USING MICROBES, FUNGI, AND PLANTS IN PASSIVE REMOVAL OF POLYCYCLIC AROMATIC HYDROCARBONS AND HEAVY METALS: POLLUTION REMEDIATION FOR THE RAIL INDUSTRY

by

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ABSTRACT

Railroads haul the majority of crude oil, hazardous materials, and industrial waste products in the US and are themselves generators of hydrocarbon and heavy metal wastes. With derailments, illegal dumping, spills during transloading, and a multitude of other incidents happening regularly, the industry needs a simple, passive method to control the environmental impact of these events. Operationally, shutting down production every time a spill happens is not feasible, but the long-term cost in leaving these toxins in the ground to accumulate over decades can cause serious liability concerns for the industry and potential health concerns for the surrounding communities. There have been many studies extolling the virtues of mycelia, phytoremediation, and microbes in remediating PAH-contaminated soil and water. Combining these systems and embedding them in railroad roadbeds that cannot be dug up and remediated in traditional ways may save the industry significant time, money, and production by keeping the tracks live while putting in biological systems, which would encourage less delay in remediation and cleanup efforts. In new roadbeds under track that is being repaired or expanded, the system can be embedded underneath the track itself, and positive feedback loops of biological development as passive pollution uptake systems can be established in territories or yards that lack access to wastewater treatment or pollution prevention technologies. Combining multiple biological systems can minimize variability in the survivability of these organisms in situ. The interdependence of each organism with the others allows for potential nutrient transfer from one to another, potentially enhanced protection from specific environmental stressors, and creation of more beneficial growing environments for themselves, decreasing the need for active remediation and wholesale removal of soils for disposal, in addition to potentially improving toxin degradation over time. This project will review the established literature supporting this hypothesis as well as detail a potential methodology to test species' viability in the implementation of such a designed system that would still be consistent with established railroad building techniques for roadbeds. We are looking for the best combination of biological systems that will remove the greatest amount of contaminants without damaging our roadbed in the greatest range of environmental conditions in order to save resources and ameliorate potential safety concerns. In the future, this proposed design may be accepted for a pilot study at Union Pacific Railroad; however, at this time, it is only a proof-of-concept proposal.

INTRODUCTION

Bioremediation Overview

The purpose of this project is to develop a proof-of-concept methodology to test the idea of using biological systems as a passive remediation tool for the removal of PAH and heavy metals in and near railroad operations. This paper will be used as a research tool to demonstrate a potential cost-benefit analysis for the implementation of a future study that will include an expanded experimental design covering a species screening test, a bench test for applicability, and finally, a pilot study to be located in situ at a yet to be determined location. Using the principles outlined here, we, as an industry, hope to perfect a modular design that could be selected for by climate and installed concurrently with our current structural engineering techniques as part of standard railroad operations and maintenance activities. In order to demonstrate this proof-of-concept potential, we have assumed that the species enumerated in the following chapters will pass our screening selection for survivability under potential railroad conditions and will be implemented in the bench test described in Chapter 3: Methodology, which should provide the majority of the feasibility analysis as well as take up the bulk of the cost for the prospective project. Current industry standards support the use of microbial remediation, but this paper may provide a new standard for passive remediation technologies that would significantly reduce our costs and labor requirements against long-term remediation projects.

Over the past 20 years, several researchers have experimented with the use of microbial consortia, fungal growth and colonization, and phytoremediation in the in situ treatment of areas contaminated with petrochemicals and metals, such as zinc, lead, and cadmium. Singularly, the application of plants, fungi, or bacteria shows positive results that are limited by the survivability of the organism, the medium and type of contamination, and the level of disturbance, including rapid changes in the structure or chemical composition of the contamination media. Less research, however, has been conducted on how the combinations of systems would encourage growth and resilience under adverse conditions that would normally destroy any single element of the system. And while there are still major components of each system's metabolic strengths and weaknesses that are hidden to researchers, synergistic interactions could result in a greater success rate when combined with other compatible biological systems. This project attempts to capitalize on the integration of four systems to create a unified, modular design of a passive remediation tool that can be installed in railroad roadways for long-term extraction and metabolism of PAH and heavy metals. These four systems of interest are (1) microbial consortia, particularly focusing on the growth of *Pseudomonas aerginosa*; (2) fungal and hyphae growth within the soil column, particularly the interaction between saprophytic fungi (specifically, *Pleurotus ostreatus*) and their mineralization of polycyclic aromatic hydrocarbons (PAH); and (3) the subsequent translocation of secondary metabolites to arbuscular mycorrhizal fungi (particularly *Glomus claroideum*) that can assist in the uptake and sequestration of those metabolites within the roots of specific plants; and (4) the resultant translocation of nutrients to specialized plants, which allows for the sequestration of toxic compounds in the roots or vacuoles of hyperaccumulating plants, such as *Elsholtzia splendens,* that can then be managed as solid waste through normal maintenance activities with no effect on production. Thus, not only is the system less expensive than incinerating contaminated soil (and the subsequent shutting down of railroad operations in order to excavate) but also it reduces the impact on train operations and encourages a long-term efficient system of remediation.

The most commonly used form of biological remediation is the application of microbial consortia. Olajire and Essien (2014) demonstrated that the most successful degradation events occurred with a community of microbes, including *Pseudomonas, Rhodococcus, Arthrobacter,* and *Bacillus* spp., with *Pseudomonas* being the most extensively studied and flexible metabolism of the group. *Pseudomonas aeruginosa* readily degrades PAH under much more toxic conditions than do other families that seem to favor aliphatic molecules. This ability seems to be increased when in the presence of non-microbial-generated enzymes from white rot, for example, that were resistant to PAH toxicity and shared similar lignin-degrading capability (Bacosa and Suto, 2011). Bezalel et al. (1996) conjectured that the most effective enzyme for this degradation of PAH was laccase; however, Pickard et al. (1999) found a greater degradation effect from cytochrome P-450 when paired with the microbes in the breakdown of the chemically similar lignin. But given the right conditions, microbes can be extremely effective at PAH breakdown, demonstrating a 97% degradation rate over 110 hours (Bacosa and Suto 2011). These microbes, however, require a significantly high proportion of oxygen and moisture to maintain a colony size sufficient for reproduction and pollutant degradation. Also, once the toxic substrate overwhelms the colony, the microbes' metabolic capacity to reduce those toxins was significantly degraded (from 80% to 20% in Shi et al.'s 2002 study).

If, however, the microbes are paired with a saprophytic fungus, such as *Pleurotus ostreatus*, the fungus produces an excess of manganese-independent peroxidase, laccase, cytochrome P-450, and epoxide hydrolase, both within the fungal cells and excreted from hyphae structures, to initiate metabolism outside the cell walls: subsequent nutrients generated move into the cells, followed by mineralization to carbon dioxide (Bezalel et al., 1996). Adongbede (2014) demonstrated a 98% degradation of PAH compounds using only saprophytic *Agaricus campestris*, while Bezalel et al. (1996) also demonstrated a 90% degradation and mineralization of PAH into carbon dioxide with *Polyporus* spp. and *Pleurotus ostreatus*. Other secondary metabolites were detected by Adongbede (2014), including sulfates and glutathione (Bezalel et al., 1997), all water soluble and capable of being metabolized by higher-order plants (if there were not so many residual metallic compounds resistant to degradation by the microbial-fungal colony). *Pl. ostreatus* demonstrated the fastest decomposition of PAH compared to other common white rot fungi (Bezalel et al., 1996) as well as the most flexibility in degrading multiple types of PAH, including polychlorinated biphenols (Kubátová et al., 2001). This combination of speed and flexibility in the presence of complex molecules made *Pl. ostreatus* the best choice for this macro test of microbial-fungal-plant synergy in metabolizing industrial pollutants. Importantly, Gaur and Adholeya (2004) note that even in highly toxic environments where the fungus was unable to thrive, spores were always detected within the system, allowing for spontaneous regrowth under a return to positive conditions.

To move these metallic and complex secondary metabolites out of the soil column and prevent toxicity thresholds from being reached within the microbe-saprophytic interaction, an arbuscular mycorrhizal (AM) fungus, such as *Glomus claroideum*, can be added (as shown by Dankhar [2011]) to increase the biosorption of the saprophytic fungus (*Pl. ostreatus*), allowing cationic metallic adsorption to the surface of the cell walls without affecting metabolic rates. The AM fungi demonstrate a complex structure with nonpathogenic pathways that can precipitate or accumulate heavy metals extracellularly, allowing for increased contact time for the translocation of metals and potentially toxic secondary metabolites created by the saprophytic fungal metabolism. This cellular mobility is more common for traditional nutrients such as nitrogen, but the same mechanisms may be possible to exploit for the pollutants of interest in this study. Wang et al. (2005) showed an increase of 125%, 205%, 114%, and 95% for (respectively) zinc, lead, cadmium, and copper uptake into cellular structures when *Elsholtzia splendens* was inoculated with *Glomales* spp. under greenhouse conditions, while Bürkert and Robson (1994) demonstrated the extraradical hyphal translocation of zinc from soil to clover under AM infection with *Glomales* spp. Further, Gaur and Adholeya (2004) mention that the infection of herbaceous plants with effective AM fungi such as *Glomales* spp. can confer tolerance to plants, as enzymatic interactions spontaneously incorporate secondary metabolites and heavy metals as part of a chelation process using siderophores, metallothioneins, phytochelatins, and phytates as part of normal plant metabolism.

As Guar and Adholeya (2004) summarize, AM fungi increase plant access to immobile nutrients, improve soil texture, help provide resistance to erosion, bind metals into roots (preventing translocation into growing shoots and increasing survivability of herbaceous plants in highly contaminated environments), and stimulate biomass growth (again, increasing survivability and increasing the plants' toxic threshold limits); most importantly, the combination of AM fungi in the system increases the volume of soil exposed to remediation effects as the hyphae expand beyond the root zone. The clover tested in the above example demonstrated a 3.15 root to shoot translocation ratio when infected with mycorrhizal species, compared to a 1.66 ratio in noninoculated plants (Guar and Adholeya, 2004). Moreover, the inoculated clover was able to sequester eight times more cadmium and three times more zinc with no change in biomass and no net metal concentration changes in the root systems.

