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Ecological Remediation Using Bacterial, Fungal, and Plant Microcosms: An Effective Solution for Bunker C Crude Oil Contamination in Waterways

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ECOLOGICAL REMEDIATION USING BACTERIAL, FUNGAL, AND PLANT MICROCOSMS: AN EFFECTIVE SOLUTION FOR BUNKER C CRUDE OIL CONTAMINATION IN WATERWAYS

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ABSTRACT

Factory legacy pollutants are an increasing concern for waterways as old infrastructure begins to deteriorate and contaminate nearby environments. The Fisherville Mill in Grafton, Massachusetts, USA, exemplifies this problem since it has now fallen into disrepair and is leaking Bunker C crude oil into the adjoining Blackstone River, a third order stream. My research examines how effectively an ecologically engineered system (EES), consisting of anaerobic bacteria environments, fungal microcosms, and aquatic plant environments, can break down petroleum hydrocarbons (PH), specifically aliphatic and polycyclic aromatic hydrocarbons (PAH), in this river environment.

I designed, built, and tested an ecologically engineered system that pumps polluted waters from a segment of the Blackstone River to a filtration station on land, before returning the water to the river upon remediation. My testing protocol involved taking water samples before and after each filtration stage monthly from June through October 2012. Water samples were analyzed at the Brown University Superfund Research Lab using mass spectrometry to determine aliphatic and PAH concentrations.

To evaluate system effectiveness, I hypothesized that aliphatic hydrocarbons and PAH post-filtration levels would be significantly lower ($p<0.05$) compared to the baseline. A secondary hypothesis was that each treatment sampling point would have significantly different water aliphatic hydrocarbon and PAH levels, indicating sequential treatment as contaminates move through the EES. My results showed that post-treatment aliphatic oil concentrations were significantly different from baseline concentrations ($p=0.005$), with an average reduction of 95.2%. Post-treatment PAH concentrations were also significantly different from baseline concentrations ($p=0.001$), with an average reduction of 91%.

I conclude that this EES provided effective treatment of Bunker C crude oil, even though some filtration stages did not achieve their intended objectives. This type of filtration may be scaled, and therefore may be considered in larger remediation efforts regarding Bunker C crude oil.

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ACRONYMS AND ABBREVIATIONS

Bunker C	Bunker C crude oil
B.U.S.R. Lab	Brown University Superfund Research Lab
EES	Ecologically Engineered System
EPA	United States Environmental Protection Agency
EPH	Extracted Petroleum Hydrocarbons
JTED	John Todd Ecological Design
MADEP	Massachusetts Department of Environmental Protection
PAH	Polycyclic Aromatic Hydrocarbons
PH	Petroleum Hydrocarbons
TPH	Total Petroleum Hydrocarbons
UCM	Unresolved Complex Mixture
WRF	White Rot Fungi

CHAPTER 1: THE ROLE OF ECOLOGICAL SYSTEMS IN THE REMEDIATION OF BUNKER C CRUDE OIL. A LITERATURE REVIEW.

1.1 Introduction

There is now considerable hydrocarbon pollution present in freshwater environments (U.S. EPA 2014) that is harmful to aquatic communities and human health (Canton and Wegman 1983, Kuhn *et al.* 1989, Nisbet and LaGoy 1992, Lilius *et al.* 1994, Bofetta *et al.* 1997, Dejmek *et al.* 2000). Hydrocarbon contamination is often recalcitrant in nature and needs applied restoration technologies for efficient cleanup (Sung *et al.* 2003). Ecologically engineered systems, an arrangement of ecological systems rather than man-made materials that have the ability to degrade contaminants, have proven effective in breaking down hydrocarbons and show promise in remediating wastewater, nutrient excesses, and factory waste (Todd and Josephson 1996). Ecologically engineered systems (EES) can also breakdown newly synthesized contaminants such as endocrine disruptors and pharmaceutical waste (Snyder *et al.* 2004). EES's should be considered for hydrocarbon remediation because of their tolerance for a diversity of waste, as well as their proven ability to remediate hydrocarbons in the literature.

1.2 Ecological Restoration as a Solution Forward

Ecological remediation, the removal of pollutants from the environment using ecological systems, is one type of application incorporated into a larger vision that brings ecological services to the forefront of dealing with societal problems. This vision more broadly involves the massive restructuring of our urban environment so that individual

buildings and structures can function like organisms, and open spaces can regenerate landscapes by providing recycling services for pollution (Todd 1994). Each building might generate its own air purification through individual plant greenhouse circulation systems, as well as degrade waste generated on-site through ecological remediation methods. Canal filtration devices and building-specific waste degradation might be me more commonplace, so that every major structure is functioning to reduce water pollution levels to satisfy Federal EPA water standards (U.S. EPA 2014). Examples existing today include ecologically engineered wastewater treatment technologies, applied in the Baima Canal, China, that aerate city canals while also decreasing harmful nutrient and chemical concentrations from daily waste disposal sites. One other project is the Four Seasons Hawaii Lagoon project that circulates water within a wetland system to reduce nutrient and chemical concentrations during runoff events (U.S. EPA 2001, John Todd Ecological Design 2014). Incorporation of remediation services will be important in the management of polluted environmental sites, but also for the future stability of our water resources (Todd and Josephson 1996).

As we consider more widespread utilization of ecological remediation practices, it is important to maintain connection to the field's founding principles. Foremost among these are symbiosis of organisms and the use of subecosystems when creating ecologically engineered systems. Symbiosis is important because specific waste products of one organism can be the substrate of another, producing process chains that heighten the efficiency and speed of chemical breakdown or immobilization (Todd and Josephson 1996). An example of this is an inorganic carbon requirement of nitrifying bacteria that if not met slows the degradation of nitrogenous wastes and allows toxic levels of

ammonia build up (Todd and Josephson 1996). Water characteristics such as pH and dissolved oxygen can also affect nutrient cycles, especially if they impact decomposition, in which microorganisms convert organic P to the soluble phosphate form required for plant uptake (Todd and Josephson 1996). These types of relationships should be considered when using ecological remediation practices.

The use of subecosystems, particularly a photosynthetically driven system linked to an animal consumer system, is also important in creating a high-functioning EES to remediate nutrient and hydrocarbon pollution (Mitsch and Jorgenson 1989, Todd and Josephson 1996). These principles help guide EESs to reduce chemical oxygen demand in water environments, ultimately leading to a healthier, oxygen-rich environment for organisms. These principles also provide system resiliency by reducing total suspended solids, neutralizing acids, nitrifying high ammonia levels, and reducing the phosphorus load that can lead to algal blooms (Todd and Josephson 1996). By following these principles of design, EES's are provided more opportunity for effective remediation.

Ecological remediation techniques also offer an opportunity to investigate how natural processes function and how water pollution concerns can be better addressed. Specifically, EES's can give the opportunity to research nutrient uptake regimes and eutrophication reversal (Wang *et al.* 2009), heavy metal contamination, bioaccumulation and degradation of pollutants (Hashim *et al.* 2011), in both soil and water environments (Aprill and Sims 1990, Frick *et al.* 1998, Ceccanti *et al.* 2006). These investigations could offer more information on how to quickly rebound damaged environments through contamination reduction and nutrient balancing.

Ecological remediation experiments can also further our knowledge of how

organisms function under stress. For example, bioremediation techniques are now beginning to demonstrate that certain microbial communities, specifically *Pseudomonas*, *Bacillus*, *Corynebacterium*, and *Enterobacter* species have a resistance to lead and cadmium while others do not (Roane and Kellogg 1996). This adaptation could have been naturally selected for because of certain heavy metal environmental stressors. Further investigation is needed to understand why resistances evolve in only certain microbial communities. As environmental remediation continues to use novel organisms and communities, we will discover new adaptations to harness for remediation purposes.

1.3 Petroleum Hydrocarbons, Bunker C Crude Oil, as a Major Concern

Factory legacy pollutants, specifically chemicals with long residual times, are becoming a larger concern for waterways as old storage infrastructure begins to deteriorate and leak contaminates to nearby environments. There are now many hydrocarbon pollutants in freshwater environments (U.S. EPA 2014) that are harmful to aquatic communities and human health (Canton and Wegman 1983, Kuhn *et al.* 1989, Nisbet and LaGoy 1992, Lilius *et al.* 1994, Bofetta *et al.* 1997, Dejmek *et al.* 2000). Bunker C crude oil (Bunker C) is a contaminant of specific interest due to its continued occurrence at many industrial sites active in the 20th century (U.S. EPA 2013).

Bunker C is a high-viscosity residual oil, used in many factories at the turn of the 20th century and still used for certain machinery in the textile industry, as well as factory heating and lighting (Fuel Oil 2013). Bunker C is often considered the heaviest of oil types, and is good for heat generation since it consists of large hydrocarbon compounds that release high amounts of energy upon combustion (Fuel Oil 2013). This oil contains

many types of petroleum hydrocarbons, including aliphatic and polycyclic aromatic hydrocarbons (PAH), which were found to be major pollutants in major catastrophic spills in Bemidji, Minnesota and the Gulf of Mexico (Delin *et al.* 1998, Robertson and Kaufman 2010). Bunker C should be examined thoroughly so that remediation strategies can be discovered for spill events such as these.

