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Bioremediation and Phytoremediation Systems for Breaking Down Total Petroleum Hydrocarbons (TPH) in Contaminated Sandy Soil

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Bioremediation and Phytoremediation Systems for
Breaking Down Total Petroleum Hydrocarbons (TPH) in
Contaminated Sandy Soil

Patrick M. McIntosh, B.S., B.S.

University of Connecticut 2008

A Thesis

Presented in Partial Fulfillment for the Requirements for

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2014

APPROVAL PAGE
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Bioremediation and Phytoremediation Systems for
Breaking Down Total Petroleum Hydrocarbons (TPH) in
Contaminated Sandy Soil

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2014

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Bioremediation and Phytoremediation Systems for Breaking Down Total Petroleum Hydrocarbons (TPH) in Contaminated Sandy Soil

ABSTRACT

The applications of bio- and phytoremediation systems were used to breakdown low-level total petroleum hydrocarbons (TPH) in a contaminated sandy soil. The overall aim was to determine which soil regimes, amendments, and plant species enhance the breakdown of TPH. The first project included planted mixtures of cool and warm-season grasses with different willows, mono and intercropped with various fertilizer and carbon additions. The second project introduced different soil regimes of saturation, intermittent oxygenation, installment of aboveground covers, as well as the addition of switchgrass and fertilizer. The reduction of TPH in both projects was measured by using gas chromatography, and indigenous microbes quantified by bacterial colony-forming units (CFU). Phytoremediation of low-level TPH contamination was observed in treatments with cool and warm seasons grasses and/or willow combinations, or through fertilization of the soil with NPK. The statistical analysis indicated that having plant species present with a fertilizer amendment was the most efficient method for breaking down aged TPH. There was no statistical significance between grasses and willows in their efficiency to degrade hydrocarbons. Furthermore, during the second experiment bioremediation was more advantageous than phytoremediation when the freshly spiked contaminant was added to sandy

soils. Optimizing the system with soil amendments (soil moisture, air supply, and fertilizers) generated favorable conditions for breaking down TPH.

LIST OF ACRONYMS

ACE	Acenaphthene
ACY	Acenaphthylene
ANOVA	Analysis of Variance
ANT	Anthracene
As	Arsenic
ASTDR	Agency for Toxic Substances and Disease Registry
Ba	Barium
BAA	Benz[<i>a</i>]anthracene
BAP	Benzo[<i>a</i>]pyrene
BBF	Benzo[<i>b</i>]fluoranthene
BKF	Benzo[<i>k</i>]fluoranthene
BPL	Benzo[<i>ghi</i>]perylene
BTEX	Benzene toluene ethylene and xylene
°C	Celsius
°C/min	Celsius Per Minute
C	Control treatment
CC	Covered Control treatment
Cd	Cadmium
CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act
CFUs	Colony Forming Units
CFU g ⁻¹	Colony Forming Units per gram of soil
CHR	Chrysene
cm	Centimeter
cm/sec	Centimeter Per Second
Cr	Chromium
CRD	Complete Random Design
CSG	Cool Season Grasses
CT	Connecticut
CTDEEP	Connecticut Department of Energy and Environmental Protection
DBA	Dibenz[<i>a,h</i>] anthracene
DCM	Dichloromethane

DDT	Dichlorodiphenyltrichloroethane
DI	Deionized Water
E	Exponential
EC	Electrical Conductivity
EPA	Environmental Protection Agency
F	Fertilized Treatment
FID	Flame Ionization Detector
FLR	Fluoranthene
FLU	Fluorine
FM	Fertilized and Molasses Treatment
g	Gram
g/kg	Gram Per Kilogram
g/L	Grams Per Liter
g/pot	Gram Per Pot
g pot ⁻¹	Gram Per Pot
GLM	General Linear Models
GC	Gas Chromatography
GF	Grass Fertilized Treatment
GFO	Grass Fertilized and Oxygenated Treatment
ha	Hectare
HFC	Hydrogen – Fluorine – Carbon Atoms
Hg	Mercury
LEP	Licensed Environmental Professional
IARC	International Agency for Research on Cancer
IPY	Indeno[1,2,3- <i>cd</i>]pyrene
kg	Kilogram
kg/pot	Kilogram Per Pot
LSD	Least Significant Difference
LUST	Leaky Underground Storage Tanks
m	Meter
μL	Microliter
mg/kg	Milligram per Kilogram
mg kg ⁻¹	Milligram per Kilogram
mL	Milliliter
mL/min	Milliliter Per Minute
mm	Millimeter
mS/cm	Millisiemens Per Centimeter
n	Number of Replications
N ₂	Nitrogen Gas
Na ₂ SO ₄	Anhydrous Sodium Sulfate
NAP	Naphthalene
NAPLs	Non-Aqueous Phase Liquids
NHRY	New Haven Rail Yard
N-P-K	Nitrogen Phosphorous Potassium
O	Oxygenated Treatment
p	Probability

PAHs	Polycyclic Aromatic Hydrocarbons
Pb	Lead
PCB	Polychlorinated Biphenyls
pH	Power of Hydrogen
PHE	Phenanthrene
PPM	Parts Per Million
PR	Perennial Ryegrass
psi	Pounds Per Square Inch
PYR	Pyrene
RCBD	Random Complete Block Design
RCRA	Resource Conservation and Recovery Act
<i>S</i>	<i>Salix</i>
S	Saturated Treatment
SC	Saturated Covered Treatment
Sd	<i>Salix dasyclados</i>
Se	<i>Salix ericophela</i>
SF	Sheep Fescue
SPF	Separatory Funnel
SG	Switchgrass
SGSd	Switchgrass/ <i>Salix dasyclados</i>
Sm	<i>Salix miyabeana</i>
Sp	<i>Salix purpurea</i>
TCE	Trichloroethylene
TF	Tall Fescue
TNT	Trinitrotoluene
TPH	Total Petroleum Hydrocarbons
T-RFLP	Terminal Restriction Fragment Length Polymorphism
USDA	United States Department of Agriculture
VOC	Volatile Organic Carbon
WSG	Warm Season Grasses
Zn	Zinc

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CHAPTER I

Introduction

1.1 Background

Natural and anthropogenic contamination polluting air, soil, and water resources are increasing daily because of industrialization, urbanization, and growth of the human population (Shukla et al., 2010). Hazardous waste such as inorganic metals (As, Ba, Cd, Cr, Hg, Pb, Zn), organic pollutants [herbicides (Atrazine), insecticides (DDT), polychlorinated biphenyls (PCB), volatile organic carbons (VOC), total petroleum hydrocarbons (TPH) including polycyclic aromatic hydrocarbons (PAH)], and radionuclides contaminate ecological communities and transform landscapes to ecologically degraded and unusable spaces in society (Pilon-Smits, 2005; Walker, 2009). Complexity in assessing and remediating these impacted locations is derived from the difficulties with economic, regulatory, and scientific contention towards environmental resources (Weisman, 1998). The organic pollutant TPH, has gained more research attention in recent decades because of its persistence in nature, overall health risks to the human population, and complications in remediation of rural and urban environments.

The TPH contaminants can enter ones body through touch, swallowing of contaminated water or food, and breathing it in air. The TPH is taken in most rapidly through air into the bloodstream where the contaminant is transformed into different harmful constituents, and a majority may be released from the body in either urine or exhaling air. There is no medical test to determine extent of exposure, however TPH compounds affect the body in different ways. Most TPH derivatives such as xylene, toluene, and benzene have a damaging effect on various organs, as well as the immune and central nervous systems. Benzene in particular has been shown to cause cancer and determined by the International Agency for Research on Cancer

(IARC) to be carcinogenic. However, mineral oils are non toxic TPH compounds used in food (Todd et al., 1999). To preserve human health and protect the public from TPH exposure, phytoremediation technology, which implies the use of plants to sequester, volatilize, or degrade soil organic pollutants, is a low-cost, solar driven, environmentally sustainable alternative to current costly practices of excavation and incineration of contaminated soil (Pilon-Smits, 2005).

Phytostimulation technology, a subset of phytoremediation, uses roots and associated microbes within the rhizosphere to degrade hydrophobic pollutants into smaller inert forms of contamination (Pilon-Smits, 2005). Microbiological activity in the rhizosphere enhances mineral and water uptake, promotes plant and root development, while decomposing organic material (Sylvia et al., 1998). Fast growing plant species with high biomass and vigorous root system are more advantageous for microbial growth and contaminant degradation due to the increase in root surface area. However, there are challenges in using this technology that can draw concern toward application and implementation.

The limitations to phytostimulation include various factors such as species dependent toxicity level of the contamination, climate, soil moisture, soil texture, organic matter, and pH (Pilon-Smits, 2005). The efficiency of this technology is limited by the extent of plant root depth and microbiological activation within the rhizosphere. Phreatophytes have extensive deep root systems that can target polluted deep soil and groundwater contamination. Other limiting factors depend on the bioavailability of the pollutants. If only a fraction of the pollutant is available to be remediated, this may not comply with regulatory cleanup criteria. Also, phytoremediation of organic contamination could take months or years to achieve certain levels required by state and federal standards (Pilon-Smits, 2005).

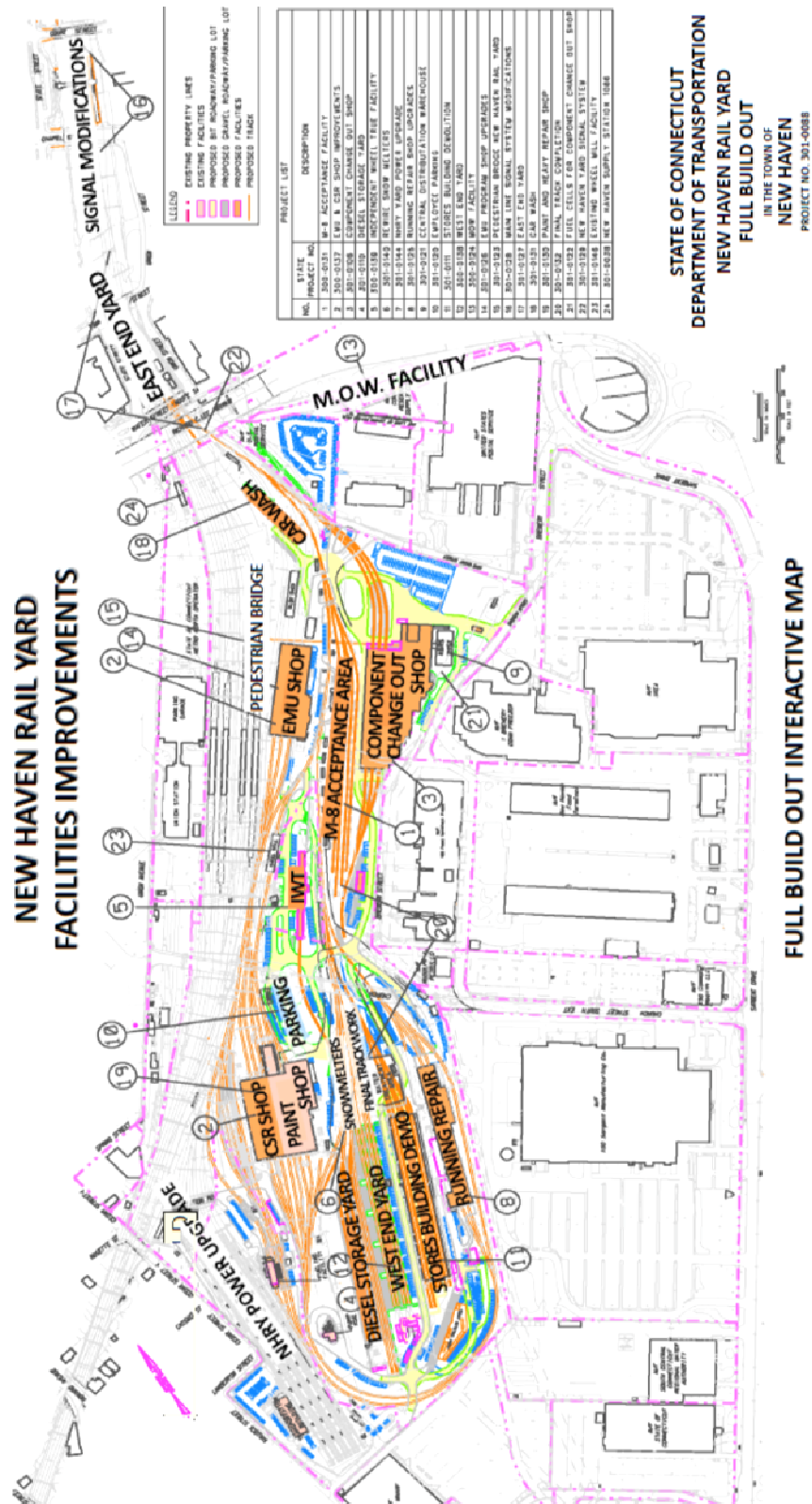
1.2 Problem Statement

New Haven Rail Yard (NHRV)

Since the late 1800s, the NHRV has been a pioneer for freight and passenger rail services, and by the early 1900s was a construction site for freight cars, repairs, and maintenance on locomotives. During these times and until 1985 on the 74-acre lot that contains the NHRV, at least 8 industrial warehouse-type and numerous other smaller buildings were constructed which processed and released hazardous waste material. The NHRV property was first listed under the Federal Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) in 1989, and as a small quantity generator of hazardous waste under Resource Conservation and Recovery Act (RCRA). This site had no Leaking Underground Storage Tank (LUST) records; however, during its history it had multiple 10,000 gallon gasoline or diesel fuel tanks removed before CERCLA Preliminary Assessment in 1989, and this rail yard was listed to have PCBs released to the soil. Since 1990 to present over 50 spills, primarily diesel fuel, were recorded that were assigned a high risk designation due to the visible, noted, and suspected chemical releases and on-site activities (NHRV, 2008).

With help from Licensed Environmental Professionals (LEP) at NHRV and Connecticut Department of Energy and Environmental Protection (CTDEEP) we received approximately two tons of sandy soil contaminated with TPH, which was used for these ex-situ remediation studies. The site from which the soil was taken had multiple diesel fuel discharges over several decades, while also having many underground storage containers that leaked. The course of action in response to the cleanup of this location is excavation and incineration. To study the ability of different grasses and willows to breakdown TPH, an ex-situ greenhouse study was conducted with the soil from the NHRV. Plant species were evaluated for suitability and survivability in the

contaminated sand based soil. *Poaceae* (grasses) species were selected based upon attributes of quick establishment, tolerance to different environmental conditions, and ability of their root-associated microorganisms to degrade petroleum hydrocarbons. *Salix* (woody plants) species were chosen for their high biomass and extensive root systems, large diversity of microbial communities, and high tolerance to inorganic and organic constituents. Furthermore, we analyzed which vegetated and unvegetated conditions of saturation, intermittent oxygenation, installment of aboveground covers, as well as the addition of switchgrass and fertilizer, have the most profound effect on degrading TPH in sandy soils.



1.3 Objectives and Hypothesis

The primary goal of this study was to identify which bio- or phytoremediation system will best remediate existing contamination in the soil from the rail yard. Objectives of this study included:

- To establish which grass and willow species, singular and in combination would be the best to degrade TPH.
- To determine which soil moisture regimes, fertilizer, and/or carbon amendment can be used to enhance TPH remediation.
- To surmise the effect of bio- and phytoremediation systems on different stages of aged contaminants.

A more inclusive understanding of soil-plant-water-contaminant interactions within polluted rail yard media will be gained from this research work. We hypothesize that the addition of plants and soil amendments will enhance contaminant degradation.

1.4 Overall Structure

Chapter 2 of this thesis is a comprehensive literature review that describes TPH origins, derivatives and fate in the soil. This review also describes various bio- and phytoremediation mechanisms responsible for different methods of contaminant removal. Chapter 3 and Chapter 4 outlines research conducted to answer the current objectives. Chapter 3 describes the first year experiment regarding degradation of low level TPH in a contaminated sandy soil using grasses and willows with and without soil amendments. Chapter 4 describes the second year project targeting bio- and phytoremediation of TPH using different soil regimes. Chapter 5 states conclusions and future research directions.

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CHAPTER II

Literature Review

2.1 Soil Composition

Soil is classified as a natural dynamic open system composed of three main phases: gases, liquids, and both mineral and organic solids. These phases also coincide with living organisms to serve as the medium to support plant life (Brady and Weil, 2002). Physical, mineralogical, and chemical properties of soils reflect the ability of the medium to be classified into different groups (Buol et al., 2003). These groups are also divided by different soil textures of sand, silt, and clay. The space between soil particles supports aerobic and anaerobic microorganism functions and survival. Within thin water films in soil pore spaces is where oxygen is present and replenished through diffusion to support aerobic microbes, as well as where these spaces support anaerobic microbes when saturated. In both aerobic and anaerobic pore filled regimes, plants and microorganisms can assimilate nutrients.

Soil organic matter retains nutrients and water retention to preserve soil structure (Willey et al., 2011). In some cases organic matter can exceed the inorganic fractions within the soil medium, where by this process is influenced by climate, biotic activities, topography, and time (Brady and Weil, 2002). Living organisms generate plant litter, root exudates, animal wastes and microbial polysaccharides to produce part of the organic matter in soil. The remainder of organic material is comprised of lysed microbial cells, animal carcasses, and dead plant debris (Davet, 2004). The soil microbial community that exists in soils consist of microflora (which includes bacteria, actinomycetes, fungi, and algae) and macrofauna (which includes nematodes, earthworms, and other larger organisms) (Sylvia et al., 1998).

2.2 Different Types of Organic Contamination

The majority of organic contaminants are carbon chain sequences that are independent, ring constructed, and/or with the addition of elemental halides with different axial formations (Mahanty et al., 2010). These carbon-based derivatives can be carcinogenic, causing problems to human health. There are many non-carcinogenic compounds generated as well, and these pollutants cause potential health hazards when in excessive amounts in soil and water.

Organic contamination occurs in many formulations, such as naturally formed in the environment or anthropogenically produced from industry or other manufacturing processes. Organic contaminants exist in solid, liquid, or volatile gas phases. These compounds generally persist for long periods of time in our environment, bonding within the soil medium, or adhering to surface or groundwater. They have the ability to resist dissipation into the environment due to their strong chemical bond properties (Walker, 2009).

Many organic pollutants contaminate soil and ground water resources within the environment. Some common organic pollutants that have been observed to contaminate the environment and cause health problems to humans include herbicides (atrazine), insecticides (DDT), trichloroethylene (TCE), munitions waste such as (trinitrotoluene (TNT)), polychlorinated biphenyls (PCB), volatile organic carbons (VOC), and total petroleum hydrocarbons (TPH). The TPHs include benzene, toluene, ethylbenzene, and xylene (BTEX) and more recalcitrant derivatives, such as polycyclic aromatic hydrocarbons (PAH) (Todd et al., 1999, Walker, 2009, Nwoko, 2013).

Herbicides (Atrazine)

The herbicide atrazine (2-chloro, 4-ethylamino, 6-isopropylamino, 1,3,5-triazine), (Figure 2.1) was used in agriculture as a non-selective weed control on waste land (Singh and Cameotra, 2014), as well as on sugarcane and corn fields to control broad-leaf weeds (Sagarkar et al., 2014). Atrazine has the ability to affect both the nervous, immune, and endocrine systems within the human body (Sagarkar et al., 2014). Residues of atrazine and its metabolites have been contaminating soil, surface and groundwater due to these compounds' long half-life and mobility within the soil medium (Zhang et al., 2014). Microbes *Acinetobacter*, *Agrobacterium*, *Arthrobacter*, *Nocardioides*, *Pseudomonas*, and *Rhodococcus* have been identified for removal of this harmful organic contaminate by utilizing atrazine as a carbon source (Singh and Cameotra, 2014). The efficacy of combinations of different microbial species rather than single monocultures for enhanced microbial degradation of atrazine are not yet known (Zhang et al., 2014).

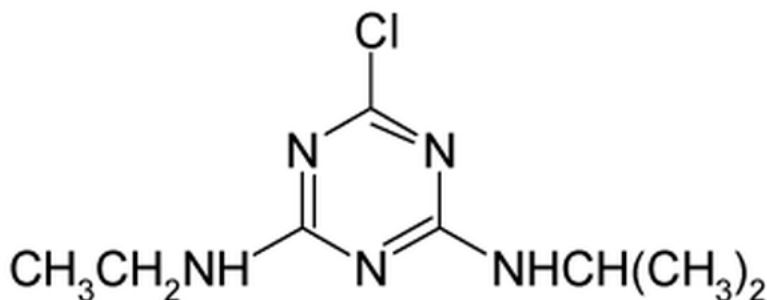


Figure 2.1: The chemical structure of atrazine (Sene et al., 2010).

Insecticides (DDT)

The insecticide DDT (1, 1, 1-trichloro-2, 2-bis (p-chlorophenyl) ethane) was created in the 1930s and was used worldwide to control pests on agriculture plants (Ortíz et al., 2013). Currently DDT (Figure 2.2) is still in use in Africa to control malaria due to its low cost. However, exposure to DDT causes endocrine disruption, liver damage and cancer (hepatocarcinogenesis) in humans and animals (Huang and Wang, 2013). Current research is studying the use of fungi for breaking down DDT into less harmful derivatives while enriching the soil medium (Huang and Wang, 2013). The causes of natural anaerobic degradation of DDT in marine sediments from historical residues by different microbial species is also being investigated (Da et al., 2014). The use of other remediation methods to remove DDT and other organochlorine pesticides are still being researched (Liang et al., 2014).

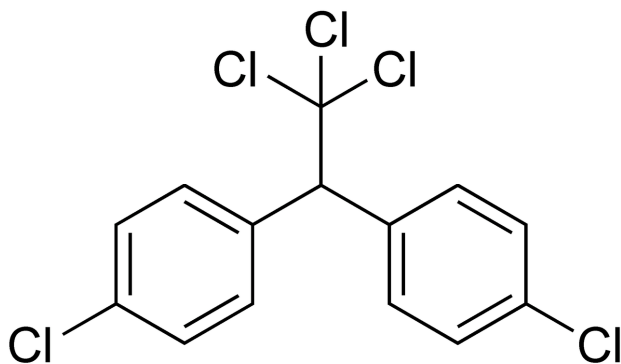


Figure 2.2: The chemical structure of DDT. (Organochlorine Pesticides, 2014)

Trichloroethylene (TCE)

Trichloroethylene (TCE) (Figure 2.3) was produced for large scale industrial uses in the chemical industry (Megharaj et al., 2011). This chemical was imported in the tens of millions of pounds into the United States between the years 2000 to 2004 as a metal degreaser, but mostly as a feedstock for HFC-134a refrigerant (ASTDR, 2013). It has polluted environments worldwide including surface and ground water due to improper management (Chen et al., 2014). Reviews of different remediation efforts have shown plants are effective in the removal of linear halogenated hydrocarbon compounds such as TCE in the environment (Nwoko, 2013).

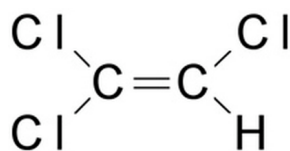


Figure 2.3: The chemical structure of trichloroethylene (TCE) (EPA, 2011).

Munitions Waste: Trinitrotoluene (TNT)

One persistent toxic organic compound 2,4,6-trinitrotoluene (TNT) is a nitroaromatic explosive that has been used for more than 100 years (Figure 2.4). Former explosives and ammunition plants, as well as military areas are the sources of soil contamination and ground water pollution from TNT (Nölvak et al., 2013). Many different approaches to enhance microbial activity to breakdown TNT have been tested, and at least one review discusses the use of transgenic plants derived from microbial pollutant-degrading genes (Abhillash et al., 2012). Bacteria under aerobic and anaerobic conditions with *in-situ* approaches can break down TNT, however remediation with fungi can only be done *ex-situ* with the addition of supplemental nutrients (Nölvak et al., 2013).

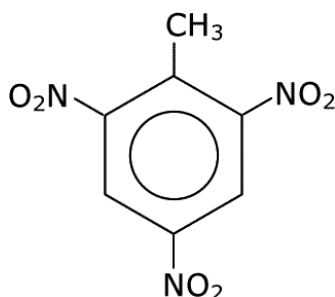


Figure 2.4: The chemical structure of 2,4,6-trinitrotoluene (TNT) (Rylott et al., 2010).

Volatile Organic Carbons (VOCs)

Volatile organic carbons (VOCs) are a vast array of various families of compounds encompassing benzene, toluene, ethylbenzene, and xylene from aromatic hydrocarbons, and TCE and tetrachloroethylene from chlorinated solvents (Meyer-Monath et al., 2014). These VOCs are emitted from industrial sources, most notably petrochemical plants and petroleum refineries, as well as from vehicular traffic that affect atmospheric quality. Eighty percent of benzene emissions and 35% of the total VOCs in the atmosphere are from vehicle emissions and losses due to evaporation (Lerner et al., 2014). Toluene in gasoline and paints, as well as ethylbenzene directly affect indoor air quality (Sriprapat et al., 2014). Many remediation methods are being researched to reduce VOC's presence since they have been linked to problems with the liver, kidneys, nervous and respiratory systems as well as fetotoxic and embryotoxic effects in humans (Meyer-Monath et al., 2014; Sriprapat et al., 2014). Continuing research has been using different plant species to remove VOCs to improve indoor and outdoor air quality (Nwoko, 2013; Sriprapat et al., 2014).

Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) were first produced for commercial sale around the 1930s (Figure 2.5). These organic pollutants act as very stable viscous liquids of low electrical conductivity and vapor pressure, and were used as lubricating oils, plasticizers in paints, and in maintenance and operation of hydraulic systems (Walker, 2009). The organic pollutants PCBs are not very soluble in water, but are instead very soluble in organic solvents. As a result, in soil PCBs can strongly adsorb to organic matter and directly affect the food chain through bioaccumulation (Kacálková and Tlustoš, 2011). Highly chlorinated compounds of PCBs are harmful to humans. They degrade slowly and have more chlorine existing on the dual ring structured compound than other chlorinated solvents (Megharaj et al., 2011). The uptake of these compounds by plants has been researched in a few studies. Kacálková and Tlustoš, (2011) found that the highest uptake of PCBs was observed in the roots of maize and sunflower (Kacálková and Tlustoš, 2011).

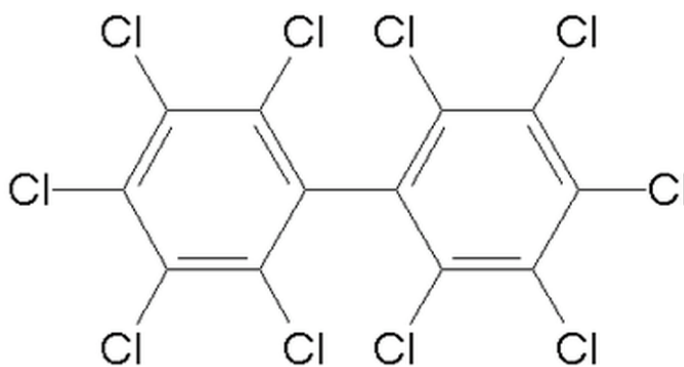


Figure 2.5: The chemical structure of a polychlorinated biphenyls (PCB). (Fiedler, H., 2013)

2.3 Total Petroleum Hydrocarbons

Total petroleum hydrocarbons (TPH) is the term associated with environmental sampling and analytical results defined as the gross quantity of measurable petroleum based hydrocarbons (Todd et al., 1999). Total petroleum hydrocarbons are aliphatic and aromatic petroleum-based hydrocarbon molecules with different composition and axial orientations. These contaminants are derived from mixtures of fuels and crude oil byproducts released into the environment through mining, manufacturing procedures, and accidental spills. Carbon fractions can be identified analytically by their relative boiling points. Aliphatic (open-chained carbon structures) petroleum hydrocarbons consist of hexane, gasoline, kerosene, and mineral oils. Aromatic (ringed) petroleum hydrocarbons consist of lower molecular weight compounds, such as benzene, toluene, ethylbenzene, and xylene (BTEX), as well as higher molecular weight lubricants, greases, and polycyclic aromatic hydrocarbons (PAHs) that are recalcitrant to natural attenuation (Todd et al., 1999). These constituents of TPH have a vast array of resilient compounds that can cause carcinogenic and mutagenic effects in humans (Todd et al., 1999).