Introduction to Railroading Applications

The ultimate goal of this review is to find biological combinations that could serve as passive agents for wide-scale remediation applications in industry, specifically, for the rail industry. However, since rail currently does not use any but the most remedial microbial processes for small spills, this paper and experiment may be able to serve as a proof-of-concept experiment leading to future pilot studies and eventual large-scale applications for the industry. With this ultimate goal in mind, it would be useful to give the reader a brief introduction to railroading to see how this biological system may help find a place in train operations.

Union Pacific transports more than 100,000 railcars per month, and all the rail lines together transport more than 7.2 million. These cars move bulk commodities like coal, oil, grain, and most hazardous materials. As a common carrier, each of the rail lines is required to accept these commodities (Union Pacific Railroad, 2014). There are nine major rail companies: BNSF (formerly the Burlington Northern and Santa Fe Railway), CN (Canadian National Railway), CP (Canadian Pacific Railway), CSX, Ferromex, Kansas City Southern, KCS (Kansas City Southern) de Mexico, Norfolk Southern, and Union Pacific. Trains average approximately 69 cars, with a weight of 3,115 tons, and travel an average of 18.6 mph over 58,000 net ton miles on Class I rail. The average haul distance per commodity is more than 890 miles (Vantuono, 2008). Union Pacific, for example, can move one ton of freight 475 miles on one gallon of diesel fuel, more than four times more efficient than trucks on a ton by mile basis. Railroads account for 2.1% of US greenhouse gas emissions and haul more than 20% of all hazardous chemicals transported in the northern hemisphere. More than 99.9% of these trips occurred without loss in 2013 (Union Pacific Railroad, 2014).

The first step in this journey is building the train, or, putting specific cars of commodities with specific locomotives. There are intermodal, flat switch, hump, trim, and gravity yards that take customer commodities from their facilities on ‘locals’ and ‘switch’ them onto through-freight trains by various methods. In gravity and hump yards (Figure 1), crews shove cars up a raised section of track, and automatic or manual switches controlled by a humpmaster in the ‘bowl’ direct the car to the right track, usually characterized by direction of destination. After the cars are put in the right tracks, a trim crew will drag out the required number of cars and build the train according to trainmaster or trimmaster instruction. At this type of yard, the most likely areas of contamination are at the locomotive or diesel shops, at the repair-in-track locations, and in the bowl as derailments occur. At flat-switch yards (Figure 1), crews manually pull cars into their outbound tracks. These crews are supervised by a trainmaster or yardmaster, who tells them how big the train can be, where it will need to depart from, and any special makeup requirements for proper handling of hazardous materials. In this type of yard, the above locations are still problem areas; however, these yards have an additional concern of mobile fueling units, as they do not have established fueling facilities. These mobile fueling operations move around the yard ‘topping up’ locomotives and can contribute to small spills and releases almost anywhere within the tracks. Figure 1 shows a sample layout of a flat-switch and a hump or gravity switch yard (Vantuono, 2008).

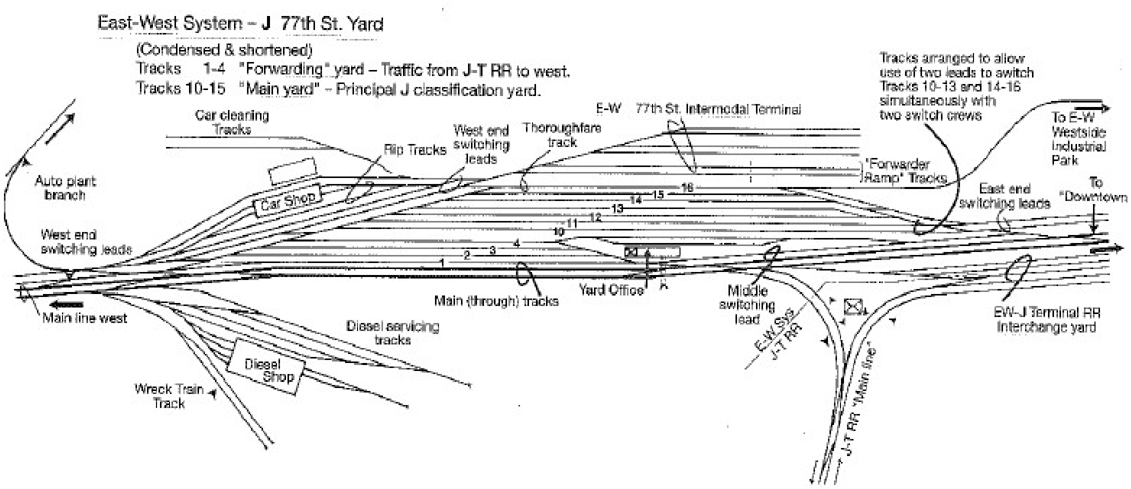

Figure 1. Example of rail yard (Vantuono, 2008).

There can sometimes be many yards (as shown above) in any one terminal. That terminal is run by either a trainmaster or an assistant superintendent, depending on the number and production of yards. Each terminal has a roadmaster in charge of maintenance on the right of way. These are civil engineers responsible for building and maintaining new track within the yards and across the roads. These roadmasters build track the same way across the system and across companies, so that the equipment is interchangeable and exchangeable between lines and states. The railroads get their efficiency from this standardization—any rated car or locomotive can go on any Class I track without concern that the stress or strain on the rail will cause a derailment, and any roadmaster can repair any section of track with some basic knowledge of grade, hydraulic action, and soil characteristics. This is an important aspect with regard to the current project (Vantuono, 2008). The consistency and universality of track building provide a very clear idea of the stresses on any prospective sub-grades based on a thorough table of constants for almost every soil type across the United States. For the purposes of this paper, I have chosen to use Union Pacific's field manual (Union Pacific, 2015); however, all track from any Class I railway will yield very similar results.

Another consistent aspect of rail yards is the list of pollutants typically found near any terminal. These pollutants tend to be polycyclic aromatic hydrocarbons, BTEX (benzene, toluene, ethylbenzene, and xylene), and heavy metals (particularly zinc and cadmium). In larger terminals, these pollutants are currently managed through large wastewater treatment plants; road territories (those areas composed purely of mainline track and small, crew change stations, not a full terminal as illustrated in Figure 1) do not usually have any pollution prevention technologies available to them. Because of past practices before the introduction of the Clean Air Act (CAA), Clean Water Act (CWA), Resource Conservation and Recovery Act (RCRA), Toxic Substances Control Act (TSCA), and other environmentally friendly legislation, most rail yards are contaminated by decades of derailments, spills (particularly of diesel and lube oil), and illegal dumping near tracks. The cost of ripping up track to repair and remediate land below grade is astronomical (at more than $1 million per mile), and the cost to production is similarly high. As a result, more remediation is required in active yards and inactive sites. An inexpensive, easily installed solution is needed to conduct repair and remediation not only for current areas of contamination, where precipitation may contact significantly contaminated areas and migrate off-property, but also for older areas of pollution, where a self-perpetuating system could stabilize and heal soil systems over time.

Biological methods offer great potential to fill this need. These types of systems could be installed into track as a part of regular maintenance to reduce contaminant levels and encourage balanced soil ecosystems under active track, installed next to track in available right-of-way corridors to prevent pollution migration, and allowed to propagate in inactive yards to change the soil structure and gradually remediate pollutants with very little cost in financial responsibility or manpower. For the installation of such a biological remediation system to be feasible, however, inoculation methods and structures would have to be ‘plug and play,’ allowing for equipment and process standardization across all railroad terminals, appropriate to the stresses and strains encountered in situ under track and ballast, with attention paid to the effect that changing sub-grade could have on track maintenance, particularly as this concerns drainage and vertical thrust. Reviews of each system’s benefits and drawbacks (as well as how the system interacts with both soil conditions and other biological agents) with a discussion of what emplacement would look like as a final product in situ with expected pollutant load reductions will be the goal of this paper. Measuring effectiveness requires testing for oil and grease, dissolved oxygen, pH, total suspended solids (TSS), and heavy metals at current permitted National Pollutant Discharge Elimination System (NPDES) outfalls at major terminals. Testing standards are compared in this paper against Federal Clean Water Act standards, in lieu of site-specific and state-governed benchmarks that could create an inaccurately restrictive minimum for some pollutants.

Contaminants and Mechanisms of Metabolism

A brief overview of two particularly common contaminant families may be useful to gain an understanding of the physical constraints required for the selection of any biological organism for the remediation of said contaminants. The primary pollutants of concern (as mentioned in the previous paragraph) are oil, grease, and heavy metals.

Oil and grease can be measured as polycyclic aromatic hydrocarbons (PAH) and are commonly referred to as BTEX compounds (Zhang, 2007). These organic pollutants tend to accumulate in soils similar in composition and behavior to lignin (Paul, 2007). These molecules are very dense, hydrophobic, and variable in shape. Because of these characteristics, there are few enzymes capable of changing the structures of these molecules to shapes more amenable to metabolic incorporation into micro-flora and -fauna, leading to their persistence in the soil column for many years. The common assumption is that lignin (or the correspondingly difficult to degrade PAH) must be broken into smaller, more manageable, and more consistently shaped molecules before anaerobic decomposition can take place. Depolymerization under these conditions tends to produce a water-soluble, acidic substrate similar to soil humic acids; however, there is some question as to whether bacteria can effect complete decomposition of these large molecules alone. Fungi are the most efficient metabolizers of these substances in nature but are typically present only in the rhizosphere and have difficulty penetrating more deeply into the soil column to reach those nutrients (Paul, 2007). Both bacteria and fungi have the ability to cleave aromatic rings, cleave phenylpropane units, and convert aldehyde groups to carboxyl groups through the use of an enzyme, typically lignin peroxidase or manganese oxidase. Fungi use extracellular enzymes, including phenol oxidase laccase, in addition to the previously mentioned structures. The end result of this process is the production of carbon dioxide and water (Paul, 2007). In the process of this digestion, some fungi use metals to facilitate the reaction as free radicals or as part of a lipid peroxidation (Paul, 2007). This provides an excellent opportunity to exploit this requirement as part of a heavy metal remediation process.

