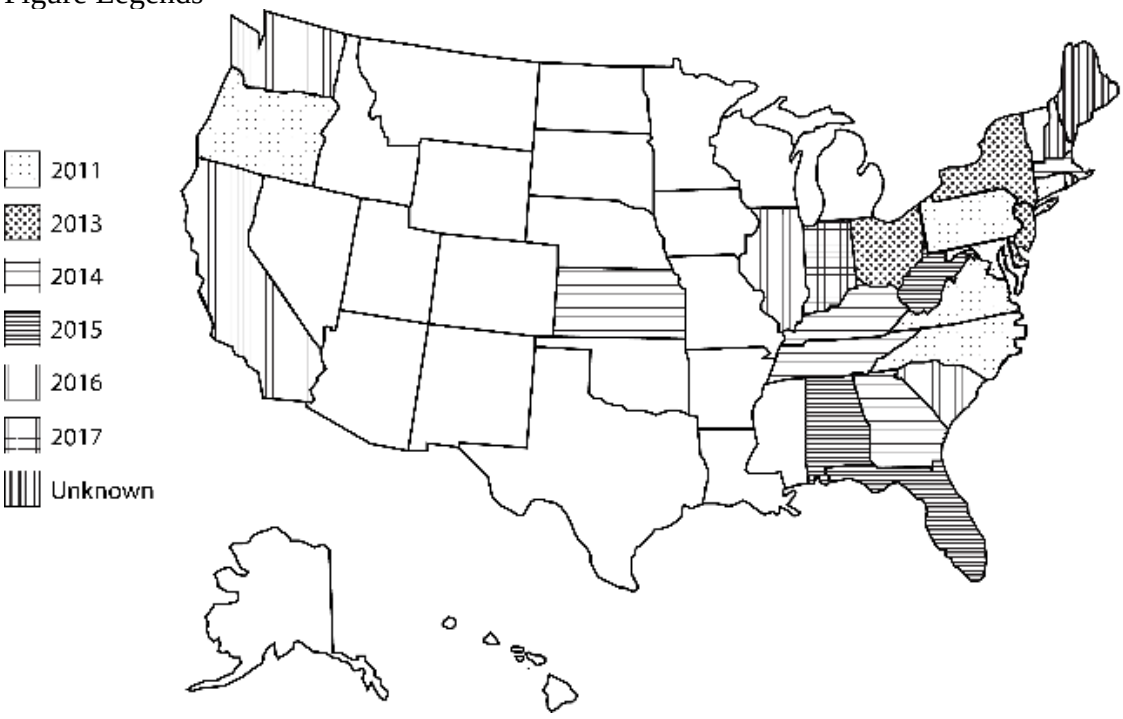

Fig. 2-1

States that have reported boxwood blight in the United States as of January 2018.

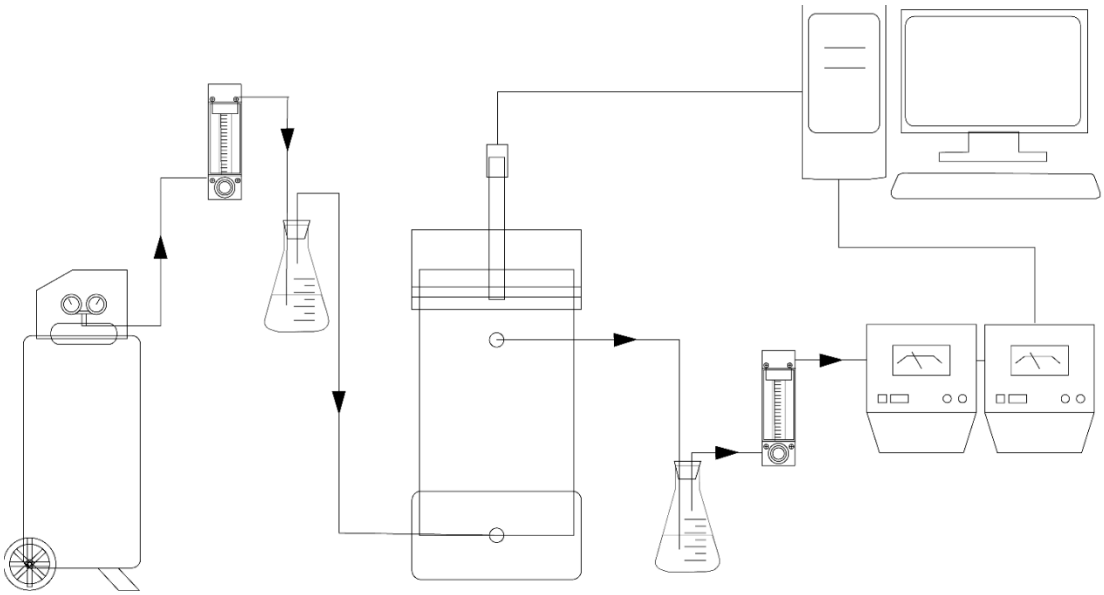

Fig. 2-2
Composting bioreactor schematic. Air flows from an air compressor through a flow restrictor and into humidification flasks. Humidified air enters the reactor vessel from the bottom and out through the top. A temperature probe sends data to a computer. Reactor effluent enters a 0.67 M boric acid trap cooled to 4 °C. Air then flows through flow meter to confirm the inflow and outflow values are the same, and finally enters a carbon dioxide and oxygen detector which relay data to a computer.

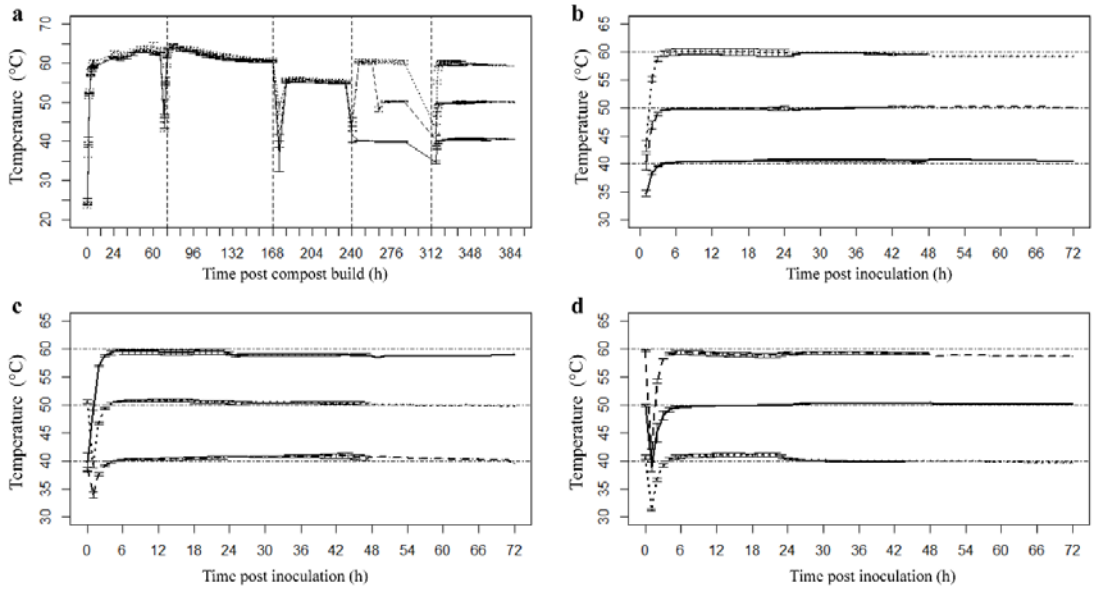

Fig. 2-3

(a) Mean (+/- standard deviation) temperature for a complete composting run. Vertical dotted lines represent points when the compost was turned. The vertical line at 312 h represents both a compost turning and pathogen inoculation. (b)-(d) Temperature data specific to the 72-h time period where survival was accessed. Due to destructive sampling, the sample size decreased by 1 every 24-hour sample time (24, 48, 72 h; n = 3, n = 2, n = 1 respectively). Horizontal dotted lines represent the temperature set point. Error bars represent mean ± standard deviation of the reactors in each of the 3 incubators.

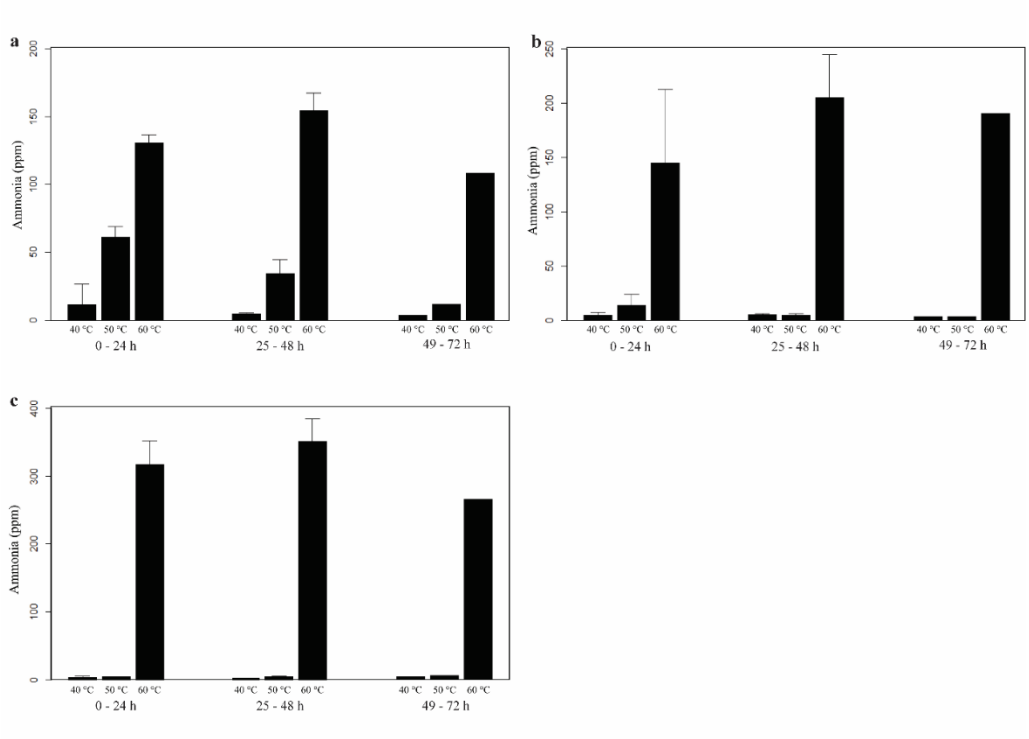

Fig. 2-4

Compost ammonia mean (+/- standard deviation) levels determined in the three replicate composting experiments. (a) Replicate 1. (b) Replicate 2. (c) Replicate 2. Due to destructive sampling, n decreases from 3 at 24 hours sequentially to 1 at 72 hours. Units of mg of ammonia represent the amount of ammonia determined to be in one boric acid trap flask.

Chapter 3. Evaluation of ammonia impacts on *Calonectria pseudonaviculata* and *Calonectria henricotiae,* causal agents of boxwood blight.

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Abstract

Boxwood blight is a devastating disease found in at least 26 states in the US. Previous work has reported that the microsclerotia produced by *Calonectria pseudonaviculata* do not survive composting regimes comparable to well managed municipal operations. Perhaps partly due to ammonia, a common fungitoxic gas in composting operations. The objectives of this paper were twofold: (i) to evaluate the ammonia-sensitivity differences of *C. pseudonaviculata* vs. *C. henricotiae,* a species not known currently in the U.S., and (ii) to determine if gaseous ammonia at temperatures above ambient impacts *C. pseudonaviculata* survival. There was little difference in the ammonia-sensitivity between the two species. Survival was less in treatments of gaseous ammonia and heat than with heat alone. Both species demonstrated similar sensitivity to ammonia levels indicating that survival of *C. henricotiae* in compost would not be expected, assuming the species share similar thermotolerances.

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Introduction

Boxwood blight, caused by *Calonectria pseudonaviculata* (U.S. and Europe) and *Calonectria henricotiae* (Europe) is a devastating fungal disease impacting the boxwood industry. *C. pseudonaviculata* has been detected in 26 states across the U.S. Boxwood blight has also been identified in multiple countries in Europe, as well as several provinces in Canada (Akilli et al. 2012; Cech et al. 2013; Crepel and Inghelbrecht 2003; Elmhirst et al. 2013; Gorgiladze et al. 2011; Ivors et al. 2012; Mirabolrathy et al. 2013; Saurat et al. 2012; and Varela et al. 2009). Boxwood blight was originally reported to be caused by only one pathogen (*C. pseudonaviculata*), but in 2015 Gehesquière et al. identified that "clade G2" was in fact another species capable of causing disease. The authors named the newly elucidated species *C. henricotiae* in honor of Beatrice Henricot, one of the original researchers working on this disease. *C. henricotiae* is currently only found in Europe. However, this second species demonstrated resistance to fungicides, causing concern about control if this species was introduced to the United States (Gehesquière et al. 2015).

In 2012, Weeda and Dart worked to determine if microsclerotia were present within infected plant tissue, as was common with other *Calonectria* species (Crous 2002). Utilizing leaf-clearing techniques, they identified numerous microsclerotia in leaf tissue infected with *C. pseudonaviculata*, but not in tissue infected with two previously known fungal pathogens of boxwoods, *Volutella buxi* and *Macrophoma candollei*. With this confirmation of microsclerotia production in boxwoods infected with blight, others began to assess epidemiological implications. Shishkoff (2016) reported that microsclerotia of both species exhibited low susceptibility to 0.6% sodium hypochlorite, but that exposure

to 70% ethanol led to rapid death for both. This information was crucial to proposed disease management strategies. Survival at psychrophilic and mesophilic temperatures was evaluated by Shishkoff and Camp (2016), who demonstrated that microsclerotia could survive at least 30 months at temperatures between 0 and 20 °C. Henricot and Culham (2002) reported survival of the pathogen for 5 years within infected plant material, possibly as mycelium due to the lack of observed microsclerotia. However, Weeda and Dart's (2012) work suggests that the pathogen was likely surviving as microsclerotia rather than mycelium.

Possible extended survival of *Calonectria* microsclerotia caused alarm in the compost industry, since microsclerotia could be accidentally disseminated if infested compost was moved or transported to a new location. This question led to initial studies where *C. pseudonaviculata* microsclerotia survival was evaluated in compost maintained at temperatures of 40, 50, and 60 °C, for times of 24, 48, and 72 h as well as 40 °C for 24, 48, and 78 h in incubators. A discrepancy in the survival results regarding 40 °C was noted, whereas survival was observed through 72 h when the microsclerotia were subjected to head alone in the incubators, survival in compost was extremely limited, with only minimal survival observed at 24 h (Chapter 2).

Based on these preliminary results, the objective of the following experiments was to further evaluate possible relationships between ammonia and pathogen eradication by composting. Previous work has demonstrated the toxic effects of ammonia on fungi. Tenuta and Lazarovits (2002) evaluated the impact of ammonia on survival of *Verticillium dahliae* microsclerotia, to further characterize how nitrogenous waste soil amendments lead to pathogen eradication. They noted significant impacts of increasing

ammonia concentration on *Verticillium* microsclerotia subjected to both Petri plate agar concentrations and gaseous ammonia in the headspace of Mason jars. Candole and Rothrock (1997) also challenged pathogens with ammonia, demonstrating complete mortality of *Thielaviopsis basicola* chlamydospores in a Petri plate assay containing 0.4 ppm ammonia in the headspace.

Materials and Methods

Ammonia sensitivity. To evaluate the ammonia sensitivity of both *C. pseudonaviculata* and *C. henricotiae*, an ammonia concentration gradient was created using various concentrations of ammonium chloride at various pH levels. Ammonia sensitivity was measured as a decrease in growth rate of the fungus in response to the concentration of ammonia (mm growth/mM ammonia). A sterile stock solution of ammonium chloride was added to flasks of glucose yeast extract agar (GYET) agar to obtain final concentrations of 0, 25, 50, 75, and 100 mM. To obtain the desired pH, MES buffer, titrated to the desired pH, was added to each flask to a final concentration of 55 mM. Combined, this resulted in 25 unique ammonia concentrations. The ammonia level was determined using the Henderson-Hasslebalch equation (Eq. 1) (Tenuta and Lazarovits 2002). After GYET agar solidification, one plate was pulled per treatment and the pH was measured using a Vernier pH probe. The procedure of Emerson et al. (1975) was used to determine the pKa: pKa = 0.09018 + 2729.92/T, T is temperature in Kelvin.

$$mM(NH3) = mM(NH_3 + NH_4^+) - \frac{mM(NH_3 + NH_4^{+1})}{\left(\frac{10^{(-pKa)}}{10^{(-pH)}} + 1\right)} \quad \textit{Equation 1}$$

Since *C. henricotiae* has not yet been reported in the United States, all research with this pathogen was conducted in containment at the USDA-ARS Foreign Disease-

Weed Science (FDWS) facility housed at Ft. Detrick (Frederick, Maryland). Six isolates, three of each species, were used in the experiment (Table 3-1). Agar plugs were removed from the edge of 3-week-old plates of each culture, and used to inoculate treatment plates, with three replicates/isolate/treatment. Plates were incubated in darkness, at room temperature, and growth measured at 5, 10, and 15 days post-inoculation. Growth was measured radially in two random perpendicular directions for each plate.

The raw data were plotted in R as a scatter plot (growth ~ ammonia concentration, by isolate) to evaluate any trend in the data. Data scatter plots were non-linear and exhibited a shape like that observed in radioactive decay. Therefore, an exponential decay model was fitted to the data. Data were separated into two sections based on the curve of the model line, similar to de Castro Montini et al. (2006). Section 1 encompassed the rapid decline observed at the beginning of the graph, and section 2 included the slow decay seen in the tailing end of the model line. As previously stated, ammonia sensitivity was defined as the decrease in growth per unit ammonia, i.e. the rate. Rate can be determined for any point in the model by taking the derivative of the model equation. Therefore, the equation for the first derivative was obtained for each data set and solved for the halfway point of each section. These values, obtained on an isolate basis, were combined into two datasets (n = 3 each) by species, and compared using a Mann-Whitney U Test in R. By first obtaining the derivatives, followed by the u-test, we were able to directly compare the mean ammonia sensitivity as determined for each species based on the subset of isolates. This procedure was performed for each time point. In addition to assessing possible differences between species, a non-parametric one-way analysis

med1way(), package: WRS2, was performed in R to determine if there were significant differences in mean sensitivities for among species over the three measured time points.