Fate of TPH Contamination

The TPH contaminants exist in solid, liquid, or volatile gas phases, and have the ability to adhere to mineral fractions and organic matter (Figure 2.6). Organic pollutants with higher molecular weights can be highly recalcitrant to volatilization or removal, whereas lower molecular weight carbon derivatives have low boiling points and volatilize more rapidly, making the constituents easier to break down (Walker, 2009). Over time, recalcitrant fractions become non-extractable as bioavailability of the contaminant decreases (Figure 2.7) (Semple et al., 2003).

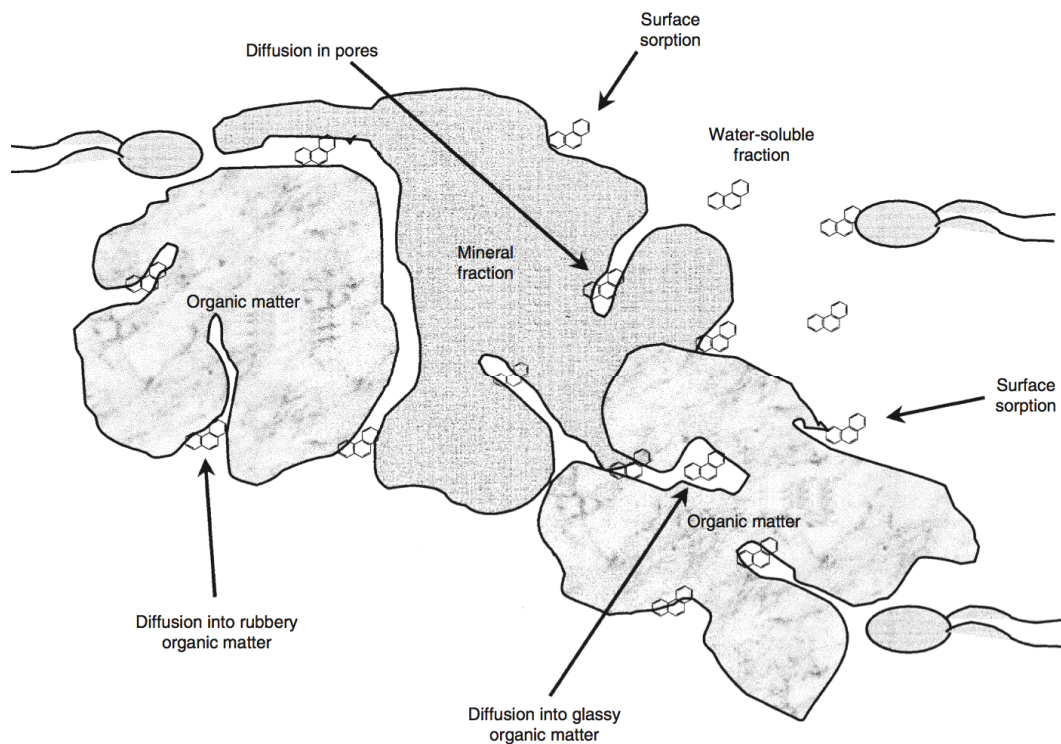


Figure 2.6: Organic contaminant TPH's physical behavior within soils (Semple et al., 2003).

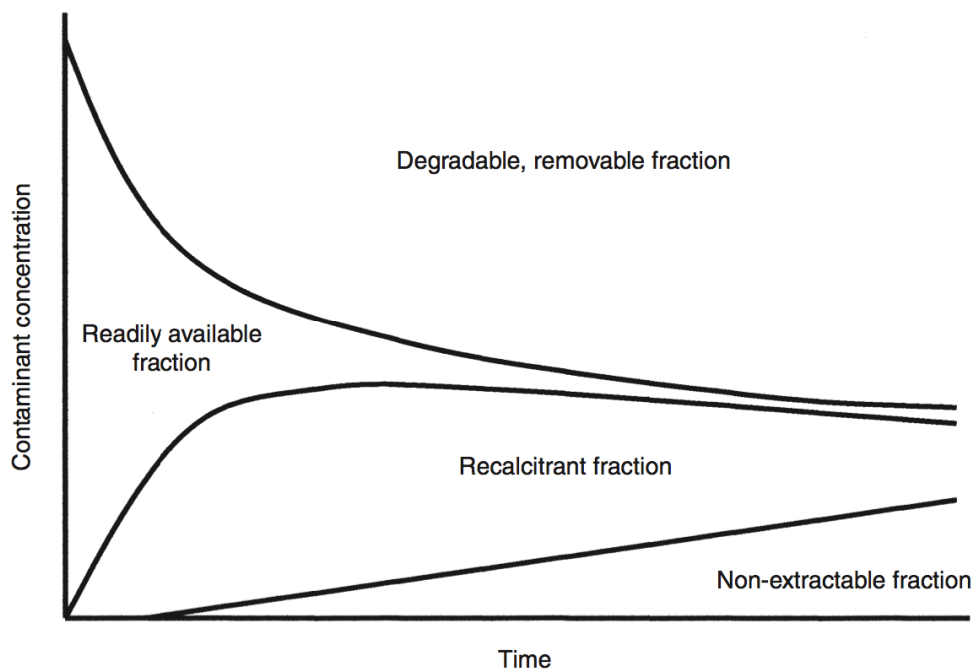


Figure 2.7: Contaminates bioavailability and extractability through the effect of contact time within soil (Semple et al., 2003).

Non-aqueous phase liquids (NAPL) are mixtures of hydrocarbon compounds that usually form liquid or semi-liquid sludges such as diesel fuel or oil that are immiscible in water (De Jonge et al., 1997; Todd et al., 1999). The NAPLs proliferate extensively within the environment, and are mainly present as dispersed droplets or a film layer on soil particles (De Jonge et al., 1997; Namkoong et al., 2002). Bioavailability and degradation rate of NAPL depends on the TPH constituent's size, which directly affects their solubility in water (De Jonge et al., 1997; Namkoong et al., 2002). Since NAPLs adhere to mineral and organic soil particles, they have a greater affinity to bind to the soil substrate and become more obstinate to transformation or degradation (Semple et al., 2003; Coulon et al., 2010). Additionally, their bioavailability is reduced by denaturing into more recalcitrant and persistent carbon derivatives through physicochemical processes, such as dissolution, diffusion, sorption, and desorption (Boopathy, 2000; Johnsen et al., 2005). Furthermore, as the contaminants age, the bioavailability of the derivatives declines over time, eventually generating components that are non-extractable for microbial degradation (Semple et al., 2003).

Derivatives of TPH

Each petroleum product varies in composition and has its own mixture of different constituents. There are hundreds of diverse chemical formulations generated to form NAPLs. The larger derivatives of TPH that are analytically identified by molecular weight are compounds characterized by their toxicity and effects on human health. The two main TPH representative compounds that are identified and targeted for their health effects are BTEX and PAHs (Todd et al., 1999). The major hydrocarbons in petroleum products and fuel are benzene, toluene, ethylbenzene, and three isomers of xylene that collectively create the acronym BTEX

(Zhao et al., 2014). Natural sources of PAHs include volcanic eruptions, forest fires, and natural oil seeps, while anthropogenic sources come from burning fossil fuels or wood, and accidental oil discharges (Mahanty et al., 2011). The most recalcitrant form of hydrocarbon PAHs have been predominantly researched due to the continuing effect on the health of the human population. The pollutant PAHs refer to hydrocarbons containing two or more fused benzene rings of varying orientation, formed as a byproduct of combustion. Hydrophobicity increases with the number of fused rings, making PAHs less bioavailable for degradation, and more persistent in the environment (Mahanty et al., 2011). PAHs composed of two or three benzene rings have lower molecular weight, and are more readily degraded than higher molecular weight benzenes that have four or more rings (Sun et al., 2010).

The United States Environmental Protection Agency (U.S. EPA) references 16 PAHs as priority pollutants, shown in Figure 2.8 in increasing molecular weight from lower-molecular-weight naphthalene (NAP), acenaphthylene (ACY), acenaphthene (ACE), fluorine (FLU), phenanthrene (PHE), anthracene (ANT), and higher-molecular weight pyrene (PYR), fluoranthene (FLR), chrysene (CHR), benz[*a*]anthracene (BAA), benzo[*b*]fluoranthene (BBF), benzo[*k*]fluoranthene (BKF), benzo[*a*]pyrene (BAP), indeno[1,2,3-*cd*]pyrene (IPY), benzo[*ghi*]perylene (BPL), and dibenz[*a,h*]anthracene (DBA) (Douben, 2003).

Eight PAHs are listed as carcinogenic, seven as primary pollutants (BAA, BBF, BKF, BAP, CHR, DBA, and IPY), where benzo[*j*]fluoranthene is carcinogenic but not a primary pollutant (ASTDR 1995). In addition, BAP is the most aggressive mutagen and oxidizes into many different phenolic groups, where one form binds to DNA (Walker, 2009). Increased exposure from acute to obtuse toxicity in the environment can lead to higher induced health risks, such as cancer or death (ASTDR 1995). Due to the wide spread abundance of PAHs in the

environment, bioremediation has been considered a viable option process to reduce or eliminate organic contamination.

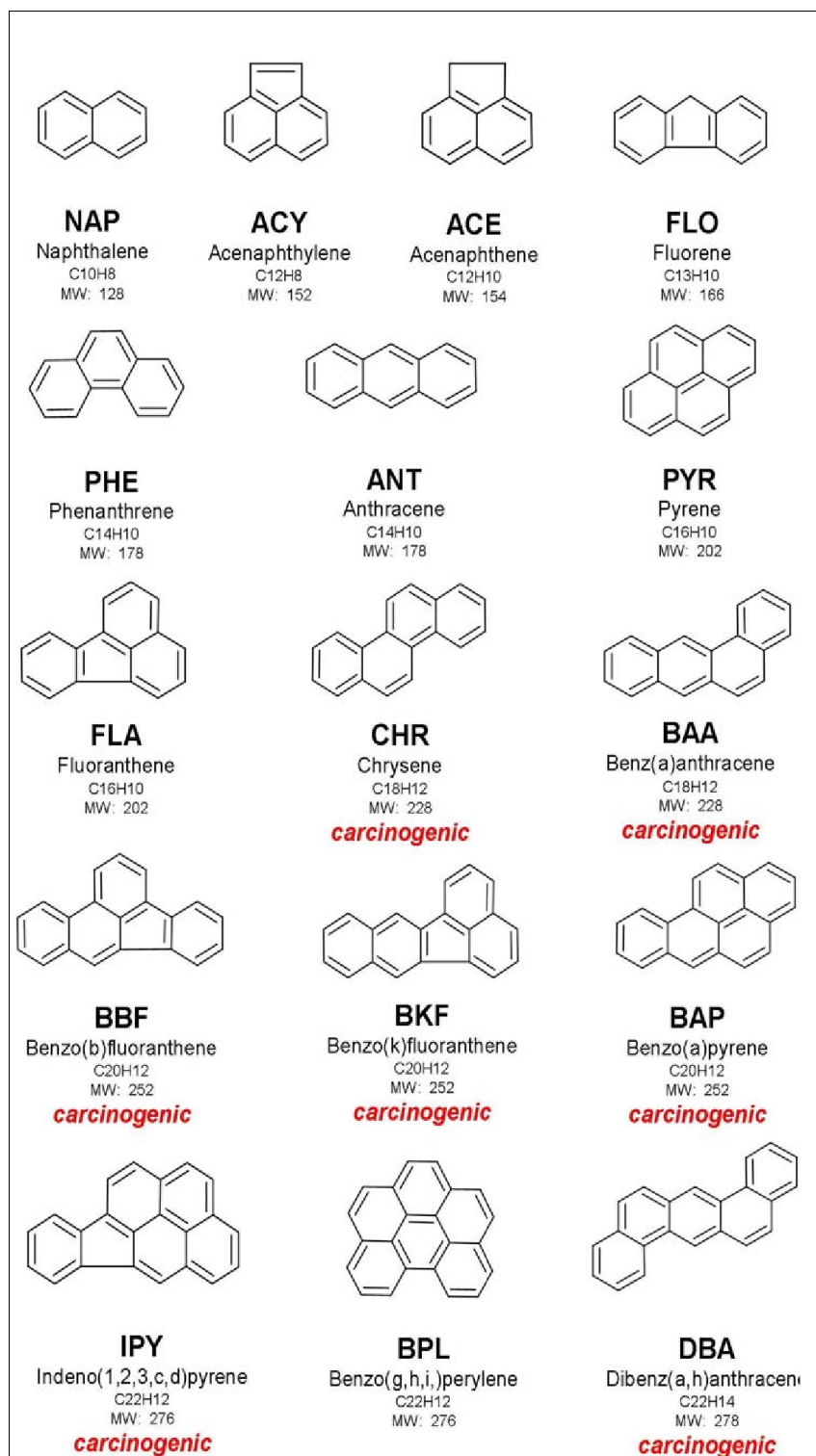


Figure 2.8: EPA's 16 Priority Pollutant PAHs (Douben, 2003).

2.4 Bioremediation

Bioremediation technology utilizes microbial associations to transform and reduce TPH within soil or liquid phase matrices (Van Hamme et al., 2003). This technology has been an important economical technique over the last several decades (Vidali, 2001; Shukla et al., 2010). Various bioremediation approaches have been classified in a variety of different *ex-situ* (land farming, composting, bioreactors, biofilters, pump and treat) and *in-situ* (biosparging, bioventing, bioaugmentation, biostimulation, phytoremediation and natural attenuation) methods to accomplish specific remedial needs (Boopathy, 2000; Shukla et al., 2010). Bioremediation (Figure 2.9) is a technology that uses biotic properties within a range of different physiochemical substrates to promote microbial processes of oxidation and metabolization, which in turn transforms, diminishes, or eliminates harmful organic pollutants present in the environment (Diplock et al., 2009; Shukla et al., 2010). Abiotic factors that affect the efficiency of bioremediation are soil pH, soil moisture and temperature, nutrient availability (e.g., nitrogen and phosphorus), oxidation potential, and chemical composition of the contaminants (Shukla et al., 2010).

At higher concentrations, NAPLs hinder the ability of microorganisms to reduce petroleum contaminants in soil substrates. However, at low enough concentrations, NAPLs are transformed into simple carbon fragments that are adsorbed within the soil matrix and are able to breakdown easier (De Jonge et al., 1997; Namkoong et al., 2002). Microorganisms have the ability to lower the concentrations of NAPL in the soil, thus breaking down TPH more effectively. Microorganisms use extracellular enzymes (such as peroxidases and laccases) to cleave the contaminate, making carbon fragments more soluble for assimilation by the cells (Mougin, 2002). By generating a more uniform distribution of the contaminant in soil, its

bioavailability increases, which, in turn, makes the remediation of hydrocarbons with microorganisms more effective (De Jonge et al., 1997; Namkoong et al., 2002).

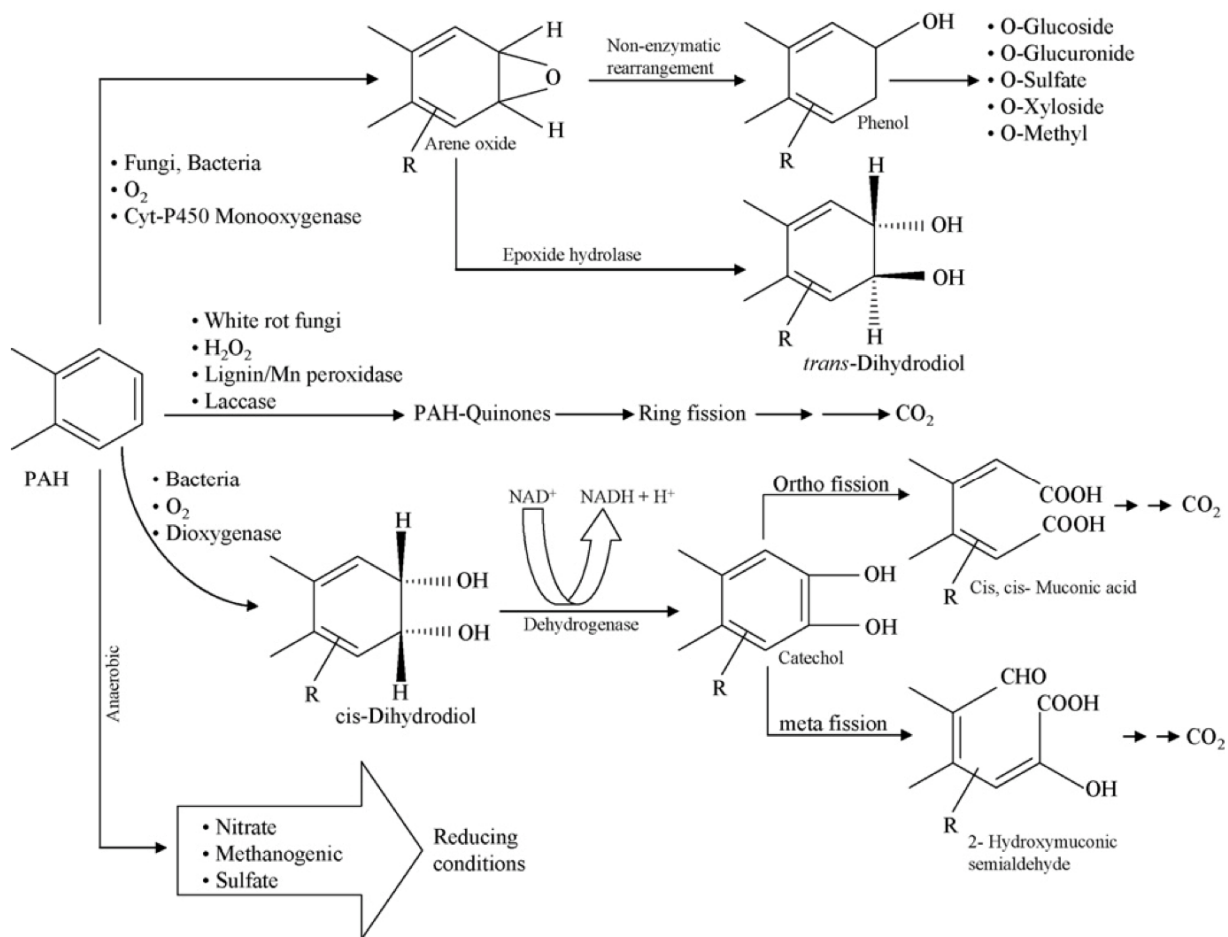


Figure 2.9: Suggested pathways for microbial catabolism of PAHs (Haritash and Kaushik, 2009).

Ex-situ Bioremediation

Land farming

Land farming is a solid phase treatment system that provides degradation of soil hydrocarbons through activation of existing microorganism communities. Land farming is a noninvasive, cost effective method that treats both soil and water. This is done by the incorporation of periodic tilling to aerate the soil, supplying water through some means of irrigation, and incorporating inorganic fertilizers (Ros et al., 2010). By mixing the soil periodically, oxygen increases contact between the pollutants, water, and the microbial degraders of the organic contaminant (Lin et al., 2010). However, there are some limitations with this method, including site specificity and the extended treatment time for remediation (Shukla et al., 2010).

Composting

Composting is a thermophilic aerobic management process, where microorganisms break down organic wastes in either static or aerated piles (Boopathy, 2000). This method is dependent on the type of contaminant and waste material used for composting. Efficiency of this method is dependent on the substrate temperature and soil/waste amendment ratio. The amendments and bulking agents (such as peat moss, pine wood shavings, bran flakes, or a mixture of these agents) may increase the efficiency of biodegradation of hydrocarbons (Megharaj et al., 2011). The typical temperature range for composting is between 55° to 65 °C. Microorganisms produce heat during the breakdown of organic materials in the waste, resulting in increasing temperature in the system. Besides keeping the system at an optimum temperature range, the compost is also monitored for moisture and pH (Shukla et al., 2010). The organic pollutants can undergo many different degradation mechanisms during the composting process. These mechanisms include

volatilization, transformation into different derivative compounds, mineralization, and formation of nonextractable bound residues (Megharaj et al., 2011). The limitations of this procedure include the extended time for remediation from months to years, and the need for nitrogen supplementation (Shukla et al., 2010).

Biopiling

Constructing biopiles to degrade hydrocarbons requires above ground heaps or cells, where water, nutrients, and aeration within the heap stimulates aerobic microbial activity to breakdown organic contaminants such as TPH (Coulon et al., 2010). These piles are covered with impermeable membranes and leachate collection systems to prevent release of pollutants to the surrounding environment (Coulon et al., 2010). The main objective is to reduce hydrocarbons to carbon dioxide and water. Besides aeration, irrigation and the addition of nutrients, heat and pH are also controlled to enhance biodegradative processes inside the biopile, which can take from 3 to 6 months (Shukla et al., 2010). Biopiles operate more effectively in temperate climates. In cooler climates, however, aeration with warm air should be implemented to achieve effective breakdown of the contaminants (Coulon et al., 2010). Limitations of this procedure include extended treatment time, bioavailability of the contaminant, and high abiotic losses through evaporation and volatilization (Shukla et al., 2010).

In-situ Bioremediation

Biosparging

Biosparging is a remediation technology that is used to breakdown organic pollutants that are adsorbed in the soil and dissolved in groundwater within the capillary fringe and the saturated zones (Waudby and Nelson, 2004). This technology is the most cost effective, noninvasive treatment method that utilizes the biodegradation abilities of indigenous microbial communities (Shukla et al., 2010). In biosparging, air (or oxygen) is injected into the saturated zone to stimulate aerobic biodegradation by existing microorganisms. Permeability of contaminated soil and biodegradability of the pollutant are the two primary environmental constraints that affect the efficiency of this technology (Waudby and Nelson, 2004). Certain contaminants (such as benzene, a derivative of TPH) degrade most efficiently under aerobic conditions, which is why this technology relies on the rate in which oxygen permeates through the soil (Bennett, 1999; Waudby and Nelson, 2004).

Bioventing

There are many ways to increase the amount of air for enhancing microbial activity. Bulman et al. (1993) documented several methods tested to break down a diesel spill from the State Railway (Westrail) Marshalling Yard, Australia, in 1989. One method that is commonly used is the lowering of the water table by a dewatering process to increase the amount of air in the residual pore spaces. Bioventing utilizes oxygen by infusing air into residual contaminated sites to stimulate the microorganisms' ability to degrade hydrocarbons (Boopathy, 2000; Shukla et al., 2010). Bioventing and biosparging are similar techniques that utilize oxygen to stimulate microbial degradation, however bioventing targets contamination in the unsaturated vadose zone (Höhener and Ponsin, 2014). The application of bioventing systems injects air at low flow rates

to sustain microbial activity. Optimizing flow rates maximizes biodegradation and minimizes volatilization of contamination while vapors move slowly through the biologically active soil (Shukla et al., 2010).

Bioaugmentation

Bioaugmentation is the addition of microbial cultures to a polluted medium (Boopathy, 2000). The added cultures represent specialized and highly concentrated bacterial populations, either single strains or groups that are specific to the type of toxic contaminant targeted for remediation. This method works best in situations where native populations of microbial organisms do not have the ability to metabolize the targeted compounds and where the site does not have sufficient microbial numbers (Tyagi et al., 2011). Bioaugmentation has been applied in surface soils and fractured geological media, but it has not been very effective in fine-grained porous media such as sand or silts. This is due to the media's ability to filtrate the microbial cells, which diminishes the ability to transport the cultures deeper into the medium (Höhener and Ponsin, 2014). Even in favorable soil conditions, nutrient enrichment is necessary to achieve the high rate of degradation and to maximize catabolic/ enzymatic activity (Kasi et al., 2013).

Biostimulation

Biostimulation focuses on enhancement of indigenous microbial communities within soil or groundwater, and it can be performed either in place or by removing the soil for treatment (Boopathy, 2000). Biostimulation is the most widely used bioremediation procedure based on the introduction of nutrients to the soil profile. Rapid depletion of available inorganic nutrients such as nitrogen and phosphorus occurs during remediation (Margesin and Schinner, 2001). *In-situ* biostimulation projects focus on enhancing N-P-K in the soil with fertilizers or organic

amendments, such as molasses or compost, to introduce labile carbon sources which increase indigenous microbial communities that degrade the contaminant (Margesin and Schinner, 2001, Masciandaro et al., 2013, Nölvak et al., 2013). *Ex-situ* biostimulation allows for optimization of soil conditions, as well as improved incubating parameters for the contaminated media with the aim of making the system more efficient toward microbial remediation (Mougin, 2002). This is achieved by the addition of surfactants or soil amendments that improve the efficiency of microbial degradation (Boopathy, 2000; Mougin, 2002). For example, the addition of N-P-K in a 3-year monitored study with field lysimeters showed significant decreases in TPH at the end of the study compared to natural attenuation (Margesin and Schinner, 2001). However, in some instances the addition of more nutrients to the matrix can negatively impact microbial biodegradation of contaminants on unsuitable soil types (Johnson and Scow, 1999; Chigbo, 2013). Never the less, the use of biostimulation technology is generally very successful depending on soil conditions, nutrient availability, and optimizing the soil for the most efficiency to breaking down hydrocarbons.

2.5 Phytoremediation

Phytoremediation is the use of plants and associated biodegrading microorganisms to remove or detoxify environmental contaminants. It has been an emerging green technology over the past few decades (Pilon-Smits, 2005; Cofield et al., 2007). Plants have the ability to uptake inorganic pollutants (such as Fe, Cd and Pb), and to breakdown organic contaminants TCE, PCBs, TPH, and PAHs. Organic pollutants are most often reduced in the rhizosphere, but can also be sequestered, volatilized, and degraded by the plant. Phytoremediation is appropriate for fixed budgets as an environmentally sustainable alternative to current costly practices of excavation and incineration (Pilon-Smits, 2005). Aboveground (phytoextraction/phytomining, phytovolatilization, phytodegradation) and belowground (phytostabilization and phytostimulation/rhizodegradation) phytoremediation techniques are the two common areas of focus of current methods for removal of TPH.