The term “heavy metal” is not a meaningful phrase without context. The chemical composition of these metals in question is not the problem—in fact, without some presence of these materials, the soil would not be healthy. It is only the excessive amount of a concentrated form of any of the metals that may contribute to high levels of water hardness (which may lead to a buildup of residue or damage pipes and equipment), undesirable taste of food grown on the soil or of water from runoff, or serious health risks. Some of the heavy metals, like lead and cadmium, are considered toxic due to their predilection to bioaccumulate up the food chain, significantly reducing biological activity on microbial and ecological scales, and, subsequently, threatening human health (Peavy et al., 1985). The term, however, has not been officially defined by any authority, and there seems to be a measure of inconsistency in its usage across research. Some metals loosely considered ‘heavy’ are actually metalloids (Dhankhar and Hooda, 2011). For the purposes of this paper, I use the EPA definition of toxic metals and assume that the regulatory level listed in Table 1 under 40 CFR 261.24 applies to this class of material and that any remediation success is best defined as the achievement of limits below those listed (again, under 40 CFR 261.24). Conventional treatments for these chemicals rely on ion exchange, evaporation recovery, membrane technologies, electrochemical technologies, solvent extraction, etc.; however, some of these technologies are ineffective at the concentrations present in industrial stormwater effluent, while others are prohibitively expensive for widespread, consistent application (Dhankhar and Hooda, 2011).

The advantage of translocating these metallic compounds into living tissues (i.e., surface vegetation) is that several plant species are capable of sequestering toxic levels of these compounds into vacuoles or chelating these materials into more benign metabolites through the cytochrome P-450 enzyme (Pickard et al., 1999). In addition, several species of microorganisms also show the ability to perform biotic alkylation with the coenzymes *N*-methyltetrahydrofolate, *S*-adenosylmethionine, and methylcobalamin. This methylation can "foster detoxification, deposition, and bioaccumulation" (Paul, 2007), with the specific results depending on the metal. Microbes and fungi also adsorb metals on the surface of their cell walls, instead of taking up toxic levels. This immobilizing of the metals on their cellular surfaces is actually more effective in sequestering heavy metals out of the soil column than their translocation properties. Thus, although the translocation can be useful in removing small amounts of potential pollutants, the biosorption these microbes can perform is more useful in protecting the survivability of the system and preventing bioaccumulation for animals consuming vegetation at or above the soil surface (Dhankhar and Hooda, 2011). The metals that are translocated to the receptive plants are thus capable of being handled as solid waste through the periodic maintenance and landscaping of the surface systems, which will prevent the metals entering back into the food chain (Figure 2).

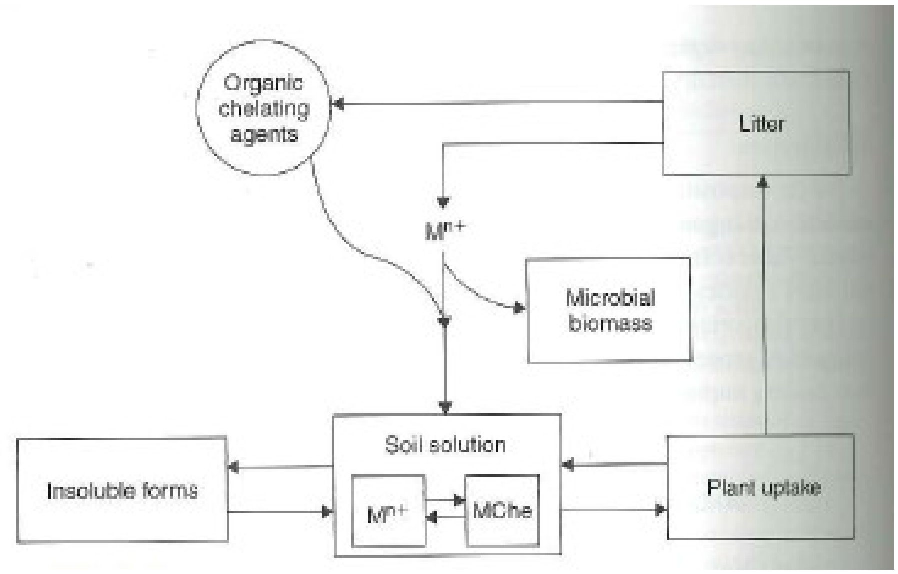

Figure 2. Microbial micronutrient cycle diagram (Paul, 2007).

CHAPTER 2

APPLICABLE BIOLOGICAL SYSTEMS

Mycelia are uniquely suited for pollution remediation. The enzymes they create for metabolism of organic nutrients are particularly adept at breaking down large, unwieldy molecules that bacteria will not metabolize (such as lignin, previously described [see Figure 3 for typical morphology]). The best results in breaking down the most stubborn molecules are from saprophytic fungi (Aust, 1993). Lignin and PAH share similar structures that prove very resistant to traditional phytoremediation or bacterial remediation. White rot fungi have been documented to dispose of both these molecules quickly and efficiently under a wide range of conditions (Adongbede and Sanni, 2014) and have even been shown to consume polychlorinated biphenyls (PCBs) and dioxins (Takada et al., 1996; Kubátová et al., 2001). These organisms are also being tested for use as wastewater filters and barrier technologies in other industries (Gaur and Adholeya, 2004; Khalesi et al., 2014). Their predilection for biosorbing heavy metals would also be significantly useful for our purposes. Any chosen biological organisms may best be combined with geotextile liners, currently used for erosion control, which are adaptable to giving the hyphae structure and protection from the stress of the vertical load under the track.

Because mycelium grows in the form of dense, expansive mats, inoculation in a geotextile liner would encourage that expansive growth along the entire extent of the roadbed and even beyond, into surrounding vegetation that could benefit from the nutrient exchange, thus facilitating phytoremediation. Arbuscular mycorrhizal fungi provide the highest rates of nutrient translocation and the most diverse species symbiosis with herbaceous plants (Raskin et al., 1997). This means that the saprophytic fungi could draw out the metals, water, and degraded PAH, producing secondary metabolites that could be subsequently consumed or utilized by AM fungi either for their own growth and propagation or to be transferred to infected plants. Once metals were secured in the vacuoles or metabolites of plants, the roadbed soils would have fewer contaminants and foster an environment encouraging a positive feedback loop of greater reproduction for the biological denizens. By effectively limiting the buildup of excess water against the track structure, removing PAH compounds, and increasing the total bioaccumulation that would be available for the uptake of heavy metals over time (Wang et al., 2005), the railroad may see a net gain in cost reductions, track stability, and gradual reductions in contaminant concentrations in the long term. In this relationship, the fungi act as an obligate heterotroph and produce a type of hormone resulting in fungal hyphae production, further strengthening the mycelium and the host plants (Miransari, 2014). The production of these chemicals creates physiological and morphological changes in the host plants' roots as well and is nonspecific to the type of plant. Thus, almost any plant will be able to act as a host plant and assist in pollutant uptake (Miransari, 2014).

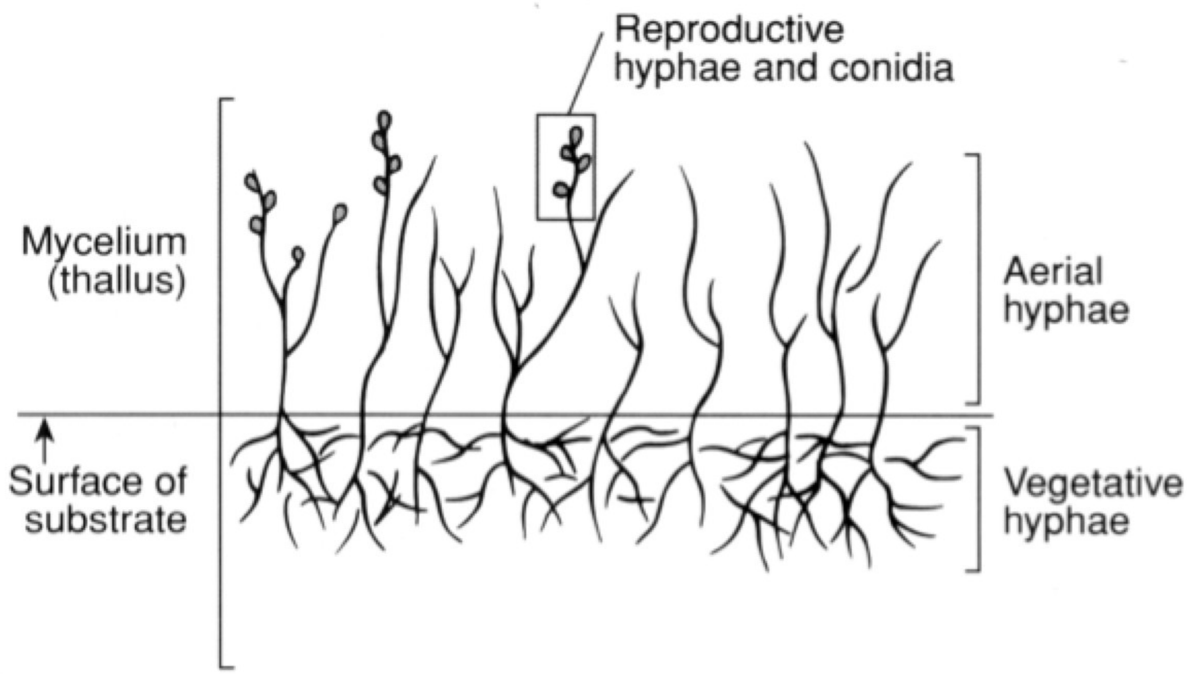

Figure 3. Mycelia morphology (Paul, 2007).

