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Effects of *in situ* Bioremediation Strategies on the Biodegradation of Polycyclic Aromatic Hydrocarbons and Microbial Community Dynamics in Soil

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

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by

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ABSTRACT OF THE DISSERTATION

Effects of *in situ* Bioremediation Strategies on the Biodegradation of Polycyclic Aromatic Hydrocarbons and Microbial Community Dynamics in Soil

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Polycyclic aromatic hydrocarbons (PAHs) are a class of compounds of environmental and public health concern because of their toxicity and environmental ubiquity that will be further exacerbated by increasing anthropogenic pollution. *In situ* bioremediation is a common cleanup technique for PAH-contaminated soils because it is considered to be cost-effective and environmentally-friendly. However, PAHs are often bound to nonpolar soil domains and become resistant to microbial degradation, the primary PAH removal pathway. This dissertation addresses these limitations by evaluating bioremediation-enhancement technologies such as biosurfactant amendment, bioaugmentation, and phytoremediation to increase PAH bioavailability and/or soil microbial activity. The use of biosurfactants to increase PAH bioavailability has the potential to be an environmental alternative to synthetic surfactants. Therefore, rhamnolipid biosurfactant was compared to Brij-35 surfactant in two soils contaminated with ^{14}C-pyrene that were also bioaugmented with a PAH-degrading microbe, *Mycobacterium vanbaalenii* PYR-1. The effect of the surfactants and bioaugmentation on PAH biodegradation and soil microbial

community dynamics was evaluated. The addition of Brij-35 increased ^{14}C-pyrene mineralization in both soils, but the rhamnolipid biosurfactant inhibited PAH degradation in a dose-dependent manner, which was likely due to preferential utilization of the biosurfactant as an easier carbon source by the degrading microorganisms. The bioaugmentation of *M. vanbaalenii* PYR-1 resulted in efficient ^{14}C-pyrene dissipation. Using 16S rRNA analysis, it was determined that the pyrene biodegradation was associated with changes in the soil microbial communities. The addition of pyrene resulted in a large increase in *Bacillus*, a genus associated with PAH degradation. However, the addition of rhamnolipid biosurfactant decreased the abundance of *Bacillus* microorganisms, which was reflected in ^{14}C-pyrene mineralization. These bioremediation-enhancement technologies were further assessed in a phytoremediation setting in PAH-contaminated soil from a shooting range site due to the accumulation of clay target fragments. Bermudagrass and switchgrass enhanced soil enzyme activity and PAH biodegradation. The bioaugmentation of *M. vanbaalenii* PYR-1 enhanced high-molecular-weight PAH biodegradation. The decrease in PAH concentrations was also reflected in lettuce seed germination toxicity assays. Overall, this research highlights the importance of physical and biological mechanisms in the evaluation and implementation of *in situ* bioremediation-enhancement technologies for successful PAH remediation of contaminated soils.

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Chapter 1 Introduction

1.1 Physicochemical Properties and Toxicity of Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a group of persistent organic pollutants that are composed of two or more fused aromatic rings in linear, angular, or cluster arrangements (ATSDR, 1995). Depending upon the structure of the rings, PAHs are classified as either alternant or non-alternant. Alternant PAHs contain only fused six-membered rings (e.g., phenanthrene, anthracene, and pyrene), while non-alternant PAHs contain four- or five-membered rings in addition to the six-membered rings (e.g., fluorene, fluoranthene, and benzo[b]fluoranthene) (Fetzer and Kershaw, 1995) (Fig. 1.1). The aromatic structure of PAHs results in increased thermodynamic and chemical stability due to electron delocalization in the π orbitals, which plays a critical role in the environmental fate and toxicity of these contaminants (Harvey, 1997). There are 16 PAHs designated as priority pollutants by the United States Environmental Protection Agency (U.S. EPA) due to their occurrence in the environment and toxicity. The physicochemical properties of the 16 priority PAHs are detailed in Table 1.1 (Aitken and Long, 2004). These compounds are all hydrophobic, as demonstrated by their relatively high octanol-water partition coefficients (K_{ow}) and low solubility in water (Table 1.1). The impact of the PAH structure on its chemical behavior is primarily dependent upon molecular size (i.e., number of rings) and angularity. Typically, an increase in the number of rings and angularity results in increased electrochemical stability and hydrophobicity (Kanaly and Harayama, 2000). For example, low-molecular-weight (LMW) PAHs

(compounds with 2 or 3 aromatic rings) are considerably more water soluble and volatile than high-molecular-weight (HMW) PAHs (compounds with 4 or more aromatic rings) (Table 1.1).

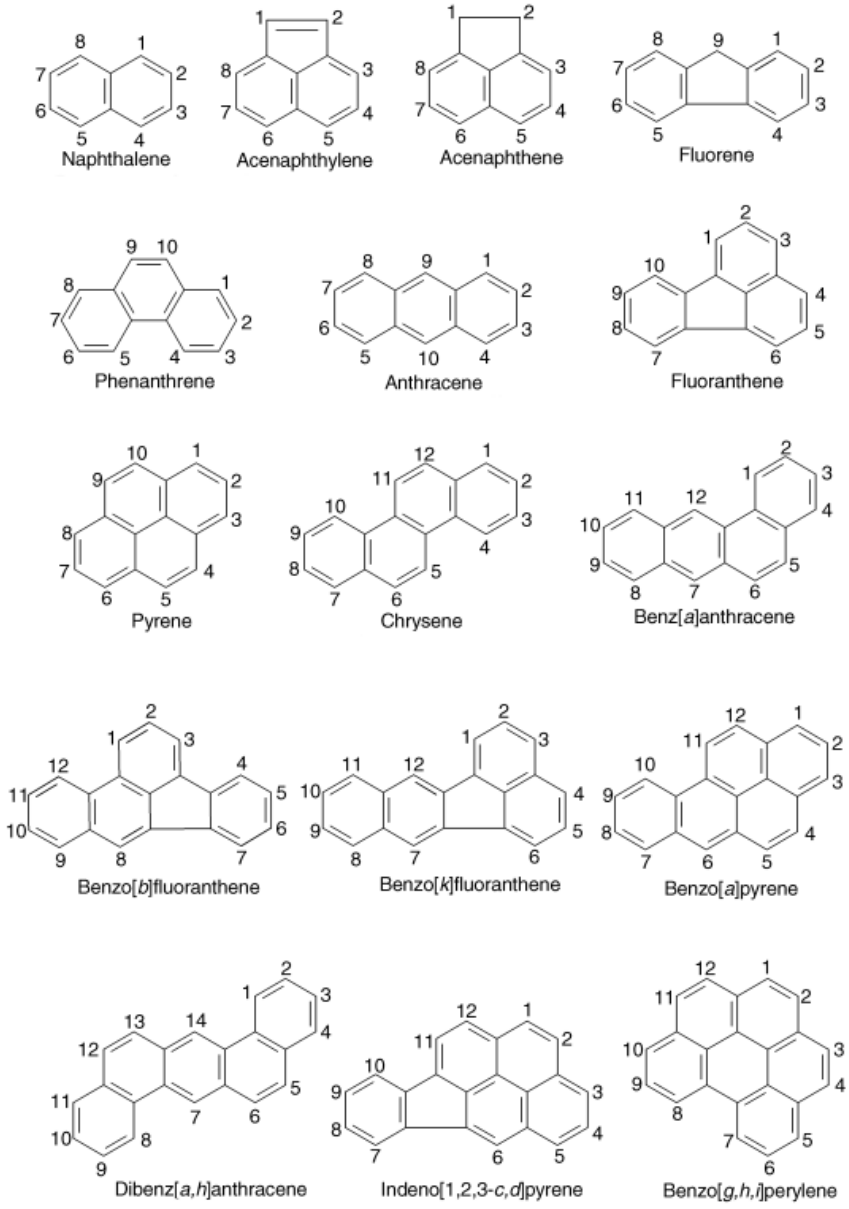

Figure 1.1. Chemical structure of the 16 United States Environmental Protection Agency priority polycyclic aromatic hydrocarbons (adapted from Aitken and Long, 2004).

Table 1.1. Properties of United States Environmental Protection Agency 16 priority polycyclic aromatic hydrocarbons.[ab]

	----------Physicochemical Properties----------						Toxicology
Compound (SPL Rank)[c]	Ring Number (5-membered)	Molecular Weight	Solubility (mg/L)	Log K_{OW}	Log K_{OC}	Vapor pressure (mm Hg)	IARC[d]
Naphthalene (81)	2	128	31.0	3.30	3.17	8.25×10^{-2}	2B
Acenaphthene (171)	2 (1)	154	1.93	3.98	3.66	4.5×10^{-3}	3
Acenaphthylene (NR)	2 (1)	152	3.93	4.07	3.40	2.9×10^{-2}	NC
Fluorene (NR)	2 (1)	166	1.68	4.18	3.86	3.2×10^{-4}	3
Phenanthrene (252)	3	178	1.20	4.45	4.15	6.8×10^{-4}	3
Anthracene (NR)	3	178	0.076	4.45	4.15	1.7×10^{-5}	3
Fluoranthene (138)	3 (1)	202	0.20	4.90	4.58	5.0×10^{-6}	3
Pyrene (260)	4	202	0.077	4.88	4.58	2.5×10^{-6}	3
Benzo[a]anthracene (38)	4	228	0.010	5.61	5.30	2.2×10^{-8}	2B
Chrysene (141)	4	228	0.0028	5.16	5.30	6.3×10^{-7}	2B

Benzo[b]fluoranthene (10)	4 (1)	252	0.0012	6.04	5.74	$5.0x10^{-7}$	2B
Benzo[k]fluoranthene (61)	4 (1)	252	0.0008	6.06	5.74	$9.6x10^{-11}$	2B
Benzo[a]pyrene (8)	5	252	0.0023	6.06	6.74	$5.6x10^{-9}$	1
Benzo[g,h,i]perylene (NR)	6	276	0.0003	6.50	6.20	$1.0x10^{-10}$	3
Dibenzo[a,h]anthracene (15)	5	278	0.0005	6.84	6.52	$1.0x10^{-10}$	2A
Indeno[1,2,3-c,d]pyrene (176)	5 (1)	276	0.062	6.58	6.20	$1.0x10^{-11}$	2B

[a]Abbreviations: NR = compound not ranked on SPL; K_{ow} = octanol-water partitioning coefficient; K_{oc} = organic carbon-normalized partitioning coefficient; IARC Classification - 1 = carcinogenic to humans; 2A = probably carcinogenic to humans; 2B = possibly carcinogenic to humans; 3 = not classifiable as carcinogenic to humans; NC = not classified.

[b]All data from ATSDR (1995), unless otherwise noted: [c]data from ATSDR (2017); [d]data from IARC (2019).

In addition to increases in hydrophobicity and environmental persistence with increasing PAH molecular size, PAH genotoxicity generally increases and toxicological concern shifts towards chronic toxicity, primarily carcinogenesis (Cerniglia, 1993). Numerous studies have indicated that LMW PAHs exhibit acute toxicity to humans, whereas HMW PAHs exhibit chronic effects such as genotoxicity (Gupta et al., 2015). The acute effects of PAHs on human health such as nausea, vomiting, and respiratory and skin irritation depend primarily on the extent of exposure, the route of exposure (e.g., inhalation, ingestion, or dermal contact), and the concentration and toxicity of the individual PAHs (Kim et al., 2013; Rengarajan et al., 2015). Polycyclic aromatic hydrocarbons can be widely distributed throughout the human body and have been detected in almost all internal organs, especially adipose tissues due to their lipophilicity (Abdel-Shafy, 2016). Once they enter the body, PAHs undergo metabolism primarily through the cytochrome P450 mixed-function oxidase system. This metabolic pathway transforms PAHs into polar epoxide intermediates that are further converted to dihydrodiol derivatives and phenols, which then form glucuronide and sulfate conjugates that are finally excreted in the bile and urine (Campo et al., 2010). However, this metabolic transformation can also result in the formation of electrophiles that elicit deleterious human health effects (Gelboin, 1980). Because of this, PAHs are considered procarcinogens because they do not directly induce DNA damage, but require metabolic activation to exert their genotoxic, mutagenic, or carcinogenic effects (Moorthy et al., 2015). There are three major pathways for PAH carcinogenic activation: the bay region dihydrodiol epoxide pathway, the radical cation pathway, and the *o*-quinone pathway,

which result in the formation of radical cations, diol epoxides, and electrophilic and redox-active *o*-quinones, respectively, all of which may react with DNA to produce DNA adducts (Fig. 1.2) (Xue and Warshawsky, 2005; Zhang et al., 2012a). Following extensive and systemic studies on the toxic effects of individual PAH metabolites in animals, it has been determined that the vicinal or bay-region diol epoxides are considered the ultimate mutagenic and carcinogenic species of PAHs (WHO, 2000) (Fig. 1.2).

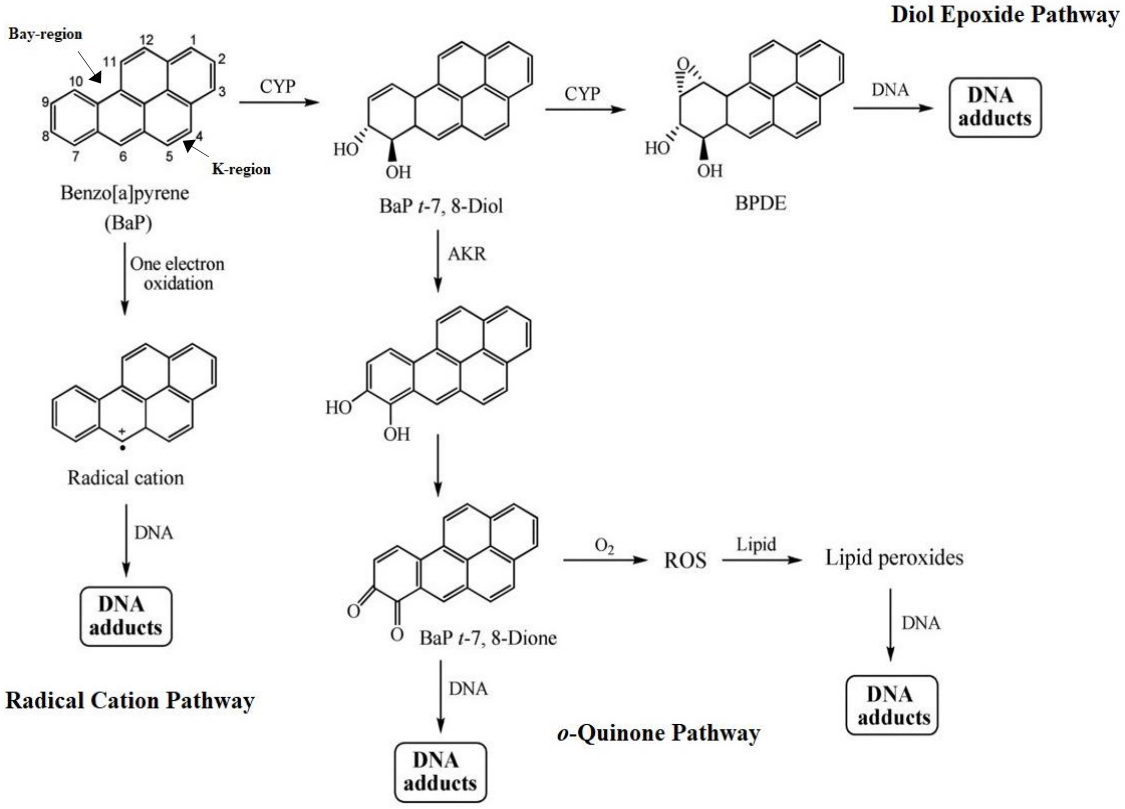

Figure 1.2. Three pathways of metabolic activation of benzo[a]pyrene leading to cancer initiation (adapted from Zhang et al., 2012a).

The International Agency for Research on Cancer (IARC) classifies numerous PAHs as known, probably, or possibly carcinogenic to humans (Group 1, 2A, or 2B) (Table 1.1). The IARC has determined that benzo[a]pyrene is one of the most potent carcinogenic PAHs, benzo[a,h]anthracene is a probable human carcinogen, and naphthalene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, and indeno[1,2,3-c,d]pyrene are possible human carcinogens (IARC, 2019). Altogether, PAHs rank as #9 on the Agency for Toxic Substances and Disease Registry's (ATSDR) Substance Priority List (SPL), which ranks contaminants based on a combination of their toxicity, frequency, and potential for human exposure at National Priority List (NPL) sites (ATSDR, 2017). Of the approximately 1,400 NPL sites that are targeted for remediation by the U.S. EPA, more than 700 sites are contaminated with PAHs (Duan et al., 2015; U.S. EPA, 2017).

1.2 Sources and Environmental Fate of PAHs

Polycyclic aromatic hydrocarbons are formed primarily during the incomplete thermal decomposition of organic substances and their subsequent recombination (Haritash and Kaushik, 2009). Thus, the composition of the PAHs formed is dependent upon the temperature and the starting organic material (Ghosal et al., 2016). Polycyclic aromatic hydrocarbons occur as complex mixtures in the environment instead of single compounds due to their differing physicochemical properties during the incomplete combustion process (Aitken and Long, 2004). There are three primary sources of PAHs in the environment: pyrogenic, petrogenic, and diagenetic/biogenic. Pyrogenic PAHs are

produced from the rapid oxygen-depleted, high-temperature (>700°C) incomplete combustion of fossil fuels and organic materials (Abdel-Shafy and Mansour, 2016). These pyrogenic PAHs are formed from the breakdown of organic matter to LMW radicals during pyrolysis, which is then followed by rapid reassembly into PAH structures (Neff, 1979). Pyrogenic PAHs are typically found at greater concentrations in urban areas because the major sources of pyrogenic PAHs are the incomplete combustion of gasoline and diesel in vehicles, the production and use of coal tar and asphalt, heat and power generation, and discharges from aluminum smelters and manufactured gas plants (MGP) (Hailwood et al., 2001; Stout et al., 2001). The most abundant pyrogenic PAHs are typically fluoranthene and pyrene (Page et al., 1999). Petrogenic PAHs originate from diagenetic processes at relatively low temperatures (100-300°C) over a long duration, leading to the formation of petroleum and other fossil fuels containing PAHs (Saber et al., 2006; Boehm and Saba, 2008). Petrogenic PAHs are introduced into the environment through accidental oil spills, discharge from tanker operations, and underground and aboveground storage tank leakage (Zakaria et al., 2002; Abdel-Shafy and Mansour, 2016). Diagenetic/biogenic PAHs are produced from biogenic precursors by plants, algae/phytoplankton, and microorganisms (Stogiannidis and Laane, 2015). For example, concentrations of perylene, naphthalene, and phenanthrene concentrations have been found in hydromorphic soils, *Magnolia* flowers, and *Coptotermes formosanus* termite nests (Azuma et al., 1996; Chen et al., 1998; Wilcke et al., 1999). While diagenetic/biogenic PAHs are often found at background levels in recent sediments, they

are frequently the primary PAHs in older sediments deposited before increased industrial activity (Gschwend and Hites, 1983).

The environmental ubiquity of PAHs is due to their chemical stability and numerous natural and anthropogenic sources. Natural sources of PAHs include volcanic eruptions, forest and prairie fires, and seeps of crude oil deposits (Zakaria et al., 2002; Alegbeleye et al., 2017). Anthropogenic sources, which contribute the vast majority of PAH contamination in the environment, include the production and use of fossil fuels such as coal, oil, and natural gas, refinement of crude oil, heat and power generation, wood treatment preservation processes, landfills, residential wood burning, and improper industrial waste disposal or spillage (Tobiszewski and Namieśnik, 2012; Stogiannidis and Laane, 2015; Abdel-Shafy and Mansour, 2016). Over the past century, there has been a substantial increase in environmental concentrations of PAHs following increased anthropogenic sources from industrialization, which can be demonstrated by PAH levels being the greatest in urban areas followed by agricultural and rural environments (Edwards, 1983; Wilcke, 2007). Even the lowest PAH concentrations in temperate soils are approximately 10 times greater than PAH concentrations assumed to have been present before global industrialization (Wilcke, 2000).

Once PAHs are emitted to the environment primarily through the combustion of fossil fuels, they are distributed atmospherically and deposited onto terrestrial, lacustrine, and marine surfaces (Baek et al., 1991; Wilcke, 2000). However, unlike most persistent organic pollutants like polychlorinated biphenyls that follow the global distillation

transportation effect, PAH concentrations generally decrease as the distance from the initial source increases (Scott et al., 2012). Atmospheric PAHs are generally more abundant at night than daytime, and during the winter months compared to the summer months due to greater deposition at lower temperatures and increased coal combustion for heating (Wang et al., 2011; Zhang et al., 2012b, Wang et al., 2018). Polycyclic aromatic hydrocarbons are semi-volatile organic compounds; therefore, PAHs can be found in both vapor and particle phases depending on the vapor pressure of the PAH, temperature, and size and surface area of suspended particles (Wang et al., 2017). However, given the physicochemical properties of PAHs, they tend to more readily sorb to atmospheric particulates than be present in the gas phase (Baek et al., 1991; Abdel-Shafy and Mansour, 2016). Because PAHs are commonly adsorbed onto atmospheric particulates, PAH transformation and degradation by thermal or photodecomposition and reactions with O_3, SO_2, NO_x, or OH radicals are reduced and can even be completely inhibited (Wilcke, 2000). In the absence of major decomposition pathways, PAHs are removed from the atmosphere by dry and wet deposition, which is considered the major source of PAHs in soil (Jones et al., 1989; Abdel-Shafy and Mansour, 2016; Wang et al., 2016). Although PAHs are considered amongst the most widespread organic pollutants in numerous environmental matrices such as soils, sediments, water, and wastewater, the ultimate sink of atmospheric PAHs is soil (Puglisi et al., 2007; Wang et al., 2010). Wild and Jones (1995) conducted an investigation to quantify the production, cycling, and storage of PAHs in the United Kingdom and determined that more than 90% of the total PAH burden resided in the surface soil.

In addition to atmospheric deposition, PAHs can also enter soil systems through disposal of waste materials, creosote use, road runoff, and fossil fuel spills (Maliszewska-Kordybach, 1999). The fate of PAHs in soil systems is primarily influenced by PAH hydrophobicity and the physicochemical properties of the soil. Due to their strong hydrophobicity and environmental recalcitrance, PAHs are typically associated with nonaqueous phases in soil where they associate into four main compartments: 1) organic matter; 2) the mineral compartment, which includes exposed soil surfaces and surfaces within pore spaces; 3) nonaqueous-phase liquids; and 4) combustion residues such as soot (Luthy et al., 1997). The degree to which PAHs are retained within the soil system is controlled primarily by soil properties such as organic matter and soil texture (silt and clay content) as well as the PAH physicochemical properties (e.g., the organic carbon-normalized equilibrium distribution coefficient, K_{oc}) (Table 1.1) (Duan et al., 2015). Because of the nonpolar, hydrophobic nature of PAHs, soil organic matter is considered the most important sorbent of PAHs (Wilcke, 2000; Liao et al., 2013). The organic matter or total organic carbon can also act as a carrier for the vertical migration of PAHs from the soil surface (Liao et al., 2013). Polycyclic aromatic hydrocarbons have a very strong affinity for soil organic matter via π-π interactions between the aromatic structure of PAHs and aromatic moieties of organic matter (Gautheir et al., 1987; Kubicki and Aptiz, 1999).

In soils with low amounts of organic matter or total organic carbon content, the soil texture plays a critical role in the environmental fate of PAHs. For example, Karickhoff et al. (1979) reported an increase in pyrene adsorption coefficients with an

increasing clay content (sand, 9.4-68; fine silt, 1,500-3,600; and clay, 1,400-3,800). In addition, decreasing particle size is typically associated with concomitant increases in the proportion of HMW and decrease in the proportion of LMW PAHs (Wilcke, 2000). The greatest PAH soil concentrations in numerous studies have been observed in the silt-sized soil fraction, which was potentially due to the silt fraction containing the greatest concentration of soil organic matter and its associated aromatic structures for binding (Baldock et al., 1992; Wilcke et al., 1997; Chiou et al., 1998). In addition, clay fractions are characterized by a very high specific surface area, abundant surface charge, and a high organic matter density, all of which provide a large number of sorption sites for PAHs (Amellel at al., 2001; Liao et al., 2013). Sorption of PAHs to soils generally entails an initially rapid and reversible phase followed by a period of slow sorption occurring over a period ranging from weeks to years, and this slow sorption leads to a chemical fraction that resists desorption and biodegradation (Hatzinger and Alexander, 1995). Increasing contact times between PAHs and soil organic matter or fine soil fractions can also result in the "aging" effect or sequestration of PAHs (Northcott et al., 2001). This process involves the continuous diffusion and retention of PAHs within the solid phase of organic matter and also in nanopores or voids in the organic matrix, thus blocking PAHs from abiotic and biotic loss processes (Nam et al., 1998; Alexander, 2000).

As a result of the strong association of PAHs with the nonpolar soil organic fractions, PAH bioavailability, or PAH concentrations in the aqueous phase that are directly available to soil microbes for degradation, is generally low (Aitken and Long, 2004; Okere and Semple, 2012). The bioavailability of PAHs is determined by two main

factors, which are the rate of transfer of PAHs from the soil to the living cell (mass transfer) and the rate of uptake and metabolism (intrinsic activity of the cell) (Semple et al., 2003). Bioavailability is an important concept with regards to PAH-contaminated soil remediation and risk assessment as microbial degradation constitutes the major dissipation pathway for PAHs compared to other processes such as evaporation, photolysis, and plant uptake (Cerniglia, 1993; Agnello et al., 2014).

1.2.1 Soil Microbial Degradation of PAHs

The extent and rate of microbial degradation of PAHs in the terrestrial environment is influenced by a variety of abiotic and biotic factors which include temperature, pH, aeration, accessibility of nutrients, microbial population, contaminant bioavailability, and physicochemical properties of the PAH (Cerniglia, 1993; Haritash and Kaushik, 2009). Typically, the rate of PAH biodegradation is inversely proportional to the number of aromatic rings or molecular weight of the PAH (Cerniglia, 1993). For example, half-lives of phenanthrene (3 aromatic rings) in soil may range from 16 to 126 days, while half-lives of HMW PAHs such as benzo[a]pyrene (5 aromatic rings) may range from 229 to 1,400 days (Shuttleworth and Cerniglia, 1995; Duan et al., 2015). Aerobic PAH biodegradation by soil bacteria is considered the most common and rapid PAH degradation pathway in soils; however, anaerobic PAH biodegradation as well as biodegradation by fungi has been observed in soils (Bamforth and Singleton, 2005; Lu et al., 2011). Despite the xenobiotic properties of PAHs, PAH-degrading soil microorganisms are ubiquitously distributed in the soil environment (Abdel-Shafey and

Mansour, 2016). Some of the major PAH-degrading genera in soils include *Mycobacterium*, *Sphingomonas*, *Bacillus*, *Pseudomonas*, and *Rhodococcus* (Bamforth and Singleton, 2005). The ability of soil microbes to degrade PAHs is determined by 1) the ability of bacteria to transport the PAH into the cell, 2) the physicochemical properties of the PAH as a substrate for available microbial enzymes, and 3) the suitability of the PAH as an inducer for the appropriate transport or degradative enzymes (Fewson, 1988; Juhasz and Naidu, 2000).

As shown in Figure 1.3, there are two primary mechanisms involved in the aerobic metabolism of PAHs by soil bacteria (Cerniglia, 1993). The principal mechanism for aerobic PAH degradation by soil bacteria involves the dioxygenase/monooxygenase enzymes, which incorporates a hydroxyl group derived from molecular oxygen into the aromatic nucleus, resulting in the oxidation of the aromatic ring to form *cis*-dihydrodiols (Heider et al., 1998; Samanta et al., 2002). This initial ring oxidation is considered to be the rate limiting step of the PAH biodegradation process in soil systems (Heitkamp and Cerniglia, 1989). The *cis*-dihydrodiols are stereoselectively dehydrogenated by *cis*-dihydrodiol dehydrogenases to form dihydroxylated intermediates, called catechols (Patel and Gibson, 1974). Subsequently, the catechol may then be cleaved by intradiol or extradiol ring-cleaving dioxygenases through the *ortho* or *meta*-cleavage pathway to tricarboxylic acid (TCA) intermediates such as succinic, fumaric, pyruvic, and acetic acids and acetaldehyde. These TCA intermediates are utilized for cell-protein synthesis and energy by microorganisms with the final production of carbon dioxide and water (Sims and Overcash, 1983; Wilson and Jones, 1993; Chauhan et al., 2008). Soil bacteria

can also degrade PAHs via the cytochrome P450-mediated pathway to form *trans*-dihydrodiols (Fig. 1.3) (Ghosal et al., 2016).

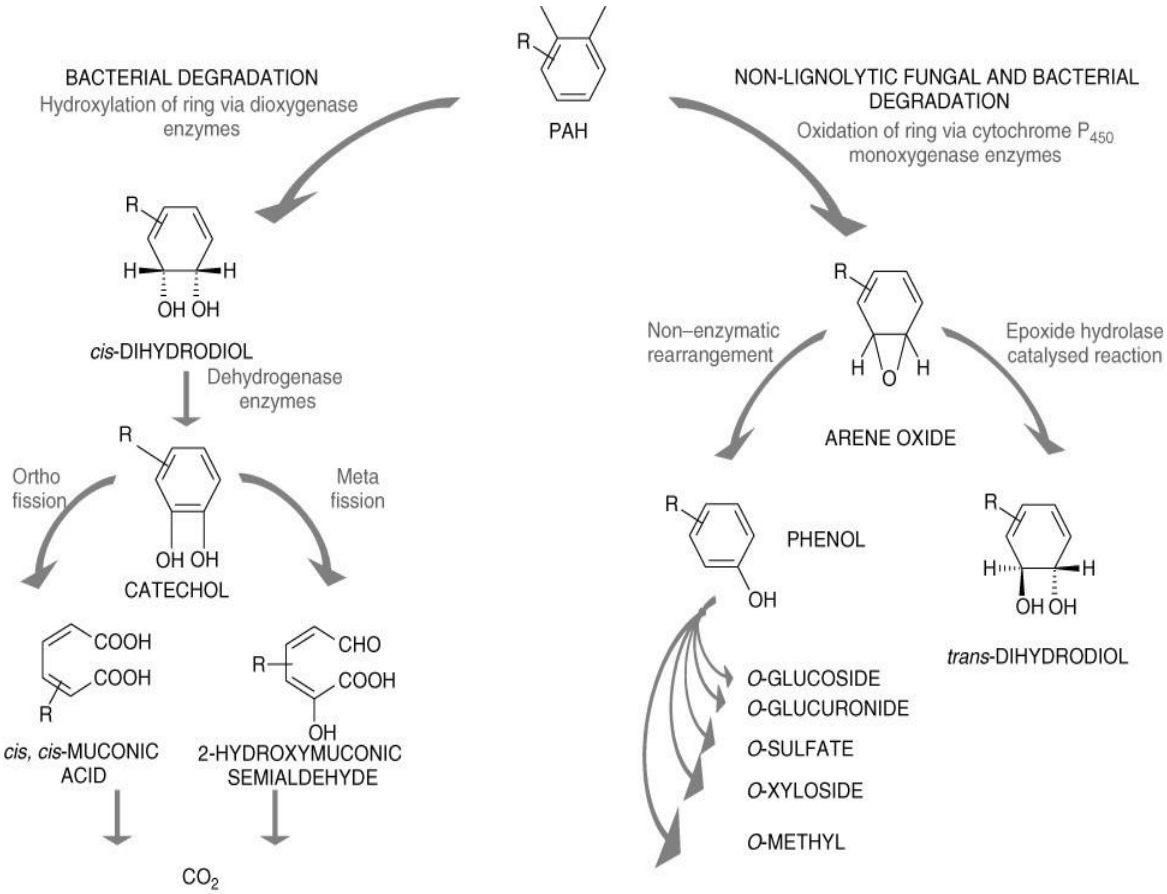

Figure 1.3. Two primary pathways for aerobic polycyclic aromatic hydrocarbon degradation by soil bacteria (adapted from Bamforth and Singleton, 2005).

Polycyclic aromatic hydrocarbons are biodegraded by soil microbes in one of two ways, either as the sole carbon and energy source or by cometabolism (Wilson and Jones, 1993). Because PAHs occur in the environment as complex mixtures of LMW and HMW PAHs, cometabolism is an important interaction that transforms non-growth substrate PAHs, particularly HMW PAHs, in the presence of growth substrates to enhance PAH degradation (Zhong et al., 2007). Numerous soil microbes have been isolated that utilize

LMW PAHs such as naphthalene and phenanthrene as their sole carbon source and throughout the past decade, multiple soil bacteria have been discovered and isolated that are capable of utilizing HMW PAHs as sole carbon sources (Chauhan et al., 2008). For example, *Mycobacterium vanbaalenii* PYR-1, an isolate from an oil-contaminated estuary near the Gulf of Mexico, has been utilized to determine the complete pyrene degradation pathway using various metabolic, genomic, and proteomic approaches (Fig. 1.4) (Khan et al., 2002; Kim et al., 2007). Pyrene is often used as a model compound for HMW PAH biodegradation because it is structurally similar to several carcinogenic HMW PAHs (Peng et al., 2008). The primary pathway for pyrene degradation by *M. vanbaalenii* PYR-1 is deoxygenation by dioxygenase and monooxygenase at the C-4 and C-5 positions to produce both *cis*- and *trans*-4,5-pyrenedihydrodiol, respectively. The metabolite undergoes further metabolization involving more than 20 enzymatic steps utilizing rearomatization, decarboxylation, and oxygenation to produce phthalate that is further transformed to the TCA cycle via the β-ketoadipate pathway (Fig. 1.4) (Wang et al., 2000; Kim et al., 2007; Peng et al., 2008). Another pyrene degradation pathway exists for *M. vanbaalenii* PYR-1 that involves the oxidation of pyrene at the C-1 and C-2 positions to form O-methylated derivatives of pyrene-1,2-diol as a detoxification step (Fig. 1.4) (Kim et al., 2007). *Mycobacterium vanbaalenii* PYR-1 is also capable of degrading or transforming biphenyl, naphthalene, phenanthrene, anthracene, fluoranthene, benzo[a]anthracene, and benzo[a]pyrene, thus making this bacterium an effective candidate for the bioremediation of PAH-contaminated soils (MacLeod and Daugulis, 2003; Kim et al., 2007; Wolf and Gan, 2018).

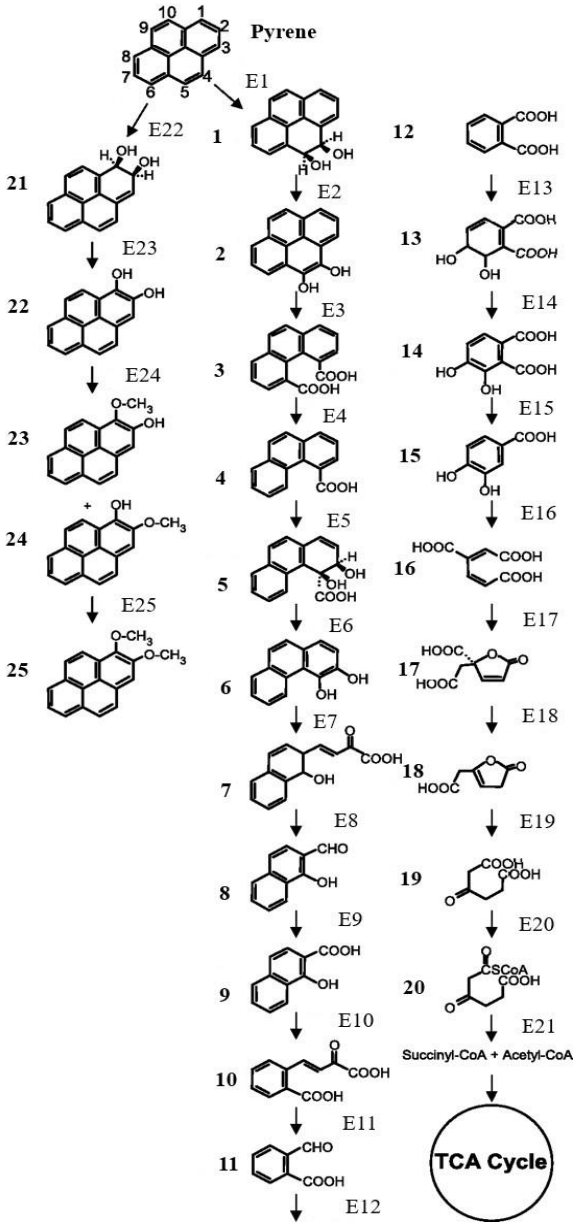

Figure 1.4. Complete pyrene biodegradation pathway by *Mycobacterium vanbaalenii* PYR-1 (adapted from Kim et al., 2007). The prefix "E" indicates an enzymatic step and numbers represent pyrene metabolites. The pyrene metabolic intermediates are as follows: 1) pyrene cis-4,5-dihydrodiol; 2, 4,5-dihydroxypyrene; 3, phenanthrene-4,5-dicarboxylate; 4, phenanthrene-4-carboxylate; 5, cis-3,4-dihydroxyphenanthrene-4-carboxylate; 6, 3,4-dihydroxy-phenanthrene; 7, 2-hydroxy-2H-benzo[h]chromene-2-carboxylate; 8, 1-hydroxy-2-naphthaldehyde; 9, 1-hydroxy-2-naphthoate; 10, trans-2'-carboxybenzalpyruvate; 11, 2-carboxylbenzaldehyde; 12, phthalate; 13, phthalate 3,4-dihydrodiol; 14, 3,4-dihydroxyphthalate; 15, protocatechuate; 16, β-carboxy-cis, cis-muconate; 17, γ-carboxymuconolactone; 18, β-ketoadipate enol-lactone; 19, β-ketoadipate; 20- β-ketoadipyl-CoA; 21, pyrene cis-1,2-dihydrodiol; 22, 1,2-dihydroxypyrene; 23, 1-methoxy-2-hydroxypyrene; 24, 1-hydroxy-2-methoxypyrene; 25, 1,2-dimethoxypyrene.

1.3 Bioremediation of PAH-contaminated Soils

Because PAHs are toxic, ubiquitous pollutants that are highly resistant to degradation in contaminated soils, remediation of PAH-contaminated sites is critical for protecting human health and the environment. Several physical and chemical PAH remediation technologies such as incineration, excavation and landfilling, UV oxidation, and solvent extraction have been used to clean up PAH-contaminated soils. However, these remediation methods have several negative aspects including cost, regulatory burden, and that some of these conventional methods do not result in PAH dissipation, but rather transfer from one environmental compartment or form to another (Ghosal et al., 2016). These limitations of conventional treatment methods have led to the increased use of bioremediation techniques at PAH-contaminated sites as they are considered to be safe, environmentally-friendly, and cost-effective (Mohan et al., 2006).

Bioremediation involves the utilization of biological processes or activity of microorganisms to remove pollutants from contaminated matrices to achieve concentrations that are acceptable according to health and regulatory standards. Bioremediation technologies can be classified into two main categories, *in situ* or *ex situ*. *In situ* bioremediation technologies target contaminant removal or attenuation under natural environmental conditions without the need for excavation, whereas *ex situ* bioremediation processes involve the physical removal of the contaminated material for remediation off-site (Pandey et al., 2009). Therefore, *in situ* remediation practices are particularly effective for widely dispersed contaminants and are typically less expensive

than *ex situ* approaches (Kuppusamy et al., 2016). Additionally, exposure to site workers to hazardous pollutants is minimal and *in situ* treatments also allow for remediation in inaccessible environments (Pandey et al., 2009). Because of these advantages, *in situ* bioremediation constitutes approximately 25% of all remediation projects for contaminated sites (Jørgensen, 2007). However, because *in situ* biodegradation does not disturb the contaminated soil, remediation has been found to be more variable due to the natural environmental conditions (Carberry and Wik, 2007). Although *ex situ* bioremediation is less economical compared to *in situ* treatments, *ex situ* bioremediation methods are less limited by environmental factors that could adversely affect the remediation efficacy and the physical and chemical conditions can be manipulated before and during degradation (Pandey et al., 2009). *Ex situ* bioremediation generally requires less time to achieve efficient contaminant remediation since optimal remediation conditions can be monitored and modified as needed (Kuppusamy et al., 2016).

The successful implementation of PAH-contaminated soil bioremediation treatments depends on a multitude of factors that can be categorized into three main domains encompassing PAHs, environmental conditions, and soil microbial communities (Sims and Overcash, 1983; Wang, 2007). Factors involving PAHs include the physicochemical properties of PAHs, concentration and toxicity, and the length of time the PAHs have been in contact with the soil and their associated bioavailability. Because *in situ* biodegradation treatments are currently a common type of remediation practice, environmental conditions in soil that must be evaluated for effective PAH biodegradation include soil type, organic matter content, nutrient availability, moisture, temperature, pH,

and presence of oxygen or alternative electron acceptors. Soil microbial transformations are the major process governing degradation of PAHs in contaminated soil, and therefore critical factors related to soil microbes include the presence of a soil microbial community capable of degrading PAHs, which encompasses microbial type, abundance, distribution, acclimation or previous exposure, and metabolic rate.