Aboveground Phytoremediation

Phytoextraction/Phytomining

Phytoextraction refers to the ability of plants to accumulate pollutants in their aboveground tissue, which is then harvested to remove the contaminants. The aboveground plant material can be used in the manufacturing of cardboard and other non-food purposes. The remaining plant tissue is ashed and subsequently disposed in a suitable landfill. Under some circumstances phytomining can be used for pollutants deposited in the aboveground biomass that can be extracted from the plant and recycled (Pilon-Smits, 2005). This technique is useful for remediation of contaminated soils where both inorganic and organic pollutants are present in the soil. Plants selected for these environments stabilize, transform, and accumulate inorganics,

while also breaking down organics. However, the bioavailability and accumulation of metals in the plants biomass can be toxic if there is too much contamination present. This will lead to limiting dissipation of both forms of contamination (Glick, 2010; Chigbo, 2013).

Phytovolatilization

Phytovolatilization refers to the plants' ability to uptake contaminants that are water-soluble and release them through transpiration of water into the atmosphere (Batty and Anslow, 2008; Ferro et al., 2013; and Masciandaro et al., 2013). The use of this technology works with low molecular weight pollutants in the soil such as single-chain hydrocarbons that are more soluble (Gerhardt et al., 2009). With larger hydrocarbons such as PYR and PHE, volatilization by various plant species will be negligible compared to the breakdown of these pollutants in the soil (Gao and Zhu, 2004).

Phytodegradation

Plant can also breakdown organic pollutants using there own enzymatic mechanisms and uptake the metabolites into the plant tissue. This process is called phytodegradation (Pilon-Smits, 2005; Batty and Anslow, 2008). Some non-volatile high molecular weight organic pollutants that can be broken down or rendered by enzymatic modification to non-toxic byproducts and be sequestered by plants (Gerhardt et al., 2009). However, loss of TPHs from volatilization, plant uptake, and accumulation are presumed to be negligible compared to breakdown of TPHs in soils and root zones (Chen et al., 2003; Cheema et al., 2009).

Belowground Phytoremediation

Phytostabilization

The concept of phytostabilization refers to the use of plants to stabilize contaminants in soils (Pilon-Smits, 2005). This technique is suitable for both inorganic and organic pollutant remediation (Batty and Anslow, 2008; Nwoko, 2013). The principle of this technology involves plants acting as hydraulic barriers in soil to prevent runoff and leaching of contaminants (Pilon-Smits, 2005; Nwoko, 2013). Trees can act as buffer strips that redirect water flow upward from polluted underground plumes by intercepting horizontal migration of contaminants (Nwoko, 2013). This technique is advantageous for both inorganic and organic contaminants at different depths in polluted soil, however it is used more often for inorganic pollutant stability and uptake.

Phytostimulation

Phytostimulation and rhizodegradation rely on plants and associated microorganisms in the rhizosphere to degrade hydrophobic organic pollutants, such as TPH, into smaller inert forms of contamination (Pilon-Smits, 2005). Phytostimulation is dependent on a variety of factors, which can be optimized to improve efficiency of breaking down TPH. Soil textual properties, temperature, moisture, amount of organic matter, and pH in the rhizosphere are factors that limit bioavailability of TPH pollutants for effective microbial remediation (Pilon-Smits, 2005). Influencing these factors within the soil substrate can affect microbial survival and sustainability (Boopathy, 2000; Shukla et al., 2010). Microbiological activity in the rhizosphere enhances mineral and water uptake, promotes plant and root development, while decomposing organic material (Sylvia et al., 1998). Optimizing the soil substrate through the addition of carbon and plant nutrients can enhance microbial diversity and plant establishment (Masciandaro et al.,

2013). Some studies revealed that nutrient additions and a proper irrigation regime have a “synergistic effect” when enhancing microbial degradation (Keller et al., 2008; Nedunuri et al., 2009). By applying nutrients N-P-K, lime, or different carbon sources such as sawdust, compost, or biosolids, the existing soil conditions can be improved to enhance plant and microbial associations, which in turn facilitate phytostimulation of organic contaminant degradation more efficiently (Vawdrey et al., 2002; Chen et al., 2003; Gerhardt et al., 2009; Zhou et al., 2009; Nedunuri et al., 2009; Shukla et al., 2010).

2.6 Plants used in Phytoremediation

Fast growing plant species with high biomass and vigorous root systems are more advantageous for microbial growth and TPH contaminant degradation due to the increase in root surface area (Pilon-Smits, 2005). In a vegetated soil substrate, microbial activity within the rhizosphere is enhanced by orders of magnitude compared to unvegetated soil (Ho and Banks, 2006; Euliss et al., 2008; Cheema et al., 2009; Hultgren et al., 2009). Some grass and woody plant species have specific bacteria and fungi in their rhizosphere that are better adapted to attenuate hydrocarbons (Euliss et al., 2008). Woody plant species have been used predominately to breakdown BTEX, while grass species *Festuca*, and *Lolium*, and others have been used to degrade TPHs and their derivative PAHs (Cook and Hesterberg, 2013).

Populus

The phreatophytic *Populus* species within the family *Salicaceae* have high transpiration rates that make this plant advantageous as a biological pump for cool temperate regions with contaminated soils (Ferro et al., 2003). Poplar species have been used primarily for metal (Cd and Zn) accumulation in their biomass (Santos Utmazian and Wenzel, 2007). However, these

plants have the ability to breakdown TPHs through rhizodegradation (Smith et al., 2008). Phreatophytic tree species are more desirable for breaking down TPHs when there are oxygen and water limiting conditions, and when deeper rooting depth is necessary for contamination that goes beyond shallow rooting depths (Cook and Hesterberg, 2013). Compared to bulk soil, soils vegetated with poplar trees have been found to have higher populations of monoaromatic petroleum hydrocarbon (BTEX) degraders, denitrifiers, and pseudomonads in their rhizosphere when in contaminated soil (Weishaar et al., 2009). In addition, rhizodegradation can be enhanced by irrigating trees with water containing with high concentrations of plant nutrients from contaminated sewage, sewage sludge, leachates or wood-ash mixtures. This makes poplar species advantageous for both phytostimulation and phytostabilization of inorganic and organic pollutants (Justin et al., 2010).

Salix

The genus *Salix* within the same family *Populus*, are phreatophytic woody plants that occur predominately in arctic and temperate zones. Physiological characteristics of *Salix* include large biomass production, high transpiration rates, and efficient nutrient uptake (Kuzovkina and Volk, 2009). *Salix* requires moist, slightly acidic soils, and full sunlight for optimal growth (Kuzovkina and Quigley, 2005). Pollutant remediation research with *Salix* species has been focused predominantly on inorganic metals (Vervaeke et al., 2003; Santos Utmazian and Wenzel, 2007). However, in the last decade, there has been an increased interest in organic phytoremediation of VOCs, PCB's, and more recently PAHs (Spriggs et al., 2005; Denys et al., 2006; Euliss et al., 2008; Hultgren et al., 2009; Kacálková and Tlustoš, 2011; Shi, 2011)

Salix nigra — a tree species with a fibrous root system that excels in moist soils — can be used for phytoremediation of heavy metals and organic pollutants (Lyyra et al., 2005). In

addition, *S. nigra* had greater degradation of PAHs over other woody plants, such as poplar and ash, in manufactured gas plant-impacted soil (Spriggs et al., 2005). Many shrub type species (such as *S. purpurea*, *S. exigua*, and *S. miyabeana*) have extensive fibrous root systems, mostly located in the upper 40-45 cm of the soil profile (Kuzovkina and Quigley, 2005), and have been used in various phytoremediation applications (Justin et al., 2010; Euliss et al., 2008). Phytoremediation research using *S. dasyclados* and *S. miyabeana* is limited in remediation of hydrocarbons, but these species showed promise for remediation of inorganic metals (Wieshammer et al., 2007; Pitre et al., 2010).

Poaceae

The grass genera *Festuca*, *Lolium*, and *Panicum* within the family *Poaceae* have a wide range of distribution throughout temperature regimes, moisture gradients, acidity, and alkalinity (Turgeon, 2005; Jimmy Carter Plant Materials Center, 2011). Cool-season types of grasses such as tall fescue, perennial ryegrass, and sheep fescue establish quickly making them advantageous in phytoremediation (Turgeon, 2005). Warm-season switchgrass has the ability to generate large amounts of biomass, and to adapt to different environmental conditions which are beneficial in pollutant remediation (Jimmy Carter Plant Materials Center, 2011).

Fescues, ryegrasses, and *Panicum* species have very dense, deep root systems with a large root surface area and many microbial degraders that are advantageous for phytostimulation, including breaking down TPH (Pilon-Smits, 2005). For example, grass species (such as switchgrass) promote degradation of recalcitrant carbon fractions within its rhizosphere by stimulating microbial groups by higher orders of magnitude compared to the bulk soil (Pilon-Smits, 2005). In a pot study with different grass species including switchgrass, degradation of

TPH contaminants occurred better than with tree species (Euliss et al., 2008; Cook and Hesterberg, 2013). Grass species are more desirable for breaking down TPHs when there are organic carbon limiting conditions and shallow contamination, and for establishing ground covers to reduce erosion and migration of pollutants (Cook and Hesterberg, 2013).

2.7 Phytoremediation Today

Salix species used in phytoremediation research have received success with TPH degradation, but have mostly been used in applications for inorganic contamination due to high biomass yields (Lyyra et al. 2005; Spriggs et al., 2005; Justin et al., 2010; Euliss et al. 2008). *Festuca*, *Lolium* and *Panicum* have been utilized more often for remediation due to quick establishment and hardiness, and have shown better remediation of pollutants over woody plants (Pradhan et al., 1998; Euliss et al., 2008; Cheema et al., 2009; Zhou et al., 2009). Plant species with rapid physiological development and the ability to compete for resources will result in higher efficiency of contaminant degradation. Phytoremediation applications using different grasses or tree type species are both appropriate in accomplishing remediation. Limitations of this technology and plant selection will always be dependent on a variety of factors when remediating contaminants in the environment, such as soil type, tolerance to environmental conditions and contaminant, bioaccumulation, and plant productivity and survival (Cook and Hesterberg, 2013).

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CHAPTER III

Breakdown of low-level Total Petroleum Hydrocarbons (TPH) in Contaminated Soil Using Grasses and Willows

ABSTRACT

An *ex-situ* phytoremediation greenhouse study targeting total petroleum hydrocarbons (TPH) was conducted using three cool-season grasses [perennial ryegrass (*Lolium perenne*), sheep fescue (*Festuca ovina*), tall fescue (*F. arundinacea*)], one warm-season grass [switchgrass (*Panicum virgatum*)] and four willow cultivars (*Salix dasyclados* ‘SV1’, *S. eriocephala* ‘S25’, *S. miyabeana* ‘SX67’, and *S. purpurea* ‘S99113-012’) which were mono and intercropped in pots filled with contaminated soil from the New Haven Rail Yard, New Haven, CT. Efficiencies of the treatments on TPH degradation were assessed in a 90-day experiment using fertilizer and fertilizer with molasses amendments. In addition, control treatments without plants or soil amendments were included in the experiment. Plant biomass, TPH concentrations measured by gas chromatography, and indigenous microbes quantified with colony-forming units (CFU), were assessed at the end of the study. Primary shoot lengths of the plants were measured throughout the study. Grasses and combinations of ‘SV1’ willow and switchgrass grown with soil amendments produced the highest aboveground biomass compared to treatments without amendments. Switchgrass had the highest aboveground biomass, and longest primary shoot lengths while willows produced the lowest aboveground biomass, and shortest primary shoot lengths. Bacterial CFU’s were in orders of magnitude higher in all vegetated pots compared to unvegetated, and the highest in willows with soil amendments. The TPH degradation was higher in treatments with plants and soil amendments, but not statistically different between plant species. Also, unvegetated treatments with amendments had a similar level of TPH degradation as treatments with plants and soil amendments. Thus, soil amendments enhanced TPH

degradation equally efficiently with and without plant species. Phytoremediation of low-level TPH contamination is achievable through the addition of cool and warm seasons grasses and/or willow combinations, or through fertilization by NPK of existing soil.

Keywords: Phytoremediation, rhizodegradation, organic contaminants, total petroleum hydrocarbons, *Festuca*, *Lolium*, *Panicum*, *Salix*

INTRODUCTION

Total petroleum hydrocarbons (TPH) are a group of organic contaminants derived from a mixture of fuels and crude oil byproducts released into the environment through mining, manufacturing procedures, and accidental spills. Constituents of TPH have a vast array of resilient compounds that can cause carcinogenic and mutagenic effects on humans (Todd et al., 1999). Different techniques and strategies have been developed to preserve human health and promote ecological safety through terrestrial and aquatic reclamation from these harmful pollutants.

Bioremediation is a technology that uses biotic properties within a range of different physiochemical substrates to assist microbial processes of oxidation and metabolization, which in turn transforms, diminishes, or eliminates harmful organic pollutants present in our environment (Diplock et al., 2009; Shukla et al., 2010). Abiotic factors that affect the efficiency of bioremediation are soil pH, soil moisture and temperature, nutrient availability (e.g., nitrogen and phosphorus), oxidation potential, and structure of the contaminates (Shukla et al., 2010). There are different *in-situ* and *ex-situ* strategies that optimize microbial degradation in a soil medium depending on environmental settings. Phytoremediation, defined as the use of plants to sequester, volatilize, or degrade soil pollutants, has received a great deal of attention in the past decade (Pilon-Smits, 2005).

Rhizodegradation and phytostimulation rely on plant associated microorganisms in the soil rhizosphere to degrade hydrophobic organic pollutants such as TPH (Pilon-Smits, 2005). In a soil substrate with vegetation, microbial activity within the rhizosphere is enhanced by orders of magnitude compared to unvegetated soil substrates (Ho and Banks, 2006; Euliss et al., 2008; Cheema et al., 2009; Hultgren et al., 2009). Some woody and grass species have specific bacteria

and fungi within their rhizosphere that are better adapted to attenuating petroleum hydrocarbons (Euliss et al., 2008). Woody plants have been used predominately to degrade BTEX, while grass species *Festuca* and *Lolium* have been used to degrade TPHs and their derivative PAHs (Cook and Hesterberg, 2013).

A mixture of two or more species as opposed to monocultures is more advantageous for optimum plant establishment and microbial associations (Enkhtuya et al., 2005). Different combinations of herbaceous plants such as rapeseed (*Brassica napus*), alfalfa (*Medicago sativa*), tall fescue (*Festuca arundinacea*) and perennial ryegrass (*Lolium perenne*) degrade TPHs more efficiently than if independently sowed (Euliss et al., 2008; Cheema et al., 2010). Few studies have compared the effectiveness of grass and woody plant species for TPH degradation (Cook and Hesterberg, 2013). Treatments with switchgrass and willows had lower levels of TPHs than poplar (*Populus*) or no plants (Euliss et al., 2008). Further, combinations of willows and grasses are more conducive than monocultures to remediate a multitude of different inorganic and organic forms of contaminants in polluted substrates (Wenzel, 2009). Willows have the ability to remediate pollutants (Vervaeke et al., 2003; Santos Utmazian and Wenzel, 2007). Thus, the simultaneous introduction of both plant groups — willows for accumulation of inorganics contaminants, and grasses for microbial degradation of hydrocarbons — can better fulfill the objective of contaminant removal from polluted sites (Masciandaro et al., 2013).

Phytoremediation projects have traditionally utilized a variety of species from the Poaceae family (grasses), as well as some phreatophytic woody species (deep-rooted plants with high transpiration rates). Cool- and warm-season grass species *Festuca*, *Lolium*, and *Panicum* have been used more frequently due to their quick establishment, hardiness, and enhanced remediation of organic pollutants at shallow rooting depths (Cofield et al., 2007; Rezek et al.,

2008; Euliss et al., 2008; Cheema et al., 2009; Kang et al., 2010). *Festuca* and *Panicum* have been shown to be effective for TPH degradation, including 3–6 ringed PAHs, due to their fibrous root systems and microbial associations that stabilize hydrocarbons in the rhizosphere (Chen et al., 2003; Cofield et al., 2007; Batty and Anslow, 2008; Euliss et al., 2008; Cheema et al., 2009). *Lolium* has been used for both degrading persistent hydrocarbons, and exploring the species' ability to be inoculated with different bacteria and mycorrhizae that enhance the reduction of TPHs in soil (Rezek et al., 2008; Kang et al., 2010; Yu et al., 2011).

The phreatophytes, such as poplars and willows, have proven to be effective at removal of inorganic (Vervaeke, 2003; Santos et al., 2007; Tlustoš et al., 2007; Zhivotovsky et al., 2011) and organic pollutants unavailable to shallow rooted Poaceae species (Lyyra et al., 2006; Euliss et al., 2008; Smith et al., 2008; Justin et al., 2010). In recent years, *Salix* species have been utilized to attenuate hydrocarbons due to their extensive root systems and enhanced rhizospheric biodegradation (Vervaeke, 2003; Hultgren et al., 2009). On contaminated soils of former manufacturing gas plants, willows degrade petroleum hydrocarbons more efficiently than poplars, ash (*Fraxinus*), or unvegetated controls (Spriggs et al., 2005). Overall, *Salix* species have performed better than other woody plants, with certain limitations depending on the nature of the soil substrate, nutrient abundance, and contaminate concentration (Hakulinen et al., 1995; Justin et al., 2010).

Phytoremediation efficiency is also dependent on soil characteristics that affect plant growth, such as nutrient abundance of nitrogen –phosphorus – potassium (NPK), soil pH and salinity (Ferro et al., 2003). Soil moisture is important for plant establishment and survival in contaminated ecosystems (Keller et al., 2008; Nedunuri et al., 2009), and soil texture directly influences soil moisture as well as nutrients, pH and organic matter. Optimization of the soil

substrate through the addition of plant nutrients and carbon can enhance plant establishment and microbial diversity (Masciandaro et al., 2013). Some studies revealed that nutrient additions and a proper irrigation regime have a “synergistic effect” when enhancing microbial degradation

(Keller et al., 2008; Nedunuri et al., 2009). By applying nutrients, lime, or different carbon sources such as sawdust, compost or biosolids, the existing soil conditions can be improved to establish enhanced plant and microbial associations, which in turn facilitate organic contaminant degradation (Vawdrey et al., 2002; Chen et al., 2003; Gerhardt et al., 2009; Zhou et al., 2009; Nedunuri et al., 2009; Shukla et al., 2010).

Molasses as a soil carbon amendment can change C:N ratios, increase microbial densities, and promote various enzymatic activity in a medium with organic pollutants (Lamichhane et al., 2012; Nölvak et al., 2013). In the presence of alfalfa hay, pea or wheat straw, and other various plant residues, soil microbial communities were 300 times higher when breaking down hydrocarbons (Shahsavari et al., 2013). However, Euliss et al. (2008) suggest that adding superfluous amounts of an organic carbon source to a soil substrate can theoretically limit remediation because of the microbial associations within the rhizosphere with the more labile carbon rather than with the recalcitrant hydrocarbons. Unvegetated soils with carbon additions have the potential to enhance hydrocarbon degradation as well as or better than vegetated soils depending on a variety of factors influencing soil remediation (Euliss et al., 2008).

Optimizing plants and soil amendments that affect the degradation of organic contamination in soil is an important step toward improving the efficiency of phytoremediation. The focus of this study was to evaluate the ability of different grasses and woody plants, as well as plant combinations, to facilitate TPH degradation in anthropogenic fill from the New Haven Rail Yard using various soil amendments.

MATERIALS AND METHODS

Soil Source and Characteristics

Since the late 1800s, the 74-acre New Haven Rail Yard (NHRY) has been a pioneer for freight and passenger services, and by the early 1900s was a construction site for freight cars, repairs, and maintenance on locomotives. Until 1985 at least 8 industrial warehouses and numerous smaller buildings were constructed on this site, which either processed or released hazardous waste material. Since 1990 to present, over 50 spills, primarily diesel fuel, were recorded on this site. This site, under the Comprehensive Environmental Response, Compensation, and Liability Act and Resource Conservation and Recovery Act, is designated to have a high-risk potential of environmental contamination release and is a small quantity generator of hazardous waste (NHRY, 2008). Initial assessments of inorganic and organic constituents from the NHRY soils were conducted by Phoenix Environmental Laboratories, Inc., Manchester, CT (Tab. 2.1). The pollutants on site were primarily heavy metals and petroleum hydrocarbons, where TPHs consisted of a large array of aliphatic compounds and a few semi-volatile ringed aromatic hydrocarbons.

Two tons of soil was removed directly from NHRY in large double-bagged plastic containers in December 2011. The soil was sieved to 6.35 mm, homogenized, and stored within university facilities. Soil characteristics (Tab. 2.2) were assessed using the University of Delaware Soil Test Laboratory specifications (Sims and Eckert, 2011). A homogenized soil sample was collected with a core sampler from the initial starting material. At the start of the study, soil was cored randomly and combined from 46 experimental pots. Three hundred grams were taken the 10th of May 2012, sealed and stored at -20°C, for TPH analysis of initial values.

Experimental Design

The experiment was assembled in the greenhouse at the Plant Science Research and Education Facilities at the University of Connecticut located in Storrs, CT: lat.41°81 N', long. 72°26 W. During the study the temperatures fluctuated from 20°C to 40°C /day and 15°C to 25°C /night with a natural photoperiod. Prior to planting, the soil was mixed in an industrial soil mixer to ensure homogeneity before amendment application, air dried, and crushed to pass through 6.35 mm sieve, then thoroughly mixed with 3661.8 kg/ha granular lime to optimize soil pH for plant establishment (16.8 g/pot). Soil (9.25 kg) was added to a lined 7.34 liter C900 series (24.13-cm diameter by 23.18-cm height) plastic containers. All containers had 30 cm diameter 5 cm deep saucers to collect leachate that was added back daily to the pot to minimize potential leaching of TPH in the system.

The seeds and cuttings were planted on the 10th of May, 2012. Four Poaceae species, represented by three cool-season grasses (CSG), including tall fescue (*Festuca arundinacea* 'Mustang 4'), perennial ryegrass (*Lolium perenne* 'Fiesta 4'), sheep fescue (*F. ovina* 'Azay') (Charles C. Hart Seed Company, Wethersfield, CT), and one warm-season grass (WSG) – switchgrass (*Panicum virgatum* 'Blackwell') (Ernst Conservation Seed, Meadville, PA.) were broadcasted at a rate of 292.95 kg/ha in each pot (1.34 grams seed/pot). Four taxonomically distant willows cultivars *Salix dasyclados* 'SV1', *S. eriocephala* 'S25', *S. miyabeana* 'SX67', *S. purpurea* 'S99113-012' developed at the State University of New York for biomass production (Abrahamson et al., 2002) were cut from the biomass trials at the Plant Science Research and Education Facilities, Storrs, CT. The dormant cuttings, 30 cm in length and 1–1.2 cm in diameter, were planted one in each pot. In addition, the following two combinations were planted at the same time: tall fescue with sheep fescue (50:50 of each application rate as in monoculture

treatments), and *S. dasyclados* ‘SV1’ with switchgrass (Combinations).

Soil fertilizer amendments of 20–20–20 (N-P-K) Jack’s water-soluble fertilizers (JR Peters Inc., Allentown, PE.) were applied at a rate of 195.3 kg/ha/week (375 mg/pot/week) for grasses and 97.65 kg/ha/week (180 mg/pot/week) for willows and combination treatments weekly. A half rate of fertilizer was applied to willows and combination treatments compared to grasses to promote optimization of the willow species without causing physiological stress by over application of nitrogen as willow growth can be hindered by the overabundance of nitrogen (Hakulinen et al., 1995; Justin et al., 2010). A 1.1% solution grandma’s unsulfured blackstrap molasses (B&G Foods, Inc. Parsippany, NJ.) was applied weekly at a rate of 9.58 kg/ha/week (44 mg molasses/pot/week) as a carbon addition to stimulate microbial development based on an industry standard for amenity turf (Plant Food Company, Inc., Cranbury, NJ). Starting on Week 6 in all treatments, soil pH was adjusted with a pelletized lawn lime (Soil Doctor, Haines, FL) as necessary every two weeks at a rate of 1220.6 kg/ha per pot (25 g/pot). All pots were drip irrigated, where each experimental unit received 150–200 mL of water daily. Additional hand watering was provided as needed to promote plant establishment and growth through the study. Leachate that percolated into the collection saucer, if any, was added back to the system. The watering rates were determined from a small preliminary leachate experiment (data not shown).

A total of 138 pots were included in the experiment representing the following treatments: 120 pots with plants (10 plant treatments [3 CSG, 1 WSG, 4 Willows, 2 Combinations] × 3 soil treatments [no amendment, fertilizer, fertilizer and molasses] × 4 replications), and 18 pots with no plants as a control (2 controls dependent on the different nitrogen rates for willows and grasses × 3 soil treatments × 3 replications). This greenhouse study was set out in a completely random design (CRD); pot positions on the bench were re-

randomized every three weeks for twelve weeks. Soil pH and electrical conductivity were measured at the end of the experiment at Week 12. Plant shoot lengths (for *Salix* the longest shoot and for grasses the longest standing grass blade) were measured every three weeks starting Week 6. Phytotoxicity symptoms such as leaf yellowing and burning were monitored and recorded throughout the experiment.

At the end of the experiment on the 8th of August 2012, plant aboveground biomass was harvested. Grasses were cut 2–3 cm above soil surface and willow shoots were separated from the original cuttings. Plant biomass was separated by taxa with the combination treatments. All samples were oven dried for 72 hours at 70°C and dry weight was recorded. Each experimental pot unit was destructively sampled at the end of the study to be analyzed for pH, microbial activity, loss on ignition, and petroleum hydrocarbons. Soil samples were collected from the middle of the pot 12 hours after the last watering event in the morning to provide for uniform water content. Soil pH was measured on the same day in a 1:1 (10 grams of soil to 10 mL DI water) aqueous suspension after sieving (2 mm) (Sims and Wolf, 2011). To determine total organic matter, 30 g of soil was collected from randomly selected pots with and without plants, weighed and placed in an oven at 105°C for 5 hours, then reweighed and placed in an oven at 400°C for 16 hours (Sparks et al., 1996). Approximately 300 g of soil from a depth of 10–13 cm from the bottom of each pot were collected and stored at -20°C for TPH extraction. For bacteria enumeration, 30 g of bulk soil was collected in the same area of the pot, and rhizospheric samples were taken within 10 mm from the plant roots. During soil sampling, all tools and containers were sterilized with 70% ethanol spray solution. All bulk and rhizospheric soil samples were immediately placed into plastic capped containers and stored at 4°C until microbial analysis was performed within a week of sample collection.

Soil Bacterial Enumeration

A preliminary study was conducted to quantify bacterial colony-forming units (CFU's) in the samples. The serial dilutions were assessed with samples from unvegetated pots at 10^{-3} , 10^{-4} , and 10^{-5} , and for vegetated pots at 10^{-4} , 10^{-5} , and 10^{-6} (Page, 1982). Bacteria were plated on beef extract agar plates with the following nutrient concentrations: 0.3% beef extract, 0.5% peptone, and 1.5% agar per liter at pH 6.8. One gram of soil was placed into autoclaved falcon tubes with a generated M9 saline solution (Geerlof, 2010) that consisted of 75.2 g/L Na_2HPO_4 , 30.0 g/L KH_2PO_4 , 5 g/L NaCl, and 5 g/L NH_4Cl at pH 7.2. The microbes were extracted from the soil by shaking each falcon tube for 20 seconds during each dilution, and 1:10 solution ratio was added to the next dilution prepared with saline solution. Then one milliliter of remaining solution was added to each plate, spread with a sterilized glass rod, and then incubated at 20°C in dark conditions for 4 days to target each particular dilution level. CFU's were quantified visually within duplicates on a scale from 30–300 culturable bacteria per gram of soil (Page, 1982).