Many different hydrocarbons make up Bunker C, and certain groups are more hazardous than others. PAHs are common groundwater contaminants, employed as water quality indicators because of their ubiquitous occurrence, recalcitrance and bioaccumulation potential. Heavy exposure can cause increased risk of lung, skin, and bladder cancers and has also been found to affect fetal growth during pregnancy (Bofetta *et al.* 1997, Dejmek *et al.* 2000). PAHs have also been found to effect the juvenile development of many aquatic organisms including rainbow trout and *Daphnia magna* among other organisms (McCann *et al.* 1975, Canton and Wegman 1983, Lilius *et al.* 1994). These contaminants require comprehensive remediation considerations because of the considerable harm they cause.

PAHs are especially harmful compounds because they have low water solubility. This characteristic causes them to readily adsorb to sediments, often settling the pollutant near the bottom of the water column where degradation occurs slowly due to frequently low dissolved oxygen concentrations (Perelo 2010). For this reason, remediation strategies for PAHs were examined thoroughly within the past several decades and will be highlighted below. Generally, it was found that PAHs degrade readily in aerobic conditions with the help of bacteria, while an anoxic environment slows their breakdown (DeLaune *et al.* 1981). Natural breakdown has also been found to occur through

volatilization, or photolysis (Haritash and Kaushik 2009). PAHs and other hydrocarbons are also generally believed to be difficult to break down with the absence of oxygen because of resonance energy associated with their aromatic rings, and the strength of their C-H and C-C bonds (Widdel and Rabus 2001). However, specific metabolites of anaerobic PAH degradation such as naphthyl-2-methylsuccinate indicate anaerobic degradation of 2-methylnaphthalene in situ (Rainer *et al.* 2004). These types of environmental factors and byproducts of degradation should be considered in experimentation aimed at breaking down PAHs.

1.4 Bioremediation

Bioremediation, the microbial breakdown of compounds using chemical pathways, has the potential to play a much larger role in hydrocarbon remediation than it has to date (Haritash and Kaushik 2009). Microorganisms in the bioremediation process transform compounds into non-hazardous forms with less input of chemicals, energy and time than traditional methods like dredging, capping, or natural attenuation (Haritash and Kaushik 2009, Perelo 2010). The literature outlining bioremediation applications below is extensive and useful for targeting specific hydrocarbon compounds. There is also the potential for new bioremediation strategies to include 'unculturable' microbial wealth, which has been estimated at 1,000 to 10,000 unknown prokaryotes per 1g of soil (Handelsman *et al.* 1998).

Bioremediation applications often take advantage of cultured microorganism species to target a specific pollutant or utilize species that occur readily in our degraded environments (Löffler and Edwards 2006). Anaerobic microbes, for example, can live in

heavily contaminated water that has turned anoxic (Lovley 2001). These microbes have evolved chemical pathways for energy generation and growth using oxygen substitutes (Lovley 2001). For example, microbes oxidize organic compounds to carbon dioxide and use electron acceptors such as nitrate, sulfate, or Fe^{3+} oxides (Lovley 2001). Natural anaerobic microbe capabilities should be harnessed for more efficient clean up of contaminants in a variety of environments.

Alternatively, aerobic degradation has other chemical pathways for hydrocarbon breakdown and energy generation. Aerobic degradation is initiated by the introduction of oxygen atoms into the aromatic ring of these pollutants to produce cis-dihydrodiols and eventual breakdown of the structure (Chakraborty and Coates 2004). We also now know that saturated alkanes, made up of C-H single bonds, are more susceptible to aerobic bacterial attack than unsaturated hydrocarbons such as alkenes or alkynes, which have C-H double and triple bonds. The straight long carbon chain structures found with many hydrocarbons also make them more prone to aerobic biodegradation because of the exposed nature of the molecule (Chakraborty and Coates 2004).

Given the ubiquitous nature of bacteria to degrade hydrocarbons both aerobically and anaerobically, it is important to learn which of the numerous bacteria strains have been identified most effective at doing so (Gibson and Parales 2000). *Pseudomonas aeruginosa*, *Pseudomons fluoresens*, *Mycobacterium*, *Haemophilus*, *Rhodococcus*, and *Paenibacillus* are some commonly known PAH-degrading bacteria, which have been studied extensively in lab environments (Haritash and Kaushik 2009). Bacteria strains can also display wide versatility in bioremediation. For example, *Pseudomonas paucimobilis* grown in fluoranthene was capable of degrading 80.0, 72.9, 31.5, 33.3, and

12.5% of pyrene, benzanthracene, chrysene, benzopyrene and benzofluoranthene respectively (Ye *et al.* 1995).

Significant numbers of petroleum-degrading aerobic microorganism species were found petroleum-polluted soils examined by Chaillan *et al. (*2004). Researchers collected and analyzed 33 species, eight bacteria, and twenty-one fungi in the process. Bacterial strains belonged to the genera *Gordonia, Brevibacterium, Aeromicrobium, Dietzia, Burkholderia* and *Mycobacterium*. Fungi belonged to *Aspergillus, Penicillium, Fusarium, Amorphoteca, Neosartorya, Paecilomyces, Talaromyces* and *Graphium*. All of these strains were cultivated in a synthetic liquid media with crude oil, and use of the oil carbon for growth determined (Chaillan *et al.* 2004). This research exemplifies the diversity that organisms possess, and also brings into question which combination of organisms would lead to a more efficient hydrocarbon remediation strategy. This topic should be explored further with experimentation.

Many of these hydrocarbon-degrading anaerobes can now be cultured, and their breakdown pathways studied (Chakraborty and Coates 2004). An example of newly identified pathways is the fumarate addition reaction used as the activation step for catabolic processes regarding monoaromatic hydrocarbon compounds. It has also been found that the type of electron acceptor utilized, either aerobic or anaerobic, for compound degradation alters breakdown pathways (Chakraborty and Coates 2004).

Cyanobacteria can also break down hydrocarbons in water environments, and are important to consider in ecological remediation research. A recent study isolated five species with this capacity in Suez and Ismailia, Egypt (Ibraheem 2010): *Phormidium*, *Nostoc*, *Anabaena*, *Aphanothece conferta,* and *Synecho-cystis aquatilis*. These species

were able to biodegrade two aliphatic compounds (n-octadecane and pyrene) and two aromatic compounds (phenanthrene and dibenzothiophene) very well. For the two aliphatic compounds treated with *A. conferta* there was a 64% reduction of n-octadecane and a 78% reduction of pyrene. Using *S. aquatilis* in treatment there was an 85% reduction in n-octadecane and a 90% reduction in pyrene (Ibraheem 2010).Cyanobacteria species should be considered for utilization in ecologically engineered systems.

1.5 Mycoremediation

The white rot fungi (WRF) group, including litter-decomposing fungi capable of extensive aerobic lignin depolymerization, also degrades hydrocarbons in soil environments through cometabolism (Cabana *et al.* 2007). It secretes unique lignin-modifying enzymes (LMEs), that are quite reactive and nonspecific, that allow them to oxidize and degrade a larger range of compounds difficult for bacteria to handle, including dyes, PAHs, PCBs, phenols, and pesticides (Cabana *et al.*2007). Its filamentous growth structure also offers a unique and beneficial way of reaching pollutants that may be inaccessible to bacteria (Reddy 1995). This unique set of capabilities should be incorporated into EES design and remediation efforts when contaminants are hard to reach.

To further examine how WRF mycoremediation occurs, it is important to understand when LMEs are produced during fungal lifecycles. WRFs produce LMEs during their secondary metabolism since lignin oxidation provides no net energy to the fungus. Low levels of limiting nutrients such as carbon or nitrogen in the soil environments prompt increased synthesis and secretion of these enzymes so that

mushrooms can gather more nutrients for growth and survival. LMEs specifically include lignin peroxidases (LiP), manganese-dependent peroxidases (MnP), and laccases. These are the essential LMEs while a suite of other enzymes complement the degradation process (Cabana *et al.* 2007). Auxiliary enzymes include glyoxal oxidase and superoxide dismutase, which aid in the breakdown process and ultimately the nutritional needs of the fungi (Leonowicz *et al.* 1999). While the WRF group secretes these enzymes for degradation purposes, the amount of enzymatic activity also varies based on species and environmental conditions such as pH and temperature (Hatakka 1994, Leung and Pointing 2002, Maganhotto de Souza Silva *et al.* 2005). Production of Lip and MnP are optimal when there is high oxygen partial pressure, but suppressed when organisms are agitated. However, laccase production increases with agitation of the mycelia organisms (Cabana *et al.* 2007). These environmental disturbances should be considered when employing WRF enzymes for degradation. WRF are capable of PAH mineralization, at rates of mineralization correlating with the intensity of production of LMEs (Sack *et al.* 1997). Besides hydrocarbons, these LMEs can also breakdown pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, bleach plant effluent, synthetic dyes, synthetic polymers, and wood preservatives (Pointing 2001). LMEs are unique enzymes to the WRF group and should be examined further.

WRF can behave differently when put into competitive environments. For example, laccase activity can be increased when mushroom species come in contact with other WRF species, bacteria, or yeasts. In cultures of *Trametes versicolor* and *Pleurotus ostreatus* in sterile soil, there was a significant 2-25 fold increase of laccase activity after the introduction of soil fungi, or bacteria (Baldrian 2004). When *Trichoderma harzianum*

was added to cultures of *Trametes versicolor*, laccase enzymatic activity increased 40 fold (Baldrian 2004). This type of intergroup competition between WRF species, or with bacteria and yeast, should be incorporated into remediation management plans when WRF groups are involved.

