

# **THE ROLE OF PLANT-BACTERIAL-FUNGAL INTERACTION IN REMEDIATION OF TERRESTRIAL OIL SPILLS**

Milan C. Vavrek, Wes Colgan III and William J. Campbell  
School of Biological Sciences  
Louisiana Tech University

## **ABSTRACT**

Facilitating interactions of plants, bacteria and fungi may accelerate oil degradation. These organisms potentially improve each other's performance and act on oil directly. Their contribution to degradation was examined using one hundred – 75 L microcosms in a greenhouse study. Microcosms (n = 10) consisted of sterilized forest soil, re-inoculated with native bacteria, fungi, or bacteria and fungi. Treatments also included 0 or 6 L m<sup>-2</sup> of crude oil and plants. After 32 weeks, interactions appeared to be antagonistic as reduction in total petroleum hydrocarbons ranged from 73.6% in microcosms with bacteria only (69.8% with fungi only) to 25.6% with plants and bacteria (0.18% with plants and fungi). Bacteria also reduced plant size and reproduction. Competition for limiting resources and an altered microbial community may account for these negative interactions. Thus, restoration may require monitoring of soil nutrients, repeated applications of fertilizer and consideration of interactions among biological components.

# INTRODUCTION

A terrestrial oil spill perturbs both the abiotic and biotic portions of the environment. Soils may become hydrophobic, exhibit altered albedo and possess high carbon to nitrogen ratios. Plant, animal, bacterial and fungal abundance and diversity are generally reduced. Further loss of biodiversity and ecosystem function may also occur as a result of disturbance of the complex interactions among the biotic components (1). Thus, bioremediation of residual petroleum occurs in an environment with an altered physical character and a reduced biological community. The rate at which the hydrocarbons degrade is a function of the interaction between the hydrocarbons, the altered soil physical properties, the remaining microbial and plant community, and the local climate (2).

The altered soil environment may limit the metabolism of oil by bacteria and fungi. Changed soil texture may limit biologically available oxygen and moisture. Sufficient nutrients, relative to the high carbon content, may also not be available (3, 4). Extreme ambient and soil temperatures and precipitation will also reduce the rate of oil degradation.

The indirect contribution of plants to oil degradation is also reduced by a decline in abundance of these species. Plants normally interact with bacteria by providing resources through sloughed cells, root exudates, and diffusion of oxygen through roots. Exudates and sloughed cells may comprise 7 to 27% of a plant's mass (5). An oxygen diffusion rate of  $0.5 \text{ mol O}_2 \text{ m}^{-2} \text{ soil surface day}^{-1}$  from roots has been recorded (6). Additional nitrogen may be provided by biological fixation (7, 8). Soybean, clover and alfalfa in conjunction with *Rhizobium*, for example, adds 50 to 200 kg of nitrogen per hectare to the rhizosphere each year (8). Greater reduction in residual petroleum in the presence of vegetation provides evidence for these positive interactions. Therefore, the indirect contribution of plants to oil degradation is potentially significant. The contaminated soil environment may also reduce plant performance, however. Reduced soil oxygen and moisture availability in addition to direct oil exposure may reduce plant survival, growth and reproduction.

Fungal species sensitivity to oil may also diminish fungal contribution to hydrocarbon degradation. In addition to direct degradation of oil, fungi may enhance oil degradation indirectly by improving plant performance (9, 10) and the environment for bacteria (11, 12). Mycorrhizal fungi augment plant uptake of water and nutrients increasing drought tolerance (13, 14) and plant growth as well.

Thus, rates of oil degradation are expected to be low as a result of perturbation of the normal interactions between bacteria, plants and fungi. Improving restoration via accelerated biodegradation may involve a complete understanding of these interactions and perhaps manipulating the biotic and abiotic components (and their interactions) of the environment. Unfortunately, most studies have examined only portions of the biodegradation process without considering the entire system (with the exception of reference 12).

This project has three objectives that address the biodegradation process as a whole by examining the importance of plant-bacteria-fungi interactions in oil bioremediation:

- 1) To quantify the contribution of plants, bacteria and fungi, singly and in combination, to reduction in residual petroleum hydrocarbons;

2) To test whether planting a suite of plant species enhances biodegradation of oil; and

A review of literature and an experiment (15) found that no single plant species provided all the limiting resources to soil microbes. Other researchers have also found that individual traits have not successfully predicted a plant's contribution to biodegradation. Aprill and Sims (16), and Anderson *et al.* (17) suggest that species such as alfalfa with large, dense, fibrous root systems contribute significantly to oil degradation. In contrast, Banks *et al.* (2) demonstrated that *Trifolium repens*, a species possessing a coarse root system, also contributed significantly to bioremediation. Therefore, we hypothesized that planting a suite of plant species possessing an array of traits would enhance plant-microbial interaction, accelerating biodegradation of oil.

3) To assess the tolerance to oil of ectomycorrhizal species from oak-hickory-pine soils.

Ectomycorrhizal species were specifically selected because the lack of these species has been shown to limit re-establishment of woody species in highly disturbed soils (9, 10). If exposure to oil significantly reduces the presence of ectomycorrhizae on woody plant species and an oil tolerant fungus species can be identified, they may be useful as an inoculant on seedlings before outplanting. Commercial fungal inoculum are already available for some species.