Soil TPH Analyses

Soil TPH concentrations were analyzed using a modified EPA Method 3550C ultrasonic extraction procedure (Khan et al., 2005). Ten grams of soil were removed from -20°C storage and added to a clean pre-weighed 125 mL Erlenmeyer flask. Then, 25-mL of dichloromethane (DCM) was added to each flask and covered with parafilm. The flasks were ultrasonicated for five minutes and the remaining extract solution was decanted into a clean DCM washed, pre-weighed 100-mL beaker. This extraction method was repeated three times. Ten grams of anhydrous Na_2SO_4 were added to each beaker to remove residual water from the extract solution. Each sample was vacuum filtered through glass microfiber filters into pre-weighed DCM

washed 30-mL test tubes. Final extracted solution was recorded for each sample before being concentrated to one test tube (about 10–15 mL) under N₂ gas. Samples were then weighed, parafilmed, covered with foil, and placed in 4°C storage until analyzed by gas chromatography (EPA, 2007).

Analytical Specifications for Gas Chromatography System

Gas chromatography (GC) was used to determine the TPH concentration in the soil and leachate samples. Standards of 125 and 250 mg/kg in DCM were generated from 50,000 mg/kg diesel in methylene chloride stock solution. About 1–1.4 µL of standards were injected into an Agilent 6890 series GC system (Agilent Technologies, Santa Clara, CA) with flame ionization detector (GC-FID) and used to generate chromatographs to evaluate total peak area under the standard curve. For each extracted sample a similar volume of 1–1.4 µL was consecutively injected manually with three repetitions. A 12 m x 0.25 mm minimum inner diameter Rt[®]-PAH fused silica capillary column (ResTek, Penn Eagle Industrial Park, CA) was used to quantify retention of carbon over time. The GC temperature was set at 80°C for two minutes, and then increased to 280°C at a rate of 8°C/min to 200°C followed by 16°C/min, and finally held for five minutes. The total run time for each sample was 30 minutes. Helium was used as the carrier gas at a pressure of 8.3 psi, flow rate 1.7 mL/min, and average constant flow velocity of 46 cm/sec. The split/splitless injection port was held at 280°C, FID was a 50:50 mix of 40 mL/min hydrogen and 400 mL/min air at 350°C, and all gases were ultra pure grade. The chromatogram was integrated from 4 to 18 minutes using OpenLAB CDS EZChrom (version 4.1a, Agilent Technologies, Wilmington, DE).

Leachate Analyses

Leachate samples were analyzed during 10 days for TPH content using a separate set of pots filled with NHRY soil and placed in the same greenhouse to evaluate the potential for the contaminate to leach into water that drained from each pot. Three unvegetated pots (9.25 kg) representing replications were setup in a CRD and were re-randomized daily. Three additions of 150 mL of water were added to each pot daily at 9:00, 12:00, and 15:00 hours. The total volume of these water additions was three times higher than in the main experiment with plant and various soil amendments. In addition, three control pots with no irrigation were added to this experiment to measure the amount of TPH volatilization within the soil sample. All leachate was collected in a saucer, its volume was measured, and the leachate samples were placed in glass beakers covered with foil every two days for TPH analyses. Soil samples were taken the first and the last day of the experiment from each pot to measure the amount of change in TPH volume within the soil sample. All samples were stored at 4°C until analyzed.

TPH Extraction Procedure

The leachate samples were analyzed using a modified EPA Method 3510C Separatory Funnel Liquid-Liquid Extraction procedure. Approximately 150–200 mL of the leachate sample was placed in a DCM cleaned 500 mL separatory funnel (SPF). Twenty-five milliliters of DCM was added to the SPF, and shaken vigorously for two minutes, with the cap was opened every 5 to 10 seconds to release excess pressure. Phase separation of leachate and extractant occurred after SF was upright in a ring stand for ten minutes. After phase separation, the remaining extract solution was drained into a clean DCM washed, pre-weighed 100-mL beaker. This extraction method was repeated three times. The rest of the extraction procedure is exactly as stated previously for contaminated soil (EPA, 1996). Flasks containing the remaining solvent were

ultrasonicated for five minutes and the remaining extract solution was decanted into a clean DCM-washed, pre-weighed 100-mL beaker. This extraction method was repeated three times. Ten grams of anhydrous Na₂SO₄ were added to each beaker to remove residual water from the extract solution. Each sample was vacuum filtered through glass microfiber filters into pre-weighed DCM-washed 30-mL test tubes. Final extracted solution was recorded for each sample before concentrating them down to one test tube (about 10–15 mL) under N₂ gas. After samples were concentrated they were weighed, parafilmed, covered with foil, and placed in 4°C storage until analyzed by GC (EPA, 2007).

Statistical Analysis

Three-way ANOVA for 2 x 3 x 10 orthogonal contrast in factorial comparisons were analyzed to find statistical differences within treatment effects, where unvegetated (n=3) and vegetated (n=4) replications were unequal evaluated by the MIXED procedure sliced by group using PDMIX800 macro. Plants were grouped as follows: (1) CSG including tall and sheep fescue, and perennial ryegrass; (2) WSG including switchgrass; (3) Willows including four cultivars ‘SV1’, ‘S25’, ‘SX67’, and ‘S99113-012’, and (4) Combinations of species – tall and sheep fescue, and ‘SV1’ and switchgrass. All assumptions were tested on each data set for violations with the GLM procedure for independence of the residuals and homogeneity of variances using the HOVTEST of Brown-Forsythe analysis. Normality of the distribution using Shapiro-Wilk was evaluated by the UNIVARIATE procedure (PC SAS version 9.3; SAS Institute, Cary, NC). No variables violated parametric assumptions. Where significant, comparisons of group means were performed using Fisher’s least significant differences ($p = 0.05$). Repeated measures were used to analyze primary shoot length in each experimental unit

with plants comparing differences in plant growth every 3rd week of the experiment. Post-hoc analysis was used at the end of the experiment to evaluate the data sets with the MIXED procedure slicing all the data means for biomass, CFUs, and TPH by group and soil amendment.

RESULTS

Total Petroleum Hydrocarbon Concentrations in Leachates

The TPH leachate concentrations are presented in Figure 3.1. The first leaching event on Day 1 had the highest concentration of TPH (1.90 mg/kg) in the leachate, followed by the lower levels of TPH in leachate during the following days, where Day 3, 5 and 7 were not statistically different ($p \geq 0.05$). The low amount of hydrocarbons in solution suggested that the majority of detectable carbon structures were hydrophobic, and did not readily dissolve. Although there was a slight increase in reported TPH on Day 10, the levels are relatively insignificant to the measurable amounts in each experimental unit after Day 3.

Soil pH and EC

During Week 6, soil pH across all pots with no amendment was 5.15 ± 0.06 , in fertilizer 5.12 ± 0.08 , and fertilizer/molasses 5.14 ± 0.06 . Beginning on Week 6, soil pH was adjusted with a pelletized lawn lime (Soil Doctor, Haines, FL) as necessary every two weeks at a rate of 1220.60 kg/ha per pot (25 grams/pot). The average electrical conductivity (EC) during Week 6 across all pots was 0.7 ± 0.4 (mS/cm) (well water was used for irrigation). At the end of the study during Week 12, the average EC across all pots was 0.1 ± 0.01 (mS/cm) and soil pH across all pots with no soil amendment was 5.24 ± 0.11 , in fertilizer treatments 5.19 ± 0.11 , and fertilizer/molasses treatments 5.25 ± 0.07 .

Plant Growth

Throughout the experiment, all plants continued active growth and there was 100% survival in all treatments. During the first three weeks no plants showed any signs of

physiological stress in any treatment (Fig. 3.2). The first visual symptoms of stress were observed in WSG and CSG during Week 6 in control pots [A], while pots with fertilizer and fertilizer/molasses [B] [C] were substantially greener. Tip burn in CSG was apparent predominantly in pots that contained perennial ryegrass and WSG comprising of switchgrass (Fig. 3.3). Yellowing of leaf blades was less apparent in willows even without soil amendments [D] [E] [F].

During Week 9, leaf yellowing and tip burn became more apparent in CSG, WSG, and Combinations in treatments with soil amendments [B] [C] (Fig. 3.4), and willows started showing chlorosis on leaves in pots with no soil amendments [D]. During the final week of the study, extensive chlorosis was present in all pots that had CSG, WSG, and Combinations (Fig. 3.5). At the end of the experiment in Week 12, Combinations with no soil amendments were the most chlorotic and less vigorous than any other treatment.

Plant Biomass

The mean dry weights of aboveground tissues were similar ($1\text{--}1.8\text{ g pot}^{-1}$) and not significantly different ($p \geq 0.05$) between all plant groups in treatments without soil amendments (Fig. 3.6 and 3.7). Within the groups CSG, WSG, and Combinations aboveground biomass was significantly greater in treatments with fertilizer and fertilizer/molasses than in treatments with no amendments (Fig. 3.6). WSG produced the highest aboveground biomass ($9.6\text{--}9.7\text{ g pot}^{-1}$) with fertilizer and fertilizer/molasses amendments, followed by CSG (7.1 g pot^{-1}), and Combinations ($3.9\text{--}4.1\text{ g pot}^{-1}$) with fertilizer and fertilizer/molasses amendments (Fig. 3.7). Willows produced the lowest aboveground biomass ($0.8\text{--}1.0\text{ g pot}^{-1}$) across all treatments and willow aboveground biomass was not significantly different between soil amendments ($p \geq 0.05$).

Plant Shoot Length

Shoot length in plant groups with no soil amendments were not statistically ($p \geq 0.05$) different across weeks (Fig. 3.8). Week 6 and 9 primary shoot lengths were significantly ($p \leq 0.05$) different between all groups (Fig. 3.9 and 3.10), while during Weeks 9 and 12, WSG in fertilizer and fertilizer/molasses treatments had the longest primary shoots (46–48 cm) of all plants ($p \leq 0.05$). In other plant groups, primary shoot lengths were not significantly different (Willows 21–25 cm, Combinations 18–20 cm, CSG 16–18 cm).

Soil Bacterial Enumeration

The total number of bacterial CFUs without plants (bulk soil) and with plants (rhizosphere soils) in different soil amendments at the end of the experiment is presented in Figures 3.11 and 3.12. Addition of soil amendments significantly increased CFUs in Willows and Combination treatments, but in WSG, CSG and unvegetated bulk soil there were no significant differences ($p \geq 0.05$) in CFUs (Fig. 3.11). There were no significant differences in CFUs between plant groups and unvegetated bulk soil without soil amendments (Figure 3.12). However, addition of fertilizer resulted in the highest bacterial CFUs in Willows ($8.07\text{E}+06 \text{ CFU g}^{-1}$), and the addition of fertilizer/molasses increased the CFU in Combinations, Willows and WSG ($7.24\text{E}+06 \text{ CFU g}^{-1}$, $4.31\text{E}+06 \text{ CFU g}^{-1}$ and $5.98\text{E}+06 \text{ CFU g}^{-1}$ respectively).

Total Petroleum Hydrocarbon Concentrations

Concentrations of TPH in soil from various treatments are presented in Figures 3.13 and 3.14. The addition of fertilizer and fertilizer/molasses resulted in significantly lower levels of TPH in all treatments including unvegetated (Fig. 3.13). The average levels of TPH were lower in all treatments with plants than in unvegetated treatments. There were no differences between CSG, WSG, Willows and Combinations in fertilizer and fertilizer/molasses soil amendments

(Fig. 3.14). Percent reduction of TPH levels at the end of the experiment compared to the initial TPH levels in soil is presented in Table 3.3. Unvegetated pots without soil amendments had the lowest reduction of TPH at 7.94%, while the addition of fertilizer and fertilizer/molasses resulted in higher reduction (42.23 % and 38.09 % respectively). A similar trend was observed in all treatments with plants where soils with amendments had significantly lower TPH concentrations. The greatest percent reduction in TPH occurred in all vegetated treatments with fertilizer (66.45 – 75.45%) and fertilizer/molasses (65.95 – 74.06%), while treatments with just soil amendment or vegetation alone had reduced less.

Soil TPH Reducing Factors

Comparison of TPH concentrations from the soil samples taken on the 13th of March, before the start of the study on the 10th of May, 2012, and at the end of the study on the 8th of August, 2012, from two different treatments (unvegetated pot without amendments, vegetated pot with amendment) are presented in Figure 2.15. Before planting, randomized core sampling was conducted from 46 of the 138 pots from the whole depth of the experimental unit, homogenized and measured for loss of TPH on the 10th of May, 2012. In addition, a chromatogram from an unvegetated pot with no soil amendment and a pot with a combination treatment - *Salix dasyclados*/switchgrass with fertilizer/molasses analyzed from August 8th, 2012 were added for comparison.

Analysis of the original soil sample showed a large array of aliphatic hydrocarbons found in diesel fuel with a smaller amount of different semi-volatile ringed aromatic hydrocarbons such as PAHs. A little over 50% (Data not shown) of petroleum hydrocarbons were lost from the original sample to the pre-planting sample taken on the 10th of May 2012 before planting in soil

pots (Figure 3.15). This TPH loss likely occurred through bioremediation and volatilization. In Table 2.3 all experimental units in the study are compared to a baseline generated by the samples taken on the 10th of May 2012. This baseline is a measurement of TPH before the beginning of the plant and amendment introduction. Furthermore, the substantial loss in peak heights over time was greatly reduced throughout the course of the study.

DISCUSSION

Plant Growth

Phytotoxicity symptoms. Phytotoxicity of the contaminant is an important factor that can inhibit the effectiveness of remediation by affecting plant growth. Adam and Duncan (2002) reported that elevated concentrations of TPH represented by high molecular weight hydrocarbons may inhibit seed germination in some species, while low molecular weight carbon fractions are less toxic to seed establishment due to volatility and transformation in the soil substrate. The observed ability of all grasses and willows to germinate or initiate growth from dormant cuttings and survive throughout the study in the presence of TPH demonstrated their resistance to this pollutant. However, the initial concentrations of TPH present in our study expressed as mg/kg represent lower contamination levels than values tested by Adam and Duncan (2002), expressed as g/kg.

The yellowing of the leaves and leaf tip burns observed during this study were likely related to low pH levels as these symptoms were less prominent after the addition of lime. Low pH buffering capacity as well as low nutrient concentrations was attributed to the sandy soil used in these experiments. The occasionally suboptimal soil moisture can also explain yellowing of the leaves as the experiment was conducted in the greenhouse during summer time. Frequently soil temperature exceeded 30°C resulting in increased water evaporation from the soil surface. As a result of this, the plant may have had partial closure of stomata, which in turn result in less water movement from roots up the plant. This created an internal water deficit followed by reduction of CO₂ uptake and photosynthesis causing stress and nutrient uptake deficiencies (Ferro et al., 2003). Ho and Banks (2006) reported similar yellowing and physiological stresses in tall fescue grown in hydrophobic soils.

Aboveground biomass. Aboveground biomass is an important parameter that may affect the efficiency of contaminant degradation. During our study, the highest aboveground biomass was recorded for switchgrass, while willows had the lowest. Switchgrass is a warm-season grass, and its survivability and vigor due to heat and drought tolerance exceeds most of the plant species used in this study. Conversely, it is likely that high air temperature observed during this experiment negatively affected aboveground growth of willows, and under these unfavorable conditions more fixed carbon was allocated to the belowground portion of the plant. Also, willow is a perennial woody species, and its belowground biomass appears to increase rapidly during the first few years while more biomass is allocated to the aboveground portion in the following years (Volk et al., 2011). Thus, due to the short time of this study and limiting rooting volume and depth in the small pots, we did not observed the optimal growth rate of *Salix* species.

The positive effects of fertilizer applications on grass growth were previously documented (Samson et al., 2005), and during this study all grasses produced significantly higher aboveground biomass in fertilized treatments. Even though it has been previously documented that willows respond to fertilizer applications with increased aboveground biomass even during a short-term study (Saska and Kuzovkina, 2014), during this experiment there were no significant differences between fertilized and unfertilized treatments. Again, it is likely that high air and soil temperatures as well as the sandy substrate present in this experiment had a negative effect on aboveground willow growth. Similarly, Euliss et al. (2008) observed that within a 13 month greenhouse study switchgrass aboveground biomass under 26-8-8 (NPK) fertilization regime was significantly higher than biomass of *Salix exigua* under 12-12-12 (NPK) fertilization. Euliss et al. (2008) suggested that the soil substrate of sediments was not conducive for growth and establishment of the willow species. Spriggs et al. (2005) observed in both 9 months and 18

months significant shoot growth of willows over other woody plant species (green ash, *Fraxinus pennsylvanica*, and hybrid poplar, *Populus nigra* x *P. deltoids*) grown in soil from a contaminated wetland. Their soil characteristics were not reported, but wetland substrates are more conducive for willow growth. In summary, the short duration of this study and sandy substrate, limited rooting volume and depth in the pots, combined with hot air and soil temperatures during the experiment did not promote aboveground willow growth, but resulted in sufficient growth of fast germinating grasses.

Soil Bacterial Enumeration

Vegetated vs. Unvegetated Treatments. At the end of the 3-month greenhouse study the bacteria CFUs were higher by orders of magnitude between vegetated and treatments without plants, while willows and combination treatments had the greatest populations of bacteria CFUs. Similar results were reported by Ho and Banks (2006) who observed significantly higher CFUs in treatments with tall fescue than in unvegetated pots in a 6-months experiment, and higher PAH degrading bacteria at the end of a 8-months experiment. Euliss et al. (2008) assessed PAH degrading bacteria in TPH contaminated soils with broadleaf arrowhead (*Sagittaria latifolia*) and upright sedge (*Carex stricta*) and found significantly higher levels of bacterial populations than in unvegetated treatments. In a greenhouse pot study of aged creosote-contaminated soil, Hultgren et al. (2009) reported significantly higher bacterial counts in the presence of basket willow (*Salix viminalis*) than in unvegetated soil. These experiments confirm that the addition of plants and soil amendments will increase bacterial counts compared to unvegetated soils.

Soil Moisture. Keller et al. (2008) suggest that optimization of soil moisture is critical for plant and microbial survival, which provide effective TPH degradation. Soils with lower water content can adversely affect microbial activity, bioavailability of contaminants and microbial degradation (Masciandaro et al., 2013). Nedunuri et al. (2009) showed that over watering also has negative effects on contaminant degradation by generating anaerobic conditions resulting in change in microbial community composition that are less effective for degradation of TPH. Cofield et al. (2007) documented that low soil moisture content presents major problems when establishing plants in sandy loam contaminated with petroleum hydrocarbons. However, after amending their potting mix at a ratio 75:25 to reduce substrate hydrophobicity, plant establishment and microbial counts were greatly improved compared to un-amended treatment (Cofield et al., 2007). During our study, soil moisture was very difficult to optimize. A combination of sandy soil texture and very high ambient soil and air temperature observed in a closed greenhouse system resulted in high evapotranspiration rates and soil water deficiency. It is possible that with a sufficient and stable moisture regime the microbial count could have been increased and result in a higher degradation of TPH efficiency. Instead, however, our soil moisture values were not measured in our experiment toward field capacity because of the difficulty in optimization.

Soil Amendments. Soil nutrients affect the density of microbial populations. Zhou et al. (2009) showed that the density of microbial populations participating in degradation of PAHs is lower in high phosphorus than in low phosphorus soil planted with tall fescue and alfalfa. In soils with more favorable characteristics (such as with the addition of a loam to increase pH and higher organic matter) larger bacteria counts in pots with and without plants are reported compared to non-amended treatments (Cofield et al., 2007; Keller et al., 2008; Cheema et al., 2009). The

addition of plant residues as soil amendments also increases hydrocarbon utilizing microorganisms, resulting in higher rates of TPH degradation (Shahsavari et al., 2013). For instance, pots with just a straw amendment have 3 times higher bacterial counts than pots with willows without amendment, and 20 times higher than unvegetated pots without amendment (Hultgren et al., 2009). Thus, these studies suggest that soil amendments could enhance microbial counts with and without plant species to improve reduction of TPH. In our study, similar observations were observed in the presence of plant species, where microbial counts were enhanced with and without amendments. However, no differences in counts were observed with treatments without plants.

Addition of plants. During our study, bacteria CFU's were in orders of magnitude higher in all vegetated pots compared to unvegetated. Euliss et al. (2008) detected different bacteria densities in the bulk (unplanted) and rhizosphere (planted) soils in relation to TPH degradation using terminal restriction fragment length polymorphism (T-RFLP). In our experiment, the bacteria populations were higher in Willows and Combinations, with the highest in Willows with fertilizer and Combinations with fertilizer/molasses treatments. Even though willows had the lowest aboveground biomass, it is likely that they produced an extensive root system during the first few months that promoted bacterial growth. In our experiment, the bacteria populations were higher in treatments that contained willow cultivars.

Soil TPHs Decrease in System

Leaching potential of TPH. Semple et al. (2003) conveyed that, even though hydrocarbons may not be present in the leachate in significant amounts, their fate has several other pathways in the system (volatilization, degradation, bioaccumulation, or sequestration). To minimize pollutants

from leaving the planted systems, all water that leached from the pots in our study was added back to the soil. Additionally, preliminary analyses of the leachates were conducted in a separate experiment, and the low amounts of hydrocarbons in the leachates detected during this study suggested that the majority of carbon structures present in the soil were hydrophobic. It has been previously reported that leaching of hydrocarbons in a loamy soil is minimal (0-0.015%) (Keller et al., 2008). While the sandy soil substrate used in our experiment had a high potential for soil water leaching through gravitational water flow, the TPH levels in the leachate were not high. The aged contamination from the NHRY was likely bonded onto organic and mineral materials in the soil over time making TPH more recalcitrant to degradation and generating non-extractable components.

Volatilization of TPH: Volatilization of TPH is another factor that often needs to be addressed during a phytoremediation project. We recorded a 50% TPH loss when comparing the soil samples taken on the 13th of March 2012 when the substrate was delivered from NHRY, with samples taken on the 10th of May 2012, just before assembling the experiment in the greenhouse. This loss maybe related to volatilization and bioremediation of hydrocarbons due to high ambient air temperatures. Our soil contained a large array of both aliphatic and aromatic hydrocarbons. If so, then the losses were primarily small aliphatic hydrocarbons (n-alkanes less than C₁₈), where larger aromatic ring hydrocarbons (>C₁₈) likely remained more recalcitrant (Todd et al., 1999; Coulon et al., 2010).

Many factors influence hydrocarbon volatility, which reflect the ability of carbon structures to vaporize from the soil substrate to the atmosphere. Volatilization of TPH can be influenced by a variety of environmental factors such as thickness of the oil layer, oil

composition, solar radiation, moisture content, soil type, wind speed, humidity, and air and soil temperature (Todd et al., 1999). For instance, within an exposed oily substrate, loss of TPH through volatilization after a nine-week study was 10.8 %. However, in a control, where turf cover was present throughout the study, loss of TPHs due to volatilization was only 0.01% (Mahmoud et al., 2010). A majority of these factors mentioned previously by Todd et al. (1999) can also affect the ability of microbial populations to degrade TPH (Boopathy, 2000). Mahmoud et al. (2010) measured microbial populations in Bermuda and American grass and showed higher order of magnitude differences in contaminated soil than pristine clean uncontaminated soil with Bermuda grass. Also, the introduction of oil contamination to the same turf grass environments shifted microbial population percentages to more active biodegraders (Mahmoud et al., 2010).

Thus, hydrocarbons can be lost from the soil through volatilization depending on temperature, aeration, and low water content when these conditions are not conducive for microbial degradation. In a constructed biopile, aromatic ringed hydrocarbons degraded primarily aerobically in the bulk soil through microbial degradation, while very small percentages were lost to advection into the air or volatilization (Coulon et al., 2010). For biopiles to be successful, the regulation of moisture in the system is very important as low moisture content limits the efficiency of microbial degradation and increases volatilization. Their experiments suggest that providing turf cover and sufficient water in a system will mitigate or even eliminate volatilization of the hydrocarbons. Thus, by conducting best management practices and influencing different environmental factors, one can control or significantly reduce the ability of TPH to volatilize from the soil substrate. In our study, bioremediation took place with volatilization and other factors. That is why our data set is evaluated at 10th of May 2012 as

our initial starting point for analysis of the break down of TPH present from the start of the experiment.

Soil Amendments

There are a few critical steps important to improve the success of phytoremediation projects. Optimization of plant growth through the addition of soil amendments stimulates soil metabolic processes resulting in higher percentages of microbial activity within the rhizosphere, driving the main mechanism of breaking down TPHs in the soil (Masciandaro et al., 2013). According to Nedunuri et al. (2009), TPH is broken down even more efficiently with the “synergistic effect” of combining nutrient amendments and more frequent watering regimes. In our study, the addition of fertilizer and fertilizer/molasses amendments resulted in lower levels of TPH at the end of the experiment compared to treatments with no amendments. Interestingly, the lower levels of TPH were observed even in unvegetated treatments at the end of the experiment. There was no significant difference in TPH degradation between fertilizer and fertilizer/molasses treatments.

Planted vs. unplanted treatments. Within our experiment, removal of TPH was more efficient in vegetated treatments compared to treatments without plants. This concurs with findings made in many other studies. Cheema et al. (2009) reported that TPH was broken down more efficiently in treatments planted with *Festuca arundinacea* than in treatments without plants (Cheema et al., 2009). Spriggs et al. (2005) also found that breakdown of TPH in presence of willows and poplars were greater than in unvegetated contaminated soil. Euliss et al. (2008) similarly observed that in treatments planted with sedge (*Carex stricta*), switchgrass, willow, and eastern gamagrass (*Tripsacum dactyloides*), TPH reduction was greater than in unvegetated soil

controls. Rezek et al. (2008) report that the additions of both nutrients and plants (*Lolium perenne*) promoted degradation of high molecular hydrocarbons than unvegetated controls. Nevertheless, after 18 months the unvegetated control had significantly lower TPH levels than the original soil sample suggesting natural attenuation degrades TPH over time (Rezek et al., 2008).

Depending on soil characteristics, it is essential to select plant species adapted to the specific soil substrate to optimize microbial associations within these plant communities to degrade TPH more efficiently (Batty and Anslow, 2008). Cook and Hesterberg (2013) summarize that plant selection between grasses and woody species depends on the factors of plants suitability to soil type, low bioaccumulation and transferring of pollutant, resistance to contaminant, and tolerance to site conditions. Importantly, vegetating a soil substrate that is less conducive to supporting plant life will result in excessive maintenance to promote plant long-term survival and suboptimal colonization of microbial associations to degrade organic pollutants. However, in spite of higher bacteria populations, we did not observe higher degradation of TPH in treatments with willows than in other plant species used in the experiment.

Addition of plant and nutrients. In our study, the breakdown of TPH was greatly enhanced within the presence of both plants and amendments. This increased efficiency of TPH degradation in treatments with plants and soil amendments compared to unplanted soil was previously documented in various studies (Spriggs et al., 2005; Rezek et al., 2008; Euliss et al., 2008; Cheema et al., 2009). Cheema et al. (2010) studied monocultures and combinations of tall fescue, ryegrass, alfalfa, and rape seed planted in a sandy loam soil and found that combinations

of tall fescue and ryegrass, and alfalfa and rapeseed degraded PAHs more efficiently than the species independently planted (Cheema et al., 2010). We were able to record similar differences, where addition of plants and soil amendments resulted in greater breakdown of petroleum hydrocarbons than in unvegetated or in non-amended treatments. However, all plant groups in fertilizer and fertilizer/molasses treatments were not statistically different from each other.