The best fungi to consider for this use would be a saprophytic species paired with an arbuscular mycorrhizal (AM) fungus, such as *Glomus claroideum* (Dhankhar and Hooda, 2011), which show the best results when paired with *Pleurotus ostreatus.* Because AM fungi are associated with more than 80% of terrestrial plant species (as a conservative estimate), the number of choices of combination with traditional phytoremediation plant species creates a significant amount of flexibility (Miransari, 2014). This relationship has been found in every type of climate, temperature, soil pH, salinity, etc. (Hakeem et al., 2015). Plants use the phosphorus, zinc, and nitrogen that may otherwise be inaccessible in depleted soils or synthetic soil columns such as track roadbed and can have their tolerance to drought and pathogens increased significantly under the AM influence. More specifically to this application, however, natural bioaccumulators of heavy metals have demonstrably been shown to increase uptake levels in the presence of AM symbiosis (Miransari, 2014), as well as exhibiting higher growth rates and greater biomass (Paul, 2007). This is most likely due to an increased root or hyphal surface area as a result of the infection, enhancing the availability of nutrients (Paul, 2007). The one significantly limiting factor to AM growth is salinity: high salt levels decrease growth rates and limit nutrient transfer to host plants (Miransari, 2014). Effective communities of mycelium would most likely be composed of AM fungi, filamentous fungi (such as *A. niger,* which showed the highest PAH and BTEX degradation [Olajire and Essien, 2014]), and the saprophytic fungi *Pleurotus ostreatus*.

AM fungi also respond well to low water potential. Mycorrhizal roots demonstrate higher hydraulic conductivity at lower water potentials, stimulate host plants to increase stomatal conductance, and improve plant osmotic adjustment, allowing host plants to use water more efficiently; in some cases, AM fungi can even transfer water to drought-stressed host plants (Hakeem et al., 2015). This means that the system can be effective at the lower water content that an effectively compressed track structure has to have for mechanical stability, as well as during rates of high water flow (although efficiency is reduced under those conditions, it is still present). Host plant choice may also increase that efficiency, as plants characterized by high transpiration levels or even aquatic plants may be chosen, specific to the site and the expected Q value of water flowing out of the roadbed (where Q is the resultant discharge from underneath the track).

It is important to note that a significant limitation to the implementation of biological systems in an industrial setting, such as this one, is that organisms have specific growing conditions, nutrients, and disturbance requirements. As Smith and Collins (2007) argue,

> The complexity of the system dictates that introduced organisms must find a niche to survive and be persistent. In general, organisms introduced into a soil system do not usually survive more than a period of days to weeks. The reason for poor survival rates could be due to nutrient competition, susceptibility to predation and chemical attack due to differences in cell chemistry from culturing, or simply nutrient deficiencies.

It should be noted that there is some question on the overall outcome of the symbiosis—some systems show mutualism, while others approach parasitism and can change depending on scale and time in situ. There is currently not enough research to permit an understanding of the full complexity of the relationship, but a commonly held thought is that "long-term benefits of the symbiosis, derived at times of stress or disturbance, may outweigh negative consequences that are transient in nature" (Smith and Collins, 2007). The strength of the proposed system lies in the interconnectedness of fungi to host plants, mycelium to bacteria, bacteria to host plants, and so on. When these systems are bound together, the efficacy of each is improved and each group's resistance to environmental stressors is presumed to be increased.

Potential bacterial species appropriate for combination with mycelium mats include *Geobacter sulfurreducens*, a dominant metal-reducing microorganism that is particularly happy in aquatic and subsurface environments. It also displays an affinity for oxidizing aromatic hydrocarbons, encouraging their solubility and subsequent uptake by bioaccumulators. Bacteria tend to require 30–90% water saturations, a narrow pH range of 6.5–8, and 10–40% oxygen (Smith and Collins, 2007). This specificity, although it makes them complicated to use by themselves, becomes less of an issue as they are paired with applicable host plants and fungi, such as *Pleurotus ostreatus* (oyster mushrooms). Indeed, *Phanerochaete chrysosporium* requires fungal symbiosis to produce the requisite amount of oxygen under low oxygen soil conditions to degrade large molecules of interest, such as PAH. *Pseudomonas, Alcaligenes, Sphingomonas, Rhodococcus,* and *Mycobacterium* all share similar requirements and increase in efficiency when inserted into a system inhabited by the mycelium (Paul, 2007). The combination of mycelium and bacteria increases bioaugmentation, enhancing survivability and transforming metabolisms in order to endure additional stresses and pollutants. Both organisms encourage growth and maximize metabolic rates, with the surface of the microbes actually providing metal-binding peptides and proteins that can further encourage fungal accumulation and translocation of nutrients (Gaur and Adholeya, 2004; Miransari, 2014; Hakeem et al., 2015).

Particularly good at this resource cycling are the plant-growth-promoting rhizobacteria (PGPR). These organisms not only help the fungi but can also fix nutrients, help synthesize plant hormones to increase growth and metabolism (which is particularly helpful for bioaccumulation of heavy metals), solubilize minerals for transfer to plants, synthesize enzymes to further break down pollutants, defend against pathogens, and encourage reproduction (Miransari, 2014; Hakeem et al., 2015). One notable species is *Kluyvera ascorbata* SUD165, which is very resistant to heavy metals and allows plants to markedly increase their toxic thresholds without decreasing uptake rates (Hakeem et al., 2015). Another species displays compatibility with fungal systems, marked affinity for PAH degradation, and heavy metal toxicity metabolism—this is *Pseudomonas* *aeruginosa* PA01 (Hakeem et al., 2015), which serves as the keystone species for the current project. In one study measuring the efficacy of bacterial consortia degradation of crude oil, *Pseudomonas aeruginosa* showed a 97.6–99.9% removal of oil within seven days (Olajire and Essien, 2014). The assistance of bacteria in the mineralization of organic nutrients, producing oxygen in the process, can create a balanced soil environment that in turn encourages the growth and sustainability of other bacteria that can interconnect the various photolithotrophs, chemoorganotrophs, chemolithotrophs, and photoaquatrophs. This enables nutrient cycling to become part of a positive feedback loop through the vertical expanse of the soil column, encouraging metabolism of various chemical species at depths and expanses that would not be sustainable with a single type of bacterial species (Paul, 2007). The greater diversity of the microbiology in the system, the greater potential exists for energy transfer and the metabolism of more-complex chemical forms without the need to continuously inject the soil with bacteria. This provides a level of redundancy to the system (once established) that can facilitate the expansion of the biology to the whole soil column, even in the oxygen-deprived sub-grade that fungi may not be able to penetrate or in the water-rich sub-ballast that may damage fungal systems in flood conditions. The additional influence of local microbes on the introduced consortia for the current project would help to potentially metabolize secondary metabolites produced by the fungi, both saprophytic and arbuscular. A higher diversity of microbes creates a greater likelihood of their being able to respond to changes in environment and nutrients under seasonal conditions and changes in plant-fungal interactions. This redundancy is a key factor in promoting survivability of the introduced consortia, as most foreign bacteria do not survive in situ and have to be continuously reintroduced to maintain desired digestion rates. Many bacteria, particularly cyanobacteria, demonstrate the ability to follow water vertically to and from the soil surface, and deep subsurface bacterial populations have been found to a depth of greater than 10 meters. This flexibility would allow for sustained pollution mitigation activity in the entire roadbed of the built structures for this project, even to the field water line present in the interface between the sub-grade and the sub-ballast. With the bacteria mineralizing nutrients, the fungi would have to compete less strenuously at a lower depth belowground to access those nutrients (Paul, 2007). This access would also facilitate the transfer of relevant metabolites to phytoremediating plants at less distance and with greater efficacy. It is also worth noting that every subsurface habitat is different. Providing a foreign consortia instead of a single species would also increase the likelihood of those introduced microbes exploiting different microbial habitats, promoting different physical, chemical, and biological conditions that should increase the likelihood of survival (Paul, 2007). As Smith and Collins (2007) observe, “Bacteria and fungi have been shown to break down practically all hydrocarbon contamination in the environment.” Their natural tendencies simply need to be synchronized.

The final piece of the puzzle in creating an effective biological symbiosis for this project is the application of bioaccumulating plant species. These phytoremediators will be the final home for those toxic metals that cannot be metabolized by the fungal-bacterial systems as well as the pools of water and nutrients translocated by the mycelial mats. For this purpose, the plants must be characterized by an efficient translocation factor (root-to-shoot metal concentration ratio) in addition to a good biological absorption coefficient (BAC>1), rapid growth, high biomass production, a good assimilation rate (high pKow), a high metal toxicity tolerance threshold, an expansive root system, and a growth form that is easy to manage (e.g., through mechanized mowing, trimming, and cutting back). Some of the common metals of interest that the railroad is concerned about are zinc, nickel, lead, cadmium, and mercury. The plant must be able to tolerate high toxicity levels for all these metals as well as having a root system appropriate for rhizofiltration, i.e., one that extends more than 15 inches into the soil and exploits the most ground to be able to cover as much of the contaminated soil as possible (Hakeem, 2015). The selected AM fungi, in turn, must be able to translocate contaminants from deep within the soil column to the rhizosphere and into the roots for sequestration of pollutants into plant vacuoles (Raskin et al., 1997). Any plants considered for the current project could also be able to sequester airborne contaminants as well: as much as 43–85% of soil pollution comes from air contamination attributable to emissions from painting, combustion engines, industrial plants, etc. (Hakeem et al., 2015).