Many types of fungi are now also known to breakdown and oxidize PAHs. It was found that a whole diversity of fungi including *Aspergillus ochraceus, Cunninghamella elegans, Phanerochaete chrysosporium, Saccharomyces cerevisiae,* and *Syncephalastrum racemosum*, can oxidize PAHs (specifically anthracene, benz[a]anthracene, benzo[a]pyrene, fluoranthene, fluorene, naphthalene, phenanthrene, pyrene) (Sutherland 1992). Several of these taxa including *Phanerochaete chrysosporium* (Bumpus 1989), *Pleurotus* sp. (Bezalel *et al.* 1996), and *Trametes versicolor* (Morgan *et al.* 1991) carried out PAH degradation in soil not just in culture. It was also found in one study that *Trametes versicolor* reduced hydrocarbon concentrations from 32 g/kg to 7 g/kg within 12 months in soil microcosms (Yateem *et al.* 1998). The effectiveness of these fungi species should be noted and utilized in future hydrocarbon remediation efforts.

White rot fungi have also demonstrated the ability to breakdown hydrocarbons in marsh conditions. Among 40 fungal species isolated in wetlands contaminated with PAH, 33 species showed an ability to degrade fluoranthene (60–99%), albeit only two species were able to degrade anthracene by more than 70% (Giraud *et al.* 2001). Mushroom species effective in doing so included *Absidia cylindrospora*, *Mucor hiemalis*, *Aspergillus fumigatus*, *Cladosporium cladosporoides*, *Fusarium solani*, and *Trichoderma viride* (Haritash and Kaushik 2009). These species should be considered when remediating in water or marsh conditions.

1.6 Phytoremediation

Phytoremediation is the process in which plants are used to remove pollutants from an environment to render them harmless (Haritash and Kaushik 2009). Plants usually remove pollutants by storing them in their plant roots or foliage. This is a particularly effective strategy where there are large areas of surface contamination (Shimp *et al.* 1993). There are also microorganisms associated with the plant rhizosphere, such as the genera *Pseudomonas, Arthobacter,* and *Micrococcus*, that are known to biodegrade a wide variety of contaminants including hydrocarbons (Shimp *et al.* 1993).

Certain terrestrial grass species show a significant effectiveness in remediating harmful hydrocarbons in soil environments (Aprill and Sims 1990, Reilley *et al.* 1996, Haritash and Kaushik 2009). Several grass species degrade PAHs, such as *Agropyron smithii*, *Bouteloua gracilis*, *Cyanodon dactylon*, *Elymus Canadensis*, *Festuca arundinacea*, *Festuca rubra*, and *Melilotus officinalis* (McCutcheon and Schnoor 2003). Grasses and legumes also have the potential to remediate hydrocarbon contamination in soils. When alfalfa and three grasses, tall fescue, sudangrass, and switchgrass, were planted in PAH contaminated soils, these plants' associated rhizospheres broke down pyrene and anthracene 30-40% more than soils that were left unplanted (Reilley *et al.* 1996). Prairie grasses are thought to make a superior vehicle for this kind of remediation because of their fibrous root systems, which have a high root surface area (Aprill and Sims 1990).

Aquatic plants can also break down hydrocarbons in water environments. Aquatic plants, *Typha* spp. and *Scirpus lacustris,* were used in a macrophyte-based

wetlands to treat PAHs, with removal rates for phenanthrene of up to 99.9% (Machate 1997). Using these plants as biological filters, while also employing their beneficial rhizosphere properties, is a favored way to remediate hydrocarbon pollution (Haritash and Kaushik 2009).

Plants are also known for removing nutrients and polishing residual biochemical oxygen demand from wastewater treatment plant effluents. They may also remove emerging organic pollutants such as pharmaceutical and personal care products with over 90% efficiency (Matamoros *et al.* 2008). It was even found that many types of plants could handle heavy strength waste from dairy factories. Researchers found that plants reduced the dairy waste PO_4-P by 41%, TN by 79%, and NH_4-N by 70% (Morgan and Martin 2008). While certain plants are effective at removing hydrocarbons, species should also be considered effective at removing other contaminants simultaneously.

Plants often can remediate water pollution in much colder climates than microorganisms. Phytoremediation was examined with two cold-hardy plants, Arctared red fescue and annual ryegrass, planted together in soil contaminated with crude oil (Reynolds and Wolf 1999). Results indicated that plots with the two species had significantly lower concentrations of total petroleum hydrocarbons compared with unplanted controls. The initial oil concentration for planted treatments and controls was approximately 6200 mg of total petroleum hydrocarbons (TPH)/kg soil. After 640 days, the two species planted on crude oil contaminated soil contained 1400 mg TPH/kg soil or 77% less than initially while the unplanted control contained 2500 mg TPH/ kg soil, 60% less than before (Reynolds and Wolf 1999). This shows that many natural degradation processes are occurring in soil but that by adding certain plant species, the recovery

period can be hastened. Another example of cold climate remediation involved an engineered wastewater treatment project designed for operation in Sweden and constructed in 1989. After 4 years of operation, results showed that the wastewater from 34 people has been treated in an effective manner. The nutrient uptake in the aquaculture included 10% of the nitrogen and 8% of the phosphorus in the total wastewater flow (Guterstam 1996). Plant systems seem ideal for nutrient and hydrocarbon remediation with their flexibility and tolerance of extreme environments. This information analyzed in laboratory and field experiments should make phytoremediation a consideration when remediating hydrocarbon-polluted sites.

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ECOLOGICAL REMEDIATION USING BACTERIAL, FUNGAL, AND PLANT MICROCOSMS: AN EFFECTIVE SOLUTION FOR BUNKER C CRUDE OIL CONTAMINATION IN WATERWAYS

A Manuscript Draft for Future Publication

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CHAPTER 2: ECOLOGICAL REMEDIATION USING BACTERIAL, FUNGAL, AND PLANT MICROCOSMS: AN EFFECTIVE SOLUTION FOR BUNKER C CRUDE OIL CONTAMINATION IN WATERWAYS

2.1 ABSTRACT

Factory legacy pollutants are an increasing concern for waterways as old infrastructure deteriorates and contaminates nearby environments. The Fisherville Mill in Grafton, Massachusetts, USA exemplifies this problem since it has now fallen into disrepair and is leaking Bunker C crude oil into the adjoining Blackstone River, a third order stream. Our research examines how effectively an ecologically engineered system (EES), consisting of anaerobic bacteria environments, fungal microcosms, and aquatic plant environments, can break down petroleum hydrocarbons, specifically aliphatic and polycyclic aromatic hydrocarbons (PAH), in this river environment.

Our testing protocol involved taking water samples before and after each filtration stage monthly from June through October 2012. Water samples were analyzed at the Brown University Superfund Research Lab using mass spectrometry to determine aliphatic and PAH concentrations.

Post-treatment aliphatic oil concentrations were significantly different from baseline concentrations ($p=0.005$), with an average reduction of 95.2%. Post-treatment PAH concentrations were also significantly different from baseline concentrations ($p=0.001$), with an average reduction of 91%. We conclude that this EES provided effective treatment for Bunker C crude oil, even though some filtration stages did not achieve their intended objectives. This type of filtration arrangement might be scaled up for use in larger remediation efforts regarding Bunker C crude oil.

2.2 KEY WORDS

Ecological Remediation, Bunker C Crude Oil, Eco-Machine, Bioremediation, Mycoremediation, Phytoremediation, Ecologically Engineered Systems.

2.3 INTRODUCTION

Factory legacy pollutants, specifically chemicals with long residual time, are an increasing concern for waterways as old containment infrastructure deteriorates. There is now considerable hydrocarbon pollution present in freshwater environments (U.S. EPA 2014), which are harmful to aquatic communities and human health (Canton and Wegman 1983, Kuhn *et al.* 1989, Nisbet and LaGoy 1992, Lilius *et al.* 1994, Bofetta *et al.* 1997, Dejmek *et al.* 2000).

The Fisherville Mill in Grafton, MA, USA is one example, having fallen into disrepair after several decades of disuse, releasing thousands of gallons of Bunker C crude oil (Bunker C) into the Blackstone River, a third order stream. Our research examines how effectively an ecologically engineered system (EES), consisting of anaerobic bacteria environments, fungal microcosms, and aquatic plant environments, can break down petroleum hydrocarbons (PH), specifically aliphatic and polycyclic aromatic hydrocarbons (PAH), in this river environment. This research builds upon several previous field experiments that effectively treated hydrocarbons through bioremediation (Giraud *et al.* 2001), mycoremediation (Bezalel *et al.* 1996, Yateem *et al.* 1998), and phytoremediation (Machate *et al*. 1997, Frick *et al.* 1998). In our study, this question is specifically addressed on a small scale (i.e. 1000 gallons treated per day). The objective is to inform development of larger projects (i.e. 100,000 gallons treated per day) in similarly polluted waterways.