CONCLUSIONS

In summary, TPH degradation was higher in treatments with plants and soil amendments, but not statistically different between plant species over the duration of the experiment. Also, unvegetated treatments with amendments had a similar level of TPH degradation as treatments with plants and soil amendments. Thus, soil amendments enhanced TPH degradation equally efficiently with and without plant species. By providing a carbon source, such as molasses, indigenous microbial communities can degrade TPH in the soil without plants. However, the addition of plants to the phytoremediation system further promotes degradation of the contaminants through more microbial associations that can persist to degrade hydrocarbons. Most phytoremediation experiments are site and contaminate specific, where the addition of nutrients and plants are more conducive toward remediation than without. All these studies suggest that optimizing the soil substrate with nutrients, water, and carbon additions, is best for the most efficient degradation of petroleum hydrocarbons. The overall results of this study indicated that introduction of plants and soil amendments had a positive effect on lowering the TPH concentrations. This study also confirms that phytoremediation of TPH in excessively well drained soils can be pursued successfully if they are kept adequately moist and fertilized. These experiments with short-term duration favors grasses over willow. Perhaps in longer-term experiments, especially in field trials, willows may be more effective.

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Table 3.1: Preliminary assessments of contamination in soil collected at New Haven Rail Yard as listed in the draft Progress Report by Phoenix Laboratories Incorporated, Manchester, CT. Evaluated on December 27, 2011 (Phoenix, 2011) compared to National Primary Drinking Water Standards. *

Parameter Analyzed	Result	Units	**MCL	Units
Inorganic Compounds				
Silver	< 0.37	mg/kg	***ND	
Arsenic	12.5	mg/kg	0.01	mg/L
Barium	34.6	mg/kg	2	mg/L
Cadmium	1.24	mg/kg	0.005	mg/L
Chromium	12.1	mg/kg	0.1	mg/L
Mercury	< 0.10	mg/kg	0.002	mg/L
Lead	101	mg/kg	0.015	mg/L
Selenium	4.3	mg/kg	0.05	mg/L
TPH by GC (Extractable Products)				
Existing Petroleum HC	1700	mg/kg	ND	
Semivolatiles				
Acenaphthylene	610	ug/kg	ND	
Anthracene	710	ug/kg	ND	
Benzo(a)anthracene	1500	ug/kg	ND	
Benzo(a)pyrene	1400	ug/kg	0.0002	mg/L
Benzo(b)fluoranthene	2200	ug/kg	ND	
Benzo(ghi)perylene	670	ug/kg	ND	
Benzo(k)fluoranthene	630	ug/kg	ND	
Chrysene	1500	ug/kg	ND	
Fluoranthene	2000	ug/kg	ND	
Indeno(1,2,3-cd)pyrene	640	ug/kg	ND	
Phenanthrene	2800	ug/kg	ND	
Pyrene	2800	ug/kg	ND	
Benzene	ND		0.005	mg/L
Soil Properties				
Soil pH	7.61	PH		
Conductivity	350	umhos/cm		
Total Organic Carbon	28000	mg/kg		

*Full copy of this report and referenced analytical procedures can be downloaded from Phoenix Laboratories Incorporated website. Report says must not be reproduced except in full as defined by the attached chain of custody. **Maximum Contaminant Level (MCL) is the highest level of a contaminant that is allowed in drinking water from the National Primary Drinking Water Regulations set by United States Environmental Protection Agency (EPA, 2009). ***ND stands for no data presented by the officiated group.

Table 3.2: Soil Characteristics and Chemical Properties of New Haven Rail Yard Soil Tested by the Soil Testing Lab in Storrs, CT

Soil Characteristics*	Values
Soil Properties	
Sand (%)	90.3
Silt (%)	5.2
Clay (%)	4.5
Soil Texture	Sand
Soil pH	5.3
Organic Matter(mg/kg)	7000.0
Macronutrients (kg/ha)	
Ca	1417.7
Mg	227.3
P	11.2
K	70.6
Micronutrients (mg/kg)	
B	0.3
Cu	1.5
Fe	130.6
Mn	11.2
Zn	6.2
Al	24

*Soil textural analysis by shaker and ribbon methods; pH in 1:1 aqueous suspension; organic matter by loss on ignition; macronutrients (Ca, Mg, P, K) and micronutrients (B, Cu, Fe, Mn, Zn, Al) by Modified Morgan extractable procedure (McIntosh, 1969) and Inductive Coupled Plasma Atomic Emission Spectrometer.

Table 3.3: Percent reduction of TPH (mg/kg) concentrations in contaminated soil from New Haven Rail Yard measured after 90 days in unvegetated treatments, treatments with cool season grasses CSG (tall fescue), warm season grasses WSG (switchgrass), willows (*S. dasyclados* and *S. eriocephala*) and plant combinations (*S. dasyclados* /switchgrass) across soil amendments (Control, Fertilizer, Fertilizer/Molasses). Means unvegetated bulk soil (n=3) and vegetated soil (n=4) followed by the same uppercase letter in the same column, and same lowercase letter in the same row are not significantly different, according to Fisher's LSD ($p < 0.05$).

Soil Amendment	Unvegetated	CSG	WSG	Willows	Combination s
Control	7.94Aa	41.99Ab	50.41Ab	50.61Ab	45.81Ab
Fertilizer	42.23Ba	69.35Bb	75.45Bb	68.65Bb	66.45Bb
Fertilizer/Molasses	38.09Ba	65.95Bb	74.06Bb	68.01Bb	69.70Bb

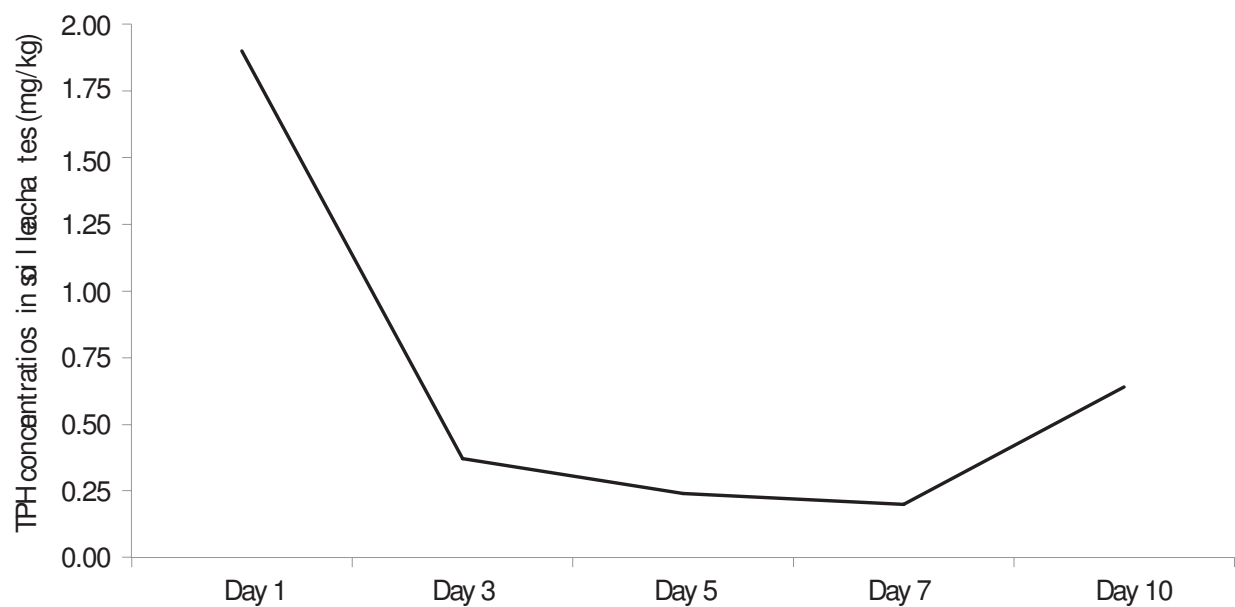


Figure 3.1: Total petroleum hydrocarbon concentrations (mg/kg) in leachate samples collected from unvegetated pots filled with New Haven Railroad contaminated soil over a 10-day period (n = 3).

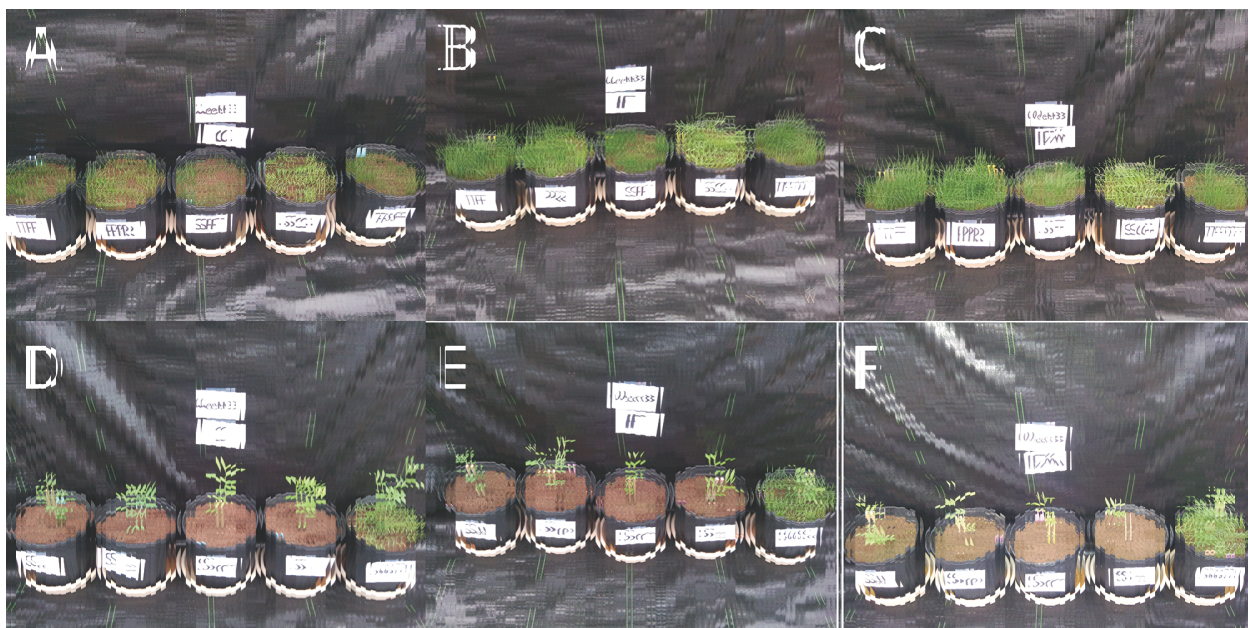


Figure 3.2: Plant growth in TPH contaminated soil from New Haven Rail Yard during Week 3 in control treatment (no soil amendments) [A][D], fertilizer [B][E], and fertilizer/molasses [C][F] (tall fescue (TF), perennial ryegrass (PR), sheeps fescue (SF), switchgrass, tall fescue and sheeps fescue combination (TFSF), and willow treatments *S. dasyclados* (Sd), *S. purpurea* (Sp), *S. miyabeana* (Sm), *S. eriocephala* (Se), and switchgrass and *S. dasyclados* combinations (SGSd))

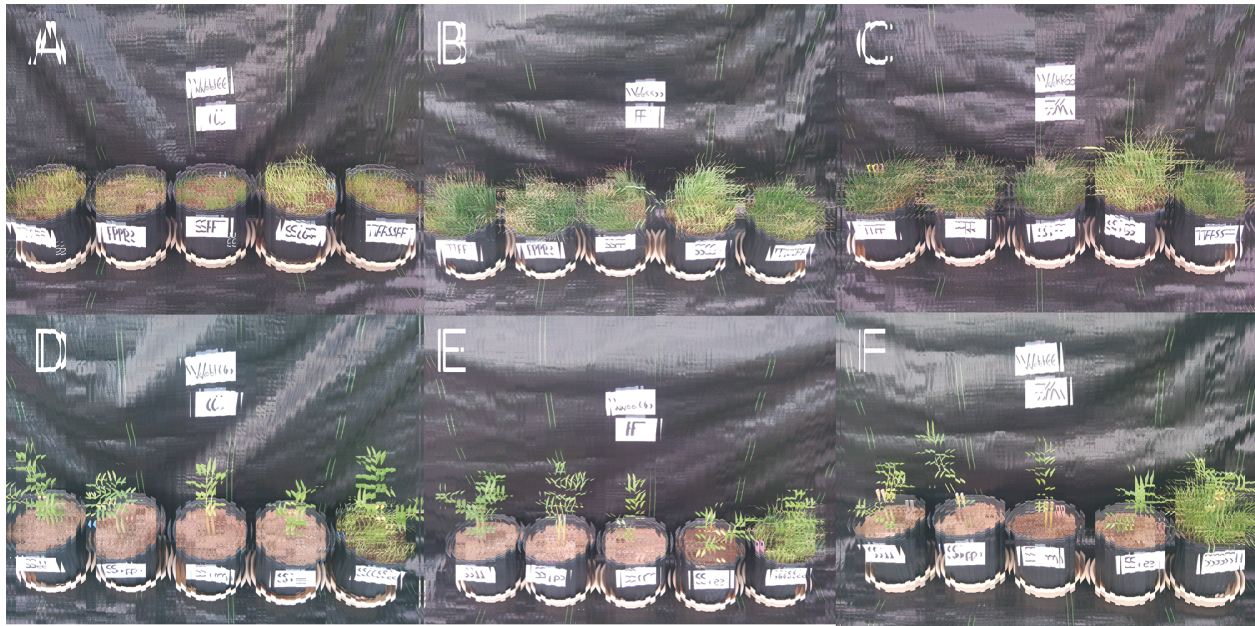


Figure 3.3: Plant growth in TPH contaminated soil from New Haven Rail Yard during Week 6 in control treatment (no soil amendments) [A][D], fertilizer [B][E], and fertilizer/molasses [C][F] (tall fescue (TF), perennial ryegrass (PR), sheeps fescue (SF), switchgrass, tall fescue and sheeps fescue combination (TFSF), and willow treatments *S. dasyclados* (Sd), *S. purpurea* (Sp), *S. miyabeana* (Sm), *S. eriocephala* (Se), and switchgrass and *S. dasyclados* combinations (SGSd))

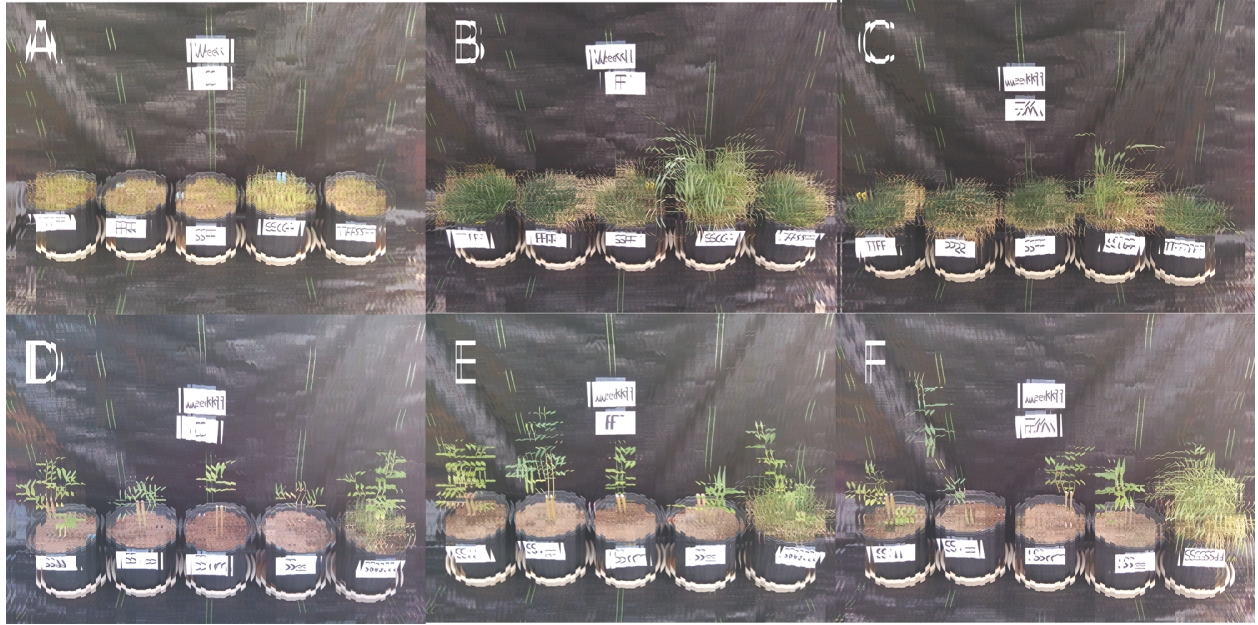


Figure 3.4: Plant growth in TPH contaminated soil from New Haven Rail Yard during Week 9 in control treatment (no soil amendments) [A][D], fertilizer [B][E], and fertilizer/molasses [C][F] (tall fescue (TF), perennial ryegrass (PR), sheeps fescue (SF), switchgrass, tall fescue and sheeps fescue combination (TFSF), and willow treatments *S. dasyclados* (Sd), *S. purpurea* (Sp), *S. miyabeana* (Sm), *S. eriocephala* (Se), and switchgrass and *S. dasyclados* combinations (SGSd))

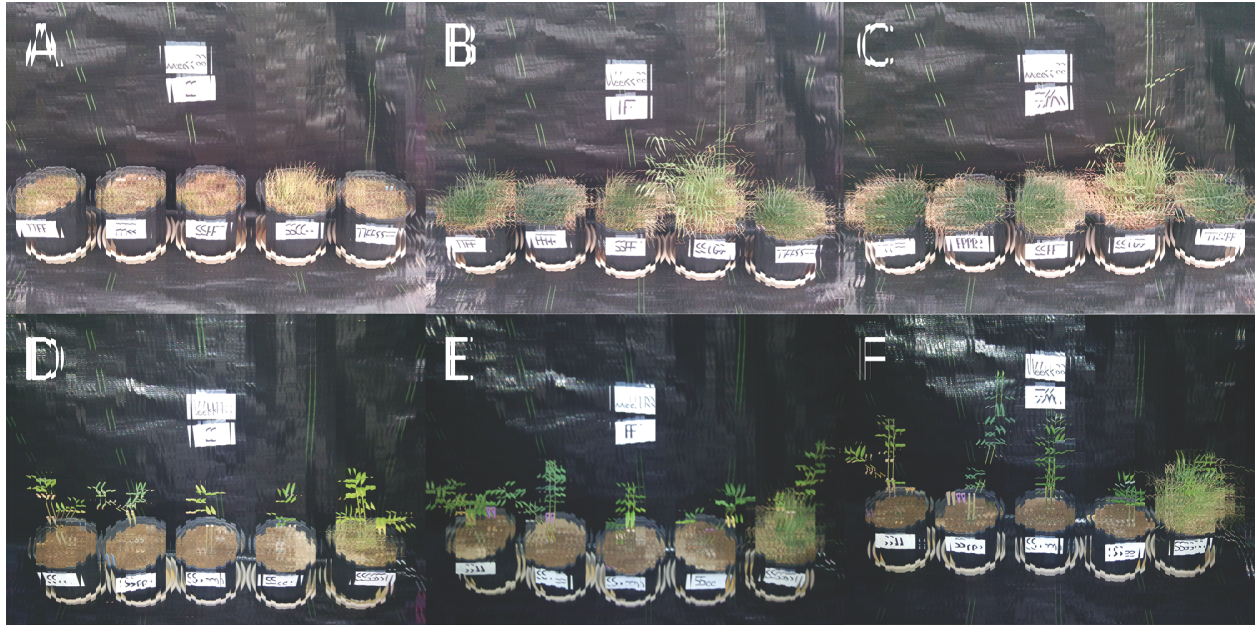


Figure 3.5: Plant growth in TPH contaminated soil from New Haven Rail Yard during Week 12 in control treatment (no soil amendments) [A][D], fertilizer [B][E], and fertilizer/molasses [C][F] (tall fescue (TF), perennial ryegrass (PR), sheeps fescue (SF), switchgrass, tall fescue and sheeps fescue combination (TFSF), and willow treatments *S. dasyclados* (Sd), *S. purpurea* (Sp), *S. miyabeana* (Sm), *S. eriocephala* (Se), and switchgrass and *S. dasyclados* combinations (SGSd))

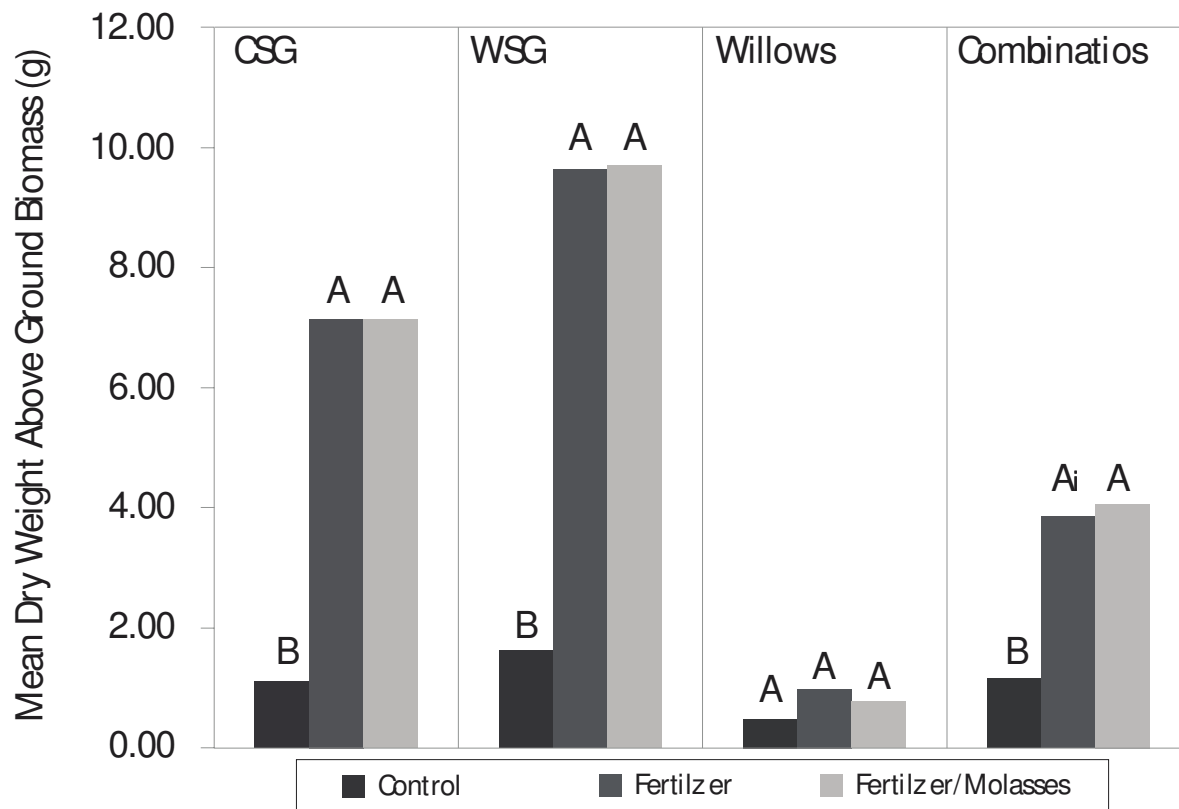


Figure 3.6: Mean dry weight (g) of aboveground biomass of cold season grasses (CSG) tall fescue, perennial ryegrass, sheep fescue), warm season grass (WSG) (switchgrass), willows (*S. dasyclados*, *S. eriocephala*, *S. purpurea*, *S. miyabeana*) and plant combinations (tall fescue/sheep fescue and *S. dasyclados*/switchgrass) with soil amendments (Control, Fertilizer, Fertilizer/Molasses) grown in pots with TPH contaminated soil from New Haven Rail Yard for 90 days. Means (n=4) followed by the same letter in the same column are not significantly different, according to Fisher's LSD, ($p < 0.05$).

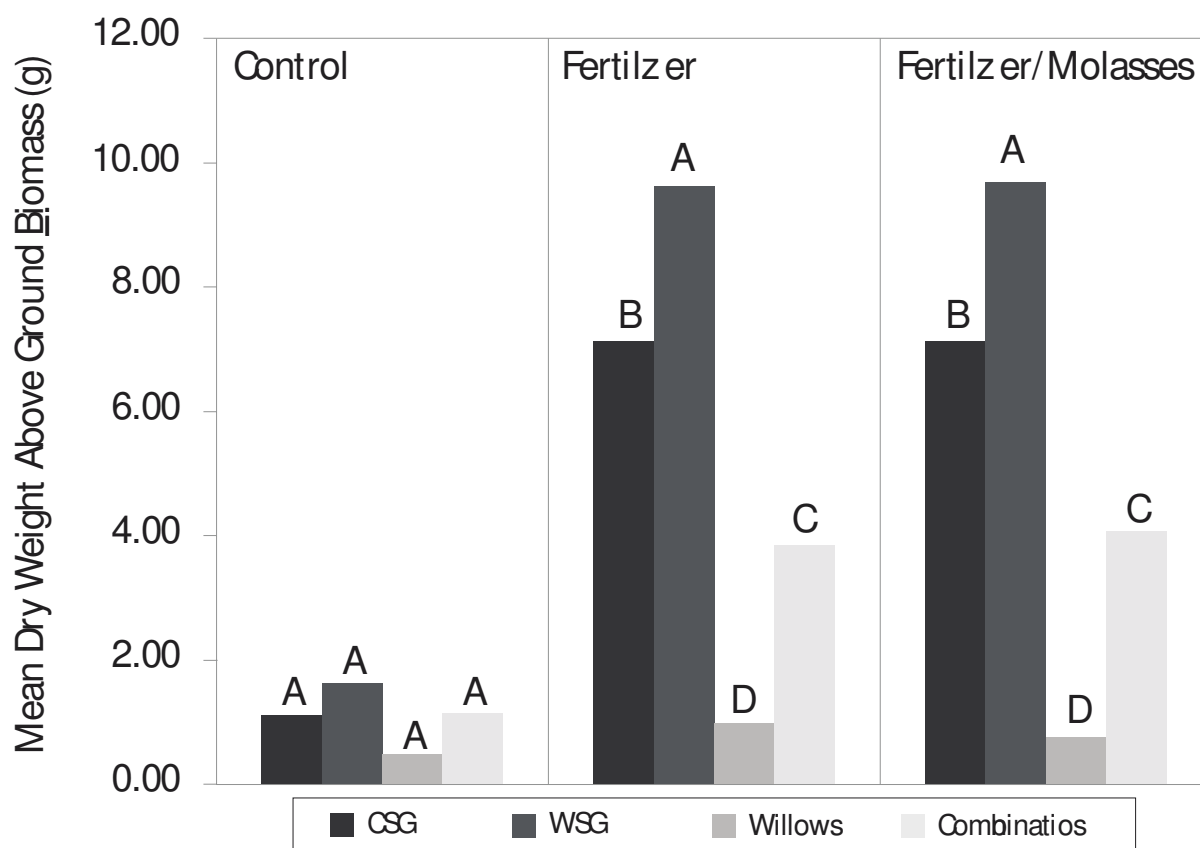


Figure 3.7: Mean dry weight (g) of aboveground biomass of cold season grasses (CSG) tall fescue, perennial ryegrass, sheep fescue), warm season grasses (WSG) (switchgrass), willows (*S. dasyclados*, *S. eriocephala*, *S. purpurea*, *S. miyabeana*) and plant combinations (tall fescue/sheep fescue and *S. dasyclados*/switchgrass) across soil amendments (Control, Fertilizer, Fertilizer/Molasses) grown in pots with TPH contaminated soil from New Haven Rail Yard for 90 days. Means (n=4) followed by the same letter in the same column are not significantly different, according to Fisher's LSD, ($p < 0.05$).