1.4 Strategies to Improve Bioremediation Efficacy of PAH-contaminated Soils

Natural or bioattenuation is an *in situ* bioremediation technique that involves passive remediation of a PAH-contaminated site without any external alterations (Smets and Pritchard, 2003). Because there are no site modifications, natural attenuation is considerably cheaper than other bioremediation methods. However, one of the major limitations of bioattenuation is that the process can take extended periods of time to achieve appreciable levels of PAH dissipation. Biodegradation is especially slow in PAH-contaminated sites that have been in contact with nonpolar soil domains for a prolonged duration, resulting in decreased PAH bioavailability (Azubuike et al., 2016). Due to the environmental stability of PAHs, the majority of PAH-contaminated sites undergo engineered or enhanced bioremediation, which involves site modifications to enhance the extent and rate of PAH degradation (Fig. 1.5) (Scow and Hicks, 2005). Typically, enhanced bioremediation techniques for PAH-contaminated soils involve bioaugmentation, biostimulation, surfactant amendment, phytoremediation, or an integrated combination of bioremediation techniques.

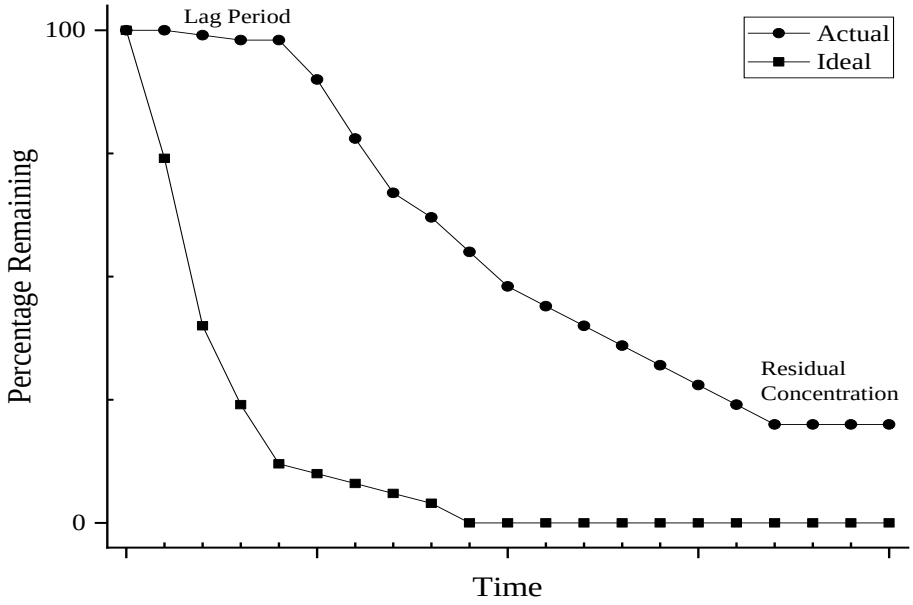

Figure 1.5. Ideal and typical biodegradation curves for PAH-contaminated soil. The lag period, slower remediation rates, and residual concentration of contaminant in the soil are typically observed in practical applications (adapted from Reynolds et al., 1999).

1.4.1 Bioaugmentation

Bioaugmentation is the process in which contaminant-degrading microorganisms are introduced into the soil as single strains or bacterial consortia to increase the rate of PAH biodegradation. The main advantage of bioaugmentation is the relatively low cost of inoculating soil microorganisms into the soil system. (Fernández-Luqueño et al., 2011). Bioaugmentation is an effective method to increase bioremediation efficacy for PAH-contaminated soils that possess low numbers of native PAH-degrading soil microbes or when the native soil microbial population does not exhibit sufficient metabolic activity to result in PAH dissipation (Forsyth et al., 1995). Bioaugmentation can also be particularly effective in aged PAH-contaminated soils that have low bioavailable PAH concentrations since the introduction of biosurfactant-producing soil

microbes such as *M. vanbaalenii* PYR-1 or *P. aeruginosa* can potentially produce biosurfactants to increase PAH desorption, thus resulting in enhanced biodegradation. The main limitation of bioaugmentation strategies is the survival of bacteria strains introduced to the contaminated soil, which is influenced by numerous abiotic and biotic factors. The primary abiotic factors include fluctuations in temperature, moisture, pH, aeration, nutrient content, and organic matter (Mrozik and Piotrowska-Seget, 2010). Biotic factors include competition between native and exogenous soil microorganisms for limited carbon sources in addition to antagonistic bacterial interactions and predation by protozoa and bacteriophages (Bisht et al., 2015). The use of microbial consortia rather than single strains of known PAH-degrading microorganisms has been shown to be more effective than single strains because the PAH intermediates during degradation of one particular strain might be further degraded by other strains in the consortia (Heinaru et al., 2005). In most bioaugmentation treatments, the bacteria are introduced into the contaminated soil in a liquid culture stage; however, this method does not guarantee appropriate bacterial distribution in the soil profile, which could greatly affect bacterial survival and metabolic activity (Mrozik and Piotrowska-Seget, 2010). To alleviate this, numerous carrier materials (e.g., peat, nylon, zeolite, and chitin) have been used to maintain sufficient bioaugmented microbial activity over a prolonged period since these materials provide the introduced microbe with a temporary protective niche and nutrients for sufficient growth (van Veen et al., 1997; Mrozik and Piotrowska-Seget, 2010).

1.4.2 Biostimulation

Biostimulation is the addition of one or multiple rate-limiting nutrients to increase the proportion of native soil microbial biomass that is capable of PAH degradation and accelerate biodegradation rates. Nutrient additions such as C, N, P, K, and S, in addition to modification of soil conditions (i.e., pH, moisture content, and aeration), have been used to provide the soil microbial population with an optimal environment for growth and increased PAH dissipation (Mohan et al., 2006). The optimal C:N:P ratio recommended for bioremediation applications is 100:10:1, and typical nutrient amendments used to achieve this nutrient ratio are inorganic fertilizers; however, other agents such as municipal sewage, biosolids, and sunflower oil have also been used to enhance the abundance and activity of soil microbial populations (Gong et al., 2005; Betancur-Galvis et al., 2006; Kirkpatrick et al., 2006). Some limitations of biostimulation include the fact that the native soil microbes must be able to degrade the contaminant, the delivery of nutrient additives to subsurface microbes might be difficult depending on site conditions, and the addition of nutrients can also promote the growth of soil microbes that are not capable of degrading PAHs, resulting in competition between PAH-degrading microbes (Adams et al., 2014; Adams et al., 2015).

1.4.3 Surfactant Amendment

Surfactant-enhanced bioremediation of PAH-contaminated soils is an effective *in situ* bioremediation technique that uses surfactants to increase the bioavailability of PAHs to PAH-degrading soil microbes, thus resulting in enhanced biodegradation. Surface

active substances or surfactants are amphiphilic compounds comprised of a polar (hydrophilic) “head” and nonpolar (hydrophobic) “tail” and this structure is responsible for their tendency to accumulate at the interface of polar and nonpolar materials, such as the interface between the aqueous phase and soil (Pletnev, 2001). Depending on the chemical nature of the hydrophilic head, surfactants are classified as nonionic (no charge) or ionic. Ionic surfactants can be further classified as cationic, anionic, or zwitterionic if the surfactant has a positive, negative, or both positive and negative charge, respectively. In order to maximize energetically-favorable hydrogen bond interactions, water excludes the hydrophobic tail of the surfactant, which normally aggregate as monomers on the surface of nonpolar materials such as soil and organic matter. With increasing surfactant concentrations, surfactant molecules gradually replace the interfacial solvent (e.g., water), resulting in lower polarity of the aqueous-phase and decreasing surface tension (Mao et al., 2015). When surfactant concentrations further increase, the surfactant molecules begin to aggregate and form micelles in an aqueous solution at or greater than a certain aqueous-phase concentration, known as the critical micelle concentration (CMC) (Vishnyakov et al., 2013). The micelle is a spherical structure in which the hydrophilic head is in contact with the polar aqueous liquid, while the hydrophobic tail remains sequestered in the center (Pletnev, 2001). The CMC is specific to each surfactant and depends on the chemical structure of the surfactant, but typically nonionic surfactants have lower CMC levels than ionic surfactants (Ying, 2006). The nonpolar tails in the micelles of surfactants at or above the CMC can interact with PAHs in contaminated soils

and increase their water solubility, thus increasing the bioavailability of PAHs to degrading soil microorganisms (Fig. 1.6) (Gao et al., 2007).

Surfactants may increase the rate of PAH desorption through two primary mechanisms: micellar solubilization and direct modification of the contaminant matrix (Edwards et al., 1991). Micellar solubilization will only occur in the presence of micelles and refers to the partitioning of PAH molecules into the hydrophobic core of the micelle which enhances PAH desorption by maximizing the concentration gradient at the soil-water interface (Edwards et al., 1991). Direct modification of the contaminant matrix can occur at concentrations below and above the surfactant CMC and involve increased PAH diffusivity as well as increased soil-water phase interfacial surface area (Deshpande et al., 1999). Increased PAH diffusivity occurs when monomers accumulate at the PAH-contaminant and soil-water interfaces and increase the contact angle between the soil and PAH, resulting in modification of the wettability of the system (Deshpande et al., 1999). Wettability describes the tendency of a liquid to spread over a soil particle surface. Once the contact angle of the PAH on the soil particle has sufficiently increased due to the repulsion from the hydrophilic head and hydrophobic soil particle, the adhesive forces are weak enough that the PAH can be separated (Mao et al., 2015). Increased soil-water phase interfacial surface area can result in the direct dispersion of soil particles due to surfactant addition, which results in increased particle surface area that can enhance PAH desorption (Adrion et al., 2016).

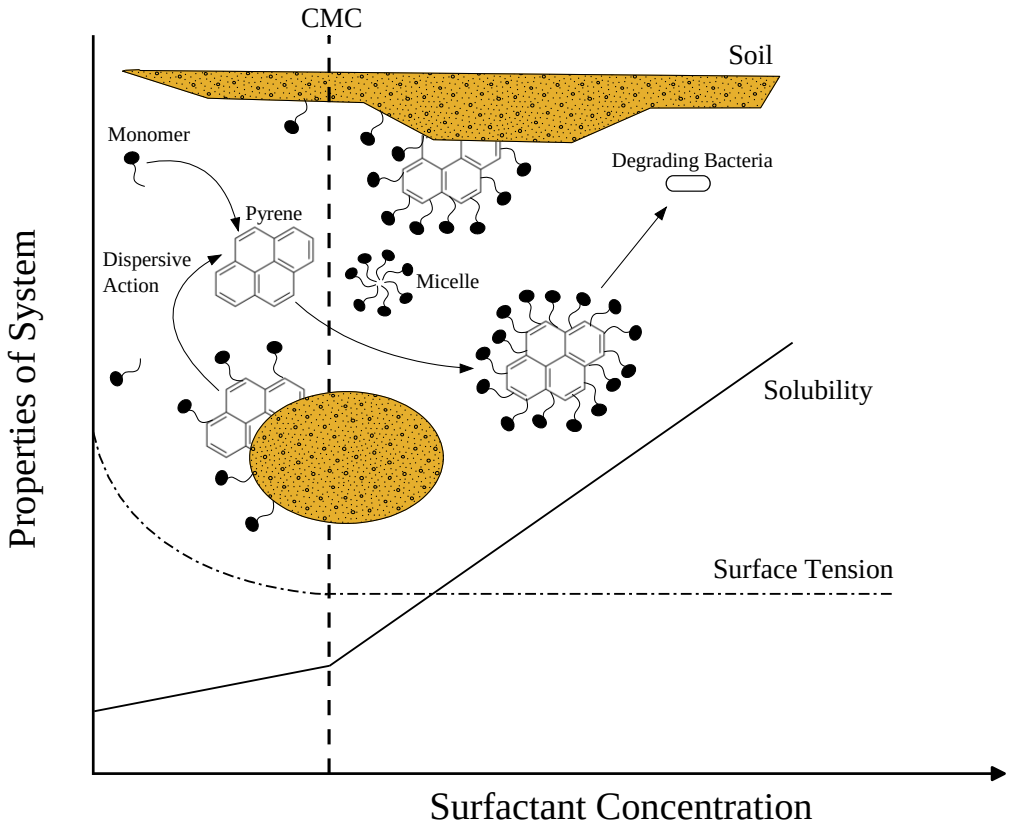

Figure 1.6. Effects of surfactant concentration on pyrene desorption and biodegradation in pyrene-contaminated soils (adapted from Zeng et al., 2018).

In most practical applications of surfactant-enhanced PAH bioremediation, a mixture of surfactants, rather than individual surfactants, are utilized because the individual surfactants can display synergetic effects. For example, mixtures of ionic and nonionic surfactants generally give a stronger solubilization effect compared to individual surfactants because the nonionic surfactants disperse the ionic surfactants and also reduce the electrostatic repulsion between ionic surfactant molecules (Paria, 2008). In addition, the use of an anionic-nonionic surfactant mixture in PAH-contaminated sites was determined to be more effective than individual surfactants due to the increased effective surfactant concentration in soil (i.e., the reduced soil sorption capacity of nonionic

surfactants and the reduced CMC of the surfactant mixture) (Zhao et al., 2005; Lu et al., 2019). However, at greater concentrations, particularly for ionic surfactants, surfactants may become toxic to soil microbes by forming mixed micelles with the phospholipid bilayer of the cell membrane, resulting in enzymatic disorder and even cell apoptosis, thus decreasing PAH bioremediation (Rosen et al., 2001).

Biosurfactants are produced by bacteria, fungi, and yeast and offer an environmentally-friendly alternative to synthetic surfactants and are becoming more economical to produce by using low-cost or renewable substrates (Zeng et al., 2018). Biosurfactants have similar properties to synthetic surfactants, including high surface-active properties and micelle formation; however, biosurfactants offer several advantages compared to synthetic surfactants such as reduced toxicity, high biodegradability, relatively low soil-sorption tendency, and greater stability when exposed to extreme temperature, pH, and salinity conditions (Bustamante et al., 2012; Congiu and Ortega-Calvo, 2014; Bezza and Chirwa, 2015). The three main classes of biosurfactants include sophorolipids, surfactins, and rhamnolipids (Agnello et al., 2014). Rhamnolipids are the most extensively used biosurfactant in PAH-contaminated soils and are primarily produced by *Pseudomonas aeruginosa* (Abdel-Mawgoud et al., 2011). Rhamnolipids are classified as glycolipids and are comprised of one or two sugar rhamnose molecules as the surfactant head and β-hydroxy-decanoic acid chains as the nonpolar tail (Agnello et al., 2014). Because of rhamnolipid biosurfactants' high biodegradability, numerous studies have determined that the rhamnolipid biosurfactant was preferentially degraded by the PAH-degrading soil microbes instead of the target PAH, resulting in inhibited

PAH biodegradation efficiency (Maslin and Maier, 2000; Ghosh and Mukherji, 2016; Wolf and Gan, 2018).

1.4.4 Phytoremediation

Phytoremediation is defined as the use of vegetation (grasses, plants, and trees) and its associated microorganisms to remove pollutants from the environment or render them harmless (Haritash and Kaushik, 2009). Although phytoremediation is considered one of the most time-consuming remediation technologies, it is typically the most cost-effective bioremediation approach as very large volumes of contaminated soils can be remediated *in situ* (Fig. 1.7) (Mench et al., 2010). There are also several advantages to using phytoremediation processes compared to traditional physical and chemical remediation technologies including relatively low cost, low maintenance after plant establishment, ability to remediate sites with mixed contaminants, low environmental impact, and high public acceptance (Agnello et al., 2014; Liu et al., 2015).

The primary type of microbe-assisted phytoremediation for PAH-contaminated soils is rhizoremediation, the degradation of recalcitrant organic contaminants in the rhizosphere, which is defined as the area around a plant root that is inhabited by a unique community of soil microorganisms influenced by the secretions released by plant root exudates (Broeckling et al., 2008). The primary goal of rhizoremediation is enhanced PAH bioremediation by rhizospheric microorganisms, whose growth is enhanced by plant roots (Agnello et al., 2014). Rhizoremediation encompasses numerous complex interactions involving plant roots, root exudates, microbes associated with the

rhizosphere that ultimately results in enhanced degradation of contaminants to less toxic compounds (Gerhardt et al., 2009). Because of the numerous benefits provided by the rhizosphere, low-maintenance grass species such as bermudagrass [*Cynodon dactylon*], tall fescue [*Festuca arundinacea]*, and alfalfa [*Medicago sativa*] are preferred due to their extensive fibrous root systems and large root surface area which results in more interactions between PAHs and the rhizosphere microbial community (Kirkpatrick et al., 2006; Alagić et al., 2016).

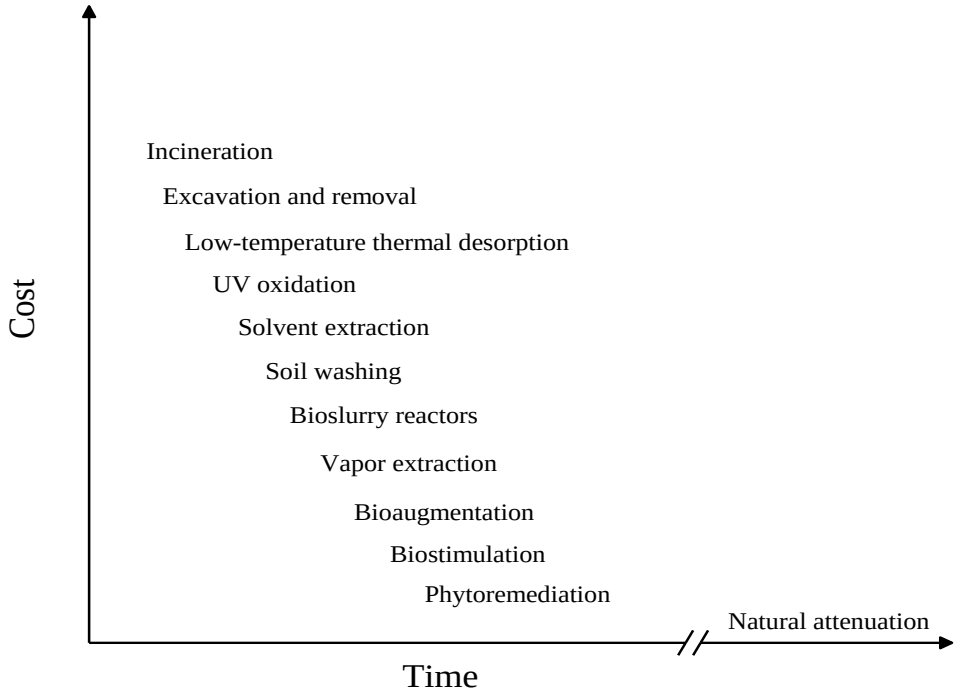

Figure 1.7. Cost and time relationship for various remediation techniques for soils contaminated with polycyclic aromatic hydrocarbons (adapted from Sylvia et al., 2005).

The increased soil microbial abundance and activity is due to the release of root exudates (complex mixture of soluble organic substances that contain sugars, amino acids, and organic acids) that are used as readily degradable carbon and energy sources by soil microbes (Koo et al., 2005). Kumar et al. (2006) and Fan et al. (2008) estimated

that approximately 20-40% of the plant's photosynthates synthesized by the plant can be deposited in the root system as exudates that increase microbial biomass, diversity, and activity, eventually leading to enhanced PAH degradation through direct metabolism or cometabolism (Yu et al., 2011). On a per gram basis, it is estimated that soil microbial abundance in the rhizosphere is 10-100 times greater than unvegetated soils (Lynch, 1990). In addition, the plant root system can also spread bacteria throughout the soil matrix and penetrate otherwise impermeable soil layers as well as provide oxygen to stimulate aerobic PAH biodegradation (Chauhan et al., 2008).

In addition to enhancing the development of soil bacteria already present in the rhizosphere, organic acids released from the root exudates can act as chemoattractants and induce motile microorganisms to move toward the plant roots (Barbour et al., 1991). Organic acids in the root exudates such as acetic, citric, malic, and oxalic acids can also enhance PAH bioavailability in contaminated soils (Gao et al., 2010). The mechanism behind enhanced PAH desorption by LMW organic acids involves the dissolving of metallic cations that act as "bridges" between organic matter-soil mineral surfaces (Gao et al., 2003). The addition of LMW organic acids can break these "bridges" resulting in the release of soil organic matter into the soil solution phase, promoting the desorption of PAHs bound to the soil organic matter (An et al., 2010).

Another role that plants have in the biodegradation of PAHs in soils is the release of enzymes that play a key role in the degradation of PAHs including oxygenases, dehydrogenases, phosphatases, peroxidase, and laccase (Cunningham and Ow, 1996;

Bisht et al., 2015). As previously mentioned, enzymes play a critical role in the microbial degradation of PAHs and the enzymes released by the root exudates have been shown to directly degrade LMW PAHs (Rentz et al., 2005). The enzymes released into the soil system after plant death can also continue to play a role in PAH dissipation (Liu et al., 2014). Soil dehydrogenase and polyphenol oxidase are oxidoreductase enzymes that are critical in the PAH degradation pathway and have been shown to increase in soils due to the release of root exudates (Soleimani et al., 2010; Sun et al., 2011; Liu et al., 2014)

Although there are numerous advantages for the implementation of phytoremediation in PAH-contaminated soils, there are also limitations to this remediation technology that must be taken into account. For example, limitations include the time necessary for acceptable remediation effects, the limited depth of the root system and beneficial rhizosphere effects, and the contaminated site must have characteristics that can support initial and continual plant growth (i.e., weather, soil nutrient availability, pH, texture, and moisture) (Khan et al., 2000; Kuiper et al., 2004).

1.4.5 Integrated Bioremediation Approaches

Although the previously mentioned bioremediation methods have been shown to be effective in remediation of PAH-contaminated sites, there are limitations for each individual treatment, especially at contaminated sites with aged, recalcitrant PAHs. To address these various individual treatment limitations, the utilization of two or more remediation technologies may overcome these limitations and result in more effective remediation of PAH-contaminated soils. Examples of integrated remediation

technologies include: physical-biological (solvent extraction and bioremediation), physical-chemical (solvent extraction and chemical oxidation), and biological-biological (e.g., bioaugmentation and phytoremediation). The most commonly implemented integrated remediation techniques that are currently being used for PAH-contaminated soils are: biological-biological (44%), chemical-biological (27%), physical-chemical (21%), physical-chemical-biological (5%), and thermal-chemical treatments (5%) (Kuppusamy et al., 2017).

1.5 Summary

Polycyclic aromatic hydrocarbons are a class of compounds of environmental and public health concern because of their known or suspected toxicity and genotoxicity as well as their ubiquitous environmental presence that will be exacerbated by increasing industrialization and associated anthropogenic pollution (Cerniglia, 1993). Due to their strong soil sorption capacity, molecular stability, and hydrophobicity, PAHs interact with nonpolar soil domains such as organic matter and become highly resistant to soil microbial degradation that is the primary removal pathway in contaminated soils (Bamforth and Singleton, 2005). Several bioremediation-enhancement technologies have been applied to increase PAH bioavailability and/or soil microbial abundance and activity such as surfactant amendment, bioaugmentation of known PAH-degrading microorganisms, and phytoremediation, to overcome the bioavailability limitations and environmental stability of PAHs. However, bioremediation is a complex process involving numerous abiotic and biotic interactions that can result in the inhibition of soil

microbial activity and lead to ineffective PAH degradation. Therefore, the effects of various bioremediation techniques that are commonly used for PAH-contaminated soils must be systematically investigated to address these issues and explore effective PAH remediation options. Because microbial degradation plays a vital role in PAH dissipation, the effects of remediation treatments, individually or in combination, on the soil microbial community must also be understood to ensure the protection of the environment and human health.

1.6 Research Objectives

The overall goal of the research described in this dissertation was to investigate the effectiveness and biological interactions for PAH biodegradation using *in situ* "green" bioremediation technologies such as biosurfactant amendment, bioaugmentation, and phytoremediation. The specific objectives are given below:

Objective 1: Compare the effect of a commonly used nonionic surfactant, Brij-35, and rhamnolipid biosurfactant, on PAH biodegradation in soils with varying physical and chemical properties. Biodegradation of PAHs was evaluated in both native soils and soils bioaugmented with a known PAH-degrading microorganism, *M. vanbaalenii* PYR-1, using ^{14}C-pyrene as a model HMW PAH.

Objective 2: Evaluate the effect of Brij-35 and rhamnolipid biosurfactant amendment and *M. vanbaalenii* PYR-1 bioaugmentation on the native soil microbes associated with PAH biodegradation. The 16S rRNA gene high-throughput sequencing was used to

analyze the shifts in soil microbial taxa due to the introduction of surfactants and bioaugmentation. Phylogenetic investigation of communities by reconstruction of unobserved states analysis (PICRUSt) was used to assess the soil bacterial populations and functional genes responsible for PAH biodegradation.

Objective 3: Assess the interactions of phytoremediation (bermudagrass [*Cynodon dactylon* (L) Pers] and switchgrass [*Panicum virgatum*]), *M. vanbaalenii* PYR-1 bioaugmentation, and surfactant amendment (Brij-35, rhamnolipid biosurfactant, and sodium dodecyl sulfate/Brij-35 mixture), when used alone or in combinations, on the remediation of PAH-contaminated soil due to accumulation of clay target fragments at an outdoor shooting range.

1.7 Dissertation Organization

This dissertation is compiled into five chapters. Chapter 1 is a review of the literature to-date that is relevant to the specific objectives of the dissertation. Chapters 2, 3, and 4 are each dedicated to one of the specific objectives. The first manuscript (Chapter 2) and second manuscript (Chapter 3) were published in *Environmental Pollution* and *Chemosphere*, respectively (Wolf and Gan, 2018; Wolf et al., 2019), while the third manuscript (Chapter 4) is being prepared for submission to *Science of the Total Environment*. Chapter 5 provides conclusions and recommendations for future research.

1.8 References

Abdel-Mawgoud, A.M., Hausmann, R., Lépine, F., Müller, M.M., Déziel, E., 2011. Rhamnolipids: Detection, Analysis, Biosynthesis, Genetic Regulation, and Bioengineering of Production, in: Soberón-Chávez, G. (Ed.), Biosurfactants: From Genes to Applications. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 13–55.

Abdel-Shafy, H.I., Mansour, M.S.M., 2016. A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation. *Egyptian Journal of Petroleum* 25, 107–123.

Adams, G., Tawari-Fufeyin, P., Igelenyah, E., 2014. Bioremediation of spent oil contaminated soils using poultry litter. *Research Journal in Engineering and Applied Sciences* 3, 124–130.

Adams, G.O., Fufeyin, P.T., Okoro, S.E., Ehinomen, I., 2015. Bioremediation, biostimulation and bioaugmentation: A Review. *International Journal of Environmental Bioremediation & Biodegradation* 3, 28–39.

Adrion, A.C., Nakamura, J., Shea, D., Aitken, M.D., 2016. Screening nonionic surfactants for enhanced biodegradation of polycyclic aromatic hydrocarbons remaining in soil after conventional biological treatment. *Environmental Science & Technology* 50, 3838–3845.

Agency for Toxic Substances and Disease Registry, 1995. Toxicological profile for polycyclic aromatic hydrocarbons. Available at: https://www.atsdr.cdc.gov/toxprofiles/tp69.pdf. (Accessed 1 May 2019).

Agency for Toxic Substances and Disease Registry, 2017. ATSDR's Substance Priority List. Available at: https://www.atsdr.cdc.gov/SPL/. (Accessed 3 May 2019).

Agnello, A.C., Huguenot, D., Van Hullebusch, E.D., Esposito, G., 2014. Enhanced phytoremediation: A review of low molecular weight organic acids and surfactants used as amendments. *Critical Reviews in Environmental Science and Technology* 44, 2531–2576.

Aitken, M.D., Long, T.C., 2004. Biotransformation, Biodegradation, and Bioremediation of Polycyclic Aromatic Hydrocarbons, in: Singh, A., Ward, O.P. (Eds.), Biodegradation and Bioremediation. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 83–124.

Alagić, S.Č., Jovanović, V.P.S., Mitić, V.D., Cvetković, J.S., Petrović, G.M., Stojanović, G.S., 2016. Bioaccumulation of HMW PAHs in the roots of wild blackberry from

the Bor region (Serbia): Phytoremediation and biomonitoring aspects. *Science of The Total Environment* 562, 561–570.

Alegbeleye, O.O., Opeolu, B.O., Jackson, V.A., 2017. Polycyclic aromatic hydrocarbons: A critical review of environmental occurrence and bioremediation. *Environmental Management* 60, 758–783.

Alexander, M., 2000. Aging, bioavailability, and overestimation of risk from environmental pollutants. *Environmental Science & Technology* 34, 4259–4265.

Amellal, N., Portal, J.-M., Vogel, T., Berthelin, J., 2001. Distribution and location of polycyclic aromatic hydrocarbons (PAHs) and PAH-degrading bacteria within polluted soil aggregates. *Biodegradation* 12, 49–57.

An, C., Huang, G., Yu, H., Wei, J., Chen, W., Li, G., 2010. Effect of short-chain organic acids and pH on the behaviors of pyrene in soil–water system. *Chemosphere* 81, 1423–1429.

Azubuike, C.C., Chikere, C.B., Okpokwasili, G.C., 2016. Bioremediation techniques–classification based on site of application: Principles, advantages, limitations and prospects. *World Journal of Microbiology and Biotechnology* 32, 180.

Azuma, H., Toyota, M., Asakawa, Y., Kawano, S., 1996. Naphthalene—a constituent of *Magnolia* flowers. *Phytochemistry* 42, 999–1004.

Baek, S.O., Field, R.A., Goldstone, M.E., Kirk, P.W., Lester, J.N., Perry, R., 1991. A review of atmospheric polycyclic aromatic hydrocarbons: Sources, fate and behavior. *Water, Air, and Soil Pollution* 60, 279–300.

Baldock, J.A., Oades, J.M., Waters, A.G., Peng, X., Vassallo, A.M., Wilson, M.A., 1992. Aspects of the chemical structure of soil organic materials as revealed by solid-state ^{13}C NMR spectroscopy. *Biogeochemistry* 16, 1–42.

Bamforth, S.M., Singleton, I., 2005. Bioremediation of polycyclic aromatic hydrocarbons: Current knowledge and future directions. *Journal of Chemical Technology & Biotechnology* 80, 723–736.

Barbour, W.M., Hattermann, D.R., Stacey, G., 1991. Chemotaxis of *Bradyrhizobium japonicum* to soybean exudates. *Applied and Environmental Microbiology* 57, 2635–2639.

Betancur-Galvis, L., Alvarez-Bernal, D., Ramos-Valdivia, A., Dendooven, L., 2006. Bioremediation of polycyclic aromatic hydrocarbon-contaminated saline–alkaline soils of the former Lake Texcoco. *Chemosphere* 62, 1749–1760.

Bezza, F.A., Chirwa, E.M., 2015. Desorption kinetics of polycyclic aromatic hydrocarbons (PAHs) from contaminated soil and the effect of biosurfactant supplementation on the rapidly desorbing fractions. *Biotechnology & Biotechnological Equipment* 29, 680–688.

Bisht, S., Pandey, P., Bhargava, B., Sharma, S., Kumar, V., Sharma, K.D., 2015. Bioremediation of polyaromatic hydrocarbons (PAHs) using rhizosphere technology. *Brazilian Journal of Microbiology* 46, 7–21.

Boehm, P., Saba, T., 2008. Identification and allocation of polycyclic aromatic hydrocarbons (PAHs). *Exponent Environmental Forensics Notes* 4, 1–5.

Broeckling, C.D., Manter, D.K., Paschke, M.W., Vivanco, J.M., 2008. Rhizosphere Ecology, in: Jørgensen, S.E., Fath, B.D. (Eds.), Encyclopedia of Ecology. Academic Press, Oxford, pp. 3030–3035.

Bustamante, M., Durán, N., Diez, M.C., 2012. Biosurfactants are useful tools for the bioremediation of contaminated soil: A review. *Journal of Soil Science and Plant Nutrition* 12, 667–687.

Campo, L., Rossella, F., Pavanello, S., Mielzynska, D., Siwinska, E., Kapka, L., Bertazzi, P.A., Fustinoni, S., 2010. Urinary profiles to assess polycyclic aromatic hydrocarbons exposure in coke-oven workers. *Toxicology Letters* 192, 72–78.

Carberry, J.B., Wik, J., 2001. Comparison of *ex situ* and *in situ* bioremediation of unsaturated soils contaminated by petroleum. *Journal of Environmental Science and Health, Part A* 36, 1491–1503.

Cerniglia, C.E., 1993. Biodegradation of polycyclic aromatic hydrocarbons. *Current Opinion in Biotechnology* 4, 331–338.

Chauhan, A., Oakeshott, J.G., Jain, R.K., 2008. Bacterial metabolism of polycyclic aromatic hydrocarbons: Strategies for bioremediation. *Indian Journal of Microbiology* 48, 95–113.

Chen, J., Henderson, G., Grimm, C.C., Lloyd, S.W., Laine, R.A., 1998. Termites fumigate their nests with naphthalene. *Nature* 392, 558–559.

Chiou, C.T., McGroddy, S.E., Kile, D.E., 1998. Partition characteristics of polycyclic aromatic hydrocarbons on soils and sediments. *Environmental Science & Technology* 32, 264–269.

Congiu, E., Ortega-Calvo, J.-J., 2014. Role of desorption kinetics in the rhamnolipid-enhanced biodegradation of polycyclic aromatic hydrocarbons. *Environmental Science & Technology* 48, 10869–10877.

Cunningham, S.D., Ow, D.W., 1996. Promises and prospects of phytoremediation. *Plant Physiology* 110, 715.

Deshpande, S., Shiau, B.J., Wade, D., Sabatini, D.A., Harwell, J.H., 1999. Surfactant selection for enhancing *ex situ* soil washing. *Water Research* 33, 351–360.

Duan, L., Naidu, R., Thavamani, P., Meaklim, J., Megharaj, M., 2015. Managing long-term polycyclic aromatic hydrocarbon contaminated soils: A risk-based approach. *Environmental Science and Pollution Research* 22, 8927–8941.

Edwards, D.A., Luthy, R.G., Liu, Z., 1991. Solubilization of polycyclic aromatic hydrocarbons in micellar nonionic surfactant solutions. *Environmental Science & Technology* 25, 127–133.

Edwards, N.T., 1983. Polycyclic aromatic hydrocarbons (PAH's) in the terrestrial environment: A review. *Journal of Environmental Quality* 12, 427–441.

Fan, S., Li, P., Gong, Z., Ren, W., He, N., 2008. Promotion of pyrene degradation in rhizosphere of alfalfa (*Medicago sativa* L.). *Chemosphere* 71, 1593–1598.

Fernández-Luqueño, F., Valenzuela-Encinas, C., Marsch, R., Martínez-Suárez, C., Vázquez-Núñez, E., Dendooven, L., 2011. Microbial communities to mitigate contamination of PAHs in soil—possibilities and challenges: A review. *Environmental Science and Pollution Research* 18, 12–30.

Fetzer, J.C., Kershaw, J.R., 1995. Identification of large polycyclic aromatic hydrocarbons in a coal tar pitch. *Fuel* 74, 1533–1536.

Fewson, C.A., 1988. Biodegradation of xenobiotic and other persistent compounds: The causes of recalcitrance. *Trends in Biotechnology* 6, 148–153.

Forsyth, J., Tsao, Y., Bleam, R., 1995. Bioremediation: When is augmentation needed? Battelle Press, Columbus, OH.

Gao, Y., He, J., Ling, W., Hu, H., Liu, F., 2003. Effects of organic acids on copper and cadmium desorption from contaminated soils. *Environment International* 29, 613–618.

Gao, Y., Ren, L., Ling, W., Kang, F., Zhu, X., Sun, B., 2010. Effects of low-molecular-weight organic acids on sorption–desorption of phenanthrene in soils. *Soil Science Society of America Journal* 74, 51–59.

Gao, Y.-Z., Ling, W.-T., Zhu, L.-Z., Zhao, B.-W., Zheng, Q.-S., 2007. Surfactant-enhanced phytoremediation of soils contaminated with hydrophobic organic contaminants: Potential and assessment. *Pedosphere* 17, 409–418.

Gauthier, T.D., Seitz, W.R., Grant, C.L., 1987. Effects of structural and compositional variations of dissolved humic materials on pyrene Koc values. *Environmental Science & Technology* 21, 243–248.

Gelboin, H.V., 1980. Benzo[a]pyrene metabolism, activation and carcinogenesis: Role and regulation of mixed-function oxidases and related enzymes. *Physiological Reviews* 60, 1107–1166.

Gerhardt, K.E., Huang, X.-D., Glick, B.R., Greenberg, B.M., 2009. Phytoremediation and rhizoremediation of organic soil contaminants: Potential and challenges. *Plant Science* 176, 20–30.

Ghosal, D., Ghosh, S., Dutta, T.K., Ahn, Y., 2016. Current state of knowledge in microbial degradation of polycyclic aromatic hydrocarbons (PAHs): A review. *Frontiers in Microbiology* 7, 1369.

Ghosh, I., Mukherji, S., 2016. Diverse effect of surfactants on pyrene biodegradation by a *Pseudomonas* strain utilizing pyrene by cell surface hydrophobicity induction. *International Biodeterioration & Biodegradation* 108, 67–75.

Gong, Z., Alef, K., Wilke, B.-M., Mai, M., Li, P., 2005. Assessment of microbial respiratory activity of a manufactured gas plant soil after remediation using sunflower oil. *Journal of Hazardous Materials* 124, 217–223.

Gschwend, P.M., Hites, R.A., 1981. Fluxes of polycyclic aromatic hydrocarbons to marine and lacustrine sediments in the northeastern United States. *Geochimica et Cosmochimica Acta* 45, 2359–2367.

Gupta, S., Pathak, B., Fulekar, M.H., 2015. Molecular approaches for biodegradation of polycyclic aromatic hydrocarbon compounds: A review. *Reviews in Environmental Science and Bio/Technology* 14, 241–269.

Hailwood, M., King, D., Leoz, E., Maynard, R., Menichini, E., Moorcroft, S., Pacyna, J., Ballesta, P., Schneider, P., Westerholm, R., 2001. Ambient air pollution by polycyclic aromatic hydrocarbons (PAH). Available at:

http://ec.europa.eu/environment/archives/air/pdf/pp_pah.pdf. (Accessed 1 May 2019).

Haritash, A.K., Kaushik, C.P., 2009. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): A review. *Journal of Hazardous Materials* 169, 1–15.

Harvey, R.G., 1997. Polycyclic Aromatic Hydrocarbons. Wiley-VCH, Inc. New York.

Hatzinger, P.B., Alexander, M., 1995. Effect of aging of chemicals in soil on their biodegradability and extractability. *Environmental Science & Technology* 29, 537–545.

Heider, J., Spormann, A.M., Beller, H.R., Widdel, F., 1998. Anaerobic bacterial metabolism of hydrocarbons. *FEMS Microbiology Reviews* 22, 459–473.

Heinaru, E., Merimaa, M., Viggor, S., Lehiste, M., Leito, I., Truu, J., Heinaru, A., 2005. Biodegradation efficiency of functionally important populations selected for bioaugmentation in phenol- and oil-polluted area. *FEMS Microbiology Ecology* 51, 363–373.

Heitkamp, M.A., Cerniglia, C.E., 1989. Polycyclic aromatic hydrocarbon degradation by a *Mycobacterium* sp. in microcosms containing sediment and water from a pristine ecosystem. *Applied and Environmental Microbiology* 55, 1968.

International Agency for Research on Cancer, 2019. Agents classified by the IARC monographs, volumes 1-123. Available at: https://monographs.iarc.fr/wp-content/uploads/2018/09/ClassificationsAlphaOrder.pdf. (Accessed 3 May 2019).

Jones, K.C., Stratford, J.A., Waterhouse, K.S., Furlong, E.T., Giger, W., Hites, R.A., Schaffner, C., Johnston, A.E., 1989. Increases in the polynuclear aromatic hydrocarbon content of an agricultural soil over the last century. *Environmental Science & Technology* 23, 95–101.

Jørgensen, K.S., 2007. *In situ* bioremediation. *Advances in Applied Microbiology* 61, 285–305.

Juhasz, A.L., Naidu, R., 2000. Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: A review of the microbial degradation of benzo[a]pyrene. *International Biodeterioration & Biodegradation* 45, 57–88.

Kanaly, R.A., Harayama, S., 2000. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. *Journal of Bacteriology* 182, 2059.

Karickhoff, S.W., Brown, D.S., Scott, T.A., 1979. Sorption of hydrophobic pollutants on natural sediments. *Water Research* 13, 241–248.

Khan, A.A., Kim, S.-J., Paine, D.D., Cerniglia, C.E., 2002. Classification of a polycyclic aromatic hydrocarbon-metabolizing bacterium, *Mycobacterium* sp. strain PYR-1, as *Mycobacterium vanbaalenii* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 52, 1997–2002.

Khan, A.G., Kuek, C., Chaudhry, T.M., Khoo, C.S., Hayes, W.J., 2000. Role of plants, mycorrhizae and phytochelators in heavy metal contaminated land remediation. *Chemosphere* 41, 197–207.