2.3.1 Bunker C, Petroleum Hydrocarbons, and Consequences

In this study we build upon previous research to explore the potential for

bioremediation (the microbial breakdown of compounds using chemical pathways), mycoremediation (the degradation of contaminants using mushrooms), and phytoremediation (the process in which plants are used to remove pollutants from an environment), to breakdown Bunker C in a water environment. Bunker C is a highly viscous residual oil that contains many types of petroleum hydrocarbons (PH), including aliphatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs). These types of petroleum hydrocarbons are common groundwater contaminants, employed as water quality indicators because of their ubiquitous occurrence, recalcitrance, and bioaccumulation potential. Heavy exposure to hydrocarbons can cause increased risk of lung, skin, and bladder cancer and affects fetal growth during pregnancy (Bofetta *et al.* 1997, Dejmek *et al.* 2000). PAHs also affect the juvenile development of many aquatic organisms including rainbow trout and *Daphnia Magna* (McCann 1975, Canton and Wegman 1983, Lilius *et al.* 1994). Bunker C requires thorough remediation because it is so severely harmful. It is important to consider new comprehensive approaches to remediate Bunker C, as it becomes a more common contaminant in waterways.

2.3.2 Bioremediation

Bioremediation, the microbial breakdown of compounds using chemical pathways, has the potential to play a significant role in Bunker C remediation (Haritash and Kaushik 2009). This type of remediation was important to consider for our EES because microorganisms transform compounds into non-hazardous forms with less input of chemicals, energy and time than such conventional methods as dredging or capping (Haritash and Kaushik 2009). These traditional methods were also not considered

because of the widespread nature of the contamination plume, making it difficult to stop flow from the source.

Bioremediation also carries the additional advantage of employing microorganisms that are highly tolerant of degraded environments (Löffler and Edwards 2006). Heavily contaminated water sources, such as the Blackstone River, often become oxygen depleted permitting the survival of only anaerobic microbes (Lovley 2001). Our EES design takes into account the presence of these anaerobic microbial communities in the Blackstone River, providing them abundant artificial habitat for colonization and thus utilization as a filter.

Many bacteria species capable of remediating hydrocarbons have now been identified (Gibson 2000). Commonly studied PAH-degrading bacteria include *Pseudomonas aeruginosa*, *Pseudomons fluoresens*, *Mycobacterium*, *Haemophilus*, *Rhodococcus*, *Paenibacillus* (Haritash and Kaushik 2009). Each bacteria species can also display a wide versatility in degrading PAHs. For example, *Pseudomonas paucimobilis* grown in fluoranthene was capable of degrading 80.0, 72.9, 31.5, 33.3, and 12.5% of pyrene, benzanthracene, chrysene, benzopyrene and benzofluoranthene, respectively (Ye *et al.* 1995). This analysis indicates that certain bacteria species are effective at breaking down a range of hydrocarbons in anaerobic soil and water environments effectively, making bioremediation a suitable candidate for tackling Bunker C that is made up of several dozen hydrocarbons. Our study goes beyond consideration of individual species effectiveness to look at how native bacterial communities can be encouraged to colonize in-situ filters.

2.3.3 Mycoremediation

The white rot fungi (WRF) group, more generally including litter-decomposing mushrooms capable of secreting lignin-modifying enzymes (LMEs), has been found to degrade hydrocarbons in soil environments and is thus an important group to consider for Bunker C remediation in an EES (Cabana *et al.* 2007). Many types of fungi are now known to breakdown and oxidize PAHs including *Aspergillus ochraceus, Cunninghamella elegans, Phanerochaete chrysosporium, Saccharomyces cerevisiae,* and *Syncephalastrum racemosum*. Among the PAHs oxidized are anthracene, benz[a]anthracene, benzo[a]pyrene, fluoranthene, fluorene, naphthalene, phenanthrene, pyrene (Sutherland 1992). Several taxa including *P. chrysosporium* (Bumpus 1989), *Pleurotus* sp. (Bezalel *et al.* 1996), and *Trametes versicolor* (Morgan *et al.* 1991) also degrade PAHs using secreted LMEs.

The ability of white rot fungi to breakdown hydrocarbons in water and marsh conditions also has been demonstrated. Among 40 fungal species isolated from wetlands contaminated by PAH, 33 showed an ability to degrade fluoranthene (60–99%), although only two degraded anthracene by more than 70% (Giraud *et al.* 2001). Several mushroom species capable of degrading hydrocarbons include *Absidia cylindrospora*, *Mucor hiemalis*, *Aspergillus fumigatus*, *Cladosporium cladosporoides*, *Fusarium solani*, and *Trichoderma viride* (Haritash and Kaushik 2009). These studies were considered when selecting fungal microcosm filters. Our species utilized as fungal microcosm filters in our EES included *Irpex lacteus (*milk-white toothed polypore*), Pleurotus ostreatus (*oyster*), Stropharia rugosoannulata (*garden giant) and *Trametes versicolor (*turkey tail).

Enzyme secretion regimes for the WRF group are also important to consider for

optimizing remediation capabilities. Standard LMEs secreted include lignin peroxidases, manganese-dependent peroxidases, and laccases (Cabana *et al.* 2007). While the WRF group secretes these enzymes for degradation purposes, the amount of enzymatic activity also varies based on species and environmental conditions, responding poorly when pH or temperature are extreme (Maganhotto de Souza Silva *et al.* 2005, Hatakka 1994, Leung and Pointing 2002). These environmental requirements were considered when arranging our fungal microcosm layout and growth regime. Our study will take the knowledge gained about PAH degradation and enzyme activity, and arrange fungal microcosms in a way that permits testing of their usefulness as drip filters. The effectiveness of WRF in complete aquatic environments is still unknown, and this arrangement will determine that capability.

2.3.4 Phytoremediation

Plant species are also important to consider when developing an EES to break down Bunker C. Phytoremediation is the process in which plants are used to remove pollutants from an environment to render them harmless by storing the compounds in roots or foliage (Haritash and Kaushik 2009). This is a particularly effective strategy where surface contamination covers a large area (Shimp *et al.* 1993, Frick *et al.* 1998). There are also microorganisms associated with the plant rhizosphere, such as *Pseudomonas, Arthobacter,* and *Micrococcus*, known to biodegrade a wide variety of contaminants including hydrocarbons (Shimp *et al.* 1993).

Plant communities, grasses and legumes, can remediate harmful pollutants including hydrocarbons, with particular effectiveness in soil environments (Reilley *et al.*

1996, Aprill and Sims 1990, Haritash and Kaushik 2009). Several grass species, including *Agropyron smithii*, *Bouteloua gracilis*, *Cyanodon dactylon*, *Elymus Canadensis*, *Festuca arundinacea*, *Festuca rubra*, and *Melilotus officinalis* degrade PAHs (McCutcheon and Schnoor 2003). Alfalfa and three grasses: tall fescue, sudangrass, and switchgrass, planted in PAH contaminated soils, were effective in breaking down pyrene and anthracene 30-40% more than soils that were left unplanted (Reilley *et al.* 1996). Prairie grasses are thought to make a superior vehicle for this kind of remediation because of their fibrous root systems, which can have a high surface area (Aprill and Sims 1990). This research was considered when selecting local grass species for phytoremediation purposes in our EES.

Aquatic plants have also been shown to break down hydrocarbons in water environments. For example, *Typha* and *Scirpus lacustris* have been used to treat PAHs, with success in reducing phenanthrene by up to 99.9% (Machate 1997). Using these plants as biological filters, while also encouraging chemical processes associated with their root zones may be an effective strategy to reduce hydrocarbon pollution at vegetated sites (Haritash and Kaushik 2009). This research was incorporated into our EES. We planted *Typha* (cattail), *Scirpus lacustris* (bulrush), *Medicago sativa* (alfalfa)*, Festuca arundinacea* (tall fescue), and *Panicum virgatum* (switchgrass).

Many species of bacteria, mushrooms, and plants have the ability breakdown hydrocarbons. In this study we investigate how these three types of ecological techniques based on specific biological groups can be incorporated into an EES to most effectively breakdown Bunker C pollution in the Grafton Canal, MA, USA. We hypothesized that an EES with anaerobic bacteria environments, fungal microcosms, and aquatic plant

environments will effectively sustain the break down of PHs, specifically aliphatic hydrocarbons and PAHs over a period of several months. A secondary hypothesis was that each treatment contributed to pollutant degradation. Thus we expected statistically significant aliphatic hydrocarbons and PAH decrease along the post-treatment sampling points.

2.4 METHODS

2.4.1 Site History and Layout

Our study site is located at the former historic Fisherville Mill in South Grafton, Massachusetts and is immediately down river of the confluence of the Quinsigamond and Blackstone Rivers (42.177355, -71.689998). The Blackstone River flows for 48 miles from Worcester, Massachusetts to Providence, Rhode Island (Figure 1). The Blackstone River corridor is known as the birthplace of the American Industrial Revolution, and is a river of national significance, as evidenced by the designations of the John H. Chafee Blackstone River Valley National Heritage Corridor, an American Heritage River and Urban River Restoration Initiative Pilot. Water quality in the Blackstone River continues to be impaired because of legacy structures and pollutants associated with the rivers industrialized history and lack of cleanup. The entire length of the Blackstone River in Massachusetts is on the state's list of impaired waters. Our remediation site and EES are on land near the Fisherville Mill, which has now burned down. This gives us direct water access to the Bunker C pollution source buried in holding tanks that were associated with the Mill, since tanks have not been removed or altered beyond initial damages.