The choice of plant should be based on environmental factors such as the amount of moisture in the soil and the average temperature range and precipitation regime of the climate, as well as plant-specific parameters such as the average depth of root penetration and the rate of transpiration. The plants would be more likely to succeed if planted near the roadbed in the discharge for our drainage systems or if we expanded the roadbed to include additional space for planting. This would give us the opportunity to have some freedom in the above-mentioned conditions and would also allow for greater choice in plant selection without damaging the track bed itself. Some common plants that are compatible with previously mentioned bacterial and mycelial species as well as fulfilling all the above-mentioned criteria are *Populus*, *Salix*, *Festuca*, *Medicago*, *Myriophyllum aquaticum*, *Typha*, *Helianthus*, and *Elsholtzia splendens*. These are just a few possibilities, however. Hyperaccumulators are defined as organisms having the potential to accumulate concentrations of more than 100 ppm of cadmium (Cd); 1,000 ppm of nickel (Ni), cobalt (Co), copper (Cu), lead (Pb), or selenium (Se); or 10,000 ppm of zinc (Zn) or manganese (Mn) (Hakeem, 2015). More than 400 species around the world have one or more of these characteristics, so there is a reasonable likelihood of finding local, applicable plants for every site (Raskin et al., 1997; Hakeem et al., 2015).

These biological systems provide the opportunity to remove contaminants that would never be excavated due to cost and also provide an in-place method of removal for immediate spills as well as a long-term solution for significantly degraded environments with very little capital investment and no deleterious effect on operations or production. These types of systems can be built for a cost of up to 80% less than traditional methods. Methods like soil flushing, soil vapor extraction, soil washing, and pump and treat all require significant capital investment and mechanical assistance, and in addition they all interfere with operations (Raskin et al., 1997; Hakeem et al., 2015). With a biological system, even if the mycelium cannot be placed directly under the track, the biological system can certainly be implemented next to the track and in adjacent areas, including roadways, right of ways, and abandoned property. The expansive growth pattern of the mycelium facilitates application of its benefits anywhere the hyphae can gain purchase (and will thereby increase remediation ability even without direct inoculation) and self-perpetuate (Raskin et al., 1997; Gaur and Adholeya, 2004; Khalesi et al., 2014). Bacteria tend to prefer small soil pores, making them perfect for penetrating the compacted sand and small gravel of the sub-grade and sub-ballast that is so difficult currently to remediate. As the bacteria mineralize nutrients and the expansive hyphae of mycelia transfer oxygen, water, and food to each other and to the roots of surface vegetation, many species of fauna and flora become capable of exploiting an expansive collection of habitats in the soil column in a cohesive positive feedback loop that not only takes up the contaminants of concern but also creates a thick layer of organically rich, useful soil over time in the roadbed near the tracks.

The end state for this project would be a modular system that could clearly identify the best-case installation scenarios for biological selection based on soil moisture content, ambient temperature/climate, and potential pollutants of concern at a local level. So, in order to be able to clearly give the structural engineers guidance for which system to install where, we need to have three separate tests. The first, conducted in 'conetainers,' would allow for researchers to screen for AMF, microbial, and plant interactions in various soil moistures and under various contaminant loads. If a species is successful and survives this initial screening, it could be used in the bench test described in Chapter 3 below. If a set of species is successful in the bench test, the third and final test would be a pilot in the prospective local region. This paper will assume that the selected species described below have been successful in the screening phase and are ready to be tested under the macro-environmental, or bench, conditions. Any potential in situ pilot test of successful combinations of macro-environments would be the next logical step, but it is outside the scope of this project. Here, we will focus purely on the bench test using the methodology described in Chapter 3.

CHAPTER 3

METHODOLOGY

Petroleum is a complex mixture of many different substances, including aliphatic hydrocarbons, such as alkanes and cycloalkanes, and mono- and polyaromatic hydrocarbons, such as benzene, toluene, and ethylbenzene. It also contains compounds like asphaltenes, resins, and heavy metals such as cadmium that are notoriously difficult to bioremediate. This complexity of substrate (and resultant level of toxicity) means that the biological systems for the current project will have to be established with initial growing conditions that are as stable and favorable as possible to create positive remediation conditions (Olajire and Essien, 2014). The following will be a proposed study design to determine which biological systems can survive in the contaminated soil, which can work with others to increase metabolic and accumulation effects, and what the greatest removal rate of both PAH and heavy metals will be from that interaction. If this methodology is accepted by the industry, we may have the opportunity to put a three-stage study in place that comprises an initial screening (or 'conetainer' test) for survivability in the contaminant of concern, a larger-scale 'bench-scale' test, or macro-environmental test which will be the primary focus of this paper's "Methodology" section, and a final pilot test where species with the best results from the 'bench-scale' test will be emplaced at a yet-to-be-determined railroad site for in situ results on a local level. Here, we are assuming that the species described below passed the initial screening tests for survivability and will now be put through the 'bench-scale' test to determine appropriate combinations for the final pilot study.

Summary of Inoculation and Application Process

The fungi and bacteria will be introduced to any prospective site and to our macro-environments through the process of inoculation. Most traditional inoculation methods depend on growth in a substrate of plant-fiber ropes or straw wrapped around dowels, or in logs of trees having medium-thick bark, usually oaks (Stamets, 2005). Because the mycelia for a project such as this have to be emplaced into construction areas or on large-scale remediation surfaces at grade, the delivery system must be large in scale (and thus easy, quick, and inexpensive to install in quantity) but of a type that will still support growth. A geotextile may provide the necessary flexibility for a large-scale delivery system, because rolls of material can be inoculated at controlled temperatures and stored until ready for installation, at which time an even and relatively quick application is feasible (*UPRR Engineering Track Manual*, 2015).

Pleurotus ostreatus spores are added to a glucose-based growth medium and mixed at low stirrer speed until the spores are evenly distributed (the mixture needs to be removed before forming small pellets or taking on a pulp-like consistency). The spore-infused inoculum is then applied to a soft textile, such as cheesecloth. The large pore spaces of cheesecloth provide a growing platform, without constricting the hyphae (Stamets, 2005). To ensure an even distribution of the spores, the cheesecloth is dipped in the glucose-based growth medium, then wrapped around a large oak branch and left in a fermenting vessel. At the cultivation stage, the fabric should be unwound over the tub and covered with 5 inches of biomass (leaves, straw, and manure), then heavily watered and left to grow. Finally, *Sedum alfredii,* chosen to act as the phytoremediation component to the system, will be planted into the soil column. *Sedum alfredii* is a small, herbaceous plant easily containable in the tub (Appendix B, Figure B3); it favorably hyperaccumulates zinc and cadmium, in particular (Wu et al., 2007; Hakeem, 2015).

Materials and Methods

The methods and materials (following Davoust and Hansson, 1991) are applicable to strains of the following fungi and bacteria: *Pleurotus ostreatus* (available commercially from Seeds and Things, sold by Amazon at http://www.amazon.com/Grams-Pleurotus-ostreatus-Mushroom-Spawn/dp/B003UI593M)*, Pseudomonas aeruginosa, Agaricus campestris, Kluyvera ascorbata* (available commercially at the CBS-KNAW Fungal Biodiversity Centre collection at http://www.cbs.knaw.nl/Collections/DefaultInfo.aspx?Page=Home). The growth medium will consist of 20 g/L of glucose, 6 g/L of yeast extract, and a mixture of nutrients comprising $(NH_4)_2SO_4$ (11 g/L), KH_2PO_4 (3 g/L), $MgSO_4$ (0.6 g/L), $ZnSO_4 \cdot 7H_2O$ (1.8 mg/L), $MnSO_4 \cdot H_2O$ (0.3 mg/L), and $CuSO_4 \cdot 5H_2O$ (0.4 mg/L).

The saprophytic inoculum will consist of *Pleurotus ostreatus* spores at a concentration of approximately 5.8×10^7 per liter, added to 1 liter of growth medium in a stirred tank reactor with a volume of 1 liter, measuring 100 mm in diameter, with four steel baffles and two impellers. The fungal spores will be mixed in the tank with the glucose-based growth medium under gentle agitation. Once the mixing is complete, rolls of cheesecloth will be soaked in the medium and left to ferment in a separate, sterile tank. There, they will be maintained at 25°C and allowed to grow for 12 hours, or until the first mycelium shows biomass.