Atmospheric ammonia. Three replicate experiments were performed to determine if gaseous ammonia interacts with temperature to decrease *C. pseudonaviculata* survival below values observed with temperature alone. Ten-week-old cultures of isolate PA1 were grown on cellophane using procedures from Chapter 2 Approximately 0.75 cm^2 pieces of colonized cellophane were placed on 60 x 15 mm Petri dishes and floated on the surface of the buffer in a treatment jar. This experiment included four exposures to four concentrations: 0, 100, 300 mM NH_4Cl, and a water control. Atmospheric ammonia was created using methods of Tenuta and Lazarovits (2002). Sodium carbonate and sodium bicarbonate were combined in a 6:4 mixture (1M each) to create a pH 10 solution. The 0 mM solution contained only buffer and served as a control for any inhibiting compounds contained in, or by, the buffer. Water served as a positive control to ensure there were no other unintended stressors. To subject the microsclerotia to ammonia, the lid of each Petri dish was removed, and the Petri plate bottom containing GYET agar and colonized cellophane was floated on the solution in a 230 mL jar. This method prevented desiccation, which preliminary experiments indicated would kill microsclerotia. Five replicate plate/jar systems were used for each treatment for each time point. Samples were pulled at 24, 48, and 72 h similar to the compost and heat experiments reported in Chapter 2. All incubators were set to 40 °C based on previous survival data observed at this temperature (Chapter 2). At the assigned time point, samples were removed and subjected to a post-wash (Chapter 2), followed by plating on GYET plates to be assessed for survival after 20 days. In addition to the 100

and 300 mM concentrations, a series of lower concentrations, 1, 5, and 20 mM NH_4Cl were evaluated for survival at 24 h, under similar experimental conditions. Ammonia ppm values of these lower concentrations were estimated using Hydrion® ammonia test strips according to manufacturer instructions (Table 3-3). Results were analyzed using a logistic regression followed by an ANOVA Chi-Squared on the model.

Results

Ammonia sensitivity. Although the media was buffered with MES, the actual pH of the media in each treatment varied based on the amount of ammonium chloride added. This difference became more pronounced as the target pH level increased. There was a 0.6 pH discrepancy between the target and actual pH for the pH 8 treatment with no added ammonium chloride, which indicated that factors outside of the ammonium chloride likely influenced the pH, such as the acidic nature of the GYET media. Similar trends were observed in the second experiment as well. Although the actual pH values differed from the target values, due to the experiment being evaluated using regression, the difference had no adverse impact on the experiment. It was crucial, however, to measure the actual pH of each treatment and use these values to calculate ammonia concentrations.

By combining the various pH and ammonium chloride concentrations, a gradient of ammonia concentrations was created, on which growth could be compared. In the first replicate, ammonia ranged from 0.00804 mM to 0.590 mM. In comparison, the second experiment had a much greater range, from 0.0101 to 1.21 mM ammonia.

The scatter plots comparing mycelial growth versus ammonia concentration all displayed curves resembling that of exponential decay. Also, the overall, R^2 values supported use of the exponential decay model. The lowest R^2 value was 0.60, and the highest 0.89. All models showed a significant correlation at the 0.05 alpha level.

Ammonia sensitivity was defined as the decrease in the rate of fungal growth related to ammonia concentration (mm growth/mM ammonia). This value is represented by the derivative of the exponential decay equation. Little significant difference in overall ammonia sensitivity was observed between the two species of *Calonectria*. In both experiments there were no significant difference between the species sensitivity for both growth curve sections (Mann-Whitney U Test; Table 3-2).

Both species displayed a significant increase in ammonia sensitivity over the sampling days for section 1 (med1way, alpha = 0.05). Conversely, all but one of the species/experiment combinations for section 2 were not significant, the exception being *C. henricotiae* in the first experiment. While not below the 0.05 alpha, the second replicate for *C. henricotiae*/section 2 was 0.088.

Atmospheric Ammonia. In the initial higher concentration of ammonia, survival was not observed in the 100 or 300 mM treatments. However, survival was noted in both the 0 mM and water control treatments, where survival rates were 93.3% ± 11.5 (mean ± standard deviation) for both after 24 h. The water treatment maintained this mean after 48 h. However, the 0 mM treatment fell to 41.7% ± 17.6 after 48 h. After 72 h mean survival was similar between the 0 mM and water treatments, 33.3% ± 23.1 and 33.3% ± 30.6 respectively.

Reducing the starting concentration of ammonium chloride led to headspace ammonia levels still conducive to survival (Table 3-4). Consistent with the higher concentration experiments, the 0 mM control showed no impact on the pathogen's survival, indicating mortality could be attributed to headspace ammonia. With a headspace ammonia measurement of 5 ppm (1 mM treatment), survival was reduced to approximately 25%. Increasing the concentration to 20 ppm (5 mM treatment) induced 50% mortality rates. At 100 ppm (20 mM treatment), survival was reduced by 90% compared to the control (Table 3-4).

While comparisons could not be conducted statistically between the higher ammonia treatments due to no survival, results from comparing the water and buffer controls indicated that while time had a significant impact on survival ($p = 2.463 \times 10^{-6}$), there was no significant influence of treatment on survival ($p=0.1444$). This indicates that there was no detrimental impact from the buffer on survival that could be mistaken for ammonia mortality. Results from the ANOVA Chi-Square of the lower concentration experiment demonstrated that increasing ammonia concentration had a negative impact on survival of the microsclerotia ($p = 8.012 \times 10^{-8}$).

Discussion

An aspect of this experiment to consider is that ammonia concentration tested are not comparable to those typically found in compost. The goal of this experiment was to challenge both *C. pseudonaviculata* and *C. henricotiae* using similar techniques to determine ammonia sensitivity differences. Since *C. henricotiae* has not been reported in the United States, it could not be utilized in the composting bioreactor system described

in Chapter 2. While *C. henricotiae* is not known in the United States, this work was important to establish a baseline comparison to *C. pseudonaviculata*. The design of the ammonia sensitivity experiment allowed us to make direct comparisons between the two species on an equal playing field. In that regards, the difference, or lack thereof, in ammonia sensitivity could be accurately assessed.

Based on our results, we were able to successfully evaluate the difference between both species, demonstrating little difference in sensitivity to ammonia. If anything, the data indicated that *C. henricotiae* became more sensitive over time. This is important, as it gives insight into the differences between the species, leading to putative management ideas. The observed increased sensitivity is similar to the findings of Shishkoff (2016) where she reported that *C. henricotiae* microsclerotia were more sensitive to various sanitizers compared to *C. pseudonaviculata.* Conversely, Gehesquière et al. (2015) reported that *C. henricotiae* was more resistant to fungicides and more thermotolerant. Combining the results from these studies is important for researchers to make a hypothesis on the possible differences in management strategies that would need to be implemented if *C. henricotiae* was ever discovered in the United States. Increased globalization vastly increases the odds of its eventual arrival to the US, making research focusing on both species very important.

The consistent exponential decay trend in mycelial growth versus ammonia resembled a Michaelis-Menten enzyme kinetic plot, if rotated 90° and mirrored, which could indicate a similar mechanism. With enzyme kinetics, the reaction velocity starts to approach and become asymptotic, due to all enzymatic active sites being utilized (Berg et al. 2012). While the exact mechanisms for ammonia toxicity are not known, it is thought

to relate to passive diffusion of ammonia through the cell membrane impacting pH gradients, cAMP production, and other physiological processes (DePasquale and Montville 1990). Eventually, addition of more ammonia does not significantly impact mycelial growth, since ammonia saturation is occurring in the cell. Therefore, increased amounts of ammonia no longer passively diffuse due to concentration gradients, or all active sites have already been "occupied" by other ammonia molecules, similar to how an enzyme active site in occupied by substrate. This would explain the rapid rate in section 1 of the function curve, compared to the slow rate in section 2. A strong, seemingly linear, downward trend was observed when plotting the calculated rates over time (Fig. 3-1a, b). This demonstrates that the sensitivity of both species was positively related to duration of ammonia. Based on these findings, and the possible implications due to the shape of the function, comparing the difference between both sections is important. That is, a faster rate (greater ammonia sensitivity) in section one indicates that smaller values of ammonia will induce more eradication, while a faster rate in section 2 indicate that it may take longer to reach ammonia saturation, therefore, adding more ammonia is still effective. Both suggestions have implications for possible pathogen eradication strategies relying on ammonia, for example a faster initial growth decrease implies that less ammonia would have to be utilized to achieve control.

When subjected to heat alone, in Chapter 2, there was 100% pathogen survival (Table 2-1), but under composting conditions there was little survival (26.7% ± 42.2). When considering the 20 ppm and 5 ppm headspace data, comparable to the mean recorded ppm from compost in Chapter 2 held at 40 °C for 24 h (6.44 ppm), an interesting story emerges. While the compost survival results were not statistically

different from 20 ppm (χ^2 test, p = 0.4486), they were significantly different compared to 5 ppm survival rates (χ^2 test, p = 0.04148). These results help support the following conclusions: (i) ammonia combined with heat increased eradication when compared to heat alone and (ii) despite this acceleration, ammonia addition still did not match the compost results in terms of pathogen eradication. Thus, while heat and ammonia are two important factors for pathogen eradication in compost, other factors may be involved that speed eradication.

In summary, the European species (*C. henricotiae*) has ammonia sensitivities that were the same, or slightly higher than the species found in the United States (*C. pseudonaviculata*). This suggests that the results obtained for gaseous ammonia toxicity for *C. pseudonaviculata* can serve as a proxy for *C. henricotiae* until further research can be conducted. When these results can be combined with work evaluating the survival of *C. henricotiae* compared to *C. pseudonaviculata* at elevated temperatures, a proxy determination can be made as to whether or not *C. henricotiae* would survive composting. If the thermotolerances of the two species are similar, and the compost reaches the conditions set forth in Chapter 2, *C. henricotiae* survival should not occur in compost.

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Table 3-1. Isolates used in the growth rate experiments

Isolate	**Species**
PA1	*C. pseudonaviculata*
CT1	*C. pseudonaviculata*
261	*C. pseudonaviculata*
G2	*C. henricotiae*
55	*C. henricotiae*
78	*C. henricotiae*

Table 3-2. Statistical comparisons between ammonia sensitivity values and experimental replications

Replicate 1						
		Section 1			Section 2	
	Cap[1,2]	*Cah*[2,3]	P-Value[4]	*Cap*[1]	*Cah*[1]	P-Value[4]
5 Days	-18.8 ± 7.16	-11.8 ± 3.12	0.2	-6.20 ± 3.53	-8.69 ± 0.98	0.4
10 Days	-27.5 ± 3.14	-27.4 ± 5.60	1.0	-12.1 ± 2.26	-16.1 ± 1.66	0.1
15 Days	-34.9 ± 2.58	-39.4 ± 6.67	0.7	-13.9 ± 4.89	-23.1 ± 2.05	0.1
Replicate 2						
		Section 1			Section 2	
	Cap[1,2]	*Cah*[2,3]	P-Value[4]	*Cap*[1,2]	*Cah*[2,3]	P-Value[4]
5 Days	-9.64 ± 1.36	-9.92 ± 1.89	1.0	-3.32 ± 0.47	-3.91 ± 1.21	0.7
10 Days	-18.6 ± 2.17	-21.9 ± 2.71	0.2	-4.87 ± 1.22	-5.78 ± 1.43	0.7
15 Days	-23.4 ± 1.61	-32.0 ± 3.91	0.1	-6.47 ± 2.12	-7.95 ± 2.01	0.2

[1] *Calonectria pseudonaviculata*
[2] Mean ± standard deviation ammonia sensitivity (mm growth/mM ammonia)
[3] *Calonectria henricotiae*
[4] From Mann-Whitney U Test

Table 3-3. Estimated ammonia concentrations (ppm) in jar headspace

NH_4Cl concentration (mM)	**Headspace ammonia (ppm)[1]**
20	100
5	20
1	5

[1] Determined using Hydrion® ammonia test strips

Table 3-4. Survival of microsclerotia subjected to gaseous ammonia

[NH_4Cl] (mM)	**[NH_3] (ppm)**	**Percent survival[1]**
0	0	100% ± 0
1	5	73.3% ± 46.2
5	20	46.7% ± 23.1
20	100	6.67% ± 11.5

[1] Mean ± standard deviation of 3 replicate experiments

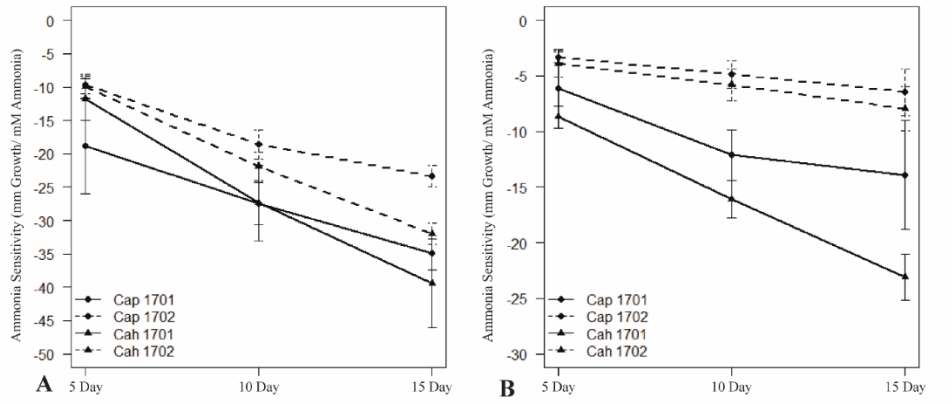

Figure 3-1.

A. Calculated mean (+/- standard deviation, n=3) ammonia sensitivity values for Section 1 of the exponential decay curve. Cap = *C. pseudonaviculata*; Cah = *C. henricotiae*. B. Mean (+/- standard deviation, n=3) ammonia sensitivity values for Section 2 of the determined exponential decay curve.

Chapter 4. Evaluation of constructed wetland impacts on *Verticillium dahliae* and *Calonectria pseudonaviculata*

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Abstract

Constructed wetlands have been utilized in wastewater treatment for decades. Originally designed for human wastewater, as well as reducing nutrient pollution , these systems have shown promise in nursery and greenhouse applications. Recirculation of irrigation water is utilized by greenhouse and nurseries. However, this practice could potentially reintroduce pathogens into the system. The objective of this chapter was to evaluate the impact that a constructed wetland system would have on pathogen survival, specifically *V. dahliae* and *C. pseudonaviculata.* The system consisted of three treatments: planted gravel, gravel, and planted gravel/spent mushroom compost mix, with three replicates of each treatment. Inoculated *V. dahliae* microsclerotia did not sustain substantial mortality over 7 days in any of the treatments. When challenged with discs soaked in the wetland effluent, *C. pseudonaviculata* mycelia grew over the discs with no observed inhibition.

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Introduction

Originally designed in Germany by Käthe Seidel, the purpose of constructed wetlands was to treat human and livestock wastewater (Vymazal 2008). There are three different systems, (i) free water surface (FWS), (ii) horizontal subsurface flow (HSSF), and (iii) vertical flow (VF) wetlands. Conventionally, HSSF wetlands are constructed with a rectangular shape and an impermeable liner to retain water. Wastewater enters at one end of the system and is collected at the opposite end (Vymazal 2008). HSSF wetlands typically consist of a gravel substrate planted with water tolerant plants. These plants serve multiple roles: insulation in colder months (Vymazal 2008), nutrient uptake (Berghage et al. 1999), and providing carbon for the microbes (Berghage et al. 1999, Gruyer et al. 2013).

While the initial development of constructed wetland systems focused on human and livestock wastewater, these systems also show promise for use in mitigating nursery and greenhouse wastewater (Arnold et al. 2003; Berghage et al. 1999; Fernandez et al. 1999; Gruyer et al. 2013). These “green” industries use large amounts of water and chemicals (pesticides, fertilizers, etc.) that can then be carried off site in the runoff. When evaluating the percent loss of applied herbicides in a nursery, Gilliam et al. (1992) reported that even when adjacent pots were tightly arranged, 30% of the applied liquid was lost. When pot spacing was increased to 30 cm, the amount of lost herbicide almost tripled, with 80% being lost as runoff. These authors demonstrated that nursery wastewater can be high in both volume and concentration of chemicals.

Legislators have recently passed laws that require nurseries to manage their runoff (Berghage et al. 1999; Lea-Cox et al. 2001; Huett et al. 2005). Such laws have forced

many nurseries and greenhouse operations to explore recycling their wastewater (Berghage et al. 1999, Huett et al. 2005), which would diminish the threat to outside ecosystems (Arnold et al. 2003). However, recycling nursery wastewater can lead to a buildup and recycling of harmful contaminants (Berghage et al. 1999; Lévesque et al. 2011; Stewart-Wade 2011). Multiple studies have demonstrated that constructed wetlands could successfully serve as a management solution to minimize nutrients and chemical runoff (Arnold et al. 2003; Berghage et al. 1999; Davison et al. 2005; Fernandez et al. 1999; Huett et al. 2005; Lévesque et al. 2011).

There has been extensive research on the survival of human pathogens in constructed wetlands, but little research involving plant pathogens. Whereas data from human pathogen survival studies can be useful, especially in terms of bacterial survival, additional research needs to be performed evaluating plant pathogen survival in constructed wetlands. For example, Diaz et al. (2012) determined that *E. coli* populations were reduced by 66-91%, and general enterococcus bacteria were reduced by 86-94% in constructed wetlands. These data could be used as an approximation for plant pathogenic bacteria that are similar to *E. coli*.

Working with plant pathogenic fungi, Gruyer et al. (2013) evaluated removal of *Pythium ultimum* and *Fusarium oxysporum* from constructed wetland systems. Their system utilized three types of HSSF wetlands: (i) planted gravel, (ii) planted gravel supplemented with sucrose, and (iii) planted gravel/compost mixture. Overall, they observed significant reduction (99.61-99.99%) in pathogen loads within all systems. They also evaluated the wetland microbial communities for production of enzymes capable of breaking down fungal cell walls and discovered that the highest level of

enzymes production was found in the gravel/compost mix (Gruyer et al. 2013). Their research established that constructed wetlands can serve as a viable tool to reduce large loads of pathogen propagules like spores, thus lessening the risk of plant infection by fungal pathogens if reusing the water.

