Bioremediation of Soils by Plant–Microbe Systems

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ABSTRACT

Sustainable ecosystems can be designed to eliminate environmental toxins and reduce pathogen loads through the direct and indirect consequences of plant and microbial activities. We present an approach to the bioremediation of disturbed environments, focusing on petroleum hydrocarbon (PHC) contaminants. Treatment consists of incorporating a plant-based amendment to enhance ecosystem productivity and physiochemical degradation followed by the establishment of plants to serve as oxidizers and foundations for microbial communities. Promising amendments for widespread use are entire plants of the water fern Azolla and seed meal of Brassica napus (rapeseed). An inexpensive byproduct from the manufacture of biodiesel and lubricants, rapeseed meal is high in nitrogen (6% wt/wt), stimulates >100-fold increases in populations of resident Streptomyces species, and suppresses fungal infection of roots subsequently cultivated in the amended soil. Synergistic enzymatic and chemical activities of plant and microbial metabolism in root zones transform and degrade soil contaminants. Emphasis is given to mechanisms that enable PHC functionalization via reactive molecular species.

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INTRODUCTION

The objective of this review is to describe the mechanisms whereby plants and plant residues alter soil microbial activities to effect beneficial changes in natural or agricultural habitats. By means of example, we focus primarily on remediation of soils contaminated with petroleum hydrocarbons (PHC). However, the concepts presented here can be extended to programs that convert soils from a high pathogenic potential (caused by monoculture agricultural practices) into a disease-suppressive state. Just as treatments can lessen soil toxicity by degrading or detoxifying PHCs, so too can they suppress soilborne diseases by killing pathogens, interfering with the process of infection or enhancing plant systemic defenses.

Herein we use the term bioremediation to encompass treatments of environments that rely on biological processes. Introducing judiciously selected plants and plant residues into a soil can bring about the complementary goals of bioaugmentation (adding metabolically active microbes) and biostimulation (improving physicochemical conditions of the soil to stimulate microbial metabolism and growth). The commonly used terms phytoremediation (Harvey et al., 2002; Morikawa and Erkin, 2003) and rhizoremediation (Kuiper et al., 2004) (rhizo = root zone) are generally applied to situations wherein plant metabolism contributes to the remedial process. In certain cases continued survival of the plant is clearly necessary for successful bioremediation. For instance, transpiration by deep-rooted poplar trees enables treatment of contaminated aquifers by bringing water into contact with plant enzymes that oxidize the contaminant (Doty et al., 2000). On the other hand, plant survival is often not necessary or may not be desirable such as in the application of the water fern Azolla, which releases its stores of nitrogen only upon death.

This short review is intended to serve environmental engineers seeking to design efficient plant-based bioremediation systems. We provide specific recommendations for materials and approaches to bioremediation and a conceptual framework for rationally managing the gaps in knowledge that are certain to be encountered in this nascent field.

PHC DEGRADATION BY MICROBES

Of all the contaminants arising from industrial activities PHCs are perhaps the best suited for bioremediation. Enzymatic pathways for the degradation of PHCs, which include alkanes, single-ring aromatics, and poly-aromatic hydrocarbons (PAH), are found in an array of bacterial and fungal taxa, and reports of newly discovered catabolic pathways are still common. Even those PHCs that do not induce degradative pathways in microbes, and thus cannot serve as C-sources in
axenic culture, can become mineralized in microbial communities via the process of co-metabolism (Dalton and Stirling, 1982). With few exceptions, such as certain fatty acid products of alkane degradation (Atlas, 1981), the susceptibility of PHCs to biodegradation increases following initial oxidation. Some larger PHCs display long-term persistence in the environment, with half-lives of months to years, mainly due to their inaccessibility within oily conglomerates or adsorption on soil particles (Eriksson et al., 2000). Biosurfactants produced by microbes can increase this accessibility but only to a limited extent (Ron and Rosenberg, 2002). The activities of plant roots, as we shall see, can further extend the susceptibility of PHCs to microbial degradation.

**SOURCES OF PHC-DEGRADERS**

Upon exposure to PHCs, virtually any soil or water habitat can ultimately develop a microbial community capable of mineralizing PHC contaminants. A survey of geographically diverse soils by Mueller et al. (1997) recovered several species of PAH-degrading bacteria, primarily pseudomonads and nocardioforms, from PHC-contaminated soils but none from uncontaminated agricultural soil. These observations raise an important question: From where do PHC-degraders in contaminated soils originate?

