PHYTOREMEDIATION OF HYDROCARBON-CONTAMINATED SOIL USING PHENOLIC-EXUDING HORTICULTURAL PLANTS

A Thesis

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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December 2015

Major Subject: Soil Science

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ABSTRACT

A greenhouse experiment was designed to test phenolic-exuding horticultural plant species for their phytoremediation potential in soils contaminated with polycyclic aromatic hydrocarbons (PAHs). Species included high-phenolic-exuding plants: *Malus* sp., *Osmanthus fragrans*, *Sambucus nigra*, *Castanea pumila*, *Morus alba*, and *Myrica cerifera* and low-polyphenol-exuding plants: *Ziziphus jujube*, *Ribes aureum*, and *Cassia fistula*. The species were planted in soil amended with benzo[a]pyrene, phenanthrene, and pyrene. After 7 months, nitrogen, phosphorus, and potassium amendments were added each month for three months. Plant roots were harvested; polyphenols were ethanol-extracted and quantified using the Folin-Ciocalteu method. Rhizosphere DNA was extracted, quantified, and the 16S rRNA gene and ITS region were sequenced for bacteria and fungi, respectively. In addition, qPCR was conducted that targeted the 16S rRNA and ITS region of bacteria and fungi, respectively. The highest and lowest concentrations of phenolics were from *Sambucus nigra* and *Ribes aureum*, respectively. There were no significant differences between 16S rRNA and ITS abundance among treatments. Sequencing showed a significant difference between the rhizosphere bacterial community compositions on a global level. Specifically, several *Actinobacteria* were over-represented in the low-phenolic-exuding plants, while high-phenolic-exuding plants were over-represented by several *Proteobacteria*. There were no significant differences between the fungal populations of treatments and all were dominated by *Ascomycota*. However, high-phenolic exuders had a higher abundance of *Basidiomycota,* than the low-phenolic

exuders. Results showed that different microbial communities were selected for by high polyphenol-exuding plants and low polyphenol-exuding plants. This selection was more pronounced in bacteria than in fungi. The drastic ways in which bacteria respond to phenolic inputs highlight their importance in phytoremediation.

DEDICATION

This work is dedicated to my Father in heaven and my mother on Earth.

ACKNOWLEDGEMENTS

I would like to thank my co-chairs, Dr. Terry J. Gentry and Dr. Arthur P. Schwab, for their guidance and support throughout the course of this research. Your mentorship, advice, and expertise were truly invaluable to me.

I would like to thank my committee member, Dr. Raghupathy Karthikeyan, and substitute committee member, Dr. Robin Autenrieth for helping me finish strong.

A special thanks goes to Dr. Pauline Wanjugi for teaching me all of the techniques I used to complete the microbial aspects of my research. I would also like to thank you for your mentorship and sound advice.

I want to acknowledge Lauren Pitts, Ammani Kyanam, Keya Howard, Mariana Valdez, Dylan Laird, Nadezda Ojeda, the Black Graduate Students' Association, the friends I made in College Station, Chardai Grays, Ngozi Okorafor and all my friends and family. My research would have progressed very slowly if it had not been for your friendship, moral support, and assistance.

Finally, I would like to thank Texas A&M for their financial support. Thank you for considering me a high achieving scholar and investing in my future.

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INTRODUCTION

When two or more aromatic rings are fused in a linear or clustered fashion, they are known as polycyclic aromatic hydrocarbons (PAHs) (Cerniglia, 1992). High molecular weight PAHs (HMW PAHs) contain at least four rings, while those with fewer are known as low molecular weight PAHs (LMW PAHs) (Lee *et al.*, 2013). Polycyclic aromatic hydrocarbons are created by the natural or industrial combustion of organic material (Cerniglia, 1992). PAHs have been known to occur as a result of automobile exhaust, coal-burning, forest fires, and volcanic eruptions (Lee and Vu, 2010; EPA; Haritash and Kaushik, 2009; Killops and Massoud, 1992; Liu *et al.*, 2001). PAHs are ubiquitous compounds and have been found in such innocuous places as parking lots and can be present in food products (Mahler *et al.*, 2005; Cerniglia, 1992; Van Metre *et al.*, 2006). PAHs are even a main component of creosote, a wood preservative (National Library of Medicine, 2002).

The properties of PAHs include low water solubility, high lipophilicity, non-polar, high melting and boiling points, and low vapor pressure (Table 1) (Haritash and Kaushik, 2009; Boffetta *et al.*, 1997). These properties become more pronounced as the number of fused benzene rings increase (Cerniglia, 1992). Therefore, in addition to possessing a higher molecular weight, 5-ringed benzo(a)pyrene is less water soluble, more lipophilic, and has higher melting and boiling points than 2-ringed naphthalene (Cerniglia, 1992). Lipophilicity is measured using the octonal-water partition coefficient (K_{ow}). K_{ow} is the

ratio of the concentration of a chemical in octanol [C_{oc}] to its concentration is water [C_w] (Dzantor and Beauchamp, 2002).

$$K_{ow} = \frac{[C_{oc}]}{[C_w]}$$

Low K_{ow} values are associated with compounds that are soluble in water while those with high K_{ow} are more lipophilic (Dzantor and Beauchamp, 2002). The range of K_{ow} for PAHs is $10^{3.4}$ to $10^{7.4}$, but often represented in the logarithmic form as log K_{ow} = 3.4 to 7.4 (Dzantor and Beauchamp, 2002). The most mobile PAHs have a log K_{ow} less than 4 and readily bioaccumulate. Bioaccumulation is the term used to describe the propensity of pollutants to enter the food chain and exist at higher concentrations at the top of the food chain than at the bottom or entry point. PAHs with K_{ow} values greater than 4 are not considered mobile in the environment (Harvey *et al.*, 2002).

The ubiquity of PAHs does not negate their toxicity, and their aforementioned properties make them a high-priority health risk. Many PAHs are carcinogenic (Mastrangelo *et al.*, 1996). Nine different PAHs, including benzo(a)pyrene, are considered when assessing the cancer risk to people from contaminated soil (James *et al.*, 2011). In fact, benzo(a)pyrene is considered one of the most toxic, carcinogenic, and mutagenic PAHs and is often used as a marker of PAH exposure (Boffetta *et al.*, 1997; Juhasz and Naidu, 2000). Primary routes of human exposure include inhalation from urban air and tobacco smoke, ingestion by drinking water and grilled food, and skin contact. The result of PAH exposure can be cancer of the lungs, skin, or bladder (Figure 1) (Boffetta *et al.*, 1997; Agency for Toxic Substances and Disease Registry (ATSDR), 1995). In addition to being carcinogens, many PAHs are lipid-soluble which allow them to be readily absorbed

by the gastrointestinal tract of mammals and localized in body fat at an average of 597 μg/kg (Samanta *et al.*, 2002; Diggs and Huderson, 2011; Kim *et al.*, 2013). These compounds also have the ability to produce fetal malformations. The teratogenicity of PAHs have been experimentally determined with several projects. One determined that the risk of a newborn with a neural tube defect was 4 to 5 times greater when the levels of PAHs were above the average known to be in lipids (Ren *et al.*, 2011). Kristensen et al., (1995) determined that offspring had birth defects and decreased body weight when mothers ingested high levels of benzo(a)pyrene during pregnancy. All of the health risks associated with PAHs are a result of the bioactive parent compounds and their metabolites. Their metabolites (dihydrodiols, glutathione conjugates, and phenols), especially epoxides, are responsible for the carcinogenic activities of the compounds (Jerina *et al.*, 1978; Sims and Grover, 1974). The interactions between the compounds and the affected tissue occur via metabolic activation by Cytochrome P450 (Cavalieri and Rogan, 1992; Shimada and Fujii-Kuriyama, 2004; Shimada *et al.*, 2013). Cytochrome P450 is an enzyme family characterized by strong absorbance at 450 nm and heme-containing monooxygenases (Alexander *et al.*, 2013). The enzymes facilitate the binding of PAHs to DNA. Once the PAHs have been activated, their metabolites are able to attack cellular DNA and cause extensive damage (Shimada *et al.*, 2013). The likelihood of a PAH forming carcinogenic metabolites can be attributed to the presence of the K-region, L-region, or Bay-region (Figure 2).

Table 1 - Structure and physicochemical properties of common polycyclic aromatic hydrocarbons

Structure	Name Molecular Formula	Molecular Weight (g/mol)	Log K_{ow}	CAS #
	Naphthalene $C_{10}H_8$	128	3.17	91-20-3
	Fluorene $C_{13}H_{10}$	166	4.02	86-73-7
	Phenanthrene $C_{14}H_{10}$	178	4.35	85-01-8
	Anthracene $C_{14}H_{10}$	178	4.35	120-12-7
	Pyrene $C_{16}H_{10}$	202	4.93	129-00-0
	Benzo(a)pyrene $C_{20}H_{12}$	252	6.11	50-32-8
	Dibenz(a,h)anthracene $C_{22}H_{14}$	278	6.70	53-70-3

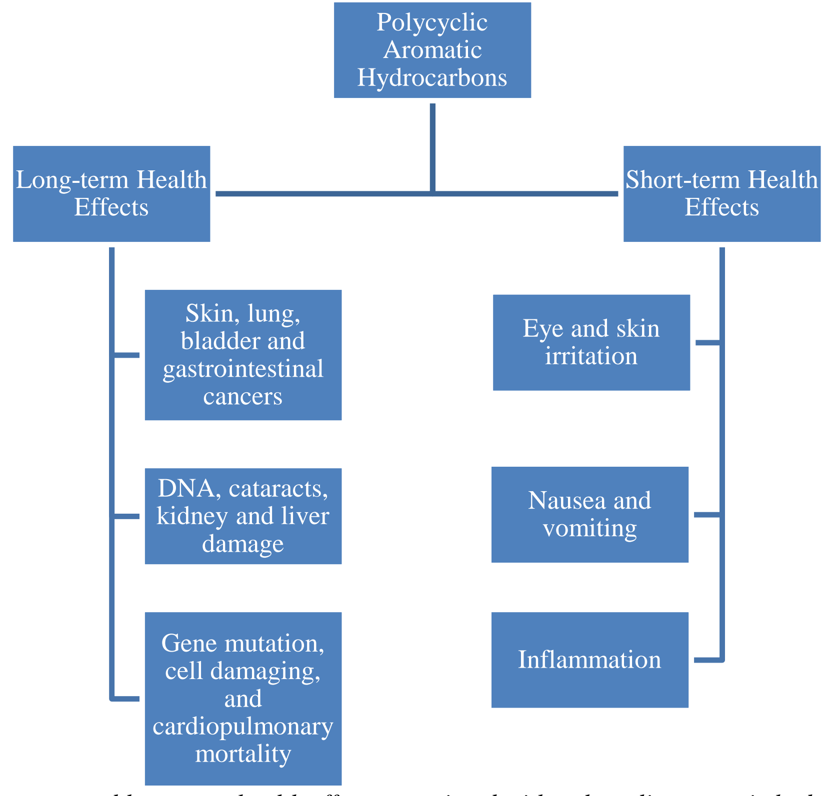

Figure 1-Short-term and long-term health effects associated with polycyclic aromatic hydrocarbons

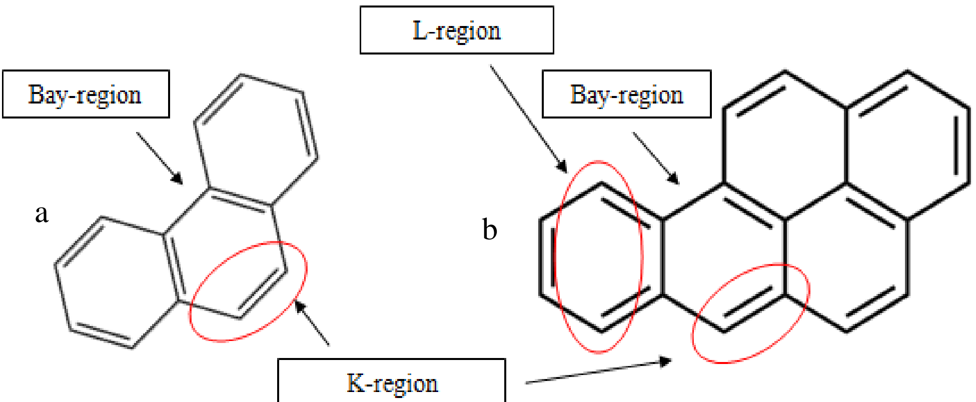

Figure 2-Bay-, L-, and K-region examples on polycyclic aromatic hydrocarbons

Figure 3-Example of cancer causing structures in polycyclic aromatic hydrocarbons: dihydrodiol and K - region epoxide

In unsubstituted PAHs, the highly reactive L-region undergoes metabolic methyl –substitution and electrophilic substitution. These substitutions enhance the carcinogenic effects of the PAH. The bay-region theory postulates that the carcinogenic properties of PAHs are a result of the creation of a trans-dihydrodiol by the addition of water to the epoxide that was created in the region. It is possible for these epoxides to be metabolized into dihydrodiols that may form soluble detoxification products or be oxidized to diol-epoxides (Figure 3) (Samanta *et al.*, 2002). According to the bay-theory, a PAH must contain two bonds in an end ring capable of undergoing metabolic reactions (Flesher *et al.*, 2002). The reactivity of a bay-region epoxide stems from its position as part of an angular benzo-ring that forms part of the bay region (Jerina *et al.*, 1978).

PAHs are highly recalcitrant. With time, PAHs become less available to undergo processes that would remove them from the soil environment (Erickson *et al.*, 1993). Interactions between physicochemical, physiological, and toxicological processes influence the bioavailability of the compounds (Cachada *et al.*, 2014). The parameters that control these three processes are outlined in Table 2. These factors include, but are not limited to, chemical structure, pH, temperature, oxygen level, nutrient availability, moisture level, presence of organisms capable of degrading the PAHs, and organic content of the soil (Bamforth and Singleton, 2005; Luo *et al.*, 2012).