The objective of this chapter is to build from Gruyer et al.'s research and to evaluate survival of resistant fungal microsclerotia in a constructed wetland system. Microsclerotia are produced by multiple pathogens that threaten the nursery industry. *C. pseudonaviculata* is the microsclerotia-forming causal agent of boxwood blight, a disease threatening nurseries nationwide (see Chapter 2). Because *C. pseudonaviculata* is a pathogen regulated by the USDA, the fungus could not be used for these constructed wetland studies. Instead, the common soil-borne pathogen *Verticillium dahliae* was chosen as the model organism for this study, since this fungus also produces microsclerotia and causes disease in nursery plants (Hong et al. 2014**)**. This proxy will have to suffice until United States regulatory restrictions to allow for the use of *C. pseudonaviculata* testing in a wetland system. To address these restrictions, *in vitro* assays were performed on *C. pseudonaviculata* within laboratory settings.

Wetland Construction

To fulfill the objectives of this study nine wetland cells were constructed: three cells each for three treatments. As in Gruyer et al. (2013), treatments were created to alter the carbon supply available to microbes. The treatments were as follows: (i) gravel alone, (ii) gravel with plants, and (iii) a 60/40 v/v mixture of spent mushroom compost and gravel. The gravel substrate for all treatments consisted of limestone rocks approximately

12-mm in diameter, obtained in State College, PA, probably originating from the Ordovician Coburn Formation. The spent mushroom compost (post-cropped pasteurized mushroom compost) was obtained from the Penn State Mushroom Research Center. Wetland plants were donated by Aquatic Resource Restoration Company, and consisted of pickerelweed (*Ponderosa cordata* L.), and softstem bulrush (*Schoenoplectus tabernaemontani* (C.C. Gmel.) Palla).

The wetland frames were constructed from wood 2x4's, creating an interior dimension of 1.09 x 0.33 m, and a height of 0.406 m. A pond-liner was placed inside the frame to contain the substrate. Marine plywood was attached to the base of the frame for extra support, and the structure placed on cinder blocks. Each wetland was adjusted to ensure a 5% slope towards the water collection system and housed in a greenhouse to regulate environmental conditions. To collect and remove the water, a series of PVC pipes and bulkheads were used. Two holes (approx. 2 cm) were cut in the bottom front and left sections of the pond liner to facilitate this collection system, and bulkheads were used to prevent leakage. A 3/4" PVC pipe drilled with holes was used as the collection bar, with a T-junction in the middle. A PVC union was installed to allow for easy removal and maintenance (Fig. 4-1). A standpipe was used to adjust the water level in the wetlands, and a PVC ball valve used to drain the systems. For ease of sampling, one sampling port was placed in the center of the wetland cells. On the lower bulkhead, a threaded cap was placed on the outside of the bulkhead, allowing for removal and cleaning of the collection system, if needed, to remove possible biofouling. This port consisted of a 3/4" PVC pipe drilled with holes to allow for complete water exchange

within the wetland system. A male threaded adapter and cap were placed at the top to seal the port until needed.

Due to the small size of the wetland cells, a batch feed system was utilized for filling. Eighty-five grams of Peter's Professional® Peat Lite Special fertilizer was added to 340.7 liters of water in a 378-L tank, leading to a target nutrient concentration of approximately 20 mg/L ammonium and 30 mg/L nitrate. Actual measurements of ammonium and nitrate concentrations were taken after mixing and recorded for each run. The mixture was added to the drained wetland cells until water flowed from the standpipe. This procedure was repeated every 7 days, establishing the hydraulic residence.

Inoculation experiments

Microsclerotia were produced following the procedure described in Chapter 2. Glucose yeast extract tyrosine (GYET) agar plates were poured, and after solidification sterilized cellophane sheets were aseptically placed on the agar surface. Agar plugs were transferred from cultures of *V. dahliae* onto the surface of the cellophane. After 5 weeks, cultures were considered mature and ready for use.

To inoculate the microsclerotia into the wetlands, the cellophane was cut into squares, approximately 0.75 cm x 0.75 cm in size. Squares were placed in nylon biopsy pouches (Electron Microscopy Services), with five bags connected vertically in a linear fashion. A metal washer was attached to the bottom pouch to act as a weight, and nylon line attached to the top pouch to aid in retrieval. This vertical strip of five pouches was inserted into the sampling port in the center of each of the wetlands, with care to ensure

that all pouches were fully submerged under water. At 3, 5, and 7 days samples were pulled from one wetland of each treatment.

Each strip of five pouches was surfaced sterilized with a post-sample wash to minimize contamination. Individual pouches were separated, rinsed with distilled water, and placed in 50 mL tubes. Each tube contained 40 mL of 0.4125% sodium hypochlorite bleach and were rocked for 10 min to enhance exposure to the bleach. Samples were removed from the bleach and transferred to tubes containing 40 mL of sterile water and rocked for 40 min to rinse the cellophane squares. After the wash, each cellophane square was transferred, with the microsclerotia facing upwards to a GYET plate. After 3 days the squares were rated positive or negative for survival based on sporulation.

None of the three wetland treatments significantly influenced *V. dahliae* microsclerotia survival. Throughout the entire 7-day evaluation period, corresponding to the hydraulic residence time of the systems, survival rates never decreased below 90%. In fact, only the gravel and plant treatments had less than 100% survival, after being submerged for 7 days. These results seemly gave different conclusions compared to the results of Gruyer et al. (2013), who reported removal of plant pathogenic fungi from effluent in their system. However, they evaluated pathogen removal from the system effluent, not pathogen survival. Their detection relied, in part, on recovery of viable spores. This contrasts with our study, where the focus was not on the efficiency of a wetland system to filter out pathogen propagules, but whether those propagules, when trapped in the wetlands, would survive. Combined, these two studies help provide information as to what may be happening with fungi in these systems. Gruyer et al.'s (2013) work demonstrated that constructed wetlands serve as a sufficient filter for plant

pathogens, while the results from our study demonstrate that though not discharged, the pathogens could still remain viable within the wetland for at least 7 days.

Water Properties

Temperature, pH, oxidation/reduction potential (ORP), conductivity, ammonium concentration, and nitrate concentrations were monitored in the wetlands for the duration of the experiment. A Hach® Sension^{TM} portable meter with a MM150 probe was used to measure pH, ORP, and conductivity, while a Vernier Labquest® system was used to measure ammonium and nitrate concentrations, as well as temperature. While these parameters were measured during the inoculation experiments, only one cell per treatment was analyzed at each time point. Therefore, a separate experiment was performed and replicated three times to measure the water parameters. After mixing the irrigation water, chemical parameters were measured before the wetlands were filled. After filling, on days 3, 5, and 7 the pH, ORP, conductivity, and pH levels were measured by inserting the appropriate probe into the sampling port. On day 7, water samples were collected during the draining process after 30 seconds of draining and analyzed for ammonium and nitrate levels.

Both the pH and oxidative-reductive potential (ORP) of the starting irrigation water were consistent between experiments, 6.97 ± 0.175 and 256 mv ± 22.8 (n=3) respectively. In contrast, variability for electrical conductivity (EC), 691 µS/cm ± 124, was considerably greater.

Examination of the EC results revealed that both the compost and plant treatments had similar values, although compost EC was more variable and higher after 3 days.

However, the variability decreased by day 7 at which point it was comparable to the plant treatment. The EC was consistently lower in the gravel treatments, in congruence with the average level observed in the irrigation water. The compost and plant treatments both increased EC levels above the original irrigation water level, but the levels remained constant in the gravel treatment. While this trend was constant through 7 days, at each of the sampling dates the EC value decreased in all three treatments.

The pH in the gravel treatment was higher than in both the compost and plant treatments, as well as the irrigation water. This trend was observed over all 3 sampling periods. The construction of the wetlands provides insight into why this could have occurred. Limestone gravel was used, which would make water alkaline and cause the pH to rise. Conversely, in both the compost and plant wetlands the production of organic acids would lead to a lower pH in the water.

The ORP data did not reveal a clear trend, as compared to both the EC and pH data. ORP values after 3 days were similar in the compost and gravel treatment, whereas the plant treatment had a slightly higher ORP value. At 5 days, the plant treatment still had the highest ORP value, while the compost value was only slightly higher than in the gravel treatments. During the final sampling period after 7 days, the variability of the compost ORP increased greatly, ranging from < 100 mV to 256 mV. There were also low outliers in both the gravel and plant treatments. The variability in the ORP data makes it difficult to draw conclusions regarding cause and effect. However, after 7 days the ORP values decreased in the compost treatment compared to the other treatments. Although statistical comparisons could not be made on the chemistry of the wetlands, the data is still useful as background information to aid future studies.

Kirby-Bauer Assay

As mentioned previously, regulatory restrictions prevented use of *C. pseudonaviculata* in the wetland inoculation work within an open greenhouse. Therefore, a Kirby-Bauer style experiment was devised to evaluate the impact of the wetland effluent on *C. pseudonaviculata*. Five-mm disks were punched from #4 Whatman filter and autoclaved. Water samples (approx. 30-40 mL) were retrieved during draining, after 30 seconds, and frozen at 0 °C. Half of the water samples were filter-sterilized and half left unfiltered to evaluate if anti-fungal compounds were present in the effluent compared to the unfiltered water. A sample of the irrigation water was also obtained to serve as a control. For both treatments, three Petri plates, inoculated in the center with *C. pseudonaviculata*, represented a sample unit. Each Petri plate received a saturated disc from the three wetland treatments: (i) gravel, (ii) planted gravel, and (iii) planted compost/gravel. The irrigation water control was handled the same as the effluent samples and was placed on one of the Petri plates in the sample unit. This sample unit was then replicated 10 times. If present, zones of inhibition around the discs were measured and recorded.

Inhibition was not observed in any assay, or in any of the controls. Thus, it appears that no anti-fungal compounds, effective against *C. pseudonaviculata*, were produced in any wetlands. This result provides insight into the lack of eradication in the inoculation experiments. Antagonistic organisms provide one of the possible routes for eradication (Kadlec et al. 2009), and their absence would contribute to increased survivability in this system. Future work could examine this concept by isolating bacterial and fungi from the wetlands, and screening for isolates that inhibit *C.*

pseudonaviculata. A more detailed analysis would help confirm that there are not significant levels of antagonistic bacteria or fungi present that are antagonistic to *V. dahliae* or *C. pseudonaviculata*. Yang and Hong (2018) demonstrated that *C. pseudonaviculata* inhibiting microbes are present in recycled irrigation water. Of the 1547 cultures screened, they concluded that 153 showed promise for fungal inhibition, with a majority (121) belonging to *Pseudomonas* spp. Of the 153 isolates, they further identified 3 strains for possible use as a biocontrol product, all *Pseudomonas protegens*. The authors postulate that the production of anti-fungal compounds is an important mode of action and recommend further research into the effectiveness of these strains in controlling *C. pseudonaviculata*.

Conclusions

Unfortunately, due to legal regulations we were unable to directly assess the survival of *C. pseudonaviculata* microsclerotia in the wetland system. However, *V. dahliae* served as a proxy, and is also a fungal plant pathogen that produced microsclerotia. The results from both the inoculation and Kirby-Bauer experiments demonstrated that microsclerotia survival is expected for at least 7 days in our batch-fed wetland system. Survival might be different in a continuously-fed wetland system, since batch-fed systems might have a different chemistry due to the drain and fill process. However, batch-fed systems would work well for nurseries that do not have a constant stream of wastewater. Although other research has demonstrated a reduction in pathogen levels within wetland effluent, our study indicated that, while reduced in numbers, the pathogen may still be present and viable when retained within the wetland system.

A potential problem with a small-scale study like this, is the issue of scaling in the system. The surface area to volume ratio is lower in our system compared to what it would be in a commercial system. Essentially, if blown up to a large scale, the wetland would appear be filled with large boulders instead of small gravel. This type of scaling is hard to account for, since using smaller particle sizes would clog the system. However, this could be part of the reason no fungal inhibiting compounds were found. The surface area ratio is lower than what would be expected in a full-scale system. Perhaps this is one of the reasons that no inhibition was observed in these experiments. However, another explanation could be that the anti-fungal compounds were not produced in levels high enough to be detected using out assay. Yang and Hong (2018) used pure cultures in their inhibition assays, allowing for higher concentrations of any inhibitory compounds. However, only 10% of the near 1500 isolates that they screened showed any sort of inhibition, and of the 150 exhibiting inhibition, only three were identified as having potential for use as a biocontrol.

Though not the primary objective of this research, an interesting insight was gathered pertaining to the removal of nitrate and ammonium from the wetland system. The gravel alone treatment did not reduce nitrate levels, whereas both the compost/plant and gravel/plant treatments demonstrated reduced nitrate levels in the effluent. While not conclusive, it appears that the compost/planted treatments performed better compared to the planted gravel treatments in nitrate removal (Fig. 4-2a). contrasting with the ammonium results (Fig. 4-2b). It is well established that constructed wetlands are an excellent method for removing nitrogen from wastewater, our findings indicate that the

addition of spent mushroom compost could further increase this ability, meriting future research.

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Vymazal, J. 2008. Constructed Wetlands, Subsurface Flow. In *Applications in Ecological Engineering*, ed. S. E. Jørgensen. Amsterdam: Elsevier.

Yang, X., and Hong, C. 2018. Biological control of boxwood blight by *Pseudomonas protegens* recovered from recycling irrigation systems. Biological Control (in press)

Table 4-1. Microsclerotia survival in the wetland systems

Treatment	3 Days	5 Days	7 Days
Gravel	100% ± 0	100% ± 0	93.3% ± 11.5
Planted	100% ± 0	100% ± 0	91.7% ± 14.4
Compost	100% ± 0	100% ± 0	100% ± 0

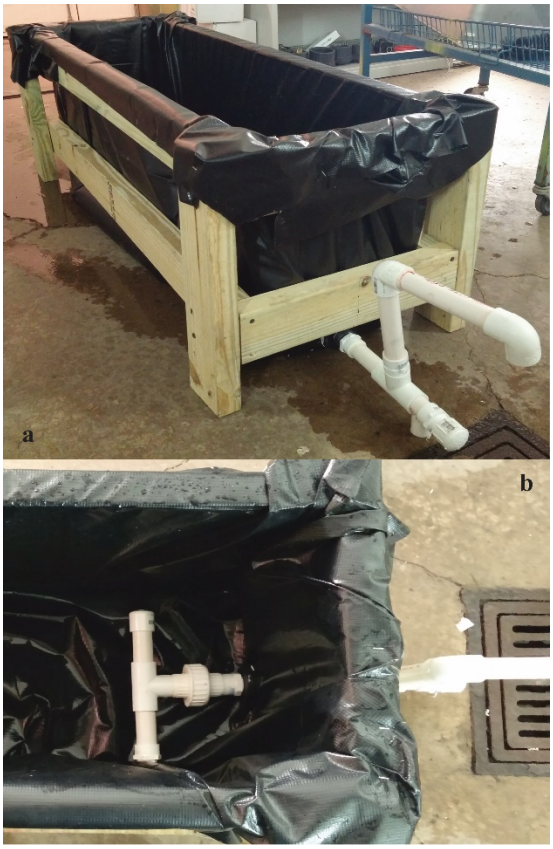


Fig. 4-1. Wetland construction images

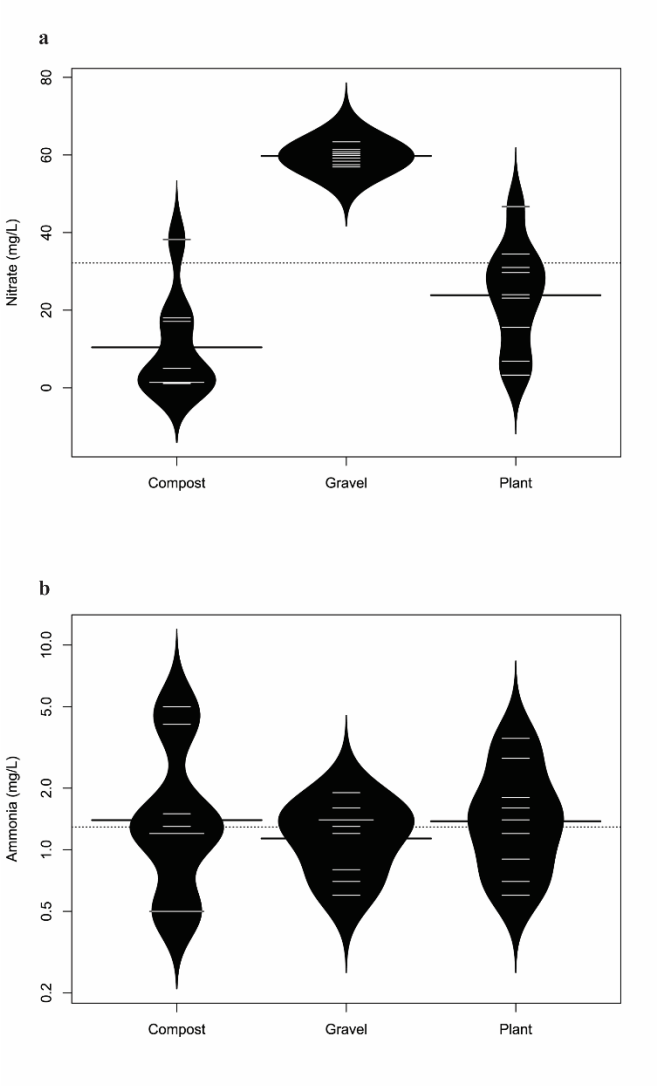


Figure 4-2. Both nitrate (a) and ammonium (b) levels measured in the wetland cells after 7 days. (a) Both the compost and plant treatment showed lower levels of nitrate when compared to the gravel treatment, indicated better treatment. (b) This was not seen with ammonium, where all three treatments had similar values. Dotted line represents overall mean, solid black lines indicate treatment mean. Note: The function beanplot() extrapolates to form the end points of the distribution.

Chapter 5. Retrospective and Future Directions

The overall theme of this research was to evaluate the effectiveness of compost and constructed wetlands in eradicating the plant pathogen *Calonectria pseudonaviculata*. The use of these, and other, sustainable practices combined with conventional methods represent the future of disease management. This integration allows for optimal pathogen management, while reducing environmental impact. However, before using these sustainable methods for pathogen control, the methods must first be evaluated to ensure that they can successfully manage the pathogen in question. This was the overall objective of my research. Composting and constructed wetlands were specifically selected due to active interest, as well as high probability of use.