Petroleum products are not sterile and, thus, any spill is likely to contain bacteria already well adapted to the particular contaminant. From a crude oil storage cavity Hino et al. (1997) have isolated two PHC-degrading *Pseudomonas* species that produce copious amounts of exopolysaccharides, a trait that generally promotes long-term survival.

Plants inhabiting a contaminated environment can also serve as a source of PHC-degrading bacteria. *Pinus*-associated fungal/bacterial consortia in uncontaminated lignin-rich forest humus soils have been shown to be capable of degrading PHCs (Heinonsalo et al., 2000). In addition, we have found that sterile 4% diesel-supplemented medium inoculated with laboratory-cultivated *Azolla pinnata* water ferns developed a bacterial consortium capable of lowering the concentration of diesel aromatics by approximately one-half within 19 days (Cohen et al., 2002). In a field experiment, *A. pinnata* plants were killed by low concentrations of diesel but released viable PHC-degrading microbes, greatly increasing the rate of degradation (Cohen et al., 2002). These findings imply that at least some microbes colonizing PHC-contaminated sites are derived from indigenous plant-associated microbial communities rather than from the bulk soil. Plant rhizospheres have substantially greater numbers and diversity of microbes compared to bulk soil and, since many plant secondary metabolites are structurally similar to PHCs (Fig. 1), these microbes are adapted to PHCs (Leahy and Colwell, 1990).

**INITIAL APPLICATION OF PLANT-BASED AMENDMENTS**

Use of organic amendments is preferable to inorganic fertilizers that can temporarily lower microbial activity, leach into groundwater, and are less successful...
in reducing the toxicity of PHC-contaminated soils, as measured by toxicity tests such as seed germination and earthworm survival (Harvey et al., 2002; Phillips et al., 2000). Incorporation of decaying plants into soil improves porosity and water holding capacity, provides foci for microbial colonization, and encourages functionalization of PHCs. Sunlight facilitates direct electron transfer from phytoorganics to PHCs (Mill et al., 1980) as well as the transfer of electrons from oxalate to iron, leading to production of highly reactive hydroxyl radicals (by the Fenton reaction) (Gadd, 1999) that can attack the PHC skeletons. White- and brown-rot fungi utilize oxalate to stimulate radical attack of biopolymers, such as lignin, by enzymatic means that do not require light (Shimada et al., 1997). The added polar and charged functional groups increase the hydrophilicity of large PHCs to make them more accessible to microbes. Functionalization can also stimulate intermolecular cross-linking that may effectively detoxify the compounds but at the same time limits their susceptibility to degradation, a phenomenon referred to as biostabilization (National Research Council Water Science and Technology Board, 2003).

Azolla possesses several attributes that warrant its consideration for widespread use as an amendment for bioaugmentation and biostimulation of contaminated sites. For centuries farmers in Asia have depended on mulched Azolla to fertilize their rice crops. Azolla plants are small (~1 cm frond diameter; ~4 cm root length), reproduce quickly (2.5–4d doubling times) and are distributed world-wide in aquatic habitats. Owing to their associated N₂-fixing symbiotic bacteria, Azolla plants do not require an external source of combined N. When they are grown in phosphate rich waters, such as in secondary sewage treatment effluent, Azolla plants can accumulate up to 1.6% phosphate (wt/dry wt) (Mandal et al., 1999). Microbial growth and activity in PHC-contaminated environments are likely to be limited by the availability of nitrogen and phosphorous (Leahy and Colwell, 1990). Thus, an

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**Figure 1.** Examples of aromatic-ring structures found in *Azolla pinnata* (left) and diesel fuel (right).
important benefit resulting from Azolla amendment is the release of substantial nitrogen and phosphorous stores during decomposition.

Another widely available amendment that is high in nitrogen (6% wt/dry wt) and easy to apply is seed meal of Brassica napus (rapeseed), a by-product of the oil extraction process. In a variety of soils treated with 0.3 to 1.0% (vol/vol) seed meal, populations of Streptomyces spp. increase by two to three orders of magnitude (Mazzola et al., 2001) (unpublished results). As a group, Streptomyces produce an extensive repertoire of degradative enzymes (Barabas et al., 2001; Park and Kim, 2003) and many are important agents of root disease suppression (Crawford et al., 1993; Rothrock and Gottlieb, 1984; Tahvonen, 1982) and plant growth promotion (Tokala, 2002). Currently, the main impediment to the production of clean-burning, low toxicity rapeseed-based biodiesel is the low price received for the meal (Bender, 1999). Thus, a major advantage of expanding the use of B. napus seed meal over other prospective bioremediation soil amendments is the consequent reduction in petroleum-based diesel usage and the decreased environmental contamination resulting from accidental spills.