Kim, K.-H., Jahan, S.A., Kabir, E., Brown, R.J.C., 2013. A review of airborne polycyclic aromatic hydrocarbons (PAHs) and their human health effects. *Environment International* 60, 71–80.

Kim, S.-J., Kweon, O., Jones, R.C., Freeman, J.P., Edmondson, R.D., Cerniglia, C.E., 2007. Complete and integrated pyrene degradation pathway in *Mycobacterium vanbaalenii* PYR-1 based on systems biology. *Journal of Bacteriology* 189, 464.

Kirkpatrick, W.D., White, P.M., Wolf, D.C., Thoma, G.J., Reynolds, C.M., 2006. Selecting plants and nitrogen rates to vegetate crude-oil–contaminated soil. *International Journal of Phytoremediation* 8, 285–297.

Koo, B.J., Adriano, D.C., Bolan, N.S., Barton, C.D., 2005. Root exudates and soil microorganisms, in: Hillel, D. (Ed.), Encyclopedia of Soils in the Environment. Elsevier, Oxford, pp. 421–428.

Kubicki, J.D., Apitz, S.E., 1999. Models of natural organic matter and interactions with organic contaminants. *Organic Geochemistry* 30, 911–927.

Kuiper, I., Lagendijk, E.L., Bloemberg, G.V., Lugtenberg, B.J.J., 2004. Rhizoremediation: A beneficial plant-microbe interaction. *Molecular Plant-Microbe Interactions* 17, 6–15.

Kumar, R., Pandey, S., Pandey, A., 2006. Plant roots and carbon sequestration. *Current Science* 91, 885-890.

Kuppusamy, S., Palanisami, T., Megharaj, M., Venkateswarlu, K., Naidu, R., 2016. Ex-situ remediation technologies for environmental pollutants: A Critical perspective, in: de Voogt, P. (Ed.), Reviews of Environmental Contamination and Toxicology Volume 236. Springer International Publishing, Cham, pp. 117–192.

Kuppusamy, S., Thavamani, P., Venkateswarlu, K., Lee, Y.B., Naidu, R., Megharaj, M., 2017. Remediation approaches for polycyclic aromatic hydrocarbons (PAHs) contaminated soils: Technological constraints, emerging trends and future directions. *Chemosphere* 168, 944–968.

Liao, X., Ma, D., Yan, X., Yang, L., 2013. Distribution pattern of polycyclic aromatic hydrocarbons in particle-size fractions of coking plant soils from different depth. *Environmental Geochemistry and Health* 35, 271–282.

Liu, R., Dai, Y., Sun, L., 2015. Effect of rhizosphere enzymes on phytoremediation in PAH-contaminated soil using five plant species. *PLOS ONE* 10, e0120369.

Liu, R., Xiao, N., Wei, S., Zhao, L., An, J., 2014. Rhizosphere effects of PAH-contaminated soil phytoremediation using a special plant named Fire Phoenix. *Science of The Total Environment* 473–474, 350–358.

Lu, H., Wang, W., Li, F., Zhu, L., 2019. Mixed-surfactant-enhanced phytoremediation of PAHs in soil: Bioavailability of PAHs and responses of microbial community structure. *Science of The Total Environment* 653, 658–666.

Lu, X.-Y., Zhang, T., Fang, H.H.-P., 2011. Bacteria-mediated PAH degradation in soil and sediment. *Applied Microbiology and Biotechnology* 89, 1357–1371.

Luthy, R.G., Aiken, G.R., Brusseau, M.L., Cunningham, S.D., Gschwend, P.M., Pignatello, J.J., Reinhard, M., Traina, S.J., Weber, W.J., Westall, J.C., 1997. Sequestration of hydrophobic organic contaminants by geosorbents. *Environmental Science & Technology* 31, 3341–3347.

Lynch, J.M., 1990. The rhizosphere. John Wiley and Sons Ltd, Chichester, West Sussex.

MacLeod, C.T., Daugulis, A.J., 2003. Biodegradation of polycyclic aromatic hydrocarbons in a two-phase partitioning bioreactor in the presence of a bioavailable solvent. *Applied Microbiology and Biotechnology* 62, 291–296.

Maliszewska-Kordybach, B., 1999. Persistent Organic Contaminants in the Environment: PAHs as a Case Study, in: Baveye, P., Block, J.-C., Goncharuk, V.V. (Eds.), Bioavailability of Organic Xenobiotics in the Environment: Practical Consequences for the Environment. Springer Netherlands, Dordrecht, pp. 3–34.

Mao, X., Jiang, R., Xiao, W., Yu, J., 2015. Use of surfactants for the remediation of contaminated soils: A review. *Journal of Hazardous Materials* 285, 419–435.

Maslin, P., Maier, R.M., 2000. Rhamnolipid-enhanced mineralization of phenanthrene in organic-metal co-contaminated soils. *Bioremediation* 4, 295–308.

Mench, M., Lepp, N., Bert, V., Schwitzguébel, J.-P., Gawronski, S.W., Schröder, P., Vangronsveld, J., 2010. Successes and limitations of phytotechnologies at field scale: Outcomes, assessment and outlook from COST Action 859. *Journal of Soils and Sediments* 10, 1039–1070.

Mohan, S.V., Kisa, T., Ohkuma, T., Kanaly, R.A., Shimizu, Y., 2006. Bioremediation technologies for treatment of PAH-contaminated soil and strategies to enhance process efficiency. *Reviews in Environmental Science and Bio/Technology* 5, 347–374.

Moorthy, B., Chu, C., Carlin, D.J., 2015. Polycyclic aromatic hydrocarbons: From metabolism to lung cancer. *Toxicological Sciences* 145, 5–15.

Mrozik, A., Piotrowska-Seget, Z., 2010. Bioaugmentation as a strategy for cleaning up of soils contaminated with aromatic compounds. *Microbiological Research* 165, 363–375.

Nam, K., Chung, N., Alexander, M., 1998. Relationship between organic matter content of soil and the sequestration of phenanthrene. *Environmental Science & Technology* 32, 3785–3788.

Neff, J.M., 1979. Polycyclic aromatic hydrocarbons in the aquatic environment: Source, fate, and biological effects. Applied Science Publishers Ltd. London, U.K.

Northcott, G.L., Jones, K.C., 2001. Partitioning, extractability, and formation of nonextractable PAH residues in soil. 1. Compound differences in aging and sequestration. *Environmental Science & Technology* 35, 1103–1110.

Okere, U., Semple, K., 2012. Biodegradation of PAHs in 'pristine' soils from different climatic regions. *Journal of Bioremediation & Biodegradation* S 1.

Page, D.S., Boehm, P.D., Douglas, G.S., Bence, A.E., Burns, W.A., Mankiewicz, P.J., 1999. Pyrogenic polycyclic aromatic hydrocarbons in sediments record past human activity: A case study in Prince William Sound, Alaska. *Marine Pollution Bulletin* 38, 247–260.

Pandey, J., Chauhan, A., Jain, R.K., 2009. Integrative approaches for assessing the ecological sustainability of *in situ* bioremediation. *FEMS Microbiology Reviews* 33, 324–375.

Paria, S., 2008. Surfactant-enhanced remediation of organic contaminated soil and water. *Advances in Colloid and Interface Science* 138, 24–58.

Patel, T., Gibson, D., 1974. Purification and properties of (+)-cis-naphthalene dihydrodiol dehydrogenase of *Pseudomonas putida*. *Journal of Bacteriology* 119, 879–888.

Peng, R.-H., Xiong, A.-S., Xue, Y., Fu, X.-Y., Gao, F., Zhao, W., Tian, Y.-S., Yao, Q.-H., 2008. Microbial biodegradation of polyaromatic hydrocarbons. *FEMS Microbiology Reviews* 32, 927–955.

Pletnev, M.Y., 2001. Chemistry of surfactants, in: Fainerman, V.B., Möbius, D., Miller, R. (Eds.), Studies in Interface Science. Elsevier, pp. 1–97.

Puglisi, E., Cappa, F., Fragoulis, G., Trevisan, M., Del Re, A.A.M., 2007. Bioavailability and degradation of phenanthrene in compost amended soils. *Chemosphere* 67, 548–556.

Rengarajan, T., Rajendran, Peramaiyan, Nandakumar, N., Lokeshkumar, B., Rajendran, Palaniswami, Nishigaki, I., 2015. Exposure to polycyclic aromatic hydrocarbons with special focus on cancer. *Asian Pacific Journal of Tropical Biomedicine* 5, 182–189.

Rentz, J.A., Alvarez, P.J.J., Schnoor, J.L., 2005. Benzo[a]pyrene co-metabolism in the presence of plant root extracts and exudates: Implications for phytoremediation. *Environmental Pollution* 136, 477–484.

Reynolds, C.M., Wolf, D.C., Gentry, T.J., Perry, L.B., Pidgeon, C.S., Koenen, B.A., Rogers, H.B., Beyrouty, C.A., 1999. Plant enhancement of indigenous soil micro-organisms: A low-cost treatment of contaminated soils. *Polar Record* 35, 33–40.

Rosen, M.J., Li, F., Morrall, S.W., Versteeg, D.J., 2001. The relationship between the interfacial properties of surfactants and their toxicity to aquatic organisms. *Environmental Science & Technology* 35, 954–959.

Saber, D., Mauro, D., Sirivedhin, T., 2006. Environmental forensics investigation in sediments near a former manufactured gas plant site. *Environmental Forensics* 7, 65–75.

Samanta, S.K., Singh, O.V., Jain, R.K., 2002. Polycyclic aromatic hydrocarbons: Environmental pollution and bioremediation. *Trends in Biotechnology* 20, 243–248.

Scott, H.E.M., Aherne, J., Metcalfe, C.D., 2012. Fate and transport of polycyclic aromatic hydrocarbons in upland Irish headwater lake catchments. *Scientific World Journal* 2012, 828343–828343.

Scow, K.M., Hicks, K.A., 2005. Natural attenuation and enhanced bioremediation of organic contaminants in groundwater. *Current Opinion in Biotechnology* 16, 246–253.

Semple, K.T., Morriss, A.W.J., Paton, G.I., 2003. Bioavailability of hydrophobic organic contaminants in soils: Fundamental concepts and techniques for analysis. *European Journal of Soil Science* 54, 809–818.

Shuttleworth, K.L., Cerniglia, C.E., 1995. Environmental aspects of PAH biodegradation. *Applied Biochemistry and Biotechnology* 54, 291–302.

Sims, R.C., Overcash, M., 1983. Fate of polynuclear aromatic compounds (PNAs) in soil-plant systems, in: Residue Reviews. Springer, pp. 1–68.

Smets, B.F., Pritchard, P., 2003. Elucidating the microbial component of natural attenuation. *Current Opinion in Biotechnology* 14, 283–288.

Soleimani, M., Afyuni, M., Hajabbasi, M.A., Nourbakhsh, F., Sabzalian, M.R., Christensen, J.H., 2010. Phytoremediation of an aged petroleum contaminated soil using endophyte infected and non-infected grasses. *Chemosphere* 81, 1084–1090.

Stogiannidis, E., Laane, R., 2015. Source characterization of polycyclic aromatic hydrocarbons by using their molecular indices: An overview of possibilities, in: Whitacre, D.M. (Ed.), Reviews of Environmental Contamination and Toxicology. Springer International Publishing, Cham, pp. 49–133.

Stout, S.A., Uhler, A.D., Boehm, P.D., 2001. Recognition of and allocation among multiple sources of PAH in urban sediments. *Environmental Claims Journal* 13, 141–158.

Sun, M., Fu, D., Teng, Y., Shen, Y., Luo, Y., Li, Z., Christie, P., 2011. *In situ* phytoremediation of PAH-contaminated soil by intercropping alfalfa (*Medicago sativa* L.) with tall fescue (*Festuca arundinacea* Schreb.) and associated soil microbial activity. *Journal of Soils and Sediments* 11, 980–989.

Sylvia, D.M., Fuhrmann, J.J., Hartel, P.G., Zuberer, D.A., 2005. Principles and applications of soil microbiology. Pearson Prentice Hall Upper Saddle River, NJ.

Tobiszewski, M., Namieśnik, J., 2012. PAH diagnostic ratios for the identification of pollution emission sources. *Environmental Pollution* 162, 110–119.

United States Environmental Protection Agency, 2017. FOIA 4 – U.S. EPA Superfund Program – FOIA-004 all final NPL sites. Available at:

https://www.epa.gov/superfund/superfund-data-and-reports. (Accessed 1 December 2017).

van Veen, J.A., van Overbeek, L.S., van Elsas, J.D., 1997. Fate and activity of microorganisms introduced into soil. *Microbiology and Molecular Biology Reviews* 61, 121.

Vishnyakov, A., Lee, M.-T., Neimark, A.V., 2013. Prediction of the critical micelle concentration of nonionic surfactants by dissipative particle dynamics simulations. *Journal of Physical Chemistry Letters*. 4, 797–802.

Wang, B., 2007. Environmental biodegradation research focus. Nova Science Publishers, New York, N.Y.

Wang, C., Wu, S., Zhou, S., Shi, Y., Song, J., 2017. Characteristics and source identification of polycyclic aromatic hydrocarbons (PAHS) in urban soils: A review. *Pedosphere* 27, 17–26.

Wang, Q., Liu, M., Li, Y., Liu, Y., Li, S., Ge, R., 2016. Dry and wet deposition of polycyclic aromatic hydrocarbons and comparison with typical media in urban system of Shanghai, China. *Atmospheric Environment* 144, 175–181.

Wang, R., Cao, H., Li, W., Wang, Wei, Wang, Wentao, Zhang, L., Liu, J., Ouyang, H., Tao, S., 2011. Spatial and seasonal variations of polycyclic aromatic hydrocarbons in Haihe Plain, China. *Environmental Pollution* 159, 1413–1418.

Wang, R.F., Wennerstrom, D., Cao, W.-W., Khan, A.A., Cerniglia, C.E., 2000. Cloning, expression, and characterization of the *katg* gene, encoding catalase-peroxidase, from the polycyclic aromatic hydrocarbon-degrading bacterium *Mycobacterium* sp. strain PYR-1. *Applied and Environmental Microbiology*. 66, 4300.

Wang, W., Massey Simonich, S.L., Xue, M., Zhao, J., Zhang, N., Wang, R., Cao, J., Tao, S., 2010. Concentrations, sources and spatial distribution of polycyclic aromatic hydrocarbons in soils from Beijing, Tianjin and surrounding areas, North China. *Environmental Pollution* 158, 1245–1251.

Wang, X., Shen, Z., Zeng, Y., Liu, F., Zhang, Q., Lei, Y., Xu, H., Cao, J., Yang, L., 2018. Day-night differences, seasonal variations and source apportionment of PM10-bound PAHs over Xi'an, Northwest China. *Atmosphere* 9.

Wilcke, W., 2000. Polycyclic aromatic hydrocarbons (PAHs) in soil: A review. *Journal of Plant Nutrition and Soil Science* 163, 229–248.

Wilcke, W., 2007. Global patterns of polycyclic aromatic hydrocarbons (PAHs) in soil. *Geoderma* 141, 157–166.

Wilcke, W., Amelung, W., Zech, W., 1997. Heavy metals and polycyclic aromatic hydrocarbons (PAHs) in a rural community leewards of a waste incineration plant. *Journal of Plant Nutrient and Soil Science* 160, 369–378.

Wilcke, W., Müller, S., Kanchanakool, N., Niamskul, C., Zech, W., 1999. Polycyclic aromatic hydrocarbons in hydromorphic soils of the tropical metropolis Bangkok. *Geoderma* 91, 297–309.

Wild, S.R., Jones, K.C., 1995. Polynuclear aromatic hydrocarbons in the United Kingdom environment: A preliminary source inventory and budget. *Environmental Pollution* 88, 91–108.

Wilson, S.C., Jones, K.C., 1993. Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): A review. *Environmental Pollution* 81, 229–249.

Wolf, D.C., Cryder, Z., Gan, J., 2019. Soil bacterial community dynamics following surfactant addition and bioaugmentation in pyrene-contaminated soils. *Chemosphere* 231, 93–102.

Wolf, D.C., Gan, J., 2018. Influence of rhamnolipid biosurfactant and Brij-35 synthetic surfactant on ^{14}C-pyrene mineralization in soil. *Environmental Pollution* 243, 1846–1853.

World Health Organization, 2000. Polycyclic aromatic hydrocarbons (PAHs). Available at: http://www.euro.who.int/__data/assets/pdf_file/0015/123063/AQG2ndEd_5_9PAH.pdf. (Accessed 1 May 2019).

Xue, W., Warshawsky, D., 2005. Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: A review. *Toxicology and Applied Pharmacology* 206, 73–93.

Ying, G.-G., 2006. Fate, behavior and effects of surfactants and their degradation products in the environment. *Environment International* 32, 417–431.

Yu, X.Z., Wu, S.C., Wu, F.Y., Wong, M.H., 2011. Enhanced dissipation of PAHs from soil using mycorrhizal ryegrass and PAH-degrading bacteria. *Journal of Hazardous Materials* 186, 1206–1217.

Zakaria, M.P., Takada, H., Tsutsumi, S., Ohno, K., Yamada, J., Kouno, E., Kumata, H., 2002. Distribution of polycyclic aromatic hydrocarbons (PAHs) in rivers and

estuaries in Malaysia: A widespread input of petrogenic PAHs. *Environmental Science & Technology* 36, 1907–1918.

Zeng, Z., Liu, Y., Zhong, H., Xiao, R., Zeng, G., Liu, Z., Cheng, M., Lai, C., Zhang, C., Liu, G., Qin, L., 2018. Mechanisms for rhamnolipids-mediated biodegradation of hydrophobic organic compounds. *Science of The Total Environment* 634, 1–11.

Zhang, K., Zhang, B.-Z., Li, S.-M., Zhang, L.-M., Staebler, R., Zeng, E.Y., 2012b. Diurnal and seasonal variability in size-dependent atmospheric deposition fluxes of polycyclic aromatic hydrocarbons in an urban center. *Atmospheric Environment* 57, 41–48.

Zhang, L., Jin, Y., Huang, M., Penning, T., 2012a. The role of human aldo-keto reductases in the metabolic activation and detoxication of polycyclic aromatic hydrocarbons: Interconversion of PAH catechols and PAH o-quinones. *Frontiers in Pharmacology* 3, 193.

Zhao, B., Zhu, L., Gao, Y., 2005. A novel solubilization of phenanthrene using Winsor I microemulsion-based sodium castor oil sulfate. *Journal of Hazardous Materials* 119, 205–211.

Zhong, Y., Luan, T., Wang, X., Lan, C., Tam, N.F., 2007. Influence of growth medium on cometabolic degradation of polycyclic aromatic hydrocarbons by *Sphingomonas* sp. strain PheB4. *Applied Microbiology and Biotechnology* 75, 175–186.

Chapter 2 Influence of Rhamnolipid Biosurfactant and Brij-35 Synthetic Surfactant on ^{14}C-Pyrene Mineralization in Soil

2.1 Introduction

Polycyclic aromatic hydrocarbons are aromatic hydrocarbons with two or more fused benzene rings and are ubiquitous environmental contaminants that are formed during the thermal decomposition of organic molecules. Approximately 700 sites on the United States Environmental Protection Agency National Priorities List are polluted with PAHs, of which more than 100 are PAH-contaminated soil sites (U.S. EPA, 2017). Bioremediation, the use of microorganisms to transform hazardous compounds to nonhazardous forms, has been recognized as an efficient, cost-effective, and versatile approach to clean up PAH-contaminated soils (Haritash and Kaushik, 2009). However, due to their hydrophobicity, PAHs are strongly associated with nonpolar soil domains such as soil organic matter or soil micropores, and therefore may not be bioavailable to degrading microorganisms (Riding et al., 2013).

Synthetic surfactants at concentrations both above and below their critical micelle concentration (CMC) have been used to enhance the availability of PAHs to the degrading microbes and increase PAH biodegradation (Jahan et al., 1997; Tiehm et al., 1997; Pinto and Moore, 2000; Kim et al., 2001; Li et al., 2015). At greater concentrations, a surfactant may become toxic to soil microbes by forming mixed micelles with the phospholipid bilayer of the cell membrane and solubilizing the cell membranes, inducing enzymatic disorders, cell penetration and even cell apoptosis

(Rosen et al., 2001). Because of the detrimental effects of synthetic surfactants, biosurfactants, notably rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa*, have become known as environmentally benign alternatives to synthetic surfactants (Congiu et al., 2015). Biosurfactants have similar properties to synthetic surfactants, including high surface-active properties and micelle formation; however, biosurfactants possess distinct advantages over synthetic surfactants. For example, rhamnolipid biosurfactants typically have better biocompatibility with cell membranes, resulting in lower toxicity to microorganisms, stability under extreme environmental conditions such as temperature, pH, and salinity, and relatively low soil-sorption tendency (Congiu and Ortega-Calvo, 2014; Bezza and Chirwa, 2015). The production of surface-active compounds by microorganisms is a critical microbial process that affects the bioavailability of PAHs and other hydrophobic organic contaminants in soil. For example, *Mycobacterium vanbaalenii* PYR-1, an isolate from an oil-contaminated estuary near the Gulf of Mexico, has been shown to produce trehalose-containing glycolipids, a type of surface-active biosurfactant, to enhance PAH solubility and degradation (Kim et al., 2015).

Bioremediation of PAHs is a complex process involving numerous abiotic and biotic interactions. Environmental stressors such as chemical toxicity have the potential to inhibit microbial activity that can lead to ineffective PAH biodegradation (Kathi and Khan, 2011). While the use of synthetic surfactants for bioremediation of PAH-contaminated soils is well documented, albeit with inconsistent results, the application of biosurfactants in bioremediation is relatively limited. In particular, few researchers have

compared the performance of synthetic and biosurfactants in PAH biodegradation in native or bioaugmented soils, leading to inclusive views on their respective advantages and limitations.

The objective of this study was to evaluate the effect of a commonly used synthetic surfactant, Brij-35, and rhamnolipid biosurfactant, at three amendment rates on PAH biodegradation in two soils. Biodegradation was determined in both native soils as well as soils augmented with a known PAH degrader, *M. vanbaalenii* PYR-1. ^{14}C-Pyrene was used as a model PAH compound and mineralization to ^{14}CO2 was measured as an endpoint of biodegradation.

2.2 Materials and Methods

2.2.1 Chemicals

Unlabeled pyrene (98%) was purchased from Sigma Aldrich (St. Louis, MO) and [4, 5, 9, 10–^{14}C]-pyrene (98.6% purity; 58.5 mCi mmol^{-1}) was purchased from Moravek (Brea, CA). Brij-35 nonionic synthetic surfactant was purchased from Sigma Aldrich and R-95 rhamnolipid nonionic biosurfactant (95%), isolated from *P. aeruginosa* NY3, was purchased from AGAE Technologies (Corvallis, OR). Microbiological supplies for *M. vanbaalenii* PYR-1 were purchased from Hardy Diagnostics (Springboro, OH). Ultima Gold XR scintillation cocktail was purchased from PerkinElmer (Waltham, MA).

2.2.2 Soil and Surfactant Properties

A San Emigdio fine sandy loam soil and Perry clay soil with substantial differences in organic matter and soil texture and initially free of PAH contamination were collected from the 0–30 cm depth at the South Coast Research and Extension Center in Irvine, California and Crossett, Arkansas, respectively. The soils were sieved through a 2-mm mesh before use. Soil particle-size distribution and textural classification were determined using the 12-h hydrometer method (Gavlak et al., 2003). The sand, silt, and clay percentages of the clay soil and sandy loam were 3%, 19%, 78% and 66%, 17%, and 17%, respectively. The soil total organic carbon (TOC) was analyzed by high-temperature combustion using an Elementar Vario MAX C/N instrument (Elementar Americas, Mt. Laurel, NJ) after the addition of HCl for carbonate removal (Schumacher, 2002). The clay soil had a TOC of 2.42% and the sandy loam soil had a TOC of 0.66%.

The CMC of the two surfactants was determined using a Du Noüy ring-tensiometer (Krüss Tensiometer 10, Hamburg, Germany). Briefly, the force required to raise a platinum wire ring from the surface of serial dilutions of surfactant stock (500 mg L^{-1}) was measured and the CMC was determined from the inflection point plotting the surface tension and logarithm of surfactant concentration. The CMC for the rhamnolipid biosurfactant was determined to be 70 mg L^{-1}, which was similar to previous studies using rhamnolipid biosurfactants (Bai et al., 2017). The emulsification activity of the surfactants was determined by mixing 2 mL surfactant with 2 mL n-hexadecane and vortexed for 2 min. The emulsion stability and the emulsification index (E_{24}) were

determined according to Youssef et al. (2004). An emulsion was considered stable if E_{24} was ≥50% (Bosch et al., 1988); both Brij-35 (58%) and rhamnolipid biosurfactant (65%) exhibited good emulsion stability.

2.2.3 Microbial Incubation

Mycobacterium vanbaalenii PYR-1 was stored at -80 °C in a 30% (v/v) glycerol stock. To prepare the inoculum, the stock suspension was transferred to a 1 L Erlenmeyer flask containing 0.1 g L^{-1} pyrene as a carbon source in 600 mL minimal basal salts (MBS) solution (15.13 g L^{-1} Na_2HPO_4, 3.0 g L^{-1} K_2HPO_4, 0.5 g L^{-1} NaCl, 1.0 g L^{-1} NH_4Cl, 0.491 g L^{-1} $MgSO_4•7H_2O$, 0.26 g L^{-1} $CaCl_2•2H_2O$) at 23 °C on a rotary incubator shaker (New Brunswick Scientific, New Brunswick, NJ) at 100 rpm (Reid et al., 2001). After 4 d incubation (late exponential phase growth), the culture was centrifuged at 10,000×g for 30 min. The supernatant was decanted and the final inoculum was resuspended in 250 mL fresh MBS twice, resulting in approximately 10^8 cells mL^{-1} (Doick and Semple, 2003). Colony-forming units (CFUs) were counted following a serial dilution of the inoculum broth plated in triplicate on plate count agar (5.0 g L^{-1} tryptone, 2.5 g L^{-1} yeast extract, 1.0 g L^{-1} dextrose, 9.0 g L^{-1} agar) incubated at 23 °C for 3 d (Doick and Semple, 2003). Briefly, the original inoculum was serially diluted and 1 mL inoculum was placed in the center of a sterile Petri dish and 20 mL cooled agar was poured onto the Petri dish. After incubation, plates with 30–300 colonies per plate were used for CFU mL^{-1} determination.

To evaluate the potential toxic effects of Brij-35 surfactant and rhamnolipid biosurfactant amendments on microbial activity, Brij-35 and rhamnolipid surfactants at 0.1X CMC and 10X CMC were added to *M. vanbaalenii* PYR-1 cultures in MBS as the sole carbon source. A positive control using glucose as a carbon source and a negative control with no carbon source were included. Viable *M. vanbaalenii* PYR-1 CFUs were determined by plating the culture on plate count agar after 1, 7, and 14 d on a rotary shaker operating at 100 rpm and 23 °C.

2.2.4 Pyrene Mineralization Experiments

The ^{14}C-pyrene mineralization assays were conducted using 250-mL Erlenmeyer flask respirometers, each consisting of a rubber stopper covered with aluminum foil (to minimize losses due to sorption from headspace into the rubber) and a 7 mL glass scintillation vial containing 1 mL 1.0 M NaOH suspended from the stopper. The NaOH acted as a trap for ^{14}CO2 and hence offered a measurement of mineralization of ^{14}C-pyrene (Guha and Jaffe, 1996; Oyelami et al., 2014). For each sample, 2 g soil (dry weight) was weighed into each respirometer and spiked with 10 mg kg^{-1} non-labeled pyrene and 1800 Bq of ^{14}C-pyrene in 1.0 mL acetone. The treated flask was closed for 1 h to allow the chemical to disperse throughout the soil sample and then opened to allow the solvent to evaporate overnight (Brinch et al., 2002). An additional 8 g soil (dry weight) was added and mixed with a stainless steel spatula over a period of 5 d. Following the chemical treatment, 10 mL sterilized MBS solution was added to the native soil treatment, followed by the addition of 10 mL surfactant solution at 0.1X, 1.0X, or 10X

CMC, resulting in initial amendment rates of 21.6, 216, and 2160 μg surfactant g-dry soil^{-1} for Brij-35 and 14, 140, and 1400 μg surfactant g-dry soil^{-1} for rhamnolipid biosurfactant, respectively. For the bioaugmented treatment, 5 mL of sterilized MBS solution and 5 mL *M. vanbaalenii* PYR-1-MBS solution were added to yield approximately 10^7 cells g^{-1} soil, and followed by the addition of 10 mL surfactant solution at 0.1X, 1.0X, or 10X CMC. All treatments contained soil slurries in a 1:2 soil:water ratio as Doick and Semple (2003) determined that this soil:water ratio resulted in greater overall mineralization and improved reproducibility as compared to other solid-water ratios.

After spiking, the respirometers were sealed and placed in an incubator at 23 °C and mixed at 100 rpm. The $^{14}CO_2$ traps were changed every 12 h for the first 2 d, and then daily for 2–7 d, followed by sampling approximately every 3 d thereafter. The trapped ^{14}C-activity was measured after the addition of 6 mL Ultima Gold XR scintillation cocktail and subsequent liquid scintillation counting (LSC) on a Beckman LS 5000TD Liquid Scintillation Counter (Fullerton, CA). Respirometers were opened briefly on a weekly basis for aeration. Following the 50-d incubation period, the total ^{14}C activity was determined by sample oxidation on an OX-500 Biological Oxidizer (R.J. Harvey, Hillsdale, NJ) to determine ^{14}C-activity remaining in the soil. Briefly, an aliquot of soil sample was air-dried in a fume hood and combusted at 900 °C for 4 min. The evolved $^{14}CO_2$ was trapped in 15 mL Ultima Gold XR scintillation cocktail, followed by quantification on LSC (Rhodes et al., 2008).

The biodegradation kinetics of ^{14}C-pyrene followed first-order kinetics, given in Eq. (1):

$$C = C_0 e^{-kt} \quad [1]$$

where C was the residual ^{14}C-pyrene concentration in the soil matrix at time t (mg L^{-1}), C_0 was the initial ^{14}C-pyrene concentration in the soil matrix (mg L^{-1}), k is the first-order biodegradation rate constant (d^{-1}), and t is the time (d). Plotting the logarithm of ^{14}C-pyrene concentration vs. time yields a linear relationship, from which k may be calculated (Kuppusamy et al., 2016). The biodegradation rate constant was calculated as the slope of the regression line belonging to the phase of maximum mineralization, which occurred between the end of the lag period (time required for 5% mineralization to occur) and the beginning of the degradation "plateau" using Origin®2018 (OriginLab, Northampton, MA) (Bueno-Montes et al., 2011).

2.2.5 Quality Assurance and Quality Control

Blank soil samples were spiked with a known amount of ^{14}C-pyrene in preliminary experiments and combusted to test the ^{14}C recovery of the combustion method. The mean recovery was ≥90%. The mass balance of ^{14}C-pyrene activity (residual + cumulative mineralization of ^{14}C remaining in the soil at the end of incubation) was greater than 80% among all soil treatments. Both untreated respirometers (i.e., no ^{14}C-pyrene) and mercuric-chloride-sterilized controls were analyzed as analytical blanks and

indicated no abiotic losses of ^{14}C-activity to the $^{14}CO_2$ trap. Triplicate samples for each treatment were used in both surfactant toxicity evaluation and ^{14}C-pyrene mineralization experiments. Statistical analyses including analysis of variance (ANOVA) and post-hoc Tukey's range test were performed using SAS® 9.3 (SAS Institute Inc., Cary, NC). Least square means for significant effects were compared using a protected least significant difference procedure at $\alpha = 0.05$.

2.3 Results and Discussion

2.3.1 Influence of Surfactant Amendment

The addition of Brij-35 at all concentrations in the native clay soil treatments resulted in the shortest lag period before PAH mineralization commenced (9 d for all Brij-35 amendment rates) as compared to the unamended clay soil treatment (19 d) (Table 2.1). After 25 d of incubation, the addition of Brij-35 surfactant at all rates in the native clay soil resulted in greater ^{14}C-pyrene mineralization than the unamended native clay soil treatment (Fig. 2.1A). In the native sandy loam soil, the addition of Brij-35 at the low or high rate resulted in a longer lag period (25 and 31 d, respectively) compared to the unamended control (23 d), while amendment of the Brij-35 surfactant at the medium rate shortened the lag period to 21 d (Table 2.1 and Fig. 2.2A). However, after 50 d of incubation, all Brij-35 surfactant treatments had greater ^{14}C-pyrene mineralization as compared to the unamended native sandy loam soil, and the enhancement may be attributed to increased bioavailability of ^{14}C-pyrene to the native soil microorganisms (Fig. 2.2A).

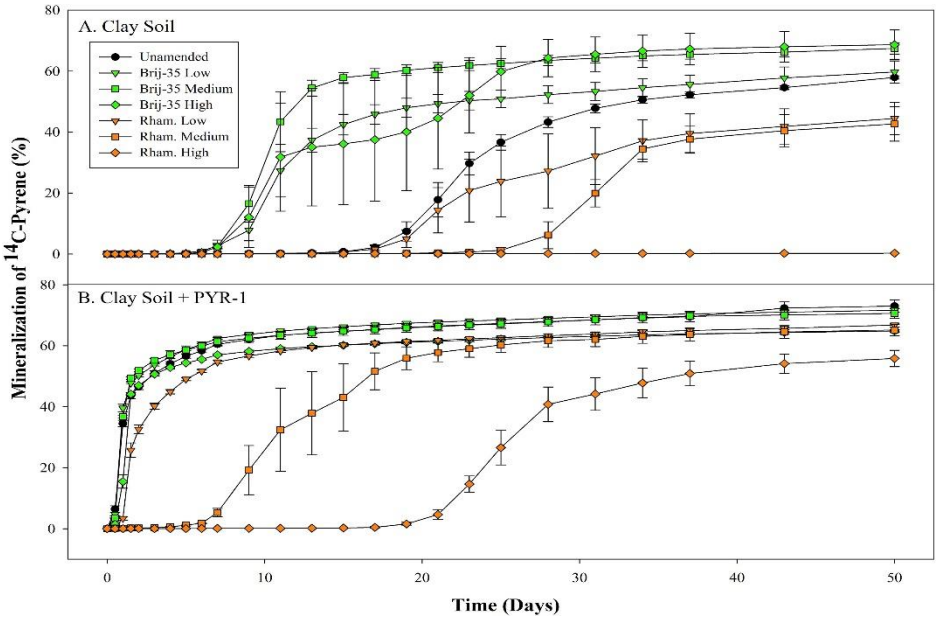

Figure 2.1. Mineralization of ^{14}C-pyrene by (A) native clay soil microbes and (B) clay soil bioaugmented with *Mycobacterium vanbaalenii* PYR-1 after the addition of Brij-35 at 21.6, 216, and 2160 µg surfactant g-dry soil^{-1} or rhamnolipid biosurfactant at 14, 140, and 1400 µg surfactant g-dry soil^{-1} (low, medium, and high), respectively, during a 50-d incubation.

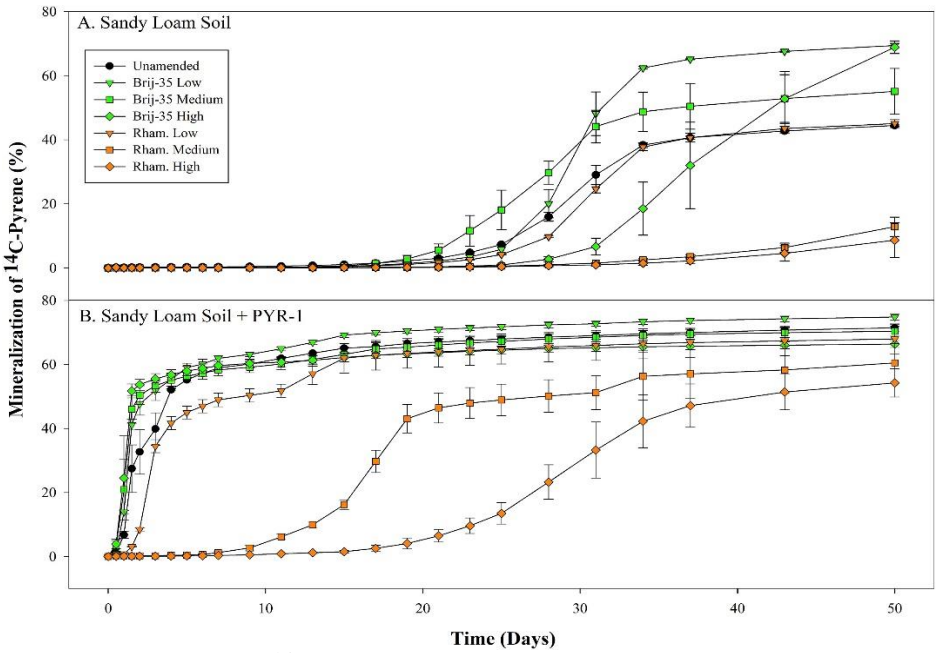

Figure 2.2. Mineralization of ^{14}C-pyrene by (A) native sandy loam soil microbes and (B) sandy loam soil bioaugmented with *Mycobacterium vanbaalenii* PYR-1 after the addition of Brij-35 at 21.6, 216, and 2160 µg surfactant g-dry soil^{-1} or rhamnolipid biosurfactant at 14, 140, and 1400 µg surfactant g-dry soil^{-1} (low, medium, and high), respectively, during a 50-d incubation.

Several studies showed that the addition of Brij-35 surfactant and rhamnolipid biosurfactant enhanced PAH desorption in PAH-contaminated soil systems, making the contaminants more bioavailable. Bueno-Montes et al. (2011) used Tenax extraction to measure the bioaccessible fractions of PAHs in aged, creosote-polluted and MGP soils and showed that the addition of Brij-35 at 25X CMC resulted in significant PAH solubilization in both soils. Congiu and Ortega-Calvo (2014) also used Tenax extraction and ^{14}C-pyrene to evaluate the effect of rhamnolipid biosurfactant on PAH solubilization and concluded that the rhamnolipid biosurfactant significantly increased the solubilization of pyrene that was initially sorbed to soil. Additionally, Adrion et al. (2016) examined the effects of four nonionic surfactants (Brij-30, Span 20, Ecosurf EH-3, and polyoxyethylene sorbitol hexaoleate) and rhamnolipid biosurfactant below their respective CMCs on PAH desorption in PAH-contaminated soil from a former MGP site. They observed that all surfactants increased PAH desorption; however, all surfactants except the rhamnolipid biosurfactant significantly increased PAH biodegradation, possibly due to rhamnolipid biosurfactant having the least effect on PAH desorption.

In this study, the addition of rhamnolipid biosurfactant at the medium and high rates resulted in significant inhibition of ^{14}C-pyrene mineralization as compared to the unamended soils (Figs. 2.1 and 2.2). The application of rhamnolipid biosurfactant at the medium and high rates resulted in an increased lag period in all native and bioaugmented soil treatments, as well as smaller biodegradation rates in the bioaugmented soil treatments as compared to the unamended treatments (Tables 2.1 and 2.2). Because of its biological origin, it is likely that the rhamnolipid biosurfactant is more amendable as a

substrate for soil microorganisms. In this case, the rhamnolipid biosurfactant likely acted as a more favorable carbon source compared to pyrene in the soil system, and the native or inoculated biodegraders exhausted the rhamnolipid biosurfactant as a carbon and energy source first, before resorting to pyrene as a substrate. This biphasic growth and sequential use of two different carbon substrates, where the easier carbon source supporting faster microbial growth is mineralized first, is referred to as "diauxie" (Alexander, 1994). This possibility was supported by the eventual increased ^{14}C-pyrene mineralization in the bioaugmented soils amended with rhamnolipid biosurfactant (Figs. 2.1 and 2.2).

Several previous studies also showed preferential degradation of the externally added rhamnolipid biosurfactant instead of the target compound. Vipulanandan and Ren (2000) observed the preferential degradation by a *Pseudomonas* sp. of rhamnolipid biosurfactant over solubilized naphthalene in biosurfactant and Triton X-100 solutions. The addition of rhamnolipid biosurfactant increased the time for complete naphthalene degradation to 40 d, as compared to only 100 h for Triton X-100, even though the presence of rhamnolipid biosurfactant clearly increased the solubility of naphthalene by 30 times its aqueous solubility. Ghosh and Mukherji (2016) compared the effects of two nonionic surfactants (Triton X-100 and Tween 80) and rhamnolipid biosurfactant at 10X CMC on mineralization of pyrene (100 mg L^{-1}) in liquid culture and found that the rhamnolipid amendment resulted in decreased mineralization compared to the unamended control due to the preferential degradation of the biosurfactant over pyrene, causing a decreased specific growth rate and pyrene utilization by *P. aeruginosa*. Bezza

and Chirwa (2016) also observed a decrease in PAH degradation in the rhamnolipid and nutrient supplemented liquid culture treatments because of preferential microbial consumption of the biosurfactant.