Table 2 - Factors that influence the bioavailability of PAHs

Physicochemical	Physiological Uptake	Toxicological
Soil organic matter	Receptor type	Metabolism
Soil quality	Anatomy	Detoxification capacity
Soil inorganic constituents	Feeding strategy	Accumulation capacity
Lipophilicity of compounds	Lipid content of organism	

Luo et al. (2012) studied the diffusion of PAHs in soils from labile to non-labile domains and found that it was dependent on pore size and organic carbon content. When PAHs were added to a microcosm that was filled with contaminated soil, added PAHs were rapidly lost via biodegradation while the concentration of the original PAHs remained static (Erickson *et al.*, 1993). The researchers concluded that the original PAHs were "bound to soil in a way that made them unavailable for degradation." Amellal *et al*. (2001) determined that low and high molecular weight PAHs were localized in the fine soil fraction and present in low concentration in the larger fraction. The inaccessibility of PAHs can cause the overestimation of health-risk if the total amount of PAHs is quantified without considering actual bioavailability (Alexander, 2000).

Bioavailable is a term that encompasses the portion of PAHs that are able to be transformed by organisms, and there are a variety of chemical methods and bioassays used to determine its quantity. These methods can be exhaustive, non-exhaustive, or biomimetic (Table 3) (Cachada *et al.*, 2014). Still the most accurate method to assess bioavailability is to use actual organisms as part of a bioassay. However, it is not always feasible to collect

PAH measurements from the organisms' tissue. The effectiveness of chemical techniques for bioavailability determinations are often measured by their correlation to biological bioassay measurements. A chemical method that is strongly positively correlated to the bioassays is considered accurate. Methods that are exhaustive can overestimate the bioavailable portion of PAHs by up 10,000 times and are weakly correlated to the assays that utilize organisms to determine bioavailability (Cachada *et al.*, 2014; Gomez-Eyles *et al.*, 2010).

Extensive reviews have been completed by Cui *et al.* (2013) and Cachada *et al.*, (2014) concerning the principles and conventions associated with each of these extraction methods. Assessing the bioavailability of PAHs is necessary to determine the best remediation techniques for their removal and to accurately describe their health risk.

Table 3 - Techniques used to assess the bioavailability of PAHs

Exhaustive	Non-exhaustive extractions	Biomimetics	Bioassays
Hot solvent (Soxhlet)	Mild solvent extraction	Solid phase microextraction (SPME)	Earthworms
Ultrasonic solvent extraction	Subcritical water extraction (SWE)	Polyoxymethylene solid phase extraction (POM-SPE)	Plants
Accelerated solvent extraction	Supercritical fluid extraction (SFE)	Extraction disks	
	Solid phase extraction (SPE) from soil water Solubilizing agents Cyclodextrin extraction Tenax extraction Persulfate oxidation	Composite membranes	

REMEDIATION OF PAHS

To develop technologies that can effectively remediate petroleum contaminated soil, understanding the properties of PAHs that facilitate soil sorption/desorption and impact ease of extraction is imperative. Previous research concerning PAH sorption and desorption, kinetics, and soil chemistry has led to the development of several PAH remediation methods. These methods take advantage of the physico-chemical properties of the compounds, biological processes or, in some cases, both (Pazos *et al.*, 2010).

Biopiling/Composting

Biopiling uses indigenous microbial populations, biostimulation, and aeration to help degradation of PAHs (Van Hamme *et al.*, 2003). Biopiling is an *ex situ* bioremediation technology that simulates microbial degradation by providing optimal growth conditions (Germaine *et al.*, 2014). As the name suggests, biopiling gathers the contaminated soil into piles, about 2 to 4 m high, and selects for aerobic degradation process due to the introduction of oxygen (Jørgensen *et al.*, 2000). Other environmental controls include maintaining optimal pH levels, moisture levels, and biostimulation or the addition of nutrients (Germaine *et al.*, 2014). Soil texture influences the soil permeability, moisture content, and bulk density of the soil and thereby impacts the effectiveness of biopiling. Optimum pH levels are usually close to neutral, but pH between 5 and 9 are acceptable, and temperature is set between 20 and 45 °C (Bamforth and Singleton, 2005; Chemlal *et al.*, 2013). Although degradation can occur at more extreme temperatures,

mesophilic temperatures are the most studied (Bamforth and Singleton, 2005). The temperature requirement makes it difficult to control this remediation effort year-round. Other drawbacks include slow degradation rates and the potential to contaminate ground and surface water (Van Hamme *et al.*, 2003). Like temperature, non-optimal pH does not completely inhibit PAH degradation. Various species of microorganisms have been determined to complete degradation at pH 5.5, but degradation increased by 40% when pH was raised to 7 (Bamforth and Singleton, 2005). Mono- and di-oxygenase enzymes in the microorganism are activated by the addition of oxygen to a biopile. These enzymes initiate the oxidation of the aromatic ring by the addition of hydrodiols (Bamforth and Singleton, 2005). However, the process is often limited by nutrient levels. Nitrogen and phosphates are added to the biopile to increase microbial activity (Bamforth and Singleton, 2005). One hundred m^3 of soil was used to create a biopile that dissipated 4,600 mg/kg of total petroleum hydrocarbon (TPH) to 691 mg/kg TPH at the end of 66 days (Iturbe *et al.*, 2004). After 40 days, 70% of diesel was removed from a contaminated site by biopiling whereas a year was required to reach a comparable result through natural attenuation (Chemlal *et al.*, 2012). A biopile was able to remove 85.5% of TPH after 76 days, but an aeration pipe burst on day 20 and researchers mention that as another drawback to biopiling (Chemlal *et al.*, 2013).

When organic matter, such as wood chips or bark, is added to a biopile, the technology is termed composting (Jørgensen *et al.*, 2000).

Landfarming

Landfarms are above-ground, engineered systems that rely on oxygen to stimulate microbial degradation of hydrocarbons and are, in that regard, similar to biopiles (United States *et al.*, 1995). Landfarms treat oil waste by adding oil sludge and nutrients to agricultural land and mixing by agricultural practices such as tilling to improve distribution of contaminants and supply oxygen (Jørgensen *et al.*, 2000; Wick *et al.*, 2011). For optimal microbial degradation, soil moisture should be maintained between 30-90% of the field moisture capacity (FMC). The FMC was described as the moisture content of the soil expressed as the percentage of the oven-dry weight after the free water has drained away (Straube *et al.*, 2003). Landfarming is an *in situ* remediation technique, but remediating contaminated soil by land-farming is difficult on a year-round basis and there remains the potential for ground and surface water contamination. Landfarming is not considered environmentally acceptable, and other remediation techniques are being considered more often (Van Hamme *et al.*, 2003).

Natural Attenuation

Natural attenuation of contaminated soil occurs without human intervention (Van Hamme *et al.*, 2003). This process takes advantage of the intrinsic biodegradation capacity of the indigenous microorganisms and can be quite slow (Chemlal *et al.*, 2013). Natural attenuation is the least invasive method of remediation but requires continued monitoring to ensure that remediation is occurring or, at the very least, contamination is not spreading (Van Hamme *et al.*, 2003; Romantschuk *et al.*, 2000). Natural attenuation allows for the

natural evolution of genes capable of degrading aromatic hydrocarbons. These genes can then be mined and utilized in advanced remediation approaches. However, this remediation method can take a long time.

Bioremediation

Polycyclic aromatic hydrocarbons have several fates in the environment. PAHs may be adsorbed to the soil particles, volatilized, photolysed, chemically degraded, or microbially degraded. Microbial degradation is the most common degradation process, and its occurrence depends on a number of factors including environmental conditions, microbial composition and population, and chemical structure. In addition, the microbe must express chemotaxis to and uptake of the PAH (Gibson and Parales, 2000). The PAHs are metabolized into simpler metabolites and mineralized to non-toxic inorganic minerals like carbon dioxide and water.

The rate of degradation depends on pH, temperature, oxygen, microbial composition and population, nutrients, and chemical structure (Haritash and Kaushik, 2009). For example, HMW PAHs are degraded at a slower rate by bacteria, fungi, and algae than their LMW counterparts. In addition, the rate of degradation can be impacted by the catabolic pathway utilized by an organism. Fungi utilize Cytochrome P450 monooxygenases while bacteria use various enzymes of the Rieske non-heme iron oxygenases family; these enzymes catalyze the initial oxidation of aromatic hydrocarbons to create *cis*-diols and can be either a dioxygenase or monooxygenase system (Gibson and Parales, 2000).

There are four dioxygenase families: Naphthalene, Toluene/Biphenyl, Phthalate, and Benzoate/Toluate. Their electron transfer and catalysis is carried out by the α-subunits common to the multicomponent dioxygenases (Iwai *et al.*, 2011). Each family is responsible for the degradation of a subset of PAHs. Naphthalene 1,2-dioxygenase (NDO) serves as the prototype for Rieske non-heme iron oxygenases (RniO) and contains a Rieske [2Fe-2S] cluster, non-heme iron (II), a water molecule and other components. It is similar to Cytochrome P450, but P450 does not catalyze the *cis*-hydroxylation of arenes and NDO does not oxidize alkenes to epoxides. For all RniO, dihydroxylation occurs at the bay-region, which has the lowest electron density. Juhasz & Naidu (2000) wrote an extensive, but not exhaustive, list of PAH degrading microorganisms which is classified by hydrocarbon substrate. A few other microorganisms that were not listed on the table have been added to Table 4 below.

Table 4 - Polycyclic aromatic hydrocarbons oxidized by different genera of bacteria

Compound	**Organism (Bacteria)**	**References**
Naphthalene	*Paenibacillus*	(Daane *et al.*, 2002)
Anthracene	*Anthrobacter* sp.	(Dean-Ross *et al.*, 2002)
Phenanthrene	*Rhodotorula*	(Romero *et al.*, 1998)
	Paenibacillus	(Daane *et al.*, 2002)
Fluoranthene	*Pseudomonas* sp.	(Dean-Ross *et al.*, 2002)
Pyrene	*Rhodococcus* sp.	(Rehmann *et al.*, 2008)
Chrysene	*Rhodococcus* sp.	(Walter *et al.*, 1991)
Benzo(a)pyrene	*Agrobacterieum*	(Ye *et al.*, 1996)
	Bacillus	(Aitken *et al.*, 1998)
	Burkholderia	(Romero *et al.*, 1998)
	Pseudomonas	(Trzesicka-Mlynarz and Ward, 1995)
	Flavobacterium	(Schneider *et al.*, 1996)
	Rhodococcus	(Walter *et al.*, 1991)

Phytoremediation

Phytoremediation is also referred to as plant-assisted bioremediation and can take advantage of the intrinsic degradation capabilities of a contaminated area (Badri *et al.*, 2009). In addition, specific plants and microbes can be paired and planted in a specific environment to elicit speedier remediation of the area. Phytoremediation is an attractive alternative to traditional remediation techniques due to its environmental, aesthetic, energy, and economic value. The cost associated with traditional remediation techniques have reached as much as $500 million whereas the cost to phytoremediate a site can range from $15,000 to $694,000 (Russell, 2005). Table 5 provides the cost of phytoremediation compared to other techniques (Schnoor, 1997). However, only natural attenuation takes longer to establish remediation results as significant as the more invasive and expensive remediation techniques. Also, this technology hinges on the ability of the plant to access the contaminants; remediation is limited to the depth of the root.

Several phytoremediation mechanisms exist by which pollutants can be treated in soil. These mechanisms include, but are not limited to: phytodegradation, phytoextraction, phytostabilization, rhizodegradation, and phytovolatilization. A description of each mechanism is listed in Table 6. The plant that is used at the site depends on the contaminant present and the specific needs of the treatment. The requirements for metal and organic contaminants are different and vary even more depending on the phytoremediation mechanism utilized. For organic pollutants, like PAHs, the plant should be fast growing, easy to plant and maintain, and, depending on the need, provide the ability

to enhance microbial degradation via root exudation (Schnoor, 1997; Interstate Technology and Regulatory Cooperation Work Group, 2001).

Table 5 - Cost of various remediation techniques (Schnoor, 1997; Interstate Technology and Regulatory Cooperation Work Group, 2001)

Treatment Type	Cost per Ton
Phytoremediation	$10-35
In situ bioremediation	$50-150
Ozonation	$20-220
Soil washing	$80-200
Stabilization	$240-340
Solvent extraction	$360-440
Incineration	$200-1,500

Table 6 - Description of phytoremediation mechanisms (Pivetz, 2001)

Phytodegradation	Contaminants are metabolically transformed within the plant tissue
Phytoextraction	Plants accumulate contaminants in above ground tissue and the plants are taken from the treated area
Phytostabilization	The movement of contaminants is inhibited by the plant through adsorption
Rhizodegradation	Microbial activity is enhanced in the root zone and the increased activity breaks down contaminants
Phytovolatilization	Plants uptake contaminants then release them into the air

LITERATURE REVIEW: ROOT EXUDATES, MICROBIAL COMMUNITIES, AND PAH DISSIPATION

Root exudates are compounds that are released by plants in the form of amino acids, organic acids, sugars, phenolic acids, secondary metabolites (low-molecular weight), mucilage and proteins (high-molecular weight) (Badri and Vivanco, 2009). Root exudates are a major source of soil organic carbon as up to 40% of carbon fixed by plants can be exuded by the roots (Badri and Vivanco, 2009; Ziegler *et al.*, 2013; Vranova *et al.*, 2013). The quantity and composition of root exudates depends on a number of physiological and environmental factors such as plant species and age (Gransee and Wittenmayer, 2000; Fletcher and Hegde, 1995), soil pH, soil type, and nutritional status (Marschner *et al.*, 2004). In addition, root exudates are involved in plant-plant and plant-microbe interactions by serving as chemical signals for communication.