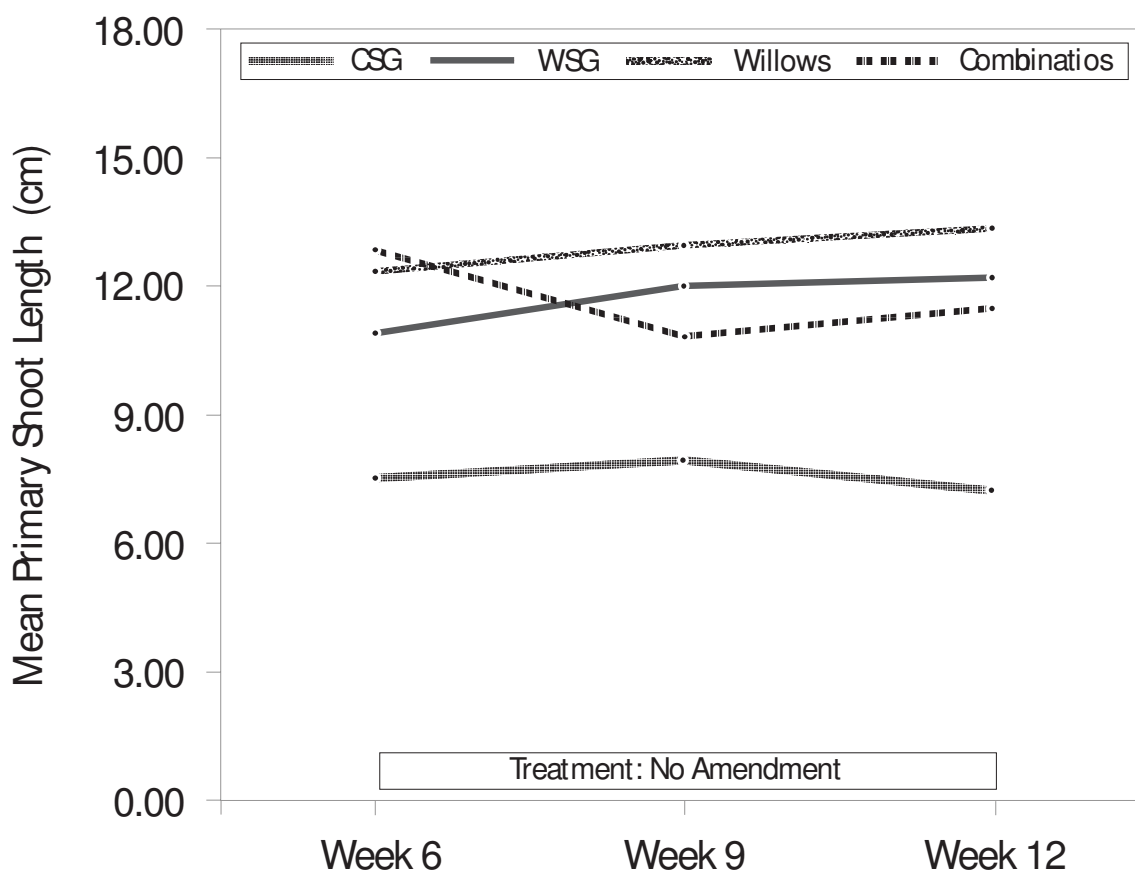


Figure 3.8: Mean (n=4) of plant shoot lengths (cm) of cold season grasses CSG (tall fescue, perennial ryegrass, sheep fescue), warm season grasses WSG (switchgrass), willows (*S. dasyclados*, *S. eriocephala*, *S. purpurea*, *S. miyabeana*) and plant combinations (tall fescue/sheep fescue and *S. dasyclados*/switchgrass) grown in pots with TPH contaminated soil from New Haven Rail Yard for 90 days without amendments and measured at week 6, 9 and 12.

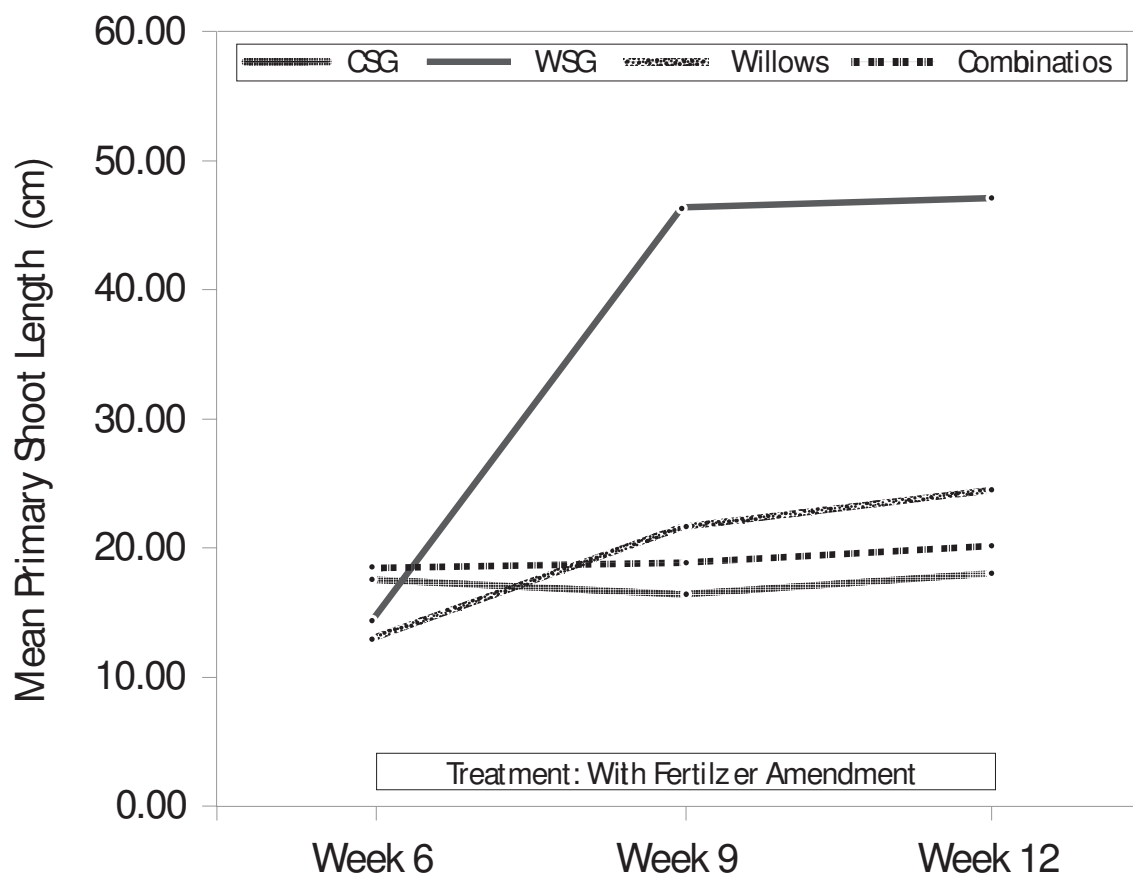


Figure 3.9: Mean (n=4) of plant shoot lengths (cm) of CSG (tall fescue, perennial ryegrass, sheep fescue), WSG (switchgrass), willows (*S. dasyclados*, *S. eriocephala*, *S. purpurea*, *S. miyabeana*) and plant combinations (tall fescue/sheep fescue and *S. dasyclados*/switchgrass) grown in pots with TPH contaminated soil from New Haven Rail Yard for 90 days with fertilizer amendments and measured at week 6, 9 and 12.

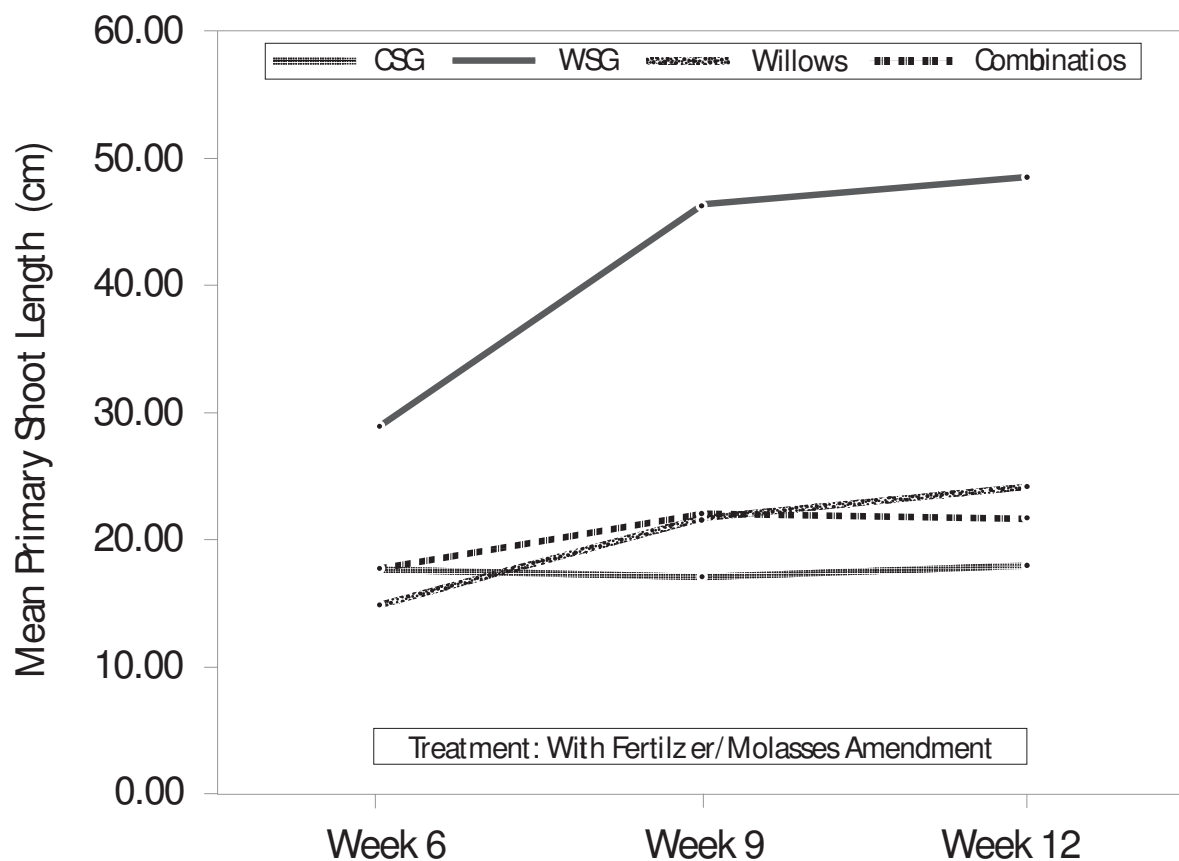


Figure 3.10: Mean (n=4) of plant shoot lengths (cm) of CSG (tall fescue, perennial ryegrass, sheep fescue), WSG (switchgrass), willows (*S. dasyclados*, *S. eriocephala*, *S. purpurea*, *S. miyabeana*) and plant combinations (tall fescue/sheep fescue and *S. dasyclados*/switchgrass) grown in pots with TPH contaminated soil from New Haven Rail Yard for 90 days with fertilizer/molasses amendments and measured at week 6, 9 and 12.

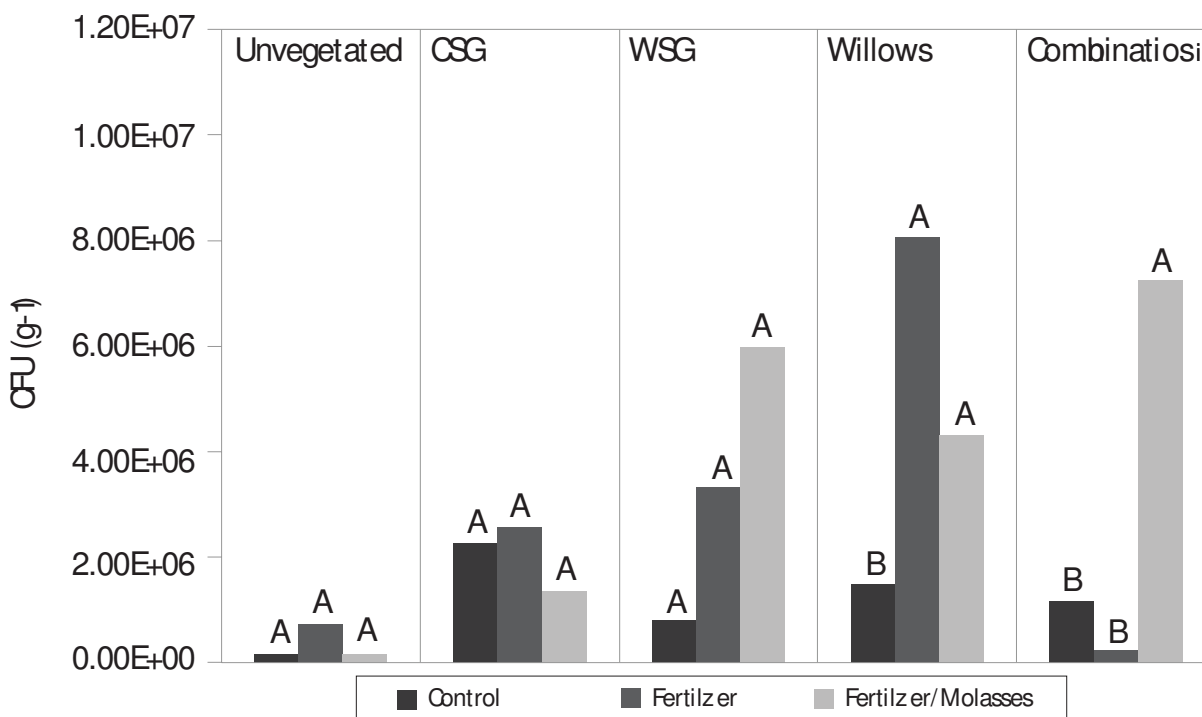


Figure 3.11: Bacteria colony-forming units per gram of soil (CFU g⁻¹) sampled across unvegetated treatments and plant groups: cool season grasses CSG (tall fescue, perennial ryegrass, sheep fescue), warm season grasses WSG (switchgrass), willows (*S. dasyclados*, *S. eriocephala*, *S. purpurea*, *S. miyabeana*) and plant combinations (tall fescue/sheep fescue and *S. dasyclados*/switchgrass) within soil amendments (Control, Fertilizer, Fertilizer/Molasses) grown in pots with TPH contaminated soil from New Haven Rail Yard for 90 days. Means of unvegetated bulk soil (n=3) and vegetated rhizospheric soil (n=4) followed by the same letter in the same column are not significantly different, according to Fisher's LSD, (p < 0.05).

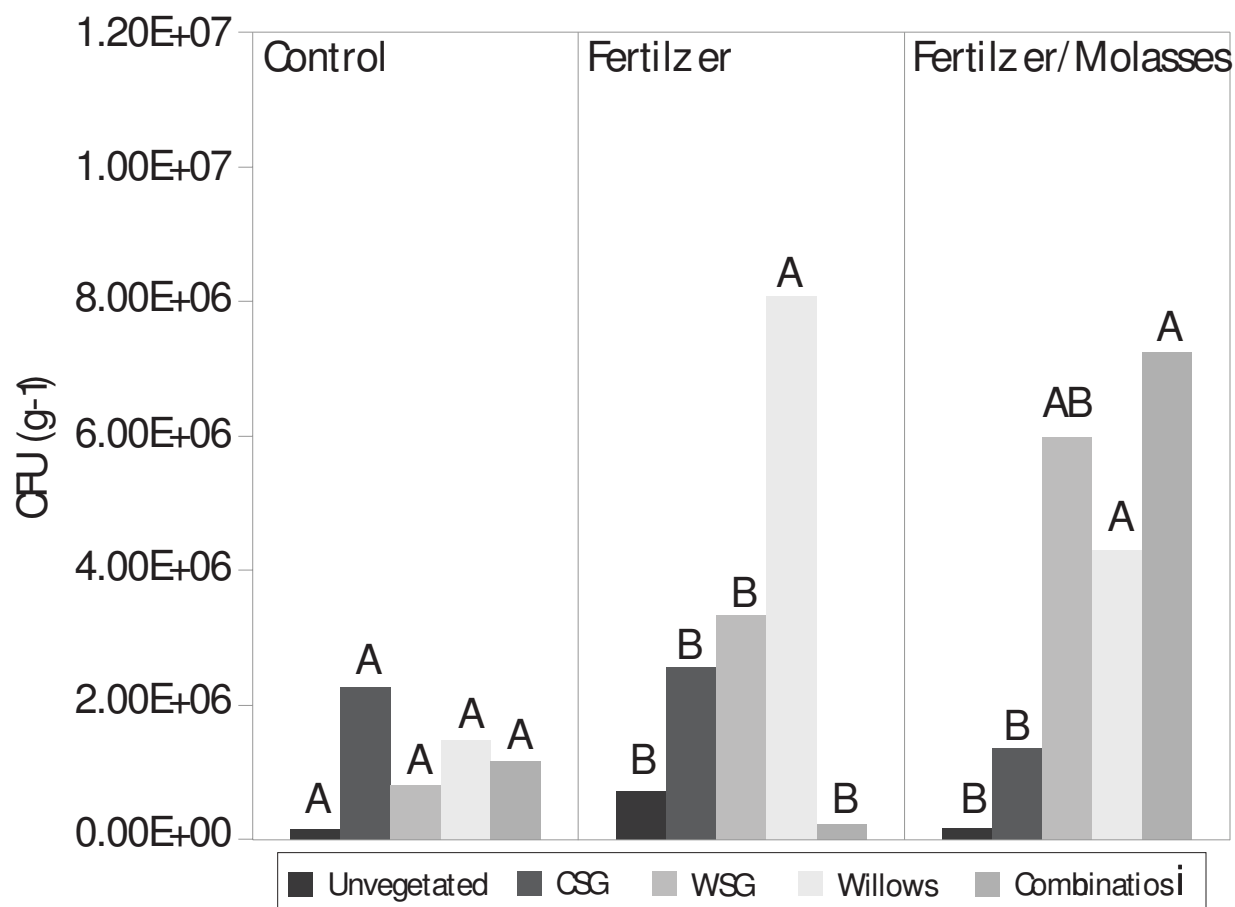


Figure 3.12: Bacteria colony-forming units per gram of soil (CFU g⁻¹) sampled within unvegetated treatments and plant groups: cool season grasses CSG (tall fescue, perennial ryegrass, sheep fescue), warm season grasses WSG (switchgrass), willows (*S. dasyclados*, *S. eriocephala*, *S. purpurea*, *S. miyabeana*) and plant combinations (tall fescue/sheep fescue and *S. dasyclados*/switchgrass) across soil amendments (Control, Fertilizer, Fertilizer/Molasses) grown in pots with TPH contaminated soil from New Haven Rail Yard for 90 days. Means of unvegetated bulk soil (n=3) and vegetated rhizospheric soil (n=4) followed by the same letter in the same column are not significantly different, according to Fisher's LSD, (p < 0.05).

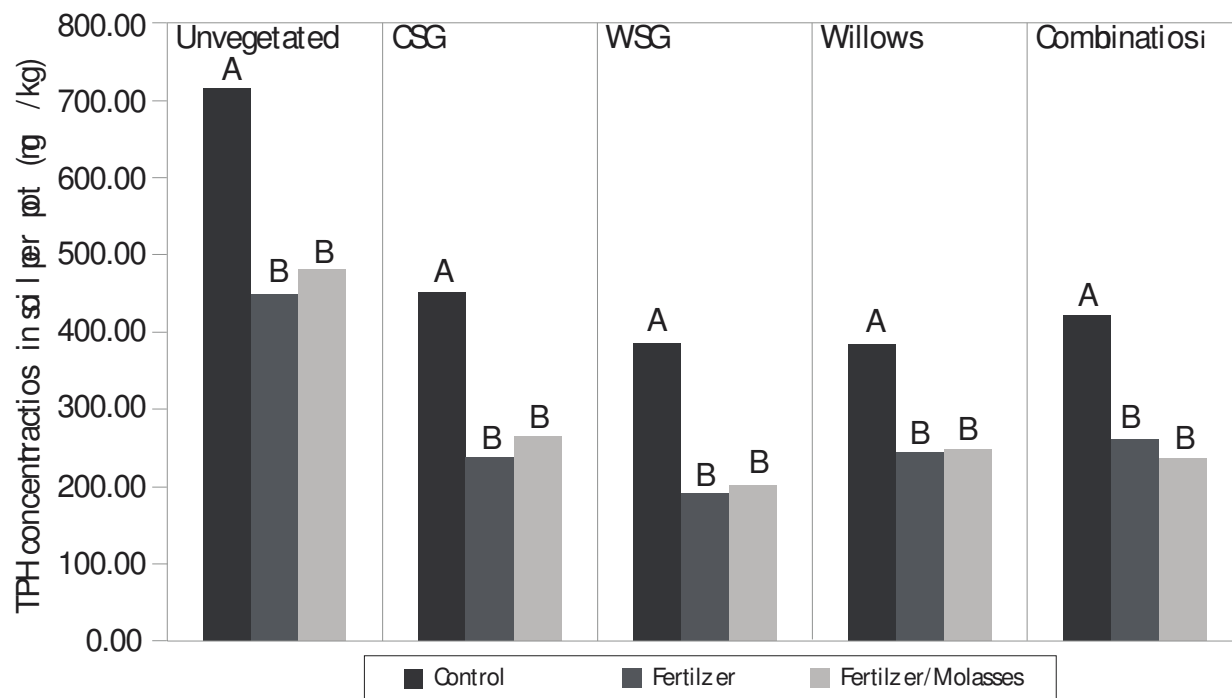


Figure 3.13: Mean concentrations of TPH (mg/kg) in contaminated soil from New Haven Rail Yard in unvegetated treatments and cold season grasses CSG (tall fescue), warm season grasses WSG (switchgrass), willows (*S. dasyclados* and *S. eriocephala*) and plant combinations (*S. dasyclados*/switchgrass) within soil amendments (Control, Fertilizer, Fertilizer/Molasses) grown in pots with TPH contaminated soil from New Haven Rail Yard for 90 days. Means of unvegetated soil (n=3) and vegetated soil (n=4) followed by the same letter in the same column are not significantly different, according to Fisher's LSD ($p < 0.05$).

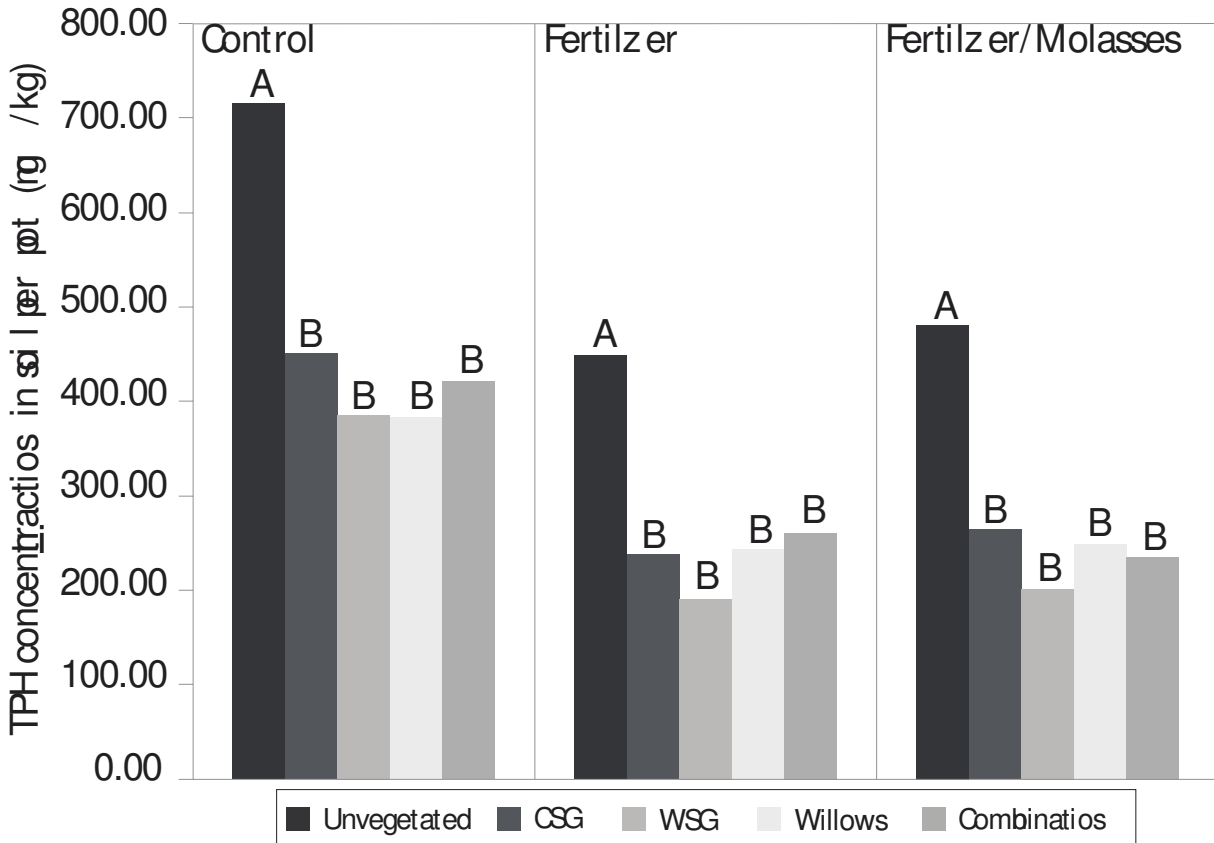


Figure 3.14: Mean concentrations of TPH (mg/kg) in contaminated soil sampled within unvegetated treatments and plant groups: CSG (tall fescue), WSG (switchgrass), willows (*S. dasyclados* and *S. eriocephala*) and plant combinations (*S. dasyclados*/switchgrass) across soil amendments (Control, Fertilizer, Fertilizer/Molasses) grown in pots with TPH contaminated soil from New Haven Rail Yard for 90 days. Means unvegetated bulk soil (n=3) and vegetated soil (n=4) followed by the same letter in the same column are not significantly different, according to Fisher's LSD, ($p < 0.05$).