Most of the studies examining the preferential utilization of surfactants by PAH-degrading microorganisms were accomplished in liquid culture, and relatively few studies have examined this phenomenon in a soil matrix that would have a more realistic representation of bioremediation practices. For example, while nonionic surfactants are less likely to adsorb to soil surfaces compared to cationic surfactants due to the negative charge of the soil, some of the Brij-35 or rhamnolipid biosurfactant added to the mineralization assays was likely sorbed to the soil (Ishiguro and Koopal, 2016). Lladò et al. (2012) concluded that rhamnolipid biosurfactant applied to a microbial consortium resulted in increased PAH biodegradation in liquid culture; however, the same rhamnolipid biosurfactant application did not improve PAH biodegradation after 200 d when applied to a soil matrix. Maslin and Maier (2000) suggested that rhamnolipid biosurfactant was used as a preferential carbon source over phenanthrene by the native soil microbial populations when the rhamnolipid was present at higher concentrations. Deschênes et al. (1996) observed that the biodegradation of three-ringed PAHs (fluorene, phenanthrene, and anthracene) was not affected by the addition of rhamnolipid biosurfactant at concentrations below and above the CMC; however, four-ringed PAHs (fluoranthene, pyrene, benzo(a)anthracene, and chrysene) had a 4–6 week lag phase before biodegradation commenced in aged PAH-contaminated soil.

Table 2.1. Influence of soil, *Mycobacterium vanbaalenii* PYR-1 bioaugmentation, and surfactant amendment on ^{14}C-pyrene lag period[†] in clay and sandy loam soil after a 50-d incubation.

		Surfactant Amendment						
		None	Brij-35 (CMC)			Rhamnolipid (CMC)		
Soil	Microbe		0.1X	1.0X	10X	0.1X	1.0X	10X
		----------d----------						
Clay	PYR-1	0.5 k[*]	0.5 k	0.5 k	0.5 k	1 jk	7 i	21 e
	Native	19 ef	9 h	9 h	9 h	19 ef	28 b	> 50[‡]
Sandy Loam	PYR-1	1 jk	1 jk	0.5 k	0.5 k	2 j	11 g	21 e
	Native	23 d	25 c	21 e	31 b	25 c	43 a	43 a

[†]Time required (d) for significant (≥5%) mineralization to occur
[*]Means followed by different letters are significantly different at α = 0.05.
[‡]Not included in statistical analysis

Table 2.2. Influence of soil, *Mycobacterium vanbaalenii* PYR-1 bioaugmentation, and surfactant amendment on ^{14}C-pyrene biodegradation rate (d^{-1}) in clay and sandy loam soil after a 50-d incubation.

		Surfactant Amendment						
		None	Brij-35 (CMC)			Rhamnolipid (CMC)		
Soil	Microbe		0.1X	1.0X	10X	0.1X	1.0X	10X
		----------d^{-1}----------						
Clay	PYR-1	2.78 cd[*]	3.88 ab	4.55 a	3.03 bc	2.60 cd	0.32 g	0.34 g
	Native	0.37 g	0.64 fg	0.73 fg	0.30 g	0.24 g	0.25 g	ND[‡]
Sandy loam	PYR-1	1.51 ef	2.37 cde	2.40 cde	1.96 de	1.44 ef	0.30 g	0.18 g
	Native	0.15 g	0.24 g	0.24 g	0.17 g	0.22 g	0.13 g	0.11 g

[*]Means followed by different letters are significantly different at α = 0.05.
[‡]Not determined

2.3.2 Surfactant Toxicity Evaluation

Mycobacterium vanbaalenii PYR-1 utilized Brij-35 surfactant and rhamnolipid biosurfactant at 0.1X and 10X CMC as the sole carbon source during a 14-d study, indicating that the presence of the nonionic surfactant or rhamnolipid biosurfactant was

not toxic to the soil microorganisms (Fig. 2.3). After 1 d of incubation, the ranking of viable *M. vanbaalenii* PYR-1 CFUs was glucose ≈ rhamnolipid 10X CMC > rhamnolipid 0.1X CMC > Brij-35 0.1X CMC ≈ Brij-35 10X CMC > unamended control. At the end of 14-d incubation, the ranking of viable *M. vanbaalenii* PYR-1 CFUs was Brij-35 0.1X CMC > Brij-35 10X CMC > glucose ≈ rhamnolipid 0.1X CMC > rhamnolipid 10X CMC > unamended control (Fig. 2.3). The enumeration of viable *M. vanbaalenii* PYR-1 CFUs in the rhamnolipid-amended treatments had a similar pattern compared to the treatments amended with glucose, an easily degraded monosaccharide, with substantial growth at the beginning of the incubation, followed by significant decrease in CFUs as the carbon substrate used for microbial growth was depleted. Yu et al. (2014) also observed rapid PAH-degrading bacteria growth using rhamnolipid biosurfactant up to 1000 mg L^{-1}, which resulted in a 40-fold increase in the maximum bacteria population as compared to the unamended control. Bezza and Chirwa (2017) concluded that the addition of a lipopeptide biosurfactant produced by *Bacillus cereus* SPL-4 resulted in a significant increase (up to two orders of magnitude) in the number of CFUs in PAH-contaminated soil during a 64-d incubation.

While nontoxic to *M. vanbaalenii* PYR-1, Brij-35 at both surfactant concentrations showed increased resistance to degradation as compared to the rhamnolipid biosurfactant based upon viable *M. vanbaalenii* PYR-1 CFUs. In the Brij-35 10X CMC-amended culture, viable CFUs were significantly lower compared to the Brij-35 0.1X CMC treatments after 14 d of incubation, potentially due to the limited bioavailability of the surfactant in the micellar phase at 10X CMC as compared to the

monomeric surfactant phase at 0.1X CMC (Zhang, 1997). In another study, Zhang et al. (1999) showed that the small radius size (2–4 nm) and structure characteristics of the micellized nonionic surfactants could prevent close contact between the microorganism and surfactant, resulting in limited degradation.

Previous studies also showed preferential degradation of biosurfactants compared to synthetic surfactants by microorganisms. Zeng et al. (2007) compared the microbial degradation of a nonionic surfactant (Triton X-100), cationic surfactant (Cetrimonium bromide), anionic surfactant (sodium dodecyl sulfate), and biosurfactant (rhamnolipid) and observed that the rhamnolipid biosurfactant was the most readily biodegradable (90% degradation) among all surfactants in the study. Mohan et al. (2006) compared the biodegradability of Triton X-100 and rhamnolipid biosurfactant as sole carbon sources to *Vibriocyclotrophicus* sp. nov., a PAH-degrading microorganism, and showed that the rhamnolipid was readily biodegradable under aerobic conditions while Triton X-100 was only partially biodegradable.

Based on results from the ^{14}C-pyrene mineralization experiment and the effect of surfactants on cell growth, the amendment of Brij-35 or rhamnolipid biosurfactant at the highest rate was not toxic to the PAH-degrading soil microorganisms. Therefore, it may be concluded that in this study, the rhamnolipid biosurfactant was preferentially used by the PAH-degrading soil microorganisms and only after the eventual depletion of the biosurfactant, the mineralization of ^{14}C-pyrene in both soils would start. This was

consistent with the observation that the lag time of pyrene mineralization increased with increasing rhamnolipid amendment rate (Table 2.1 and Figs. 2.1 and 2.2).

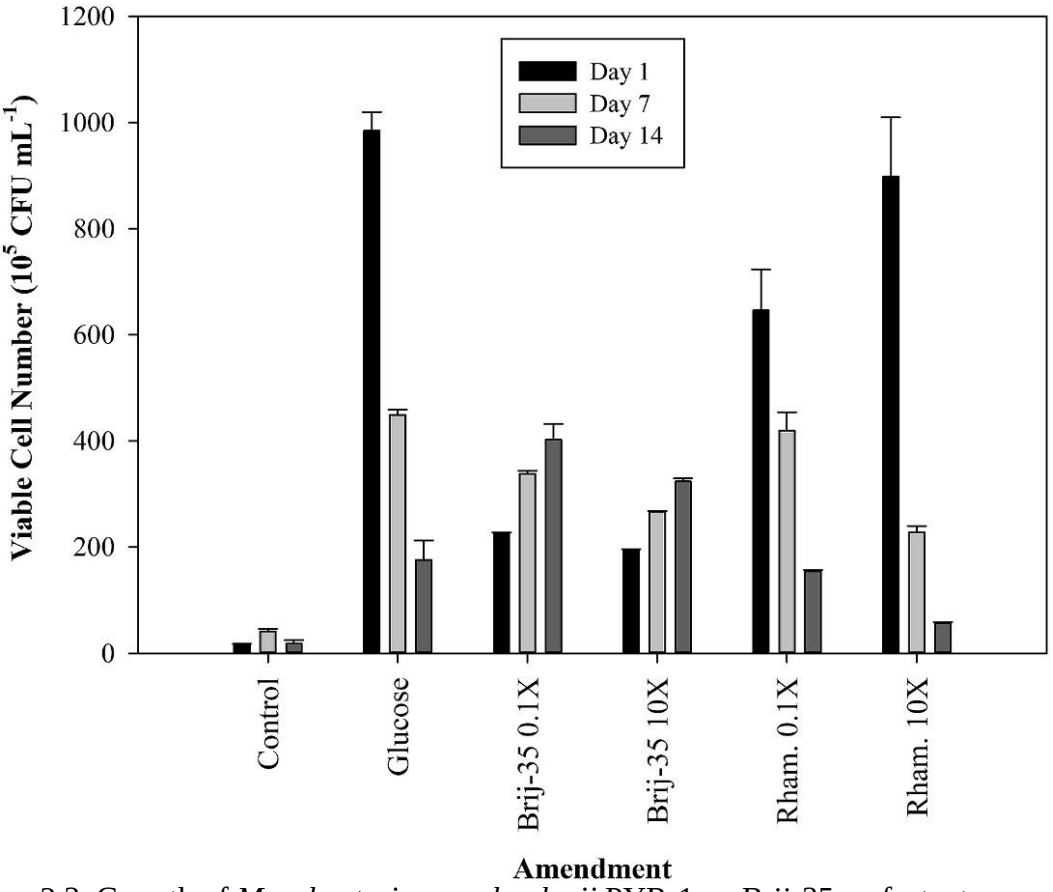

Figure 2.3. Growth of *Mycobacterium vanbaalenii* PYR-1 on Brij-35 surfactant or rhamnolipid biosurfactant at 0.1X CMC and 10X CMC during a 14-d incubation.

2.3.3 Influence of Soil Physical Properties

Compared to the native sandy loam soil treatments, the native clay soil treatments had a significantly shorter lag period in the unamended and all surfactant-amended treatments, except for rhamnolipid at the high rate (Table 2.1). The differences between the clay and sandy loam soil treatments in ^{14}C-pyrene mineralization by the native microbes may be attributed to the higher TOC in the clay soil. The quantity and quality of

soil organic matter has been previously shown to regulate the soil microbial community diversity and activity, as organic matter acts as energy and nutrient sources for heterotrophic soil microorganisms, stimulating microbial biomass growth and activity (Murphy et al., 2011). Additionally, the finer silt and clay fractions could provide more sites for bacteria or organic matter attachment, leading to a larger microbial biomass in the clay aggregate and better utilization of ^{14}C-pyrene (Cui et al., 2011).

2.3.4 Influence of Microbial Bioaugmentation

Bioaugmentation of *M. vanbaalenii* PYR-1 significantly increased the mineralization of ^{14}C-pyrene in both PAH-contaminated soils (Figs. 2.1B and 2.2B). Because of the rapid mineralization, bioaugmentation of *M. vanbaalenii* PYR-1 in soils with or without surfactant resulted in a shortened lag period before ^{14}C-pyrene mineralization commenced as compared to all corresponding soil treatments without the bacterial augmentation (Table 2.1). There were no differences in the length of lag period between the clay and sandy loam bioaugmentation treatments, except for the medium rate rhamnolipid treatment where the clay soil had a shorter lag period (7 d) than the sandy loam soil (11 d) (Table 2.1). Additionally, bioaugmentation of *M. vanbaalenii* PYR-1 in both soils resulted in an increased biodegradation rate in all soil treatments as compared to the native soils, except for the rhamnolipid-amended treatments at medium and high rates (Table 2.2). The bioaugmentation of *M. vanbaalenii* PYR-1 in the clay soil treatments resulted in greater biodegradation rates compared to the bioaugmented sandy loam soil treatments, except for treatments amended with rhamnolipid biosurfactant at the

medium and high rates (Table 2.2). The addition of Brij-35 at the low and medium rates in the bioaugmented clay soil treatments resulted in a higher biodegradation rate, with the rate constant at 3.88 d^{-1} and 4.55 d^{-1}, respectively, as compared to the bioaugmented clay soil without the surfactant (2.78 d^{-1}).

However, after 25 d of incubation, the microbes in the native clay soil amended with Brij-35 surfactant at all three levels had undergone rapid ^{14}C-pyrene mineralization and were not significantly different as compared to the bioaugmented clay soil treatments amended with Brij-35 (Fig. 2.1). This suggests that the bioaugmentation of PAH-degrading microbes may not be necessary for treating PAH-contaminated soils in environments where the native soil microbial community is capable of degrading PAHs and in situations where PAHs are readily bioavailable. After 50 d of incubation, there were no significant differences between the unamended bioaugmented soil treatments, and the bioaugmented soil amended with Brij-35 at all levels (Figs. 2.1 and 2.2). This observation may be partially attributed to the production of surface-active trehalose-containing glycolipids by *M. vanbaalenii* PYR-1 (Kim et al., 2015). It is worth noting that the mixture of the biologically produced glycolipids by *M. vanbaalenii* PYR-1 with the rhamnolipid biosurfactant or Brij-35 surfactant did not result in increased ^{14}C-pyrene mineralization. In a previous study, mixtures of synthetic surfactants such as sodium dodecyl sulfate, Tween-80, Triton X-100, and Brij-35 had a synergistic effect, causing a lower CMC as well as a significant PAH solubility enhancement in the mixed surfactant systems, leading to increased biodegradation of phenanthrene (Zhao et al., 2005).

Mycobacterium vanbaalenii PYR-1 has been extensively studied in pure cultures for the elucidation of mechanisms of PAH degradation; however, few studies have examined PAH biodegradation by *M. vanbaalenii* PYR-1 in contaminated soils where microbe survival and growth may be a limiting factor due to various environmental and microbial variables such as microbial competition with native soil populations, nutrient availability, moisture content, soil pH, and soil temperature (Lebeau, 2011). In the current study, the survival and PAH-degradation enhancement of *M. vanbaalenii* PYR-1 bioaugmentation can be clearly seen in the unamended and rhamnolipid-amended treatments by the significant decrease in lag period and total ^{14}C-pyrene mineralization (Table 2.1 and Fig. 2.2). Ghaley et al. (2013) also observed rapid PAH mineralization in pyrene-contaminated soils (700 mg kg^{-1}) bioaugmented with *M. vanbaalenii* PYR-1 with a similar lag period (0.5 d) and total pyrene mineralization (58%). A similar *Mycobacterium* sp. also exhibited effective degradation potential for PAHs, resulting in enhanced ^{14}C-pyrene mineralization (up to 40%) in three different petroleum-contaminated soils (Cheung and Kinkle, 2001).

2.4 Conclusions

Findings from this study showed that using a readily biodegradable biosurfactant such as rhamnolipids may not necessarily enhance PAH biodegradation in soil. Even though biosurfactants are capable of increasing the bioavailability of contaminants such as PAHs, they may also serve as a more favorable substrate to the degrading microorganisms than the target contaminant. Therefore, a surfactant recalcitrant to

biodegradation may have the advantage in stimulating immediate biodegradation. However, biosurfactants such as rhamnolipids may be desirable as biosurfactants do not persist to create an additional contamination scenario (Goudar et al., 1999). These results highlight the complex nature of the interactions between surfactants and biodegraders in contaminated soils in the context of contaminant remediation. The success or failure of surfactant-enhanced PAH biodegradation depends on many factors such as the type, structure, and amendment rates of the surfactant applied to the contaminated soil, in addition to the presence of the PAH-degrading microorganisms and their response to the increased bioavailable PAH concentrations and possible surfactant toxicity. Therefore, biosurfactants, especially those produced by the degrading microorganisms themselves, are likely more preferable as they act to enhance biodegradation while increasing contaminant bioavailability. In contrast, external addition of biosurfactants may not affect the microbial degradation of contaminants in a straightforward manner, as degrading microorganisms may preferentially use these materials as substrates, and while at high concentrations, the surfactants may form micelles, thus isolating the hydrophobic contaminants from the degrading microorganisms. Therefore, in practical applications of biosurfactant-enhanced bioremediation, it is necessary to find the proper balance between the biodegradability of the biosurfactant and its effects on contaminant solubilization and biodegradation. The type as well as levels of surfactants should be determined for specific contaminated sites and individual contaminants using optimization experiments prior to full-scale implementation.

2.5 References

Adrion, A.C., Nakamura, J., Shea, D., Aitken, M.D., 2016. Screening nonionic surfactants for enhanced biodegradation of polycyclic aromatic hydrocarbons remaining in soil after conventional biological treatment. *Environmental Science & Technology* 50, 3838–3845.

Alexander, M., 1999. Biodegradation and bioremediation. Academic Press, New York.

Bai, N., Wang, S., Abuduaini, R., Zhang, M., Zhu, X., Zhao, Y., 2017. Rhamnolipid-aided biodegradation of carbendazim by *Rhodococcus* sp. D-1: Characteristics, products, and phytotoxicity. *Science of The Total Environment* 590, 343–351.

Bezza, F.A., Chirwa, E.M., 2015. Desorption kinetics of polycyclic aromatic hydrocarbons (PAHs) from contaminated soil and the effect of biosurfactant supplementation on the rapidly desorbing fractions. *Biotechnology & Biotechnological Equipment* 29, 680–688.

Bezza, F.A., Chirwa, E.M.N., 2017. The role of lipopeptide biosurfactant on microbial remediation of aged polycyclic aromatic hydrocarbons (PAHs)-contaminated soil. *Chemical Engineering Journal* 309, 563–576.

Bezza, F.A., Chirwa, E.M.N., 2016. Biosurfactant-assisted bioremediation of polycyclic aromatic hydrocarbons (PAHs) in liquid culture system and substrate interactions. *Polycyclic Aromatic Compounds* 37, 1–20.

Bosch, M.P., Robert, M., Mercade, M., Espuny, M., Parra, J., Guinea, J., 1988. Surface active compounds on microbial cultures: Investigation and production of surface active compounds on microbial cultures. *Tenside Detergents* 25, 208–211.

Brinch, U.C., Ekelund, F., Jacobsen, C.S., 2002. Method for spiking soil samples with organic compounds. *Applied Environmental Microbiology* 68, 1808.

Bueno-Montes, M., Springael, D., Ortega-Calvo, J.-J., 2011. Effect of a nonionic surfactant on biodegradation of slowly desorbing PAHs in contaminated soils. *Environmental Science & Technology* 45, 3019–3026.

Cheung, P.-Y., Kinkle, B.K., 2001. *Mycobacterium* diversity and pyrene mineralization in petroleum-contaminated soils. *Applied Environmental Microbiology* 67, 2222.

Congiu, E., Ortega-Calvo, J.-J., 2014. Role of desorption kinetics in the rhamnolipid-enhanced biodegradation of polycyclic aromatic hydrocarbons. *Environmental Science & Technology* 48, 10869–10877.

Congiu, E., Parsons, J.R., Ortega-Calvo, J.-J., 2015. Dual partitioning and attachment effects of rhamnolipid on pyrene biodegradation under bioavailability restrictions. *Environmental Pollution* 205, 378–384.

Cui, X., Hunter, W., Yang, Y., Chen, Y., Gan, J., 2011. Biodegradation of pyrene in sand, silt and clay fractions of sediment. *Biodegradation* 22, 297–307.

Deschênes, L., Lafrance, P., Villeneuve, J.-P., Samson, R., 1996. Adding sodium dodecyl sulfate and *Pseudomonas aeruginosa* UG2 biosurfactants inhibits polycyclic aromatic hydrocarbon biodegradation in a weathered creosote-contaminated soil. *Applied Microbiology and Biotechnology* 46, 638–646.

Doick, K.J., Semple, K.T., 2003. The effect of soil:water ratios on the mineralisation of phenanthrene:LNAPL mixtures in soil. *FEMS Microbiology Letters* 220, 29–33.

Gavlak, R., Horneck, D., Miller, R.O., Kotuby-Amacher, J., 2003. Soil, plant and water reference methods for the western region. WCC-103 Publication, Fort Collins.

Ghaly, A., Yusran, A., Dave, D., 2013. Effects of biostimulation and bioaugmentation on the degradation of pyrene in soil. *Journal of Bioremediation & Biodegradation*. S 5.

Ghosh, I., Mukherji, S., 2016. Diverse effect of surfactants on pyrene biodegradation by a Pseudomonas strain utilizing pyrene by cell surface hydrophobicity induction. *International Biodeterioration & Biodegradation* 108, 67–75.

Goudar, C., Strevett, K., Grego, J., 1999. Competitive substrate biodegradation during surfactant-enhanced remediation. *Journal of Environmental Engineering* 125, 1142–1148.

Guha, S., Jaffé, P.R., 1996. Biodegradation kinetics of phenanthrene partitioned into the micellar phase of nonionic surfactants. *Environmental Science & Technology* 30, 605–611.

Haritash, A.K., Kaushik, C.P., 2009. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): A review. *Journal of Hazardous Materials* 169, 1–15.

Ishiguro, M., Koopal, L.K., 2016. Surfactant adsorption to soil components and soils. *Advances in Colloid and Interface Science* 231, 59–102.

Jahan, K., Ahmed, T., Maier, W.J., 1997. Phenanthrene mineralization in soil in the presence of nonionic surfactants. *Toxicological & Environmental Chemistry* 64, 127–143.

Kathi, S., Khan, A.B., 2011. Phytoremediation approaches to PAH contaminated soil. *Indian Journal of Science and Technology* 4, 56–63.

Kim, I.S., Park, J.-S., Kim, K.-W., 2001. Enhanced biodegradation of polycyclic aromatic hydrocarbons using nonionic surfactants in soil slurry. *Applied Geochemistry* 16, 1419–1428.

Kim, S.-J., Kweon, O., Sutherland, J.B., Kim, H.-L., Jones, R.C., Burback, B.L., Graves, S.W., Psurny, E., Cerniglia, C.E., 2015. Dynamic response of *Mycobacterium vanbaalenii* PYR-1 to BP Deepwater Horizon crude oil. *Applied and Environmental Microbiology* 81, 4263.

Kuppusamy, S., Palanisami, T., Megharaj, M., Venkateswarlu, K., Naidu, R., 2016. In-Situ Remediation Approaches for the Management of Contaminated Sites: A Comprehensive Overview, in: de Voogt, P. (Ed.), Reviews of Environmental Contamination and Toxicology Volume 236. Springer International Publishing, Cham, pp. 1–115.

Lebeau, T., 2011. Bioaugmentation for *In situ* Soil Remediation: How to Ensure the Success of Such a Process, in: Singh, A., Parmar, N., Kuhad, R.C. (Eds.), Bioaugmentation, Biostimulation and Biocontrol. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 129–186.

Li, S., Pi, Y., Bao, M., Zhang, C., Zhao, D., Li, Y., Sun, P., Lu, J., 2015. Effect of rhamnolipid biosurfactant on solubilization of polycyclic aromatic hydrocarbons. *Marine Pollution Bulletin* 101, 219–225.

Lladó, S., Solanas, A.M., de Lapuente, J., Borràs, M., Viñas, M., 2012. A diversified approach to evaluate biostimulation and bioaugmentation strategies for heavy-oil-contaminated soil. *Science of The Total Environment* 435–436, 262–269.

Maslin, P., Maier, R.M., 2000. Rhamnolipid-enhanced mineralization of phenanthrene in organic-metal co-contaminated soils. *Bioremediation Journal* 4, 295–308.

Mohan, P.K., Nakhla, G., Yanful, Ernest.K., 2006. Biokinetics of biodegradation of surfactants under aerobic, anoxic and anaerobic conditions. *Water Research* 40, 533–540.

Murphy, D.V., Cookson, W.R., Braimbridge, M., Marschner, P., Jones, D.L., Stockdale, E.A., Abbott, L.K., 2011. Relationships between soil organic matter and the soil microbial biomass (size, functional diversity, and community structure) in crop and pasture systems in a semi-arid environment. *Soil Research* 49, 582–594.

Oyelami, A., Elegbede, B., Semple, K., 2014. Impact of different types of activated carbon on the bioaccessibility of ^{14}C-phenanthrene in sterile and non-sterile soils. *Environments* 1, 137-156.

Pinto, L.J., Moore, M.M., 2000. Release of polycyclic aromatic hydrocarbons from contaminated soils by surfactant and remediation of this effluent by *Penicillium* spp. *Environmental Toxicology and Chemistry* 19, 1741–1748.

Reid, B.J., MacLeod, C.J.A., Lee, P.H., Morriss, A.W.J., Stokes, J.D., Semple, K.T., 2001. A simple ^{14}C-respirometric method for assessing microbial catabolic potential and contaminant bioavailability. *FEMS Microbiology Letters* 196, 141–146.

Rhodes, A.H., Carlin, A., Semple, K.T., 2008. Impact of black carbon in the extraction and mineralization of phenanthrene in soil. *Environmental Science & Technology* 42, 740–745.

Riding, M.J., Doick, K.J., Martin, F.L., Jones, K.C., Semple, K.T., 2013. Chemical measures of bioavailability/bioaccessibility of PAHs in soil: Fundamentals to application. *Journal of Hazardous Materials* 261, 687–700.

Rosen, M.J., Li, F., Morrall, S.W., Versteeg, D.J., 2001. The relationship between the interfacial properties of surfactants and their toxicity to aquatic organisms. *Environmental Science & Technology* 35, 954–959.

Schumacher, B.A., 2002. Methods for the determination of total organic carbon (TOC) in soils and sediments. Ecological Risk Assessment Support Center 1–23.

Tiehm, A., Stieber, M., Werner, P., Frimmel, F.H., 1997. Surfactant-Enhanced Mobilization and Biodegradation of Polycyclic Aromatic Hydrocarbons in Manufactured Gas Plant Soil. *Environmental Science & Technology* 31, 2570–2576.

United States Environmental Protection Agency, 2017. FOIA 4 – U.S. EPA Superfund Program – FOIA-004 all final NPL sites Available at: https://www.epa.gov/superfund/superfund-data-and-reports (Accessed 1 December 2017).

Vipulanandan, C., Xupeng, R., 2000. Enhanced solubility and biodegradation of naphthalene with biosurfactant. *Journal of Environmental Engineering* 126, 629–634.

Youssef, N.H., Duncan, K.E., Nagle, D.P., Savage, K.N., Knapp, R.M., McInerney, M.J., 2004. Comparison of methods to detect biosurfactant production by diverse microorganisms. *Journal of Microbiological Methods* 56, 339–347.

Yu, H., Huang, G.-H., Xiao, H., Wang, L., Chen, W., 2014. Combined effects of DOM and biosurfactant enhanced biodegradation of polycyclic aromatic hydrocarbons (PAHs) in soil–water systems. *Environmental Science and Pollution Research* 21, 10536–10549.

Zeng, G., Fu, H., Zhong, H., Yuan, X., Fu, M., Wang, W., Huang, G., 2007. Co-degradation with glucose of four surfactants, CTAB, Triton X-100, SDS and rhamnolipid, in liquid culture media and compost matrix. *Biodegradation* 18, 303–310.

Zhang, C., 1997. Removal of hydrophobic organic compounds and surfactants from wastewater: Polyaphron-enhanced solvent extraction and biodegradation. PhD Dissertation, Louisiana State University, Baton Rouge, LA.

Zhang, C., Valsaraj, K.T., Constant, W.D., Roy, D., 1999. Aerobic biodegradation kinetics of four anionic and nonionic surfactants at sub- and supra-critical micelle concentrations (CMCs). *Water Research* 33, 115–124.

Zhao, B., Zhu, L., Li, W., Chen, B., 2005. Solubilization and biodegradation of phenanthrene in mixed anionic–nonionic surfactant solutions. *Chemosphere* 58, 33–40.

Chapter 3 Soil Bacterial Community Dynamics Following Surfactant Addition and Bioaugmentation in Pyrene-contaminated Soils

3.1 Introduction

Polycyclic aromatic hydrocarbons are a class of fused-ring aromatic compounds and 16 PAH compounds are designated as priority pollutants by the U.S. EPA because of their known or suspected toxicity and genotoxicity as well as frequent environmental occurrence (Tong et al., 2018). Bioremediation, the utilization of microorganisms to biologically degrade hazardous organic compounds to levels below concentration limits established by regulatory authorities, is considered an effective technique to remediate soils contaminated with PAHs (Kuppusamy et al., 2017). In a 2007 U.S. EPA report on contaminant treatment technologies, 37 out of 145 PAH remediation projects utilized bioremediation applications (U.S. EPA, 2007). However, biological treatment of PAH-contaminated soils is dependent upon the presence and degradation activity of soil microbes capable of transforming the priority pollutants (Wang et al., 2017). Additionally, bioremediation efficacy for PAH-contaminated soil may also be limited by the bioavailability of soil-bound PAHs due to their physical and chemical properties, resulting in low aqueous solubility and high solid-water distribution ratios that promote PAH accumulation in the solid phases of the terrestrial environment (Lu et al., 2019). Surfactant-amended bioremediation has been proposed to increase the rate of PAH desorption from the soil to the aqueous phase through micellar solubilization and/or by direct modification of the soil matrix (Singleton et al., 2016). In recent years, interest in

and feasibility of biosurfactant-enhanced bioremediation has increased because rhamnolipid biosurfactants offer several advantages over their synthetic counterparts such as increased stability in pH and temperature extremes and environmental compatibility, while still offering similar PAH desorption effects (Liu et al., 2017).

Under situations where PAH bioavailability is limited, surfactants and biosurfactants have been shown to increase transport of PAHs from the soil matrix into the aqueous phase, resulting in increased bioavailability to PAH-degrading microorganisms and enhance PAH biodegradation (Adrion et al., 2016). However, surfactants may also negatively affect the PAH-degrading activity of the soil microbial community in PAH-contaminated soils (Bezza and Chirwa, 2017). For example, some surfactants, especially anionic surfactants, can bind to peptides, enzymes, and DNA and change the biological function of soil microorganisms (Singh et al., 2018). Conversely, surfactants can also be preferentially utilized as carbon and energy sources for growth by PAH-degrading microorganisms, resulting in a decrease in metabolism of the target contaminant, thus inhibiting PAH biodegradation (Ghosh and Mukherji, 2016). However, the effects of surfactant amendments on the PAH degradation capacities of the soil microbial community has been rarely examined. A better understanding of the soil microbial community dynamics in response to amendment of different surfactants is valuable for successful surfactant-enhanced bioremediation of PAH-contaminated soils.

We previously reported that amendment of the synthetic surfactant Brij-35 enhanced pyrene mineralization in soils, while amendment of rhamnolipid biosurfactant

significantly inhibited the mineralization and also increased the lag period before mineralization commenced (Wolf and Gan, 2018). As the differences in pyrene degradation likely resulted from changes in the soil microbial communities due to surfactant application, we hypothesized that the PAH-degrading soil microorganisms that maintained a high relative abundance could be identified by analysis of 16S rRNA gene sequences. The objectives of this study were to evaluate the effects of Brij-35 and rhamnolipid amendment on the native soil microbes associated with PAH mineralization and to determine whether such effects were dose dependent. The 16S rRNA gene high-throughput sequencing and phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) were employed to analyze the shifts in soil microbial taxa due to the presence of surfactants and to assess the bacterial species and functional genes responsible for PAH biodegradation.

3.2 Materials and Methods

3.2.1 Chemicals and Materials

Pyrene (98%) and Brij-35 surfactant were purchased from Sigma Aldrich (St. Louis, MO). Rhamnolipid biosurfactant (R-95), isolated from *P. aeruginosa* NY3, was purchased from AGAE Technologies (Corvallis, OR). A clay soil and sandy loam soil were collected from Crossett, Arkansas and Irvine, California, respectively. The soil total organic carbon (TOC) was analyzed according to Schumacher (2002) by high-temperature combustion using an Elementar Vario MAX C/N instrument (Elementar Americas, Mt. Laurel, NJ). The Perry clay soil had sand, silt, and clay percentages of 3%,

19%, and 78% and a TOC content of 2.4%. The San Emigdio fine sandy loam soil had sand, silt, and clay percentages of 66%, 17%, and 17% and a TOC content of 0.7%.

3.2.2 Pyrene Mineralization Experiment

The pyrene mineralization experiment was conducted using flask respirometers. For each sample, 2 g soil was spiked with 10 mg kg^{-1} pyrene according to Brinch et al. (2002). An additional 8 g soil was added to the treated soil and then mixed over a 5-d period. Following pyrene spiking, 10 mL sterilized minimal basal salts (MBS) solution was added to the native (i.e., without bioaugmentation) soil treatment, followed by the addition of 10 mL surfactant solution resulting in initial amendment rates of 14, 140, and 1,400 µg g^{-1} for rhamnolipid biosurfactant and 21.6, 216, and 2,160 µg g^{-1} for Brij-35, respectively. For the bioaugmentation treatment, *M. vanbaalenii* PYR-1-MBS solution was added to yield approximately 10^7 cells g^{-1} soil and then 10mL of surfactant solution was added. Additional details of the pyrene mineralization study may be found in Wolf and Gan (2018).

3.2.3 Soil DNA Extraction and 454-Pyrosequencing Data Analysis

At the end of the 50-d mineralization experiment, genomic DNA was extracted from 250 mg soil samples in triplicate using a bead-beating protocol with SurePrep™ Soil DNA Isolation Kit (Thermo Fisher Scientific, Waltham, WA) in accordance with the manufacturer's instructions, except that cell lysis was achieved by horizontally vortexing tubes at a maximum speed for 6 min (Singleton et al., 2016). Extracts belonging to the

same soil replicates were pooled prior to subsequent molecular analyses and the concentration and quality of DNA was measured using a Nanodrop 2000C Ultramicrospectrophotometer (Thermo Fisher Scientific) (Lladó et al., 2015).

The soil microbial community was assessed by Ion Torrent Tag-encoded FLX amplicon pyrosequencing (bTEFAP) of the 16S rRNA gene by MR DNA (Shallowater, TX) on an Ion Torrent Personal Genome Machine™ (PGM) following the manufacturer's instructions. Briefly, the 16S variable region V4 was amplified using barcoded forward primer 515F GTGCCAGCMGCCGCGGTA, reverse primer 806R GGACTACHVGGGTWTCTAAT, and HotStarTaq Plus Master Mix Kit (Qiagene, Valencia, CA). A single-step 30 cycle PCR program consisted of 94 °C for 3 min, followed by 28 cycles of 94 °C for 30 sec, 53°C for 40 sec, and 72 °C for 1 min, after which a final elongation step at 72 °C for 5 min was performed. Following PCR, multiple samples were pooled in equal proportion based on their molecular weight and DNA concentrations, and purified using the Agencourt AMPure XP purification system (Beckman Coulter, Brea, CA).

The barcoded 16S rRNA gene sequences were trimmed into libraries using Quantitative Insights Into Microbial Ecology (QIIME) (version 1.9.1) (Caporaso et al., 2010). The following steps were then performed using QIIME: Denoising using Denoiser (Reeder and Knight, 2010); operational taxonomic unit (OTU) clustering at 97% similarity using UCLUST (Edgar, 2010); sequencing alignment using PyNAST (Caporaso et al., 2009); and taxonomic assignment using BLASTn against a curated

GreenGenes/RDP/NCBI derived database (DeSantis et al., 2006). Alpha-diversity analyses and beta-diversity analyses using UniFrac were analyzed using the smallest library count (Lozupone and Knight, 2005). Statistical analysis was performed using NCSS 12 Statistical Software 2018 (NCSS, Kaysville, UT) and phyloseq R package (McMurdie and Holmes, 2014).

PICRUSt was used to predict the mean relative abundance of gene functions related to PAH-biodegradation. PICRUSt uses evolutionary modeling to predict metagenomes that were then collapsed into Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp/) Orthology (KO) from the analyzed 16S rRNA data compared against Greengenes reference database (version 13_8) (McDonald et al., 2011; Langille et al., 2013). Briefly, the OTUs from the 16S data were normalized by PICRUSt and KO profiles related to PAH degradation were calculated using the PICRUSt algorithm and visualized using Statistical Analysis of Metagenomic Profiles (STAMP) (Langille et al., 2013; Parks et al., 2014). KO profiles of different treatments were pairwise compared, where Welch's *t* test gave a $p<0.05$ associated confidence interval for each gene (Parks et al., 2014).

Linear discriminant analysis Effect Size (LEfSe) software was used to determine taxa for which the relative abundance was significantly different among the various treatments and can be used as potential metagenomic biomarkers (Segata et al., 2011). Briefly, LEfSe v1.0 performed a nonparametric Wilcoxon sum-rank test followed by linear discriminant analysis (LDA) to assess the effect size of each differentially

abundant taxon (Segata et al., 2011). For this analysis, the alpha parameter significance threshold for the Wilcoxon sum-rank test was set to 0.04 and the log value for the LDA score cut-off was set to 2.0 (Mukherjee et al., 2017). All analyses carried out through LEfSe were performed through the Galaxy server (Goecks et al., 2010).

3.2.4 Sequence Deposition

16S rRNA gene amplicon library data from 454-pyrosequencing were submitted to the Sequence Read Archive of the National Center for Biotechnology Information (NCBI) under BioProject accession number PRJNA524706.

3.3 Results and Discussion

The effects of Brij-35 nonionic surfactant and rhamnolipid biosurfactant amendment and the addition of *M. vanbaalenii* PYR-1 bioaugmentation on pyrene mineralization in the two soil systems were reported elsewhere (Wolf and Gan, 2018). Briefly, the addition of Brij-35 surfactant increased pyrene mineralization in the clay and sandy loam soils compared to the unamended soils at the end of the 50-d mineralization study. The addition of rhamnolipid biosurfactant substantially inhibited pyrene mineralization and resulted in a longer lag period in a dose-dependent manner before pyrene mineralization commenced. The application of rhamnolipid at the high rate in the native clay and sandy loam soil resulted in 0.2±0.1% and 13±0.8% pyrene mineralization after 50 d, respectively. The inoculation of *M. vanbaalenii* PYR-1 had an immediate impact on pyrene mineralization in both unamended and Brij-35-amended soils. The

objectives of the current study were to discern if the addition of the rhamnolipid biosurfactant or Brij-35 synthetic surfactant as well as the bioaugmentation of *M. vanbaalenii* PYR-1 had significant effects on the soil microbial community dynamics related to PAH biodegradation in soils.

3.3.1 Bacterial Community Structure in Untreated Soils

The sequence and soil microbial diversity indices in both soil systems, before any treatment, are shown in Table 3.1. The 16S rRNA gene 454-pyrosequencing analysis showed the highest bacterial diversity in the untreated clay and sandy loam soils, where *Proteobacteria* was the dominant phylum in the clay and sandy loam soil, comprising 29% and 33% of all bacterial OTUs, respectively (Table 3.1 and Fig. 3.1). The second-most abundant phylum in the untreated clay and sandy loam soil systems was *Gemmatimonadetes* and *Actinobacteria*, accounting for 15% and 24%, respectively. Although there is substantial variability in the abundance of different phyla in different soils based on numerous physical and chemical soil properties, *Proteobacteria* and *Actinobacteria* are commonly the most abundant phyla in soils according to 16S rRNA analysis (Janssen, 2006; Delgado-Baquerizo et al., 2018). Lladó et al. (2015) and Wu et al. (2017) observed similar results where *Proteobacteria* and *Actinobacteria* were the most abundant phyla in the untreated soils before PAH or surfactant amendment.

Table 3.1. 454-pyrosequencing analysis and diversity indices of the pyrene-contaminated soil systems after a 50-d mineralization study.