Carbon compounds can be exuded from the root in several ways. Low-molecular weight substances are released via passive diffusion and different plant metabolic processes release a variety of high-molecular weight substances (Vranova *et al.*, 2013). These carbon compounds are used by microbes as a nutrient source that lead to an increase in microbial biomass and activity near the root. The physical and chemical alterations surrounding the root coupled with the increase in microbial mass and activity are known as the rhizosphere effect (Ziegler *et al.*, 2013). The rhizosphere effect can be either positive or negative, with positive effects being symbiotic associations with beneficial microbes and negative effects including associations with parasitic or pathogenic bacteria (Badri

and Vivanco, 2009). Hartwig et al. (1991) determined that flavonoids exuded from alfalfa seeds enhanced the growth rate of *Rhizobium meliloti* and characterized those flavonoids. Bais et al. (2006) wrote an extensive review concerning the various roles of root exudates since they are numerous. The function of root exudates, specifically phenolics, that is most critical to this project concerns their effect on microbial communities and PAH dissipation.

Phenolic compounds are involved in rhizogenesis, vitrification, resistance to biotic and abiotic stress, and redox reaction in soils (Kevers *et al.*, 1984; Makoi and Ndakidemi, 2007). They serve as flower pigments, act as constitutive protection agents against invading organisms, function as signaling molecules, act as allelopathic compounds, affect cell and plant growth and are an important natural animal toxicant. Time, depth, root age, soil type, and nutrient level all influence the release of exudates. For example, *Lupinus albus* and *Brassica napus* can release large amounts of phenolics in response to phosphorus deficiency. In addition, the phenolic concentration in *Cistus albidus* green leaves can range from 66.5 to 95.9 mg gallic acid per gram of soil dissolved matter. (Castells and Peñuelas, 2003; Makoi and Ndakidemi, 2007).

Phenolic exudation enhances PAH degradation by influencing the microbial population. Due to their structural similarity to PAHs, phenolics can increase the expression of genes that code for enzymes that are necessary for hydrocarbon degradation pathways or increase the total microbial population thereby increasing the abundance of genes associated with PAH degradation. (Dzantor, 2007). Although *nahG* expression, one of the genes responsible for naphthalene dioxygenase production, was repressed in the

presence of root exudates from sorghum, switchgrass, osage orange, red mulberry, hybrid willow, hybrid poplar, and kou, the treatments experienced increased microbial growth that is likely responsible for the faster PAH degradation reported in planted soils (Kamath *et al.*, 2004). Lee *et al.* (2008) found that after 80 days, pyrene and phenanthrene dissipation was greater in soils planted with *Panicum bisulcatum, Echinogalus crus-galli, Astragalus membranaceus,* and *Aeschynomene indica* compared to unplanted soil.

Miya & Firestone (2001) added slender oat plant root debris and root exudates, individually and in conjunction, to phenanthrene-amended soils for a total of three experimental treatments (four including control). The researchers analyzed heterotrophic microbial populations and phenanthrene degrader populations separately and observed an overall positive effect of plant-based amendments on both communities. The microbial analysis was conducted using a most probable number (MPN) assay. Within the first four days of plant amendments heterotrophic microbial populations increased. The increase for the phenanthrene-degrader populations occurred within the first six days. The largest effects of plant-based amendments were seen when both plant root exudates and plant debris were added. When both plant residue types were added jointly, at least 75% of phenanthrene was removed from the soil almost a week sooner that the control.

Another experiment corroborated the findings of Miya and Firestone (2001) when four bacterial isolates were able to utilize D-mannitol as a sole carbon source. This indicated the bacteria's likely increase in population when the compound was exuded by plants because D-mannitol is a common component of root exudates. The bacterial isolates were detected by MPN assays conducted on rhizosphere sediment samples of pyrene-

exposed *Phragmites australis* collected from 3 freshwater sites in Japan. Partial 16S rRNA gene sequences identified them as *Mycobacterium gilvum* (Toyama *et al.*, 2011). In another study, *Sphingomonas yanoikuyae* experienced an increase in growth while using root extracts from willow, poplar, slender oat, mulberry, and kou as the sole carbon and energy sources. The growth was validated by calculating the change in optical density (OD600) (Rentz *et al.*, 2005). In the presence of ryegrass root exudates, *Pseudomonas* sp. and *Arthrobacter* sp. were the major microbial players in a study that utilized stable isotope probing to identify the effects of root exudates on microbial diversity of a PAH-contaminated soil. The study found that the key microbes in the soil amended with exudates differed greatly from the soil without exudates. The unamended soil contained *Pseudoxanthomonas* sp. and *Microbacterium* sp. as the dominant microorganisms (Cébron *et al.*, 2011).

Leigh et al. (2002) were able to show that *Burkholderia* sp. colonies were able to utilize naturally occurring mulberry exudates as a sole carbon source. These exudates were specifically flavones: morusin, morusinol, and kuwanon C. This fact hints at an expected increase in *Burkholderia* sp. populations in soil in response to mulberry root exudates because the microorganisms can utilize them as alternative nutritional sources. In a study that analyzed 16S rRNA samples from a diverse group of microorganisms, researchers found that less than 5% of taxa responded negatively to the introduction of organic acids (quinic, lactic, and maleic) which were used as model root exudates in the experiment. Using 16S rRNA gene PhyloChip analysis, researchers found 10 to 22 more taxa of microorganisms in soils amended with organic acids as compared to the control.

Actinobacteria, Proteobacteria and *Firmicutes* dominated within each treatment (Shi *et al.*, 2011).

Mycobacterium gilvum (strain IPF) grew in response to the addition of *Phragmites australis* root exudates (Toyama *et al.*, 2011). The growth of the microbes equated to a comparable degradation of pyrene when compared to the control. Plant residues like bamboo leaves, orange peels, and wood chips enhanced the bio-dissipation of PAH in a soil environment. The reduction of PAH correlated positively with the growth of the two microorganisms used in the study, B1 (*Pseudomonas putida*) and B2 (uncharacterized isolate) (Chen and Yuan, 2012). The effect was still more or less prominent depending on the plant and the microbe. Orange peel extracts were a more effective growth medium and PAH remover when B1 was involved, but bamboo leaves were better for B2.

Corgié et al. (2003) spiked sand with phenanthrene and found that remediation of PAH was a function of proximity to ryegrass root where organic substrates were up to 65% of the root exudates.

Gao et al. (2010) amended brown-red, yellow-brown, and red soil collected from the A-horizon in China with phenanthrene and pyrene and used artificial root exudates: glucose, fructose, sucrose, succinic acid, malic acid, serine, arginine, and cysteine to study the desorption of PAH from soils by root exudates. Desorption of PAH from soil increases bioaccessibility and microbial degradation of the hydrocarbons. The desorption of phenanthrene and pyrene in soils, and the effects depended on concentration, aging time, and soil properties with more desorption occurring in soils with lower organic matter content.

Overall, the research showed a net positive effect of root exudates on microbial communities and PAH dissipation, but the effect depended widely on the origin of the exudation, the affected microbes and soil conditions.

Table 7 provides an incomplete, but representative list of plant species used in root exudation and PAH dissipation studies and, if known, their respective contaminant media. In addition, many studies rely on artificial root exudates to study their influence on microbial communities and PAH dissipation. The effect of those root exudates on PAH dissipation is described in Table 8 below as neutral (=) or positive (+).

Table 7 - Representative list of plant species used in root exudation and PAH dissipation studies and their respective contaminant media

Scientific Name	Common Name	PAH Media	Reference
Miscanthus x *giganteus*	perennial grass	Soil sample characterized by total PAH contamination of 1480 mg kg^{-1} dry soil sample from a former coal mine	(Techer *et al.*, 2012)
Populus deltoids X *nigra* DN34	hybrid poplar	Naphthalene added to mineral medium Benzo(a)pyrene added to solution	(Kamath *et al.*, 2004) (Rentz *et al.*, 2005)
Salix alba X *massudana*	hybrid willow	Naphthalene added to mineral medium Benzo(a)pyrene added to solution	(Kamath *et al.*, 2004) (Rentz *et al.*, 2004)
Avena sativa	slender oat	Benzo(a)pyrene added to solution	(Rentz *et al.*, 2005)
Maclura pomifera	osage orange	Naphthalene added to mineral medium Benzo(a)pyrene added to solution	(Kamath *et al.*, 2004) (Rentz *et al.*, 2005)
Morus rubra	red mulberry	Naphthalene added to mineral medium Benzo(a)pyrene added to solution	(Kamath *et al.*, 2004) (Rentz *et al.*, 2005)
Morus alba	white mulberry	Benzo(a)pyrene added to solution	(Rentz *et al.*, 2005)
Festuca arudinacea	tall fescue	Pre-treated contaminated soil	(Parrish *et al.*, 2004)
Melilotus officinalis	yellow sweet clover	Pre-treated contaminated soil	(Parrish *et al.*, 2004)
Cordial subcordata	kou	Naphthalene added to mineral medium Benzo(a)pyrene added to solution	(Kamath *et al.*, 2004) (Rentz *et al.*, 2005)
Sorghum bicolor (L.) Moench	sorghum	0 or 100mg of phenanthrene added to water Phenanthrene concentrations were applied to heat sterilized quartz sand	(Liste and Alexander, 1998) (Muratova *et al.*, 2009)
Triticum aestivum L.	wheat	0 or 100mg of phenanthrene added to water	(Liste and Alexander, 1998)

Table 7 cont.

Scientific Name	**Common Name**	**PAH Media**	**Reference**
Zea mays L.	corn	0 or 100mg of phenanthrene added to water	(Liste and Alexander, 1998)
		clay loam from an abandoned pasture Soil from a petroleum refinery land treatment	(Yoshitomi and Shann, 2001)
Helianthus annuus L.	sunflower	0 or 100mg of phenanthrene added to water	(Liste and Alexander, 1998)
Raphanus sativus L.	radish	0 or 100mg of phenanthrene added to water	(Liste and Alexander, 1998)
Pisum sativum L.	pea	0 or 100mg of phenanthrene added to water	(Liste and Alexander, 1998)
Glycine max L.	soybean	0 or 100mg of phenanthrene added to water	(Liste and Alexander, 1998)
Panicum bisulcatum	grass	Phenanthrene and Pyrene added to "clean" soil	(Lee *et al.*, 2008)
Echinogalus crus-galli	grass	Phenanthrene and Pyrene added to "clean" soil	(Lee *et al.*, 2008)
Astragalus membranaceus	legume	Phenanthrene and Pyrene added to "clean" soil	(Lee *et al.*, 2008)
Aeschynomene indica	legume	Phenanthrene and Pyrene added to "clean" soil	(Lee *et al.*, 2008)
Medicago sativa L.	alfalfa	phenanthrene, anthracene, fluorene and fluoranthene added to mineral medium	(Zhang *et al.*, 2012)
Lolium perenne L.	ryegrass	Sand spiked with phenanthrene	(Corgié *et al.*, 2003)
		Pre-treated contaminated soil	(Parrish *et al.*, 2004)

Table 8 - Effect of selected root exudates on polycyclic aromatic hydrocarbon dissipation. A neutral effect is designated by (=) and positive effects are designated by (+).

Name of Root Exudate	PAH	Effect
Quercetin	Phenanthrene[4]	=
	Fluorene[4]	=
	Pyrene[4]	=
	Fluoanthene[4]	=
Rutin	Phenanthrene[4]	=
	Fluorene[4]	=
	Pyrene[4]	=
	Fluoranthene[4]	=
Catechin	Naphthalene[2]	+
	Fluoranthene[2]	+
	Phenanthrene[2]	+
	Pyrene[2]	+
	Chrysene[2]	+
	Benzo[a]pyrene[2]	+
Guaiacol	Phenanthrene[3]	+
	Pyrene[3]	+
Salicylic acid	Fluoranthene[1]	+
	Pyrene[1]	+
	Naphthalene[1]	+
	Benz[a] anthracene[1]	+
	Chrysene[1]	+
	Benzo[a]pyrene[1]	+
Gallic acid	Naphthalene[2]	+
	Fluoranthene[2]	+
	Phenanthrene[2,3]	+
	Pyrene[2,3]	+
	Chrysene[2]	+
	Benzo[a]pyrene[2]	+

1. (Singer *et al.*, 2003)
2. (Nam *et al.*, 2001)
3. (Ting *et al.*, 2011)
4. (Techer *et al.*, 2012)

PURPOSE AND METHODOLOGY

Purpose

The purpose of this research is to screen horticulturally significant phenolic-producing plants for their potential use as bioremediation technology. We will do this by observing the ability of the plants to persist in contaminated soil, to produce phenolic exudates, and to impact microbial abundance and community composition.

We propose the following objective and supporting hypotheses:

Objective: To examine the role of exudation of phenolics in the rhizosphere on microbial community composition in the presence of PAH contaminated soil

Hypotheses:

1. *Malus* sp., *Osmanthus fragrans*, and *Castanea pumila* will exude more phenolics than *Ziziphus jujuba*, *Ribes aureum*, and *Cassia fistula*.
2. Overall rhizosphere bacterial and fungal abundance will positively correlate with an increase in phenolic root exudation.
3. The dominant rhizosphere microbial populations will differ among plant species with varying root phenolic concentrations
4. In the presence of high-phenolic input, there will be an increase in the relative abundance of known PAH-degraders such as *Proteobacteria* for bacteria and *Basidiomycoa* for fungi
5. Plant species with similar phenolic exudation will show similar microbial community structures

Methodology

Selection of plants

Plants were chosen based on their reported production or non-production of polyphenol exudates as observed in various studies. Because polyphenols are primarily studied for their antioxidant properties, the original plant list originated from a large screening by health scientists (Fujii et al., 2003; Katalinic et al., 2006; Li et al., 2008). We used these studies to select plant species with high concentrations of phenolics and those that had close to no detectable amounts of polyphenols. Plants were then accepted for further consideration or rejected based on their confirmed allelopathic properties. A plant that utilizes allelopathy exudes compounds that generally create a hostile environment for other organisms (Lovett, 1985). Polyphenols have been confirmed as an aspect of the root exudates mixture; therefore, it is to be believed that plants with strong allelopathic properties also exude polyphenols (Zhang et al., 2007; Alsaadawi et al., 2012). On the other hand, the plants that lack allelopathic properties may not exude as much polyphenols and were also accepted for further consideration in the experiment as a control.