2.4.2 Overview of Ecologically Engineered System

There are three general components to the EES we tested (Figure 2). First, contaminated water from the canal is drawn through four anaerobic microbial filters ('Bottom Filters'). The Bottom Filters were placed near the middle of the canal one foot above the canal bottom and drew between 500 to 1000 gallons of water per day. They specifically consisted of PVC piping (8 inches wide, 250 feet long) filled with quarter inch gravel and sand. The gravel and sand environment promoted anaerobic bacteria colonization of this area as the Bottom Filters pumped in bacteria and water from the anoxic environment in the canal. This filter arrangement also prevented canal debris from entering the system. This treatment was first in our EES because bacteria have displayed the strongest capabilities in degrading hydrocarbons in the literature, and thus were most suitable to encounter the highest concentrations of Bunker C. Our constructed piping system also provided a suitable bacteria environment that our other treatments could not exist in.

The water was then pumped to the Eco-Machine (John Todd Ecological Design 2014), a patented living technologies system consisting of two sub-components: fungal microcosm filters ('Myco-Reactors') and an aquatic plant environment ('Aquatic Cells'). Contaminated water was first dispersed in the Myco-Reactors, which have different mycelia species that produce lignin-modifying enzymes capable of degrading hydrocarbons. The mycelia species used in the Myco-Reactors included *Irpex lacteus (*milk-white toothed polypore*), Pleurotus ostreatus (*oyster*), Stropharia rugosoannulata (*garden giant) and *Trametes versicolor (*turkey tail). *Irpex lacteus,* and *Pleurotus ostreatus* were chosen for their ability to break down PAHs in water (Haritash and

Kaushik 2009), *Trametes versicolor* for its ability to breakdown PAHs in soils (Morgan *et al*. 1991, Yateem *et al*. 1998), and *Stropharia rugosoannulata* for its ability to thrive at warmer temperatures, such as those in a greenhouse environment. These mushroom species were grown on a wood chip medium, and placed in separate containers. Canal water entered these containers from above with a drip nozzle, passed through the filter media, and was collected below before being pumped to the Aquatic Cell treatment. These containers were covered at all times, and shaded to reduce the mycelia medium temperature to approximately 85°F. This treatment was second in our EES because these mushroom species have shown capabilities to degrade hydrocarbons more effectively than our selected plant species, and thus were more suitable to encounter higher Bunker C concentrations.

The water then encountered the Aquatic Cells treatment, a series of six 700-gallon tanks with plant species including *Typha* (cattail), *Scirpus lacustris* (bulrush), *Medicago sativa* (alfalfa), *Festuca arundinacea* (tall fescue), and *Panicum virgatum* (switchgrass). These tanks were connected together with level tubing, so as water was pumped into the first tank, water was sequentially forced to move through all six tanks and back out into the canal. Since the water moved through the EES in this fashion, the pumping in of 500 to 1000 gallons of water per day meant that this same amount was also exiting the system per day. Each tank area was divided in half, with one side planted with the previously mentioned species on a floating material so that plant roots were in contact with the water, and the other half left unplanted and open. All tanks were aerated and the Aquatic Cells experienced a normal fluctuation of summer temperatures from 70°F to 100°F. The contaminated water interacted with these plant species and their rhizosphere networks for

phytoremediation and bioremediation purposes. The Aquatic Cells were placed as our third treatment because of their intermediate ability to degrade hydrocarbons.

After treatment in the Eco-Machine, water was dispersed on a floating raft system ('Canal Restorer') planted with native plant species with established root systems in the water column. Predominate species included *Typha* and *Scirpus lacustris,* as well as added mycelia media. This Canal Restorer was put in place to provide further phytoremediation and mycoremediation treatment in the river.

2.4.3 Water Sampling Protocol

To verify the performance of this EES, we took water samples for analysis of aliphatic and PAH from five locations on-site monthly from June to October 2012. Point #1 (Baseline) was the baseline variable in this experiment since it was upstream of the study site, with no contact to filtered water. Point #2 (Intake) was the water intake for the filtration system and is different from Point #1 because it is downstream of the canal restorer treatment. Point #3 Post-Bacteria, #4 Post-Mushroom, and #5 Post-Plants were sampling points taken after each respective treatment (Figure 3). We took samples over this extended time period to determine the average effectiveness of EES treatments, rather than looking at individual sampling differences. Long-term averages were also analyzed because of sample size limitations and lack of replication.

No sampling was conducted within 24 hours of a precipitation event above 0.25 inches of rainfall. Since there was a small sample size with this study, we used a time composite sampling method, in which each sample was composed of four discrete aliquots collected within a 30-minute interval. Tyvec suits and nitrile gloves were used to

collect all samples. We collected samples south to north, so that there was no sampling interference within the canal or EES. Once samples were collected, they were preserved with additions of hydrochloric acid to reduce the pH below 2. Samples were then delivered to the Brown University's Superfund Research Program (B.U.S.R.) Lab and processed using mass spectrometry to determine aliphatic and PAH concentrations.

2.4.4 Analytical Methods

The B.U.S.R. lab aliphatic hydrocarbon and PAH analysis was performed using Mass DEP's *Method for the Determination of Extractable Petroleum Hydrocarbons* (MDEC 2004). This analysis initially yielded a total petroleum hydrocarbon (TPH) measure reported as a single concentration value (#/L), before providing separate values for into aliphatic and PAH concentrations. Non-petroleum hydrocarbons were present in these TPH analyses and were listed as "unresolved complex mixture" (UCM) within the aliphatic measurement (B.U.S.R. Lab 2012, Figure 4).

We compared aliphatic and PAH concentrations among treatments using IBM SPSS statistics software Version 21 for analysis of covariance. Data were first log transformed due to unequal variances among the treatments. In subsequent analysis treatments were the group and number of days since the start of this experiment (June 15) the covariate. Means of treatments were compared in pairs using a confidence interval of 95%. We also tested normality of the residuals of the analysis of covariance. The Kolmogorov-Smirnov test was not significant, thus normality is assumed for both aliphatic hydrocarbon and PAH data. None of the calculated p-values were corrected, because of the limited number of data points.

2.5 RESULTS

Aliphatic hydrocarbon concentrations recorded throughout four months of testing show significant reductions when comparing EES treatments to the baseline (Figure 5). There is a one to two order of magnitude reduction in aliphatic hydrocarbon levels when post-treatment samples are compared to the study baseline, Point #1 (Baseline). Specifically Points #3 (Post-Bacteria), #4 (Post-Mushrooms) and #5 (Post-Plants) had oil concentrations lower than the baseline by 95.8, 96, and 95.2%, respectively. The average overall treatment reduction over the test period was 95.8%. When looking at the average additive effect of each treatment, Points #3 (Post-Bacteria) reduced aliphatic hydrocarbons from the baseline by 95.8%. Point #4 (Post-Mushrooms) reduced hydrocarbons from Point #3 by 5.2%, and #5 (Post-Plants) increased hydrocarbons from Point #4 by 22.4% (Figure 5).

Point #3 (Post-Bacteria) aliphatic levels were significantly different from those of Point #1 (Baseline) ($p=0.043$) (Table 2). This also was the case for Point #4 (Post-Mushrooms), $p=0.001$, and Point #5 (Post-Plants), $p=0.005$. All of these significant reductions indicate that Hypothesis #1 is supported.

There were also reductions following the treatments relative to Point #2 (Intake). The specific levels of significance were for Point #3 (Post-Bacteria) $p=0.008$ (Table 2) for Point #4 (Post-Mushroom), $p <0.001$, and for Point #5 (Post-Plant filter), $p=0.001$. While the different treatments lowered the Point #2 (Intake) hydrocarbon level further than the Point #1 (Baseline) level, Points #1 and #2 were not significantly different from one another (Table 2).

We did not find significant differences between the bacteria, mushroom and plant

treatments (Table 2). Point #3 (Post-Bacteria) and Point #5 (Post-Plant) treatments reduced aliphatic hydrocarbon levels similarly ($p=0.670$), while Point #4 (Post-Mushrooms) and Point #5 (Post-Plants) were less comparable ($p=0.407$). Post-Mushrooms and Post-Bacteria treatments were most different in level of pollutant reduction ($p=0.283$). Therefore, treatments did not break down aliphatic hydrocarbons to a significantly different extent, in contradiction to hypothesis #2.

PAH hydrocarbon concentrations recorded throughout four months of testing show significant one to two order of magnitude reductions on EES treatment relative to the baseline (Figure 6). Specifically the average levels of reduction Post-Bacteria, Post-Mushrooms, and Post-Plants were 82.8, 94.3% and 91%, respectively. The average overall treatment reduction was 89.4%. However, when looking at individual reductions over time, there is a decrease in reduction in the final two sampling periods. When looking at the average additive effect of each treatment, Points #3 (Post-Bacteria) reduced PAHs from the baseline by 82.8%. Point #4 (Post-Mushrooms) reduced hydrocarbons from Point #3 by 67.1%, and #5 (Post-Plants) increased hydrocarbons from Point #4 by 58.4% (Figure 6).

Post-Bacteria PAH levels were significantly different from Point #1 (Baseline) ($p=0.014$). Post-Mushrooms PAH levels were also significantly different from the baseline ($p=<0.001$), as were Post-Plants PAH levels ($p=0.001$) (Table 3). All of these significant reductions support Hypothesis #1.