Treatment	Size[a]	OTUs[b]	Chao1[c]	Shannon[d]	Coverage[e]
Clay soil					
Untreated	23,493	4,720	10,479	9.99	0.88
Native + Pyrene	44,711	3,972	9,643	6.53	0.94
Brij-35 Low	37,415	3,990	9,892	6.87	0.93
Brij-35 Medium	76,535	4,491	11,570	7.02	0.95
Brij-35 High	47,827	3,332	8,586	6.35	0.95
Rham. Low	47,134	4,535	10,833	7.67	0.94
Rham. Medium	41,679	3,177	7,640	7.01	0.95
Rham. High	62,900	1,062	2,511	2.54	0.99
PYR-1 + Pyrene	44,563	4,616	11,999	7.27	0.93
PYR-1 + Brij-35 Low	41,167	4,550	11,739	7.05	0.93
PYR-1 + Brij-35 Medium	40,855	3,892	9,392	7.12	0.94
PYR-1 + Brij-35 High	42,360	3,440	8,770	6.99	0.95
PYR-1 + Rham. Low	51,863	4,059	10,104	7.10	0.95
PYR-1 + Rham. Medium	33,305	3,211	8,119	7.78	0.94
PYR-1 + Rham. High	56,301	2,467	5,857	5.89	0.97
Sandy loam soil					
Untreated	25,436	4,819	12,252	10.15	0.89
Native + Pyrene	46,182	3,062	6,273	7.74	0.96
Brij-35 Low	39,691	3,450	6,742	7.59	0.95
Brij-35 Medium	42,086	3,266	6,251	7.81	0.96
Brij-35 High	18,261	1,111	2,061	6.54	0.97
Rham. Low	43,833	3,606	7,387	8.51	0.95
Rham. Medium	54,144	2,727	6,239	6.57	0.97
Rham. High	57,551	1,571	3,557	4.18	0.98
PYR-1 + Pyrene	48,878	4,048	9,585	7.44	0.95
PYR-1 + Brij-35 Low	15,424	885	1,903	6.79	0.97
PYR-1 + Brij-35 Medium	25,067	1,760	3,734	7.59	0.96
PYR-1 + Brij-35 High	10,957	626	1,182	5.76	0.97
PYR-1 + Rham. Low	53,758	4,649	12,420	6.90	0.94
PYR-1 + Rham. Medium	51,382	2,401	5,527	6.23	0.97
PYR-1 + Rham. High	57,553	1,715	3,617	4.61	0.98

[a]Number of sequences for each library
[b]Calculated with UCLUST in QIIME at the 3% distance level
[c]Chao1 richness index calculated using QIIME at the 3% distance level
[d]Shannon diversity index calculated using QIIME at the 3% distance level
[e]Estimated sample coverage: C = 1 – (Nx/N; where Nx is the number of unique sequences and N is the total number of sequences

At the end of the 50-d mineralization study, the number of OTUs and Shannon diversity index in all soil treatments decreased compared to the untreated clay and sandy loam soils, which may be attributed to the introduction and interactions of environmental stressors, including pyrene, Brij-35 surfactant, and rhamnolipid biosurfactant, on the endogenous soil microbial community, resulting in the acclimation of soil microbes that were able to survive the environmental stressors (Table 3.1). These results were similar to those of Röling et al. (2002), where a dramatic reduction in bacterial community diversity during the bioremediation of beach sediment contaminated with oil was observed due to the strong selection for bacteria belonging to the alkane-degrading groups.

3.3.2 Effect of Pyrene on Soil Microbial Community

At the end of the 50-d study, the spiking of pyrene in both soils (Native+Pyrene) resulted in a substantial increase in *Firmicutes* compared to the untreated soil (Fig. 3.1). In the clay soil, the relative abundance of *Firmicutes* increased from approximately 2% in the untreated soil to 48% and 37% in the Native+Pyrene and *M. vanbaalenii* PYR-1 bioaugmented (PYR-1+Pyrene) clay soil treatments, respectively. The sandy loam soil treatments showed a similar trend, with an increase in *Firmicutes* from 6% in the untreated soil to 21% and 10% in the Native+Pyrene and PYR-1+Pyrene treatments after 50 d, respectively. Within the *Firmicutes* phylum in the Native+Pyrene clay and sandy loam soil treatments, the *Bacillus* genus accounted for approximately 90% and 79%, respectively. In the PYR-1+Pyrene clay and sandy loam treatments, *Bacillus* accounted for approximately 90% and 87% of *Firmicutes*, respectively. *Gemmatimonadetes* and

Acidobacteria phyla abundance in the untreated clay soil decreased from 15% and 14% to 1% and 2%, respectively, after pyrene contamination, most likely due to pyrene-induced proliferation of *Firmicutes* (Fig. 3.1). Chadhain et al. (2006) also observed a decrease in *Acidobacteria* abundance after the addition of PAHs in soil. Niepceron et al. (2014) reported that the *Firmicutes* phylum was significantly higher in soil microcosms spiked with phenanthrene at 300 mg kg^{-1} compared to the corresponding control throughout a 90-d biodegradation study. Sun et al. (2014) observed similar results and showed that *Bacillus* was the most abundant genus after a 90 d bioremediation study of PAH-contaminated soil.

Members of *Bacillus* have commonly been inoculated to PAH-contaminated soils because the genus is typically associated with increased PAH biodegradation efficacy (Das and Mukherjee, 2007; Patowary et al., 2015). Toledo et al. (2006) assessed PAH biodegradation in solid waste oil samples and determined that the majority of the PAH-degrading microbes (66.6%) belonged to *Bacillus* and 10 isolated *Bacillus* strains were capable of tolerating and degrading naphthalene, phenanthrene, fluoranthene, or pyrene as the sole carbon source in mineral liquid media. Ijah and Antai (2003) performed a year-long crude oil-contaminated soil biodegradation study and showed that *Bacillus* species were the predominant crude-oil-utilizing bacteria in the soil amended with 30% or 40% crude oil, most likely due to resistant endospores.

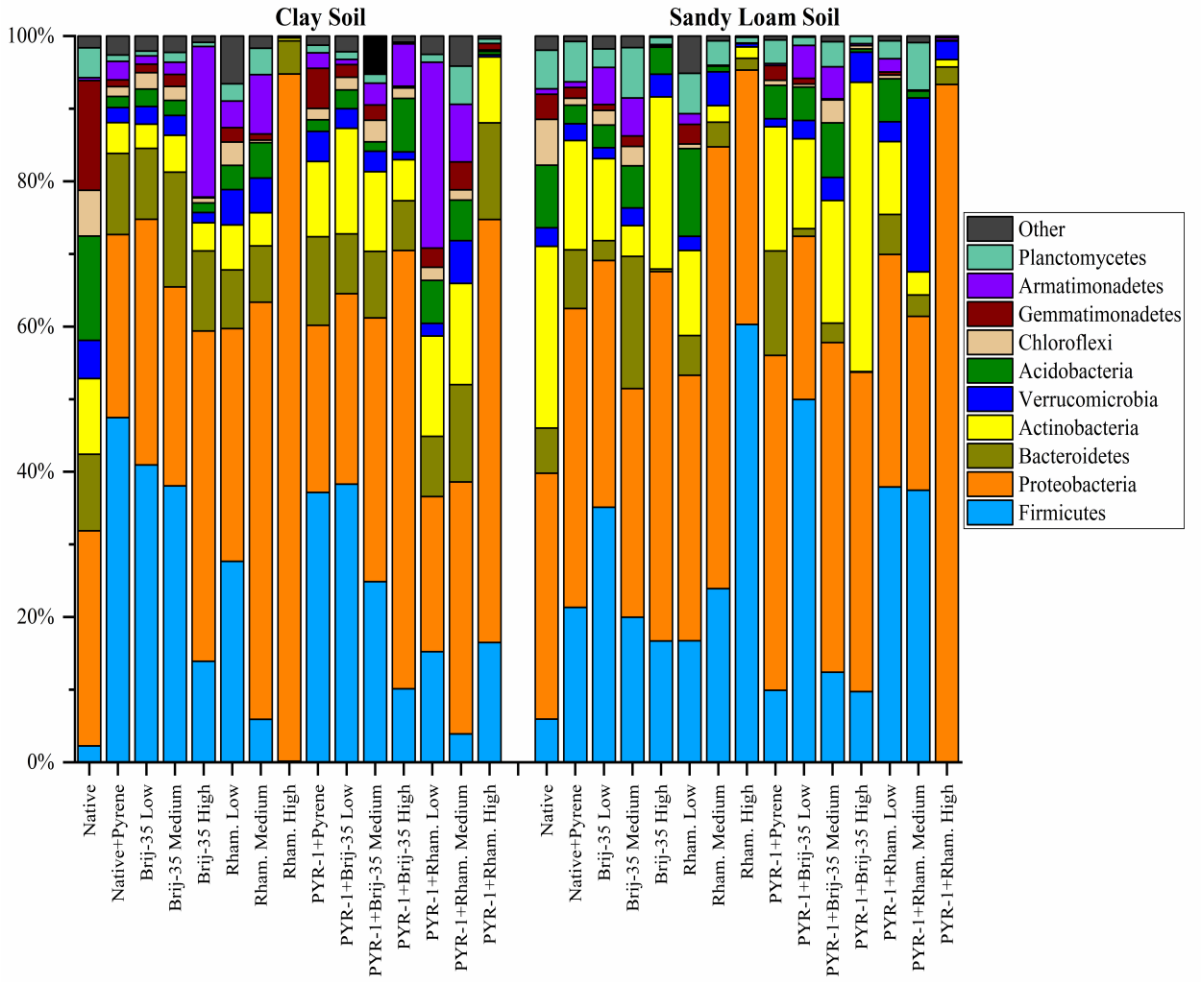

Figure 3.1. Biodiversity of bacterial phyla expressed as relative abundance (%) of the operational taxonomic units in the native or bioaugmented with *Mycobacterium vanbaalenii* PYR-1 clay and sandy loam soils amended with Brij-35 surfactant at 21.6, 216, and 2,160 μg g^{-1} or rhamnolipid biosurfactant at 14, 140, and 1,400 μg g^{-1} (low, medium, and high), respectively, after a 50-d mineralization assay.

After 50 d, the unamended clay soil displayed a shorter lag period (19 d) for pyrene mineralization and mineralized significantly more pyrene (58±1.0%) than the unamended sandy loam (23 d and 44±0.3%) (Wolf and Gan, 2018). The increased pyrene degradation in the clay soil may be potentially attributed to the greater abundance of

genera such as *Bacillus*, *Sphingomonas*, and *Kaistobacter* (43%, 10%, and 18%, respectively), as compared to the unamended sandy loam soil (17%, 2%, and 2%, respectively) (Figs. 3.2 and 3.3). Using weighted Principal Coordinates Analysis (PCoA), the differences between the soil microbial communities of the two soils can be seen by the two distinct clusters, regardless of surfactant amendment (Fig. S3.1).

Due to their broad occurrence in numerous contaminated sites and diverse metabolic pathways for xenobiotic degradation, genera of the *Sphingomonadaceae* family such as *Sphingomonas* are considered effective PAH-degrading soil microorganisms (Bastiaens et al., 2000; Leys et al., 2004; Leys et al., 2005; Lladó et al., 2015; Muangchinda et al., 2015). Bastida et al. (2016) evaluated PAH biodegradation in a semiarid petroleum-contaminated soil amended with compost and concluded that *Sphingomonadales* played a dominant role in the initial steps of PAH biodegradation, suggesting that *Sphingomonadales* were primarily responsible for the conversion of the aromatic hydrocarbons into *cis*-dihdyrodiol via dioxygenases as well as in the *meta*-cleavage pathway to catechol. *Kaistobacter* has only recently been linked with PAH degradation and their role in PAH biodegradation is still unclear; however, Li et al. (2017) utilized ^{13}C-phenanthrene and stable isotope probing in activated sludge and suggested that *Kaistobacter* was among the primary native microorganisms responsible for phenanthrene degradation. Wang et al. (2016a) utilized KEGG functional prediction and PICRUSt analysis of PAH-contaminated sediment and concluded that *Kaistobacter* contributed to the “Polycyclic aromatic hydrocarbon degradation” (rn00624; Table S3.1)

KEGG pathway, specifically, the process of metabolizing pyrene to 3,4-dihydroxy-phenanthrene.

3.3.3 Effect of Surfactant Amendment on Soil Microbial Community

Although *Firmicutes* and *Proteobacteria* phyla comprised a substantial proportion of the soil microbial community, the effects of Brij-35 and rhamnolipid surfactant application, particularly at the high rates, on soil microbial dynamics was apparent. In the pyrene-contaminated clay and sandy loam soils, the OTU numbers and Shannon diversity index were not different from the surfactant-amended treatments in both native and bioaugmented soil treatments, except for the addition of rhamnolipid at the high rate, which resulted in a dramatic decrease in OTU number and Shannon diversity index (Table 3.1). The Shannon diversity index of the Native+Pyrene clay and sandy loam soils decreased from 6.53 and 7.74 to 2.54 and 4.18, respectively, in the soils amended with rhamnolipid at the high rate.

Notably, the abundance of *Bacillus* (comprising the majority of *Firmicutes*) present after the 50-d incubation of the pyrene-contaminated clay soil, with or without bioaugmentation, was less than 2% when rhamnolipid biosurfactant was amended at the high rate (Fig. 3.2). In the native clay soil amended with rhamnolipid at the high rate, the most dominant genus was *Mycoplana* (67%). The ability of *Mycoplana* to effectively use the rhamnolipid biosurfactant as a carbon source likely resulted in a substantial decrease in the abundance of known PAH degraders, such as *Bacillus*, *Sphingomonas*, *Kaistobacter*, *Mycobacterium*, and *Rhodococcus* that were present in other soil treatments

(Fig. 3.2) (Al-Turki, 2009; Wang et al., 2016b). Although it has been shown that some species of *Mycoplana* such as *Mycoplana* sp. MWVMB2 were capable of effective PAH biodegradation in soils contaminated with phenanthrene up to 200 mg kg^{-1} with or without the use of surfactants such as Span 80, Tween 20, cetyl trimethyl ammonium bromide, sodium dodecyl sulfate, and Triton X-100, the *Mycoplana* sp. that was the dominant genus identified in this study was not able to mineralize pyrene after 50 d (Lakshmi et al., 2013; Wolf and Gan, 2018). In contrast, the native sandy loam soil amended with rhamnolipid at the high rate did not follow this trend and *Bacillus* comprised approximately 58% of the genera relative abundance (Fig. 3.3). It should be noted that at the end of the 50-d mineralization study, the native sandy loam amended with rhamnolipid at the medium or high rate was just commencing pyrene mineralization, suggesting that the rhamnolipid biosurfactant was potentially exhausted as a preferential carbon source by the soil microbes (Wolf and Gan, 2018). A study by Wang et al. (2016b) considered the influence of rhamnolipid biosurfactant, Tween 80, and sodium dodecyl benzenesulfonate at 5, 10, 50, 100, and 1,000 mg kg^{-1} on soil microbial dynamics and PAH biodegradation in aged PAH-contaminated soil. The researchers reapplied the surfactants after 42 d due to surfactant adsorption onto solid matrices as well as partial surfactant biodegradation based upon surfactant degradation results by Cserháti et al. (2002). Wang et al. (2016b) observed similar results to the sandy loam soil amended with rhamnolipid at the high rate in this study, with *Bacillus* abundance being three to five times as high as that of the other surfactant-amended PAH-contaminated soils.

Additionally, the native sandy loam soil amended with rhamnolipid biosurfactant at the medium rate as well as the bioaugmented sandy loam soil amended with rhamnolipid at the high rate contained a substantially greater relative abundance of *Pseudomonas* (22% and 31%) compared to the unamended and bioaugmented sandy loam soil (3% and 1%) (Fig. 3.3). *Pseudomonas* are known PAH-degrading soil microorganisms and have been shown to effectively degrade PAHs such as naphthalene, phenanthrene, pyrene, and anthracene in crude-oil contaminated soils. The PAH biodegradation by *Pseudomonas* was also shown to be enhanced in the presence of surfactants such as Tween 80, Triton 100, and rhamnolipid biosurfactant (Avramova et al., 2008; Bautista et al., 2009). Cébron et al. (2011) used DNA stable isotope probing in ^{13}C-phenanthrene-contaminated soil to assess the effects of ryegrass root exudates on PAH biodegradation and concluded that *Pseudomonas* sp. was one of the few soil microorganisms activated by the root exudates because the easily degradable carbon source addition provided by the root exudates favored the development of fast-growing r-strategists and copiotrophic soil microorganisms belonging to *Gammaproteobacteria* (Rentz et al., 2004). Rhamnolipid biosurfactant, which is composed of a β-hydroxy fatty acid connected by the carboxyl moiety to a rhamnose sugar molecule, has the potential to also be utilized by *Pseudomonas* as an easily degradable carbon source similar to root exudates (Randhawa and Rahman, 2014). Colores et al. (2000) investigated the effect of Witconol SN70 nonionic surfactant on the soil microbial community as well as the biodegradation of hexadecane and concluded that *Pseudomonas* populations in the soil could utilize both the surfactant and hexadecane for growth, which could have important

implications on remediation efforts. The effect of rhamnolipid at the high rate can also be seen using weighted PCoA, where the treatments in both soils clustered separate of the other unamended and surfactant-amended treatments (Fig. S3.2). Additionally, Brij-35 surfactant at the high rate resulted in a cluster separate from the unamended and surfactants amended at the low and medium rates, which were clustered together, indicating no substantial difference in the soil microbial communities (Fig. S3.2).

The amendment of either surfactant at various rates, except rhamnolipid at the high rate, to the sandy loam soil resulted in an increase in *Brevibacillus* abundance compared to the unamended native or bioaugmented sandy loam soil (Fig. 3.3). Wei et al. (2017) evaluated *Brevibacillus* in liquid culture spiked with pyrene and showed that *Brevibacillus* was able to degrade 57% of pyrene as the sole energy and carbon source; however, these findings have yet to be repeated in a soil system and warrant future research, as the increased abundance of *Brevibacillus* may be attributable to growth due to surfactant degradation in the sandy loam soil and may have important implications for surfactant-enhanced bioremediation. The addition of Brij-35 at the low rate to the native or bioaugmented sandy loam soil resulted in a dramatic increase in *Bacillus* (44% and 34%) compared to the unamended native or bioaugmented sandy loam (17% and 9%) (Fig. 3.3). The greater *Bacillus* abundance in the native sandy loam soil amended with Brij-35 at the low rate may have contributed to the increased pyrene mineralization compared to the native unamended sandy loam soil (Wolf and Gan, 2018).

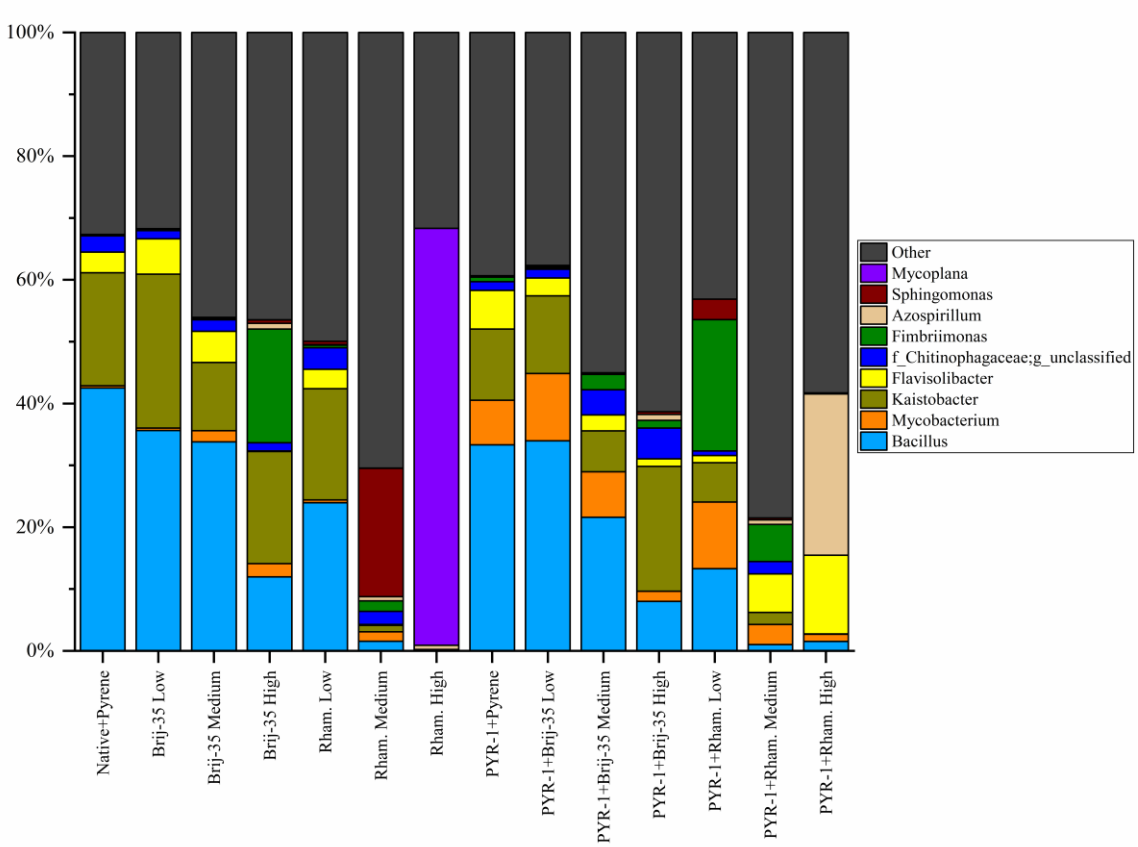

Figure 3.2. Biodiversity of select bacterial genera expressed as relative abundance (%) of the operational taxonomic units in the native or bioaugmented with *Mycobacterium vanbaalenii* PYR-1 clay soil amended with Brij-35 surfactant at 21.6, 216, and 2,160 μg g^{-1}or rhamnolipid biosurfactant at 14, 140, and 1,400 μg g^{-1} (low, medium, and high), respectively after a 50-d mineralization assay. For taxa that were defined as unclassified, the name of the higher taxon level was added for its taxon abbreviation (f = family; g = genus).

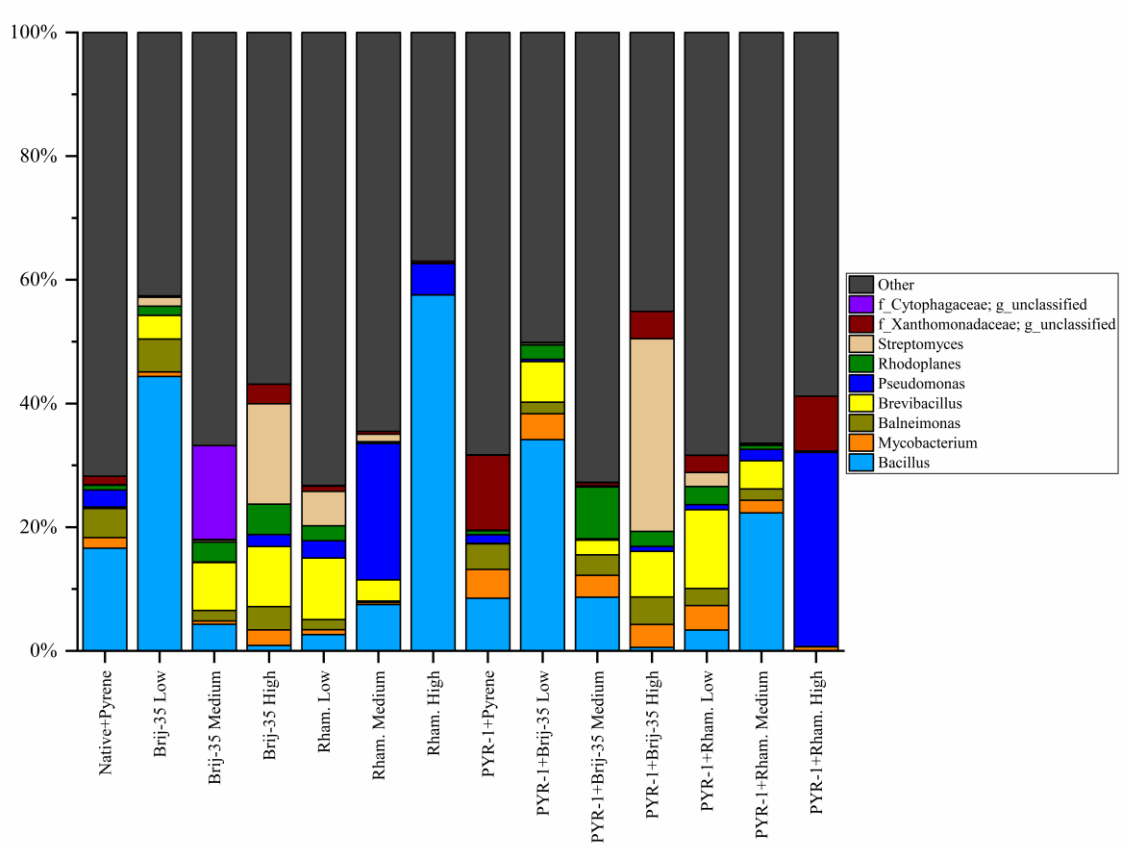

Figure 3.3 Biodiversity of select bacterial genera expressed as relative abundance (%) of the operational taxonomic units in the native or bioaugmented with *Mycobacterium vanbaalenii* PYR-1 sandy loam soil amended with Brij-35 surfactant at 21.6, 216, and 2,160 μg g^{-1} or rhamnolipid biosurfactant at 14, 140, and 1,400 μg g^{-1} (low, medium, and high), respectively, after a 50-d mineralization assay. For taxa that were defined as unclassified, the name of the higher taxon level was added for its taxon abbreviation (f = family; g = genus).

3.3.4 Effect of Bioaugmentation on Soil Microbial Community

The inoculation of PAH-degrading bacteria in a wide range of contaminated soils has been successfully implemented for the removal of priority PAH pollutants and continues to be a promising remediation method due to its low cost, lack of secondary pollution, and environmental safety (D'Annibale et al., 2005). The bioaugmentation of *M.*

vanbaalenii PYR-1, an isolate from an oil-contaminated estuary of the Gulf of Mexico, Redfish Bay, near Aransas Pass, has previously been shown to significantly enhance the initiation and rate of PAH mineralization in both PAH-contaminated soils compared to the native soils (Ghaley et al., 2013; Kim et al., 2015; Wolf and Gan, 2018). The *Mycobacterium* genus is often associated with HMW PAH biodegradation (Hunter et al., 2005). The effectiveness of the bioaugmentation of *M. vanbaalenii* PYR-1 on pyrene mineralization was evident in the unamended, Brij-35 amended at all rates, and rhamnolipid biosurfactant amendment at the low rate, in both soil systems (Wolf and Gan, 2018). *Mycobacterium vanbaalenii* PYR-1 has been studied in detail with respect to the molecular genetics of PAH degradation and has been shown to encode PAH ring-hydroxylating oxygenases *nidAB/nidA3B3*, which are utilized in the oxidation of HMW PAHs such as pyrene (Kim et al., 2006). Additionally, *M. vanbaalenii* PYR-1 has a complex and very hydrophobic rigid cell envelope that is enriched in mycolic acids (60% of the dry mass of the cell wall) and the mycolic acid wall monolayer acts as a biosurfactant (glycolipid) to enhance PAH solubility and biodegradation (Wick et al., 2003; Kim et al., 2015). Because of these characteristics, *M. vanbaalenii* PYR-1 is considered an excellent candidate for bioaugmentation in PAH-contaminated soils.

The 16S rRNA gene analysis was used in this study to determine if *M. vanbaalenii* PYR-1 (*Actinobacteria* phylum) was capable of successfully acclimating after the introduction in both soil systems with or without the addition of the surfactants at different rates. As shown in Figs. 3.2 and 3.3, the bioaugmentation of *M. vanbaalenii* PYR-1 in all clay and sandy loam soil treatments, except for rhamnolipid at the high rate

in both soils and rhamnolipid at the medium rate in the sandy loam soil, resulted in an increase in *Mycobacterium* compared to the native treatments. This increase in *Mycobacterium* was especially evident when comparing the Native+Pyrene and PYR-1+Pyrene treatments (Figs. S3.3 and S3.4). Additionally, LEfSE software was used to determine which soil microorganisms were differentially abundant between the bioaugmented and native soil systems. The abundance of *Mycobacterium* was found to be significantly greater in the bioaugmented soil treatments compared to the native soil treatments (Fig. 3.4).

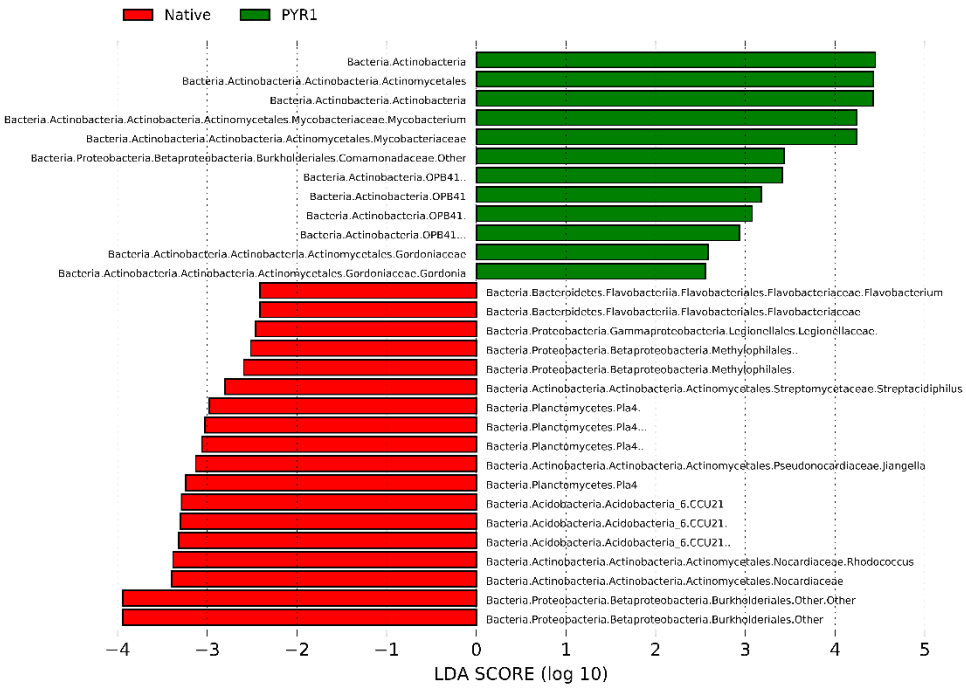

Figure 3.4. Analysis of differences in the microbiota between the bioaugmented clay and sandy loam soil treatments bioaugmented with *Mycobacterium vanbaalenii* PYR-1 and native clay and sandy loam soil treatments using LEfSe software (linear discriminant analysis [LDA] coupled with effect size measurements). Taxa enriched in the bioaugmented soil treatments are indicated with a positive LDA score (green) and taxa enriched in the native soil treatments have a negative score (red).

Functions of different OTUs and prediction of the functional composition of the metagenome in both soils was accomplished using the 16S rRNA gene data, Greengenes reference database, KEGG pathways, and PICRUSt to evaluate the effectiveness of surfactant addition as well as the bioaugmentation of *M. vanbaalenii* PYR-1 on pyrene mineralization. By analyzing soil functional genes, contributions of different bacteria involved in the biodegradation of PAHs were assessed. For instance, according to the "Xenobiotics biodegradation and metabolism" list on the KEGG website (http://ww.kegg.jp/kegg/), pyrene and phenanthrene can be degraded to 3,4-dihydroxy-phenanthrene via "Polycyclic aromatic hydrocarbon degradation", which can then be further metabolized into the TCA cycle via "Naphthalene degradation" (rn00626; Table S3.2) and "Benzoate degradation" (rn00362; Table S3.3) (Wang et al., 2016a). These KEGG pathways include numerous predicted PAH-degradation-related KOs, such as PAH oxygenase large subunit (K11943), PAH oxygenase small subunit (K11944), and extradiol dioxygenase (K11945) and determine whether bioaugmentation or surfactant addition had any significant effect on these genes and thus, PAH biodegradation in the two soils. Upon analysis of the "Polycyclic aromatic hydrocarbon degradation" KEGG pathway, *M. vanbaalenii* PYR-1 bioaugmentation in both soils significantly increased the KOs associated with the PAH biodegradation pathway compared to the native soils (Fig. 3.5). The same trend of bioaugmentation of *M. vanbaalenii* PYR-1 resulted in increased PAH-biodegradation-related KOs in the "Naphthalene degradation" and "Benzoate degradation" compared to the native soil systems (Fig. 3.5). Additionally, PICRUSt was utilized to assess which taxa contributed to the PAH-related KOs. For example, *M.*

vanbaalenii PYR-1 substantially contributed to PAH oxygenase large and small unit; however, other soil microbes in addition to *M. vanbaalenii* PYR-1 contributed to the increased extradiol dioxygenase in the bioaugmented soils compared to the native soils (data not shown). These results were in agreement with Niepceron et al. (2014) who evaluated phenanthrene biodegradation potential by assessing the PAH-ring hydroxylating dioxygenase (PAH-RHDα) sequences in PAH-contaminated soil and showed that PAH-RHDα was closely related to either *Burkholderia* or *Mycobacterium*. Wang et al. (2016a) also used PICRUSt to investigate the successions of bacterial communities in PAH-contaminated soils undergoing bioremediation and concluded that bacteria in the *Mycobacterium* genus contributed substantially to functional genes in all PAH-degradation pathways for metabolizing pyrene to the TCA cycle.

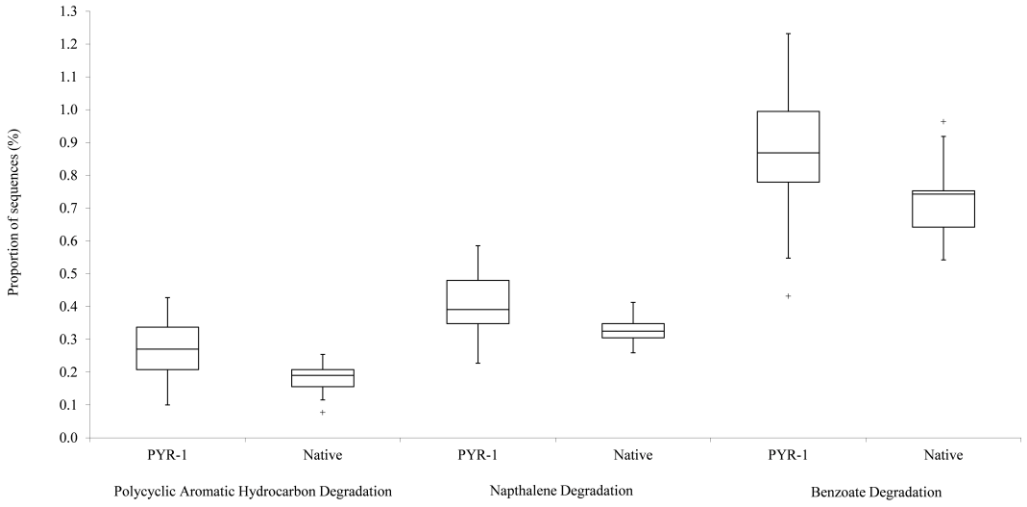

Figure 3.5. Box-and-whisker plot illustrating the "Polycyclic aromatic hydrocarbon degradation", "Naphthalene degradation", and "Benzoate degradation" KEGG Pathways predicted by phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) between *Mycobacterium vanbaalenii* PYR-1 bioaugmented and native soil treatments after a 50-d mineralization assay.

3.4 Conclusions

The soil microbial community shifts analyzed by 16S rRNA gene analysis identified the complex soil microbial population dynamics involved in pyrene biodegradation in this study. Surfactant addition shifted soil microbial populations in the pyrene-contaminated soils, especially in soils amended with rhamnolipid at the high rate. The shifts coincided with the reduced pyrene mineralization as well as the apparent disappearance of known PAH-degrading soil microbial populations. Although the mechanisms of surfactant-induced inhibition of PAH mineralization is still somewhat unclear, this study highlighted the importance of connecting soil microbial population dynamics with PAH biodegradation. The present study emphasized the need for studying the effect of surfactants and biosurfactants on complex microbial communities in PAH-contaminated soils to optimize surfactant-enhanced bioremediation operations. The bioaugmentation of *M. vanbaalenii* PYR-1 was shown to survive initial soil inoculation and result in reduction of the acclimation period and increased mineralization rate of pyrene in both soil systems. New technologies and methods that enhance survival of the bioaugmented microbes and increase PAH bioavailability using appropriate surfactants could provide an efficient and environmentally-friendly *in situ* bioremediation technique for PAH-contaminated soils.

3.5 References

Adrion, A.C., Nakamura, J., Shea, D., Aitken, M.D., 2016. Screening nonionic surfactants for enhanced biodegradation of polycyclic aromatic hydrocarbons remaining in soil after conventional biological treatment. *Environmental Science & Technology* 50, 3838–3845.

Al-Turki, A., 2009. Microbial polycyclic aromatic hydrocarbons degradation in soil. *Research Journal of Environmental Toxicology* 3, 1–8.

Avramova, T., Sotirova, A., Galabova, D., Karpenko, E., 2008. Effect of Triton X-100 and rhamnolipid PS-17 on the mineralization of phenanthrene by *Pseudomonas* sp. cells. *International Biodeterioration & Biodegradation* 62, 415–420.

Bastiaens, L., Springael, D., Wattiau, P., Harms, H., deWachter, R., Verachtert, H., Diels, L., 2000. Isolation of adherent polycyclic aromatic hydrocarbon (PAH)-degrading bacteria using PAH-sorbing carriers. *Applied and Environmental Microbiology* 66, 1834.

Bastida, F., Jehmlich, N., Lima, K., Morris, B.E.L., Richnow, H.H., Hernández, T., von Bergen, M., García, C., 2016. The ecological and physiological responses of the microbial community from a semiarid soil to hydrocarbon contamination and its bioremediation using compost amendment. *Journal of Proteomics* 135, 162–169.

Bautista, L.F., Sanz, R., Molina, M.C., González, N., Sánchez, D., 2009. Effect of different non-ionic surfactants on the biodegradation of PAHs by diverse aerobic bacteria. *International Biodeterioration & Biodegradation* 63, 913–922.

Bezza, F.A., Chirwa, E.M.N., 2017. The role of lipopeptide biosurfactant on microbial remediation of aged polycyclic aromatic hydrocarbons (PAHs)-contaminated soil. *Chemical Engineering Journal* 309, 563–576.

Brinch, U.C., Ekelund, F., Jacobsen, C.S., 2002. Method for spiking soil samples with organic compounds. *Applied and Environmental Microbiology* 68, 1808.

Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis, T.Z., Andersen, G.L., Knight, R., 2009. PyNAST: A flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26, 266–267.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters,

W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* 7, 335.

Cébron, A., Louvel, B., Faure, P., France-Lanord, C., Chen, Y., Murrell, J.C., Leyval, C., 2011. Root exudates modify bacterial diversity of phenanthrene degraders in PAH-polluted soil but not phenanthrene degradation rates. *Environmental Microbiology* 13, 722–736.

Chadhain, S.M.N., Norman, R.S., Pesce, K.V., Kukor, J.J., Zylstra, G.J., 2006. Microbial dioxygenase gene population shifts during polycyclic aromatic hydrocarbon biodegradation. *Applied and Environmental Microbiology* 72, 4078–4087.

Colores, G.M., Macur, R.E., Ward, D.M., Inskeep, W.P., 2000. Molecular analysis of surfactant-driven microbial population shifts in hydrocarbon-contaminated soil. *Applied and Environmental Microbiology* 66, 2959.

Cserháti, T., Forgács, E., Oros, G., 2002. Biological activity and environmental impact of anionic surfactants. *Environment International* 28, 337–348.

D'Annibale, A., Ricci, M., Leonardi, V., Quaratino, D., Mincione, E., Petruccioli, M., 2005. Degradation of aromatic hydrocarbons by white-rot fungi in a historically contaminated soil. *Biotechnology and Bioengineering* 90, 723–731.

Das, K., Mukherjee, A.K., 2007. Crude petroleum-oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East India. *Bioresource Technology* 98, 1339–1345.

Delgado-Baquerizo, M., Oliverio, A.M., Brewer, T.E., Benavent-González, A., Eldridge, D.J., Bardgett, R.D., Maestre, F.T., Singh, B.K., Fierer, N., 2018. A global atlas of the dominant bacteria found in soil. *Science* 359, 320–325.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L., 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology* 72, 5069.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461.

Ghaly, A., Yusran, A., Dave, D., 2013. Effects of biostimulation and bioaugmentation on the degradation of pyrene in soil. *Journal of Bioremediation & Biodegradation* S 5.

Ghosh, I., Mukherji, S., 2016. Diverse effect of surfactants on pyrene biodegradation by a *Pseudomonas* strain utilizing pyrene by cell surface hydrophobicity induction. *International Biodeterioration & Biodegradation* 108, 67–75.

Goecks, J., Nekrutenko, A., Taylor, J., The Galaxy Team, 2010. Galaxy: A comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biology* 11, R86.

Hunter, D.R., Ekunwe, I.S., Dodor, E.D., Hwang, H.-M., Ekunwe, L., 2005. *Bacillus subtilis* is a potential degrader of pyrene and benzo[a]pyrene. *International Journal of Environmental Research and Public Health* 2.

Ijah, U.J.J., Antai, S.P., 2003. Removal of Nigerian light crude oil in soil over a 12-month period. *International Biodeterioration & Biodegradation* 51, 93–99.

Janssen, P.H., 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Applied and Environmental Microbiology* 72, 1719.

Kim, S.-J., Kweon, O., Freeman, J.P., Jones, R.C., Adjei, M.D., Jhoo, J.-W., Edmondson, R.D., Cerniglia, C.E., 2006. Molecular cloning and expression of genes encoding a novel dioxygenase involved in low- and high-molecular-weight polycyclic aromatic hydrocarbon degradation in *Mycobacterium vanbaalenii* PYR-1. *Applied and Environmental Microbiology* 72, 1045.

Kim, S.-J., Kweon, O., Sutherland, J.B., Kim, H.-L., Jones, R.C., Burback, B.L., Graves, S.W., Psurny, E., Cerniglia, C.E., 2015. Dynamic response of *Mycobacterium vanbaalenii* PYR-1 to BP Deepwater Horizon crude oil. *Applied and Environmental Microbiology* 81, 4263.