I chose the word sustainable for a purpose in my dissertation title. It was the overarching connection between my varied, and seemingly different projects. Despite the differences between both the compost studies and the wetland study, the theme was the same: utilizing sustainable methods to help manage plant disease. While the use of pesticides will not be replaced (for now at least), the mindset of "spray and pray" can. We also have a duty as a society to change how we interact with our planet, to ensure that its beauty will still be around for generations to come. This can seem a daunting task, and it is easy to get lost in the assumed futility of it; however, by focusing our efforts and resources, a difference can be made. By no means will the work I described solve the world's problems alone, but they begin to address these issues. Focusing on what I know, and what my skills are, I can work to improve my little area of expertise. Whether it be convincing more growers that they can compost their waste and reapply it, to working on lowering the levels of nutrients entering the Chesapeake Bay due to implementation of

constructed wetlands in nurseries, a difference can be made. If everyone works on their immediate area, or within their specific area of expertise, the benefits will be enormous.

Sustainability is a mindset as much (if not more so) than it is a series of strategies, or best management practices. It is the idea that we, as a society, can be strategic with our resources in productive and creative ways. Many times, these practices can also help reduce costs as well. A nursery could cut down on fertilizer costs by utilizing compost from their own scraps, or a greenhouse by catching their wastewater, still full of nitrogen, and reusing it. A waste of resources is a waste of money, plain and simple. Sure, there can be an initial cost to many sustainable practices, but they will pay for themselves in the long run.

After boxwood blight became established in the United States, concern arose that it could survive composting. The results of this work demonstrate that *C. pseudonaviculata* survival is not expected in compost that is >50 °C for 24 consecutive hours or more. This lack of survival is good news for the nursery industry. Currently, the accepted methods for disposal of infected boxwoods range from landfilling, to burial and burning. However, these options have limitations. Burying the boxwoods does not guarantee removal of the inoculum. In fact, considering that the microsclerotia can survive in soil for extended periods of time, future errant excavation could reintroduce the pathogen. While burning of the infected plants would lead to death of the pathogen, updrafts from the flames could carry unburned, or partially burned leaves beyond the burn pile, spreading the pathogen. Finally, sending the plants to the landfill does eliminate any spread of the pathogen, but is not a sustainable option. However, now that it has been shown that properly managed compost will eradicate microsclerotia,

composting infected plants would serve as a green solution to the problem. Beyond reducing green waste infusion into landfills, composting would provide a source of fertilizer that a nursery could use to increase plant health and reduce dependency on inorganic fertilizers. However, one caveat is that the compost must be well managed. If there is a lack of proper turning, and/or temperature/time does not meet the requisite time (>50 °C, 24 h or longer), eradication of the pathogen cannot be guaranteed. Sanitation of equipment and the work area is also important, as lack thereof could lead to pathogen reintroduction in the newly composted material.

Composting serves as an excellent way to manage *C. pseudonaviculata*, but constructed wetlands did not perform as well. In fact, pathogen survival was observed in all of the treatments at all time periods (3, 5, and 7 days), indicating that wetlands, using a batch flow system, do not serve as an adequate way to eradicate the microsclerotia of *Verticillium dahliae*. Unfortunately, direct evaluation of *C. pseudonaviculata* was not possible due to regulatory reasons, leading to the *V. dahliae* proxy. While a definitive statement cannot be made about *C. pseudonaviculata* survival until a time exists where it can be directly evaluated, for now it should be assumed that a constructed wetland will not serve as successful tactic for eradication of *C. pseudonaviculata*. Constructed wetlands should still be considered for nursery use due to their established ability to reduce nutrient levels. While not the primary objective, data were collected on the nutrient removal in the systems. As expected the gravel treatment made no difference in the nutrient levels, but both the plant and compost treatments showed promise in nitrate removal.

The use of constructed wetlands for nutrient removal has been standard practice for decades, so these results are not unexpected. However, what is of interest is the performance of the incorporated spent mushroom compost. The nitrate data indicated that there may be more removal in the compost treatment wetlands. I believe that this is the best avenue for future work with the wetland system. Pennsylvania has a large surplus of spent mushroom compost, as well as a surplus of limestone gravel. If these two substrates combined serve better than planted gravel substrate alone, their incorporation into wetlands for wastewater treatment would help find a use of the tremendous amount of spent mushroom compost produced in PA. In addition, due to their low maintenance, wetlands would be an excellent tool for nurseries to address their runoff in an environmentally friendly way.

Like all scientific research our studies beget more questions than answers. In my opinion, this is the best part of scientific exploration. With this work, a baseline has been set for the compost survival of *C. pseudonaviculata*. However, a baseline is not the final answer. Many more avenues can be addressed, especially with the bioreactor system. One of the most important studies to follow this work would be to work on refining the time and temperature relationships. Probing of the lower temperature eradication for temperature would provide valuable information to composters, such as, knowing how many hours it takes for the pathogen to die at 30 °C. This provides information that could be present in a “worst case scenario” situation, helping build confidence to composters and regulators. Various other compost formulations could be used as well to determine if formulation effects survival. Finally, assessing survival in infected plant material is also an important next step, as this would be more representative of actual conditions.

Finally, I'd like to make a last statement to the student(s) who follows me on either of these projects. While I will not be able to directly help you get situated in the lab, or with either of these systems. I have left information in the appendices of this dissertation to assist you. You will find a general schematic of the bioreactor system, as well as a SOP for its operation. I've also included protocols for specific experiments, as well as some general lab protocols. Finally, I have included my R script used for the generation of figures as well as the statistical tests. Hopefully these can be of help if you decide to pursue R for your statistical needs. To put the final figures together, I paste the output from R into Adobe Illustrator to add in the labels, arrange the subfigures, etc. Last of all, enjoy your time here and don't forget to have fun!

Appendix A Supplemental Information for Chapter 2

Cellophane Microsclerotia Production and Processing

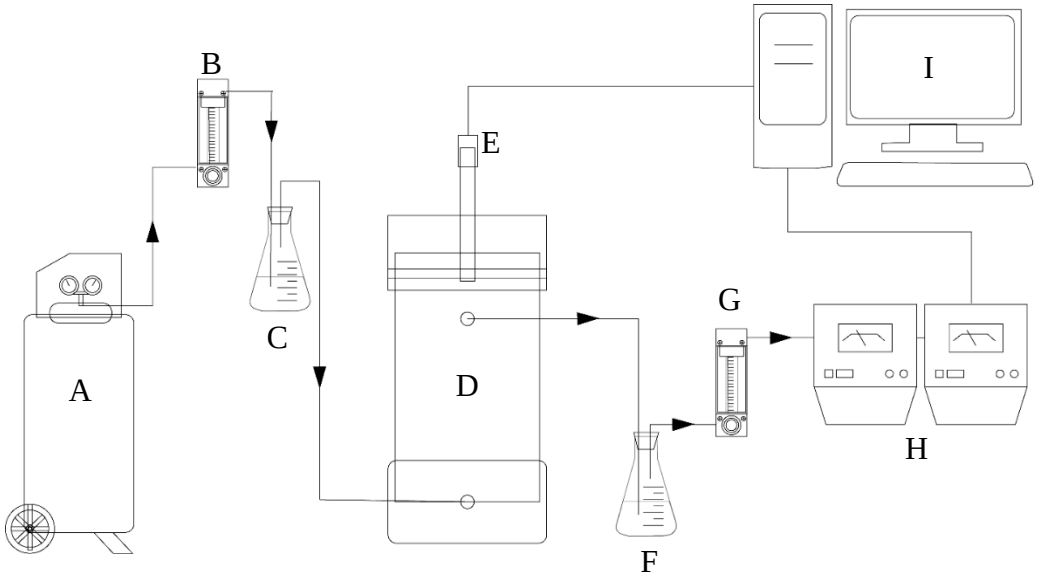
Cellophane Microsclerotia Production

1. Cut cellophane into 6.25 cm squares
2. Place cut cellophane into glass petri plates
    2-1. Place a piece of milk filter etc. between each piece
3. Fill plate with distilled water
4. Autoclave 15 minutes
5. Using tweezers, transfer each square onto a pre-pored solidified petri plate

Cellophane Surface Sterilization

- Set up two sets of falcon tubes (50 mL)
    - Set 1 will be 25 mL 1% clorox (0.08% sodium hypochlorite)
        - Solution dilution for clorox:
            - $m1v1 = m2v2$
            - $(100)(x) = (1)(25)$
            - $100x = 25$
            - $x = \frac{25}{100}$
            - 25/100=0.25
            - Add 0.25 mL of clorox to 24.75 mL water
        - Set two will be 25 mL water
        - Both sterile
            - Filter sterilize clorox
            - Autoclave water in batch, add to already sterile tubes in allotted amounts
- Using sterile petri dish lid, carefully unwrap metal pouch and place MS square in petri dish bottom
- Once all squared removed, add all squares to their respective bleach tubes
- Rock for 3 minutes (Rocker brand: Research Products International Corp)
- Transfer squares into water tube
    - Works best to use long tool to transfer squares, as they will sink to the bottom
- Rock 30 min
- Transfer to GYET plates, parafilm
- Read results 20 days later

Bioreactor SOP



A. Air compressor
B. Flow Meter
C. Humidification Flasks
D. Reactor Vessel
E. Temperature Probe
F. Boric Acid Trap
G. Flow Meter
H. Oxygen and CO_2 Detectors
I. Computer

Overview of reactor system

This bioreactor system is a series of composting vessels connected to an air compressor, allowing for simulated composting with precise controls. There are nine compost vessels housed in three incubators that can be set to independent temperatures. Air leaves the compressor and flows through a rotameter manifold, allowing for individual control of airflow in each vessel. To prevent desiccation, this air enters a humidification flask before entry into the vessel. Air enters the bottom on the vessel, flows up through a false floor, then subsequently through the compost. An outlet is present at the top, directing the air out of the incubators, and into boric acid traps cooled to 4 °C, allowing for ammonia level determination. A final flow meter is present to check for leaks in the system, before

the air can be directed into an oxygen or carbon dioxide detector. Alternatively, the effluent can simply be vented into the atmosphere. A temperature probe present in the vessel provides real time temperature data to the computer for further analysis.

Loading the Reactor Vessels

The reactor vessels are very versatile, and many varieties of compost can be used. The total mass on compost used depends on various properties like density or percent moisture.

1. Before loading, ensure that each reactor has 3 CPVC stands and a metal mesh floor
2. Place the 3 CPVC couplings upright in the base of the vessel, then place the mesh floor on top
3. The base of the floor should be level and higher than the inlet location.
4. Add compost to the vessel, ensuring that the floor does not shift. The top of the compost should be 1 in below the outlet max.
5. Place the rubber seal cap on top of the vessel, tapping it with the butt of a screwdriver if needed to ensure proper seating, then tighten.
6. The composting vessel is now ready to place in the incubators, Take care not to tip, or otherwise disturb the vessel in a way that would collapse the floor.

Setting up the vessels/Incubator operation

1. Remove all cords and tubes from the interior of the incubator as best you can.
2. Before placing the vessel in the incubator, insert the temperature probe in the sample port on top of the vessel. Hand tighten to ensure no leaks.

3. Place the vessel in the incubator and repeat step 2 for the other 2 vessels.

4. Once all three vessels are placed in the incubator with their respective temperature probes (marked with 1, 2, and 3 lines to correspond to the vessel) the air tubing can be inserted.

5. Orange marked tubing is the inflow and goes on the bottom. Blue marked tubing is outflow and goes on the top. These are marked like the temperature probes, with 1, 2, and 3 pieces of tape.

6. Shut the incubator and set the desired temperature.

7. To set the incubator temperature first press the top button that looks like an X over a W. Press until “SP” appears. SP will display for a moment, followed by a temperature. Using the arrow keys, set the temperature to the desired value. Once finished, press the X/W button again, until the display goes back to showing the actual temperature.

Determining Air Measurements

1. Currently, only one vessel can be placed in the oxygen or CO_2 sensor at a time. Due to the low air flow, accurate readings will not occur until at least 2 hours. It is recommended that one reactor be hooked up and left for an extended duration.

2. In contrast, ammonia measure can be performed simultaneously for all vessels using the boric acid flasks

3. To make the boric acid flask, first make a 0.67M boric acid solution (42g/L) plus a small amount of methyl red and add 100 mL to each flask.

4. Insert the corresponding stopper into the matching flask and let sit for the desired time.

5. Make sure that water is present in the metal bin to help facilitate temperature transfer and use red rings to weigh down the flasks.

6. After sample, record the pH of each flask, then using the following equation you can determine the mg of ammonia produced:

$$NH_3 = 10^{(-6.81170+1.99691pH-0.10297pH^2)}$$

7. This mass can be converted to ppm using the following formula

$$ppm = \frac{1000mRT}{MPV_S}$$

m = mass of ammonia (g)
R = gas constant (0.08206 $\frac{L*atm}{mol*K}$),
T = temperature (K)
M = molecular weight (g)
P = pressure (atm)
V_s = total volume of air (m^3)

Retrieval of data from Mushroom Master

1. Temperature data is recorded near continuously in the Mushroom Master software.

2. Open Mushroom Master before beginning the compost run to ensure temperate data collection.

3. To get temperature information after the program has opened, click “Actuals”

4. This will bring up a screen displaying the data. To view the compost temperature information, click “Comp.Sensors”

5. Clicking “Air-Sensors” will display air data

6. To view a graph, click “Graphic”, and right click to go back to the main data screen.

7. To export data, go back to the main screen by clicking the ^ near the top left corner.

8. Click the “Directory” heading, and if no information shows up, click “All” on the righthand side.

9. Scroll through the displayed information until you see the date for the run you would like to export.

10. Select this file and click export. Change the separator to a “.” as opposed to “,”.

11. This file will be saved in: Computer > System (C:) > MMwin > Export

12. Using the text file import wizard in Excel, this data can be imported in the standard spread sheet format.

13. Further manipulations can then be performed in Excel, especially by formatting the data as a sortable/searchable table.

Table A-1. Methods used in compost eradication studies

Organism	System	Source	Spike	Survival	Study
Botrytis aclada	Heaps	Infested Material	Nylon Bags	Bioassay, Baiting	Bollen et al. 1989
B. cinerea	Stack	Infested Material	Nylon Bags	Plating	Hoitink et al. 1976
D. ulmi	Aerated Pile	Infested Material	Ziploc Bags	Plating	Wichuk et al. 2011
F.oxysporum	Heaps	Infested Material	Nylon Bags	Bioassay, Baiting	Bollen et al. 1989
M. phaseolina	Soil	Sclerotia	Direct	Plating	Chun & Lockwood 1985
M. phaseolina	Pit	Infested Material	Nylon Bags	Plating	Lodha et al. 2002
P. cinnamomi	Stack	Infested Material	Nylon Bags	Plating, Baiting	Hoitink et al. 1976
P. nicotianae	Soil	Chlamydospores	Direct	Bioassay	Coelho et al. 2001
P. ramorum	Heat Treatment	Infested Material	Direct	Plating	Harnik et al. 2004
P. ramorum	Pile	Infested Material	Nylon Bags	Plating, Baiting	Garbelotto 2003
P. ramorum	Windrow/Aerated Pile	Infested Material	Nylon Bags	Plating, Baiting	Swain et al. 2006
Pl. brassicae	Flasks, Windrows	Infested Material	Nylon Bags	Bioassay	Fayolle et al. 2006
Pl. brassicae	Anaerobic Digester	Infected Material	Direct	Bioassay	Ryckeboer et al. 2002
Py. ultimatum	Soil	Sporangia	Direct	Plating	Chun & Lockwood 1985
Py. ultimatum	Aerated Pile	Infected Material	Muslin Bag	Plating	Suarez-Estrella et al. 2007
R. solanacearum	Anaerobic Digester	Infected Material	Direct	Plating	Ryckeboer et al. 2002
R. solani	Aerated Pile	Infected Material	Muslin Bag	Plating	Suarez-Estrella et al. 2007
S. cepivorum	Heaps	Sclerotia	Sieve Pocket	Bioassay, Baiting	Bollen et al. 1989
T. basicola	Soil	Chlamydospores	Direct	Plating	Chun & Lockwood 1985
V. albo-atrum	Aerated Pile	Infested Material	Ziploc Bags	Plating	Wichuk et al. 2011
V. dahliae	Aerated Pile	Infested Material	Ziploc Bags	Plating	Wichuk et al. 2011
Ca. pseudonaviculata	Bioreactor	Microsclerotia	Sieve Pouch	Plating	This Study