There were also pollutant reductions within the EES at Point #2 (Intake). Point #3 (Post-Bacteria) PAH levels differed from those of the intake at a significance level of $p=0.004$ (Table 3). Post-Mushrooms and Post-Plants filter aliphatic levels differed from

those of the intake at significance levels of $p < 0.001$. While the different treatments lowered the Point #2 (Intake) hydrocarbon level further than the Point #1 (Baseline) hydrocarbon level, these two canal stations were not significantly different from one another in their aliphatic levels (Table 3).

When comparing treatment options against one another, there were no significant differences between the bacteria, mushroom and plant filter phases with regard to pollutant concentrations (Table 3). PAH concentrations were most similar between Point #3 (Post-Bacteria) and Point #5 (Post-Plants) ($p=0.617$), and Point #4 (Post-Mushrooms) and Point #5 (Post-Plants) ($p=0.420$). Post-Bacteria and Post-Mushrooms PAH concentrations were wider apart, but still not significantly different ($p=0.259$). These results fail to support hypothesis #2.

2.6 DISCUSSION

We conclude from our results that the EES at the Blackstone River study site has a very strong influence on hydrocarbon concentrations. The sequence of anaerobic bacteria environments, fungal microcosms, and aquatic plant environments reduced both aliphatic and PAHs concentrations by 82-96%. Hydrocarbon reductions relative to our baseline were significant for all treatments, supporting our hypothesis #1. The breakdown levels measured are consistent with microbial remediation results reported by others. For example, *Pseudomonas paucimobilis* grown in fluoranthene, a commonly tested PAH, degraded 80.0, 72.9, 31.5, 33.3, and 12.5% of the pyrene, benzanthracene, chrysene, benzopyrene and benzofluoranthene it was exposed to, respectively (Ye *et al.* 1995). Our results may also suggest similar findings with the mushroom degradation of

hydrocarbons reported by Giraud *et al.* (2001), who found that 33 species degraded fluoranthene by 60-99%. This can be easily seen in our study with the Myco-Reactors reducing PAH concentrations by 67.1%. This could also be the case for aliphatic hydrocarbons, despite reductions of only 5.2% overall, because of large divergences between Post-Bacteria and Post-Mushroom treatments in the final two sampling points of our analysis which yielded a 53.2% reduction on average. Other experiments have shown that plants used in this study, *Typha* and *Scirpus lacustris*, can remove PAHs with efficiencies as high as 99.9% (Machate 1997). Our EES incorporated several of these previously studied species, and while our Aquatic Cell system kept hydrocarbon concentrations at a low level significantly different from the baseline, they did not contribute to further reductions beyond the Post-Mushroom treatment. The average hydrocarbon concentrations found at Point #5 (Post-Plants) actually increased by 22.4% for aliphatics and 58.4% for PAHs. This point will be discussed further below.

Our study suggests that integrating bioremediation, and mycoremediation into a combined EES is a highly effective solution for remediation of Bunker C contamination. Indeed 82-96% of the aliphatic and PAH compounds present at the test site were removed over a four month period on average. The EES also reduced the concentrations of all of the several dozen hydrocarbons analyzed. This conclusion suggests than an EES with multiple remediation techniques can be versatile in breaking down a variety hydrocarbon compounds.

We didn't, however, find a significant difference between the three treatment stages (Point #3 (Post-Bacteria), Point #4 (Post-Mushrooms), and Point #5 (Post-Plants)) in either aliphatic or PAH analysis, failing to support the hypothesis that suspected

significant sequential treatment. These results could be attributed to the initial bacteria filter removing a majority of the hydrocarbons from the water, leaving little pollution for the other two filters to reduce (Figure 5, 6). This is likely given that several genera including *Gordonia, Brevibacterium, Aeromicrobium, Dietzia, Burkholderia* and *Mycobacterium* were able to exclusively use crude oil as an energy source (Chaillan *et al.* 2004). An alternative explanation would be that while the fungal microcosms are providing hydrocarbon reductions, 5.2% reduction of aliphatics and 67.1% reduction of PAHs beyond Point #3, they are more efficient at removing hydrocarbons in a dryer environment. This conclusion is consistent with data on *P. chrysosporium* (Bumpus 1989), *Pleurotus* sp. (Bezalel *et al.* 1996), and *Trametes versicolor* (Morgan *et al.* 1991) in soil environments. Temperature may also have impacted fungal microcosm function during certain days of the summer, when temperatures exceeded 100°F, since enzymatic activity in WRF species drops rapidly in extreme temperature (Maganhotto de Souza Silva *et al.* 2005, Hatakka 1994, Leung and Pointing 2002). Nevertheless, our mushroom treatment did further reduce hydrocarbon concentrations and do provide some beneficial effect. This benefit could have possibly been significant if testing continued beyond our four month scope, as there was a large divergence between Post-Bacteria and Post-Mushroom aliphatic concentrations during the final two sampling periods.

Another interesting relationship occurred between treatment stations. When hydrocarbon concentrations were compared between treatments, it was found that these were decreased at Point #3 (Post-Bacteria) and Point #4 (Post-Mushrooms), but increased at Point #5 (Post-Plants). We infer from these results that this could be due to excess adsorption of hydrocarbons onto the plant root systems or the hyperaccumulation of

hydrocarbons that are difficult to degrade in the EES. Evapotranspiration could have also played a role by decreasing the water volume and thus increasing hydrocarbon concentrations, since tanks were half exposed to the greenhouse environment. As a result of the Post-Mushroom treatment, the water entering the Aquatic Cells also had a lower pH around 5, and this could have contributed to the poor remediation of hydrocarbons. This observation could warrant individual treatment studies, so as to determine the true effectiveness of each treatment phase. PAH concentrations also increased at all sampling locations during the testing period. This could be due to increased temperature at the study site, causing Bunker C pollution to leak more quickly from its source.

2.7 CONCLUSIONS AND RECOMMENDATIONS

Our EES design combining the practices of bioremediation, mycoremediation, and phytoremediation, demonstrated effectiveness in removing aliphatic hydrocarbons and PAHs on a small scale through several months of testing. While it is difficult to differentiate between how the treatment phases truly function individually, this EES degraded Bunker C effectively as a whole.

Our results are sufficiently robust to suggest that a system with bioremediation and mycoremediation could be scaled up to treat much higher volumes of contaminated water (100,000 gallons treated per day) but further verification will be required at this scale of application. Installing a larger system like this would require a significant amount of space adjacent to pollution sites, so that Bottom Filters and Myco-Reactors could be adequately installed. However, this type of system would only work well for a water contamination event, since it would be difficult to remediate river sediments or soil

with this filter arrangement. We recommend continued research and development of ecologically engineered systems based on the encouraging results from our system at Grafton, MA. For future utilization and efficiency of this system, it will be important to determine the bacteria taxonomic classes present in our anaerobic bacteria filter. This knowledge could highlight if certain bacteria should be supplemented or added to this filter environment. Some species of interest to add could include *Pseudomonas aeruginosa*, *Pseudomons fluoresens*, *Mycobacterium*, *Haemophilus*, *Rhodococcus*, and *Paenibacillus* for their success in degrading hydrocarbons in soil environments (Haritash and Kaushik 2009). More mushroom species could also be considered for fungal microcosm filtration in the EES. These species include *Aspergillus ochraceus, Cunninghamella elegans, Phanerochaete chrysosporium, Saccharomyces cerevisiae,* and *Syncephalastrum racemosum* (Sutherland 1992). Other wetland-tolerant mushrooms species include *Absidia cylindrospora*, *Mucor hiemalis*, *Aspergillus fumigatus*, *Cladosporium cladosporoides*, and *Fusarium solani* (Haritash and Kaushik 2009).

At this stage of research, it is not certain how these living technologies are each influencing the overall hydrocarbons levels in the Blackstone River on a larger scale. Early evidence suggests that there is a beneficial effect occurring downstream, but data collected over a relatively long period of time (possibly several years) will be necessary to confirm these impacts. It is expected that over time this EES will act as an ecological chemostat, contributing to beneficial chemical composition of the water for optimal growth of organisms that remediate pollution problems present.

2.8 ACKNOWLEDGEMENTS

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2.9 TABLES

Table 1. Summary of hydrocarbon types and terminology

Acronym for Hydrocarbon Type	Hydrocarbon Type
EPH	Extracted Petroleum Hydrocarbons
TPH	Total Petroleum Hydrocarbons
PAH	Polycyclic Aromatic Hydrocarbons
UCM	Unresolved Complex Mixture

Table 2. Analysis of covariance for all aliphatic hydrocarbon oil testing points

	#1 Baseline	#2 Intake	#3 Post-Bacteria	#4 Post-Mushrooms	#5 Post-Plants
#1 Baseline		0.365	0.043*	0.001*	0.005*
#2 Intake	0.365		0.008*	0.000*	0.001*
#3 Post-Bacteria	0.043*	0.008*		0.283	0.670
#4 Post-Mushrooms	0.001*	0.000*	0.283		0.407
#5 Post-Plants	0.005*	0.001*	0.670	0.407	

*Significance at 95% confidence interval

Table 3. Analysis of covariance for all PAH oil testing points

	#1 Baseline	#2 Intake	#3 Post-Bacteria	#4 Post-Mushrooms	#5 Post-Plants
#1 Baseline		0.503	0.014*	0.000*	0.001*
#2 Intake	0.503		0.004*	0.000*	0.000*
#3 Post-Bacteria	0.014*	0.004*		0.259	0.617
#4 Post-Mushrooms	0.000*	0.000*	0.259		0.420
#5 Post-Plants	0.001*	0.000*	0.617	0.420	

*Significance at 95% confidence interval

2.10 FIGURES

Figure 1. Bunker C remediation study site in Grafton, MA, USA

This map indicates the Bunker C remediation study site in Grafton, MA, USA. This map also demonstrates the important location of Grafton, MA at the headwaters of the Blackstone River Watershed and National Heritage Corridor.