Kuppusamy, S., Thavamani, P., Venkateswarlu, K., Lee, Y.B., Naidu, R., Megharaj, M., 2017. Remediation approaches for polycyclic aromatic hydrocarbons (PAHs) contaminated soils: Technological constraints, emerging trends and future directions. *Chemosphere* 168, 944–968.

Lakshmi, M., Anandaraj, V.P., Velan, M., 2013. Bioremediation of phenanthrene by *Mycoplana* sp. MVMB2 isolated from contaminated soil. *Clean Soil, Air, Water* 41, 86–93.

Langille, M.G.I., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A., Clemente, J.C., Burkepile, D.E., Vega Thurber, R.L., Knight, R., Beiko, R.G., Huttenhower, C., 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology* 31, 814.

Leys, N.M., Bastiaens, L., Verstraete, W., Springael, D., 2005. Influence of the carbon/nitrogen/phosphorus ratio on polycyclic aromatic hydrocarbon degradation by *Mycobacterium* and *Sphingomonas* in soil. *Applied Microbiology and Biotechnology* 66, 726–736.

Leys, N.M.E.J., Ryngaert, A., Bastiaens, L., Verstraete, W., Top, E.M., Springael, D., 2004. Occurrence and phylogenetic diversity of *Sphingomonas* strains in soils contaminated with polycyclic aromatic hydrocarbons. *Applied and Environmental Microbiology* 70, 1944.

Li, J., Zhang, D., Song, M., Jiang, L., Wang, Y., Luo, C., Zhang, G., 2017. Novel bacteria capable of degrading phenanthrene in activated sludge revealed by stable-isotope probing coupled with high-throughput sequencing. *Biodegradation* 28, 423–436.

Liu, Y., Hu, Y., Long, S., Liu, L., Liu, X., 2017. Analysis of the effectiveness of urban land-use-change models based on the measurement of spatio-temporal, dynamic urban growth: A cellular automata case study. *Sustainability* 9.

Lladó, S., Covino, S., Solanas, A.M., Petruccioli, M., D'annibale, A., Viñas, M., 2015. Pyrosequencing reveals the effect of mobilizing agents and lignocellulosic substrate amendment on microbial community composition in a real industrial PAH-polluted soil. *Journal of Hazardous Materials* 283, 35–43.

Lozoupone, C., Knight, R., 2005. UniFrac: A new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology* 73, 494–504.

Lu, L., Zheng, Z., Champagne, B., Yang, X., Wu, W., 2019. Self-regularized nonlinear diffusion algorithm based on levenberg gradient descent. *Signal Processing* 163, 107–114.

McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A., Andersen, G.L., Knight, R., Hugenholtz, P., 2011. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *The ISME Journal* 6, 610.

McMurdie, P.J., Holmes, S., 2014. Shiny-phyloseq: Web application for interactive microbiome analysis with provenance tracking. *Bioinformatics* 31, 282–283.

Muangchinda, C., Chavanich, S., Viyakarn, V., Watanabe, K., Imura, S., Vangnai, A.S., Pinyakong, O., 2015. Abundance and diversity of functional genes involved in the degradation of aromatic hydrocarbons in Antarctic soils and sediments around Syowa Station. *Environmental Science and Pollution Research* 22, 4725–4735.

Mukherjee, A., Chettri, B., Langpoklakpam, J.S., Basak, P., Prasad, A., Mukherjee, A.K., Bhattacharyya, M., Singh, A.K., Chattopadhyay, D., 2017. Bioinformatic approaches including predictive metagenomic profiling reveal characteristics of bacterial response to petroleum hydrocarbon contamination in diverse environments. *Scientific Reports* 7, 1108.

Niepceron, M., Beguet, J., Portet-Koltalo, F., Martin-Laurent, F., Quillet, L., Bodilis, J., 2014. Low impact of phenanthrene dissipation on the bacterial community in grassland soil. *Environmental Science and Pollution Research* 21, 2977–2987.

Parks, D.H., Tyson, G.W., Hugenholtz, P., Beiko, R.G., 2014. STAMP: Statistical analysis of taxonomic and functional profiles. *Bioinformatics* 30, 3123–3124.

Patowary, K., Saikia, R.R., Kalita, M.C., Deka, S., 2015. Degradation of polyaromatic hydrocarbons employing biosurfactant-producing *Bacillus pumilus* KS2. *Annals of Microbiology* 65, 225–234.

Randhawa, K.K., Rahman, P.K.S.M., 2014. Rhamnolipid biosurfactants-past, present, and future scenario of global market. *Frontiers in Microbiology* 5, 454–454.

Reeder, J., Knight, R., 2010. Rapidly denoising pyrosequencing amplicon reads by exploiting rank-abundance distributions. *Nature Methods* 7, 668.

Rentz, J.A., Alvarez, P.J.J., Schnoor, J.L., 2004. Repression of *Pseudomonas putida* phenanthrene-degrading activity by plant root extracts and exudates. *Environmental Microbiology* 6, 574–583.

Röling, W.F.M., Milner, M.G., Jones, D.M., Lee, K., Daniel, F., Swannell, R.J.P., Head, I.M., 2002. Robust hydrocarbon degradation and dynamics of bacterial communities during nutrient-enhanced oil spill bioremediation. *Applied and Environmental Microbiology* 68, 5537.

Schumacher, B.A., 2002. Methods for the determination of total organic carbon (TOC) in soils and sediments. Ecological Risk Assessment Support Center 1–23.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., Huttenhower, C., 2011. Metagenomic biomarker discovery and explanation. *Genome Biology* 12, R60.

Singh, R., Glick, B.R., Rathore, D., 2018. Biosurfactants as a biological tool to increase micronutrient availability in soil: A review. *Pedosphere* 28, 170–189.

Singleton, D.R., Adrion, A.C., Aitken, M.D., 2016. Surfactant-induced bacterial community changes correlated with increased polycyclic aromatic hydrocarbon

degradation in contaminated soil. *Applied Microbiology and Biotechnology* 100, 10165–10177.

Sun, G.-D., Xu, Y., Liu, Y., Liu, Z.-P., 2014. Microbial community dynamics of soil mesocosms using *Orychophragmus violaceus* combined with *Rhodococcus ruber* Em1 for bioremediation of highly PAH-contaminated soil. *Applied Microbiology and Biotechnology* 98, 10243–10253.

Toledo, F.L., Calvo, C., Rodelas, B., González-López, J., 2006. Selection and identification of bacteria isolated from waste crude oil with polycyclic aromatic hydrocarbons removal capacities. *Systematic and Applied Microbiology* 29, 244–252.

Tong, R., Yang, X., Su, H., Pan, Y., Zhang, Q., Wang, J., Long, M., 2018. Levels, sources and probabilistic health risks of polycyclic aromatic hydrocarbons in the agricultural soils from sites neighboring suburban industries in Shanghai. *Science of The Total Environment* 616–617, 1365–1373.

United States Environmental Protection Agency, 2007. Treatment Technologies for Site Cleanup: Annual Status Report (Twelfth Edition) Available at: https://www.epa.gov/remedytech/treatment-technologies-site-cleanup-annual-status-report-twelfth-edition (Accessed 10 February 2019).

Wang, H., Wang, B., Dong, W., Hu, X., 2016b. Co-acclimation of bacterial communities under stresses of hydrocarbons with different structures. *Scientific Reports* 6, 34588.

Wang, L., Li, F., Zhan, Y., Zhu, L., 2016a. Shifts in microbial community structure during *in situ* surfactant-enhanced bioremediation of polycyclic aromatic hydrocarbon-contaminated soil. *Environmental Science and Pollution Research* 23, 14451–14461.

Wang, Y., Xu, J., Shan, J., Ma, Y., Ji, R., 2017. Fate of phenanthrene and mineralization of its non-extractable residues in an oxic soil. *Environmental Pollution* 224, 377–383.

Wei, K., Yin, H., Peng, H., Liu, Z., Lu, G., Dang, Z., 2017. Characteristics and proteomic analysis of pyrene degradation by *Brevibacillus brevis* in liquid medium. *Chemosphere* 178, 80–87.

Wick, L.Y., Pelz, O., Bernasconi, S.M., Andersen, N., Harms, H., 2003. Influence of the growth substrate on ester-linked phospho- and glycolipid fatty acids of PAH-degrading *Mycobacterium* sp. LB501T. *Environmental Microbiology* 5, 672–680.

Wolf, D.C., Gan, J., 2018. Influence of rhamnolipid biosurfactant and Brij-35 synthetic surfactant on ^{14}C-pyrene mineralization in soil. *Environmental Pollution* 243, 1846–1853.

Wu, M., Ye, X., Chen, K., Li, W., Yuan, J., Jiang, X., 2017. Bacterial community shift and hydrocarbon transformation during bioremediation of short-term petroleum-contaminated soil. *Environmental Pollution* 223, 657–664.

3.6 Supporting Information

Table S3.1. Polycyclic aromatic hydrocarbon degradation pathway (rn00624) KEGG Orthologs.

KEGG Ortholog ID No.	Name
K00448	Protocatechuate 3,4-dioxygenase, alpha subunit
K00449	Protocatechuate 3,4-dioxygenase, beta subunit
K00480	Salicylate hydroxylase
K04100	Protocatechuate 4,5-dioxygenase, alpha chain
K04101	Protocatechuate 4,5-dioxygenase, beta chain
K04102	4,5-dihydroxyphthalate decarboxylase
K11943	PAH dioxygenase large subunit
K11944	PAH dioxygenase small subunit
K11945	Extradiol dioxygenase
K11946	Hydratase-aldolase
K11947	Aldehyde dehydrogenase
K11948	1-hydroxy-2-naphthoate dioxygenase
K11949	4-(2-carboxyphenyl)-2-oxobut-3-enoate aldolase
K14578	Naphthalene 1,2-dioxygenase ferredoxin component
K14579	Naphthalene 1,2-dioxygenase subunit alpha
K14580	Naphthalene 1,2-dioxygenase subunit beta
K14581	Naphthalene 1,2-dioxygenase ferredoxin reductase component
K14582	Cis-1,2-dihydro-1,2-dihydroxynaphthalene
K14599	Dibenzofuran dioxygenase subunit alpha
K14600	Dibenzofuran dioxygenase subunit beta
K14601	1,1a-dihydroxy-1-hydro-9-fluorenone dehydrogenase
K14602	2'-carboxy-2,3-dihydroxybiphenyl 1,2-dioxygenase large subunit
K14603	2'-carboxy-2,3-dihydroxybiphenyl 1,2-dioxygenase small subunit and ferredoxin fusion protein
K14604	2-hydroxy-6-oxo-6-(2'-carboxyphenyl)-hexa-2,4-dienoate hydrolase
K16269	Cis-1,2-dihydrobenzene-1,2-diol dehydrogenase
K18067	Phthalate 4,5-cis-dihydrodiol dehydrogenase
K18068	Phthalate 4,5-dioxygenase
K18069	Phthalate 4,5-dioxygenase reductase component
K18074	Terephthalate 1,2-dioxygenase oxygenase component alpha subunit
K18075	Terephthalate 1,2-dioxygenase oxygenase component beta subunit
K18076	1,2-dihydroxy-3,5-cyclohexadiene-1,4-dicarboxylate dehydrogenase
K15077	Terephthalate 1,2-dioxygenase reductase component
K18251	Phthalate 3,4-dioxygenase subunit alpha
K18252	Phthalate 3,4-dioxygenase subunit beta
K18253	Phthalate 3,4-dioxygenase ferredoxin component

K18254	Phthalate 3,4-dioxygenase ferredoxin reductase component
K18255	Phthalate 3,4-cis-dihydrodiol dehydrogenase
K18256	3,4-dihydroxyphthalate decarboxylase
K18257	Cis-3,4-dihydrophenanthrene-3,4-diol dehydrogenase
K18275	2-formylbenzoate dehydrogenase
K19065	3-hydroxybenzoate 4-monooxygenase

Table S3.2. Naphthalene biodegradation pathway (rn00626) KEGG Orthologs.

KEGG Ortholog ID No.	Name
K00001	Alcohol dehydrogenase
K00121	S-(hydroxymethyl)glutathione dehydrogenase / alcohol dehydrogenase
K00152	Salicylaldehyde dehydrogenase
K00480	Salicylate hydroxylase
K01670	Naphthyl-2-methylsuccinate synthase alpha subunit
K04072	Acetaldehyde dehydrogenase / alcohol dehydrogenase
K13953	Alcohol dehydrogenase, propanol-preferring
K13954	Alcohol dehydrogenase
K14578	Naphthalene 1,2-dioxygenase ferredoxin component
K14579	Naphthalene 1,2-dioxygenase subunit alpha
K14580	Naphthalene 1,2-dioxygenase subunit beta
K14581	Naphthalene 1,2-dioxygenase ferredoxin reductase component
K14582	Cis-1,2-dihydro-1,2-dihydroxynaphthalene
K14583	1,2-dihydroxynaphthalene dioxygenase
K14584	2-hydroxychromene-2-carboxylate isomerase
K14585	Trans-o-hydroxybenzylidenepyruvate hydratase-aldolase
K14586	2-naphthoate monooxygenase
K15567	Naphthyl-2-methylsuccinate synthase beta subunit
K15568	Naphthyl-2-methylsuccinate synthase gamma subunit
K15569	Naphthyl-2-methylsuccinate CoA transferase subunit
K15570	Naphthyl-2-methylsuccinate CoA transferase subunit
K15571	Naphthyl-2-methylsuccinyl-CoA dehydrogenase
K15572	Naphthyl-2-hydroxymethylsuccinyl-CoA hydratase
K15573	Naphthyl-2-hydroxymethylsuccinyl-CoA dehydrogenase BnsC subunit
K15574	Naphthyl-2-oxomethyl-succinyl-CoA thiolase subunit
K15575	Naphthyl-2-oxomethyl-succinyl-CoA thiolase subunit
K18242	Salicylate 5-hydroxylase large subunit
K18243	Salicylate 5-hydroxylase small subunit
K19958	Naphthyl-2-hydroxymethylsuccinyl-CoA dehydrogenase BnsD subunit

Table S3.3. Benzoate degradation pathway (rn00362) KEGG Orthologs.

KEGG Ortholog ID No.	Name
K00074	3-hydroxybutyryl-coa dehydrogenase
K00217	Maleylacetate reductase
K00252	Glutaryl-coa dehydrogenase
K00446	Catechol 2,3-dioxygenase
K00448	Protocatechuate 3,4-dioxygenase, alpha subunit
K00449	Protocatechuate 3,4-dioxygenase, beta subunit
K00626	Acetyl-coa C-acetyltransferase
K0632	Acetyl-coa acyltransferase
K01031	3-oxoadipate coa-transferase, alpha subunit
K01032	3-oxoadipate coa-transferase, beta subunit
K01055	3-oxoadipate enol-lactonase
K01075	4-hydroxybenzoyl-coa thioesterase
K01607	4-carboxymuconolactone decarboxylase
K01615	Glutaconyl-coa decarboxylase
K01617	2-oxo-3-hexenedioate decarboxylase
K01666	4-hydroxy 2-oxovalerate aldolase
K01692	Enoyl-coa hydratase
K01782	3-hydroxyacyl-coa dehydrogenase
K01821	4-oxalocrotonate tautomerase
K01825	3-hydroxyacyl-coa dehydrogenase
K01856	Muconate cycloisomerase
K01857	3-carboxy-cis,cis-muconate cycloisomerase
K02554	2-keto-4-pentenoate hydratase
K03268	Benzene/toluene/chlorobenzene dioxygenase subunit alpha
K03381	Catechol 1,2-dioxygenase
K03464	Muconolactone D-isomerase
K04073	Acetaldehyde dehydrogenase
K04098	Hydroxyquinol 1,2-dioxygenase
K04100	Protocatechuate 4,5-dioxygenase, alpha chain
K04101	Protocatechuate 4,5-dioxygenase, beta chain
K04105	4-hydroxybenzoate-coa ligase
K04107	4-hydroxybenzoyl-coa reductase subunit gamma
K04108	4-hydroxybenzoyl-coa reductase subunit alpha
K04109	4-hydroxybenzoyl-coa reductase subunit beta
K04110	Benzoate-coa ligase
K04112	Benzoyl-coa reductase subunit C
K04113	Benzoyl-coa reductase subunit B
K04114	Benzoyl-coa reductase subunit A
K04115	Benzoyl-coa reductase subunit D
K04116	Cyclohexanecarboxylate-coa ligase

K04117	Cyclohexanecarboxyl-coa dehydrogenase
K04118	Pimeloyl-coa dehydrogenase
K05549	Benzoate/toluate 1,2-dioxygenase subunit alpha
K05550	Benzoate/toluate 1,2-dioxygenase subunit beta
K05783	Dihydroxycyclohexadiene carboxylate dehydrogenase
K05784	Benzoate/toluate 1,2-dioxygenase reductase component
K07104	Catechol 2,3-dioxygenase
K07516	3-hydroxyacyl-coa dehydrogenase
K07534	Cyclohex-1-ene-1-carboxyl-coa hydratase
K07535	2-hydroxycyclohexanecarboxyl-coa dehydrogenase
K07536	2-ketocyclohexanecarboxyl-coa hydrolase
K07537	Cyclohexa-1,5-dienecarbonyl-coa hydratase
K07538	6-hydroxycyclohex-1-ene-1-carbonyl-coa dehydrogenase
K07539	6-oxocyclohex-1-ene-carbonyl-coa hydrolase
K07823	3-oxoadipyl-coa thiolase
K07824	Benzoate 4-monooxygenase
K10216	2-hydroxymuconate-semialdehyde hydrolase
K10217	Aminomuconate-semialdehyde
K10218	4-hydroxy-4-methyl-2-oxoglutarate aldolase
K10219	2-hydroxy-4-carboxymuconate semialdehyde hemiacetal dehydrogenase
K10220	4-oxalmesaconate hydratase
K10221	2-pyrone-4,6-dicarboxylate lactonase
K10621	2,3-dihydroxy-p-cumate/2,3-dihydroxybenzoate 3,4-dioxygenase
K10622	HCOMODA/2-hydroxy-3-carboxy-muconic semialdehyde decarboxylase
K13767	Enoyl-coa hydratase
K14333	2,3-dihydroxybenzoate decarboxylase
K14334	Carboxy-cis,cis-muconate cyclase
K14727	3-oxoadipate enol-lactonase / 4-carboxymuconolactone decarboxylase
K15511	Benzoyl-coa 2,3-epoxidase subunit A
K15512	Benzoyl-coa 2,3-epoxidase subunit B
K15513	Benzoyl-coa-dihydrodiol lyase
K5514	3,4-dehydroadipyl-coa semialdehyde dehydrogenase
K15766	2-aminobenzenesulfonate 2,3-dioxygenase subunit alpha
K15767	2-aminobenzenesulfonate 2,3-dioxygenase subunit beta
K16173	Glutaryl-coa dehydrogenase (non-decarboxylating)
K16242	Phenol hydroxylase P3 protein
K16243	Phenol hydroxylase P1 protein
K16244	Phenol hydroxylase P2 protein
K16245	Phenol hydroxylase P4 protein
K16246	Phenol hydroxylase P5 protein
K16249	Phenol hydroxylase P0 protein

K16268	Benzene/toluene/chlorobenzene dioxygenase subunit beta
K16269	Cis-1,2-dihydrobenzene-1,2-diol dehydrogenase
K16514	4-oxalomesaconate tautomerase
K16515	4-oxalomesaconate hydratase
K18089	Benzene/toluene/chlorobenzene dioxygenase ferredoxin component
K18090	Benzene/toluene/chlorobenzene dioxygenase ferredoxin reductase component
K18364	2-oxopent-4-enoate/cis-2-oxohex-4-enoate hydratase
K18365	4-hydroxy-2-oxovalerate/4-hydroxy-2-oxohexanoate aldolase
K18366	Acetaldehyde/propanal dehydrogenase
K19065	3-hydroxybenzoate 4-monooxygenase
K19066	Cyclohex-1-ene-1-carbonyl-coa dehydrogenase
K19067	Cyclohexane-1-carbonyl-coa dehydrogenase
K19515	Benzoyl-coa reductase subunit bamb
K19516	Benzoyl-coa reductase subunit bamc
K20458	3-hydroxybenzoate/4-hydroxybenzoate---coa ligase
K20941	Gamma-resorcylate decarboxylase
K20942	Resorcinol 4-hydroxylase (FADH2)
K20943	Resorcinol 4-hydroxylase (NADPH)
K20944	Resorcinol 4-hydroxylase (NADH)
K22270	3-hydroxybenzoate 6-monooxygenase
K22553	4-methoxybenzoate monooxygenase

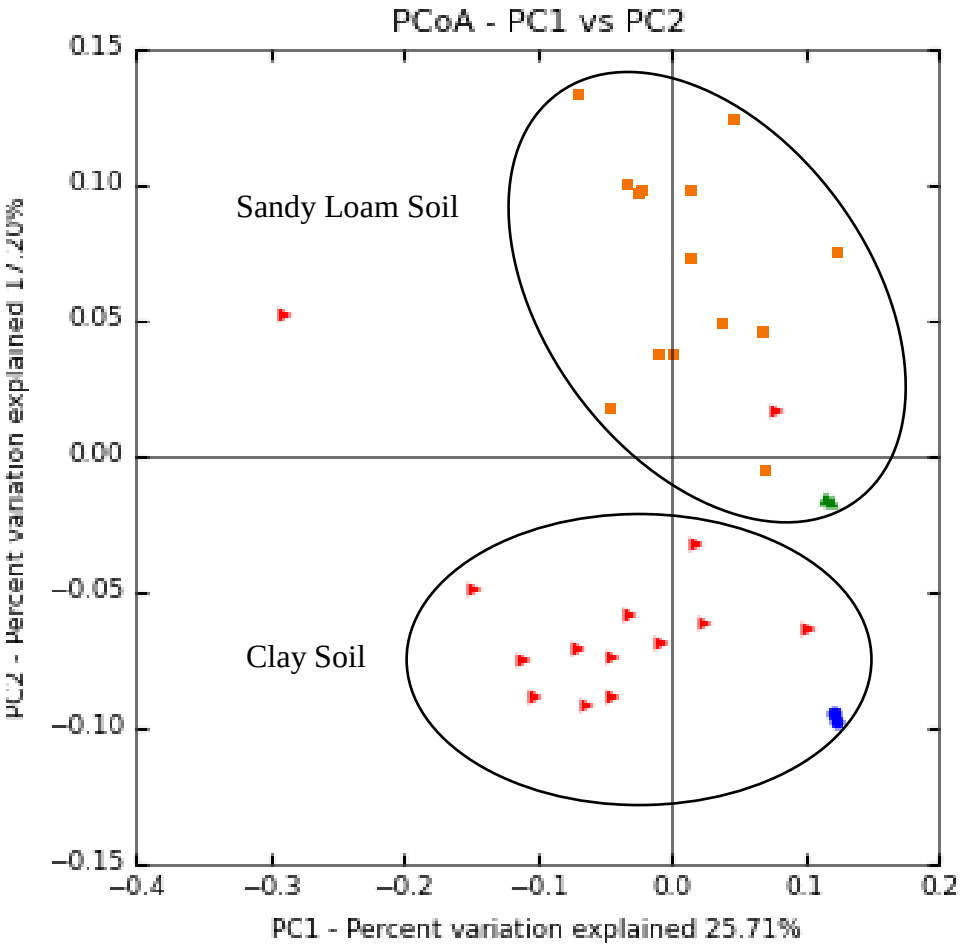

Figure S3.1. Weighted UniFrac principal coordinate analysis plot comparing sample distribution and clustering belonging to the two different soil systems after a 50-d mineralization assay (red = clay soil, blue = untreated clay soil, orange = sandy loam soil, green = untreated sandy loam soil).

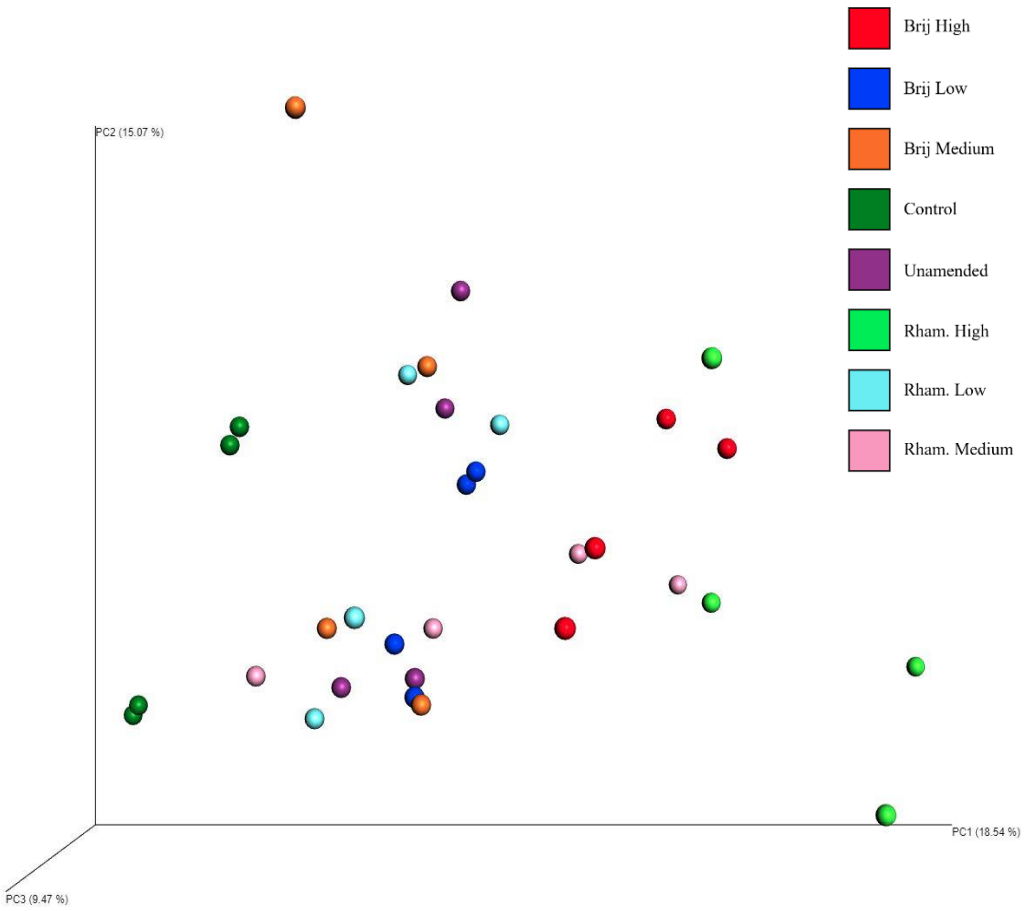

Figure S3.2. Weighted UniFrac 3D principal coordinate analysis plot comparing sample distribution and clustering of soil treatments amended with Brij-35 surfactant at 21.6, 216, and 2,160 µg g^{-1} or rhamnolipid biosurfactant at 14, 140, and 1,400 µg g^{-1} (low, medium, and high), respectively, after a 50-d mineralization assay.

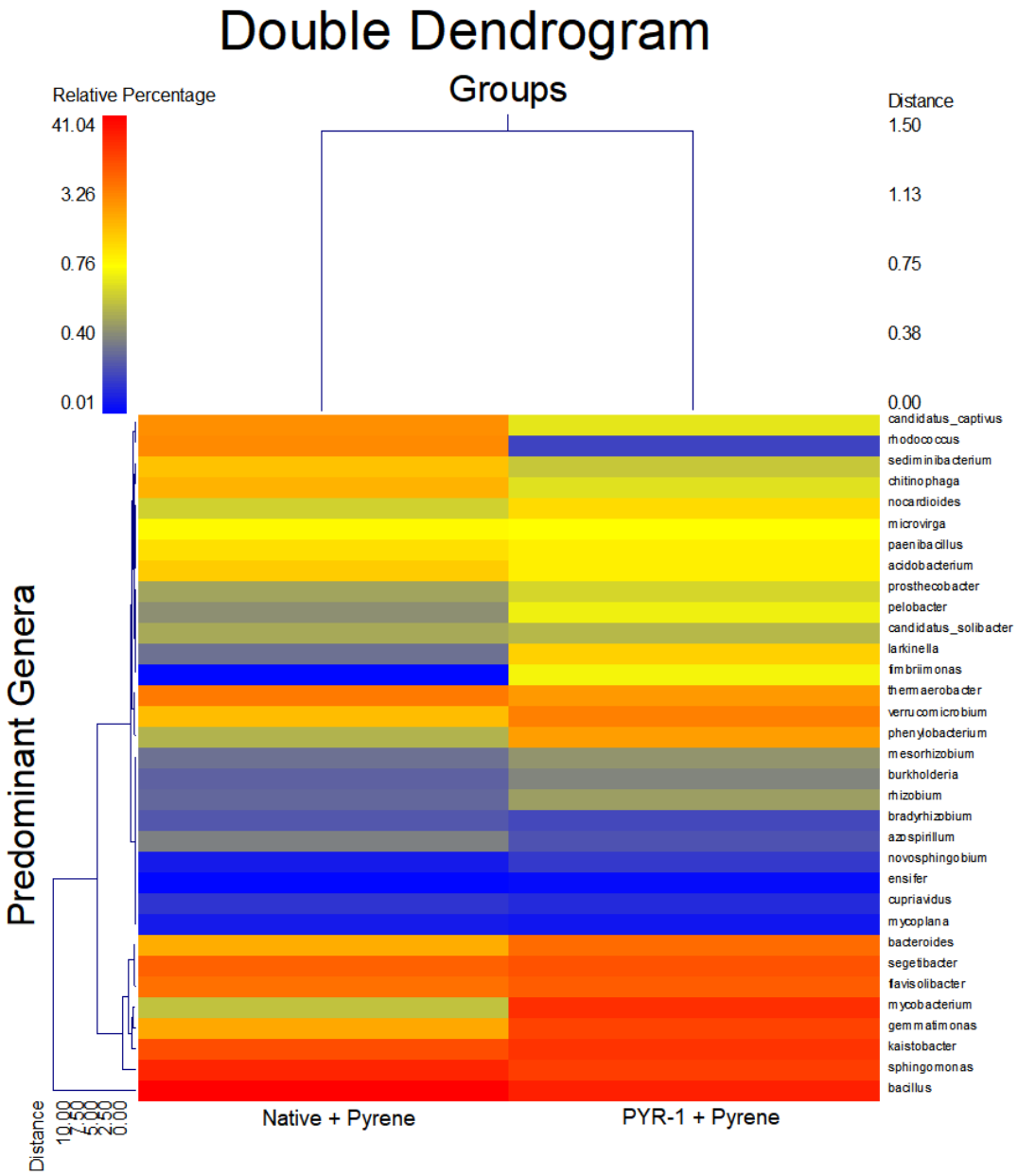

Figure S3.3. Dual hierarchical clustering dendrogram evaluation of the predominant genera among the unamended native and *Mycobacterium vanbaalenii* PYR-1 bioaugmented clay soil treatments. The heat map plot depicts the relative abundance of each genus within each sample. On top of the figure, the distance of the samples based on weighted pair linkage and Manhattan distance methods with no scaling is shown, along with a distance score.

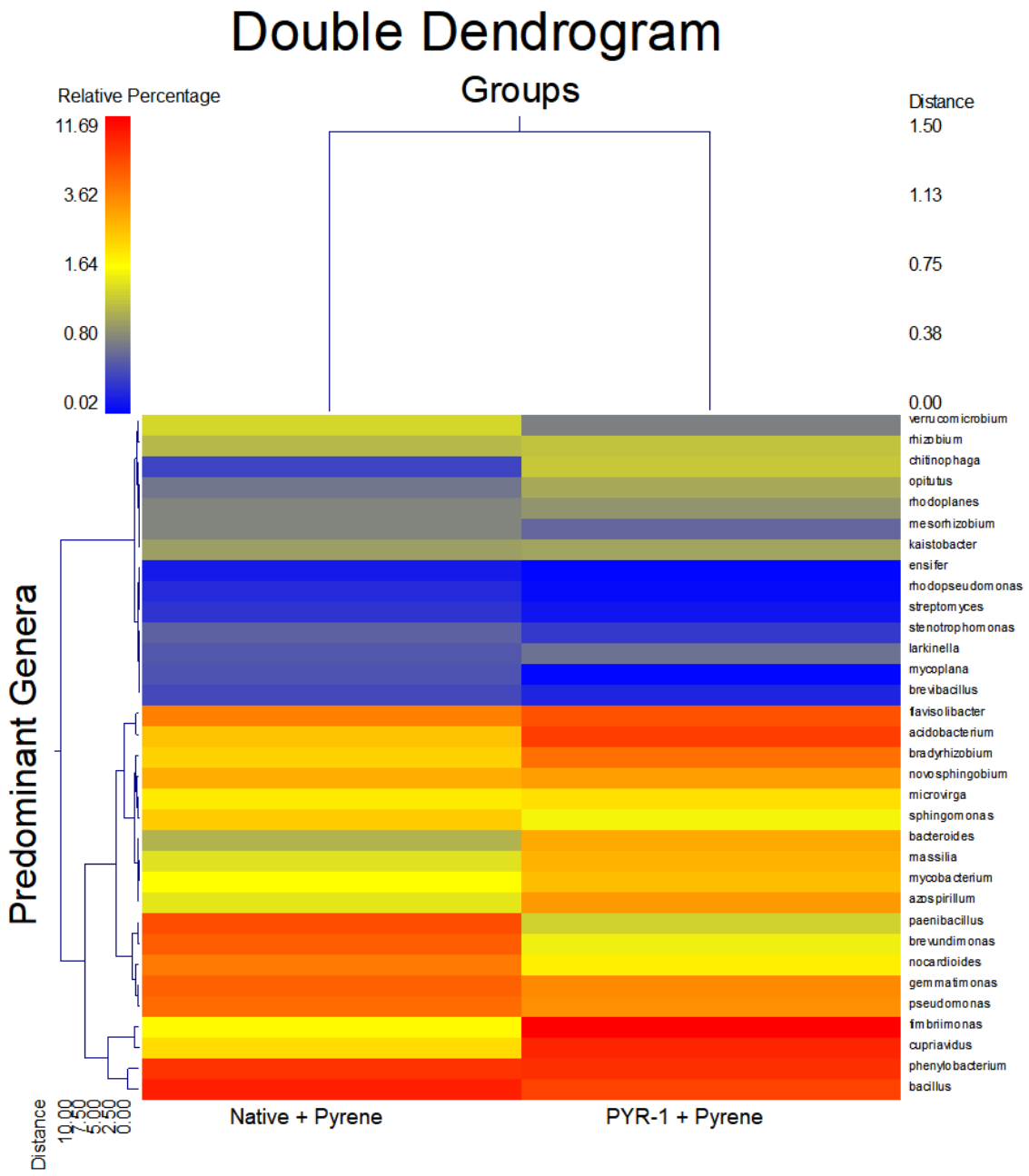

Figure S3.4. Dual hierarchical clustering dendrogram evaluation of the predominant genera among the unamended native and *Mycobacterium vanbaalenii* PYR-1 bioaugmented sandy loam soil treatments. The heat map plot depicts the relative abundance of each genus within each sample. On top of the figure, the distance of the samples based on weighted pair linkage and Manhattan distance methods with no scaling is shown, along with a distance score.

Chapter 4 Bioremediation of Polycyclic Aromatic Hydrocarbons from an Outdoor Shooting Range Soil Using Microbial-Plant Combinations

4.1 Introduction

Target shooting is an increasingly popular recreational sport with over approximately 100,000 shooting ranges worldwide (Perroy et al., 2014). In the United States (U.S.), there are an estimated 12,000 shooting ranges, consisting of 9,000 civilian and 3,000 military shooting ranges (Wan et al., 2013). Over 18 million adults participated in any type of clay target shooting (i.e., sporting clays, skeet, or trap) on these civilian shooting ranges in 2014, which was a 3.6% increase compared to participants in 2012 (U.S. National Shooting Sports Foundation, 2015). Accordingly, there have been vast numbers of clay targets used for these outdoor clay target shooting activities. Baer et al. (1995) determined that clay target use in the U.S. since 1970 has averaged approximately 560 million targets/year. Until recently, clay targets were composed of approximately 67-70% clay or dolomitic limestone, 30-32% coal tar or petroleum pitch used as a binding agent, and fluorescent paint (Rodríguez-Seijo et al., 2017). The coal tar or petroleum pitch binding agent is a large source of PAHs with concentrations up to 3,000-40,000 mg/kg clay target (Lobb, 2006). Each clay target used in these outdoor shooting activities weighs approximately 100 g each and spread into fragments of various sizes when shot. Through mechanical weathering processes, these clay fragments become incorporated into the soil and may provide a long-term source of PAH contamination in the environment (Synakorn et al., 2012).

Polycyclic aromatic hydrocarbons are ubiquitous environmental contaminants and 16 PAHs are considered priority pollutants by the U.S. EPA due to their recalcitrance to degradation and mutagenic or carcinogenic properties (Sun et al., 2010). There have been a few ecotoxicological evaluations concerning the large PAH concentrations from clay target fragments, but these studies have reported that the PAHs elicit low toxicity in aquatic organisms (Baer et al., 1995). This low toxicity was determined to be primarily due to the low bioavailability of PAHs resulting from the process of making clay targets in which the PAHs in the binding agent are bound under heat and pressure with dolomitic limestone (Baer et al., 1995). In addition, due to their aromatic nature and hydrophobicity, PAHs typically bind to nonpolar soil domains such as organic matter, further decreasing their bioavailability (Riding et al., 2013). However, a recent clay target ecotoxicity study using *Eisenia andrei* showed that the content of clay fragments in soils was correlated with PAH bioaccumulation in the terrestrial soil organism, suggesting that direct ingestion can be a more important route of exposure and potentially explain the lack of toxicity in exposed aquatic organisms (Rodríguez-Seijo et al., 2017). Clay-target-contaminated site evaluations have also concluded that the elevated PAH concentrations in the soil from the clay target fragments pose an unacceptable level of risk to future potential residents and current site workers (Lobb, 2006; Frakes et al., 2007; Synakorn et al., 2012). There are several PAH remediation strategies involving physical, chemical, biological, and thermal technologies; however, conventional PAH removal methods such as incineration, excavation, and land filling are expensive and inefficient (Gan et al., 2009; Bezza and Chirwa, 2016). Because of these issues, biological remediation practices

such as bioaugmentation and phytoremediation have become preferred *in situ* treatment technologies as they are considered to be cost-effective and more environmentally-friendly for the cleanup of PAH-contaminated soils (Mohan et al., 2006).

However, biological remediation operations can also be ineffective due to the limited PAH soil bioavailability that is a consequence of the clay target manufacturing process and the physicochemical properties of these compounds, which can be further exacerbated by the aging effect in field-contaminated soils (Wei et al., 2017). These PAH bioavailability limitations can be overcome through the use of surfactants that increase the desorption of PAHs from the soil to the aqueous phase, thus increasing their bioavailability to the degrading soil microbes (Lamichhane et al., 2017). Biosurfactants such as rhamnolipids or glycolipids offer an environmentally-friendly alternative to synthetic surfactants and are becoming more economically-feasible through the use of low-cost substrates and offer distinct advantages to synthetic surfactants such as reduced toxicity, high biodegradability, and greater stability under different temperature, pH, or salinity conditions (Olasanmi and Thring, 2018). In practical surfactant-enhanced PAH-contaminated soil remediation applications, mixtures of surfactants are commonly used to take advantage of the potential synergistic effects that can result in increased solubilization at a reduced effective surfactant concentrations (Zhao et al., 2005). The bioaugmentation of biosurfactant-producing soil microbes has also been shown to be an effective strategy for the remediation of PAH-contaminated soils. For example, *M. vanbaalenii* PYR-1, a glycolipid-producing microorganism isolated from an oil-contaminated estuary near the Gulf of Mexico, has been shown to enhance PAH

solubility and degradation in PAH-contaminated soils (Ghaly et al., 2013; Kim et al., 2015; Wolf and Gan, 2018).

Another *in situ* biological remediation treatment commonly used to increase PAH bioavailability is phytoremediation, or the use of plants and the associated rhizosphere to restore contaminated sites (Mougin, 2002). Phytoremediation is considered to be an effective, low-cost alternative to cleanup large contaminated sites (Liu et al., 2015). The PAH bioavailability is enhanced in the plant rhizosphere, as plant roots secrete root exudates (carbohydrates, organic acids, and amino acids) that promote PAH desorption from the soil matrix (An et al., 2010). In addition, plant roots may release enzymes that play a key role in the degradation of PAHs including oxygenases, dehydrogenases, phosphatases, and lignolytic enzymes (Bisht et al., 2015). Finally, plant roots also provide easily degradable carbon sources and other nutrients that increases microbial biomass, diversity, and activity, contributing to enhanced PAH degradation through direct metabolism or co-metabolism (Yu et al., 2011). Because of the numerous benefits provided by the rhizosphere, grass species are recommended for phytoremediation treatments due to their extensive fibrous root systems and large root surface area, and hence more extensive interactions between PAHs and the rhizosphere microbial community (Kirkpatrick et al., 2006).