R Script for Creation of Temperature Graphs

```
rxt1603 <-  read.table("C:\\Users\\rjh346\\Dropbox\\00_GRAD_SCHOOL_NEW\
      \R Projects\\Compost Properties\\RXT1603 Whole
      Profile.txt", header=TRUE)

rxt1603.inc1 <- subset(rxt1603, Incubator=="INC1")
rxt1603.inc2 <- subset(rxt1603, Incubator=="INC2")
rxt1603.inc3 <- subset(rxt1603, Incubator=="INC3")

plot(rxt1603.inc1$Time, rxt1603.inc1$Mean, ann=FALSE, type="o",
      pch=".", lty=1, ylim=c(20,70),axes=FALSE)
      lines(rxt1603.inc2$Time, rxt1603.inc2$Mean, type="o",
      pch=".", lty=2)
      lines(rxt1603.inc3$Time, rxt1603.inc3$Mean, type="o",
      pch=".", lty=3)
      title(ylab="Temperature (Celsius)", xlab="Time After Start
      (hrs)")
      axis(side=1, at=seq(0,408,by=12))
      axis(side=2, at=seq(20,70, by=5))
      box()
      arrows(rxt1603.inc1$Time, rxt1603.inc1$Mean-
            rxt1603.inc1$SD,rxt1603.inc1$Time,
      rxt1603.inc1$Mean+rxt1603.inc1$SD, length=0.05,
      angle=90, code=3, lty=1)
      arrows(rxt1603.inc2$Time, rxt1603.inc2$Mean-
            rxt1603.inc2$SD,rxt1603.inc2$Time,
      rxt1603.inc2$Mean+rxt1603.inc2$SD, length=0.05,
      angle=90, code=3, lty=2)
      arrows(rxt1603.inc3$Time, rxt1603.inc3$Mean-
            rxt1603.inc3$SD,rxt1603.inc3$Time,
      rxt1603.inc3$Mean+rxt1603.inc3$SD, length=0.05,
      angle=90, code=3, lty=3)
      abline(v=72, lty=2)
      abline(v=168, lty=2)
      abline(v=240, lty=2)
      abline(v=312, lty=2)

rxt1603.inoc <-
      read.table("C:\\Users\\rjh346\\Dropbox\\00_GRAD_SCHOOL_NEW\ \R
Projects\\Compost Properties\\RXT1603 Inoc.txt",      header=TRUE)
rxt1604 <-  read.table("C:\\Users\\rjh346\\Dropbox\\00_GRAD_SCHOOL_NEW\
      \R Projects\\Compost Properties\\RXT1604 Inoc.txt",
      header=TRUE)
rxt1605 <-  read.table("C:\\Users\\rjh346\\Dropbox\\00_GRAD_SCHOOL_NEW\
      \R Projects\\Compost Properties\\RXT1605 Inoc.txt",
      header=TRUE)

###Making the graphs for just the inoculated period
####RXT1603

rxt1603.inoc.1 <- subset(rxt1603.inoc, Incubator=="INC1")
rxt1603.inoc.2 <- subset(rxt1603.inoc, Incubator=="INC2")
rxt1603.inoc.3 <- subset(rxt1603.inoc, Incubator=="INC3")

plot(rxt1603.inoc.1$Time, rxt1603.inoc.1$Mean, ann=FALSE,   axes=FALSE,
pch=".", type="o", lwd=2, lty=1, ylim=c(30,65))
```

```
      lines(rxt1603.inoc.2$Time, rxt1603.inoc.2$Mean, pch=".",
      type="o", lwd=2, lty=2)
      lines(rxt1603.inoc.3$Time, rxt1603.inoc.3$Mean, pch=".",
      type="o", lwd=2, lty=3)
      axis(side=1, at=seq(0,72, by=6))
      axis(side=2, at=seq(30,65, by=5))
      box()
      abline(h=40, lty=4)
      abline(h=50, lty=4)
      abline(h=60, lty=4)
      title(ylab="Temperature (Celsius)", xlab="Hours post
      Inoculation")
      arrows(rxt1603.inoc.1$Time, rxt1603.inoc.1$Mean-
            rxt1603.inoc.1$SD, rxt1603.inoc.1$Time,
      rxt1603.inoc.1$Mean+rxt1603.inoc.1$SD, length=0.05,
      code=3, angle=90)
      arrows(rxt1603.inoc.2$Time, rxt1603.inoc.2$Mean-
            rxt1603.inoc.2$SD, rxt1603.inoc.2$Time,
      rxt1603.inoc.2$Mean+rxt1603.inoc.2$SD, length=0.05,
      code=3, angle=90)
      arrows(rxt1603.inoc.3$Time, rxt1603.inoc.3$Mean-
            rxt1603.inoc.3$SD, rxt1603.inoc.3$Time,
      rxt1603.inoc.3$Mean+rxt1603.inoc.3$SD,
      length=0.05, code=3, angle=90)


####RXT1604 Profile
  rxt1604.1 <- subset(rxt1604, Incubator=="INC1")
  rxt1604.2 <- subset(rxt1604, Incubator=="INC2")
  rxt1604.3 <- subset(rxt1604, Incubator=="INC3")

  plot(rxt1604.1$Time, rxt1604.1$Mean, ann=FALSE, axes=FALSE,
      pch=".", type="o", lwd=2, lty=1, ylim=c(30,65))
            lines(rxt1604.2$Time, rxt1604.2$Mean, pch=".",
      type="o", lwd=2, lty=2)
      lines(rxt1604.3$Time, rxt1604.3$Mean, pch=".", type="o",
      lwd=2, lty=3)
      axis(side=1, at=seq(0,72, by=6))
      axis(side=2, at=seq(30,65, by=5))
      box()
      abline(h=40, lty=4)
      abline(h=50, lty=4)
      abline(h=60, lty=4)
      title(ylab="Temperature (Celsius)", xlab="Hours post
      Inoculation")
      arrows(rxt1604.1$Time, rxt1604.1$Mean-rxt1604.1$SD,
      rxt1604.1$Time, rxt1604.1$Mean+rxt1604.1$SD,
      length=0.05, code=3, angle=90)
      arrows(rxt1604.2$Time, rxt1604.2$Mean-rxt1604.2$SD,
      rxt1604.2$Time, rxt1604.2$Mean+rxt1604.2$SD,
      length=0.05, code=3, angle=90)
      arrows(rxt1604.3$Time, rxt1604.3$Mean-rxt1604.3$SD,
      rxt1604.3$Time, rxt1604.3$Mean+rxt1604.3$SD,
      length=0.05, code=3, angle=90)
```

```
####RXT1605
  rxt1605.1 <- subset(rxt1605, Incubator=="INC1")
  rxt1605.2 <- subset(rxt1605, Incubator=="INC2")
  rxt1605.3 <- subset(rxt1605, Incubator=="INC3")

  plot(rxt1605.1$Time, rxt1605.1$Mean, ann=FALSE, axes=FALSE,
      pch=".", type="o", lwd=2, lty=1, ylim=c(30,65))
      lines(rxt1605.2$Time, rxt1605.2$Mean, pch=".", type="o",
      lwd=2, lty=2)
      lines(rxt1605.3$Time, rxt1605.3$Mean, pch=".", type="o",
      lwd=2, lty=3)
      axis(side=1, at=seq(0,72, by=6))
      axis(side=2, at=seq(30,65, by=5))
      box()
      abline(h=40, lty=4)
      abline(h=50, lty=4)
      abline(h=60, lty=4)
      title(ylab="Temperature (Celsius)", xlab="Hours post
      Inoculation")
      arrows(rxt1605.1$Time, rxt1605.1$Mean-rxt1605.1$SD,
      rxt1605.1$Time, rxt1605.1$Mean+rxt1605.1$SD,
      length=0.05, code=3, angle=90)
      arrows(rxt1605.2$Time, rxt1605.2$Mean-rxt1605.2$SD,
      rxt1605.2$Time, rxt1605.2$Mean+rxt1605.2$SD,
      length=0.05, code=3, angle=90)
      arrows(rxt1605.3$Time, rxt1605.3$Mean-rxt1605.3$SD,
      rxt1605.3$Time, rxt1605.3$Mean+rxt1605.3$SD,
      length=0.05, code=3, angle=90)
      legend(54, 40, c("INC 1", "INC 2", "INC 3"), lwd=c(2,2,2),
      pch=c(".",".","."), lty=c(1,2,3), bty="n")
```

R Script for Creation of Ammonia Graphs

```
library(gplots)
###Import of the master data sheet from excel###
masterData <-
      read.table("C:\\Users\\rharv\\Dropbox\\00_GRAD_SCHOOL_NEW\\ R
Projects\\Compost Properties\\Ammonia Data PPM.txt",  header=TRUE)

###Subset of the master by experiment###
rxt1603 <- subset(masterData, Experiment=="1603")
rxt1604 <- subset(masterData, Experiment=="1604")
rxt1605 <- subset(masterData, Experiment=="1605")

###Subsequent subsets by day and experiment###
#this generates the 9 data sets to make the 9 graphs
rxt1603.24 <- subset(rxt1603, Time=="24")
rxt1603.48 <- subset(rxt1603, Time=="48")
rxt1603.72 <- subset(rxt1603, Time=="72")

rxt1604.24 <- subset(rxt1604, Time=="24")
rxt1604.48 <- subset(rxt1604, Time=="48")
rxt1604.72 <- subset(rxt1604, Time=="72")

rxt1605.24 <- subset(rxt1605, Time=="24")
rxt1605.48 <- subset(rxt1605, Time=="48")
rxt1605.72 <- subset(rxt1605, Time=="72")



##RXT1603 Graphs##

#24 Hours
attach(rxt1603.24)
      height.03.24 <- tapply(Ammonia, Temp, mean)
      lower.03.24 <- tapply(Ammonia, Temp, sd)
      upper.03.24 <- tapply(Ammonia, Temp, sd)
      rel.03.24 <- rank(height.03.24)/length(height.03.24)
      grays.03.24 <- gray(1-rel.03.24)

###PLOT 1
barplot2(height.03.24, plot.ci=TRUE, ci.l=height.03.24,
      ci.u=height.03.24+upper.03.24,ylim=c(0,200),    names.arg=c("",
"", ""),ylab="Ammonia (ppm)",       col=c("Black", "Black", "Black"),
xlim=c(0,5), width=.5)



#48 Hours
attach(rxt1603.48)
      height.03.48 <- tapply(Ammonia, Temp, mean)
      lower.03.48 <- tapply(Ammonia, Temp, sd)
      upper.03.48 <- tapply(Ammonia, Temp, sd)
      rel.03.48 <- rank(height.03.48)/length(height.03.48)
      grays.03.48 <- gray(1-rel.03.48)

###PLOT 2
```

```
barplot2(height.03.48, plot.ci=TRUE, ci.l=height.03.48,
      ci.u=height.03.48+upper.03.48, names.arg=c("", "", ""),
      ylim=c(0,200), col=c("Black", "Black", "Black"),
      xlim=c(0,5), width=.5)

#72 Hours
attach(rxt1603.72)
      height.03.72 <- tapply(Ammonia, Temp, mean)
      rel.03.72 <- rank(height.03.72)/length(height.03.72)
      grays.03.72 <- gray(1-rel.03.72)

###PLOT 3
barplot2(height.03.72, names.arg=c("", "", ""), ylim=c(0,200),
      col=c("Black", "Black", "Black"), xlim=c(0,5), width=.5)


##RXT1604 Graphs##

#24 Hours
attach(rxt1604.24)
      height.04.24 <- tapply(Ammonia, Temp, mean)
      lower.04.24 <- tapply(Ammonia, Temp, sd)
      upper.04.24 <- tapply(Ammonia, Temp, sd)
      rel.04.24 <- rank(height.04.24)/length(height.04.24)
      grays.04.24 <- gray(1-rel.04.24)

###PLOT 4
barplot2(height.04.24, plot.ci=TRUE, ci.l=height.04.24,
      ci.u=height.04.24+upper.04.24, ylim=c(0,250),   names.arg=c("",
"", ""), ylab="Ammonia (ppm)",      col=c("Black", "Black", "Black"),
xlim=c(0,5), width=0.5)

#48 Hours
attach(rxt1604.48)
      height.04.48 <- tapply(Ammonia, Temp, mean)
      lower.04.48 <- tapply(Ammonia, Temp, sd)
      upper.04.48 <- tapply(Ammonia, Temp, sd)
      rel.04.48 <- rank(height.04.48)/length(height.04.48)
      grays.04.48 <- gray(1-rel.04.48)

###PLOT 5
barplot2(height.04.48, plot.ci=TRUE, ci.l=height.04.48,
      ci.u=height.04.48+upper.04.48, names.arg=c("", "", ""),
      ylim=c(0,250), col=c("Black", "Black", "Black"),
      xlim=c(0,5), width=.5)

#72 Hours
attach(rxt1604.72)
      height.04.72 <- tapply(Ammonia, Temp, mean)
      rel.04.72 <- rank(height.04.72)/length(height.04.72)
      grays.04.72 <- gray(1-rel.04.72)

###PLOT 6
barplot2(height.04.72, names.arg=c("", "", ""), ylim=c(0,250),
      col=c("Black", "Black", "Black"), xlim=c(0,5), width=.5)
```

```
##RXT1605 Graphs##

#24 Hours
attach(rxt1605.24)
      height.05.24 <- tapply(Ammonia, Temp, mean)
      lower.05.24 <- tapply(Ammonia, Temp, sd)
      upper.05.24 <- tapply(Ammonia, Temp, sd)
      rel.05.24 <- rank(height.05.24)/length(height.05.24)
      grays.05.24 <- gray(1-rel.05.24)

###PLOT 7
barplot2(height.05.24, plot.ci=TRUE, ci.l=height.05.24,
      ci.u=height.05.24+upper.05.24, ylim=c(0,400),   names.arg=c("40",
"50", "60"), ylab="Ammonia (ppm)",  col=c("Black", "Black", "Black"),
xlim=c(0,5), width=.5)

#48 Hours
attach(rxt1605.48)
      height.05.48 <- tapply(Ammonia, Temp, mean)
      lower.05.48 <- tapply(Ammonia, Temp, sd)
      upper.05.48 <- tapply(Ammonia, Temp, sd)
      rel.05.48 <- rank(height.05.48)/length(height.05.48)
      grays.05.48 <- gray(1-rel.05.48)

###PLOT 8
barplot2(height.05.48, plot.ci=TRUE, ci.l=height.05.48,
      ci.u=height.05.48+upper.05.48, names.arg=c("40", "50",
      "60"), ylim=c(0,400), col=c("Black", "Black", "Black"),
      xlim=c(0,5), width=.5)

#72 Hours
attach(rxt1605.72)
      height.05.72 <- tapply(Ammonia, Temp, mean)
      rel.05.72 <- rank(height.05.72)/length(height.05.72)
      grays.05.72 <- gray(1-rel.05.72)

###PLOT 9
barplot2(height.05.72, names.arg=c("40", "50", "60"),
      ylim=c(0,400), col=c("black","black", "black"),
      xlim=c(0,5), width=0.5)
```

R Script for Ammonia Model

```
model <-    read.table("C:\\Users\\rharv_000\\Dropbox\\#GRAD_SCHOOL_NEW
      \\R Projects\\Data\\AmmoniaModel.txt", header=TRUE)
#model imported

#pH predicts ammonia pH = x ammonia = y
# lm(y~x)

##This performs a simple linear regression on the untransformed ##data
model_reg <- lm(Ammonia ~ pH, data=model)
      summary(model_reg)
      plot(residuals(model_reg))

##This performs a polynomial regression, with ln() transformed
##ammonia
model_ln <- lm(log(Ammonia) ~ poly(pH,2,raw=TRUE), data=model)
      summary(model_ln)
      plot(residuals(model_ln))

##This performs a polynomial regression, with log10() transformed
ammonia
##THIS ONE IS THE ONE BEING USED
model_log <- lm(log10(Ammonia)~poly(pH,2,raw=TRUE), data =
      model)
      summary(model_log)
      plot(residuals(model_log))


##Construction of graph to validate model
graph <-    read.table("C:\\Users\\rjh346\\Dropbox\\00_GRAD_SCHOOL_NEW\
      \R Projects\\Amm_Model\\Model Stats.txt",header=TRUE)
days <- c(2,3,4,5,6,7)

actual <- subset(graph, Method=="Actual")
model <- subset(graph, Method=="Model")

plot(days, actual$Mean, pch=15, type="o", ann=FALSE, lwd=2,
      lty=1, ylim=c(0,250))
      lines(days, model$Mean, pch=16, type="o", lwd=2, lty=2)
      arrows(days, actual$Mean-actual$SD, days,
            actual$Mean+actual$SD, angle=90, code=3, length=0.05,
            lty=1)
      arrows(days, model$Mean-model$SD, days,
      model$Mean+model$SD, angle=90, code=3, length=0.05,
      lty=2)
      legend("bottomright", c("Adj R-Squared = 0.9854"), bty="n")
      legend("topright", c("Actual", "Model"), pch=c(15,16),
      lwd=c(2,2), lty=c(1,2), bty="n")
```

Selected Model Output:

```
Call:
lm(formula = log10(Ammonia) ~ poly(pH, 2, raw = TRUE), data = model)
Residuals:
     Min       1Q   Median       3Q      Max
-0.13962 -0.04377 -0.01988  0.03919  0.17086
```

```
Coefficients:
                         Estimate Std. Error t value Pr(>|t|)
(Intercept)              -6.81170    0.45706 -14.903  < 2e-16 ***
poly(pH, 2, raw = TRUE)1  1.99691    0.15340  13.018  < 2e-16 ***
poly(pH, 2, raw = TRUE)2 -0.10297    0.01272  -8.098 1.18e-10 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Residual standard error: 0.06129 on 50 degrees of freedom
Multiple R-squared:  0.9902,        Adjusted R-squared:  0.9898
F-statistic:  2529 on 2 and 50 DF,  p-value: < 2.2e-16
```

R Script for Logistic Regressions

```
heat <-     read.table("C:\\Users\\rharv\\Dropbox\\00_GRAD_SCHOOL_NEW\\
      R Projects\\AMM Experiments\\ATM Results\\Heat Data.txt",
      header=TRUE)
heatModel <- glm(Survival~Temp+Wash+Time, data=heat,
      family=binomial(link="logit"))
      summary(heatModel)
      anova(heatModel, test="Chisq")
```

Appendix B Supplemental Information for Chapter 3

Procedure for creating ammonia plates

1. Tape out treatment grid on bench top labeling rows and columns with respective pH and NH_4Cl
2. Place 1000 mL E-flasks in each treatment square
3. Fill each flask with 500 mL water and etch the glass at the line (sharpie rubs off in autoclave)
4. Reuse water for step 3, checking in between each flask with a graduated cylinder, adding water as needed to maintain 500 mL
5. Begin preparation of the stock solutions
    a. Glucose
        i. Add 15 g to each flask
    b. Yeast Extract
        i. Dissolve 3 g YE into ~80 mL water, add to 120 mL
        ii. Add 4 mL to each flask
    c. Tyrosine
        i. Add 40 mg to each flask
    d. MES (10x stock (0.55 M) - comes out to a 55 mM concentration per treatment)
        i. Dissolve 32.5 g into ~ 200 mL water (prepare as many solutions as pH treatments)
        ii. Titrate using HCl to desired pH
        iii. Add water up to 300 mL
        iv. Add 50 mL to each flask in the corresponding pH treatment
6. Add 10 g agar to each flask
7. Add milli-q water to the 400 mL mark on each flask
8. Autoclave for 15 minutes on slow exhaust, also autoclave 3L of sterile water
9. NH4Cl - after flasks cool slightly
    a. Dissolve 167.6 g into ~1400 mL water, fill to 2000 mL
        i. Probable break this into smaller amounts?
        ii. Actually, it seems I only need to make 500
    b. Using filter sterilizer:
    c. Add amount indicated based on concentration
        i. 100 mM - 32 mL
        ii. 75 mM - 24 mL
        iii. 50 mM - 16 mL
        iv. 25 mM - 8 mL
10. Fill what volume remains to the 500 mL etch mark with the sterile water
11. Pour