INFLUENCES OF PLANT–MICROBE SYSTEMS ON PHC DEGRADATION AND DETOXIFICATION

A nutritionally balanced soil amendment can support higher than typical numbers of resident microbes in the soil for several months. To help maintain microbial metabolism and to distribute microorganisms into greater depths of the soil profile, amendment should be followed by the establishment of plants on the treated site. A fallow period of 3–5 weeks is generally required to prevent infections by opportunistic pathogens that are more likely to occur during the period of microbial population expansion immediately following incorporation of an organic amendment (Papavizas and Davey, 1960). Some soil amendments, such as B. napus seed meal, lead to the establishment of a microbial community that is suppressive to root diseases whereas others, such as sawdust, can increase the infection potential of soil dwelling plant pathogens and parasites (Davey and Papavizas, 1960). Just as organic residues vary in their utility as bioremediation agents, plants vary greatly in their tolerance to environmental contaminants. Adam and Duncan (1999), for example, screened 22 species of grasses, herbs, legumes and commercial crops for germination rates in diesel-contaminated soil. At exposures of 50 g diesel/kg soil, results ranged from complete inhibition of germination for Cocksfoot grass (Dactylis glomerata) and Rough meadow grass (Poa trivialis) to no significant inhibition for two B. napus cultivars (cv. Rocket and cv. Martina) and for one of the two tested cultivars of Flax (Linum usitatissimum cv. Elise). More research directed toward screening plants for tolerances to toxicants would provide a useful database for environmental engineers.

The activities of plant roots that encourage degradation, volatilization or immobilization of pollutants have been recently reviewed (Harvey et al., 2002; Morikawa and Erkin, 2003). By virtue of their physical movement and high degree of microbial colonization, roots transport PHC-degraders into contact with the target substrate. Roots also release O$_3$, which is necessary for the operation of most PHC-biodegradative pathways. Bacteria living endophytically and on the roots of
most, but not all, plants in PHC-contaminated environments are more likely to have the ability to degrade PHCs than are bacteria resident to bulk soil (Duane et al., 2001; Siciliano et al., 2001). The plant and microbial enzymes involved in the primary and secondary oxidations of PHCs are reasonably well characterized. Less attention, however, has been paid to the activities of non-PHC-degrading organisms that may contribute indirectly to PHC degradation, particularly via free radical-mediated events.

Cultivar-specific differences in root-associated microbial communities of plants, such as wheat (Mazzola et al., 2002) and other grasses (Lucia et al., 1980), can be important determinants of phytoremediation efficacy and plant tolerance of pollutants. The molecular basis for host-microbe specificity is, for most cases, largely unknown. *Azospirillum* spp., common bacterial associates of grasses, are more likely to colonize the roots if they reduce nitrate only to nitrite (Cohen et al., 2004; Lucia et al., 1980). Though *Azospirillum* spp. are not reported to be PHC-degraders, their release of nitrite can enhance the potential of a grass-microbe soil community to degrade a pollutant. Nitrite stimulates root growth (Zimmer et al., 1988) and can be reduced to nitric oxide (*NO*) by nitric oxide reductases (Stohr and Ullrich, 2002), nitrate reductases (Yamasaki, 2000), or non-enzymatically within low-pH compartments (Bethke et al., 2004; Yamasaki, 2000). *NO* is an inducer of plant systemic resistance (Delledonne et al., 1998; Durner et al., 1998) and in roots can induce aerenchyma formation to further increase transport of oxygen into the rhizosphere (Dordas et al., 2003). *NO* combines with superoxide (O$_2^-$) to form peroxynitrite (ONOO$^-$), which along with hydroxyl (OH) and carbonate radicals (CO$_3^{2-}$) that are formed partly as a consequence of oxalate oxidase activity, will react with PHCs (Squadrito and Pryor, 1998) and initiate degradation and cross-linking reactions (Fig. 2).

Plants and plant residues support substantial *NO* production by plant-associated and soil-dwelling bacteria. Plant surfaces and internal tissues are colonized by nitric oxide synthase-containing bacteria, such as *Rhodococcus* sp. strain APG1 (Cohen and Yamasaki, 2003) and *Streptomyces lavendulae* (Cohen et al., 2003), which were isolated from aerial tissue of *A. pinnata* and roots of apple, respectively. In soil, bacterial nitrification (the oxidation of ammonia to nitrogen oxides) can be greatly enhanced by addition of organic amendment. We have observed up to a 200-fold increase in *NO*-production in a soil amended with 0.5% *B. napus* seed meal that was dependent on the activities of nitrifying bacteria (unpublished results). The consequence of intensive *NO* production in the soil on the formation of long-lived organic humic material is a worthwhile topic for future research.