Typically, the primary contaminants of concern during the remediation of outdoor shooting range soils are heavy metals (i.e., lead, zinc, antimony, nickel, and manganese) from ammunition; however, large concentrations of PAHs from the clay target fragments

remain in the contaminated soil and could possibly become more bioavailable during the remediation of the metals (Baer et al., 1995; Peddicord and LaKind, 2000). Therefore, the objective of this study was to evaluate the effect and interactions of phytoremediation (i.e., bermudagrass [*Cynodon dactylon* (L.) Pers] or switchgrass [*Panicum virgatum*]), bioaugmentation of *M. vanbaalenii* PYR-1, and surfactant application (i.e., Brij-35, rhamnolipid biosurfactant, and Brij-35/sodium dodecyl sulfate) on remediation of PAH-contaminated soil from an outdoor shooting range.

4.2 Materials and Methods

4.2.1 Chemicals

Sixteen priority PAHs were analyzed in this phytoremediation study: naphthalene, acenaphthene, acenaphthylene , fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenzo[a,h]anthracene, and benzo[g,h,i]perylene. A standard mixture of the 16 priority PAHs was purchased from Sigma-Aldrich (St. Louis, MO). A deuterated PAH standard solution containing naphthalene-d8, acenaphthene-d10, phenanthrene-d10, pyrene-d10, chrysene-d12, perylene-d12, and benzo[g,h,i]perylene-d12 (Dr. Ehrenstorfer GmbH, Augsburg, Germany) and p-terphenyl-d14 (Sigma-Aldrich) were used as recovery and internal standards, respectively. A loamy sand certified reference material (CRM) soil fortified with 12 PAHs was purchased from Sigma-Aldrich.

Brij-35 nonionic surfactant and sodium dodecyl sulfate (SDS) anionic surfactant were purchased from Sigma-Aldrich. Rhamnolipid biosurfactant (95%) isolated from *P. aeruginosa* NY3 was purchased from AGAE Technologies (Corvallis, OR). Activated alumina for PAH cleanup was purchased from GFS Chemicals (Columbus, OH). Diatomaceous earth, Ottawa sand, and all GC-MS grade solvents used in this study were purchased from Thermo Fisher Scientific (Waltham, MA). All substrates utilized for soil enzymatic analyses were purchased from Tokyo Chemical Industry Co., (Tokyo, Japan). Bermudagrass, switchgrass, and lettuce [*Lactuca sativa*] seeds were purchased from Lowe's (Mooresville, NC)

4.2.2 Study Area and Soil Characterization

A Vista coarse sandy loam was collected manually using a shovel from the 0-15 cm soil depth of an abandoned shooting range located near Lake Elsinore, California (33°43'44.9"N, 117°19'29.2"W) that was littered with clay target fragments with no prior soil remediation or waste removal from the site. The collected soil was air-dried for 5 d at approximately 23 °C and sieved through a 2-mm stainless-steel mesh screen. Representative soil subsamples were analyzed for soil physicochemical properties and initial PAH concentrations (Table 4.1). Soil particle-size distribution was determined according to the 12-h hydrometer method (Gavlak et al., 2003). The soil pH and electrical conductivity were determined potentiometrically in a 1:2 soil-to-water suspension (Sikora and Moore, 2014). Total metal analysis was carried out using an Optima 7300 DV inductively coupled, argon-plasma optical emission spectrometer (PerkinElmer,

Waltham, MA) following U.S. EPA Method 3050B after a 6-h digestion in a mixture of nitric acid, hydrogen peroxide, and hydrochloric acid at 95 °C (U.S. EPA, 1996).

4.2.3 Microbial Incubation and Surfactant Characteristics

Mycobacterium vanbaalenii PYR-1 was stored at -80 °C in a 30% (v/v) glycerol stock and the inoculum was prepared according to a previous method in MBS solution amended with pyrene as a carbon source (Wolf and Gan, 2018). The CMC of Brij-35 and rhamnolipid biosurfactant was determined previously (Wolf and Gan, 2018). The CMC of the Brij-35/SDS surfactant mixture was determined by measuring the surface tension of surfactant solutions over a concentration range using a Du Noüy ring-tensiometer (Krüss Tensiometer 10, Hamburg, Germany) and using the inflection in the plot of surface tension against surfactant concentration. The CMC was determined to be 0.099 mM at 0.5/0.5 molar fraction, which was similar to a previous study (Zhao et al., 2005).

4.2.4 Treatment Preparation and Phytoremediation Experiment

After the soil was thoroughly mixed, 1 kg soil was placed in a stainless-steel bowl and 150 mL of distilled water was added and mixed to achieve a soil water potential of approximately -33 kPa determined by a soil tensiometer. For the *M. vanbaalenii* PYR-1 bioaugmented treatments, 15 mL *M. vanbaalenii* PYR-1-MBS solution was added to yield approximately 10^6 CFU/g soil and thoroughly mixed (Chen et al., 2016). The same procedure using only the MBS solution was added to the non-inoculated, or native, soil as the control. The PYR-1-MBS solution was reapplied every 2 months by adding the

inoculum solution into the soil rhizosphere 5 cm below the soil surface (Chen et al., 2016).

Once the soil treatments were prepared, the soil was added to the phytoremediation sample containers, which consisted of 800-mL glass jars (15.9 × 8.3 cm pots) (Ball, Broomfield, CO) that were first painted on the outside with black paint, followed by aluminum enamel to prevent exposure to light (Rust-Oleum, Vernon Hills, IL) (Grover et al., 1975). The pots contained approximately 50 g of 2-cm diameter gravel at the bottom to allow for accumulation of any excess soil water (Kirkpatrick et al., 2006). Bermudagrass and switchgrass seeds were surface-sterilized by three sequential washings in 0.1% sodium hypochlorite, followed by two rinses with sterile distilled water (Balcom and Crowley, 2009). Bermudagrass and switchgrass seeds were planted at a rate of 20 seeds/pot and sealed with plastic wrap for 1 week for optimal seedling emergence conditions. After 2 weeks, plants were thinned to 8 plants/pot and amended with a commercial fertilizer for bermudagrass establishment. Treatments were then fertilized monthly with 100 mg/kg-N as urea, and 12.5 mg/kg-P as monobasic potassium phosphate (Cofield et al., 2007a).

Due to the potential toxicity of surfactants to emerging plant seedling (Wen et al., 2010; Gálvez et al., 2019), surfactant addition at 50 mg/kg was initiated 1 week after plant thinning and initial fertilizer application. Since rhamnolipid biosurfactants have been previously shown to be degraded by the soil microbial community and are considered more biodegradable than the synthetic surfactants used in this study,

surfactants at the initial rate were reapplied to the soil surface every 40 d (Szulc et al., 2014).

Each pot was placed randomly in one of four blocks, each containing one replication of all treatment combinations in a climate-controlled growth chamber (Conviron® CMP 3000) (Controlled Environments, Winnipeg, Canada). The PAH phytoremediation experiment was continued for 8 months in the growth chamber under a 12/12 hour day/night period at 23±1/19±1 °C and 40% relative humidity. The average light intensity (200 μmol/m^2s^1) was obtained through fluorescent and incandescent lighting in the growth camber (Dudley et al., 2019). Each pot was weighed daily for 8 months and the soil moisture was gravimetrically adjusted to 20% by application of distilled water (Wolf et al., 2015). The quantity of distilled water added to the soil to achieve proper soil moisture was not adjusted for vegetation biomass produced during the study. Plant shoots were trimmed to a height of 5 cm every 3 months in order to stimulate continuous plant growth (Cofield et al., 2007b).

At the end of the 8-month phytoremediation experiment, plant shoots and roots were separated from the soil as described in section 2.7. Once the vegetation was removed, the soil was sieved to pass through a 2-mm sieve and separated into two subsamples. The first soil subsample was air-dried for 7 d at approximately 23 °C in the dark and used for PAH analysis and toxicity assay (see sections 2.5 and 2.8 below). The second soil subsample was used for soil enzyme analysis (section 2.6) and kept at field-moist conditions and analyzed within 1 week after the termination of the experiment.

4.2.5 Polycyclic Aromatic Hydrocarbon Soil Extraction and Analysis

Polycyclic aromatic hydrocarbon extraction and in-cell cleanup was performed by pressurized liquid extraction (PLE) using a Dionex ASE 350 accelerated solvent extractor (Thermo Fisher Scientific) according to procedures by Liu et al. (2014). Briefly, 40 g soil samples were ground via mortar and pestle and mixed with 2 g diatomaceous earth. The 34 mL stainless-steel extraction cells were packed with two 30-mm cellulose filters and 5 g activated alumina (for in-cell cleanup). The soil sample was then transferred to the extraction cell with the addition of 500 µL deuterated PAH surrogate standard solution. The PLE program was conducted according to Dionex manufacturer procedures (Jinshui et al., 2013). Briefly, a mixture of *n*-hexane and dichloromethane (1:1, v/v) was used as the solvent at a pressure of 1,500 psi and a temperature of 100 °C. The oven heat-up time was 5 min with two extraction cycles with a 5-min static time. At the end of the cycle, the cell was rinsed with fresh solvent (60% of extraction cell volume) and purged using nitrogen for 90 s. The extract was concentrated to 1 mL under a stream of nitrogen (N-EVAP™ 111, Organomation Associates, Berlin, MA) and filtered through a 0.22-µm polytetrafluoroethylene membrane (Millipore, Burlington, MA). Prior to GC/MSD analysis, p-terphenyl-d14 was added as an internal standard to all final extracts.

The 16 priority PAHs were analyzed using an Agilent 6890N/5973B GC/MSD equipped with a DB-5 column (30 m × 0.25 mm × 0.25 µm) (Agilent Technologies, Wilmington, DE). Helium was used as the carrier gas at a flow rate of 1.0 mL/min and sample aliquots (2 µL) were injected at 280 °C. The GC oven temperature was initially

set at 60 °C (held for 1 minute) and then raised at 5°C/min to 280°C (held for 20 minutes). Quantification of PAHs was performed using an internal standard-normalized calibration curve and coefficients of determination for all calibration curves fulfilled the requirement of $R^2 \geq 0.99$.

4.2.6 Soil Enzyme Activity

Soil dehydrogenase soil activity was analyzed by the use of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazoliumchloride (INT) as a substrate (Schinner et al., 1996). A 1.0-g soil aliquot was mixed with Tris buffer (pH 7) and INT substrate in a stoppered 100-mL Erlenmeyer flask, and the mixture was incubated for 2 h at 40 °C in the dark. After incubation, the mixture was extracted using 10 mL N,N-dimethylformamide:ethanol mixture (1:1, v/v) for 1 h at 23 °C in the dark and shaken every 20 min. Immediately after filtration, iodonitrotetrazolium formazan (INTF) formation was measured colorimetrically at 464 nm against the reagent blank using a UV-Visible spectrophotometer (Varian Cary 50 Conc) (Varian, Palo Alto, CA). Soil dehydrogenase activity was expressed as µg INTF produced/g dry soil 2h.

Soil polyphenol oxidase activity was measured by the utilization of pyrogallic acid as a substrate to form purpurogallin (Shen et al., 2006; Liu et al., 2014). Ten mL of 1.0% pyrogallol was added to 1.0 g soil sample and incubated at 30 °C for 2 h at 200 rpm. Afterwards, 5 mL of citrate-phosphate buffer (pH 4.5) was added to the treatment to stop the reaction, followed by the addition of 35 mL ether and shaking for 30 min at 200 rpm. The colored ether with dissolved purple gallic prime was measured colorimetrically

at 430 nm on a UV-Visible spectrophotometer. Assays without soil and without pyrogallol addition were performed as control tests. The polyphenol oxidase activity was expressed as mg purpurogallin produced/g dry soil 2h.

Control assays for each soil enzyme activity included autoclaved soil treatments, assays without soil, and assays without substrate addition during incubation (Trevors, 1996). Results of soil enzyme activities are reported on an oven-dry-weight basis.

4.2.7 Vegetation Analysis

At the end of the phytoremediation experiment, plant shoots were cut at the soil surface and rinsed with distilled water to remove any adhering soil. Approximately 4 g (wet weight) shoot subsample was taken and freeze-dried for PAH extraction and the remaining shoots were dried to a constant weight at 55 °C and weighed to determine total shoot biomass. The freeze-dried plant shoots were ground to pass a 2-mm, stainless-steel mesh screen using a Wiley Mill Grinder (Thomas Scientific, Swedesboro, NJ) and 2 g was used to determine PAH shoot concentrations using procedures similar to those for soil PAH extraction (Wang et al., 2012). Plant roots were manually collected from the soil using forceps, placed on a 500-µm stainless-steel sieve, and thoroughly rinsed with distilled water to remove any adhering soil particles. Approximately 2 g (wet weight) root subsample was taken and freeze-dried and 1 g was used to determine PAH root concentrations similar to shoot analysis. The remaining plant roots were dried to a constant weight at 55 °C and weighed to determine total root biomass.

4.2.8 Lettuce Seed Germination Toxicity Assay

The lettuce seed toxicity assay was performed to evaluate changes in phytotoxicity before and after remediation treatments by following a method in Cofield et al. (2007a). Briefly, 100 g soil at 85% water-holding capacity was placed in a 150 mm × 15 mm Petri dish and 40 lettuce seeds were evenly distributed and pressed into the soil. Approximately 20 g of Ottawa sand was spread across the soil and the lid was placed on the Petri dish and sealed with parafilm to maintain optimal growth conditions. A sandy loam control soil containing no PAH contamination was prepared in the same manner every 20 samples. The samples were placed in a growth chamber at 22±1 °C in darkness for 2 d and then 16/8 h day/night cycle for another 3 d. Afterwards, all germinated seeds were counted for the control, pre-, and post-remediation soils.

4.2.9 Quality Control and Statistics

Method blanks were included every 10 samples to determine any potential background contamination and no PAHs were detected. Spiked blanks (PAH-standard-spiked Ottawa sand) and CRM PAH-contaminated loamy sand soil were included to determine extraction efficiency (80-112%). In addition, a deuterated PAH surrogate standard solution containing seven PAHs of various molecular weights was added to all samples prior to the PLE extraction to assess surrogate recoveries, and the surrogate recoveries were 91±11%.

The experiment was designed and analyzed as a randomized complete block design with four blocks arranged in a 4 × 3 × 2 factorial treatment structure (i.e., soil amendment × vegetation × microbial community). Least squares means for significant effects were determined using a protected least significant difference procedure at $\alpha = 0.05$. All statistical analyses were performed using SAS® 9.4 (SAS, Cary, NC). All treatments were conducted in quadruplicate and three non-vegetated mercuric-chloride-sterilized control treatments were used to indicate any abiotic PAH loss.

4.3 Results and Discussion

4.3.1 Initial Soil Characteristics and Plant Biomass

The initial soil parameters of the outdoor shooting range soil are given in Table 4.1. The heavy metal concentrations in the initial outdoor shooting range soil were substantially lower than levels in other outdoor shooting range soils (Peddicord and LaKind, 2000; Rodríguez-Seijo et al., 2016). This might be due to the fact that soil sampling was conducted at the location that contained the greatest concentration of clay target fragments and was relatively near the firing stand. Kajander and Parri (2014) suggested that shotgun ammunition travels approximately 200-250 m from the firing stand while clay target fragments typically land 20-80 m from the firing stand. Although the concentrations of PAHs ($\sum_{16}$PAH) in the initial outdoor shooting range soil were greater than typical background concentrations in urban soils, these levels were lower when compared to other outdoor shooting range soils contaminated with clay target fragments (Wilcke, 2007). For example, total soil PAH concentrations in other outdoor

shooting ranges from clay targets in California and Florida were 2,431 and 1,324 mg/kg, respectively (Lobb, 2006; Frakes et al., 2007). Similar to other studies examining PAH-contaminated soils from clay targets, acenaphthylene was not detected, and the majority of the total PAH concentrations consisted of HMW PAHs (84%). This finding was to be expected due to the PAH composition of the binding agent pitch in clay targets as well as the increased recalcitrance of HMW PAHs in aged, field-contaminated soils (Baer et al., 1995; Potin et al., 2004; Lobb, 2006; Kajander and Parri, 2014; Bartolomé et al., 2018). Based upon the individual PAH profiles in the clay target fragments on the soil surface, the binding material most likely consisted of coal tar pitch (Magee et al., 2018; Bartolomé et al., 2018). Of the 16 priority PAHs, 5 PAH compounds in the initial soil exceeded U.S. EPA regional screening levels for industrial soils; these PAHs are also classified as probably or possibly carcinogenic to humans according to the IARC (ATSDR, 1995) (Table 4.1).

Table 4.1. Initial soil physicochemical properties of the outdoor shooting range soil.

Soil Characteristic	Value
Sand (%)	62±1.5[a]
Silt (%)	22±1.9
Clay (%)	16±0.50
pH (1:2)	6.1±0.03
Electrical Conductivity (1:2) (dS/m)	0.8±0.07
Organic Matter (%)	1.86±0.06
NO_3-N (mg/kg)	10±0.3
P (mg/kg)	50±5.3
K (mg/kg)	110±5.5
Total Metals (mg/kg)	
Mn	2.4±0.15
Ni	0.07±0.01
Pb	9.9±0.59
Sb	0.06±0.01
Zn	0.80±0.05
Polycyclic Aromatic Hydrocarbons (number of rings) (mg/kg)[b]	
Naphthalene (2)	0.8±0.06
Acenaphthene (3)	7.4±2.88
Acenaphthylene (3)	ND
Fluorene (3)	8.9±1.14
Phenanthrene (3)	9.4±1.24
Anthracene (3)	31±8.91
Fluoranthene (4)	44±7.36
Pyrene (4)	41±7.4
Benzo[a]anthracene (4)	**40±6.5**[c]
Chrysene (4)	29±4.8
Benzo[b]fluoranthene (5)	**49±7.6**
Benzo[k]fluoranthene (5)	18±2.9
Benzo[a]pyrene (5)	**35±5.1**
Indeno[1,2,3-cd]pyrene (5)	**27±3.9**
Dibenzo[a,h]anthracene (6)	**6.9±0.94**
Benzo[g,h,i]perylene (6)	26±3.3
Total ($\sum_{16}$PAH)	373±43

[a]Dry-weight means ± standard deviation are reported based on three replications for soil properties

[b]Dry-weight means ± standard deviation are reported based on five replications for initial polycyclic aromatic hydrocarbon concentrations.

[c]Values in bold are greater than U.S. EPA Regional Screening Levels (U.S. EPA, 2018).

ND = not detected

Switchgrass vegetation in the native treatments amended with Brij-35/SDS and rhamnolipid surfactants did not survive after thinning and initial surfactant addition at 4 weeks. Therefore, switchgrass grown in soil without PAH contamination at similar heights to the other treatments were transplanted to the Brij-35/SDS- and rhamnolipid-amended treatments to yield 8 plants/pot and the transplanted switchgrass plants survived for the remainder of the 8-month experiment with surfactant reapplication. At the end of the 8-month phytoremediation experiment, bermudagrass root, shoot, and total biomass was far greater than switchgrass root, shoot, and total biomass amongst all vegetated treatments. Although bermudagrass and switchgrass biomass did not differ between the surfactant-amended and the unamended control treatments, the bioaugmentation of *M. vanbaalenii* PYR-1 resulted in increased bermudagrass shoot biomass and switchgrass root biomass compared to the non-inoculated vegetated treatments (Fig. 4.1). The positive effect of bioaugmentation on plant growth was potentially related to the increased dissipation of HMW PAHs due to *M. vanbaalenii* PYR-1 bioaugmentation (see section 3.2.2). Rostami et al. (2016) also observed that the bioaugmentation of *P. aeruginosa* increased great millet [*Sorghum bicolor*] root biomass after a 90-d phytoremediation experiment in pyrene-contaminated soil (150 mg/kg) due to increased pyrene biodegradation and reduced toxic effects of PAHs. Positive effects of *P. aeruginosa* bioaugmentation on alfalfa [*Medicago sativa* L.] root and shoot biomass were also demonstrated by Agnello et al. (2016), who reported increased root and shoot biomass after a 90-d phytoremediation study of a co-contaminated soil containing high levels of heavy metals (copper, lead, and zinc) and petroleum hydrocarbons (3,800

mg/kg). Chen et al. (2016) also observed increased ryegrass [*Lolium multiflorum* Lam] and *Seduce alfredii* biomass following repeated inoculation with *Microbacterium* sp. KL5 and *Candida tropicalis* C10 in a 2-yr phytoremediation study in soil spiked with phenanthrene, fluoranthene, anthracene, and pyrene.

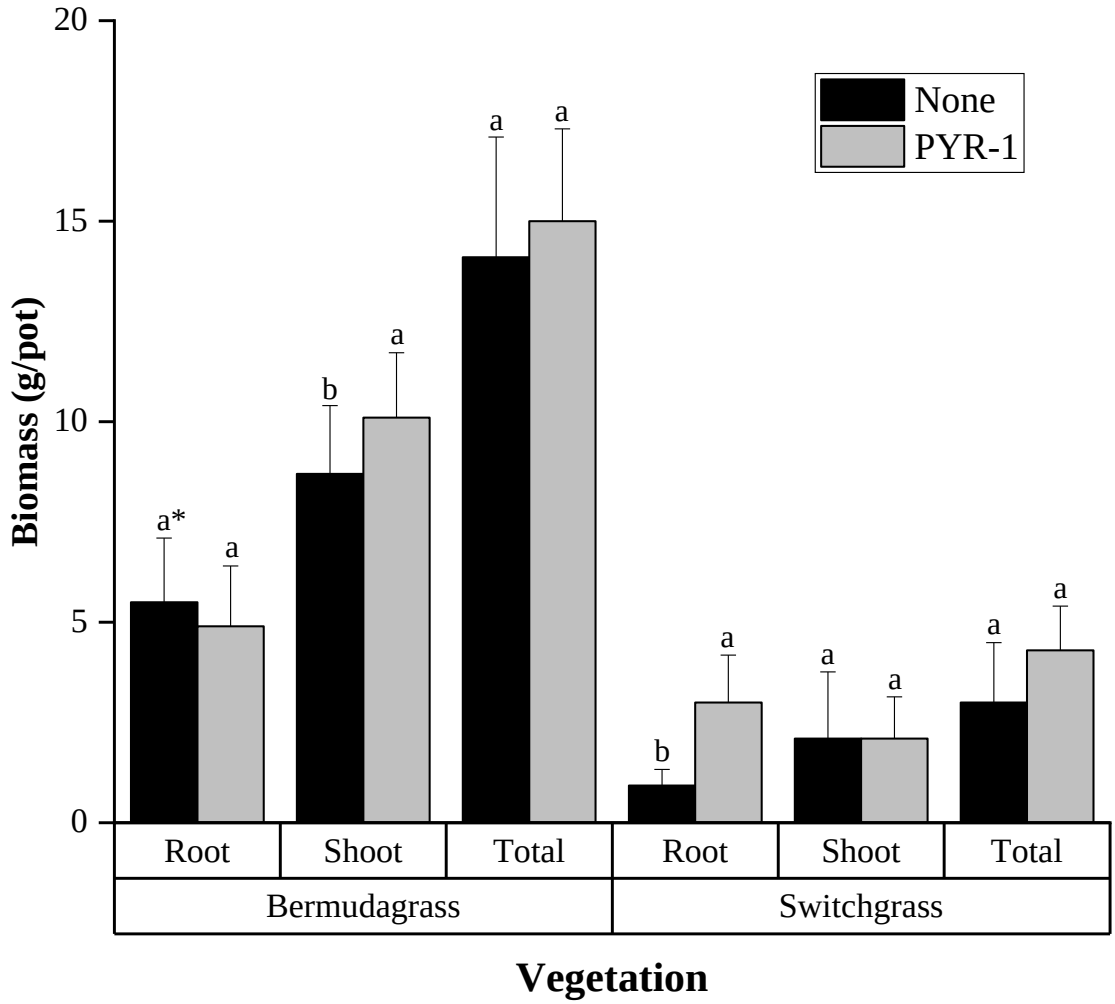

Figure 4.1. Influence of *Mycobacterium vanbaalenii* PYR-1 bioaugmentation on bermudagrass and switchgrass root, shoot, and total biomass following an 8-month greenhouse study. *Means followed by a different letter for a given vegetation parameter are significantly different ($P<0.05$).

The direct plant uptake of PAHs, especially HMW PAHs, from the soil has been previously shown to be negligible as soil microbial degradation is the primary process involved in effective PAH-contaminated site bioremediation (Cerniglia, 1992; Meng et al., 2011). However, the application of surfactant amendments to contaminated soils might increase the mass transfer of PAHs to the aqueous phase, thereby resulting in increased plant uptake of these compounds. For example, Gao et al. (2006) determined that ryegrass plant uptake of phenanthrene and pyrene in water was enhanced when amended with Brij-35 surfactant at concentrations lower than 74 mg/L. However, this was not the case in a surfactant-enhanced phytoremediation experiment of PAH-contaminated soils where pyrene concentrations in plant tissues accounted for less than 0.1% when amended with Tween 80 or Brij-35 surfactants (Gao et al., 2007). At the end of the 8-month phytoremediation experiment, plant uptake of PAHs was negligible with the maximum PAH plant uptake occurring in the native rhamnolipid-amended bermudagrass treatment and total PAH plant accumulation accounting for only 0.8% of the initial PAH amount. These results were similar to Reilley et al. (1996) that conducted a 24-wk phytoremediation study of anthracene- and pyrene-spiked soil at 100 mg/kg using four plant species (alfalfa, tall fescue [*Festuca arundinacea* Schreb.], sudangrass [*Sorghum vulgare* L.], and switchgrass). They estimated that the total accumulation of both PAHs in the four plants accounted for less than 0.03% of the initial PAH concentrations. Gao and Zhu (2004) also demonstrated that plant-promoted biodegradation was the dominant contribution to phenanthrene and pyrene dissipation

(greater than 99.7%) compared to the contribution of direct plant uptake (less than 0.01% and 0.24%, respectively) after a 45-d phytoremediation study evaluating 12 plant species.

4.3.2 Dissipation of Priority PAHs From Contaminated Soil

4.3.2.1 Impact of Vegetation on PAH Dissipation

Although bermudagrass produced significantly more root and shoot biomass than switchgrass after the 8-month phytoremediation experiment, the use of both grasses increased dissipation of low-molecular-weight (LMW) and HMW PAHs compared to the non-vegetated treatments (Tables 4.2 and 4.3). Bermudagrass has been recognized as a hydrocarbon-tolerant plant and switchgrass has been previously utilized in PAH phytoremediation studies (Reilley et al., 1996; Cofield et al., 2007b; Kaimi et al., 2007). Hutchinson et al. (2001) compared the effectiveness of bermudagrass and tall fescue during a 1-year phytoremediation study of total petroleum hydrocarbons (48,800 mg/kg) and observed that bermudagrass generated almost twice as much root and shoot biomass as tall fescue in all treatments and bermudagrass growth resulted in a 68% reduction in total petroleum hydrocarbons. Thompson et al. (2008) examined the effects of fertilizer rate on bermudagrass growth and subsequent dissipation of pyrene-contaminated soils at 1,000 mg/kg and determined that at a C:N ratio of 4.5:1, bermudagrass increased pyrene biodegradation from 31% in the non-vegetated treatment to 62% in the bermudagrass treatment after 100 d. Another study by Krutz et al. (2005) examined pyrene biodegradation spiked at 500 mg/kg in a 63-d bermudagrass phytoremediation experiment and showed that pyrene degradation was significantly greater in the

bermudagrass rhizosphere soil compared to the bermudagrass bulk soil or non-vegetated bulk soil due to the presence of bermudagrass root exudates and possible selective PAH-degrader population enrichment. Reilley et al. (1996) examined the effects of switchgrass on PAH biodegradation in aged, MGP-contaminated soil ($\sum_{16}$PAH at 2,900 mg/kg) and reported that switchgrass vegetation resulted in a total PAH reduction to 2,053 mg/kg with substantial biodegradation occurring for 4- and 5-ring PAHs after 1 yr. Pradhan et al. (1998) reported a 57% PAH reduction in MGP-contaminated soil ($\sum_{16}$PAH at 200 mg/kg) vegetated with switchgrass following a 6-month phytoremediation experiment.

Table 4.2. Influence of vegetation on polycyclic aromatic hydrocarbons in an outdoor shooting range soil following an 8-month greenhouse experiment.

	Vegetation		
PAHs	None	Bermudagrass	Switchgrass
	----------mg/kg soil----------		
Naphthalene	0.23±0.09 a*	0.14±0.08 b	0.17±0.09 ab
Acenaphthene	3.1±1.5 a	1.2±0.76 b	1.5±0.96 b
Fluorene	2.5±0.74 a	1.5±1.4 b	1.8±0.89 b
Fluoranthene	17±4.8 a	11±6.6 b	13±5.9 b
Pyrene	17±4.6 a	11±6.3 b	12±5.9 b
Benzo[a]anthracene	15±4.7 a	10±5.9 b	12±5.2 b
Chrysene	12±3.5 a	7.9±4.3 b	9.3±3.9 b
Benzo[b]fluoranthene	19±5.3 a	13±7.2 b	14±5.9 b
Benzo[k]fluoranthene	7.1±1.9 a	4.7±2.6 b	5.3±4.4 b
Low-molecular-weight ($\sum_6$PAH)	39±15 a	24±9.3 b	27±11 b
High-molecular-weight ($\sum_{10}$PAH)	122±42 a	81±26 b	90±29 b
Total ($\sum_{16}$PAH)	162±55 a	104±35 b	118±39 b

*Means followed by a different letter for a polycyclic aromatic hydrocarbon are significantly different (P<0.05).

Table 4.3. Interaction of vegetation and surfactant amendment on polycyclic aromatic hydrocarbons in an outdoor shooting range soil following an 8-month greenhouse experiment.

PAHs	Vegetation	Surfactant Amendment			
		None	Rhamnolipid	Brij-35	Brij-35/SDS
		----------mg/kg soil----------			
Phenanthrene	None	4.5±2.9 a*	2.9±0.83 ab	3.0±0.90 ab	2.1±0.93 b
	Bermudagrass	2.1±0.67 b	1.8±0.52 b	2.1±0.48 b	2.1±1.1 b
	Switchgrass	2.1±0.51 b	1.7±0.66 b	2.2±0.28 b	2.6±1.13 ab
Anthracene	None	30±12.7 a	20±6.05 ab	21±7.20 ab	16±4.70 b
	Bermudagrass	12±5.21 b	12±3.63 b	13±3.39 b	14±7.46 b
	Switchgrass	14±4.28 b	14±8.37 b	15±2.71 b	18±8.32 ab
Benzo[a]pyrene	None	18±9.4 a	13±3.4 ab	14±3.2 ab	10±2.0 b
	Bermudagrass	8.4±3.12 b	9.0±2.06 b	10±2.28 b	9.0±4.21 b
	Switchgrass	9.3±9.41 b	10±3.39 b	9.7±3.20 b	11±1.98 b
Indeno[1,2,3-cd]pyrene	None	13±6.94 a	9.7±2.16 ab	9.7±1.92 ab	7.3±1.66 b
	Bermudagrass	6.2±2.46 b	6.8±1.50 b	7.9±1.82 b	6.5±2.92 b
	Switchgrass	6.7±1.57 b	6.9±3.71 b	6.9±0.93 b	7.7±3.03 b
Dibenzo[a,h] anthracene	None	3.7±1.9 a	2.4±0.6 ab	2.5±0.6 ab	1.8±0.4 b
	Bermudagrass	1.5±0.6 b	1.7±0.4 b	2.0±0.5 b	1.7±0.8 b
	Switchgrass	1.6±0.4 b	1.8±0.7 b	1.6±0.2 b	1.9±0.9 b
Benzo[g,h,i] perylene	None	13±6.4 a	9.0±2.0 ab	9.6±1.9 ab	6.1±2.3 b
	Bermudagrass	6.1±2.4 b	6.7±1.5 b	7.7±1.7 b	6.4±2.8 b
	Switchgrass	6.2±1.4 b	7.0±2.3 b	6.5±0.7 b	7.2±2.8 b

*Means followed by a different letter for a polycyclic aromatic hydrocarbon are significantly different (P<0.05).

Bermudagrass and switchgrass significantly reduced some PAH levels in the outdoor shooting range soil compared to the non-vegetated treatments (Table 4.3). However, the application of Brij-35/SDS surfactant mixture did not result in a significant difference between the non-vegetated and vegetated treatments for some PAHs (Table 4.3). The mixed surfactant amendment treatments were not significantly different from

the Brij-35- and rhamnolipid-amended non-vegetated treatments; however, the Brij-35/SDS-amended treatments resulted in a significantly greater dissipation for phenanthrene, anthracene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenzo[a,h]anthracene, and benzo[g,h,i]perylene compared to the unamended, non-vegetated treatment (Table 4.3). In most practical applications of surfactant-enhanced PAH bioremediation, a mixture of anionic and nonionic surfactants is utilized because the mixture typically increases the effective surfactant concentration in soil due to the reduction in nonionic surfactant soil sorption, and a decrease of the surface and interfacial tension at a lower CMC, resulting in increased PAH bioavailability (Lu et al. 2019). Ni et al. (2014) evaluated the effects of sodium dodecyl benzene sulfonate (SDBS) and Tween 80 anionic-nonionic surfactant mixture at different surfactant ratios (1:1, 1:2, and 2:1) with ryegrass phytoremediation in phenanthrene- and pyrene-contaminated soils and concluded that the 1:1 surfactant mixture at less than 150 mg/kg showed the best remediation efficiency and was more effective than individual surfactants in promoting plant-microbe associated bioremediation. Even though rhamnolipid biosurfactants are a promising alternative to synthetic surfactants, the application of rhamnolipid biosurfactant was not significantly different from the unamended control in the non-vegetated treatments (Table 4.3). Szulc et al. (2014) also reported that the addition of rhamnolipids (150 mg/kg) did not contribute to diesel-oil removal at the end of a 1-yr bioaugmentation experiment using a PAH-degrading consortium. Additionally, Lin et al. (2011) observed that the biodegradation rate of rhamnolipid-amended treatments in the latter stage of diesel remediation were similar to unamended treatments.

Currently, there are limited phytoremediation studies evaluating the effects of vegetation on PAH levels in outdoor shooting range soils contaminated with clay target fragments. Wawra et al. (2018) concluded that only the combined treatment of black lotus [*Robinia pseudoacacia* Nyirsegi] and ferrihydrite-bearing material, gravel sludge, and green waste biochar (1:1:1) amendment significantly decreased $\sum_{16}$PAH from an initial concentration of 200 mg/kg. Specifically, the amendment-enhanced black lotus treatment was the only treatment that appreciably decreased phenanthrene, benzo[a]anthracene, chrysene, benzo[a]pyrene, fluoranthene, benzo[b]fluoranthene, benzo[k]fluoranthene, and indeno[1,2,3-cd]pyrene concentrations after 1 yr (Wawra et al., 2018).

4.3.2.2 Effect of Bioaugmentation on PAH Dissipation

Four applications of *M. vanbaalenii* PYR-1 during the 8-month study significantly reduced PAH levels in contaminated outdoor shooting range soil. Bioaugmentation did not reduce levels of benzo[g,h,i]perylene and the LMW PAHs (naphthalene, acenaphthene, fluorene, phenanthrene, and anthracene) that are more easily biodegradable and were initially at lower concentrations than the HMW PAHs (Table 4.1 and Fig. 4.2). *Mycobacterium vanbaalenii* PYR-1 bioaugmentation has been extensively studied in pure culture settings (Kweon et al., 2011). *Mycobacterium vanbaalenii* PYR-1 is an effective HMW PAH-degrading microbe, partially attributable to the production of surface-active trehalose-containing glycolipids that has previously been reported to be as

effective as external surfactants in the biodegradation of pyrene (Kim et al., 2015; Wolf and Gan, 2018).

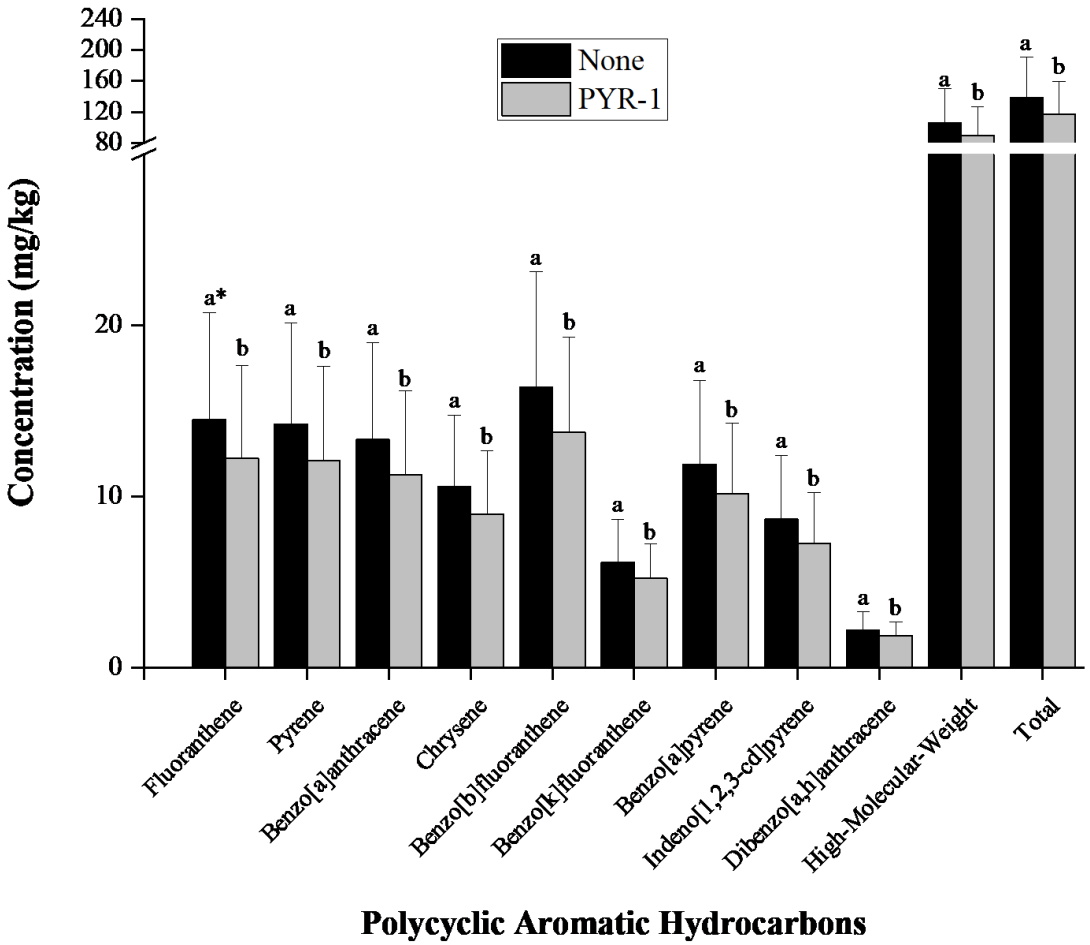

Figure 4.2. Influence of bioaugmentation of *Mycobacterium vanbaalenii* PYR-1 on polycyclic aromatic hydrocarbons in outdoor shooting range soil following an 8-month greenhouse study. *Means followed by a different letter for a polycyclic aromatic hydrocarbon are significantly different ($P<0.05$).

Child et al. (2007a) evaluated the growth of *M. vanbaalenii* PYR-1 and four other *Mycobacterium* isolates associated with barley [*Hordeum vulgare*] root surfaces after growth of the seedlings from inoculated seeds and concluded that *M. vanbaalenii* PYR-1

remained associated with the root as it grew from the inoculated seed and followed the root tip as it traveled throughout the growth matrix. In addition, *M. vanbaalenii* PYR-1 had one of the lowest contact angles of ethylene glycol on confluent layers of cells on agar, likely due to glycolipid biosurfactant production, indicating that this microorganism had a high potential to colonize the barley root tip. This is a beneficial trait for PAH remediation potential as the microbe would be distributed through contaminated soils as the roots grow (Lugtenberg et al., 2001). In another study, Child et al. (2007b) evaluated the effects on ^{14}C-pyrene mineralization using a *Mycobacterium* sp. KMS in barley rhizosphere and concluded that roots inoculated with the microbe mineralized ^{14}C-pyrene to a greater extent than treatments with solely bioaugmentation or sterile barley because the microbe was dispersed throughout the entire soil matrix as it traveled with the roots. Ma et al. (2018) reported that the bioaugmentation of *M. gilvum* CP13 in combination with mustard [*Brassica juncea*] resulted in a significant reduction of total PAH levels ($\sum_{16}$PAH from 251 to 96 mg/kg) after 183 d. The most significant increases in PAH biodegradation rate were for 4-6 ringed PAHs, indicating that the bioaugmentation of *M. gilvum* CP13 improved the degradation of recalcitrant HMW PAHs as the microbe could readily employ pyrene as a sole carbon source, similar to *M. vanbaalenii* PYR-1 (Ma et al., 2018). Additionally, dehydrogenase activity of the soil was examined to reflect the degree of PAH biodegradation and it was observed that dehydrogenase activity was significantly higher in the bioaugmented treatments compared to the control group, suggesting that PAHs or their metabolites were likely used as substrates by *M. gilvum* CP13, thus increasing the activity of dehydrogenase (Ma et al., 2018).