Protocol for setting up ammonia jars

High Concentration

1. Make up combined buffer solution
    A. Na2CO3 (1M)
        159g / 1500 mL
    B. NaHCO3 (1M)
        84g / 1000 mL
    C. Makes 2500 total
        i. 500 for NH4Cl Buffer
        Ii. 2000 for jars
2. Make stock solution of NH4Cl
    500 mL total
    1 M
    26.7 g NH4Cl into 500 mL Buffer
3. Mix all together, add amount to jar
    Control: 50 mL
    100 mM: 45 mL
    300 mM: 35 mL

Low Concentration

Buffer

- 63.6 g Na2CO3
- 33.6 g NaHCO3
- 1000 mL total volume

Ammonia Buffer

- 0.6675 g NH4Cl
- 15.9 g Na2CO3
- 12.6 g NaHCO3
- 250 mL total volume

Jar Prep

- 0 mM
    - 50 mL buffer
    - 0 mL NH4
- 1 mM
    - 49 mL buffer
    - 1 mL NH4
- 5 mM
    - 45 mL buffer
    - 5 mL NH4
- 20 mM
    - 30 mL buffer
    - 20 mL NH4

Equations and Derivatives

Replicate 1

5 Days

$$f(PA1) = 3.4879e^{-15x} + 22.9855e^{-0.1x} - 13.3264$$
$$f'(PA1) = -52.3185e^{-15x} - 2.2985e^{-0.1x}$$

$$f(CT1) = 2.7985e^{-70x} + 193.6267e^{-0.05x} - 181.3052$$
$$f'(CT1) = -195.9e^{-70x} - 9.681e^{-0.05x}$$

$$f(261) = 2.366e^{-60x} + 134.6e^{-0.05x} - 123.0855$$
$$f'(261) = -141.96e^{-60x} - 6.73e^{-0.05x}$$

$$f(G2) = 0.8092e^{-80x} + 159.883e^{-0.05x} - 148.9751$$
$$f'(G2) = -64.736e^{-80x} - 7.994e^{-0.05x}$$

$$f(78) = 2.236e^{-60x} + 171e^{-0.05x} - 161.858$$
$$f'(78) = -134.16e^{-60x} - 8.587e^{-0.05x}$$

$$f(55) = 0.9425e^{-80x} + 198.8564e^{-0.05x} - 187.9187$$
$$f'(55) = -56.55e^{-80x} - 9.943e^{-0.05x}$$

10 Days

$$f(PA1) = 4.4828e^{-60x} + 255.7091e^{-0.05x} - 234.7688$$
$$f'(PA1) = -268.968e^{-60x} - 12.7854e^{-0.05x}$$

$$f(CT1) = 5.5343e^{-60x} + 287.5745e^{-0.05x} - 264.6597$$
$$f'(CT1) = -336.06e^{-60x} - 14.379e^{-0.05x}$$

$$f(261) = 5.1578e^{-60x} + 196.9728e^{-0.05x} - 175.9598$$
$$f'(261) = -309.47e^{-60x} - 9.8486e^{-0.05x}$$

$$f(G2) = 2.1548 + 301.9502e^{-0.05x} - 280.6996$$
$$f'(G2) = -129.288e^{-60x} - 15.097e^{-0.05x}$$

$$f(78) = 5.6838e^{-60x} + 314.2011e^{-0.05x} - 295.2530$$
$$f'(78) = -341.028e^{-60x} - 15.710e^{-0.05x}$$

$$f(55) = 3.2598e^{-60x} + 365.5106e^{-0.05x} - 344.3917$$
$$f'(55) = -195.59e^{-60x} - 18.276e^{-0.05x}$$

Replicate 1

15 Days

$$f(PA1) = 7.8078e^{-60x} + 202.0835e^{-0.05x} - 172.4556$$
$$f'(PA1) = -468.468e^{-60x} - 10.104e^{-0.05x}$$

$$f(CT1) = 6.0847e^{-60x} + 394.5625e^{-0.05x} - 360.4116$$
$$f'(CT1) = -365.082e^{-60x} - 19.728e^{-0.05x}$$

$$f(261) = 6.93e^{-60x} + 254.3600e^{-0.05x} - 223.8194$$
$$f'(261) = -415.8e^{-60x} - 12.718e^{-0.05x}$$

$$f(78) = 6.7901e^{-60x} + 466.9746e^{-0.05x} - 439.0443$$
$$f'(78) = -407.41e^{-60x} - 23.349e^{-0.05x}$$

$$f(G2U1^{*}) = 5.788e^{-60x} + 513.312e^{-0.05x} - 483.306$$
$$f'(G2U1) = -347.28e^{-60x} - 25.66e^{-0.05x}$$

$$f(G2U2^{*}) = 3.426e^{-60x} + 429.899e^{-0.05x} - 399.314$$
$$f'(G2U2) = -205.56e^{-60x} - 21.495e^{-0.05x}$$

*Due to a records error it was impossible to determine G2 and 55 from each other, thus the designations

Replicate 2

5 Days

$$f(PA1) = 2.0546e^{-60x} + 64.4767e^{-0.05x} - 53.3926$$
$$f'(PA1) = -123.276e^{-60x} - 3.224e^{-0.05x}$$

$$f(CT1) = 2.3947e^{-60x} + 79.5439e^{-0.05x} - 67.9291$$
$$f'(CT1) = -143.682e^{-60x} - 3.978e^{-0.05x}$$

$$f(261) = 1.8210e^{-60x} + 300.4881e^{-0.01x} - 288.9609$$
$$f'(261) = -109.26e^{-60x} - 3.005e^{-0.01x}$$

$$f(G2) = 1.7108e^{-60x} + 52.9859e^{-0.05x} - 43.6792$$
$$f'(G2) = -102.65e^{-60x} - 2.649e^{-0.05x}$$

$$f(78) = 2.1376e^{-60x} + 87.7253e^{-0.05x} - 78.6349$$
$$f'(78) = -128.26e^{-60x} - 4.386e^{-0.05x}$$

$$f(55) = 2.0683e^{-60x} + 101.4355e^{-0.05x} - 93.3475$$
$$f'(55) = -124.10e^{-60x} - 5.0718e^{-0.05x}$$

10 Days

$$f(PA1) = 3.9861e^{-60x} + 89.2136e^{-0.05x} - 68.3026$$
$$f'(PA1) = -239.2e^{-60x} - 4.461e^{-0.05x}$$

$$f(CT1) = 4.7690e^{-60x} + 129.3826e^{-0.05x} - 106.2082$$
$$f'(CT1) = -286.14e^{-60x} - 6.469e^{-0.05x}$$

$$f(261) = 4.8787e^{-60x} + 83.1555e^{-0.05x} - 62.1312$$
$$f'(261) = -292.72e^{-60x} - 4.158e^{-0.05x}$$

$$f(G2) = 4.8608e^{-60x} + 86.4410e^{-0.05x} - 67.9297$$
$$f'(G2) = -291.65e^{-60x} - 4.322e^{-0.05x}$$

$$f(78) = 5.5977e^{-60x} + 129.2656e^{-0.05x} - 111.1925$$
$$f'(78) = -335.86e^{-60x} - 6.463e^{-0.05x}$$

$$f(55) = 5.5830e^{-60x} + 142.9060e^{-0.05x} - 124.3907$$
$$f'(55) = -334.98e^{-60x} - 7.145e^{-0.05x}$$

Replicate 2

15 Days

$$f(PA1) = 5.6094e^{-60x} + 103.0802e^{-0.05x} - 71.9230$$
$$f'(PA1) = -336.56e^{-60x} - 5.154e^{-0.05x}$$

$$f(CT1) = 4.663e^{-60x} + 183.917e^{-0.05x} - 148.356$$
$$f'(CT1) = -279.78e^{-60x} - 9.196e^{-0.05x}$$

$$f(261) = 6.4931e^{-60x} + 113.9508e^{-0.05x} - 82.1445$$
$$f'(261) = -389.59e^{-60x} - 5.698e^{-0.05x}$$

$$f(G2) = 7.266e^{-60x} + 116.317e^{-0.05x} - 88.944$$
$$f'(G2) = -435.96e^{-60x} - 5.816e^{-0.05x}$$

$$f(78) = 8.2688e^{-60x} + 184.7739e^{-0.05x} - 158.1214$$
$$f'(78) = -496.13e^{-60x} - 9.2387e^{-0.05x}$$

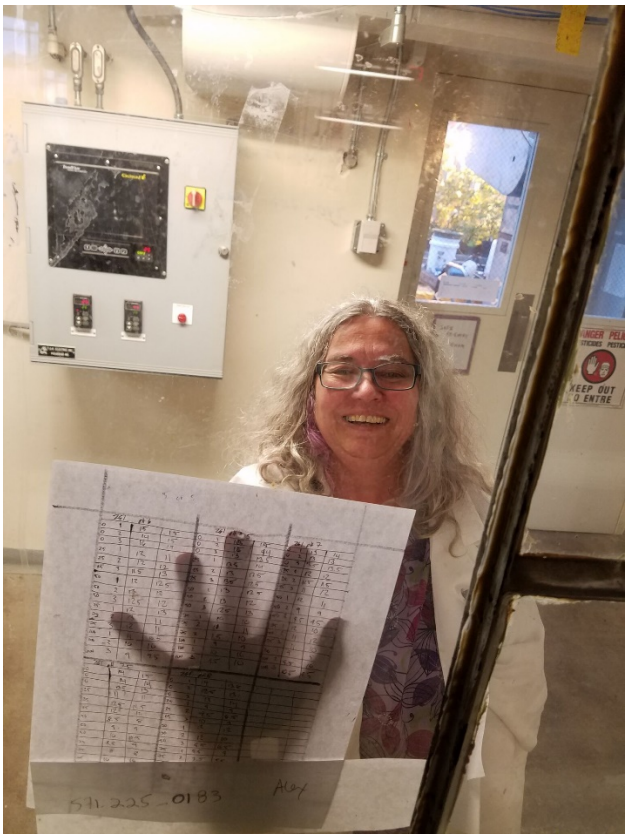
$$f(55) = 8.381e^{-60x} + 191.497e^{-0.05x} - 164.560$$
$$f'(55) = -502.86e^{-60x} - 9.575e^{-0.05x}$$

Model R^2 Values

	Replicate 1		
	5 Days	10 Days	15 Days
PA1	0.608	0.7179	0.8237
CT1	0.8014	0.8595	0.8532
261	0.7477	0.8652	0.8634
G2	0.7426	0.7712	0.8439
78	0.7986	0.8987	0.8611
55	0.7209	0.7868	0.8393
	Replicate 2		
	5 Days	10 Days	15 Days
PA1	0.6359	0.7035	0.7976
CT1	0.8285	0.7951	0.8596
261	0.6182	0.7978	0.8462
G2	0.8505	0.8366	0.8688
78	0.8527	0.7764	0.855
55	0.8526	0.8418	0.8298

Fig. B-1

Highly sophisticated data transfer method utilized to transfer data out of the Ft. Detrick Foreign Disease and Weed Research Unit hot zone to cold zone.



Calculated Derivative Values

Replicate 1						
	5 Days		10 Days		15 Days	
	Section 1	Section 2	Section 1	Section 2	Section 1	Section 2
PA1	-27.00	-2.49	-26.14	-12.56	-33.40	-9.929
CT1	-15.6	-9.51	-31.07	-14.13	-37.86	-19.38
261	-13.8	-6.61	-25.23	-9.678	-33.39	-12.50
G2	-9.159	-7.855	-21.50	-14.84	-43.57	-22.94
78	-15.24	-8.438	-32.65	-15.44	-42.88	-25.21
55	-10.45	-9.77	-27.97	-17.96	-31.68	-21.12
Replicate 2						
	5 Days		10 Days		15 Days	
	Section 1	Section 2	Section 1	Section 2	Section 1	Section 2
PA1	-9.354	-3.121	-16.359	-4.318	-21.897	-4.989
CT1	-11.122	-3.851	-20.699	-6.262	-23.102	-8.902
261	-8.443	-2.986	-18.721	-4.025	-25.080	-5.516
G2	-7.753	-2.564	-18.832	-4.184	-27.507	-5.630
78	-10.761	-4.246	-23.168	-6.256	-33.916	-8.943
55	-11.238	-4.910	-23.805	-6.916	-34.587	-9.269

Note: in replicate 1 the x values for section 1 and section 2 were 0.05 and 0.45 respectively. In replicate 2 these values were 0.05 and 0.65.