The allelopathic potential of root exudates of *Malus pumila* L., *Prunus persica* L., and *Ziziphus jujuba* Mill. against apple germination were tested and researchers found that *M. pumila* L. and *P. persica* L. inhibited growth of apple seeds while *Z. jujuba* did not. Qualitative determination of root exudates of these plants determined that the root exudates of *M. pumila* L. mainly contained organic acids, glycol, esters, and benzophenol derivatives. The root exudates of *Z. jujuba* Mill. did not contain any phenolic acids (Zhang et al., 2007). The allelopathic potential of Ribes aureum and other invasive wood plant

species in Hungary was tested by observing their ability to effect the germination rate, shoot length and root length of white mustard (*Sinapis alba* L.). Researchers found that Ribes aureum did not significantly affect the germination rate or shoot length of white mustard (Csiszár, 2009). The allelopathic activity of *Cassia fistula* was tested by observing their ability to inhibit the growth of select weeds in crop farms. *C. fistula* did impact the growth of the weeds and the plants. Another experiment tested the affect *Cassia fistula* had on the growth of radish. When compared with other treatments from the study, *C. fistula* did not significantly impact the growth of the radish (Hussain et al., 2007; Hong et al., 2003). Other experiments explicitly targeted the phenolic production of *Malus* sp., *Osmanthus fragrans*, *Castanea pumila* or their relatives.

Finally, the plants needed to be horticulturally significant because they would, ideally, be used in the construction of remediation gardens. We chose plants that were aesthetically pleasing and could be planted in various beautification projects. We used a total of 6 plant species for the study (Table 9). Three are considered to be high phenolic-producing plants while the other three are considered low phenolic-producing plants.

Table 9 - List of plants used in the study

Scientific Name	Common Name	Phenolic Production	Referencing Articles
Ziziphus jujuba	Jujube	Low	(Zhang *et al.*, 2007)
Ribes aureum	Golden Currant	Low	(Csiszar, 2009)
Cassia fistula	Cassia	Low	(Hussain *et al.*, 2007; Hong *et al.*, 2003)
Malus sp.	Crabapple	High	(Fletcher and Hegde, 1995; Zhang *et al.*, 2007; Francini and Sebastiani, 2013)
Osmanthus fragrans	Tea Olive	High	(Benavente-García *et al.*, 2000; Del Río *et al.*, 2003; Petridis *et al.*, 2012)
Castanea pumila	Dwarf Chestnut	High	(G. Osterc, M. Štefančiča, A. Solar, 2008)

The plants were ordered, bare root, if possible and transplanted to the contaminated soil contained in ~4 L pots (TyTy Nursery, TyTy, GA) in a greenhouse. The plants were hand-watered every other day with 400 mL of reverse osmosis water at 50mg/kg total dissolved solids, fertilized three times during the experiment, and allowed to grow normally for nine months. Fertilizer, at the rate of 264 mg/kg N, 26 mg/kg P, and 33 mg/kg K was applied to each plant. The fertilizer was split over three applications applied in 50 mL volumes, once a month, for three months starting during month 7 of the experiment. This fertilizer was used to decrease competition between the plant and microorganisms

for nutrients and facilitate microbial degradation of the PAHs (Fu *et al.*, 2012). At the end of the experiment, the plants were destructively sampled.

Soil

The soil was collected from Texas A&M Agrilife Research Farm, approximately 8 km southwest of College Station, TX and situated within the Brazos River Floodplain in south-central Texas. The soil is a Weswood silty clay loam with 25:57:18 sand:silt:clay, a pH of 8.2 and an organic C concentration of 0.8 g C kg^{-1} (Wight *et al.*, 2012; Dou *et al.*, 2014). The soil was sieved on site using a 1.75 mm mesh, and allowed to air-dry for 2 weeks before being contaminated. Aliquots of soil (3.5 kg) were amended with 12 mL diesel fuel, 38 mL acetone, 10 mg/kg benzo[a]pyrene, 50 mg/kg phenanthrene, and 50 mg/kg pyrene. PAHs exist in petroleum matrix and are not found alone; therefore, diesel was used as a carrier for the hydrocarbons and to emulate a petroleum matrix. The soil was shaken to homogenize the distribution of contaminants and air-dried overnight to allow the acetone to evaporate. The resulting concentrations were 35 mg/kg diesel fuel, 50 mg/kg phenantrene, 50 mg/kg pyrene, and 10 mg/kg benzo[a]pyrene. The soil was then placed in a 4 L pot as the growth medium for the plants and a small volume (50 mL) of non-contaminated soil was added to each pot to replenish the microbial population.

Sample collection and DNA extraction

To collect the rhizosphere soil, the pot was cut open to reveal the contents. Using gloved hands, the bulk soil was removed from the roots slowly. The plant, roots and

attached soil were then shaken, by hand, to allow less adhered soil to fall away. A mid-sagittal cut with ethanol-sterilized scissors divided the root in half. One half was placed in a 50 mL centrifuge tube and immediately placed on ice for microbial analysis. The other half was placed in a small envelope to be dried at 50°C for 72 hours and used for polyphenol analysis. The remaining bulk soil was dried, bagged, and placed in a -80°C freezer until PAH analysis. The 50 mL centrifuge tubes were centrifuged at 1600 rpm on an Eppendorf 5430/5430 R with F-45-30-11 rotor for 20 min. The supernatant was discarded and the remaining soil was extracted using PowerSoil DNA Isolation Kits (Mo Bio Laboratories Inc., Carlsbad, CA, USA). DNA was ethanol-precipitated and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Colorimetric phenolic assay

Root polyphenols were extracted using the Folin-Ciocalteu Method (Sulaiman *et al.*, 2011; Ainsworth and Gillespie, 2007). Gallic acid was used as the surrogate for polyphenol analysis. Gallic acid (0.05 g) was dissolved in 2 mL of 96% ethanol and filled to 50 mL with deionized water. This stock solution was then diluted to create 0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL concentrations. Each standard solution (100 µL) was placed in a test tube and 5 mL of 0.1% (v/v) 2 *N* Folin-Ciocalteu reagent was introduced. After mixing, 3.5 mL of 0.12% sodium carbonate (w/v) was added and the solution was allowed to incubate, in the dark, for 1 hour. The absorbance was read at 760 nm. The percent

transmittance (%T) was noted and plotted in a graph from which a logarithmic regression equation was derived and used for concentration determination.

Dried plant material was ground in a mortar and pestle. About 0.070 g of dried plant material was weighed and extracted with 5 mL of 80% ethanol in a 25 mL centrifuge tube. The tubes were placed in a water bath at 30°C for 30 minutes, and then allowed to steep for an hour. The samples were centrifuged at 4500 rpm on an Eppendorf 5430/5430 R with F-45-30-11 rotor for 10 minutes and the supernatants were placed in an empty 25 mL centrifuge tube. A 100 µL aliquot of each sample was added to a test tube where 5 mL of 0.1% (v/v) 2 *N* Folin-Ciocalteu Reagent was introduced. The solution was mixed then 3.5 mL of 0.12% sodium carbonate (w/v) was added. The test tubes were left in the dark for 1 hour for color development. Absorbance was measured at 760 nm.

A standard curve (0-1 mg/ml) prepared with gallic acid was used to convert percent transmittance readings into absorbance and then into phenolic concentration. Phenolic concentration results were determined using the regression equation $y = 1.3585x + 0.0498$ ($R^2 = 0.9814$), where x is the absorbance of the various samples. The phenolic data are reported in terms of gallic acid equivalents (GAE) determined by the equation $GAE = (c)(v/m)$ where c is the phenolic concentration from the regression equation in mg/ml, v is the volume used to determine the concentration (0.1 mL), and m is the mass of sample used in the determination (g) (Abdelhady, 2011). The units of GAE are mg/g of sample. The phenolic values reported are an average of triplicate extractions and determinations taken from replicate pots. Significance was determined through a one-way ANOVA ($p<0.05$) and Tukey's HSD.

Community quantitative PCR

The quantitative-PCR (qPCR) assays targeting total bacteria and fungi were performed using 1100F/1492R for bacteria and ITS1/ITS5.8S for fungi (Table 10). The assays were performed in a 22.5 µL reaction mixture containing 12.5 µL SYBR green real master mix (5Prime, Gaithersburg, MD), 1.25 µL of each primer (concentration 10 µM), 2.5 µL template, 0.5 µL bovine serum albumin (10 mg mL^{-1}), and 4.5 µL molecular grade water. Each analysis run included a set of standards, negative controls, and samples (each with three analytical replicates) on a 96-well plate. For the ITS region, the qPCR was run with the following conditions: 94°C for 5 min; 94°C for 30 sec, 57°C for 45 sec (30 cycles); 72°C for 1.5 min (Manter and Vivanco, 2007). For the 16S rRNA gene, the qPCR conditions were as follows: 95°C for 10 min; 95°C for 30 sec, 53°C for 30 sec (40 cycles); 72°C for 1 min (Fierer *et al.*, 2005). The qPCR was performed using an Eppendorf Mastercycler® ep realplex thermal cycler (Eppendorf, Hamburg, Germany).

Table 10 - List of PCR primers used in this study

Primer	**Sequence (5' – 3')**	**Annealing temperature (°C)**	**References**
1100F	GGC AAC GAG CGM GAC CC	53	(Lane *et al.*, 1985; Dorsch and Stackerbrandt, 1992)
1492R	GGT TAC CTT GTT ACG ACT T		(Turner *et al.*, 1999)
ITS1F	CTT GGT CAT TTA GAG GAA GTA A	57	(Gardes and Bruns, 1993)
ITS5.8S	CGC TGC GTT CTT CAT CG		(Vilgalys and Hester, 1990)
ITS1	TCC GTA GGT GAA CCT GCG G	53	(White *et al.*, 1990)
ITS4	TCC TCC GCT TAT TGA TAT GC		(White *et al.*, 1990)

Standards for the 16S rRNA gene qPCR assays were generated by growing *E. coli* ATCC 8739 on Brain Heart Infusion Agar (BHI Agar) for three days. Cultures were then transferred to a 15 ml centrifuge tube with 5 mL of Lysogeny broth (LB broth). After three days, the tubes were centrifuged at 1400 rpm on an Eppendorf 5430/5430 R with F-45-30-11 rotor for 10 minutes and DNA was extracted from the pellet using a Mo Bio PowerSoil Kit (Mo Bio Laboratories Inc., Carlsbad, CA). The concentration of DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA concentrations ranging from 10^{-1} to 10^{-7} ng μL^{-1} DNA were used to generate the 16S rRNA qPCR standard curves.

Standards for the ITS assays were generated by extracting ITS1-5.8S-containing plasmids from *E. coli* (Hollister *et al.*, 2013). The *E. coli* were grown in LB broth with 50 µL ampicillin for three days then the plasmids were extracted using a 5Prime PerfectPrep Spin Mini Kit (VWR International, Radnor, PA). Plasmid DNA concentrations ranging from 10^{-1} to 10^{-7} ng μL^{-1} DNA were used to generate the ITS qPCR standard curves.Errors in the estimations of relative amounts of DNA can happen at high and low C_T; therefore, they were not used in the creation of the standard curve equation (Nadkarni *et al.*, 2002). The equation used to quantify the 16S rRNA gene number was $y = -2.729x + 33.904$ ($R^2 = 0.93$) and the equation used to quantify ITS was $y = -10.098 + 76.703$ ($R^2 = 0.99$).

Sequence analysis and community comparisons

The 16S rRNA gene PCR primers 1100F/1492R with barcode on forward primer and ITS region with primers ITS1/ITS4 were used in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. PCR products were used to prepare a DNA library by using the Illumina TruSeq DNA library preparation protocol. Sequencing was performed at Mr. DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines.

MiSeq sequence data were trimmed to a similar length, aligned, quality checked and assigned to operational taxonomic units (OTUs) based on phylotype using the MOTHUR MiSeq SOP pipeline (Schloss, 2009). MOTHUR was also used to calculate the observed species (S_{obs}), Shannon's diversity index values (H'), inverse Simpson index values ($Simpson^{-1}$), and Chao1 richness estimates. Phylogenetic trees were constructed using dist.shared and tree.shared commands in MOTHUR and inter-treatment comparisons of phylogenetic structure were conducted using the Analysis of Molecular Variance (AMOVA) test in MOTHUR. P-values ≤ 0.05 were considered significant and pairwise comparisons of individual plants were conducted if the global test was found to be statistically significant. Bonferonni correction for multiple comparisons was used among the pairwise comparisons, adjusting the significance level to $P \leq 0.0017$ (Hollister *et al.*, 2010; Neter *et al.*, 1996).

Principal Coordinates Analysis (PCoA) of treatment communities based on OTU composition was conducted using the dist.shared and pcoa commands in MOTHUR. Population-level analysis to determine the organisms that were significantly different between the treatment communities (high- vs. low-phenolic exuders) was conducted using the lefse command in MOTHUR.