4.3.3 Soil Enzyme Activity

4.3.3.1 Soil Dehydrogenase and Polyphenol Oxidase Activity

Soil dehydrogenase and polyphenol oxidase are oxidoreductase enzymes that are critical in the PAH degradation pathway (Liu et al., 2014). Soil dehydrogenases are the main representatives of the oxidoreductase enzyme class and are considered one of the most important soil enzymes as they are used as an indicator of overall soil microbial activity because the enzyme occurs in all living soil microbe cells and is almost exclusively intracellular (Dawson et al., 2007; Moeskops et al., 2010; Kaczyńska et al., 2015). Dehydrogenase is a critical enzyme in the degradation and transformation of PAHs and other cyclic organic compounds as it is primarily involved in the formation of catechols (Lu et al., 2017). In this study, soil dehydrogenase activity at the end of the 8-month phytoremediation experiment was greater in the bermudagrass treatments than the non-vegetated control; however, dehydrogenase activity in the switchgrass treatments was not significantly different from the bermudagrass or non-vegetated treatments, which could potentially be due to the decreased root biomass compared to that of bermudagrass (Table 4.4). The increased dehydrogenase activity in the vegetative treatments during phytoremediation of PAH-contaminated soils has been previously reported (Sun et al., 2011; Liu et al., 2013; Lu et al., 2017). Kaimi et al. (2007) evaluated 12 plant species including bermudagrass and concluded that bermudagrass vegetation resulted in increased soil dehydrogenase activity and this activity was correlated to the remediation ability of bermudagrass. Soleimani et al. (2010) determined that the increased

dehydrogenase activity in vegetated treatments (tall fescue and meadow fescue [*Festuca pratensis* Huds.]) infected with *Neotyphodium* endophytes was due to the greater root biomass in the endophyte-infected vegetative treatments.

Soil polyphenol oxidase catalyzes the orthohydroxylation of phenols to catecholes and subsequent dehydrogenation of catecholes to orthoquinones (Worthington and Worthington, 2011). Polyphenol oxidase activity increased in the bermudagrass and switchgrass rhizosphere compared to the non-vegetated soil treatments (Table 4.4), which was in agreement with Chen et al. (2004) and Sun et al. (2010) that reported that polyphenol oxidase activity in rhizosphere soil was higher than in the non-vegetated control and concluded that polyphenol oxidase mainly originated from the plant roots. Ding et al. (2008) examined the effects of the ryegrass rhizosphere on polyphenol oxidase as well as benzo[a]pyrene biodegradation and concluded that polyphenol oxidase activity increased in the rhizosphere, which contributed to the increased benzo[a]pyrene dissipation. Liu et al. (2014) observed similar results and concluded that the increased polyphenol oxidase activity in the rhizosphere was closely related to its efficiency in remediating PAHs in the soil as the increased polyphenol oxidase activity catalyzed PAH ring-opening reactions, which then generated more readily degradable PAH intermediates that could be utilized by numerous soil microbes. Polyphenol oxidase is also actively involved in the conversion of aromatic organic compounds in the humus fractions (Liu et al., 2014). Chen et al. (2003) used ^{14}C-pyrene in a 190-d phytoremediation experiment and found that there were significant amounts of ^{14}C activity remaining for both

vegetation treatments (tall fescue and switchgrass) in the humic/fulvic fraction of soil at the end of the experiment.

Table 4.4. Influence of vegetation on soil dehydrogenase and polyphenol oxidase enzyme activity in an outdoor shooting range soil following an 8-month greenhouse experiment.

	Vegetation		
Soil Enzyme Activity	None	Bermudagrass	Switchgrass
Dehydrogenase (µg INTF/g soil 2hr)	0.09±0.06 b*	0.16±0.10 a	0.12±0.08 ab
Polyphenol oxidase (mg purpurogallin/g soil 2hr)	0.41±0.21 b	0.56±0.19 a	0.65±0.20 a

*Means followed by a different letter for each soil enzyme activity are significantly different (P<0.05).

4.3.4 Lettuce Seed Germination Toxicity Assay

Lettuce seed germination levels were below 5% in the initial PAH-contaminated soil before it had undergone any remediation, which was similar to previous studies using seed germination as a toxicity assay in heavily PAH-contaminated soils (Lagadec et al., 2000; Maila and Cloete, 2002; Cofield et al., 2007a; Dawson et al., 2007). At the end of the 8-month phytoremediation experiment, lettuce seed germination was still somewhat limited as the soil still had PAH contamination, albeit at substantially reduced levels. Bermudagrass treatments with and without *M. vanbaalenii* PYR-1 bioaugmentation exhibited greater lettuce seed germination compared to the non-vegetated control treatments (Fig. 4.3). Lettuce seed germination in the non-inoculated switchgrass treatments was significantly greater than the non-vegetated control, but less than bermudagrass treatments (Fig. 4.3). The bioaugmentation of *M. vanbaalenii* PYR-1 in the non-vegetated treatments yielded germination results that were not significantly different

from those of bioaugmented switchgrass treatments, but the non-vegetated, bioaugmented treatment had greater seed germination compared to the non-inoculated control treatment (Fig. 4.3). As shown in Tables 4.2 and4.3 and Fig. 4.2, the combination of phytoremediation and *M. vanbaalenii* PYR-1 bioaugmentation resulted in decreased PAH concentrations compared to the non-vegetated and non-inoculated control, indicating that PAHs had a substantial impact on lettuce seed germination. Maila and Cloete (2002) observed similar trends for garden cress [*Lepidium sativum*] in aged, industrial PAH-contaminated soil that had undergone phytoremediation using Guinea grass [*Panicum maximum*] after 70 d. They reported the vegetated soil had a significantly higher seed germination rate compared to the non-vegetated control (approximately 70 and 38%, respectively) and this was most likely due to the reduction in soil PAH contamination. The increased seed germination due to PAH removal was also observed by Dawson et al. (2007) and Lagadec et al. (2000) that employed surfactant application/soil turning and subcritical water remediation techniques, respectively, to remove significant levels of PAHs in soils.

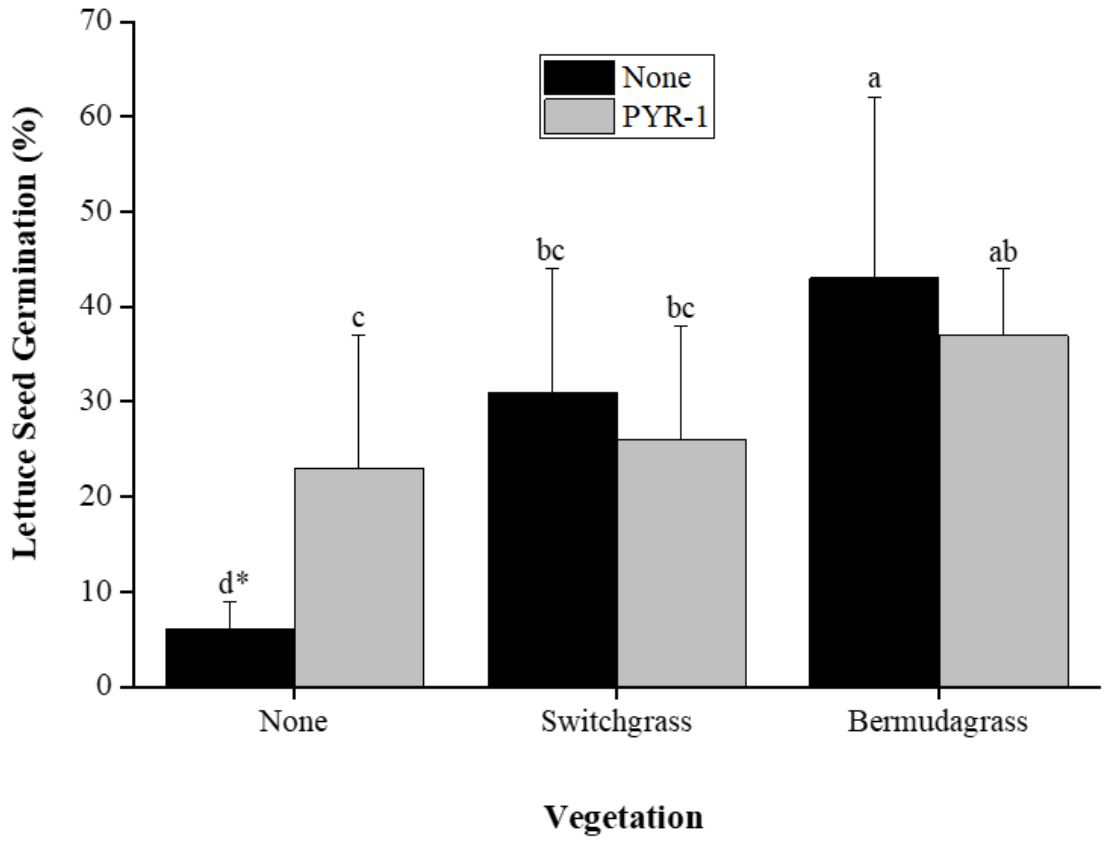

Figure 4.3. Interaction of bioaugmentation of *Mycobacterium vanbaalenii* PYR-1 and vegetation on lettuce seed germination toxicity assay in outdoor shooting range soil following an 8-month greenhouse study. *Means followed by a different letter are significantly different ($P<0.05$).

4.4 Conclusions

The results obtained at the end of the 8-month phytoremediation experiment on PAH removal in soil contaminated with clay target fragments suggested that an integrated approach towards effective *in situ* biological treatment may be achieved through the utilization of the bermudagrass and switchgrass rhizosphere environment in addition to the repeated inoculation of a known HMW PAH-degrading soil microorganism such as *M. vanbaalenii* PYR-1. Based on the results, given the selection of appropriate PAH-

tolerant plant species and provided with sufficient time and nutrients for proper plant establishment and growth, phytoremediation is an effective remediation option for aged, PAH-contaminated outdoor shooting range soils. The increased PAH dissipation in the rhizosphere, particularly in the bermudagrass rhizosphere, was observed and further evaluated through enzyme activity assays and lettuce seed germination toxicity assay. The repeated application of *M. vanbaalenii* PYR-1 notably improved HMW PAH dissipation compared to natural attenuation. Conversely, the enhancement of rhamnolipid biosurfactant and Brij-35 application were insignificant and require further research to examine optimal surfactant concentrations and reapplication intervals. The use of an anionic-nonionic surfactant mixture such as Brij-35/SDS was shown to be more effective than the individual surfactant and biosurfactant treatments evaluated in this study in the non-vegetated treatments. Overall, findings of this study highlighted the significant contributions of the plant rhizosphere and PAH-degrading soil microbes and their complementary effects in improving bioremediation of PAH-contaminated soils.

4.5 References

Agency for Toxic Substances and Disease Registry, 1995. Toxicological profile for polycyclic aromatic hydrocarbons (PAHs). Available at: https://www.atsdr.cdc.gov/toxprofiles/tp69.pdf (accessed 15 March 2019).

Agnello, A.C., Bagard, M., van Hullebusch, E.D., Esposito, G., Huguenot, D., 2016. Comparative bioremediation of heavy metals and petroleum hydrocarbons co-contaminated soil by natural attenuation, phytoremediation, bioaugmentation and bioaugmentation-assisted phytoremediation. *Science of The Total Environment* 563–564, 693–703.

An, C., Huang, G., Yu, H., Wei, J., Chen, W., Li, G., 2010. Effect of short-chain organic acids and pH on the behaviors of pyrene in soil–water system. *Chemosphere* 81, 1423–1429.

Andreoni, V., Cavalca, L., Rao, M.A., Nocerino, G., Bernasconi, S., Dell'Amico, E., Colombo, M., Gianfreda, L., 2004. Bacterial communities and enzyme activities of PAHs polluted soils. *Chemosphere* 57, 401–412.

Baer, K.N., Hutton, D.G., Boeri, R.L., Ward, T.J., Stahl, R.G., 1995. Toxicity evaluation of trap and skeet shooting targets to aquatic test species. *Ecotoxicology* 4, 385–392.

Balcom, I.N., Crowley, D.E., 2009. Pyrene effects on rhizoplane bacterial communities. *International Journal of Phytoremediation* 11, 609–622.

Bartolomé, N., Hilber, I., Schulin, R., Mayer, P., Witt, G., Reininghaus, M., Bucheli, T.D., 2018. Comparison of freely dissolved concentrations of PAHs in contaminated pot soils under saturated and unsaturated water conditions. *Science of The Total Environment* 644, 835–843.

Bezza, F.A., Chirwa, E.M., 2016. Biosurfactant-enhanced bioremediation of aged polycyclic aromatic hydrocarbons (PAHs) in creosote contaminated soil. *Chemosphere* 144, 635–644.

Bisht, S., Pandey, P., Bhargava, B., Sharma, S., Kumar, V., Sharma, K.D., 2015. Bioremediation of polyaromatic hydrocarbons (PAHs) using rhizosphere technology. *Brazilian Journal of Microbiology* 46, 7–21.

Cerniglia, C.E., 1992. Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation* 3, 351–368.

Chen, F., Tan, M., Ma, J., Zhang, S., Li, G., Qu, J., 2016. Efficient remediation of PAH-metal co-contaminated soil using microbial-plant combination: A greenhouse study. *Journal of Hazardous Materials* 302, 250–261.

Chen, Y., Wang, C., Wang, Z., Huang, S., 2004. Assessment of the contamination and genotoxicity of soil irrigated with wastewater. *Plant and Soil* 261, 189–196.

Chen, Y.-C., Banks, M.K., Schwab, A.P., 2003. Pyrene degradation in the rhizosphere of tall fescue (*Festuca arundinacea*) and switchgrass (*Panicum virgatum* L.). *Environmental Science & Technology* 37, 5778–5782.

Child, R., Miller, C.D., Liang, Y., Narasimham, G., Chatterton, J., Harrison, P., Sims, R.C., Britt, D., Anderson, A.J., June 1, 2007a. Polycyclic aromatic hydrocarbon-degrading *Mycobacterium* isolates: Their association with plant roots. *Applied Microbiology and Biotechnology* 75, 655–663.

Child, R., Miller, C.D., Liang, Y., Sims, R.C., Anderson, A.J., 2007b. Pyrene mineralization by *Mycobacterium* sp. strain KMS in a barley rhizosphere. *Journal of Environmental Quality* 36, 1260–1265.

Cofield, N., Schwab, A.P., Banks, M.K., 2007b. Phytoremediation of polycyclic aromatic hydrocarbons in soil: Part I. Dissipation of target contaminants. *International Journal of Phytoremediation* 9, 355–370.

Cofield, N., Schwab, A.P., Williams, P., Banks, M.K., 2007a. Phytoremediation of polycyclic hydrocarbon contaminated soil: Part II. Impact on ecotoxicity. *International Journal of Phytoremediation* 9, 371–384.

Dawson, J.J.C., Godsiffe, E.J., Thompson, I.P., Ralebitso-Senior, T.K., Killham, K.S., Paton, G.I., 2007. Application of biological indicators to assess recovery of hydrocarbon impacted soils. *Soil Biology and Biochemistry* 39, 164–177.

Ding, K., Luo, Y., Liu, S., 2008. Ecotoxicity effect of polycyclic aromatic hydrocarbons on wheat growth. *Journal of Nanjing Institute of Technology*, 10.

Dudley, S., Sun, C., McGinnis, M., Trumble, J., Gan, J., 2019. Formation of biologically active benzodiazepine metabolites in *Arabidopsis thaliana* cell cultures and vegetable plants under hydroponic conditions. *Science of The Total Environment* 662, 622–630.

Frakes, R.A., Boughner, E.A., Boggs, J.F., Tutton, J., Bargar, T.A., 2007. Delineation of the nature and extent of contamination at the former NAS Key West Skeet Club on Great White Heron National Wildlife Refuge. Available at:

https://ecos.fws.gov/ServCat/DownloadFile/21637?Reference=23118 (Accessed 1 May 2019).

Gálvez, A., López-Galindo, A., Peña, A., 2019. Effect of different surfactants on germination and root elongation of two horticultural crops: Implications for seed coating. *New Zealand Journal of Crop and Horticultural Science* 47, 83–98.

Gan, S., Lau, E.V., Ng, H.K., 2009. Remediation of soils contaminated with polycyclic aromatic hydrocarbons (PAHs). *Journal of Hazardous Materials* 172, 532–549.

Gao, Y., Ling, W., Wong, M.H., 2006. Plant-accelerated dissipation of phenanthrene and pyrene from water in the presence of a nonionic-surfactant. *Chemosphere* 63, 1560–1567.

Gao, Y., Zhu, L., 2004. Plant uptake, accumulation and translocation of phenanthrene and pyrene in soils. *Chemosphere* 55, 1169–1178.

Gao, Y.-Z., Ling, W.-T., Zhu, L.-Z., Zhao, B.-W., Zheng, Q.-S., 2007. Surfactant-enhanced phytoremediation of soils contaminated with hydrophobic organic contaminants: Potential and assessment. *Pedosphere* 17, 409–418.

Gavlak, R., Horneck, D., Miller, R.O., Kotuby-Amacher, J., 2003. Soil, plant and water reference methods for the western region. WCC-103 Publication, Fort Collins.

Ghaly, A., Yusran, A., Dave, D., 2013. Effects of biostimulation and bioaugmentation on the degradation of pyrene in soil. *Journal of Bioremediation & Biodegradation* S 5.

Grover, R., Gear, J.R., Zerr, R., 1975. Relative phytotoxicity of some picloram derivatives. *Bulletin of Environmental Contamination and Toxicology* 14, 721–725.

Hutchinson, S.L., Banks, M.K., Schwab, A.P., 2001. Phytoremediation of aged petroleum sludge. *Journal of Environmental Quality* 30, 395–403.

Jinshui, C., Guifeng, D., Lina, L., Kettle, A., 2013. Accelerated solvent extraction – GC-MS analysis and detection of polycyclic aromatic hydrocarbons in soil. Available at: http://tools.thermofisher.com/content/sfs/brochures/115025-AU313-ASE-Hydrocarbons-Soil-AU70846_E.pdf (Accessed 2 November 2018).

Kaczyńska, G., Borowik, A., Wyszkowska, J., 2015. Soil dehydrogenases as an indicator of contamination of the environment with petroleum products. *Water, Air, & Soil Pollution* 226, 372.

Kaimi, E., Mukaidani, T., Tamaki, M., 2007. Screening of twelve plant species for phytoremediation of petroleum hydrocarbon-contaminated soil. *Plant Production Science* 10, 211–218.

Kajander, S., Parri, A., 2014. Management of the environmental impact of shooting ranges: Best available techniques. Available at: https://www.ecde.info/sites/default/files/docs/sy_4_2014_en.pdf (Accessed 15 March 2019).

Kim, S.-J., Kweon, O., Sutherland, J.B., Kim, H.-L., Jones, R.C., Burback, B.L., Graves, S.W., Psurny, E., Cerniglia, C.E., 2015. Dynamic response of *Mycobacterium vanbaalenii* PYR-1 to BP Deepwater Horizon crude oil. *Applied and Environmental Microbiology* 81, 4263.

Kirkpatrick, W.D., White, P.M., Wolf, D.C., Thoma, G.J., Reynolds, C.M., 2006. Selecting plants and nitrogen rates to vegetate crude-oil–contaminated soil. *International Journal of Phytoremediation* 8, 285–297.

Krutz, L.J., Beyrouty, C.A., Gentry, T.J., Wolf, D.C., Reynolds, C.M., 2005. Selective enrichment of a pyrene degrader population and enhanced pyrene degradation in bermuda grass rhizosphere. *Biology and Fertility of Soils* 41, 359–364.

Kweon, O., Kim, S.-J., Holland, R.D., Chen, H., Kim, D.-W., Gao, Y., Yu, L.-R., Baek, S., Baek, D.-H., Ahn, H., Cerniglia, C.E., 2011. Polycyclic aromatic hydrocarbon metabolic network in *Mycobacterium vanbaalenii* PYR-1 *Journal of Bacteriology*. 193, 4326.

Lagadec, A.J.M., Miller, D.J., Lilke, A.V., Hawthorne, S.B., 2000. Pilot-scale subcritical water remediation of polycyclic aromatic hydrocarbon- and pesticide-contaminated soil. *Environmental Science & Technology* 34, 1542–1548.

Lamichhane, S., Bal Krishna, K.C., Sarukkalige, R., 2017. Surfactant-enhanced remediation of polycyclic aromatic hydrocarbons: A review. *Journal of Environmental Management* 199, 46–61.

Lin, T.-C., Pan, P.-T., Young, C.-C., Chang, J.-S., Chang, T.-C., Cheng, S.-S., 2011. Evaluation of the optimal strategy for *ex situ* bioremediation of diesel oil-contaminated soil. *Environmental Science and Pollution Research* 18, 1487–1496.

Liu, R., Dai, Y., Sun, L., 2015. Effect of rhizosphere enzymes on phytoremediation in PAH-contaminated soil using five plant species. *PLOS ONE* 10, e0120369.

Liu, R., Xiao, N., Wei, S., Zhao, L., An, J., 2014. Rhizosphere effects of PAH-contaminated soil phytoremediation using a special plant named Fire Phoenix. *Science of The Total Environment* 473–474, 350–358.

Liu, S.L., Cao, Z.H., Liu, H.E., 2013. Effect of ryegrass (*Lolium multiflorum* L.) growth on degradation of phenanthrene and enzyme activity in soil. *Plant, Soil and Environment* 59, 247–253.

Lobb, A.J., 2006. Potential for PAH contamination from clay target debris at shooting site: review of literature on occurrence of site contamination from clay targets: Report No. U06/81. Available at: https://api.ecan.govt.nz/TrimPublicAPI/documents/download/908516 (Accessed 1 May 2019.

Lu, H., Sun, J., Zhu, L., 2017. The role of artificial root exudate components in facilitating the degradation of pyrene in soil. *Scientific Reports* 7, 7130.

Lu, H., Wang, W., Li, F., Zhu, L., 2019. Mixed-surfactant-enhanced phytoremediation of PAHs in soil: Bioavailability of PAHs and responses of microbial community structure. *Science of The Total Environment* 653, 658–666.

Lugtenberg, B.J.J., Dekkers, L., Bloemberg, G.V., 2001. Molecular determinants of rhizosphere colonization by *Pseudomonas*. *Annual Review of Phytopathology*. 39, 461–490.

Ma, L., Deng, F., Yang, C., Guo, C., Dang, Z., 2018. Bioremediation of PAH-contaminated farmland: Field experiment. *Environmental Science and Pollution Research* 25, 64–72.

Magee, B.H., Forsberg, N.D., Meyer, A.K., 2018. Methods for deriving site-specific relative bioavailability factors from animal bioavailability data. Presented at the Society of Environmental Toxicology and Chemistry Europe 28th Annual Meeting, Rome, Italy.

Maila, M.P., Cloete, T.E., 2002. Germination of *Lepidium sativum* as a method to evaluate polycyclic aromatic hydrocarbons (PAHs) removal from contaminated soil. *International Biodeterioration & Biodegradation* 50, 107–113.

Meng, L., Qiao, M., Arp, H.P.H., 2011. Phytoremediation efficiency of a PAH-contaminated industrial soil using ryegrass, white clover, and celery as mono- and mixed cultures. *Journal of Soils and Sediments* 11, 482–490.

Moeskops, B., Sukristiyonubowo, Buchan, D., Sleutel, S., Herawaty, L., Husen, E., Saraswati, R., Setyorini, D., De Neve, S., 2010. Soil microbial communities and

activities under intensive organic and conventional vegetable farming in West Java, Indonesia. *Applied Soil Ecology* 45, 112–120.

Mohan, S.V., Kisa, T., Ohkuma, T., Kanaly, R.A., Shimizu, Y., 2006. Bioremediation technologies for treatment of PAH-contaminated soil and strategies to enhance process efficiency. *Reviews in Environmental Science and Bio/Technology* 5, 347–374.

Mougin, C., 2002. Bioremediation and phytoremediation of industrial PAH-polluted soils. *Polycyclic Aromatic Compounds* 22, 1011–1043.

Ni, H., Zhou, W., Zhu, L., 2014. Enhancing plant-microbe associated bioremediation of phenanthrene and pyrene contaminated soil by SDBS-Tween 80 mixed surfactants. *Journal of Environmental Sciences* 26, 1071–1079.

Olasanmi, O.I., Thring, W.R., 2018. The role of biosurfactants in the continued drive for environmental sustainability. *Sustainability* 10.

Peddicord, R.K., LaKind, J.S., 2000. Ecological and human health risks at an outdoor firing range. *Environmental Toxicology and Chemistry* 19, 2602–2613.

Perroy, R.L., Belby, C.S., Mertens, C.J., 2014. Mapping and modeling three dimensional lead contamination in the wetland sediments of a former trap-shooting range. *Science of The Total Environment* 487, 72–81.

Potin, O., Rafin, C., Veignie, E., 2004. Bioremediation of an aged polycyclic aromatic hydrocarbons (PAHs)-contaminated soil by filamentous fungi isolated from the soil. *International Biodeterioration & Biodegradation* 54, 45–52.

Pradhan, S.P., Conrad, J.R., Paterek, J.R., Srivastava, V.J., 1998. Potential of phytoremediation for treatment of PAHs in soil at MGP sites. *Journal of Soil Contamination* 7, 467–480.

Reilley, K.A., Banks, M.K., Schwab, A.P., 1996. Dissipation of polycyclic aromatic hydrocarbons in the rhizosphere. *Journal of Environmental Quality* 25, 212–219.

Riding, M.J., Doick, K.J., Martin, F.L., Jones, K.C., Semple, K.T., 2013. Chemical measures of bioavailability/bioaccessibility of PAHs in soil: Fundamentals to application. *Journal of Hazardous Materials* 261, 687–700.

Rodríguez-Seijo, A., Cachada, A., Gavina, A., Duarte, A.C., Vega, F.A., Andrade, M.L., Pereira, R., 2017. Lead and PAHs contamination of an old shooting range: A case study with a holistic approach. *Science of The Total Environment* 575, 367–377.

Rodríguez-Seijo, A., Lago-Vila, M., Andrade, M.L., Vega, F.A., 2016. Pb pollution in soils from a trap shooting range and the phytoremediation ability of *Agrostis capillaris* L. *Environmental Science and Pollution Research* 23, 1312–1323.

Rostami, S., Azhdarpoor, A., Rostami, M., Samaei, M.R., 2016. The effects of simultaneous application of plant growth regulators and bioaugmentation on improvement of phytoremediation of pyrene contaminated soils. *Chemosphere* 161, 219–223.

Schinner, F., Öhlinger, R., Kandeler, E., Margesin, R., 1996. Methods in soil biology. Springer-Verlag. Berlin, Germany.

Shen, R.F., Cai, H., Gong, W.H., 2006. Transgenic Bt cotton has no apparent effect on enzymatic activities or functional diversity of microbial communities in rhizosphere soil. *Plant and Soil* 285, 149–159.

Sikora, F., Moore, K., 2014. Soil test methods from the Southeastern United States. Available at http://aesl.ces.uga.edu/sera6/PUB/MethodsManualFinalSERA6.pdf. (Accessed 1 March 2019).

Soleimani, M., Afyuni, M., Hajabbasi, M.A., Nourbakhsh, F., Sabzalian, M.R., Christensen, J.H., 2010. Phytoremediation of an aged petroleum contaminated soil using endophyte infected and non-infected grasses. *Chemosphere* 81, 1084–1090.

Sun, M., Fu, D., Teng, Y., Shen, Y., Luo, Y., Li, Z., Christie, P., 2011. *In situ* phytoremediation of PAH-contaminated soil by intercropping alfalfa (*Medicago sativa* L.) with tall fescue (*Festuca arundinacea* Schreb.) and associated soil microbial activity. *Journal of Soils and Sediments* 11, 980–989.

Sun, T.-R., Cang, L., Wang, Q.-Y., Zhou, D.-M., Cheng, J.-M., Xu, H., 2010. Roles of abiotic losses, microbes, plant roots, and root exudates on phytoremediation of PAHs in a barren soil. *Journal of Hazardous Materials* 176, 919–925.

Synakorn, D., Glass, J.D., Lorenzetti, R.J., 2012. Kirtland air force base, New Mexico engineering evaluation/cost analysis (EE/CA) report military munitions response program (MMRP) sites TS775 and TS776. Available at: https://www.kirtland.af.mil/Portals/52/documents/AFD-120418-088.PDF?ver=2016-06-27-121411-607 (Accessed 1 March 2019.

Szulc, A., Ambrożewicz, D., Sydow, M., Ławniczak, Ł., Piotrowska-Cyplik, A., Marecik, R., Chrzanowski, Ł., 2014. The influence of bioaugmentation and biosurfactant addition on bioremediation efficiency of diesel-oil contaminated soil: Feasibility during field studies. *Journal of Environmental Management* 132, 121–128.

Thompson, O.A., Wolf, D.C., Mattice, J.D., Thoma, G.J., 2008. Influence of nitrogen addition and plant root parameters on phytoremediation of pyrene-contaminated Soil. *Water, Air, and Soil Pollution* 189, 37–47.

Trevors, J.T., 1996. Sterilization and inhibition of microbial activity in soil. *Journal of Microbiological Methods* 26, 53–59.

United States Environmental Protection Agency, 1996. Method 3050B. Available at: http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/3050b.pdf (Accessed 19 December 2018).

United States Environmental Protection Agency, 2018. Regional screening levels (RSLs) - generic tables. Available at: https://www.epa.gov/risk/regional-screening-levels-rsls-generic-tables (Accessed 1 May 2019).

United States National Shooting Sports Foundation, 2015. Sport shooting participation in the United States in 2014. Available at: http://www.armalot.com/wp-content/uploads/2016/08/NSSF-Sport-Shooting-Participation-Report-1.pdf (accessed 4 March 2019).

Wan, X., Tandy, S., Hockmann, K., Schulin, R., 2013. Changes in Sb speciation with waterlogging of shooting range soils and impacts on plant uptake. *Environmental Pollution* 172, 53–60.

Wang, Z., Liu, Z., Yang, Y., Li, T., Liu, M., 2012. Distribution of PAHs in tissues of wetland plants and the surrounding sediments in the Chongming wetland, Shanghai, China. *Chemosphere* 89, 221–227.

Wawra, A., Friesl-Hanl, W., Puschenreiter, M., Soja, G., Reichenauer, T., Roithner, C., Watzinger, A., 2018. Degradation of polycyclic aromatic hydrocarbons in a mixed contaminated soil supported by phytostabilisation, organic and inorganic soil additives. *Science of The Total Environment* 628–629, 1287–1295.

Wei, R., Ni, J., Li, X., Chen, W., Yang, Y., 2017. Dissipation and phytoremediation of polycyclic aromatic hydrocarbons in freshly spiked and long-term field-contaminated soils. *Environmental Science and Pollution Research* 24, 7994–8003.

Wen, J., McLaughlin, M.J., Stacey, S.P., Kirby, J.K., 2010. Is rhamnolipid biosurfactant useful in cadmium phytoextraction? *Journal of Soils and Sediments* 10, 1289–1299.

Wilcke, W., 2007. Global patterns of polycyclic aromatic hydrocarbons (PAHs) in soil. *Geoderma* 141, 157–166.

Wolf, D.C., Brye, K.R., Gbur, E.E., 2015. Using soil amendments to increase bermuda grass growth in soil contaminated with hydraulic fracturing drilling fluid. *Soil and Sediment Contamination: An International Journal* 24, 846–864.

Wolf, D.C., Gan, J., 2018. Influence of rhamnolipid biosurfactant and Brij-35 synthetic surfactant on ^{14}C-Pyrene mineralization in soil. *Environmental Pollution* 243, 1846–1853.

Worthington, K., Worthington, V., 2011. Worthington enzyme manual. Available at: http://www.worthington-biochem.com/pap/default.html (Accessed 1 May 2019).

Yu, X.Z., Wu, S.C., Wu, F.Y., Wong, M.H., 2011. Enhanced dissipation of PAHs from soil using mycorrhizal ryegrass and PAH-degrading bacteria. *Journal of Hazardous Materials* 186, 1206–1217.

Zhao, B., Zhu, L., Li, W., Chen, B., 2005. Solubilization and biodegradation of phenanthrene in mixed anionic–nonionic surfactant solutions. *Chemosphere* 58, 33–40.

Chapter 5 Conclusions and Future Work

5.1 Conclusions

The primary goal of this work was to investigate the biotic and abiotic mechanisms and interactions to improve bioremediation effectiveness of PAH-contaminated soils using "green" bioremediation technologies such as biosurfactant amendment, bioaugmentation, and phytoremediation. Conclusions for the specific objectives of this dissertation are given below:

Objective 1: Compare the effect of Brij-35 synthetic surfactant and rhamnolipid biosurfactant and *M. vanbaalenii* PYR-1 bioaugmentation on PAH biodegradation in different soils.

Due to hydrophobicity and environmental recalcitrance of PAHs in soils, the use of synthetic surfactants to increase the bioavailability of PAHs to degrading soil microbes is a common remediation procedure. However, the application biosurfactants in PAH-contaminated soils is relatively limited, even though it has the potential to provide an environmentally-friendly alternative to synthetic surfactants. The application is the Brij-35 nonionic surfactant and rhamnolipid biosurfactant was compared in two soils to potentially increase the bioavailable ^{14}C-pyrene concentrations to PAH-degrading microorganisms and enhance biodegradation. Compared to the native control treatments, the addition of Brij-35 at all three rates in the clay soil resulted in a decreased lag period and the medium and high amendment rates led to greater ^{14}C-pyrene mineralization after

50 d. In the sandy loam treatments, the addition of Brij-35 at all rates enhanced ^{14}C-pyrene biodegradation. Conversely, rhamnolipid biosurfactant amendment at all three rates in both soils inhibited ^{14}C-pyrene mineralization in a dose-dependent manner. The bioaugmentation of *M. vanbaalenii* PYR-1 in the unamended and Brij-35-amended treatments decreased the lag phase and enhanced ^{14}C-pyrene degradation in both PAH-contaminated soils. However, the addition of rhamnolipid biosurfactant again inhibited ^{14}C-pyrene mineralization in both bioaugmented soils in a dose-dependent manner. Findings from this study highlight the complex nature of the interactions between surfactants and the soil microbial community responsible for PAH degradation. As shown by this study, the external addition of biosurfactants to soil contaminated with PAHs may not affect the biodegradation of PAHs in a straightforward manner as the degrading soil microbes may preferentially use the biosurfactants as substrates rather than the target contaminant. Subsequently, a surfactant that is resistant to microbial degradation and nontoxic to soil microbes, such as Brij-35, may be considered more effective in stimulating PAH biodegradation. In practical applications of biosurfactant-enhanced remediation, the degradability of the biosurfactant to the native soil microbial community must be evaluated prior to full-scale implementation for successful PAH bioremediation.

Objective 2: Evaluate the effect of Brij-35 and rhamnolipid biosurfactant amendment and *M. vanbaalenii* PYR-1 bioaugmentation on the native soil microbes associated with PAH biodegradation.

Since aerobic PAH degradation by soil bacteria is considered the primary PAH degradation pathway in soils, the differences in ^{14}C-pyrene mineralization from Objective 1 likely resulted from changes in the soil microbial communities due to surfactant addition or *M. vanbaalenii* PYR-1 bioaugmentation. In this study, the soil microbial communities were analyzed using 16S rRNA high-throughput sequencing and PAH-degrading soil microbes were evaluated using PICRUSt. The addition of pyrene in both soils resulted in a significant increase in *Firmicutes,* a phylum associated with PAH degradation, and a decrease in microbial richness compared to the untreated soils. The negative effect of rhamnolipid biosurfactant amendment on PAH degradation in both soils could also be observed through 16S rRNA analysis. For example, in the native and bioaugmented clay soils, the relative abundance of *Bacillus* in the biosurfactant-amended treatments was substantially lower than the unamended treatments. Additionally, the native clay soil treatment amended with rhamnolipid biosurfactant at the high rate that had less than 1% ^{14}C-pyrene mineralization was dominated by *Mycoplana* sp. which was not capable of degrading pyrene. Through 16S rRNA analysis, the bioaugmentation of *M. vanbaalenii* PYR-1 was shown to survive initial soil inoculation and enhance ^{14}C-pyrene degradation. Through PICRUSt analysis, it was determined that the bioaugmentation of *M. vanbaalenii* PYR-1 resulted in increased PAH-degrading functional genes in both soils compared to the native soils. Although the mechanisms behind surfactant-induced shifts in the soil microbial community is still unclear, findings from this study highlight the importance of assessing the soil microbial community dynamics prior to full-scale PAH bioremediation implementation. For example, the contaminated soil could contain

substantial populations of PAH-degrading soil microbes, and therefore the appropriate PAH bioremediation procedure might be biostimulation to increase PAH-degrading soil microbial activity, which could be considerably more economical compared to the other remediation techniques.

Objective 3: Assess the interactions of phytoremediation (bermudagrass and switchgrass), *M. vanbaalenii* PYR-1 bioaugmentation, and surfactant amendment (Brij-35, rhamnolipid biosurfactant, and sodium dodecyl sulfate/Brij-35 mixture) on the remediation of PAH-contaminated soil due to accumulation of clay target debris from an outdoor shooting range.

Phytoremediation is an effective *in situ* remediation technology for PAH-contaminated soils because of the soil microbial enrichment in the root rhizosphere in addition to the secretion of root exudates that release enzymes critical to PAH dissipation and enhance PAH bioavailability. Polycyclic aromatic hydrocarbon pollution typically occurs at high concentrations over a large area (e.g., industrial MGP or coal combustion sites). One extreme example of high concentrations of PAH contamination occurring over a large area, but is often overlooked, is the mechanical weathering of clay target fragments that are comprised of coal tar or petroleum pitch as a binding agent that may incorporate into the soil at shooting ranges and form a long-term source of HMW PAHs in the environment. An 8-month remediation study was performed using bermudagrass or switchgrass in addition to *M. vanbaalenii* PYR-1 bioaugmentation and/or surfactant amendment in a PAH-contaminated soil collected from an abandoned outdoor shooting

range. Both vegetation treatments increased PAH dissipation compared to the non-vegetated treatments. The use of vegetation also resulted in increased polyphenol oxidase soil activity that is responsible for the hydroxylation of phenols to catechols and dehydrogenation of catecholes to quinones. Bioaugmentation of *M. vanbaalenii* PYR-1 enhanced HMW PAH degradation, but had no effect on LMW PAH dissipation as compared to the non-inoculated treatments. In the non-vegetated treatments, Brij-35/SDS surfactant mixture application was more effective than the use of individual surfactants. The enhanced PAH biodegradation was accompanied with improved lettuce seed germination in the soils that had undergone vegetation and bioaugmentation treatments. The results of this study highlight that an integrated approach using multiple remediation techniques such as combining phytoremediation and bioaugmentation has the potential to alleviate the limitations of individual bioremediation approaches and can be an effective *in situ* bioremediation option for aged, HMW PAH-dominated soils such as those from outdoor shooting ranges.

5.2 Recommendations for Future Work

Due to the human and environmental toxicity of PAHs in addition to their environmental recalcitrance and ubiquity that will only be further exacerbated as industrialization and associated anthropogenic pollution continues to increase, efficient remediation techniques must be developed. Several bioremediation-enhancement technologies have become available for increasing PAH bioavailability and/or soil microbial abundance and activity, which include surfactant amendment, bioaugmentation

of known PAH-degrading microorganisms, and phytoremediation, to overcome the bioavailability limitations and environmental stability of PAHs. However, bioremediation is a complex process involving numerous abiotic and biotic interactions that can result in the inhibition of soil microbial activity and lead to ineffective PAH degradation. Therefore, the effects of various bioremediation techniques that are commonly used for PAH-contaminated soils must be better understood to address these issues and determine effective PAH remediation technologies and their effects on the soil microbial community to ensure the protection of the environment and human health. While bench-scale and growth chamber experiments were used in this research effort to investigate the mechanisms of different PAH bioremediation treatments and their interactions, the results from this dissertation project bring important information for future full-scale applications that involve environmental heterogeneities (e.g., PAH distribution and bioavailable concentrations, soil microbial community abundance and activity fluctuations, and various soil characteristics) that are present at real-world PAH contamination sites. Based on the results of this dissertation research, the use of vegetation such as bermudagrass with surfactant amendment or bioaugmentation of PAH-degrading microorganisms such as *M. vanbaalenii* PYR-1 should be evaluated in a large-scale PAH-contaminated field site.

This dissertation research has shown that molecular tools such as 16S rRNA high-throughput sequencing and PICRUSt should be used before bioremediation implementation as these tools provide valuable information for the design and implementation of *in situ* bioremediation technologies at PAH-contaminated sites.

Although more expensive, future work should also include shotgun metagenomic analysis as this method can be used to identify sequences of fungi, which cannot be identified through 16S rRNA analysis. Fungi such as white rot fungi have also been shown to degrade PAHs and recent bioremediation operations have considered consortia of bacteria, bacteria and fungi, or bacteria and algae with successful results, although the mechanisms require further examination.