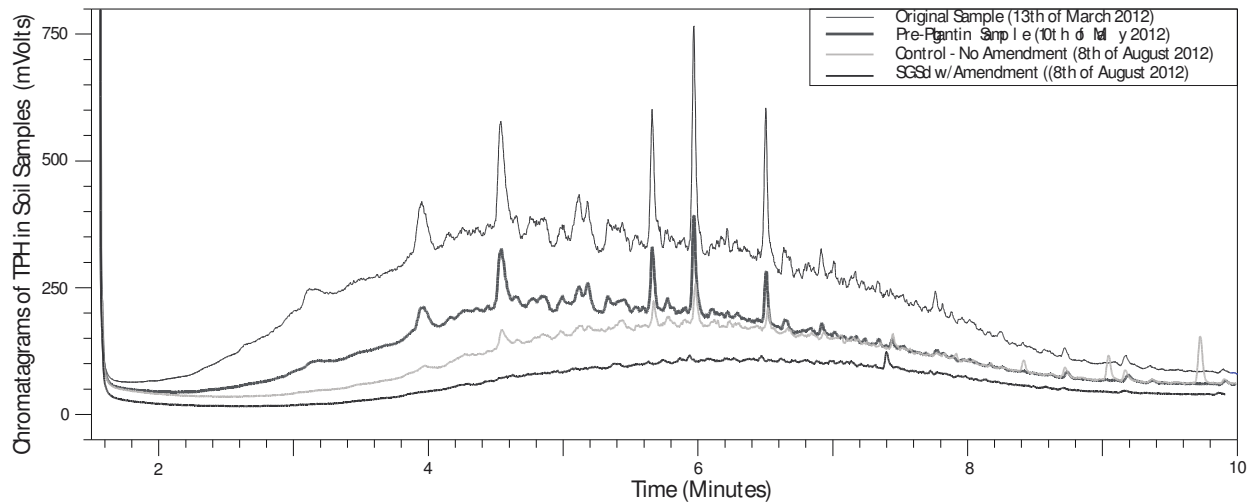


Figure 3.15: Comparison of chromatograms from the original sample on March 13th, 2012, pre-planting sample before experimental setup on May 10th, 2012, and at the end of the study unvegetated control pot with no amendment, and *Salix dasyclados* /switchgrass with fertilizer/molasses soil amendment on August 8th, 2012.

CHAPTER IV

Bio- and Phytoremediation of Total Petroleum Hydrocarbons (TPH) Under Vegetated and Unvegetated Conditions

ABSTRACT

Remediation of TPH in a contaminated sandy soil under various vegetated and unvegetated conditions, biostimulation and phytoremediation techniques was assessed in a 90-day greenhouse pot study. The soil contained residual petroleum hydrocarbons from the New Haven Rail Yard, New Haven, CT., which was previously phytoremediated, then spiked with used motor oil before the beginning of the experiment. The treatments included saturated (unvegetated), intermittently aerated (unvegetated and vegetated), with plastic sealed covers (unvegetated saturated and unvegetated at field capacity) and fertilized (unvegetated at field capacity and vegetated at field capacity). Vegetated treatments used switchgrass (*Panicum virgatum*). Hydrocarbons were measured by gas chromatography, and indigenous microbes were quantified by colony-forming units (CFU). At the end of the experiment bacteria CFU's were in orders of magnitude higher in vegetated pots compared to unvegetated, where vegetated fertilized pots had the highest number of bacteria CFU's. Final TPH concentrations were the lowest in all saturated soil treatments, and highest in the presence of switchgrass. Final TPH concentrations were also low in unvegetated pots with fertilizer. Thus, remediation of freshly introduced motor oil on pre-phytoremediated soil was most efficient in saturated, anaerobic environments.

Keywords: Bioremediation, phytoremediation, biostimulation, bioventing, petroleum hydrocarbons

INTRODUCTION

Total petroleum hydrocarbon (TPH) contamination consists of aliphatic and aromatic carbon structures that originate from various manufacturing processes, as well as combustion of diesel and crude oil byproducts. The main sources of release of these compounds into the environment are from leaking underground storage tanks, mining operations, accidental spills, or effluent discharges (Todd et al., 1999). Heavier aromatic ringed TPH constituents have been reported to cause carcinogenic effects from increased systemic exposure. However, only a relatively small number of these hydrocarbon formulations are documented regarding adverse health effects from their toxicity (Todd et al., 1999).

Non-aqueous phase liquids (NAPL) are mixtures of hydrocarbon compounds that are usually from liquid or semi-liquid sludges such as diesel fuel or oil (De Jonge et al., 1997; Todd et al., 1999). These compounds proliferate extensively within the environment, and are mainly presented as dispersed droplets or film layers on soil particles (De Jonge et al., 1997; Namkoong et al., 2002). At higher concentrations, NAPLs hinder the ability of microorganisms to break down petroleum contaminants in soil substrates. Conversely, at low enough concentrations, NAPLs are transformed into simple carbon fragments that are absorbed by the soil matrix (De Jonge et al., 1997; Namkoong et al., 2002).

While surfactants are generally used, microorganisms can also lower the concentrations of NAPL. Microorganisms use extracellular enzymes, such as peroxidases and laccases, to cleave the contaminant, making carbon fragments more soluble for assimilation (Mougin, 2002). By generating a more uniform distribution of the contaminant, its bioavailability increases and makes the remediation of hydrocarbons with microorganisms more effective (De Jonge et al., 1997; Namkoong et al., 2002).

Bioremediation technology utilizes microbial associations to breakdown TPH within soil or liquid phase matrices (Van Hamme et al., 2003), and has been an important economical technique over the last several decades (Vidali, 2001; Shukla et al., 2010). Various bioremediation approaches have been classified in a variety of different *in-situ* (bioparging, bioventing, bioaugmentation, biostimulation, phytoremediation and natural attenuation) and *ex-situ* (land farming, composting, bioreactors, biofilters, pump and treat) methods to accomplish specific remedial needs (Boopathy, 2000; Shukla et al., 2010). Bioventing emphasizes diffusing oxygen through soil to stimulate the microorganisms' ability to consume hydrocarbons (Boopathy, 2000; Shukla et al., 2010). Biostimulation focuses on enhancement of indigenous microbial communities within soil or groundwater, and it can be performed either in place or by removing the soil for treatment (Boopathy, 2000). *Ex-situ* biostimulation allows for optimization of soil conditions and incubation parameters, making the system more efficient toward microbial remediation (Boopathy, 2000; Mougin, 2002).

Similarly, phytoremediation introduces plants to enhance associated microbial communities within the rhizosphere to accomplish biostimulation remediation processes (Pilon-Smits, 2005). For example, grass species such as switchgrass (*Panicum virgatum*) promote degradation of recalcitrant carbon fractions within its rhizosphere by stimulating microbial groups and increasing populations compared to the bulk soil (Pilon-Smits, 2005; Jimmy Carter Plant Materials Center, 2011). Phytoremediation is difficult to manage in sandy soils, where lack of nutrients and moisture directly affect plant growth (Ferro et al., 2003, Keller et al., 2008; Nedunuri et al., 2009).

Both biostimulation and phytoremediation techniques are dependent on a variety of factors that can be optimized to improve efficiency and microbial sustainability (Boopathy,

2000; Shukla et al., 2010). Soil moisture content, temperature and dissolved oxygen affect microbial communities and their ability to breakdown TPH (Boopathy, 2000).

Aerobic microorganisms produce dioxygenase and monooxygenase enzymes that induce the transformation and mineralization of TPH (Van Hamme et al., 2003). In aerobically active soils, organisms use oxygen as a reactant for metabolic activities. Soil moisture directly affects the amount of oxygen in a soil system (Masciandaro et al., 2013).

Plant roots significantly enhance soil aeration by increasing porosity and lowering soil moisture content. Accordingly, plants promote a more dynamic environment for aerobic microbes to breakdown hydrocarbons (Cook and Hesterberg, 2013). Mascianaro et al. (2013) reported that the optimum moisture range for aerobic biodegradation of hydrocarbons was between 45 and 85% of the soil's water holding capacity. The environment is anaerobic at high moisture levels, while at low moisture levels the survival and capacity to breakdown hydrocarbons of aerobic bacteria is impacted (Coulon et al., 2010).

Saturation of the soil can occur with increasing depth, generating low oxygen levels within the deep soil layers (Hurst et al., 1996; Cook and Hesterberg, 2013) and limiting aerobic degradation. However, bacteria in saturated conditions have the ability to substitute oxygen for other electron acceptors (Boopathy, 2000). In anaerobic environments, microbial communities can use carbon dioxide, sulfate-reduction, iron(III)- reduction, denitrification, and manganese oxides as electron acceptors, depending on their availability and the redox potential of the matrix (Boopathy, 2000; Van Hamme et al., 2003). These communities can also utilize soil humic acids and fermentative oxidation processes to reduce organic contaminants (Van Hamme et al., 2003). In anaerobic environments, humic substances can act as electron shuttles enhancing the electron transfer speed during anaerobic biotransformation of organic contaminants (Chen et al., 2012).

Soil systems with low water content can affect microbial activity and bioavailability of contaminants, while high water content can generate anoxic conditions with lower rates of diffused oxygen (Masciandaro et al., 2013).

In summary, various microbial communities can utilize aerobic or anaerobic mechanisms to breakdown petroleum hydrocarbons in soil (Masciandaro et al., 2013). For example, aerobic systems were more effective than anaerobic systems in a bioremediation study of mangrove sediment slurries, where higher-ringed hydrocarbons were biodegraded more efficiently in the presence of low oxygen ($2\pm0.3\%$) than in non-oxygen (0%) environments (Li et al., 2010). Similarly, in a 22-day remediation study of lagoon effluents, greater breakdown of TPH was observed in the intermittent aeration treatments compared to no aeration treatments, but curiously lower break down was observed in treatments with constant aeration (Vieira et al., 2009). However, in a laboratory study of marine sediments that were held anaerobically for 60 days, followed by an aerobic 30 day interval, 91% break down of TPH was achieved, which was 30 – 40% higher than under aerobic conditions for the entire 90 days of the experiment (Rocchetti et al., 2011). Boopathy et al. (2012) found that the highest removal of TPH (78%) in anaerobic marsh sediments after an 80-day incubation period was achieved with mixed electron acceptor environments instead of nitrate-reducing or sulfate-reducing conditions singularly. However, an 8.1% reduction was reported for natural attenuation (Boopathy et al., 2012).

The objective of this study was to compare aerobic and anaerobic treatments for remediation of freshly introduced used motor oil on a sandy soil previously phytoremediated with some residual TPH contamination. This study seeks to evaluate various approaches to remediation of TPH in a greenhouse pot study, which included: saturation (unvegetated), intermittent aeration (vegetated and unvegetated), installment of plastic sealed covers

(unvegetated, saturated and field capacity) and fertilizer addition (vegetated and unvegetated at field capacity). Thus, anaerobic treatments included saturation with and without aboveground covers to mitigate surface diffusion of oxygen. Aerobic treatments consisted of intermittent aeration, as well as the addition of plants and nutrients, along with a natural attenuation.

MATERIALS AND METHODS

Soil Characteristics

Soil used in this study had aged petroleum hydrocarbons collected from New Haven Rail Yard (NHRY) and was previously phytoremediated (Chapter 3). The soil with residual hydrocarbons was then spiked with fresh NAPL (7.9 mL/pot) from used motor oil, and mixed in an industrial soil mixer to ensure homogeneity. Prior to the beginning of the experiment, the soil was air dried and crushed to pass through a 6.35-mm sieve, then thoroughly mixed with NAPL from used motor oil, and 3661.8 kg/ha (16.8 g/pot) granular lime was added to optimize soil pH for plant and microbial establishment. Before the beginning of the study three hundred grams of soil was cored from each experimental pot to be sealed and stored at -20°C for TPH analysis to evaluate initial TPH concentrations. Its average initial TPH (n=40) values and soil characteristics are presented in Table 4.1.

Experimental Design

The study was conducted in the climate controlled Floriculture Greenhouse at the University of Connecticut located in Storrs, CT: lat.41°48 N', long.72°15W'. During the study, the temperature cooling and heating threshold was set to 24°C/day and 16°C/night with a natural photoperiod. Occasionally the temperatures exceeded 24°C/day during the experiment but never fell below 16°C/night.

Soil (9.5 kg) was added to lined 7.34 liter C900 series (24.13-cm width by 23.18-cm height) plastic containers. All treatments, except saturated, had 30 cm by 5 cm saucers to collect leachate that was added back to the pot surface daily to minimize potential loss of TPH in the system. In the saturated treatments, a C900 pot was placed inside of an 11.36-liter C1200 series

(27.94-cm width by 24.13-cm height) plastic container lined with plastic, and then filled with tap water to 2.5 cm above soil level, which was maintained throughout the experiment. For treatments with covers, plastic bags were tightly wrapped with rubber bands around the top of the pots. Treatment with oxygen amendment had a 0.3175 cm inner diameter Tygon tubing inserted from the top of the soil down to 7.6 cm from the bottom of the pot and connected with a coupler to a pressurized air tank (Figure 4.1). Oxygen was applied to each experimental unit for 6 minutes daily at a rate of 71.4 mL/min. For treatment with fertilizer, amendments of 20-20-20 Jack's water-soluble fertilizers (JR Peters Inc., Allentown, PA) were applied weekly at 195.3 kg/ha (375 mg/pot/week). Fertilizer was added to both treatments to optimize plant growth as positive effects of fertilizer on C4 grass growth was previously documented (Samson et al., 2005). For treatments with plants, switchgrass seed (*Panicum virgatum* 'Cave-in-Rock') (Ernst Conservation Seed, Meadville, PA) was broadcasted at 292.95 kg/ha into each pot (1.34 grams seed/pot). Soil pH was measured weekly in these planted treatments and adjusted when necessary with a pelletized lawn lime (Soil Doctor, Haines, FL) at a rate of 488.24 kg/ha per pot (10 grams/pot).

All treatments were set up on 19 June 2013. A randomized complete block design with a total of 40 pots representing five replications was used with the following eight treatments: Saturated (S), Saturated Covered (SC) (Fig 4.1a), Control (C), Soil with aboveground cover (CC), Oxygen (O), Fertilizer (F), Switchgrass and Fertilizer (GF), and Switchgrass, Fertilizer and Oxygen (GFO) (Fig. 4.1b). All pots were mist irrigated using tap water. The threshold for the mist irrigation rate was based on the amount of evapotranspiration from solar radiation per unit of time expressed as above $600 \text{ J/s}\cdot\text{m}^2$ (Argus, 2010). That is, when light exceeded this threshold, the system would disperse 20 ml of water over 10 seconds. Water rates were regulated

during higher temperature days to water more efficiently, and during overcast days mist irrigation was manually controlled. The watering rates were designed to mitigate and evapotranspiration in plants and soil.

At the end of the experiment on 19 September 2013, switchgrass aboveground biomass was harvested at 2.5 cm above soil surface, dried in an oven for 72 hours at 70°C and weighed. Each experimental unit was destructively sampled at the end of the study to be analyzed for pH, microbial activity, soil organic matter and TPH. Soil pH values were measured on the same day in a 1:1 aqueous suspension after sieving through 2-mm mesh (Sims and Eckert, 2011). In S and SC treatments, the contaminated water remaining in the pot was weighed and a 500 mL sample was taken from each pot for leachate analysis.

Approximately 300 g of soil was collected from the middle of each pot at a depth of 10-13 cm from the bottom and another sample was collected from 2.5 cm from the bottom of each pot. Soil subsets were weighed then placed in an oven at 400°C for 16 hours to analyze the amount of organic matter by loss on ignition (Sparks et al., 1996). All samples were stored at -20°C for TPH extraction. For bacteria enumeration, 30 g of bulk soil was collected from these same areas of the pot, and rhizospheric samples were taken within 10 mm from the switchgrass roots. During soil sampling the tools and containers were sterilized with 70% ethanol. All bulk and rhizospheric soil samples were weighed and sealed in pre-autoclaved falcon tubes at room temperature until microbial analysis was performed during the same day.

Soil Bacterial Enumeration

One gram of soil from each treatment was placed into autoclaved falcon tubes with M9 saline solution (Geerlof, 2010) that consisted of 75.2 g/L Na₂HPO₄, 30.0 g/L KH₂PO₄, 5g/L

NaCl, and 5 g/L NH_4Cl at pH 7.2 and covered while being stored at room temperature until microbial analyses were performed during the same day. Several falcon tubes as controls were used to measure amount of culturable bacteria in the air of the greenhouse. The serial dilutions were conducted with samples from unvegetated and saturated pots at 10^{-3} , 10^{-4} , and 10^{-5} , and from treatments with switchgrass pots at 10^{-4} , 10^{-5} , and 10^{-6} (Page, 1982). Bacteria were plated on beef extract agar plates with subsequent nutrient concentrations: 0.3% beef extract, 0.5% peptone, and 1.5% agar per liter at pH 6.8. The microbes were extracted from the soil by shaking each falcon tube for 20 seconds during each dilution, and 1:10 solution ratio was added to the next dilution prepared with saline solution. One milliliter of remaining solution was then added to each plate, spread with a sterilized glass rod, and then incubated at 20°C in dark conditions for 3 days to target each particular dilution level. The CFU's were quantified visually within duplicates on a scale of 30–300 culturable bacteria per gram of soil (Page, 1982).

Soil TPH analyses

Soil TPH concentrations were analyzed using the modified EPA Method 3550C ultrasonic extraction procedure (USEPA, 2007). Ten grams of soil were removed from -20°C storage and added to a clean pre-weighed 125-mL Erlenmeyer flask. Then, 25-mL of dichloromethane (DCM) was added to each flask and covered with parafilm. The flasks were ultrasonicated for five minutes and the remaining extract solution was decanted into a clean DCM-washed, pre-weighed 100-mL beaker. This extraction method was repeated three times. Ten grams of anhydrous Na_2SO_4 were added to each beaker to remove residual water from the extract solution. Each sample was vacuum filtered through glass microfiber filters into pre-weighed DCM-washed 30 mL test tubes. Final extracted solution was recorded for each sample

before being concentrated down to one test tube (about 10–15 mL) under N₂ gas. Samples were then weighed, parafilmed, covered with foil, and placed in 4°C storage until analyzed by gas chromatography (GC).

Leachate Analyses

In S and SC treatments the amount of water left in the C1200 pot was weighed and a 500-mL sample was taken from each experimental unit for leachate analysis (USEPA, 2007). The leachate samples were analyzed using a modified EPA Method 3510C separatory funnel liquid-liquid extraction procedure (EPA, 1996). Approximately 250 mL of the total 500-mL leachate sample was placed in a DCM washed 500-mL separatory funnel. Twenty-five milliliters of DCM were added to the separatory funnel, and shaken vigorously for two minutes while opening the cap every 5–10 seconds to release excess pressure. Phase separation of leachate and extractant occurred after the separatory funnel was upright in a ring stand for ten minutes. After phase separation the remaining extract solution was drained into a 100-mL DCM washed beaker. This extraction method was repeated three times and the procedure was done twice to capture the entire original 500-mL leachate sample from each pot. The rest of the extraction procedure was conducted according to the EPA protocol for soil TPH analyses. Flasks containing the remaining solvent were ultrasonicated for five minutes and the remaining extract solution was decanted into a clean DCM-washed, pre-weighed 100-mL beaker. This extraction method was repeated three times. Ten grams of anhydrous Na₂SO₄ were added to each beaker to remove residual water from the extract solution. Each sample was vacuum filtered through glass microfiber filters into pre-weighed DCM-washed 30-mL test tubes. Final extracted solution was recorded for each sample before concentrating them down to one test tube (about 10–15 mL) under N₂ gas. After samples were concentrated they were weighed, parafilmed, covered with foil, and placed in 4°C storage

until analyzed by GC.

Analytical Specifications for Gas Chromatography System

Gas chromatography was used to determine the TPH concentrations in the soil and leachate samples. Standards of 125 and 250 mg/kg in DCM were generated from 50,000 mg/kg diesel fuel in methylene chloride stock solution. About 1–1.4 μL of standards were injected into an Agilent 6890 series Gas Chromatography system (Agilent Technologies, Santa Clara, CA) with flame ionization detector (GC-FID) and used to generate chromatographs to evaluate total peak area under the standard curve (Agilent Technologies, Santa Clara, CA). For each extracted sample, similar volumes of 1–1.4 μL were consecutively injected manually with three repetitions. A 12 m x 0.25 mm inner diameter Rt -PAH fused silica capillary column (ResTek, Penn Eagle Industrial Park, CA) was used to quantify retention of carbon over time. The GC temperature was set at 80°C for two minutes, and then increased to 280°C at a rate of 8°C/min to 200°C followed by 16°C/min, and finally held for five minutes. The total run time for each sample was 30 minutes. Helium was used as the carrier gas at a pressure of 8.3 psi, flow rate 1.7 mL/min, and average velocity of 46 cm/sec at constant flow. The split/splitless injection port was held at 280°C. The GC-FID was a 50:50 mix of 40 mL/min hydrogen and 400 mL/min air at 350°C. All gases were ultra pure grade. The chromatogram was integrated from 4 to 18 minutes using OpenLAB CDS EZChrom software (version 4.1a, Agilent Technologies, Wilmington, DE).

Statistical Analysis

Statistical analysis was performed using SAS software (version 9.3, SAS Institute, Cary, NC). Analysis of variance was used to analyze TPH in soil, leachates, moisture and organic matter with the MIXED procedure. All assumptions were tested on each data set for violations with the GLM procedure for independence of the residuals and homogeneity of variances using the HOVTEST of Brown-Forsythe analysis. Normality of the distribution using Shapiro-Wilk was evaluated by the UNIVARIATE procedure. No variables violated parametric assumptions. When significant, comparisons of means were examined using Fisher's least significant differences ($p \leq 0.05$). The initial soil TPH of each experimental unit was used as a covariate between the soil TPH at the end of the experiment in the middle and the bottom of the soil pots. Pearson's chi-square analysis was used with the FREQ procedure to evaluate bacteria counts likelihood of independence from treatments.

RESULTS AND DISCUSSION

Soil Organic Matter

Soil organic matter was not significantly different ($p \geq 0.05$) in the middle samples between all treatments (Table 4.2). In the bottom samples, SC and GF were significantly different from each other in organic matter content but not from the rest of treatments. All treatments had significantly similar ($p \geq 0.05$) soil organic matter content between the middle and the bottom samples taken in each experimental unit, except for treatments C, F, O, and GF ($p \leq 0.05$) which had lower values in the bottom samples. This suggests that some transformation or assimilation of organic material to more labile useable forms took place in the soil in the bottom of the soil pot in treatments C, F, O, and GF.

Contaminated media often has unfavorable environmental conditions for plant and microbial growth. Organic matter content, along with other factors, can affect microbial breakdown of hydrocarbons (Masciandaro et al., 2013). Specifically, humification of organic matter can decrease bioavailability of contaminants by redistributing soluble degradable fractions to forms less bioavailable for plants and microorganisms to assimilate. In a previous study the addition of compost and other carbon-based amendments resulted in 50% higher reduction in TPH (Masciandaro et al., 2013), and in another study mixing electron acceptors (fermenting, sulfate and nitrate reducing acceptors) with molasses as a simple carbon source facilitated 78 % higher efficiency of hydrocarbons breakdown compared to the control (Boopathy et al., 2012).

Plant Biomass and Bacterial Counts

The dry weights of aboveground tissues ($10.90 - 10.94 \text{ g pot}^{-1}$) were not significantly different ($p \geq 0.05$) between switchgrass treatments GF and GFO. Plant biomass is considered to

be sensitive to the amount of oxygen supplied from soil aeration, where the greater supply of oxygen leads to higher biomass (Letey et al., 1962). In our study, however, this was not observed presumably because the daily aeration inputs used were relatively low.

The total number of aerobic bacterial CFUs in treatments without plants (bulk soil) and with switchgrass (rhizosphere soils) is presented in Table 4.3. Pearson's chi-square analysis was used to verify the likelihood that the microbial population in each experimental unit was independent of each CFU range (1.00E+04 to 1.00E+05, 1.00E+05 to 1.00E+06, greater than 1.00E+06 CFU). Bacterial populations were not independent of treatments, and fell into specific ranges of aerobic bacterial CFU counts associated with certain treatments. There were no significant differences ($p \geq 0.05$) between treatments except for GF and GFO, which had higher numbers of bacterial CFUs (Fig. 4.2). It has been previously recorded that natural microbial communities are higher by orders of magnitude in the presence of plants than in bulk soil (Pilon-Smits, 2005; Ho and Banks, 2006; Euliss et al., 2008; Hultgren et al., 2009). Masciandaro et al. (2013) suggest that the addition of soil amendments optimizes plant growth and results in increased microbial efficiency to breakdown TPH.

Total Petroleum Hydrocarbon Concentrations in Leachates

Semple et al. (2003) states that fate of the hydrocarbons has several pathways including volatilization, degradation, bioaccumulation, sequestration and leaching. The water that gravitrophically remained from S and SC pots while saturated were evaluated for TPH. The leachate TPH concentrations during our experiment were in the range of 0.60–0.80 mg kg⁻¹ and were not significantly different ($p \geq 0.05$) between treatments S and SC. No other treatment was evaluated for leachate because the other experimental units were closed systems where by if any leachate was collected in the drainage pot saucer it was returned to the pot surface. The low

amount of hydrocarbons in solution in treatment S and SC suggested that the majority of detectable TPH did not enter the solution and was retained in the soil system. Keller et al. (2008) reported a similar observation where, leaching of hydrocarbons in a loamy soil was minimal (0 - 150 mg kg⁻¹).

Breakdown of TPH in Soil

Breakdown of soil TPH in various treatments at the end of the experiment are presented in Table 4.4. Saturated treatments S and SC as well as fertilized treatment F had the highest TPH reduction (85.9% – 88.1 %), while vegetated treatments GFO and GF had the lowest reduction of TPH in the middle samples (52.3 % – 63.0 %). Accordingly, reduction of TPH at the bottom samples was only significantly different between the SC and F treatments (88.38 – 89.52%) and the C and GFO treatments (77.7% – 89.9%).

Final concentrations of TPH in soil within the middle and bottom samples from various treatments (Fig. 4.3) were similar and not significantly different ($p \geq 0.05$) between treatments C, S, SC, F, and GF. However, in treatments CC, O, and GFO there were significant differences ($p \leq 0.05$) between their sampling depths. For the middle sampling depth, lowest TPH concentrations were in treatments S, SC, and F (Figure 4.4). Treatment F was statistically similar to O but different to all other treatments. The highest TPH concentrations at the middle sampling depth at the end of the study were in treatments GF and GFO. Concentrations of TPH at the bottom sampling depth were only significantly different ($p \geq 0.05$) between the SC and F treatments and the C and GFO treatments.

Saturated Treatments

Microbial degradation, microbial activity and bioavailability of contaminants are directly impacted by soil water content (Masciandaro et al., 2013). Nedunuri et al. (2009) argued in favor of aerobic conditions stating that anaerobic conditions induced by over watering can directly change microbial communities and develop a net negative effect on contaminant degradation. Conversely, Rocchetti et al. (2011) found that greater degradation of TPH was observed when anaerobic conditions were maintained prior to aerobic conditions. In their laboratory experiment a 60-day saturated treatment was followed by a 30-day unsaturated treatment producing 30-40% higher efficiency in breaking down of TPH, compared to unsaturated treatment alone. In our study, reductions of TPH in saturated treatments S and SC were significantly higher than in aerated treatments O (Table 4.5 Middle Sample Location).