RESULTS

Phenolic Concentration

To answer the question posed by hypothesis 1 which relates to phenolic exudation of the various plants, we used a colorimetric phenol assay to determine the phenol concentration of the plants. All measurements are in gallic acid equivalents (GAE). Crabapple had the highest phenolic concentration at 0.48 mg GAE/g, followed by tea olive at 0.39 mg GAE/g, cassia at 0.17 mg GAE/g, dwarf chestnut at 0.16 mg GAE/g, jujube at 0.13 mg GAE/g and golden currant at 0.09 mg GAE/g. The bars in Figure 4 are color-coded based on their expected characterization as either high or low-phenolic producers. The green represents purported high-phenolic producers, while red denotes supposed low-phenolic producers. Blue represents the control. Crabapple had significantly higher phenolic concentrations than the control ($p<0.01$), golden currant ($p<0.01$), jujube ($p<0.01$), chestnut, and cassia. Tea olive had significantly higher phenolic concentrations than the control ($p<0.01$) and golden currant only (Figure 4).

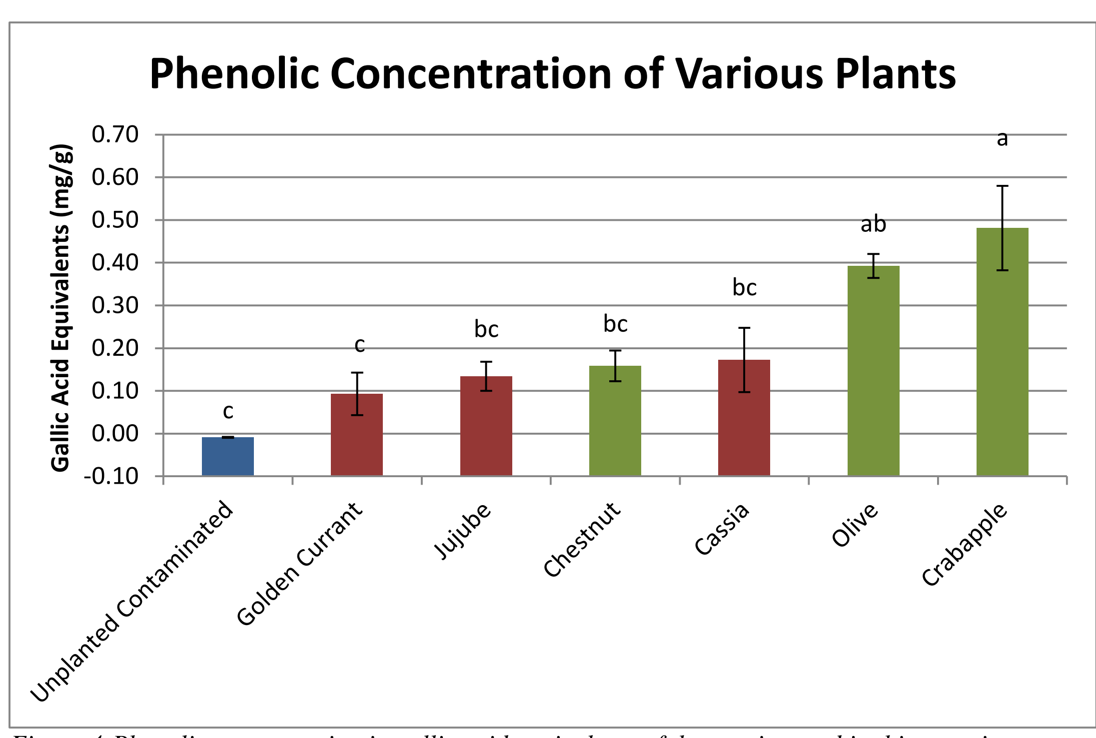

Figure 4-Phenolic concentration in gallic acid equivalents of the species used in this experiment. Data represents the mean of three replicates. Different letters indicate a significant difference at $p < 0.05$. Green represents high-phenolic exuders, red represents low-phenolic exuders, and blue represents controls. Error bars are based on standard errors of triplicate samples.

Previous literature characterized *Castanea crenata x Castanea sativa* (chestnut hybrid) as a likely candidate for high phenolic concentration (G. Osterc, M. Štefančiča, A. Solar, 2008); however, *Castanea pumila* did not follow that trend. Instead it clustered more closely with low-phenolic producers. Alternatively, *Osmathus fragrans* (tea olive) was confirmed to be a high-phenolic producer despite previous literature that does not explicitly focus on that species of olive, but mostly on a relative, *Olea europea* (Benavente-García *et al.*, 2000; Del Río *et al.*, 2003; Petridis *et al.*, 2012). These observations confirm that membership in a taxon is not an adequate measure of potential phenolic-production (Fletcher and Hegde, 1995; Phillips *et al.*, 2012). Instead, members of a taxon should be tested on an individual basis to determine their relative phenolic-production potential.

Various factors determine phenolic output, including root structure and time of plant growth. Growth stage is also a major influence on plant phenolic exudation (Sellami *et al.*, 2009; Hegde and Fletcher, 1996; Kim *et al.*, 2014). Therefore, the differences in the growth rates of plants can impact the phenolic outputs and comparisons. However, that consideration is most prominent when considering plants that are started from seed. This experiment did not account for differences in growth rates of the plants because some of the plants were received with established root systems. Differences in phenolic concentrations can also be attributed to the differences in root systems within (root size, age, etc.) and between the plants. A relatively large root within a root system can contain more bioactive compounds than a smaller root within the same system (Wang *et al.*, 2013). Therefore, selecting roots of similar size is

imperative. In addition, exudation can differ among the same plant at varying developmental stages. For example, a radish sprout contains more phenolics than the mature taproot of the same plant (Hanlon and Barnes, 2011). Also, sampling of the rhizosphere focused exclusively on total mass without regarding root order. The concentration of phenolics decreases as root order increases (Wang *et al.*, 2015). Future studies should ensure that sampling accounts for root order, root growth rate, and overall mass.

Community Quantitative PCR

Hypothesis 2 concerns the abundance of bacterial and fungal genes in the treatments as a function of phenolic exudation. Although significant differences exist in the phenolic concentrations of the plants, those results did not translate into differences in 16S rRNA gene or ITS copy numbers. Differences between the 16S rRNA gene or ITS copy numbers between samples (Figures 5 and 6) were not significantly different. Similarly, Phillips *et al.* (2012) found that the abundance of the 16S rRNA gene was consistent between treatments of mineralization microcosms amended with phenanthrene, naphthalene, or hexadecane and planted with either alfalfa or wildrye. Significant differences did not exist between these treatments and the control, but differences between the abundance of catabolic genes involved in PAH degradation were significant. Therefore, changes in microbial community structure cannot be ruled out due to insignificant changes in 16S rRNA gene abundance. Quantifying the genes involved in PAH degradation or their expression in order to identify changes in microbial communities

in PAH-contaminated soil may be helpful because overall bacterial or fungal abundance were not specific enough to recognize shifts. Attempts at quantifying functional genes in this experiment did not occur because of complications with enzyme selection, primer design, and selection of positive controls.

Competition between microbes and plants for nitrogen could lead to considerably lower microbial biomass (Kuzyakov and Xu, 2013). Because hydrocarbon substrates were added that increased carbon availability, an increase in abundance and activity of microorganisms that increased immobilization and depleted available nutrients may have occurred. This could halt the original surge in microbial activity and abundance caused by the carbon inputs. Although fertilizer was added to the plants a total of three times throughout the experiment, the competition for the nutrients may have been sufficient enough to impact the microbial population.

Many studies that note an increase in population size as it relates to phenolic input utilized individual compounds, artificial exudates, or actual root exudates applied exogenously (Técher *et al.*, 2011; Techer *et al.*, 2012; Rentz *et al.*, 2005; Phillips *et al.*, 2012). Not every experiment utilizes actual soil media (Chen and Yuan, 2012; Kamath *et al.*, 2004; Rentz *et al.*, 2005), but some do (Lee *et al.*, 2008; Chen and Yuan, 2012). This experiment utilized a spiked-soil media and root exudates provided directly from the plant through integration in the soil. Although many variables were held constant, other factors could still contribute to the statistically equal 16S rRNA gene copy results. A supplemental laboratory experiment conducted in microcosms focusing on extracted phenolics and

microbial communities could provide more information concerning the effect of these specific plant phenolics on microbial populations.

The bacterial community in the rhizosphere community is highly dependent on the host plant, but part of the community may contain microorganisms that are not dependent on plant genotype (Lambais *et al.*, 2014). These microorganisms are saprophytic and may account for the similarities in microbial and fungal populations despite the significant differences in plant exudation.

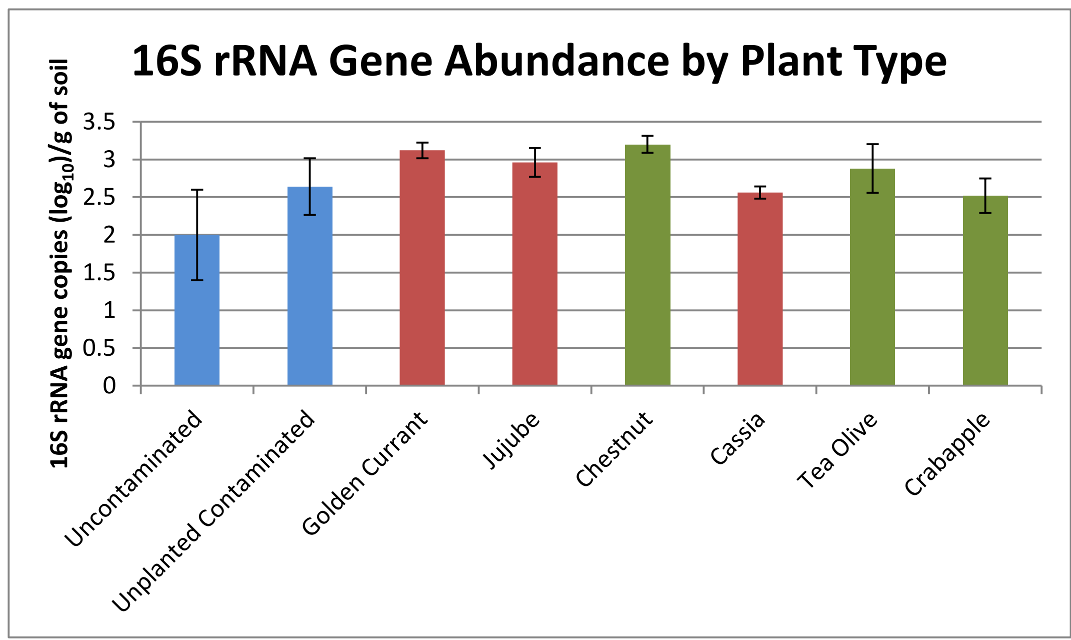

Figure 5-Rhizosphere 16S rRNA gene abundance of plants included in experiment. Green represents high-phenolic exuders, red represents low-phenolic exuders, and blue represents controls. Error bars are based on standard errors of triplicate samples.

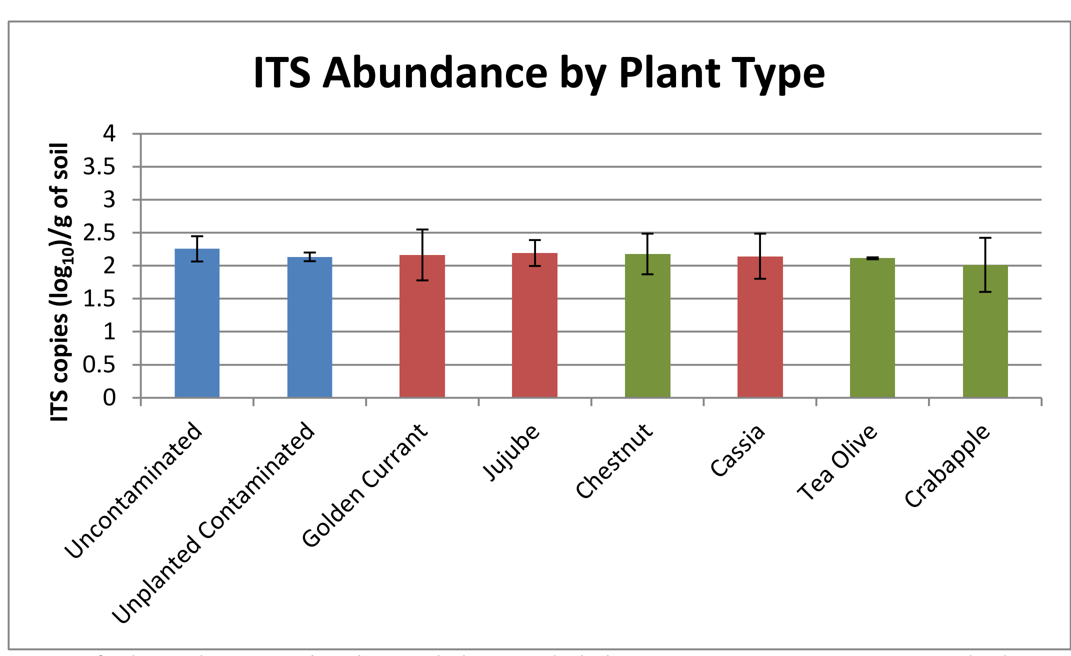

Figure 6-Rhizosphere ITS abundance of plants included in experiment. Green represents high-phenolic exuders, red represents low-phenolic exuders, and blue represents controls. Error bars are based on standard errors of triplicate samples.