Figure 2. Diagram of water flow through the ecologically engineered system

This flow diagram shows how contaminated water from the Blackstone River is circulated through the EES to filter out Bunker C crude oil. Water originally comes from the canal through two anaerobic bacteria filters (Bottom Filters), which then enters a manifold before being sent to the fungal microcosm filters (Myco-Reactors) or back out to the canal via the Canal Restorer, a combination of plant and mushroom media on a floating apparatus. Once the remaining water has gone through the Myco-Reactors, it is circulated through a myco-sump and then to six aquatic plant environments (Aquatic Cells) for further treatment. After the Aquatic Cell stage, water is returned to the canal via the Canal Restorer.

Figure 3. Water sampling locations in ecologically engineered system

This schematic visually represents how the EES is set up in Grafton, MA, USA. Each number marker represents a point that was sampled every month over a four-month interval. Sampling point 1 is labeled as Point #1 (Baseline), 2 is labeled as Point #2 (Intake), 3 is labeled as Point #3 (Post-Bacteria) Filter, 4 is labeled as Point #4 (Post-Mushroom) Filter, and 5 is labeled as Point #5 (Post-Plants) Filter. This schematic also visualizes the real life scale of this EES.

Figure 4. Relationship of total petroleum hydrocarbons, EPH, and PAH

This figure displays the step-by-step process through which the B.U.S.R. Lab extracts aliphatic hydrocarbons and polycyclic aromatic hydrocarbons (PAH) from water samples that are given to them for processing. Canal water is collected and a solvent is first added to the liquid. Extraction then takes place, taking out all petroleum hydrocarbons from this liquid, yielding total petroleum hydrocarbons (TPH). This extraction is then further divided into two different types of hydrocarbons, aliphatics including an unresolved complex mixture (UCM), and PAH. These two concentrations are then determined through mass spectrometry analysis.

Figure 5. Aliphatic hydrocarbon levels within the ecologically engineered study site

This figure displays water aliphatic hydrocarbons concentrations over a four-month sampling period. The hydrocarbon concentration scale is logarithmic due to the large reduction in concentration from before and after filtration. Five sampling points are displayed for comparison. Point #1 (Baseline), while Point #2 (Intake) is further downstream at the EES intake point. There is a one to two order reduction in

hydrocarbons during the filtration process occurring at Points #3, #4, and #5. Point #3 (Post-Bacteria) aliphatic levels were significantly different from Point #1 (Baseline) ($p=0.043$) (Table 2). Point #4 (Post-Mushrooms) aliphatic levels were also significantly different from the baseline ($p=0.001$) as well as in the case with Point #5 (Post-Plants) filter ($p=0.005$). All of these significant reductions indicate that Hypothesis #1 is supported. Specifically Points #3 (Post-Bacteria), #4 (Post-Mushrooms) and #5 (Post-Plants) had oil concentrations lower than the baseline by 95.8, 96 and 95.2%, respectively. The average overall treatment reduction over the test period was 95.8%. When looking at the average additive effect of each treatment, Points #3 (Post-Bacteria) reduced aliphatic hydrocarbons from the baseline by 95.8%. Point #4 (Post-Mushrooms) reduced hydrocarbons from Point #3 by 5.2%, and #5 (Post-Plants) increased hydrocarbons from Point #4 by 22.4%.

Figure 6. PAH levels throughout ecologically engineered study site

This figure displays water PAH hydrocarbons concentrations over the four-month sampling period. The hydrocarbon concentration scale is logarithmic due to the large reduction in concentration from before and after filtration. Five sampling points are displayed for comparison. Point #1 (Baseline), while Point #2 (Intake) is further downstream at the EES intake point. There is a one to two order reduction in hydrocarbons during the filtration process occurring at Points #3, #4, and #5. Post-Bacteria PAH levels were significantly different from Point #1 (Baseline) ($p=0.014$). Post-Mushrooms and Post-Plants PAH levels were also significantly different from the baseline ($p=<0.001$, $p=0.001$) (Table 3). All of these significant reductions support Hypothesis #1. When comparing the average baseline oil level to average of each treatment, Post-Bacteria was reduced 82.8%, Post-Mushrooms reduced 94.3%, and Post-Plants reduced 91%. The average overall treatment reduction over the testing period was 89.4%. When looking at the average additive effect of each treatment, Points #3 (Post-Bacteria) reduced PAHs from the baseline by 82.8%. Point #4 (Post-Mushrooms) reduced hydrocarbons from Point #3 by 67.1%, and #5 (Post-Plants) increased hydrocarbons from Point #4 by 58.4%.

Figure 1.


Blackstone Valley
Blackstone River Watershed
National Heritage Corridor
Worcester
MASSACHUSETTS
Blackstone River
Blackstone
Woonsocket
CONNECTICUT
RHODE ISLAND
Pawtucket Falls and Slater Mill
Central Falls
Pawtucket
Providence
Grafton, MA Study Site

Figure 2.

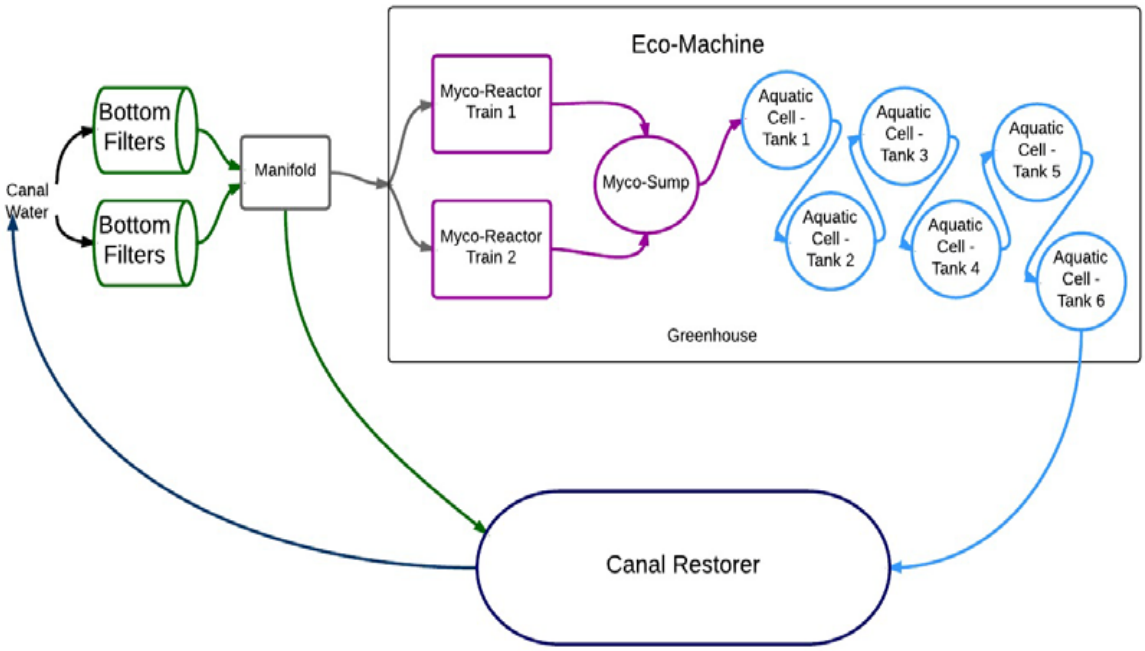

Eco-Machine
Canal Water
Bottom Filters
Bottom Filters
Manifold
Myco-Reactor Train 1
Myco-Reactor Train 2
Myco-Sump
Aquatic Cell - Tank 1
Aquatic Cell - Tank 2
Aquatic Cell - Tank 3
Aquatic Cell - Tank 4
Aquatic Cell - Tank 5
Aquatic Cell - Tank 6
Greenhouse
Canal Restorer

Figure 3.