R Script for Ammonia Models

```
masterData <-
      read.table("C:\\Users\\rharv\\Dropbox\\00_GRAD_SCHOOL_NEW\\R
      Projects\\AMM Experiments\\Master Data Sheet.txt", header=TRUE)

##########Separation of Data into Components##########

#####By Experiment#####
      AMM1701 <- subset(masterData, Experiment=="AMM1701")
      AMM1702 <- subset(masterData, Experiment=="AMM1702")


#####By Experiment and Day#####
      AMM1701_5 <- subset(AMM1701, Day=="5")
      AMM1701_10 <- subset(AMM1701, Day=="10")
      AMM1701_15 <- subset(AMM1701, Day=="15")

      AMM1702_5 <- subset(AMM1702, Day=="5")
      AMM1702_10 <- subset(AMM1702, Day=="10")
      AMM1702_15 <- subset(AMM1702, Day=="15")

#####By Species#####
      AMM1701_Cap <- subset(AMM1701, Species=="Cap")
      AMM1701_Cah <- subset(AMM1701, Species=="Cah")

      AMM1702_Cap <- subset(AMM1702, Species=="Cap")
      AMM1702_Cah <- subset(AMM1702, Species=="Cah")

#####By Day and Species#####
      AMM1701_Cap_5 <- subset(AMM1701_Cap, Day=="5")
      AMM1701_Cap_10 <- subset(AMM1701_Cap, Day=="10")
      AMM1701_Cap_15 <- subset(AMM1701_Cap, Day=="15")
      AMM1701_Cah_5 <- subset(AMM1701_Cah, Day=="5")
      AMM1701_Cah_10 <- subset(AMM1701_Cah, Day=="10")
      AMM1701_Cah_15 <- subset(AMM1701_Cah, Day=="15")

      AMM1702_Cap_5 <- subset(AMM1702_Cap, Day=="5")
      AMM1702_Cap_10 <- subset(AMM1702_Cap, Day=="10")
      AMM1702_Cap_15 <- subset(AMM1702_Cap, Day=="15")
      AMM1702_Cah_5 <- subset(AMM1702_Cah, Day=="5")
      AMM1702_Cah_10 <- subset(AMM1702_Cah, Day=="10")
      AMM1702_Cah_15 <- subset(AMM1702_Cah, Day=="15")

#####By Isolate#####
      AMM1701_PA1 <- subset(AMM1701_Cap, Isolate=="PA1")
      AMM1701_CT1 <- subset(AMM1701_Cap, Isolate == "CT1")
      AMM1701_261<- subset(AMM1701_Cap, Isolate=="261")
      AMM1701_G2 <- subset(AMM1701_Cah, Isolate=="G2")
      AMM1701_78 <- subset(AMM1701_Cah, Isolate=="78")
      AMM1701_55 <- subset(AMM1701_Cah, Isolate=="55")
      AMM1701_G2U1 <- subset(AMM1701_Cah, Isolate=="G2_U1")
      AMM1701_G2U2 <- subset(AMM1701_Cah, Isolate=="G2_U2")

      AMM1702_PA1 <- subset(AMM1702_Cap, Isolate=="PA1")
      AMM1702_CT1 <- subset(AMM1702_Cap, Isolate=="CT1")
      AMM1702_261 <- subset(AMM1702_Cap, Isolate=="261")
```

```
      AMM1702_G2 <- subset(AMM1702_Cah, Isolate=="G2")
      AMM1702_78 <- subset(AMM1702_Cah, Isolate=="78")
      AMM1702_55 <- subset(AMM1702_Cah, Isolate=="55")

#####By Isolate and Day#####
      AMM1701_PA1_5 <- subset(AMM1701_PA1, Day=="5")
      AMM1701_PA1_10 <- subset(AMM1701_PA1, Day=="10")
      AMM1701_PA1_15 <- subset(AMM1701_PA1, Day=="15")
      AMM1701_CT1_5 <- subset(AMM1701_CT1, Day=="5")
      AMM1701_CT1_10 <- subset(AMM1701_CT1, Day=="10")
      AMM1701_CT1_15 <- subset(AMM1701_CT1, Day=="15")
      AMM1701_261_5 <- subset(AMM1701_261, Day=="5")
      AMM1701_261_10 <- subset(AMM1701_261, Day=="10")
      AMM1701_261_15 <- subset(AMM1701_261, Day=="15")
      AMM1701_G2_5 <- subset(AMM1701_G2, Day=="5")
      AMM1701_G2_10 <- subset(AMM1701_G2, Day=="10")
      AMM1701_78_5 <- subset(AMM1701_78, Day=="5")
      AMM1701_78_10 <- subset(AMM1701_78, Day=="10")
      AMM1701_78_15 <- subset(AMM1701_78, Day=="15")
      AMM1701_55_5 <- subset(AMM1701_55, Day=="5")
      AMM1701_55_10 <- subset(AMM1701_55, Day=="10")
      AMM1701_G2U1_15 <- subset(AMM1701_G2U1, Day=="15")
      AMM1701_G2U2_15 <- subset(AMM1701_G2U2, Day=="15")

      AMM1702_PA1_5 <- subset(AMM1702_PA1, Day=="5")
      AMM1702_CT1_5 <- subset(AMM1702_CT1, Day=="5")
      AMM1702_261_5 <- subset(AMM1702_261, Day=="5")
      AMM1702_PA1_10 <- subset(AMM1702_PA1, Day=="10")
      AMM1702_CT1_10 <- subset(AMM1702_CT1, Day=="10")
      AMM1702_261_10 <- subset(AMM1702_261, Day=="10")
      AMM1702_PA1_15 <- subset(AMM1702_PA1, Day=="15")
      AMM1702_CT1_15 <- subset(AMM1702_CT1, Day=="15")
      AMM1702_261_15 <- subset(AMM1702_261, Day=="15")

      AMM1702_G2_5 <- subset(AMM1702_G2, Day=="5")
      AMM1702_78_5 <- subset(AMM1702_78, Day=="5")
      AMM1702_55_5 <- subset(AMM1702_55, Day=="5")
      AMM1702_G2_10 <- subset(AMM1702_G2, Day=="10")
      AMM1702_78_10 <- subset(AMM1702_78, Day=="10")
      AMM1702_55_10 <- subset(AMM1702_55, Day=="10")
      AMM1702_G2_15 <- subset(AMM1702_G2, Day=="15")
      AMM1702_78_15 <- subset(AMM1702_78, Day=="15")
      AMM1702_55_15 <- subset(AMM1702_55, Day=="15")



#########AMM1701##########
#####Day 5#####
      model.1701.pa1.5 <- lm(Growth~exp(-15*Ammonia)+exp(-.10*Ammonia),
            data=AMM1701_PA1_5)
            summary(model.1701.pa1.5)
            with(AMM1701_PA1_5, plot(Ammonia,Growth,pch=0, ann=FALSE))
            points(AMM1701_PA1_5$Ammonia, predict(model.1701.pa1.5),
            pch=15)
            title(xlab = "Ammonia (mM)", ylab="Growth (mm)")
```

```
      legend("topright", c("Actual", "Predicted"), pch=c(0,15),
      bty="n")

      plot(model.1701.pa1.5)

model.1701.ct1.5 <- lm(Growth~exp(-70*Ammonia)+exp(-.05*Ammonia),
      data=AMM1701_CT1_5)
      summary(model.1701.ct1.5)
      with(AMM1701_CT1_5, plot(Ammonia,Growth,pch=0))
      points(AMM1701_CT1_5$Ammonia, predict(model.1701.ct1.5),
      pch=15)

model.1701.261.5 <- lm(Growth~exp(-60*Ammonia)+exp(-
      .05*Ammonia),data=AMM1701_261_5)
      summary(model.1701.261.5)
      with(AMM1701_261_5, plot(Ammonia, Growth, pch=0))
      points(AMM1701_261_5$Ammonia, predict(model.1701.261.5),
      pch=15)

model.1701.g2.5 <- lm(Growth~exp(-80*Ammonia)+exp(-.05*Ammonia),
      data=AMM1701_G2_5)
      summary(model.1701.g2.5)
      with(AMM1701_G2_5, plot(Ammonia, Growth, pch=0))
      points(AMM1701_G2_5$Ammonia, predict(model.1701.g2.5),
      pch=15)

model.1701.78.5 <- lm(Growth~exp(-60*Ammonia)+exp(-.05*Ammonia),
      data=AMM1701_78_5)
      summary(model.1701.78.5)
      with(AMM1701_78_5, plot(Ammonia, Growth, pch=0))
      points(AMM1701_78_5$Ammonia, predict(model.1701.78.5),
      pch=15)

model.1701.55.5 <- lm(Growth~exp(-80*Ammonia)+exp(-.05*Ammonia),
      data=AMM1701_55_5)
      summary(model.1701.55.5)
      with(AMM1701_55_5, plot(Ammonia, Growth, pch=0))
      points(AMM1701_55_5$Ammonia, predict(model.1701.55.5),
      pch=15)


#####1701 Day 10#####
model.1701.pa1.10 <- lm(Growth~exp(-60*Ammonia)+exp(-
      .05*Ammonia), data=AMM1701_PA1_10)
      summary(model.1701.pa1.10)
      with(AMM1701_PA1_10, plot(Ammonia, Growth, pch=0))
      points(AMM1701_PA1_10$Ammonia, predict(model.1701.pa1.10),
      pch=15)

model.1701.ct1.10 <- lm(Growth~exp(-60*Ammonia)+exp(-
      .05*Ammonia), data=AMM1701_CT1_10)
      summary(model.1701.ct1.10)
      with(AMM1701_CT1_10, plot(Ammonia, Growth, pch=0))
      points(AMM1701_CT1_10$Ammonia, predict(model.1701.ct1.10),
      pch=15)
```

```
      model.1701.261.10 <- lm(Growth~exp(-60*Ammonia)+exp(-
            .05*Ammonia), data=AMM1701_261_10)
            summary(model.1701.261.10)
            with(AMM1701_261_10, plot(Ammonia, Growth, pch=0))
            points(AMM1701_261_10$Ammonia, predict(model.1701.261.10),
            pch=15)

      model.1701.g2.10 <- lm(Growth~exp(-60*Ammonia)+exp(-.05*Ammonia),
            data=AMM1701_G2_10)
            summary(model.1701.g2.10)
            with(AMM1701_G2_10, plot(Ammonia, Growth, pch=0))
            points(AMM1701_G2_10$Ammonia, predict(model.1701.g2.10),
            pch=15)

      model.1701.78.10 <- lm(Growth~exp(-60*Ammonia)+exp(-.05*Ammonia),
            data=AMM1701_78_10)
            summary(model.1701.78.10)
            with(AMM1701_78_10, plot(Ammonia, Growth, pch=0,
                  ann=FALSE))
            points(AMM1701_78_10$Ammonia, predict(model.1701.78.10),
            pch=15)
            title(xlab="Ammonia (mM)", ylab="Growth (mm)")
            legend("topright", c("Actual", "Predicted"), pch=c(0,15),
            bty="n")
            plot(model.1701.78.10)

      model.1701.55.10 <- lm(Growth~exp(-60*Ammonia)+exp(-.05*Ammonia),
            data=AMM1701_55_10)
            summary(model.1701.55.10)
            with(AMM1701_55_10, plot(Ammonia, Growth, pch=0))
            points(AMM1701_55_10$Ammonia, predict(model.1701.55.10),
            pch=15)


###1701 15 Days###
      model.1701.pa1.15 <- lm(Growth~exp(-60*Ammonia)+exp(-
            .05*Ammonia), data=AMM1701_PA1_15)
            summary(model.1701.pa1.15)
            with(AMM1701_PA1_15, plot(Ammonia, Growth, pch=0))
            points(AMM1701_PA1_15$Ammonia, predict(model.1701.pa1.15),
            pch=15)

      model.1701.ct1.15 <- lm(Growth~exp(-60*Ammonia)+exp(-
            .05*Ammonia), data=AMM1701_CT1_15)
            summary(model.1701.ct1.15)
            with(AMM1701_CT1_15, plot(Ammonia, Growth, pch=0))
            points(AMM1701_CT1_15$Ammonia, predict(model.1701.ct1.15),
            pch=15)

      model.1701.261.15 <- lm(Growth~exp(-60*Ammonia)+exp(-
            .05*Ammonia), data=AMM1701_261_15)
            summary(model.1701.261.15)
            with(AMM1701_261_15, plot(Ammonia, Growth, pch=0))
            points(AMM1701_261_15$Ammonia, predict(model.1701.261.15),
            pch=15)
```

```
      model.1701.78.15 <- lm(Growth~exp(-60*Ammonia)+exp(-.05*Ammonia),
            data=AMM1701_78_15)
            summary(model.1701.78.15)
            with(AMM1701_78_15, plot(Ammonia, Growth, pch=0))
            points(AMM1701_78_15$Ammonia, predict(model.1701.78.15),
            pch=15)

      model.1701.g2u1.15 <- lm(Growth~exp(-60*Ammonia)+exp(-
                  .05*Ammonia), data=AMM1701_G2U1_15)
            summary(model.1701.g2u1.15)
            with(AMM1701_G2U1_15, plot(Ammonia, Growth, pch=0))
            points(AMM1701_G2U1_15$Ammonia,
                  predict(model.1701.g2u1.15),pch=15)

      model.1701.g2u2.15 <- lm(Growth~exp(-60*Ammonia)+exp(-
                  .05*Ammonia), data=AMM1701_G2U2_15)
            summary(model.1701.g2u2.15)
            with(AMM1701_G2U1_15, plot(Ammonia, Growth, pch=0))
            points(AMM1701_G2U2_15$Ammonia,
                  predict(model.1701.g2u2.15), pch=15)


######TTESTS######
###1701###
      cap.1701.s1.5 <- c(-27.00,-15.6,-13.8)
      cap.1701.s2.5 <- c(-2.49,-9.51,-6.61)

      cah.1701.s1.5 <- c(-9.159,-15.24,-10.95)
      cah.1701.s2.5 <- c(-7.855,-8.438,-9.77)

      wilcox.test(cap.1701.s1.5, cah.1701.s1.5)
      wilcox.test (cap.1701.s2.5, cah.1701.s2.5)

      cap.1701.s1.10 <- c(-26.14,-31.07,-25.23)
      cap.1701.s2.10 <- c(-12.56,-14.13,-9.678)

      cah.1701.s1.10 <- c(-21.50,-32.65,-27.97)
      cah.1701.s2.10 <- c(-14.84,-15.44,-17.96)

      wilcox.test (cap.1701.s1.10, cah.1701.s1.10)
      wilcox.test (cap.1701.s2.10, cah.1701.s2.10)

      cap.1701.s1.15 <- c(-33.40,-37.86,-33.39)
      cap.1701.s2.15 <- c(-9.929,-19.38,-12.50)

      cah.1701.s1.15 <- c(-43.57,-42.88,-31.68)
      cah.1701.s2.15 <- c(-22.94,-25.21,-21.12)

      wilcox.test (cap.1701.s1.15, cah.1701.s1.15)
      wilcox.test (cap.1701.s2.15, cah.1701.s2.15)
```

```
###########AMM1702##########
#####Day 5#####
      model.1702.pa1.5 <- lm(Growth~exp(-60*Ammonia)+exp(-.05*Ammonia),
            data=AMM1702_PA1_5)
            summary(model.1702.pa1.5)
            with(AMM1702_PA1_5, plot(Ammonia, Growth, pch=0))
            points(AMM1702_PA1_5$Ammonia, predict(model.1702.pa1.5),
            pch=15)

      model.1702.ct1.5 <- lm(Growth~exp(-60*Ammonia)+exp(-.05*Ammonia),
            data=AMM1702_CT1_5)
            summary(model.1702.ct1.5)
            with(AMM1702_CT1_5, plot(Ammonia, Growth, pch=0))
            points(AMM1702_CT1_5$Ammonia, predict(model.1702.ct1.5),
            pch=15)

      model.1702.261.5 <- lm(Growth~exp(-60*Ammonia)+exp(-.01*Ammonia),
            data=AMM1702_261_5)
            summary(model.1702.261.5)
            with(AMM1702_261_5, plot(Ammonia, Growth, pch=0))
            points(AMM1702_261_5$Ammonia, predict(model.1702.261.5),
            pch=15)

      model.1702.g2.5 <- lm(Growth~exp(-60*Ammonia)+exp(-.05*Ammonia),
            data=AMM1702_G2_5)
            summary(model.1702.g2.5)
            with(AMM1702_G2_5, plot(Ammonia, Growth, pch=0))
            points(AMM1702_G2_5$Ammonia, predict(model.1702.g2.5),
            pch=15)

      model.1702.78.5 <- lm(Growth~exp(-60*Ammonia)+exp(-.05*Ammonia),
            data=AMM1702_78_5)
            summary(model.1702.78.5)
            with(AMM1702_78_5, plot(Ammonia, Growth, pch=0))
            points(AMM1702_78_5$Ammonia, predict(model.1702.78.5),
            pch=15)

      model.1702.55.5 <- lm(Growth~exp(-60*Ammonia)+exp(-.05*Ammonia),
            data=AMM1702_55_5)
            summary(model.1702.55.5)
            with(AMM1702_55_5, plot(Ammonia, Growth, pch=0))
            points(AMM1702_55_5$Ammonia, predict(model.1702.55.5),
            pch=15)


#####Day 10#####
      model.1702.pa1.10 <- lm(Growth~exp(-60*Ammonia)+exp(-
            .05*Ammonia), data=AMM1702_PA1_10)
            summary(model.1702.pa1.10)
            with(AMM1702_PA1_10, plot(Ammonia, Growth, pch=0))
            points(AMM1702_PA1_10$Ammonia, predict(model.1702.pa1.10),
            pch=15)

      model.1702.ct1.10 <- lm(Growth~exp(-60*Ammonia)+exp(-
            .05*Ammonia), data=AMM1702_CT1_10)
            summary(model.1702.ct1.10)
            with(AMM1702_CT1_10, plot(Ammonia, Growth, pch=0))
```

```
      points(AMM1702_CT1_10$Ammonia, predict(model.1702.ct1.10),
      pch=15)

model.1702.261.10 <- lm(Growth~exp(-60*Ammonia)+exp(-
      .05*Ammonia), data=AMM1702_261_10)
      summary(model.1702.261.10)
      with(AMM1702_261_10, plot(Ammonia, Growth, pch=0))
      points(AMM1702_261_10$Ammonia, predict(model.1702.261.10),
      pch=15)

model.1702.g2.10 <- lm(Growth~exp(-60*Ammonia)+exp(-.05*Ammonia),
      data=AMM1702_G2_10)
      summary(model.1702.g2.10)
      with(AMM1702_G2_10, plot(Ammonia, Growth, pch=0))
      points(AMM1702_G2_10$Ammonia, predict(model.1702.g2.10),
      pch=15)

model.1702.78.10 <- lm(Growth~exp(-60*Ammonia)+exp(-.05*Ammonia),
      data=AMM1702_78_10)
      summary(model.1702.78.10)
      with(AMM1702_78_10, plot(Ammonia, Growth, pch=0))
      points(AMM1702_78_10$Ammonia, predict(model.1702.78.10),
      pch=15)

model.1702.55.10 <- lm(Growth~exp(-60*Ammonia)+exp(-.05*Ammonia),
      data=AMM1702_55_10)
      summary(model.1702.55.10)
      with(AMM1702_55_10, plot(Ammonia, Growth, pch=0))
      points(AMM1702_55_10$Ammonia, predict(model.1702.55.10),
      pch=15)


#####15 Days#####
model.1702.pa1.15 <- lm(Growth~exp(-60*Ammonia)+exp(-
      .05*Ammonia), data=AMM1702_PA1_15)
      summary(model.1702.pa1.15)
      with(AMM1702_PA1_15, plot(Ammonia, Growth, pch=0))
      points(AMM1702_PA1_15$Ammonia, predict(model.1702.pa1.15),
      pch=15)

model.1702.ct1.15 <- lm(Growth~exp(-60*Ammonia)+exp(-
      .05*Ammonia), data=AMM1702_CT1_15)
      summary(model.1702.ct1.15)
      with(AMM1702_CT1_15, plot(Ammonia, Growth, pch=0))
      points(AMM1702_CT1_15$Ammonia, predict(model.1702.ct1.15),
      pch=15)

model.1702.261.15 <- lm(Growth~exp(-60*Ammonia)+exp(-
      .05*Ammonia), data=AMM1702_261_15)
      summary(model.1702.261.15)
      with(AMM1702_261_15, plot(Ammonia, Growth, pch=0))
      points(AMM1702_261_15$Ammonia, predict(model.1702.261.15),
      pch=15)

model.1702.g2.15 <- lm(Growth~exp(-60*Ammonia)+exp(-.05*Ammonia),
      data=AMM1702_G2_15)
      summary(model.1702.g2.15)
```

```
            with(AMM1702_G2_15, plot(Ammonia, Growth, pch=0))
            points(AMM1702_G2_15$Ammonia, predict(model.1702.g2.15),
            pch=15)

      model.1702.78.15 <- lm(Growth~exp(-60*Ammonia)+exp(-.05*Ammonia),
            data=AMM1702_78_15)
            summary(model.1702.78.15)
            with(AMM1702_78_15, plot(Ammonia, Growth, pch=0))
            points(AMM1702_78_15$Ammonia, predict(model.1702.78.15),
            pch=15)

      model.1702.55.15 <- lm(Growth~exp(-60*Ammonia)+exp(-.05*Ammonia),
            data=AMM1702_55_15)
            summary(model.1702.55.15)
            with(AMM1702_55_15, plot(Ammonia, Growth, pch=0))
            points(AMM1702_55_15$Ammonia, predict(model.1702.55.15),
            pch=15)

#####T Tests#####
      cap.1702.s1.5 <- c(-9.354, -11.122, -8.443)
      cap.1702.s2.5 <- c(-3.121, -3.851, -2.986)
      cah.1702.s1.5 <- c(-7.753, -10.761, -11.238)
      cah.1702.s2.5 <- c(-2.564, -4.246, -4.910)

      wilcox.test (cap.1702.s1.5, cah.1702.s1.5)
      wilcox.test (cap.1702.s2.5, cah.1702.s2.5)

      cap.1702.s1.10 <- c(-16.359, -20.699, -18.721)
      cap.1702.s2.10 <- c(-4.318, -6.262, -4.025)
      cah.1702.s1.10 <- c(-18.832, -23.168, -23.805)
      cah.1702.s2.10 <- c(-4.184, -6.256, -6.916)

      wilcox.test (cap.1702.s1.10, cah.1702.s1.10)
      wilcox.test (cap.1702.s2.10, cah.1702.s2.10)

      cap.1702.s1.15 <- c(-21.897,-23.102, -25.080)
      cap.1702.s2.15 <- c(-4.989, -8.902, -5.516)
      cah.1702.s1.15 <- c(-27.507, -33.916, -34.587)
      cah.1702.s2.15 <- c(-5.630, -8.943, -9.269)

      wilcox.test (cap.1702.s1.15, cah.1702.s1.15)
      wilcox.test (cap.1702.s2.15, cah.1702.s2.15)


######Importation of Derivative Data#####
######Obtained using the models above####

derivatives <-
      read.table("C:\\Users\\rjh346\\Dropbox\\00_GRAD_SCHOOL_NEW\\R
      Projects\\AMM Experiments\\AMM Master\\Derv ANOVA.txt",
      header=TRUE)
cap1701 <-
      read.table("C:\\Users\\rjh346\\Dropbox\\00_GRAD_SCHOOL_NEW\\R
      Projects\\AMM Experiments\\AMM Master\\1701 Cap.txt",
      header=TRUE)

der1701 <- subset(derivatives, Experiment=="1701")
```

```
der1701.cap <- subset(der1701, Species=="Cap")
der1701.cah <- subset(der1701, Species=="Cah")

der1702 <- subset(derivatives, Experiment=="1702")
der1702.cap <- subset(der1702, Species=="Cap")
der1702.cah <- subset(der1702, Species=="Cah")

#####ANOVA comparing the species#####

med1way(S1~Day, data=der1701.cap)
med1way(S2~Day, data=der1701.cap)


med1way(S1~Day, data=der1701.cah)
med1way(S2~Day, data=der1701.cah)


med1way(S1~Day, data=der1702.cap)
med1way(S2~Day, data=der1702.cap)


med1way(S1~Day, data=der1702.cah)
med1way(S2~Day, data=der1702.cah)

#####Preparation for graph creation#####
#####Saving standard deviations to variables#####
      der.1701.cap.sd <- c(7.16, 3.14, 2.58)
      der.1702.cap.sd.s1 <- c(1.36, 2.17, 1.61)
      der.1701.cah.sd.s1 <- c(3.12, 5.60, 6.67)
      der.1702.cah.sd.s1 <- c(1.89, 2.17, 1.61)

      der.1702.cap.sd.s2 <- c(0.47, 1.22, 2.12)
      der.1701.cap.sd.s2 <- c(3.53, 2.26, 4.89)
      der.1702.cah.sd.s2 <- c(1.21, 1.42, 2.01)
      der.1701.cah.sd.s2 <- c(0.981, 1.66, 2.05)


      day <- c(5,10,15)


      cap1701s1 <- c(-18.8, -27.48, -34.9)
      cap1702s1 <- c(-9.64, -18.6, -23.4)
      cah1701s1 <- c(-11.8, -27.4, -39.4)
      cah1702s1 <- c(-9.92, -21.9, -32.0)

      range.s1 <- range(0, cap1701s1, cap1702s1, cah1701s1, cah1702s1)

      plot(day,cap1701s1, type="o", ann=FALSE, axes=FALSE, lwd=2,
            ylim=c(-50,0), pch=16)
            lines(day,cap1702s1, type="o", lty=2, lwd=2, pch=16)
            lines(day,cah1701s1, type="o", lty=1, pch=17, lwd=2)
            lines(day,cah1702s1, type="o", lty=2, pch=17, lwd=2)
            axis(1, at=c(5,10,15), lab=c("5 Days", "10 Days", "15
                  Days"))
            axis(2, las=1, at=5*0:range.s1)
            box()
            title(ylab="mm/mM Ammonia")
```

```
      legend("bottomleft", c("Cap 1701", "Cap 1702", "Cah 1701",
            "Cah 1702"),
            pch=c(16,16,17,17), lty=c(1,2,1,2), bty="n")

      arrows(day, cap1701s1-der.1701.cap.sd, day,
            cap1701s1+der.1701.cap.sd, length=0.05, angle=90,
            code=3, lty=1)
      arrows(day, cap1702s1-der.1702.cap.sd.s1, day,
            cap1702s1+der.1702.cap.sd.s1, length=0.05, angle=90,
            code=3,  lty=2)
      arrows(day, cah1701s1-der.1701.cah.sd.s1, day,
            cah1701s1+der.1701.cah.sd.s1, length=0.05, angle=90,
            code=3, lty=1)
      arrows(day, cah1702s1-der.1702.cah.sd.s1, day,
            cah1702s1+der.1702.cah.sd.s1, length=0.05, angle=90,
            code=3, lty=2)



cap1701s2 <- c(-6.14, -12.12, -13.9)
cap1702s2 <- c(-3.32, -4.87, -6.47)
cah1701s2 <- c(-8.69, -16.1, -23.1)
cah1702s2 <- c(-3.91, -5.78, -7.95)

range.s2 <- range(0, cap1701s2, cap1702s2, cah1701s2, cah1702s2,-
      30)

plot(day,cap1701s2, type="o", ann=FALSE,  ylim=c(-30,0),
      axes=FALSE,       lwd=2, pch=16)
lines(day,cap1702s2, type="o", lty=2, lwd=2, pch=16)
lines(day,cah1701s2, type="o", lty=1, pch=17, lwd=2)
lines(day,cah1702s2, type="o", lty=2, pch=17, lwd=2)
points(c(-27,-26.14,-33.4), pch=1)
axis(1, at=c(5,10,15), lab=c("5 Days", "10 Days", "15 Days"))
axis(2, las=1, at=5*0:range.s2)
box()
title(ylab="mm/mM Ammonia")
legend("bottomleft", c("Cap 1701", "Cap 1702", "Cah 1701", "Cah
      1702"), pch=c(16,16,17,17), lty=c(1,2,1,2), bty="n")

arrows(day, cap1701s2-der.1701.cap.sd.s2, day,
      cap1701s2+der.1701.cap.sd.s2, length=0.05, angle=90,
      code=3, lty=1)
arrows(day, cap1702s2-der.1702.cap.sd.s2, day,
      cap1702s2+der.1702.cap.sd.s2, length=0.05, angle=90,
      code=3,  lty=2)
arrows(day, cah1701s2-der.1701.cah.sd.s2, day,
      cah1701s2+der.1701.cah.sd.s2, length=0.05, angle=90,
      code=3,  lty=1)
arrows(day, cah1702s2-der.1702.cah.sd.s2, day,
      cah1702s2+der.1702.cah.sd.s2, length=0.05, angle=90,
      code=3,  lty=2)

range.com <- range(0, cap1701s2, cap1702s2, cah1701s2, cah1702s2,
      cap1701s1, cap1702s1, cah1701s1, cah1702s1,-50)
```

```
plot(cap1701s1, type="o", ann=FALSE, ylim=range.com, pch=0,
            lwd=2, axes=FALSE)
            lines(cap1702s1, type="o", pch=15, lwd=2)
            lines(cah1701s1, type="o", pch=1, lwd=2)
            lines(cah1702s1, type="o", pch=16, lwd=2)
            lines(cap1701s2, type="o", lty=5, pch=0, lwd=2)
            lines(cap1702s2, type="o", lty=5, pch=15,  lwd=2)
            lines(cah1701s2, type="o", lty=5, pch=1,  lwd=2)
            lines(cah1702s2, type="o", lty=5, pch=16)
            axis(1, at=1:3, lab=c("5 Days", "10 Days", "15 Days"))
            axis(2, las=1, at=4*0:range.com)
            box()
            title(ylab="-mm/mM Ammonia")

            #legend("bottomleft", c("Cap 1701 S1", "Cap 1702 S1", "Cah
                  #1701 S1", "Cah 1702 S1"),col=c("blue", "blue",
                        #"red","red"), pch=c(0,15,1,16),
lty=c(1,1,1,1),                           #bty="n")

            legend("bottom", c("Cap 1701 S2", "Cap 1702 S2", "Cah 1701
                  S2", "Cah 1702 S2"), pch=c(0,15,1,16),
lty=c(5,5,5,5),         bty="n")
```