We were not able to specifically identify which electron acceptors were involved in the anaerobic degradation of NAPL hydrocarbons. However, at the bottom of the pots with saturated conditions, humus like material was observed, which suggests that some form of decomposition /transformation was taking place in conjunction with the bottom liner. Under anaerobic conditions, reducing environments occur and promote sulfate-reducing processes, iron (III)-reducing processes, denitrifying conditions, and/or use manganese oxides as electron acceptors (Boopathy, 2000; Van Hamme et al., 2003). For example, high molecular weight hydrocarbons, such as fluroanthene, can be anaerobically oxidized to carbon dioxide under sulfate-reducing conditions (Coates et al., 1997; Kanaly and Harayama, 2000).

Intermittently Aerated Treatments

The addition of an air supply promotes the release of root exudates and enhances the activity of microbial communities (Masciandaro et al., 2013). Plants have the ability to oxygenate soil through various means, particularly by reducing the amount of soil moisture in the substrate (Cook and Hesterberg, 2013). Various studies documented that treatments with an intermittent air supply increase the efficiency of TPH degradation, compared to non-aerated or anaerobic treatments (Vieira et al., 2009; Li et al., 2010).

Thus, aerated treatments with plants should have the highest amount of oxygen that results in creating favorable conditions for breakdown of TPHs. However, this was not observed during our experiment, suggesting that, in sandy soils rich in NAPLs, hydrocarbons are more conducive to breakdown under low to no oxygen conditions, rather than in highly oxygenated, plant assisted treatments (Fig. 4.4, middle samples). The addition of only an air supply in treatment O resulted in significantly better degradation than treatment GFO (Table 4.5). Bulman et al. (1993) and Moller et al. (1995) found similar results where the addition of both nutrients and air increased reduction in diesel fuel or residual oil.

In our study, the fertilized soils in the middle of the pots had lower concentrations of TPH than the control treatment (Table 4.5). By providing an essential nutrient supply, stimulation of indigenous microbial communities can enhance degradation of hydrocarbons (Boopathy, 2000). Similarly, in a clay soil in an 8-week laboratory incubation study, biostimulation with inorganic fertilizers and the addition of carbon and nutrients from biosolids had higher percent degradation of TPH compared to natural attenuation (Sarkar et al., 2005). Bento et al. (2005) also found higher degradation of high molecular weight hydrocarbons on a sandy loam and loamy sand when in fertilized treatments compared to natural attenuation.

Covered Treatments

Significantly different levels of TPH reductions in treatments with plastic sealed covers were recorded, where SC treatment had greater reduction than treatment CC (Table 4.5). Using covers on pots should reduce the volatilization loss of TPHs. However, in spite of this, lower TPH concentrations in saturated anaerobic treatments (S and SC) were observed than in aerobic treatments (C, CC, O, GF, GFO). This reaffirms the significance of anaerobic environments in the breakdown of TPH.

Fertilized treatments

In our study, TPH reductions in fertilized treatments were comparable to saturated treatments (Table 4.5, middle sample). However, the mechanism for TPH reductions in fertilized treatments was not identified. Some treatments had different rates of breaking down TPH between the middle and bottom of the contaminated soil pots. This could be related to the combination of availability of the TPH, soil moisture, oxygen, and nutrients as well as potential for the microbial communities to disperse TPH. Nutrients in the soil such as nitrates can be used as electron acceptors instead of oxygen because they are much more soluble, have equivalent free energy, and their byproducts of decomposition do not accumulate within the system (Macrae and Hall, 1998). Boopathy (2004) found statistically similar degradation of TPHs when either using nitrate or sulfate reducing conditions in silt loam soil columns. However, when introduced as a mixed electron system their final concentration of TPH was halved. Furthermore, fermentation oxidation can accompany denitrification under reducing conditions (Ambrosoli et al., 2005). Fermentation is possible for high molecular weight hydrocarbons if there exists a

syntrophic relationship with hydrogen consuming microorganisms, or the presence of highly oxidized molecules in anaerobic conditions.

In summary, saturated soils have more than one method that can help the matrix to breakdown hydrocarbons. The efficacy of these different methods (denitrifying, sulfate reducing, fermentation, etc.) either independent or in combination is based on the amount of nutrients and oxygen within the system. Additionally, soil type is an important factor that affects root surface area and the release of root exudates in unsaturated treatments. The ability of a plant to release root exudates is much higher in loamy sands than in sandy soils (Masciandaro et al., 2013). It is possible that our sandy soil from New Haven Rail Yard, which typically requires intensive water and nutrient management, did not receive optimum conditions for plant growth and effective phytoremediation of TPH. Conversely, soil that has been already phytoremediated and freshly spiked with NAPLs had acclimated indigenous microbial communities that were more effective in breaking down hydrocarbons than introducing new microbes with the addition of plant treatments.

CONCLUSIONS

The age of the TPH contamination should influence the selection of remediation method applied for degradation of hydrocarbons. The efficiency of our pre-phytoremediated soil system to breakdown freshly introduced hydrocarbons may be due to already pre-established microbial associations, easily bioavailable contaminants, and optimized soil conditions for microbial establishment and survival, as were also noted by Makadia et al. (2011). In our previous study, we accessed different bio-phyto mechanisms toward aged contaminant breakdown in sandy soil (Chapter 3). In that study, aerobic plant treatments degraded aged hydrocarbons more efficiently than treatments without plants. In our present study, “fresh” NAPLs were used to spike a sandy soil that had been previously phytoremediated. This “fresh” form of contamination is more easily bioavailable and degraded anaerobically. Our studies suggest that TPH is more readily bioavailable and biodegradable in spiked (fresh) samples compared to aged samples. Thus, the choice of remediation methods (aerobic phytoremediation or anaerobic bioremediation) should be based on age, amount, and availability of the contaminant.

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Table 4.1: Characteristics and chemical properties of contaminated soil from previously remediated New Haven Rail Yard soil and after the addition of fresh motor oil.

Soil Characteristics*	Values
Sand (%)	89.2
Silt (%)	6.0
Clay (%)	4.8
Soil Texture	Sand
Soil pH	5.2
Organic Matter (mg/kg)	7000
Macronutrients (kg/ha)	
Ca	500
Mg	101
P	19.0
K	198
Micronutrients (mg/kg)	
B	0.10
Cu	0.50
Fe	12
Mn	3.8
Zn	1.4
Al	130
Initial TPH (mg/kg)	1700

*Soil textural analysis by shaker and ribbon methods; pH in 1:1 aqueous suspension; organic matter by loss on ignition; macronutrients (Ca, Mg, P, K) and micronutrients (B, Cu, Fe, Mn, Zn, Al) by Modified Morgan extractable procedure (McIntosh, 1969) and Inductive Coupled Plasma Atomic Emission Spectrometer. The TPH in each experimental unit was extracted using ultrasonication with dichloromethane and averaged (n=40) (EPA, 2007).

Table 4.2: Percent soil organic matter (g) in contaminated soil treatments from New Haven Rail Yard measured after 90 days in Control (C), Saturated (S), Saturated Covered (SC), Control Covered (CC), Oxygenated (O), Fertilized (F), Switchgrass Fertilized (GF), and Switchgrass Fertilized and Oxygenated (GFO) across sampling events (Initial Sample June 19, 2013; Middle Sample and Bottom Sample, September 19, 2013) Means (n=3) followed by the same uppercase letter in the same column, and same lowercase letter in the same row are not significantly different, according to Fisher's LSD ($p < 0.05$).

Treatment	Organic Matter (%)	
	Middle Sample	Bottom Sample
C	1.58Aa	1.39ABb
S	1.50Aa	1.37ABa
SC	1.59Aa	1.44Aa
CC	1.58Aa	1.37ABa
O	1.61Aa	1.37ABb
F	1.60Aa	1.41ABb
GF	1.56Aa	1.29Bb
GFO	1.55Aa	1.40ABa

Table 4.3: Chi-Square analysis of evaluating independence of bacteria colony-forming units per gram of soil (CFU g⁻¹) sampled within treatments Control (C), Saturated (S), Saturated Covered (SC), Control Covered (CC), Oxygenated (O), Fertilized (F), and Switchgrass Fertilized (GF), and Switchgrass Fertilized and Oxygenated (GFO). Asterisks falling into the same microbial dilution range represent treatment replications that are not independent of each other (n=5).

Treatments	1.00E+04 - 1.00E+05	1.00E+05 - 1.00E+06	>1.00E+06
C		*****	
S	*****		
SC	*	****	
CC		*****	
O		*****	
F		*****	
GF			*****
GFO			*****

Table 4.4: Reduction of TPH (mg/kg) from residual and spiked contaminants measured as a percentage after 90 days in Control (C), Saturated (S), Saturated Covered (SC), Control Covered (CC), Oxygenated (O), Fertilized (F), Switchgrass Fertilized (GF), and Switchgrass Fertilized and Oxygenated (GFO) across sampling events (Initial Sample June 19, 2013; Middle Sample and Bottom Sample, September 19, 2013) Means of Middle Sample, n=5, Bottom Sample, n=3 followed by the same uppercase letter in the same column, and same lowercase letter in the same row are not significantly different, according to Fisher's LSD ($p < 0.05$).

Treatment	Middle Sample (percent loss)	Bottom Sample (percent loss)
C	67.0Ba	70.0Aa
S	88.1Da	88.3ABa
SC	85.9Da	88.4Ba
CC	64.5Ba	82.9ABb
O	74.7BCa	89.9ABb
F	82.3CDa	89.5Ba
GF	63.0ABa	77.7ABa
GFO	52.3Aa	69.8Ab

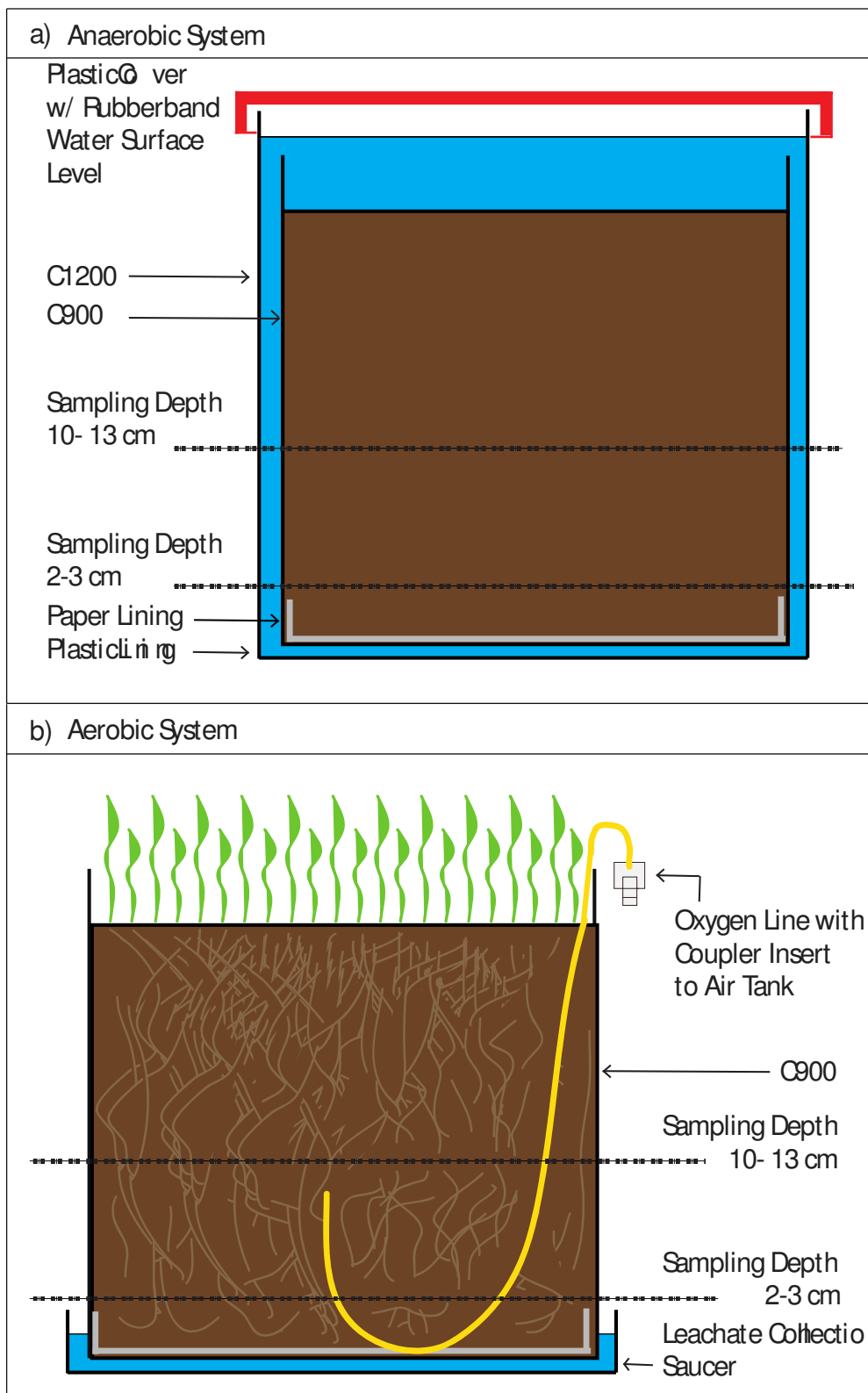


Figure 4.1: Experimental set-ups for treatments (a) Saturated Covered (SC), and (b) Oxygenated (O and GFO).

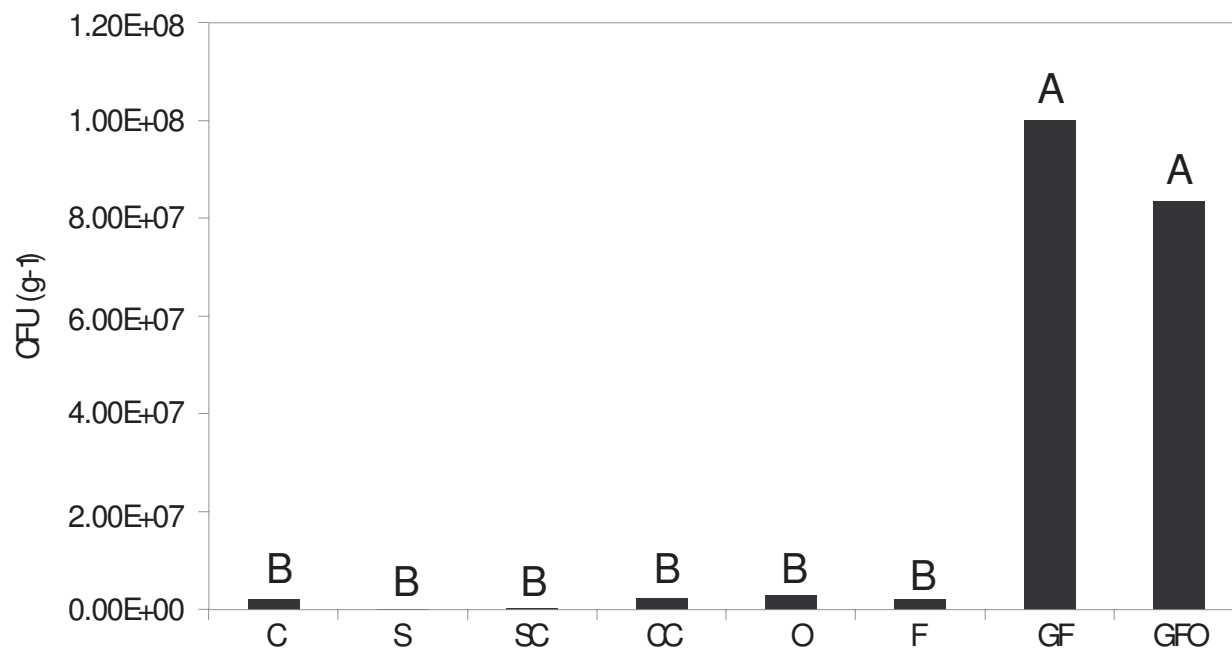


Figure 4.2: Bacteria colony-forming units per gram of soil (CFU g⁻¹) sampled within treatments Control (C), Saturated (S), Saturated Covered (SC), Control Covered (CC), Oxygenated (O), Fertilized (F), Switchgrass Fertilized (GF), and Switchgrass Fertilized and Oxygenated (GFO). Means (n=5) followed by the same uppercase letter are not significantly different, according to Fisher's LSD ($p < 0.05$).

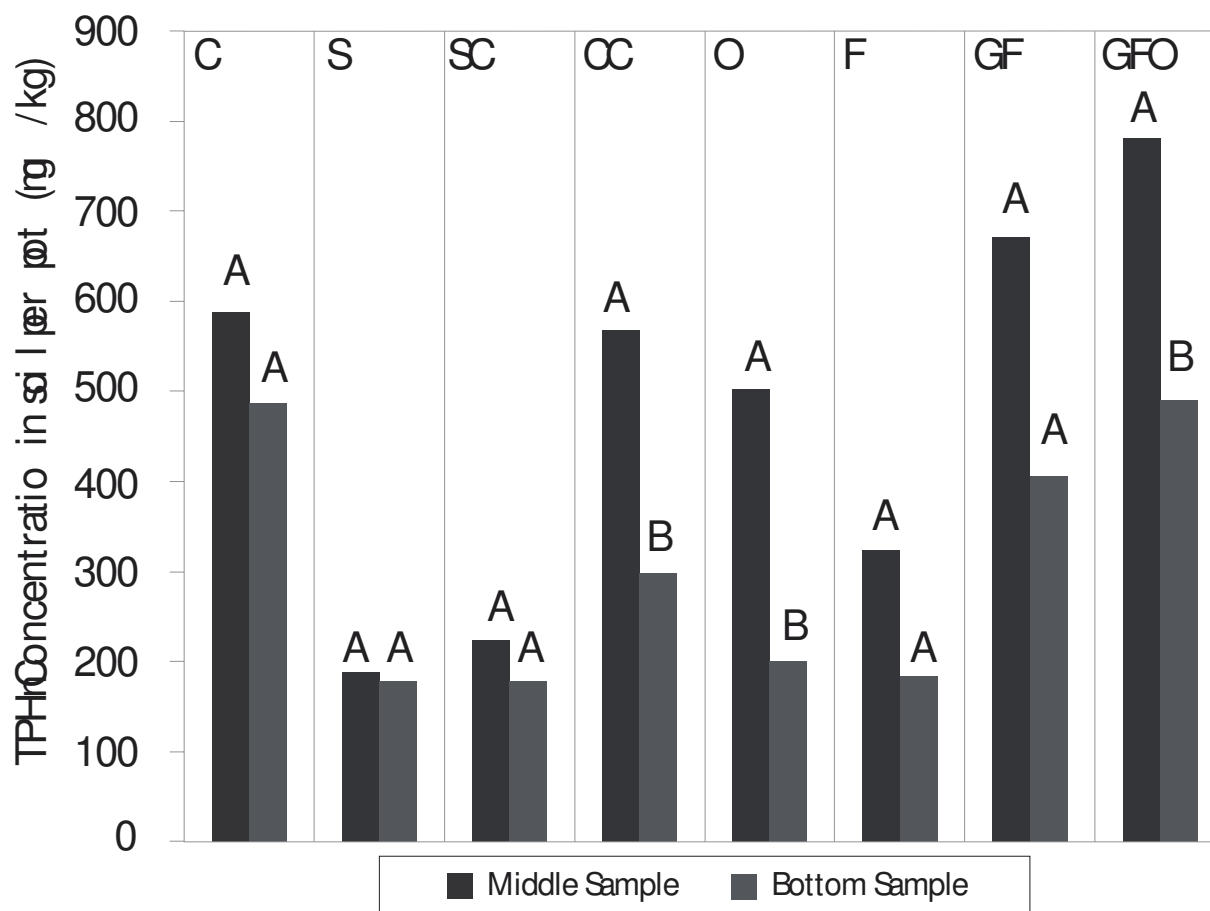


Figure 4.3: Final TPH (mg/kg) concentrations in contaminated soil treatments from residual and spiked contaminants measured after 90 days in Control (C), Saturated (S), Saturated Covered (SC), Control Covered (CC), Oxygenated (O), Fertilized (F), Switchgrass Fertilized (GF), and Switchgrass Fertilized and Oxygenated (GFO) across sampling events (Initial Sample June 19, 2013; Middle Sample and Bottom Sample, September 19, 2013) Means (n=3) followed by the same uppercase letter within the same treatment column are not significantly different, according to Fisher's LSD ($p < 0.05$).

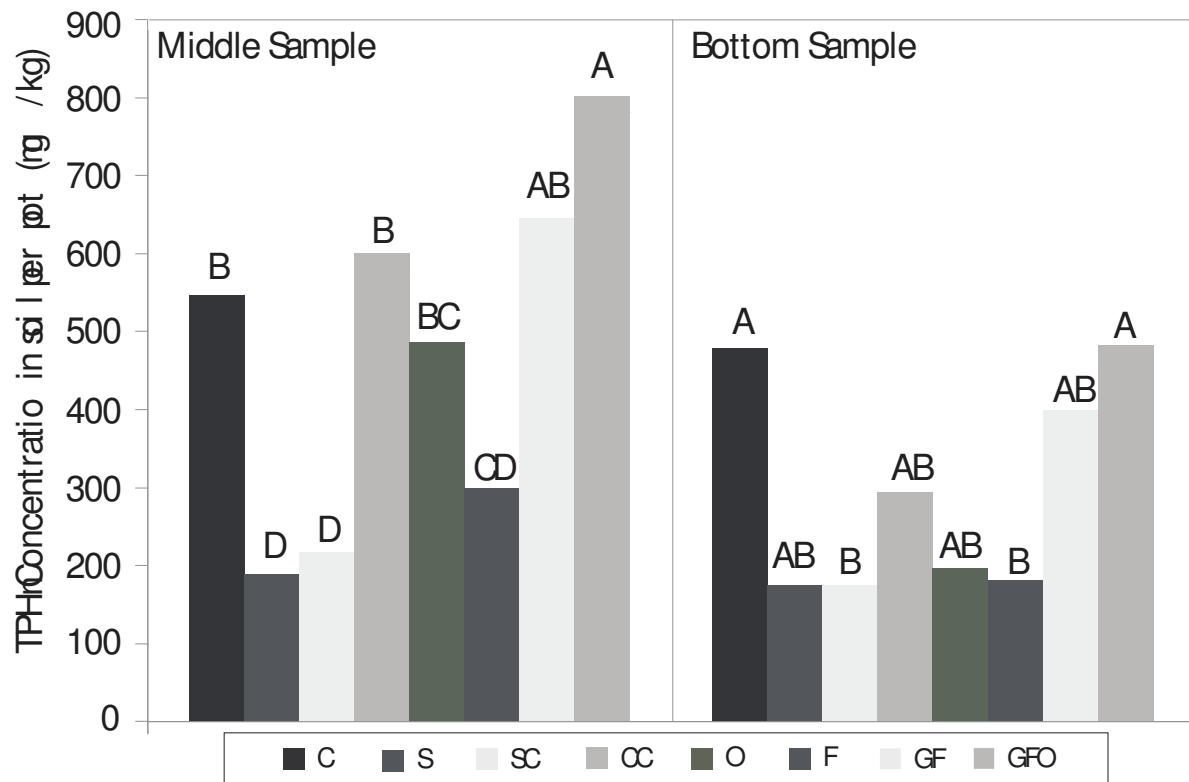


Figure 4.4: Final TPH (mg/kg) concentrations in contaminated soil treatments from residual and spiked contaminants measured after 90 days in Control (C), Saturated (S), Saturated Covered (SC), Control Covered (CC), Oxygenated (O), Fertilized (F), Switchgrass Fertilized (GF), and Switchgrass Fertilized and Oxygenated (GFO) across sampling events (Initial Sample June 19, 2013; Middle Sample and Bottom Sample, September 19, 2013) Comparison between treatment means for Middle Sample, n=5, Bottom Sample, n=3 within each sample location followed by the same uppercase letter between sample treatments are not significantly different, according to Fisher's LSD ($p < 0.05$).

CHAPTER V

Summary

Conclusion

Both TPH bioremediation and phytoremediation techniques have been shown to be effective at breaking down low levels of aged and recent contamination in sandy soils.

Administrators seeking to remove TPH from sandy soils should consider each method before implementation of a remediation project. Even though phytoremediation is an inexpensive technique for TPH degradation, the maintenance of plant health and vigor, which are vitally important for successful degradation of hydrocarbons, can be challenging in sandy soils.

In the first greenhouse experiment, phytoremediation of aged contamination was more effective in treatments with plants combined with a soil amendment. There was no statistical significance between grasses and willows in their efficiency to degrade hydrocarbons. Only when amendments optimized plant growth, a large response was noticed in the break down of hydrocarbons at the conclusion of the study.

In the second greenhouse experiment, bioremediation of spiked contaminated soil was more effective for breaking down TPH than phytoremediation. Bacterial CFU in unplanted soil amendments were lower by orders of magnitude in treatments with grass. New case treatments with full pore space saturation and with the addition of water-soluble fertilizer were the most effective at breaking down TPH. The success of the bioremediation in this study could be due to the high potential of established microbial communities that were previously acclimated to TPH rich hydrocarbons, and were thus more efficient than introducing new microbial associations from phytoremediation.

Most remediation projects are site and contaminant specific and the choice of the method would depend on the site conditions. In the case of aged contaminated sandy soil, when nutrients

and soil moisture can be optimized, phytoremediation would be the best choice to assist administrators with reduction of TPH. Furthermore, when these conditions are not optimized and the contamination is readily bioavailable as we observed in spiked soil, different forms of bioremediation can be used for the removal of TPH.

Proposals for Future Research Studies

Based on the findings from these studies the following research directions are proposed:

- Several other drought tolerant plant types including species within the grass family should be observed for phytoremediation prospects dealing with sandy soil. *Festuca* and *Panicum* were very tolerant genera during our experiment and showed promise for phytoremediation. More emphasis should be placed on the testing of combination of species rather than single cultivars for water stressed conditions.
- Field trials and long-term installations should be monitored to observe which plant species, or combination of plant species, would be more advantageous for remediation of hydrocarbons on sandy soil.
- A Gas Chromatography Flame Ionization Detector was used to detect hydrocarbons in soil. Other analytical methods (GC-ECD, GC-MS, HPLC, etc.) could be used to compare effectiveness of the technique in determining the loss of hydrocarbons. Also this could allow identification of each carbon derivative generated with GC-MS to target and isolate the more carcinogenic PAHs within the soil medium being tested.
- In both studies, bacterial CFU's were only counted but not identified. Isolating and identifying these species may help to determine which bacteria or fungi best tolerate the stress conditions of sandy soil.

- Future research studies of phytoremediation should focus on both recontaminated and post-remediated soil conditions. For example, re-harnessing indigenous microbial communities thriving during remediation could be advantageous for future research when deciding which remedial method (bioremediation or phytoremediation) is appropriate for implementation.
- Investigate various nitrogen amendments and target the reducing conditions of sulfur, nitrogen, or iron on removing TPH contamination in soil. Under different soil moisture regimes, identify the most effective intervals for treatment applications (weekly, monthly, yearly) to achieve the most effective degradation of hydrocarbons within each particular reducing condition and moisture regime.
- We tested one dilution range of molasses as a highly labile carbon source. Different carbon sources and at different rates need to be tested to identify the most effective carbon source to enhance microbial productivity. These carbon solutions can be applied to the irrigation at different rates or times to promote more microbial activity.
- While in this study soil surfactants were not used, additional research is required to understand how the use of surfactants increases the bioavailability of TPH in sandy soil during bio- or phytoremediation.