AM inoculation will concentrate on in vitro cultivation of *Glomus claroideum* (sourced from the Canadian government's "Catalogue of Arbuscular Mycorrhizal Fungi Strains," available at http://www.agr.gc.ca/eng/science-and-innovation/research-centres/ontario/eastern-cereal-and-oilseed-research-centre/the-glomeromycetes-in-vitro-collection/catalogue-of-arbuscular-mycorrhizal-fungi-strains-available-in-the-glomeromycetes-in-vitro-collection/?id=1236785110466) and *Glomus brohultii* (due to the relative unavailability of this strain, it may be worth cultivating *G. mosseae* instead or taking soil samples from the original identification locations in Cuba, Costa Rica, China, or the Democratic Republic of the Congo [Herrera-Peraza, 2003; Wu et al., 2007]) Fortunately, *G. brohultii* seems strongly correlated in presence to *P. vittata* and *S. alfredii* (Wu et al., 2007). If older plants could be sourced, they may have *G. brohultii* already infecting the roots, which may allow an inoculum to be grown from the seedlings and applied to the test tubs as described below. These two species seemed to show the greatest inclination for metal translocation appropriate for the selected plant species (Bürkert, 1994; Wang, 2005; Wu, 2007). However, if *G. brohultii* cannot be sourced or grown from culture, switching to a more common strain would still achieve the goals of the project. The AMF used should be applied to the soil as part of a powder or liquid application and allowed to grow within the macrosystem according to the testing parameter for that tub. The experimental model will consist of three phases of 32 25-gallon plastic tubs filled with packed sand and gravel, with a 4-inch layer of topsoil from the selected site and a 1-inch layer of mulch representing the field water level and basic soil column of the roadbed. For each system or combination of systems, there will be four macro-environments—a plain control, a PAH-contaminated imitation roadbed, a heavy metal–contaminated imitation roadbed, and a combined PAH/heavy metal–contaminated simulated roadbed. The first tub (all subsequent tubs will follow the general setup described here) will consist of a plain tub with a drain cut into the side and closed with an airtight stopper using a bentonite plug and filled with a proportional representation of the sub-ballast diorite. In a second tub, contaminated soil, with 50% hydrocarbon-contaminated soil (engine oil from a nearby locomotive facility) by dry weight, will be a 4-inch layer placed onto the imitation grade material. In a third tub, soil contaminated with zinc (5% by dry weight) will be layered onto the modeled grade material. In the final tub, each pollutant (engine oil and zinc) will be added in the same concentration as in tub 2 and tub 3, respectively. Please refer to Table 1 below for the experimental model summary. The fungi-cultivated, rolled cheesecloth will be cut to size and unrolled onto the surface of the appropriate test tubs and then covered with 1 inch of compost, manure, and straw. *Sedum alfredii* seedlings will be planted on top of the soil column in appropriate tubs, and the cheesecloth will be slit to allow planting (with the surface litter surrounding but not covering the seedlings), each approximately 5 weeks old. The samples will be maintained in a controlled greenhouse with natural sunlight, approximately 12 hours a day. Temperatures should be maintained as close to 25°C as possible with average day and night temperatures recorded daily. Every week, the biological system will be soaked with distilled water, using a sterile, clean weed sprayer to imitate precipitation. The resultant runoff will be tested for oil and grease (O&G), total polycyclic aromatic hydrocarbons (PAH), pH, and biochemical oxygen demand (BOD), and a toxicity characteristic leaching procedure (TCLP) performed according to ASTM standards delineated by EPA 1664A (Oil and Grease), SW8260 Mod (total PAH), EPA 150.1/SN4500-H B/SW9040, 9045 (pH), and 40CFR Part 136, Md 1311, 1312 (toxicity characteristic leaching procedure) (Zhang, 2007). Each month, 25 g of dry *Sedum alfredii* plant sample will be taken and tested for total uptake in metals; the levels will be graphed over time to demonstrate potential bioaccumulation and note any increase in mortality for the plants as potential bioaccumulation occurs. Three phases of this test cycle will occur with the 32 tubs under the previously described sampling conditions, resulting in a total of 96 tubs tested.

Since some of the interactions explored in these macrosystems are unusual and untried for industrial applications, it is important to narrow down whether it is the single organism that is effective for the given pollutant or the combination of systems that enables successful remediation. To that end, each macrosystem demonstrates single-organism efficiency or one-by-one combination efficiency. Under field conditions, it may prove that the application of all organisms as one system is actually less efficient than the selective application of one or two against either the PAH contamination or the heavy metal contamination. That drill-down into exactly which systems work best under different pollutant combinations or exposures would allow us to fine-tune the application for maximum removal efficiency as well as support (or disprove) the hypothesis that synergistic connections between biology increase remediation capabilities as compared to single systems. So, for example, if saprophytic fungi demonstrate the greatest removal efficiencies against PAH compounds, but a combination of saprophytic fungi and bacteria show the highest removal rates in the combined PAH/heavy metal medium, field applications could reflect a more efficient treatment plan depending on the local soil conditions, potentially saving time and costs associated with using the full inoculum of both types of fungi and bacteria, as well as planting. The average removal rates for each system and combination of systems will be analyzed after the third phase of testing and compared against the pollutant types to determine the greatest removal rates.

Table 1. Macrosystem sampling plan, single phase

Biosystem	**Control**	**PAH contaminant (50% dry weight engine oil)**	**Heavy metal contaminant (5% dry weight zinc)**	**Both PAH and heavy metal contaminants**
Saprophytic fungi	Tub 1	Tub 2	Tub 3	Tub 4
Bacteria only	Tub 5	Tub 6	Tub 7	Tub 8
Plants inoculated with AM fungi	Tub 9	Tub 10	Tub 11	Tub 12
Saprophytic fungi and bacteria	Tub 13	Tub 14	Tub 15	Tub 16
Bacteria and inoculated	Tub 17	Tub 18	Tub 19	Tub 20

plants				
Saprophytic fungi and inoculated plants	Tub 21	Tub 22	Tub 23	Tub 24
Saprophytic fungi, inoculated plants, and bacteria	Tub 25	Tub 26	Tub 27	Tub 28
Plants only	Tub 29	Tub 30	Tub 31	Tub 32

Extracting PAH contaminants of interest is based on Adongbede and Sanni's experiment (2014) in the biodegradation of engine oil by *Agaricus campestris*. Here, they allowed the fungus 30 days to work on the substance. The authors point out that although the fungus reduced petroleum hydrocarbons by over 97%, they also produced secondary metabolites with unknown effects into the system (Adongbede and Sanni, 2014). In this system, those metabolites may be successfully used as nutrients for either the microbial consortia or the phytoremediation plants symbiotically bonded to other AM fungi in the system. The effectiveness of hydrocarbon removal will be determined using the same structural parameters as Adongbede and Sanni's test, with the following materials and parameters:

Equipment: GC-FID from analytical grade n-hexane using a Hewlett Packard HP

Carrier gas: helium

Makeup nitrogen gas: air flow rate of 22 ml/min

Fuel: air flow rate of 45 ml/min

Fuel: H_2

Injector temperature: 220°C

Initial and final oven temperatures: 70–200°C

Detector flame ionized at 250°C with before and after levels of PAH along the peak heights of the chromatographic run.

In testing the effective removal of PAH and heavy metals, water runoff will be collected at the silicone drain and submitted to a third-party laboratory (American Interplex, North Little Rock, AR) for TCLP and O&G. Table 2 has a breakdown of regulatory standards that samples must pass in order to be considered viable for remediation application. Sampling biomass and calculating (1) mycorrhizal dependency and (2) increased or decreased heavy metal uptake according to the formulas below (in addition to empirical confirmation with the microscope) would show the ability of the selected test plant to form mycorrhizal relationships as well as show an increase or decrease in heavy metal accumulation that may be incorporated into the above estimations of in situ performance with a single species of plant, such *as S. alfredii.* For physical confirmation of inoculation efficiency, dry samples will be stained with 0.05% trypan blue in lactoglycerol and reviewed at 100x magnification consistent with procedures delineated by Albrechtova et al. (2012).

1) $MD = \frac{Dry\ weight\ of\ mycorrhizal\ plants - Dry\ weight\ of\ nonmycorrhizal\ plants}{Dry\ weight\ of\ mycorrhizal\ plants} \times 100$

2) $Increase\ or\ decrease\ of\ heavy\ metal\ uptake = \frac{Heavy\ metal\ uptake\ of\ mycorrhizal\ plants - Heavy\ metal\ uptake\ of\ nonmycorrhizal\ plants}{Heavy\ metal\ uptake\ of\ nonmycorrhizal\ plants} \times 100$

Table 2. Passing standards for successfully remediated effluent.

Contaminant	Regulatory level (mg/L)
Benzene	0.5
Cadmium	1
Chromium	5
Lead	5
Mercury	0.2
Zinc	490 micrograms
O&G	15
TSS	30
pH	6–9
BOD5	Pass

Because there will be a screening method prior to the implementation of the macrosystem test, this should provide a level of redundancy and replication needed for statistical viability in analysis. Using a small ‘conetainer,’ we can selectively test each strain of AMF, bacteria, filamentous fungi, and seedling against each contaminant of concern in approximately 15 g of soil. Then, using the Petersen method of estimating abundance, we can estimate the total microbiological population for the species of interest, its subsequent survivability, and its ability to interact synergistically with the other microbial species introduced. Each replicate will comprise a known population introduced to the test, left to grow for 6 days, and tested through the use of fluorescence in situ hybridization using a soil slurry (Christensen et al., 1999) to measure a final population. Using this data, the survivability of the population may be measured according to the population below (Krebs, 1989):

$$\hat{N} = \frac{(M+1)(C+1)}{R+1} - 1$$

Where

$M = estimated\ introduced\ microbial\ population\ to\ 'conetainer'$

$C = estimated\ population\ measured\ by\ FISH$

$R = proportion\ survived\ C/M$

If R is greater than 80%, we will consider that population to have been successful at reproducing within the ‘conetainer’ environment, and it will move to the next stage of bench testing, or implementation in the macrosystem above. A set of test samples will have to be collected prior to the initiation of screening in order to determine the requisite confidence interval and number of sample ‘conetainers’ needed for each population test. A species must demonstrate 80% or higher survivability in 100% of tests prior to selection for the bench test. Sample size for the conetainer screening test will be determined by the use of the initial test sample according to the biased Petersen sample size estimator (binomial confidence interval) shown below (from Krebs, 1989):

$$N = \frac{R}{C} \pm \{Z_{\propto}\sqrt{\frac{(1-f)(\frac{R}{C})(1-\frac{R}{C})}{C-1}} + 1/(2C)\}$$

Where

$M = estimated\ introduced\ microbial\ population\ to\ 'conetainer'$

$C = estimated\ population\ measured\ by\ FISH$

$R = proportion\ survived\ C/M$

$f = R/M$

$Z_{\propto} = standard\ normal\ deviate\ for\ (1{-}\propto)\ confidence\ level$

Hyperaccumulating plants will also be screened with this ‘conetainer’ method using methodology described above to ensure >100% zinc uptake and capacity for AMF infection prior to implementation in the macrosystem study.

Upon completion of the macrosystem test, data will be subjected to a one-way ANOVA to calculate means and standard errors for total removal of PAH and heavy metals for each macrosystem described by the tub test. Mean removal rates will be compared using Duncan’s multiple range test (Wang et al., 2005). The highest mean removal rates for both PAH and heavy metals will result in the system being selected for the prospective pilot test.

CHAPTER 4

EXPECTED RESULTS

A 24-week test of the feasibility of this integration of biological systems, beginning with 130 kg of PAH and 11 kg of zinc, will be expected to yield end values of 4 kg of PAH and 2 kg of zinc remaining. The expected values (see Table 3) are calculated based on the heavy metal accumulation rates for zinc delineated by Wu et al. (2007) of 85%, the PAH breakdown rate reported in Adongbede and Sanni's 2014 study, and the degradation rates reported by Kubátová et al. (2001) and Wu (2007).