Nature produces an abundance of nitro- and chloro-aromatic compounds but their tendency to crosslink into large polymers confounds attempts to identify them and diminishes their bioavailability. Myneni (2002) used a synchrotron method to demonstrate that most of the chloride in plant material becomes bound to hydrocarbons during the process of decay to the humic state, presumably through the action of peroxidases. Peroxidases can also catalyze the formation of nitro-aromatics by forming a NO$_2^-$ intermediate from nitrite (Sakihama et al., 2003). Such reactions apparently occur inside plants and may account for the finding from a
A survey of seven plant species that 0.4–8.1% of total plant nitrogen is bound into unidentifiable forms (Morikawa et al., 2004).

The production of oxidative enzymes by plant roots varies considerably, even between cultivars of the same species. Another class of enzymes, the phenoloxidases (tyrosinases and laccases), have broad substrate specificity that may promote co-metabolic degradation of functionalized PHCs (Fig. 3). The phenoloxidase activity in roots of the apple cultivar Hashabi is almost four-fold greater than that of cultivar MM 111 (Gur et al., 1988) and exhibits a positive correlation with phenolic levels in root tissue. Plant growth-promoting rhizobacteria may influence such cultivar-specific differences since they can stimulate the expression of phenoloxidases and accumulation of phenolics by their host plant (Chen et al., 2000). A plant may also be able to indirectly enhance the phenoloxidase activity of its associated microbial community. For instance, wheat cultivars Penawawa and Lewjain favor [...]

Figure 2. (A) A scheme emphasizing radical processes in the bioremediation of PHC by a wheat root-microbe system. (B) Formation of reactive molecular species. Nitric oxide (NO) is formed in plants from NO$_3^-$ by cytosolic nitrate reductase (NR) or plasma membrane-bound NO reductase (NOR) and in bacteria by dissimilatory nitrogen reduction or by NO synthase. Superoxide (O$_2^-$) is formed as a consequence of plant and bacterial aerobic respiratory processes. Peroxynitrite (ONOO$^-$) is formed from NO and O$_2^-$ . Hydrogen peroxide (H$_2$O$_2$) generated by oxalate oxidase (OXO), found in wheat root epidermal cell walls, splits into OH$^-$ and *OH in the presence of a transition metal. CO$_2$ formed by OXO and by plant and bacterial catabolic processes combines with water to form a bicarbonate buffering system. Bicarbonate ion (HCO$_3^-$ ) can react with ONOO$^-$ or hydroxyl radical (OH$^-$) to form carbonate radical (CO$_3^{2-}$ ).
the growth of fluorescent pseudomonad populations that are antagonistic to the fungus *Rhizoctonia solani* (Mazzola et al., 2002; Mazzola and Gu, 2002). Metabolites released from antagonistic *Pseudomonas fluorescens* strains can induce *R. solani* phenoloxidase activity (Crowe and Olsson, 2001). The relationship between plant phenolics and phenoloxidase activities has been investigated largely in the context of plant–pathogen interactions without much attention to its potential influence on biodegradation.

**CONCLUSIONS AND PROSPECTS**

Much is still unknown about tolerances, degradative capacities and ecological interactions of organisms that have potential for use in bioremediation programs. It is clear, however, that plants and microbes act cooperatively to improve the rates of biodegradation and biostabilization of environmental contaminants. Although creation of genetically modified organisms has been proposed as a way to enhance bioremediation, we have yet to fully tap the potential of extant organisms.
Future research should examine the efficacy of structurally defined natural plant products in stimulating co-metabolic degradation of specific contaminants. Knowledge of the microbial community structure resident to the rhizosphere of plants that are resistant to a given contaminant will improve the chances of successfully increasing biodegradation rates when co-inoculating plants and microbes into contaminated environments. In designing a phytoremediation program the oxidative capacity of a plant should be considered in terms of its action on the contaminant itself and for its potential to support rhizospheric microbes with the capacity to enhance biodegradation. Additional basic biological and ecological information in these areas will allow us to make better informed decisions on how to widen bottlenecks in bioremediation processes.

REFERENCES