R Script for Ammonia Logistic Regression and Compost Chi Sq Comparison

```
logReg <-   read.table("C:\\Users\\rharv\\Dropbox\\00_GRAD_SCHOOL_NEW\\
      R Projects\\AMM Experiments\\ATM Results\\Atm Log Reg.txt",
      header=TRUE)

log <- glm(Survival~Treatment, data=logReg,
            family=binomial(link="logit"))
      summary(log)
      anova(log, test = "Chisq")
```

Comparison between gaseous survival and compost survival

```
ppm20 <- c(7,8)
compost <- c(4,11)
ppm5 <- c(10,4)

tbl <- as.data.frame(rbind(ppm20,compost))

names(tbl) <- c("Alive", "Dead")

chisq.test(tbl)


tbl2 <- as.data.frame(rbind(ppm5,compost))

names(tbl2) <- c("Alive","Dead")

chisq.test(tbl2)
```

Appendix C Supplemental Information for Chapter 4

R Script used to compile figures for Chapter 4

```
dataAll <- read.table("C:\\Users\\rharv\\Dropbox\\00_GRAD_SCHOOL_NEW\\R
Projects\\Wetlands\\WT All Data.txt", header=TRUE)

##########Data Subsetting##########
      #####By Day#####
            day3.all <- subset(dataAll, Day==3)
            day5.all <- subset(dataAll, Day==5)
            day7.all <- subset(dataAll, Day==7)
      #####By Treatment/Day#####
            day3.all.gravel <- subset(day3.all, Treatment=="Gravel")
            day3.all.plant <- subset(day3.all, Treatment=="Plant")
            day3.all.compost <- subset(day3.all, Treatment=="Compost")

            day5.all.gravel <- subset(day5.all, Treatment=="Gravel")
            day5.all.plant <- subset(day5.all, Treatment=="Plant")
            day5.all.compost <- subset(day5.all, Treatment=="Compost")

            day7.all.gravel <- subset(day7.all, Treatment=="Gravel")
            day7.all.plant <- subset(day7.all, Treatment=="Plant")
            day7.all.compost <- subset(day7.all, Treatment=="Compost")


##########Boxplots##########

######pH######
      ###Day 3###
            boxplot(pH ~ Experiment, data= day3.all.gravel)
            boxplot(pH ~ Experiment, data=day3.all.plant)
            boxplot(pH ~ Experiment, data=day3.all.compost)

            boxplot(pH ~ Treatment, data=day3.all)

      ###Day 5###
            boxplot(pH ~ Experiment, data= day5.all.gravel)
            boxplot(pH ~ Experiment, data=day5.all.plant)
            boxplot(pH ~ Experiment, data=day5.all.compost)

            boxplot(pH ~ Treatment, data=day5.all)

      ###Day 7###
            boxplot(pH ~ Experiment, data= day7.all.gravel)
            boxplot(pH ~ Experiment, data=day7.all.plant)
            boxplot(pH ~ Experiment, data=day7.all.compost)

            boxplot(pH ~ Treatment, data=day7.all)

######EC######
      ###Day 3###
            boxplot(EC ~ Experiment, data= day3.all.gravel)
            boxplot(EC ~ Experiment, data=day3.all.plant)
            boxplot(EC ~ Experiment, data=day3.all.compost)
```

```
            boxplot(EC ~ Treatment, data=day3.all)

      ###Day 5###
            boxplot(EC ~ Experiment, data= day5.all.gravel)
            boxplot(EC ~ Experiment, data=day5.all.plant)
            boxplot(EC ~ Experiment, data=day5.all.compost)

            boxplot(EC ~ Treatment, data=day5.all)

      ###Day 7###
            boxplot(EC ~ Experiment, data= day7.all.gravel)
            boxplot(EC ~ Experiment, data=day7.all.plant)
            boxplot(EC ~ Experiment, data=day7.all.compost)

            boxplot(EC ~ Treatment, data=day7.all)

######Redox######
      ###Day 3###
            boxplot(Redox ~ Experiment, data= day3.all.gravel)
            boxplot(Redox ~ Experiment, data=day3.all.plant)
            boxplot(Redox ~ Experiment, data=day3.all.compost)

            boxplot(Redox ~ Treatment, data=day3.all)

      ###Day 5###
            boxplot(Redox ~ Experiment, data= day5.all.gravel)
            boxplot(Redox ~ Experiment, data=day5.all.plant)
            boxplot(Redox ~ Experiment, data=day5.all.compost)

            boxplot(Redox ~ Treatment, data=day5.all)

      ###Day 7###
            boxplot(Redox ~ Experiment, data= day7.all.gravel)
            boxplot(Redox ~ Experiment, data=day7.all.plant)
            boxplot(Redox ~ Experiment, data=day7.all.compost)

            boxplot(Redox ~ Treatment, data=day7.all)


############Temp#########################################
      tempData <-
      read.table("C:\\Users\\rharv\\Dropbox\\00_GRAD_SCHOOL_NEW\\R
      Projects\\Wetlands\\WT1702 Temp.txt", header=TRUE)

      tempData01 <-
      read.table("C:\\Users\\rharv\\Dropbox\\00_GRAD_SCHOOL_NEW\\R
      Projects\\Wetlands\\WT1701 Temp.txt", header=TRUE)

      tempData03 <-
      read.table("C:\\Users\\rharv\\Dropbox\\00_GRAD_SCHOOL_NEW\\R
      Projects\\Wetlands\\WT1703Temp.txt", header=TRUE)
```

```
plot(tempData$Time,tempData$Temperature, pch=".", type="o", lwd=2,
lty=2, ylim=c(5,35), axes=FALSE)
      lines(tempData01$Time, tempData01$Temperature, lwd=2, lty=1)
      lines(tempData03$Time, tempData03$Temperature, lwd=2, lty=3)
      axis(side=1, at=seq(0,192, by=24))
      axis(side=2, at=seq(10,40, by=5))
      legend("bottomright", c("WT1701", "WT1702", "WT1703"),
      lty=c(1,2,3), bty="n")
      box()


############Nutrient Removal#############

nutData <- read.table("C:\\Users\\rharv\\Dropbox\\00_GRAD_SCHOOL_NEW\\R
Projects\\Wetlands\\WT All Data Nut.txt", header=TRUE)
      boxplot(Nit~Treatment, data=nutData)
      boxplot(Amm~Treatment, data=nutData)
```

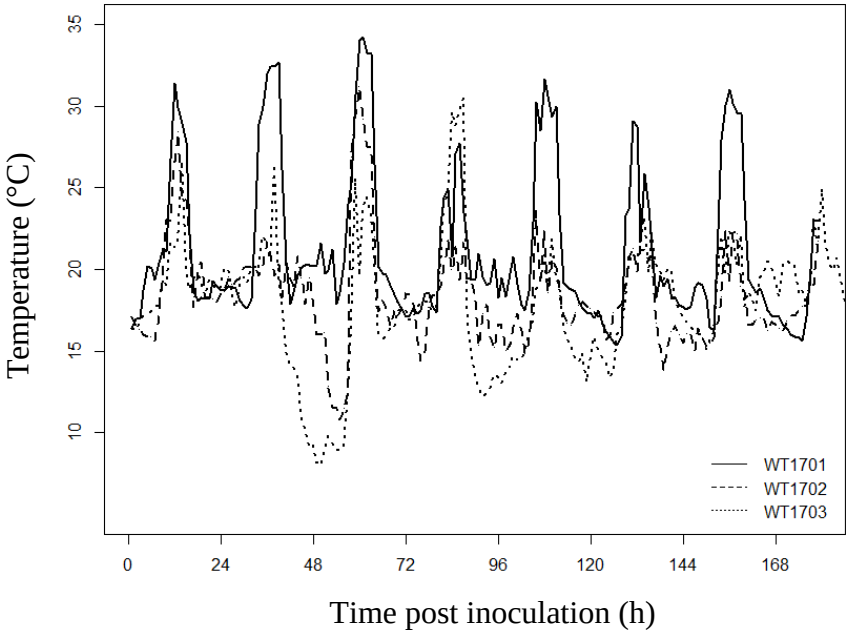


Fig. C-1.

Ambient greenhouse temperature during wetland inoculation experiments.

Appendix D. Miscellaneous Lab Protocols

PCR Protocol

Ingredient	Volume (µL)
Water	32.4
Green	5
Clear	5
dNTPs	0.5
$MgCl_2$	4
Fw	1
Rv	1
Taq	0.1

This PCR formulation is designed to work with the Promega GoTaq PCR kit. The water that is used must be pure, molecular grade water, not just distilled. Green and Clear refer to the supplied buffers of corresponding color, while Fw and Rv are the forward and reverse primers respectively. To create the master mix, multiply the supplied volumes by the number of reactions you want plus one, and add all together into the tube. Save the enzyme for last and keep it in the -20 °C freezer until needed, then return. The other reagents can be kept chilled on the benchtop during PCR prep. Load the tubes spaced out in the themocycler, then begin the cycle

Antibiotic Prep

Neomycin

1 g into 100 mL water
6 mL into 500 mL media

Streptomycin

5 g into 100 mL water
10 mL into 500 mL media

Ampicillin

1 g into 20 mL water
0.5 mL into 500 mL media

Media Recipes

Glucose Yeast Extract Agar

15 g glucose
10 g agar
100 mg yeast extract
40 mg tyrosine
Autoclave 15 minutes

V8 Agar

20 g agar
2 g $CaCO_3$
200 mL V8
800 mL water
Autoclave 30 minutes

Oatmeal Agar

10 g oatmeal
7.5 g agar
500 mL water
Autoclave 30 minutes

General note: When looking to make more experimental agars, i.e. rabbit dung, saw dust, etc., mixing in around 10 g of the desired substance and 7.5-10 g of agar is a good start. Autoclave for 45 min to ensure sterility. From here, adjust the amounts of ingredients as needed

Vita
Robert J. Harvey
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Education:

2013 - York College of Pennsylvania — Biology, B.S.
2018 - Penn State University (Anticipated) — Plant Pathology, Ph.D.

Skills/Experience:

- Construction, operation, and maintenance of small scale composting and wetland systems
- Understanding and applying biogeochemical concepts to compost and water systems
- Working knowledge of mushroom production, as well as limitations and future directions
- Design and evaluation of multifaceted rigorous experiments to find solutions to questions and problems
- Performing statistical evaluations on data sets using the open source R software
- Mycological and microbiological lab techniques, including (but not limited to): DNA extraction, PCR, culture maintenance, microscopic evaluation
- Communication of scientific concepts to the public
- Experience working in BSL3 Government Quarantine lab housed at Ft. Detrick, Frederick MD

Teaching/Outreach Experience

- PPEM318, Diseases of Forest and Shade Trees (2015-2018)
    - Teaching assistant as well as lab coordinator
- Mentoring of Undergraduate Research Students (2016, 2018)
    - Led a total of two undergraduate students through creation of experiments, followed by performance and evaluation. Projects ranged from isolation of soil bacterial to evaluation of wetland substrate perform in nutrient removal
- Pennsylvania Farm Show (2016-2017)
    - Served in the Mushroom Booth to educate the public on the importance, and well as production method, for mushrooms in the state of Pennsylvania
- Philly Students Grow with Penn State (2016)
    - Worked with high school groups from Philadelphia to discuss basic mushroom biology, as well as production
- Pennsylvania Governor's School Mushroom Research Center Tour (2016)
    - Facilitated a tour of the Penn State Mushroom Research Center to a group of approx. 20 high schoolers participated in the Governor's School Program

Invited Seminars

- "From Compost to Containment: A Journey Through Grad School:
    - York College of Pennsylvania, Richard Clark Lecture Series, Sept. 2017
- "Survival of *Calonectria pseudonaviculata* microsclerotia in compost"
    - Mt. Aloysius College, Senior Seminar Class, Feb. 2017

Honors and Awards

- First Place Oral Presentations, Alleghany Branch ASM Meeting, Erie, PA (2016)
- Lester P. Nichols Memorial Award (2016)
- James P. Roberts Award, Excellence in Mushroom Production (2013,2014)