Table 3. Expected experimental values to test feasibility (note: soil weight was calculated based on the dry density of dolomite [Chesterman, 2001], a commonly used sub-ballast material [Union Pacific Railroad, 2015]).

	Total soil weight (kg)	**PAH content (kg)**	**Zn content (kg)**	**Expected amt. remaining (kg)**	**% removal**
Tub 25	260	0	0		0.00
Tub 26	260	130	0	4	97.00
Tub 27	260	0	11	2	85.00
Tub 28	260	130	11	4 PAH and 2 metal	

Using Wang et al.'s (2005) methodology, water samples would be collected weekly for a period of 24 weeks; these samples would be run for TCLP (toxic characteristic leaching potential, also known as RCRA 8, which includes all regulated metals including BTEX as well as oil and grease [O&G], total suspended solids [TSS], etc.). Wang et al. (2005) also sampled plants weekly and, using dry biomass, calculated the heavy metal uptake for each. In theory, that mass could be subtracted from the total soil weight shown in Table 2. However, as Wang et al. (2005) clearly demonstrated, root to shoot ratios, uptake rates, and hyperaccumulation rates were very specific to plant and plant/fungi combinations. Olajire and Essien (2014) reported a lab degradation of PAH of 20–48% from microbial activity alone, but depending on strain and environmental conditions, the survival rate of the microbial consortia used varied significantly.

There are some significant unknowns in dealing with this kind of model for industrial applications. The AMF may not survive outside of native soil conditions, or the two types of AMF may not be able to coexist in the same root structures. Metabolites from *Pleurotus ostreatus* may not be taken up by the AMF and never transferred to the plants. The translocation of metals from the soil to the plants may not happen—the AMF may avoid metal uptake, or the plant may not be able to hyper-accumulate the amounts required in a single lifetime, resulting in a remediation period of millennia instead of decades and making the application impractical. The soil conditions may be so unique at different sites that it would be impractical to attempt a modular biological design for industries that reach across so many different geographical regions with such diverse temperatures, water availability, local fauna, etc. And finally, the difficulty in inoculating

AMF for large-scale applications may be cost limiting for any biological application (Rai, 2006). However, the significant potential offered by these systems is worth investigating. Reducing the complexity of the system while maintaining the potential synergistic properties inherent in the different organisms is a main reason for breaking the testing plan into three phases that look at each part of the system as well as the whole. While the whole should show the greatest removal efficiencies for both PAH and heavy metals, there is still much we do not understand about how these organisms work with and against each other. So, while the potential for success seems high, there are many pitfalls that could remove this technique from consideration, at least on an industrial scale, until the previous concerns can be addressed with actual field data.

CHAPTER 5

CONCLUSION

The combination of biological systems applied to a geotextile should allow for higher survivability and ease in application near the roadbed. Implementing the system under new track may be an option at yards and terminals; the other location for implementation would be along nearby roadbed environments. These parallel ditches, drainage ways, and access road applications would allow the biological systems to still spread underneath the sub-ballast and provide additional uptake of pollutants, in addition to treating the runoff into the drainage system, while improving survivability for the biology. Overall, this in situ biological remediation could demonstrate a 97% reduction in PAH over one week (as demonstrated in Adongbede and Sanni's [2014] experiment) as well as the successful sequestration of 85% zinc by dry weight in the nearby plants over time (as demonstrated in Wang et al., 2005; Wu et al., 2007), allowing us to harvest biomass and dispose of it as solid waste. The most effective combination may be the initial application of oyster mushrooms (*Pleurotus ostreatus*) with *Pseudomonas aeruginosa* on the surface, while inoculating sub-ballast with *Pseudomonas aeruginosa, Agaricus campestris, Geobacter sulfurreducens,* and *Kluyvera ascorbata,* followed by planting *Sedum alfredii, Pteris vittata, Arabidopsis halleri,* and *Elsholtzia splendens* that have been inoculated with *A. niger, G. claroideum,* and *G. brohultii* (see Table 4).

Table 4. Suggested combinations for consortia makeup for maximum efficacy on-site**.**

Microbes	Fungi	Type	Plants
***Pseudomonas aerginosa* PA01**	*Pleurotus ostreatus*	SAP	*Sedum alfredii*
Agaricus campestris	*Glomus claroideum*	AM	*Pteris vittata*
Geobacter sulfurreducens	*Aspergillus niger*	SAP	*Arabidopsis halleri*
***Kluyvera ascorbata* SUD165**	*Glomus brohultii*	AM	*Elsholtzia splendens*

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APPENDICES

APPENDIX A

HYDRAULIC PROPERTIES OF BALLAST, SUB-BALLAST, AND SUB-GRADE

Class I track distributes load in the shape of an inverted triangle (see figures A1 and A2). The wheel-rail stress is approximately 100,000 psi, the ballast bearing stress is approximately 85 psi, and the sub-grade stress is approximately 20 psi (Union Pacific Railroad, 2015). This set of relationships can be used to estimate that any biological system integrated into sub-ballast or sub-grade should be able to bear a maximum of 85 psi for survivability. Additionally, in areas with high freeze-thaw coefficients, it may be more efficacious to install biological systems in surrounding right of way to the track, as opposed to placing additional vertical stresses on the sub-grade, potentially causing buckling as water (at higher concentrations) within the sub-ballast freezes and moves the ballast structure. In any event, any system installed into the soil will need access to water, but not enough to drown, and access to nutrients without smothering. At this point, soil profiles will be useful in estimating how much of each (water, oxygen, and nutrients) any organisms may be exposed to. Again, this is using the engineering field manual's best-case scenarios for installation in track-suitable media. According to $q = KJ$ where q is the specific discharge, J is the slope of the energy line, and K is the hydraulic gradient (or saturated hydraulic conductivity), we can estimate $q = Q/A$ where Q is the flow rate (volume over time) and the resultant A is the amount of water present at sub-grade (Peck, 1977). In a traditional rail engineering plan, this A value would be used to calculate the type and placement of drainage; however, in this paper, it can be used to determine the amount of free water that any chosen organisms will have available for uptake. If A is too high, the fungi will be drowned; too low, and the hyphae will withdraw for conservation.

The current track design calls for a geotextile emplaced between the sub-ballast and sub-grade (see figure A4). This location is the field water line, and the standard use of a geotextile would be a simple way to emplace the biologically inoculated system into the pattern.

Figure A1. Stress-strain rail relationships (Vantuono, 2008).

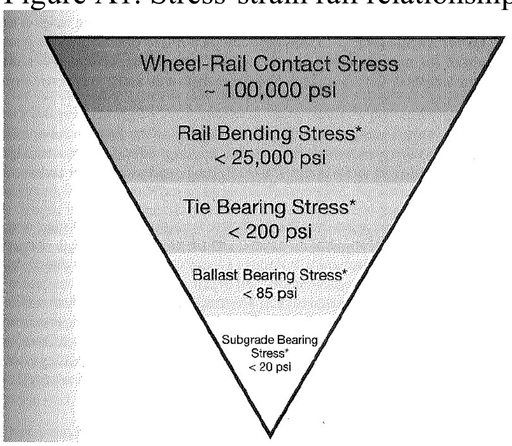


Figure A2. Rail loading relationships (Vantuono, 2008).

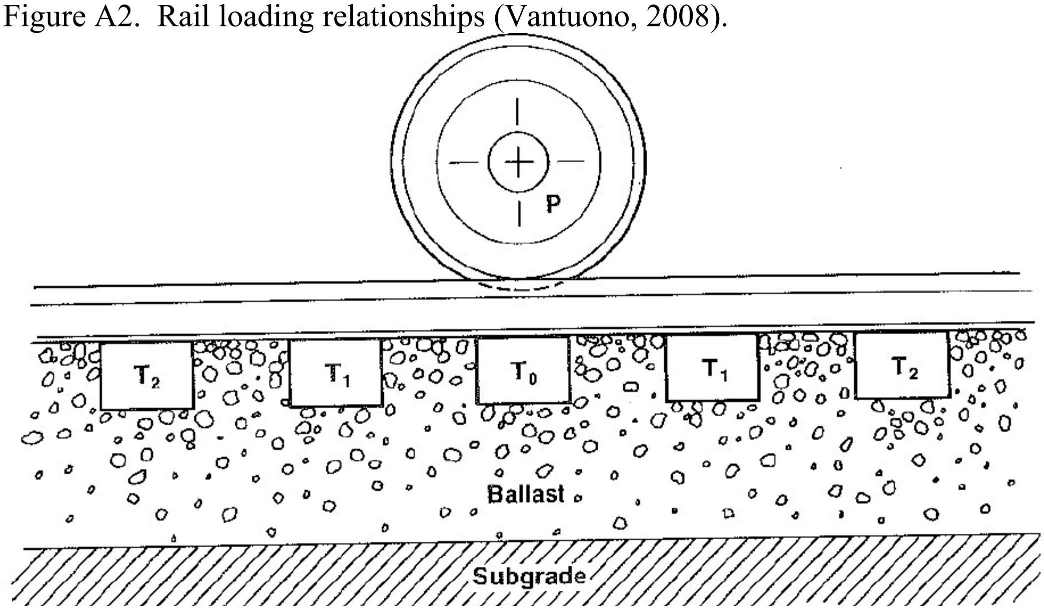


Figure A3. Track structures (UPRR Field Manual, 2015).