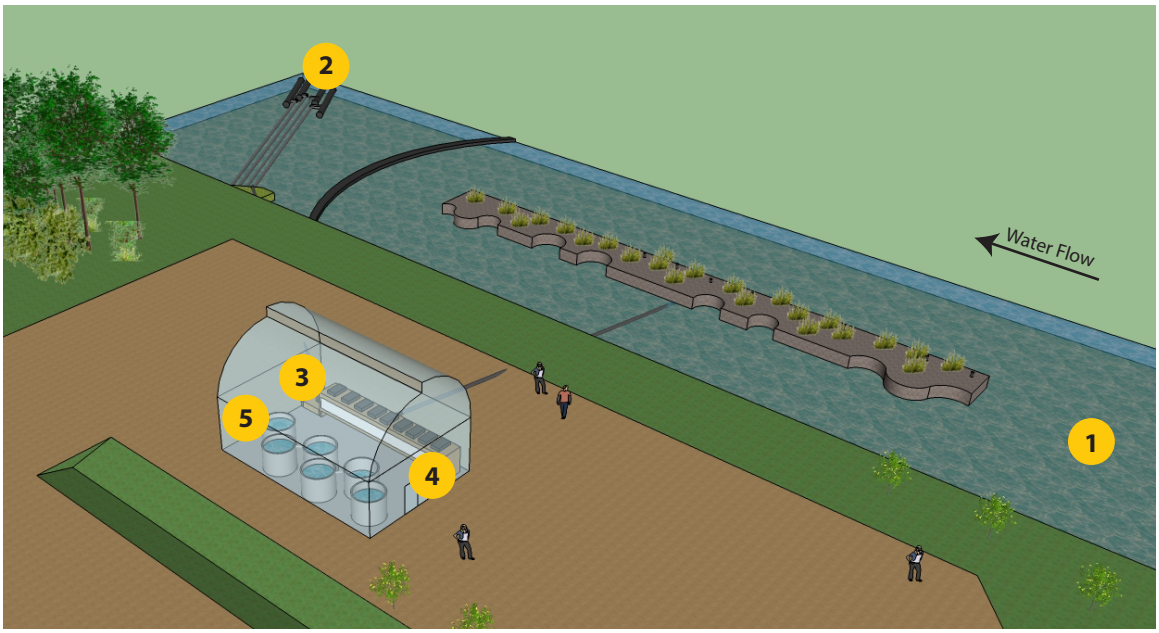


Figure 4.

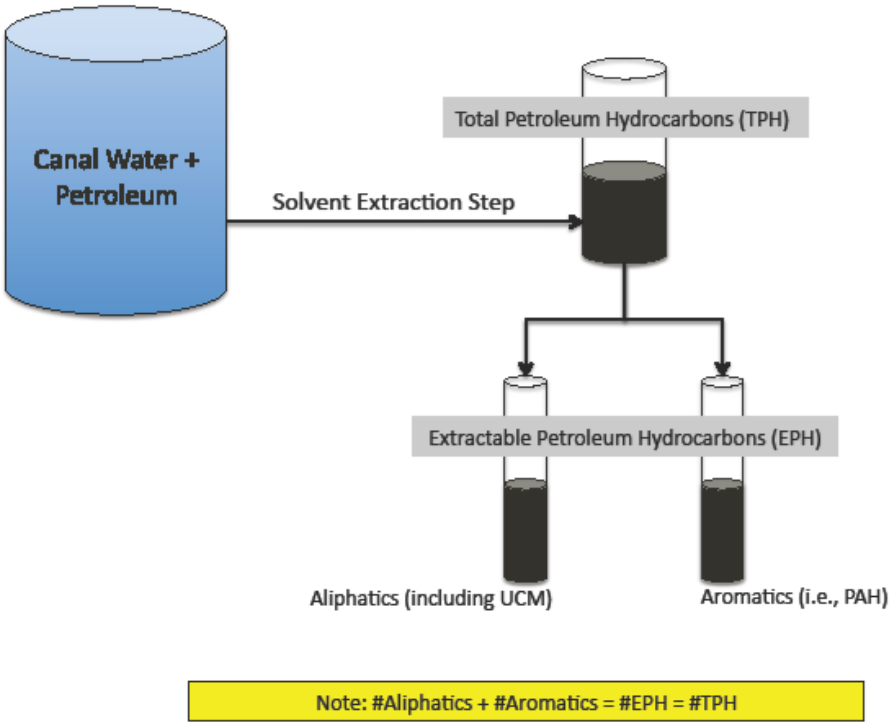

Canal Water + Petroleum
Solvent Extraction Step
Total Petroleum Hydrocarbons (TPH)
Extractable Petroleum Hydrocarbons (EPH)
Aliphatics (including UCM)
Aromatics (i.e., PAH)
Note: #Aliphatics + #Aromatics = #EPH = #TPH

Figure 5.

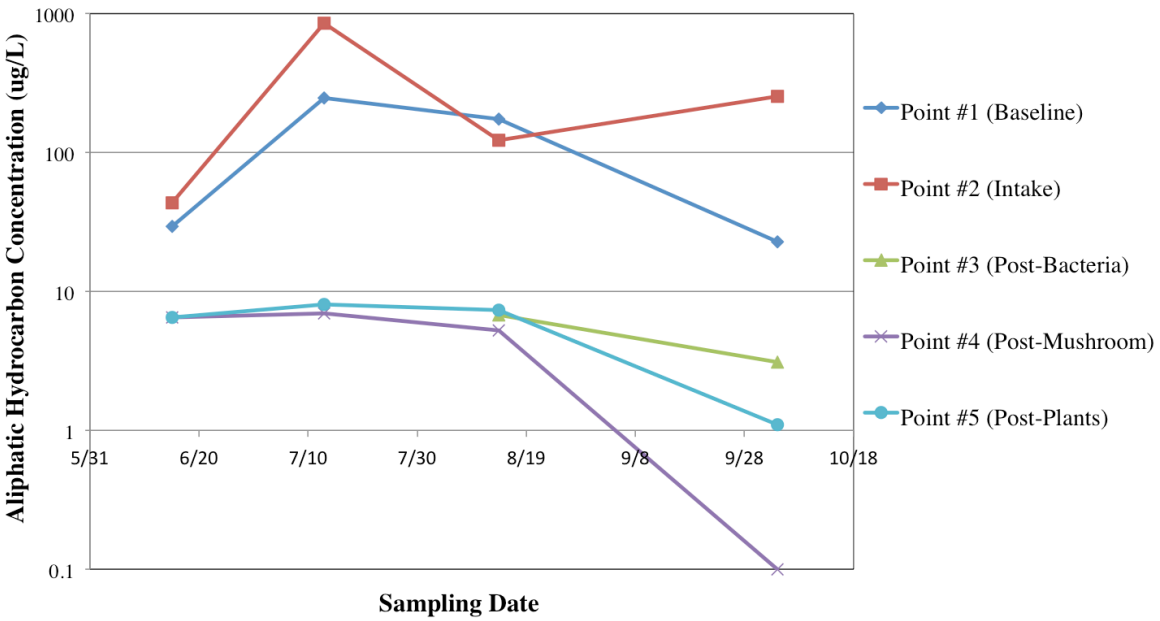
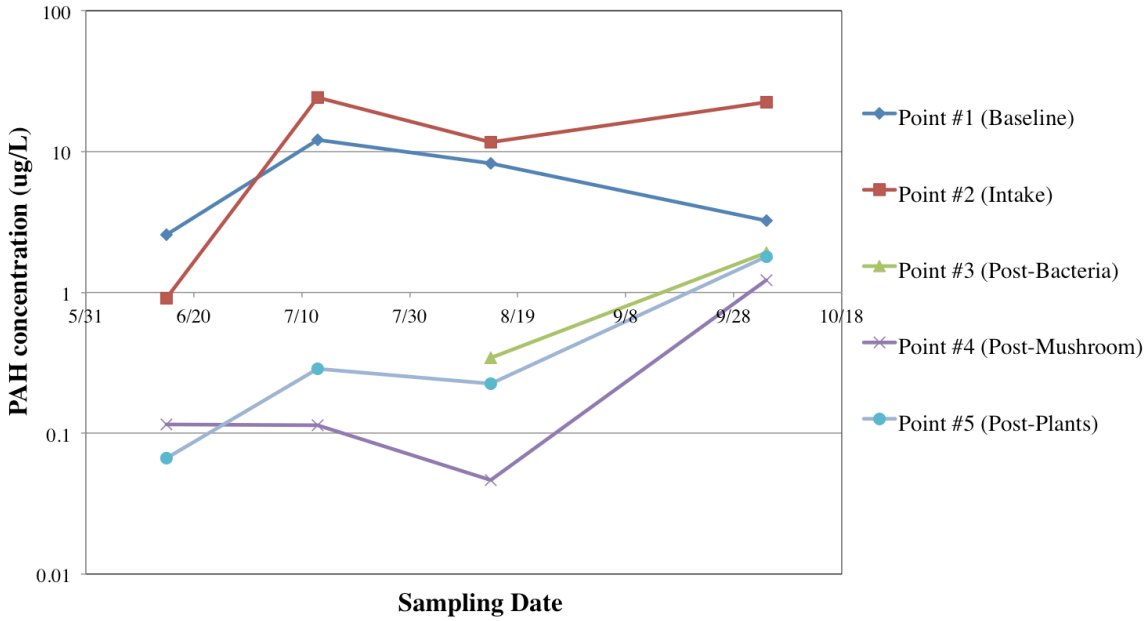

1000
100
10
1
0.1
Aliphatic Hydrocarbon Concentration (ug/L)
5/31
6/20
7/10
7/30
8/19
9/8
9/28
10/18
Sampling Date
Point #1 (Baseline)
Point #2 (Intake)
Point #3 (Post-Bacteria)
Point #4 (Post-Mushroom)
Point #5 (Post-Plants)

Figure 6.


100
10
1
0.1
0.01
PAH concentration (ug/L)
5/31
6/20
7/10
7/30
8/19
9/8
9/28
10/18
Sampling Date
Point #1 (Baseline)
Point #2 (Intake)
Point #3 (Post-Bacteria)
Point #4 (Post-Mushroom)
Point #5 (Post-Plants)

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