Community Composition, Diversity, and Estimated Richness

DNA sequencing was used to address hypotheses 3 through 5 which concerned a change in dominant microbial population, a change in relative abundance of PAH-exuders, and similarity in rhizosphere microbial structure as a result of similar phenolic exudation. A total of 2,156,177 16S rRNA gene sequences were generated through Illumina sequencing with an average read length of 392±25bp (mean±s.d.). Each treatment was sampled in triplicate and the average of those sequences reported in Table 11. Overall, a total of 863 OTUs were identified. Shannon diversity index values (H') suggest variation in diversity among the samples, but a discernible trend based on phenolic concentration was lacking. Chao 1 richness estimates suggest that over half of the estimated diversity present in these communities was identified by sequencing. While the inverse Simpson suggests an increase in diversity in planted treatments compared to the uncontaminated and the unplanted contaminated, the increase does not seem to correlate with an increase in phenolic exudation.

Members of 27 bacterial phyla were detected among the samples. *Proteobacteria* and *Acidobacteria* were encountered most frequently, representing 44 to 74% and 10 to 27% of each sample, respectively. *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* represented 3 to 22%, 1 to 11%, and 2 to 5% of the represented phyla respectively. Less than 10% of the microbial composition of each treatment was represented by the remaining 22 phyla (Figure 7).

Of the *Proteobacteria* phylum, *Gammaproteobacteria* was the most represented class in the control, jujube, chestnut, and crabapple. *Alphaproteobacteria* dominated in

cassia, tea olive, and golden currant (Figure 8). Both of these classes of bacteria have been associated with PAH degradation (Sun *et al.*, 2014).

Neither *Gammaproteobacteria* nor *Alphaproteobacteria*, as a whole, experienced a significant increase or decrease in abundance in the presence of the plants when compared to the unplanted contaminated and the uncontaminated treatments. This does not mean, however, that families, genera, or species within the class were not affected by the presence of plants in the PAH-contaminated soils. This is especially true for *Proteobacteria*, a phylum with a high amount of physiological diversity.

Previous studies have noted that *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* comprise the core rhizosphere microbiome for *Arabidopsis thaliana*. The plant is also able to select a subset of microbes at different stages of growth. (Lundberg *et al.*, 2012; Bulgarelli *et al.*, 2012; Chaparro *et al.*, 2014). In this study, *Proteobacteria* dominated in all treatments with *Acidobacteria* and *Bacteroidetes* completing the core for these treatments.

Although AMOVA found significant differences ($p = <0.001$) globally in rhizosphere bacterial community composition of the different plant species, pairwise comparisons of the treatments were not significantly different (Table 12). Next, the plants were grouped according to hypothesized phenolic exudation (high or low) and the microbial communities were distinct enough to be statistically different. Therefore distinctions that were insignificant when analyzed individually were significant when pooled together.

OTUs that were over-represented in either high-phenolic exuders or low-phenolic exuders are listed in Tables 13 and 14. These OTUs may have been responsible for the global differences between the high- and low-phenolic exuders because pairwise comparisons between treatments did not yield any significant differences in OTUs. However, when the treatments were grouped together as either high- or low-phenolic exuders, certain OTUs were shown to be over-represented in the treatments.

The low-phenolic exuders have a larger population of *Actinobacteria* compared to high-phenolic exuders which were dominated by *Proteobacteria*. This was in line with a study that stated that *Proteobacteria* outcompete *Actinobacteria* in hydrocarbon-contaminated environment (Mukherjee *et al.*, 2014). Figures 9 and 10 highlight the ways the treatments clustered together based on their microbial communities. Overall, samples within the same treatment clustered together and a distinction between high-phenolic exuders and low-phenolic exuders was evident. This suggests that rhizosphere conditions, such as phenolic exudation within treatments were similar enough to select for microbial communities that were closely related. Plants are able to select a subset of microbes to perform functions not expressed by the traditional core microbiome. The microbial community composition for these treatments may reflect a shift in functions specific to PAH degradation in the presence of increased carbon inputs.

Table 11 - Summary of bacterial (16S) sequence library sizes, operational taxonomic units (OTUs), and diversity and richness estimates. Green represents high-phenolic exuders, red represents low-phenolic exuders, and blue represents controls.

	Sequence Library Size	**Number of OTUs**	**Simpson^{-1}**	**Chao1**	**Shannon (H')**
All treatments	2,156,177	863			
Cassia	98,732 ± 6,275	491 ± 10	16.36 ± 8.97	536.11 ± 5.35	3.70 ± 0.32
Golden Currant	89,043 ± 5,552	523 ± 35	25.31 ± 1.82	519.97 ± 20.26	4.02 ± 0.09
Jujube	85,113 ± 6,775	443 ± 10	16.43 ± 6.59	531.74 ± 1.32	3.76 ± 0.19
Chestnut	74,248 ± 1,926	448 ± 10	16.37 ± 12.94	528.80 ± 18.56	3.70 ± 0.51
Tea Olive	89,149 ± 11,809	474 ± 20	26.60 ± 4.51	588.87 ± 17.97	4.12 ± 0.12
Crabapple	92,396 ± 2,644	459 ± 8	18.68 ± 12.15	501.75 ± 11.27	3.80 ± 0.25
Uncontaminated	92,130 ± 1,112	526 ± 8	16.31 ± 1.74	581.00 ± 20.35	3.92 ± 0.08
Unplanted Contaminated	97,911 ± 7,560	491 ± 24	15.41 ± 4.14	540.47 ± 26.74	3.65 ± 0.24
	Mean ± s.e.		Mean ± s.e.		

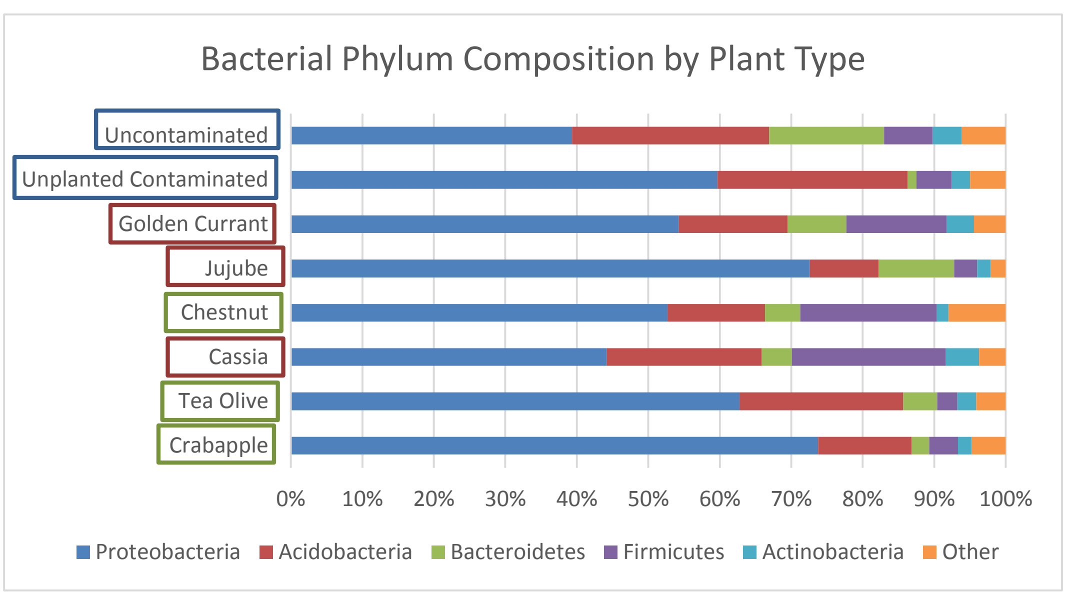

Figure 7- Bacterial rhizosphere community composition of high- and low-phenolic exuding plants. Green boxes represent high-phenolic exuders, red boxes represent low-phenolic exuders and blue boxes represent controls.

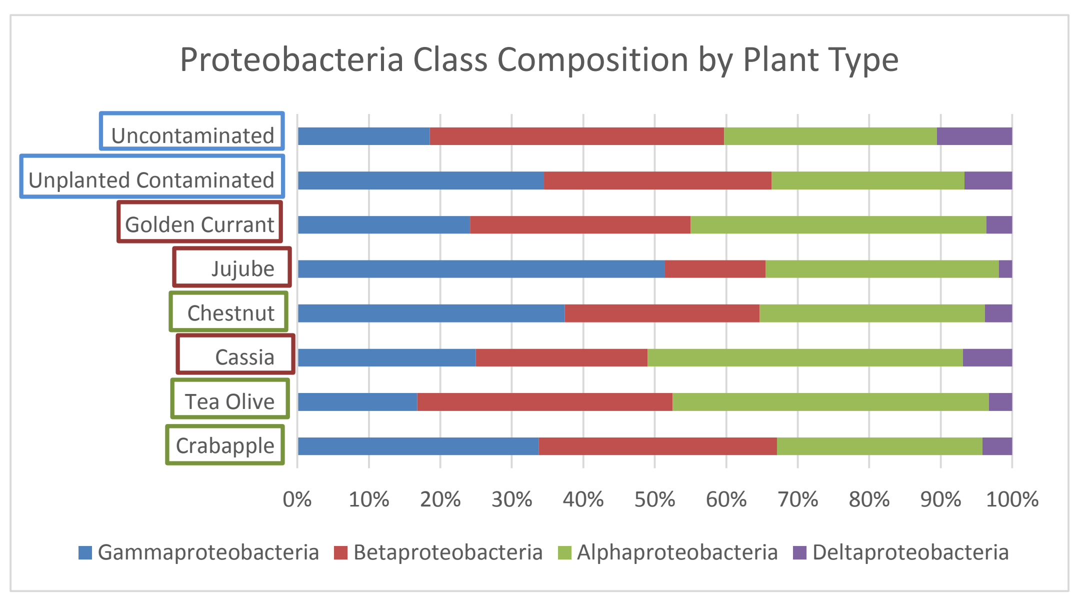

Figure 8- Rhizosphere Proteobacteria class composition of high- and low-phenolic exuding plants. Green boxes represent high-phenolic exuders, red boxes represent low-phenolic exuders, and blue boxes represent controls

Table 12 - Bacterial analysis of molecular variance (AMOVA) to determine pairwise differences between treatments. The values in the table are the p-values of the pairwiese comparisons between treatments. Green represents high-phenolic exuders, red represents low

AMOVA

	Uncontaminated	**UpC**	**Golden Currant**	**Jujube**	**Chestnut**	**Cassia**	**Tea Olive**
Unplanted Contaminated (UpC)	0.07						
Golden Currant	0.10	0.07					
Jujube	0.12	0.08	0.12				
Chestnut	0.10	0.11	0.11	0.09			
Cassia	0.10	0.10	0.10	0.20	0.06		
Tea Olive	0.10	0.08	0.10	0.10	0.08	0.05	
Crabapple	0.10	0.09	0.11	0.11	0.50	0.05	0.10

Table 13 - List of OTUs responsible for the global difference in high-polyphenol bacterial rhizosphere community composition. The OTUs listed were over-represented in the high-phenolic exuders and are organized by ascending p-value.

OTU	Class	pValue	Phylum	Class	Order	Family	Genus
Otu302	High	0.001	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	
Otu031	High	0.002	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	
Otu193	High	0.006	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonadaceae
Otu005	High	0.008	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	
Otu175	High	0.010	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	
Otu164	High	0.016	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	
Otu142	High	0.018	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	
Otu010	High	0.025	Proteobacteria	Alphaproteobacteria	Sphingomonadales		
Otu006	High	0.030	Proteobacteria	Gammaproteobacteria			
Otu239	High	0.035	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Duganella
Otu286	High	0.036	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Hydrogenophaga
Otu039	High	0.037	Proteobacteria	Deltaproteobacteria	GR-WP33-30		
Otu151	High	0.039	Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	Legionella
Otu041	High	0.040	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Phenylobacterium
Otu199	High	0.042	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Perlucidibaca
Otu075	High	0.043	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Chitinophagaceae	Niastella
Otu196	High	0.045	Proteobacteria	Deltaproteobacteria	Myxococcales	Cystobacteraceae	Hyalangiu
Otu073	High	0.046	Proteobacteria	Alphaproteobacteria	Rhodospirillales		
Otu029	High	0.046	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Curvibacter
Otu061	High	0.046	Proteobacteria	Gammaproteobacteria	Chromatiales	Chromatiaceae	

Table 14 - List of OTUs responsible for the global difference in low-polyphenol bacterial rhizosphere community composition. The OTUs listed were over-represented in the low-phenolic exuder and are organized by ascending p-value.