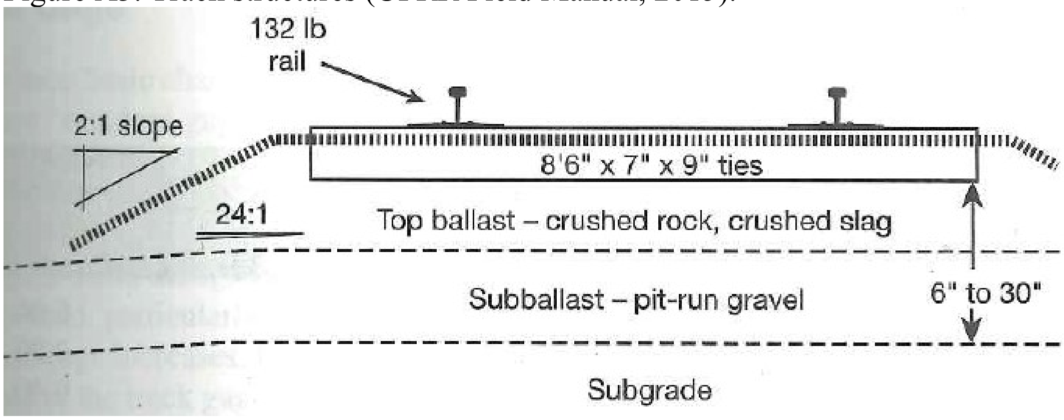
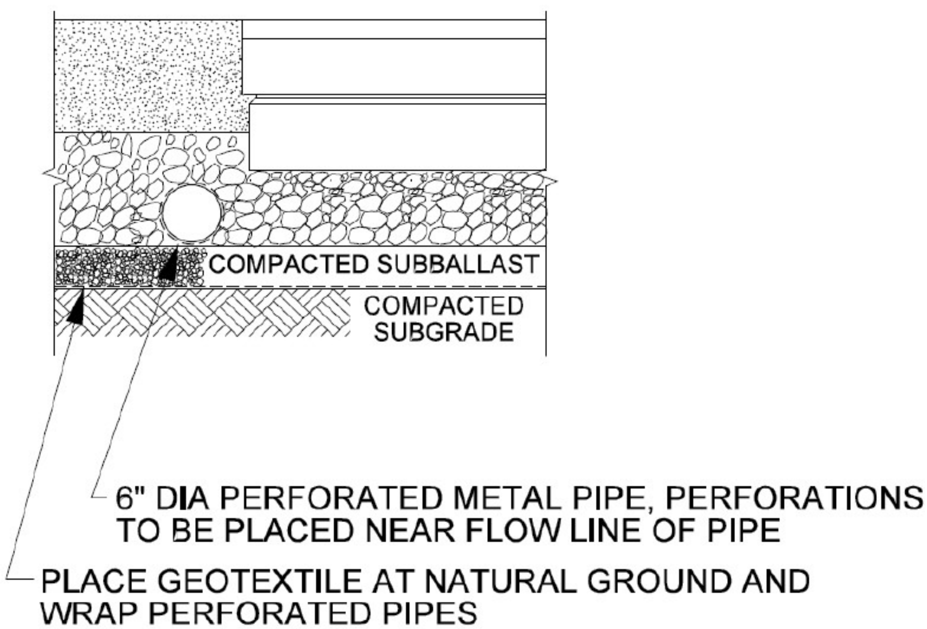


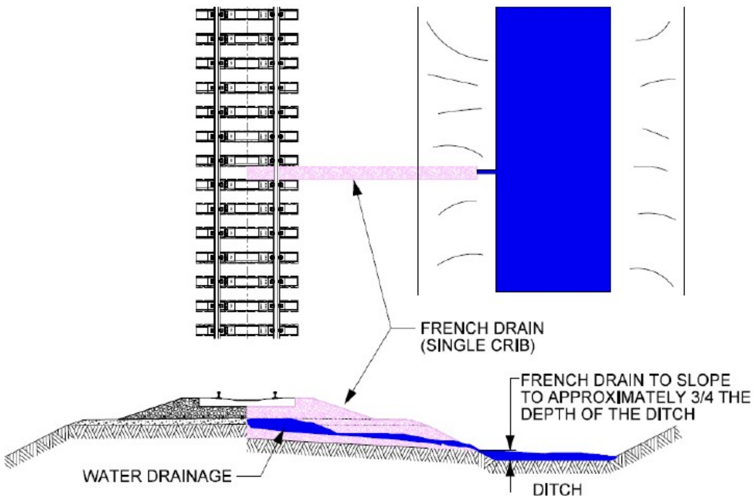
Figure A4. Roadbed soils (UPRR Field Manual, 2015).



In high *A* environments (those with high flow and saturation rates), microbial applications that tend to have higher survivability in aqueous environments may be a better choice. Alternatively, there are some aquatic fungi that could be adapted for inoculation, although those methods are significantly more difficult for system-wide implementation, and aquatic fungi do not survive dehydration as well as terrestrial fungi.

Additionally, current methods for preparing drainage systems (see figure A5) do not have any ways to reduce the amount of water flowing through the track system; our best methods only try to route water to the right of way, away from the immediate area of the track. This, however, only floods the roadbed next to the track, causing freeze-thaw events, maintenance problems, and bad customer relations as nearby property owners take the brunt of the routed water onto their lands. One of the main advantages of using biological systems in the roadbed and the right of way would be in translocating that water into the plants and fungi and taking it out of the ballast, sub-ballast, and subgrade, proactively decreasing freezing effects and flooding. Thus, any biological system we put into place would have to deal with sandy or gravelly soils, with a minimal soil moisture content of 7–10%, small pore spaces filled mostly with air, and a constant strain of approximately 85 psi in the sub-ballast and 20 psi in the sub-grade (depending on installation location and growth patterns) (Peck, 1977; Union Pacific Railroad, 2015).

Figure A5. Drainage Systems—Ballast to Sub-ballast.



Because biological activity will be directly impacted by the amount of water available within the system, we can measure the metabolic activity (to ensure survivability) with the water-filled pore space in the installed site. This measure,

$$\%WFPS = \frac{soil\ water\ content\ x\ bulk\ density\ \times\ 100}{1-(\frac{bulk\ density}{2.65})}$$

correlates to the amount of relative microbial activity. Soil water content can be determined for each site through gravimetric methods. As a result, if the %*WFPS* of the in situ soil column is known, and relative microbial activity can be determined in a lab or other controlled design particular to the site location’s soil conditions, then a regression equation would provide a good estimate of biological success when actually installed (Paul, 2007).

APPENDIX B

BIOLOGICAL ILLUSTRATIONS

Figure B1. Demonstration of AM infection with herbaceous plant (Martin, 2011). Note how the hypha penetrates through the root cell, moving nutrients from the soil into the cell itself.

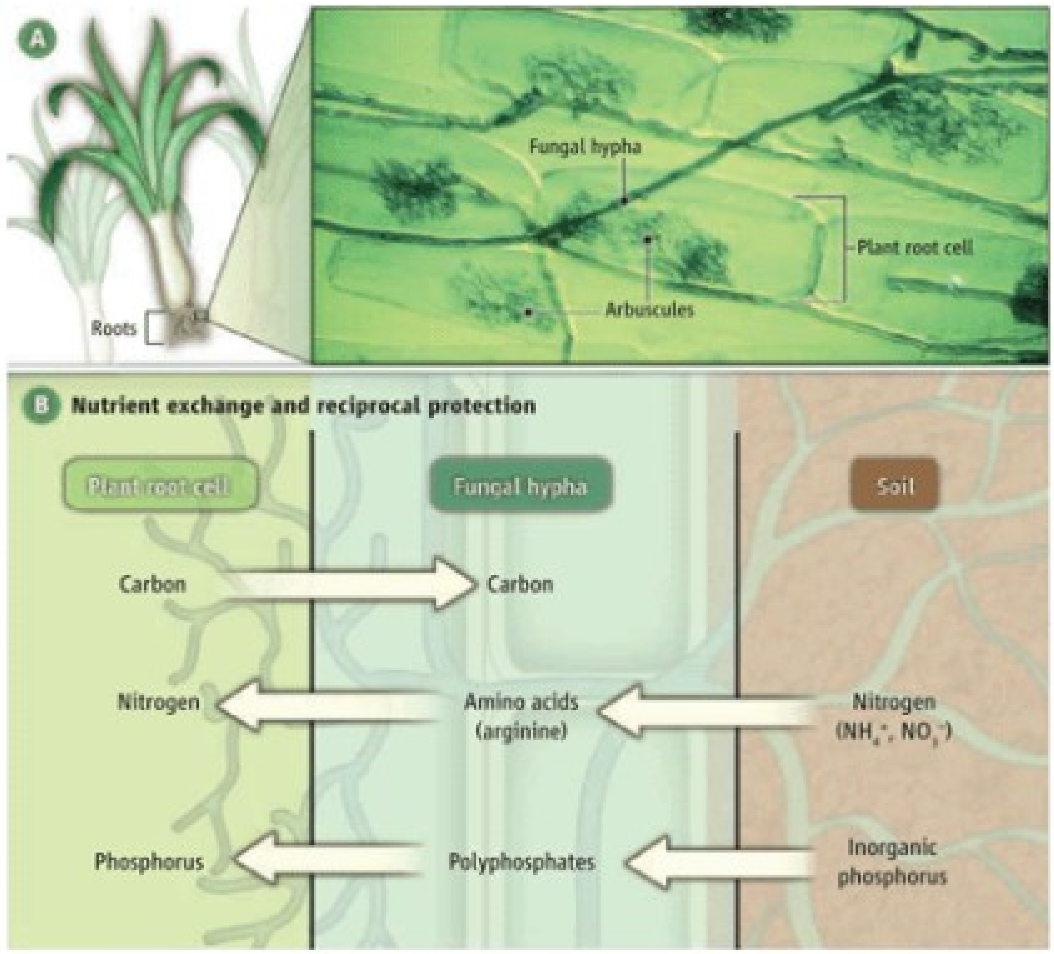


Figure B2. *Sedum alfredii* (“Sedum alfredii,” 2013).

Figure B3. *Pteris vittata* (“Pteris vittata,” 2011).

Figure B4. *Arabidopsis halleri* ("Arabidopsis halleri," 2010).

Figure B5. *Elsholtzia splendens* ("Elsholtzia splendens," 2010).