OTU	**Class**	**p-value**	**Phylum**	**Class**	**Order**	**Family**	**Genus**
Otu223	Low	0.000	Actinobacteria	Actinobacteria	Actinomycetales		
Otu213	Low	0.001	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Kribbella
Otu231	Low	0.001	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	Singulisphaera
Otu146	Low	0.001	Proteobacteria	Deltaproteobacteria	Myxococcales	Nannocystaceae	Nannocystis
Otu033	Low	0.003	Actinobacteria	Actinobacteria	Actinomycetales		
Otu149	Low	0.003	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	Isosphaera
Otu105	Low	0.005	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Chitinophagaceae	
Otu115	Low	0.009	Actinobacteria	Actinobacteria	Actinobacteridae	Actinomycetales	Micrococcineae
Otu182	Low	0.011	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Nocardioides
Otu294	Low	0.011	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter
Otu109	Low	0.012	Firmicutes				
Otu266	Low	0.013	Deinococcus-Thermus	Deinococci	Deinococcales	Trueperaceae	Trupera
Otu328	Low	0.015	Bacteroidetes	Sphingobacteria	Sphingobacteriales		
Otu139	Low	0.018	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Sphingobacteriaceae	
Otu020	Low	0.022	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Luteimonas
Otu060	Low	0.023	Firmicutes	Bacilli	Bacillales	Planococcaceae	
Otu338	Low	0.025	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus
Otu183	Low	0.025	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bacteriovoraceae	Peredibacter
Otu234	Low	0.025	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
Otu173	Low	0.029	Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardiaceae	Pseudonocardia
Otu141	Low	0.037	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium
Otu277	Low	0.039	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Nitrobacter
Otu206	Low	0.040	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter
Otu076	Low	0.040	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	
Otu079	Low	0.049	Actinobacteria	Actinobacteria	Solirubrobacterales	Solirubrobacteraceae	Solirubrobacter
Otu082	Low	0.049	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Methylibium

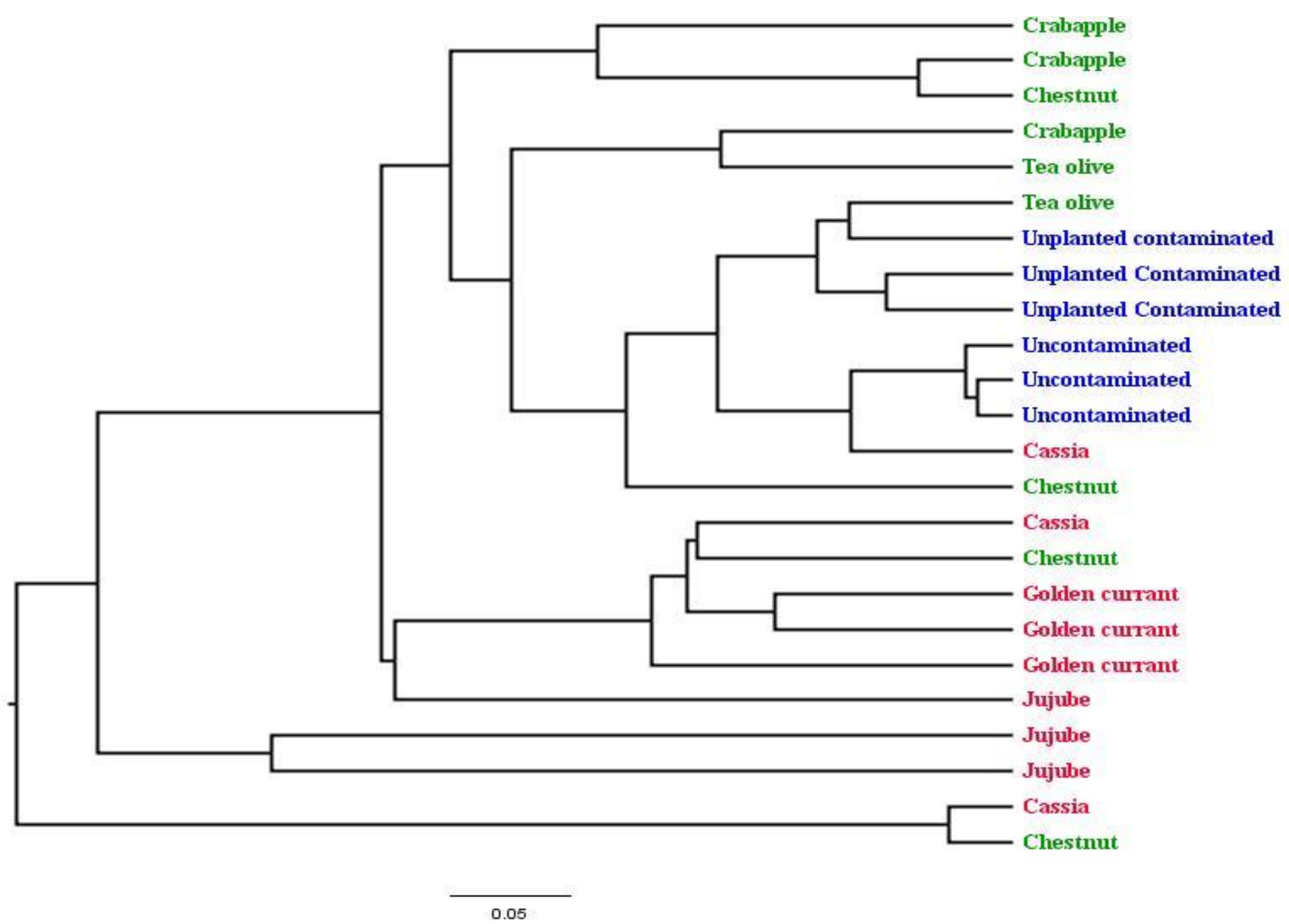

Figure 9-Phylogenetic tree of treatments based on rhizosphere bacterial community composition. Theta-YC sample tree generated using MOTHUR. Green represents high-phenolic exuders, red represents low-phenolic exuders, and blue represents controls.

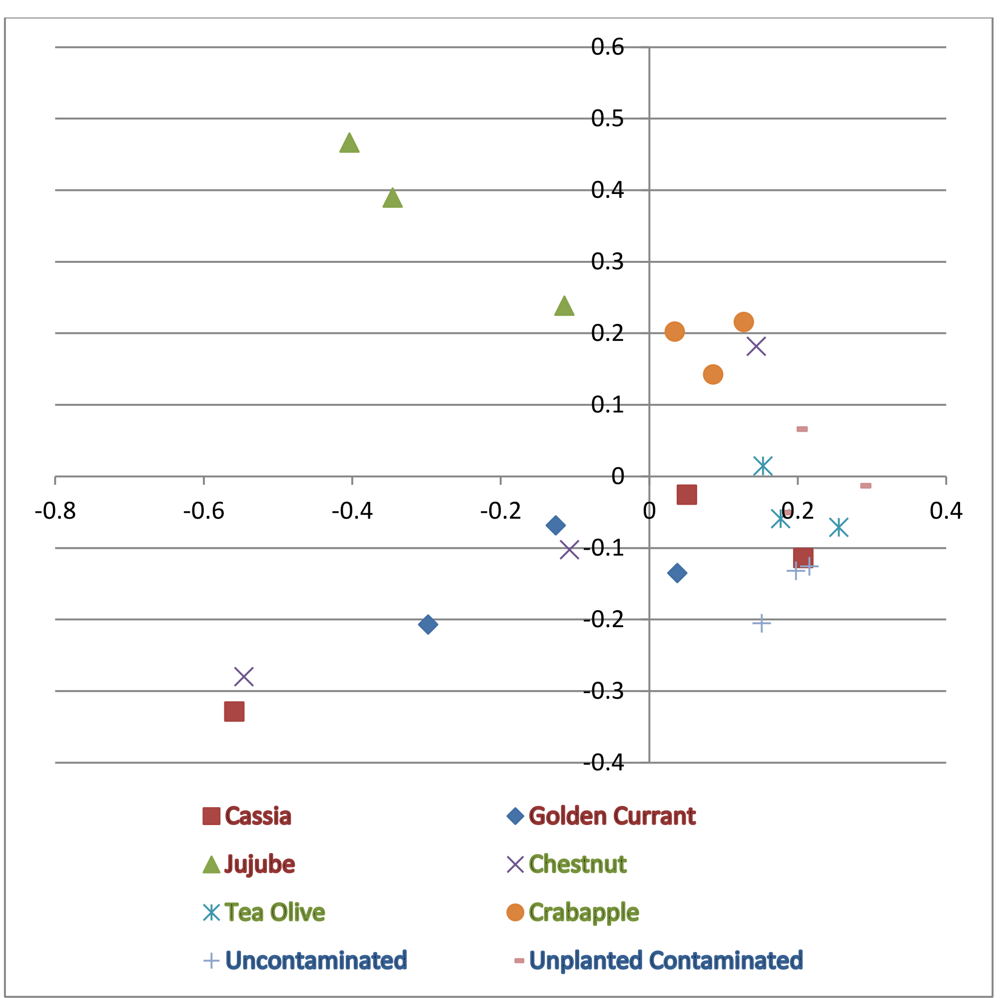

Figure 10-Principal Coordinate Analysis graph (PCoA) detailing how the treatment clustered based on rhizosphere bacterial community composition. Green represents high-phenolic exuders, red represents low-phenolic exuders, and blue represents controls

A total of 2,676,382 ITS sequences were generated through Illumina sequencing (Table 15). Overall, a total of 1217 OTUs were identified. Shannon diversity index values (H') suggest variation in diversity among the samples, but a discernible trend based on phenolic concentration was lacking. Chao 1 richness estimates suggest that at least a quarter of the estimated diversity present in these communities was identified by sequencing. While the inverse Simpson shows an increase in diversity in planted treatments compared to the uncontaminated and the unplanted contaminated, the increase in diversity is not significant and does not correlate with an increase in phenolic exudation. Figure 11 shows the fungal community composition of the rhizosphere of the various treatments. *Ascomycota* was the dominate phylum in all treatments.

Although an AMOVA test found significant differences ($p = <0.001$) globally, pairwise comparisons of the treatments were not significantly different. Significant differences were found when the AMOVA test was run using a high-phenolic/low-phenolic treatment design. While individual treatments were not distinct enough to be significant, statistically significant differences between the fungal communities of high-phenolic exuders and that of low-phenolic exuders existed ($p<0.001$) (Table 16). Despite the observed increase in *Basidiomycota* it Figure 11, the lefse command in MOTHUR did not discern any fungal OTUs that were significantly different between treatments. Figures 12 and 13 depict the clustering of the treatments based on the fungal community composition. Compared to the clustering based on bacterial composition, the samples were more dispersed indicating that the different plant types did not have as great of an

impact on fungal community composition as they did on bacteria community composition.

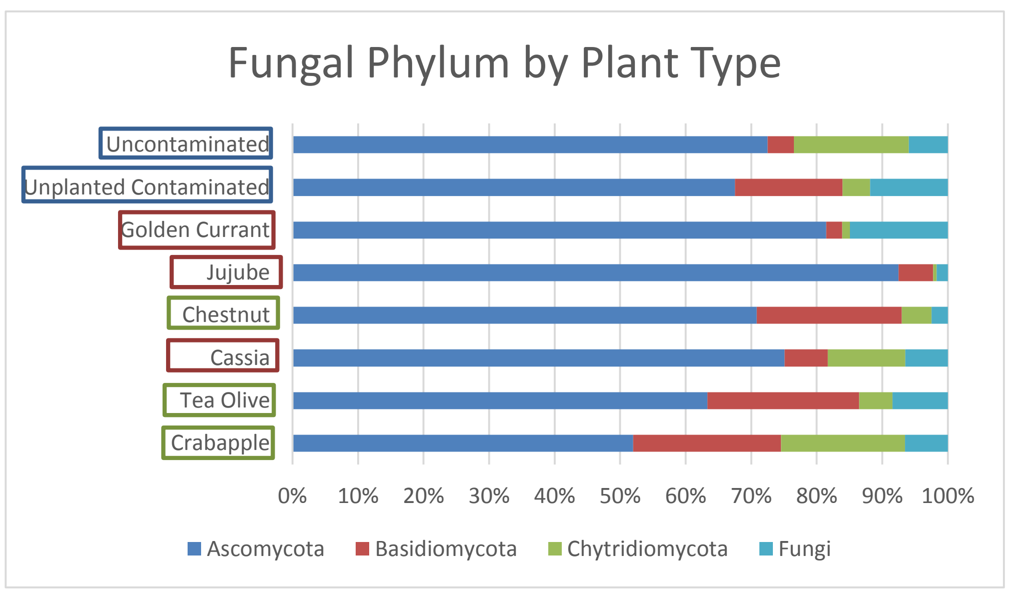

Figure 11-Fungal rhizosphere community composition of high- and low-phenolic exuding plants. Green boxes represent high-phenolic exuders, red boxes represent low-phenolic exuders, and blue boxes represent controls.

Table 15 - Summary of fungal (ITS) sequence library sizes, operational taxonomic units (OTUs), and diversity and richness estimates. Green represents high-phenolic exuders, red represents low-phenolic exuders, and blue represents controls.

	Sequence Library Size	**Number of OTUs**	**Simpson^{-1}**	**Chao1**	**Shannon (H')**
All treatments	2,676,832	1217			
Cassia	74,737 ± 6,765	459 ± 13	14.81 ± 4.79	463.40 ± 12.75	3.40 ± 0.34
Golden Currant	71,309 ± 3,418	455 ± 34	13.00 ± 3.92	476.21 ± 11.05	3.33 ± 0.22
Jujube	121,010 ± 84,030	523 ± 25	4.50 ± 2.81	348.46 ± 128.91	1.72 ± 0.90
Chestnut	165,941 ± 65,708	429 ± 50	2.73 ± 0.86	288.24 ± 100.43	1.58 ± 0.57
Tea Olive	100,440 ± 4,758	466 ± 20	12.73 ± 1.78	474.81 ± 5.45	3.45 ± 0.11
Crabapple	93,907 ± 3,656	410 ± 75	8.57 ± 3.97	401.93 ± 25.18	2.81 ± 0.39
Uncontaminated	105,252 ± 6,933	540 ± 37	18.99 ± 1.30	475.95 ± 12.20	3.64 ± 0.08
Unplanted Contaminated	159,681 ± 81,949	469 ± 51	5.92 ± 2.72	317.57 ± 127.90	2.13 ± 1.05
	Mean ± s.e.		Mean ± s.e.		

Table 16 - Fungal analysis of molecular variance (AMOVA) to determine pairwise differences between treatments. Values in table represent the p-value of the pairwise comparison between treatments. Green represents high-phenolic exuders, red represents low-phenolics

AMOVA							
	Uncontaminated	**UpC**	**Golden Currant**	**Jujube**	**Chestnut**	**Cassia**	**Tea Olive**
Unplanted Contaminated (UpC)	0.10						
Golden Currant	0.10	0.19					
Jujube	0.10	0.09	0.11				
Chestnut	0.11	0.23	0.12	0.22			
Cassia	0.10	0.30	0.09	0.11	0.10		
Tea Olive	0.10	0.10	0.10	0.12	0.21	0.10	
Crabapple	0.10	0.70	0.09	0.09	0.20	0.11	0.09

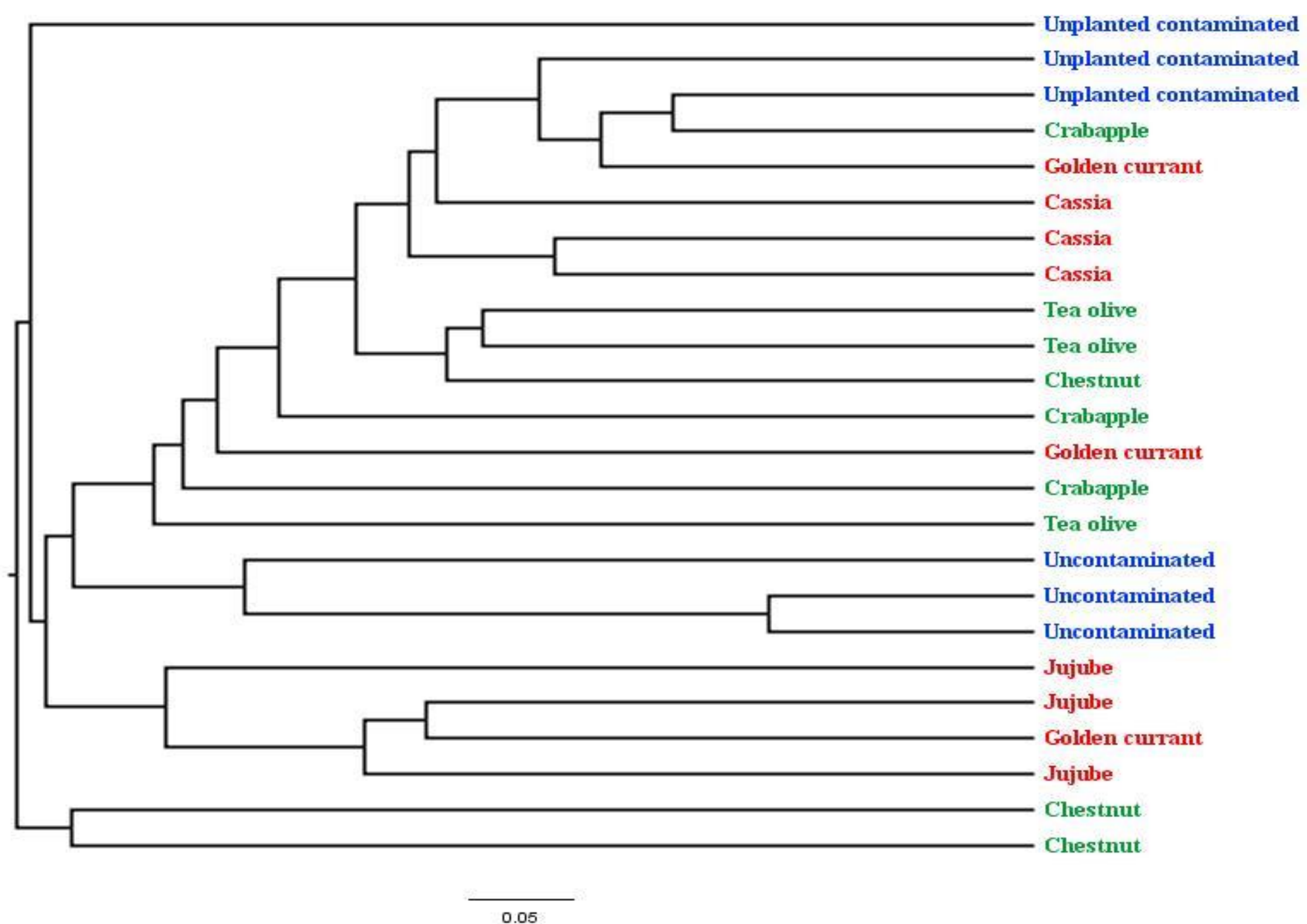

Figure 12-Phylogenetic tree of treatments based on fungal community composition. Theta-YC sample tree generated using MOTHUR. Green represents high-phenolic exuders, red represents low-phenolic exuders, and blue represents controls.

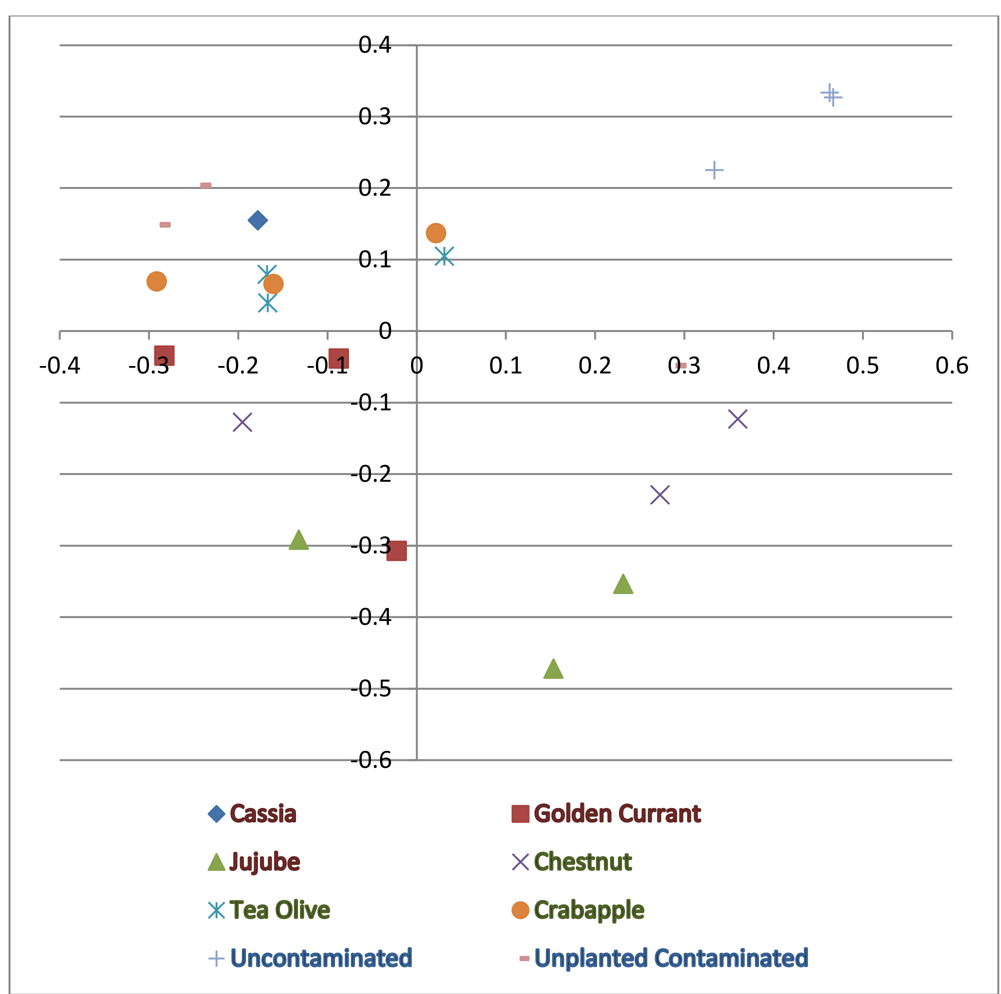

Figure 13-Principal Coordinate Analysis graph (PCoA) detailing how the treatment clustered based on fungal community composition. Green represents high-phenolic exuders, red represents low-phenolic exuders, and blue represents controls.

CONCLUSION

While many experiments have looked at how phenolic exudates impact microbial communities, this is the first to compare this suite of low- and high-phenolic exuding plants' impact on bacterial and fungal community composition in the presence of phenanthrene, pyrene, and benzo[a]pyrene.

While many experiments are able to see significant differences in the fungal and bacterial communities after the addition of phenolic exudates, in the presence of the hydrocarbons, those differences can be lost if the correct measurements are not taken. Compared to the unplanted contaminated and golden currant treatments, crabapple and tea olive exuded significantly more phenolics. This result was partially in line with hypothesis 1 which also proposed *Castanea pumila* would exude significantly higher phenolic than the low-exuders. However, this did not translate to significantly greater 16S rRNA or ITS gene copies or statistically significant differences in microbial community composition in pairwise comparisons. Therefore, hypothesis 2 was not supported.

Folin-Ciocalteu colorimetric method used for phenol determination can detect the presence of any reducing compound in a sample and ascorbic acid often can interfere with measurements, so this may have potentially impacted the phenolic exudation results (Sánchez-Rangel et al., 2013). However results are within the range of fruit phenolic content. The phenolic content of various fruits quantified using the Folin-Ciocalteu method range from 11.88 to 582.52 mg GAE/100 g of wet weight. Certain apple cultivars

have shown values such as 68.29, 73.96, and 70.57 mg GAE/100g (Francini and Sebastiani, 2013).

Monitoring specific PAH-related genes may be necessary to identify shifts in the community. *Proteobacteria* have been shown to out-compete *Actinobacteria* in a highly PAH-polluted area of a soil in Finland. Specifically, *Deltaproteobacteria* decreased in abundance as pollution increased and *Betaproteobacteria* abundance matched the patterns of PAH contaminant distribution (Mukherjee *et al.*, 2014). The dominant microbial population did not shift in any treatment and did not offer support for hypothesis 3. Despite not seeing significant shifts in microbial composition among treatments, there is evidence that the microbial composition is similar to those recorded in field remediation studies. This could be indicative of a shift in functional capabilities similar to what occurs *in situ* in petroleum contaminated soils and indicate the occurrence of remediation. In addition, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Cyanobacteria* all correlate with phenolics both positively and negatively (Chaparro *et al.*, 2014). The abundance of bacteria in these phyla can both increase and decrease as a result of phenolic exudation. Therefore, an overall increase in bacterial abundance may not be observable due to the community's contradictory reaction to phenolics. Bacteria within the same class can have opposing responses to phenolic inputs and can cause no significant changes in the global community. However, this phenomenon also means that bacteria with the ability to degrade PAHs can increase in abundance while other bacteria decrease in abundance. Therefore, degradation can still occur in the presence of no significant changes in the global community.

Gammaproteobacteria and *Alphaproteobacteria* are both associated with PAH degradation when phenolics are involved. In the presence of *Orychophragmus violaceus* and *Rhodococcus ruber* Em1, a positive correlation was observed between PAH degradation and the abundance of *Gammaproteobacteria*. The same study noted an inverse relationship existed between PAH degradation and *Alphaproteobacteria* abundance (Sun *et al.*, 2014). Therefore the increase in *Proteobacteria*, specifically the *Alphaproteobacteria* in Table 13 can hint towards similar results and partially support hypothesis 4.

Also depicted in Table 13 is an over-representation of a variety of *Burkholderiales* compared to the two in Table 14. *Burkholderiales*, *Actinomycetales*, and *Rhizobiales* were the most active microorganisms in a groundwater remediation study (Herbst *et al.*, 2013). The presence of these microorganisms in low-phenolic exuders' soils may indicate PAH degradation similar to that of natural attenuation, but the significant increase of those populations in the presence of the high-phenolic exuders is worth noting.

Generally, bacteria are considered more important than fungi in bioremediation efforts. However, fungi do have their place. Two species belonging to *Ascomycota* were able to degrade 32.9% of PAHs in a contaminated soil microcosm (Li *et al.*, 2012). However, alfalfa acted as an antagonist to its degradation efforts. *Ascomycota* was the dominant fungal phyla among all treatments, but differences were absent between fungal communities between treatments while bacteria showed some significant differences between the low- and high-phenolic exuders. This could be due to the differences in growth rates between fungi and bacteria and their ability to respond to disturbances such

as the addition of PAHs and phenolics. *Phanerochaete* is a genus in the *Basidiomycota* division of fungus and has been implicated in the degradation of PAHs. The microbial community is able to degrade PAHs that are sorbed to *Phanerochaete* more easily than PAHs that are sorbed to soil particles (Ding *et al.*, 2012). The high-phenolic exuders, crabapple, chestnut, and tea olive, had more *Basidiomycota* in their community than all of the low-phenolic exuders. This could suggest that the high-phenolic exuders are selecting for fungal populations that are functionally more capable of degrading PAHs. Still, the difference in statistically significant results can corroborate the fact that, in this context, bacteria are the major players and thus warrant further and greater attention.

The phylogeny of the treatments based on the bacterial communities indicates that phenolic outputs may have noticeable results on the community. Considering the lack of significant results in all but two treatments, the clustering in the tree and the PCoA are considerably tight. Based on those diagrams, the plants do seem to differentially impact the microbial community in the presence of PAHs. However, it may be by something other than the phenolic exudates. Furthermore, culture-independent methods for determining microbial community composition, while thorough, are not without their drawbacks. Changes in experimental conditions like repeated PCR or differences in annealing temperatures can alter the OTUs recovered in a microbial community (Schmidt *et al.*, 2013). In addition, the primer pair-barcode combinations can impact the richness and resolution of OTUs (Tedersoo *et al.*, 2015). Template concentration can also impact the microbial community identified (Kennedy *et al.*, 2014). Still, the results of the bacterial PCoA offers partial support for hypothesis 5. The lack of similar results in the fungal data

may be due to decreased diversity identified by sequencing (25% for fungi compared to 60% for bacteria).

As previously indicated, a myriad of factors that contribute to the changes in a rhizosphere community. Therefore, a major objective of microbial ecology is to understand how microbial communities relate to these factors. This experiment investigated a select group of plants, and it is evident that more research is needed to broaden the scope of potential phytoremediators. As more information is gleaned from experiments and technology advances, the objectives of microbial ecology become easier to accomplish.

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