## METHODS

A greenhouse experiment using microcosms was used to assess the contribution of plants, bacteria and fungi to bioremediation of crude oil. One hundred - 75 L greenhouse containers were used to form the microcosms. Soil from an established oak-hickory-pine community (Louisiana Tech University Arboretum, Ruston, LA) was sterilized using a steam trailer by heating until the core temperature reached 84 °C. Soils were steamed twice (separated by at least 24 hours) to control resistant spores. Soils were then mixed with perlite (*ca.* 4:1) to reduce the soil compaction that typically occurs in containerized conditions (WC III, personal observation). Before soils were placed in the containers, 0.038 m<sup>3</sup> of Turface (chipped montmorillonite clay) was added to the bottom of the container to aid in drainage. The containers possessed no holes for drainage.

A series of treatments were applied to form microcosms with individual or combined biotic components of the environment (Table 1). To apply these treatments, the sterilized soil in a microcosm was inoculated by adding 1000 ml of a slurry consisting of freshly collected oak-hickory-pine soil (from the Louisiana Tech University Arboretum) in a 0.9% salt solution. The salt solution prevented osmotic shock of bacteria and fungi. For microcosms possessing only bacteria, a slurry was added that had been treated with a fungicide (Captan). Soils possessing fungi only received slurry treated with bactericide (a mix of penicillin G and oxytetracycline; 18, 19). After several days, soils were flushed with tap water to reduce salt concentrations before seedlings were transplanted into microcosms.

A North Louisiana crude oil (Calumet Lubricants Co., Princeton, LA) was then applied to the soil surface as appropriate at the rate of 6 L m<sup>-2</sup> (mean TPH, diesel range,  $\pm$  SE = 14931  $\pm$  756 mg kg<sup>-1</sup>). Microcosms were maintained for a week to allow volatilization of light oil fractions and to more realistically simulate an actual spill. An array of plant species likely to interact synergistically with oil-degrading bacteria and fungi were transplanted into the soils (Table 2). The particular species possessing C<sub>3</sub> or

C<sub>4</sub> photosynthetic pathways, small or large root systems, or biological nitrogen fixation or lack *Rhizobium* nodules were selected based on the results from previous research (15). Five seedlings of each herbaceous species were planted in each microcosm. In addition, seedlings of *Pinus taeda* (Loblolly pine), *P. echinata* (Shortleaf pine), *Quercus nigra* (Water oak), *Q. shumardii* (Shumard oak) were included in microcosms possessing mixed plant cultures. These species are common, dominant woody species of the oak-hickory-pine system and served as 'bait' species to assess ectomycorrhizal fungal colonization. Two seedlings of each woody species were planted with the exception of *Q. nigra* of which only one seedling was placed in each microcosm. Three weeks after transplanting, seedlings were replanted as needed. To examine whether a mixed culture of plant species may enhance microbial degradation of oil and revegetation of oil spill sites, microcosms with monocultures were also formed. These microcosms received fungi, bacteria and 25 seedlings of *Avena sativa* 'Bob'.

To control inadvertent introduction of microbes, seeds of woody species were surface sterilized in 30% H<sub>2</sub>O<sub>2</sub> for 5 – 20 minutes before sowing into sterile soil (Promix BX; Premier Brands, Inc.). Seeds of woody species were sown into SC-10 Super Cells (Stuewe and Sons, Inc.; 3.8 cm diameter x 21 cm depth) to allow development of taproots. Herbaceous seeds were sown in Promix in plug flats (TLC Polyform, Inc.; 288 square flat). Herbaceous roots were surface sterilized before transplanting by dipping roots into a 0.26% sodium hypochlorite solution.

Microcosms were placed in a greenhouse in a randomized complete block design and were supplied with tap water as required. After 36 weeks, residual petroleum was quantified as Total Petroleum Hydrocarbons (TPH), diesel range organics (EPA method 8015B by ANA-Lab Corp., Kilgore, TX). Soils for TPH analysis were collected with a 2.064 cm diameter Oakfield soil sampler. Two samples were collected per microcosm, each to a depth of 15.5 cm. Soils collected from each microcosm were thoroughly mixed before analysis. To positively correlate chemical analysis of oil reduction with biological activity, a seed germination test (bioassay) was also performed at the start and finish of the experiment. The bioassay consisted of 15 lettuce seeds (*Lactuca sativa*) and 15 oat (*Avena sativa* 'Bob') seeds sown on *ca.* 10 g of contaminated or uncontaminated soils in each petri dish (n = 10). Seeds were exposed to 24 hr fluorescent lighting ( $50.46 \pm 3.08 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) and moderate temperatures (25 – 27 °C).

Plant size was used to determine plant performance. Plant growth and size is generally correlated with survival and reproduction (20, 21). Plant size was estimated using height, stem diameter and height x stem diameter of woody species, total leaf area (CI-203 portable laser area meter; CID, Inc.), above- and belowground biomass (dry weight), root area and root length. Root size was measured because of the importance of the rhizosphere for microbial degradation of oil. Soil was washed from the roots before measuring.

Fungal performance was assessed by the percent of feeder roots of harvested oak and pine seedlings that were colonized by ectomycorrhizal fungi (22). For this assessment, a randomly selected sub-set of the *Quercus shumardii* and *Pinus taeda* and *P. echinata* seedlings were harvested 24 weeks after planting. The remainder of woody species was harvested at the completion of the experiment. All roots were washed and separated from the soils. Root systems of individual seedlings were evaluated at 10-40X under a dissecting microscope. Each mycorrhiza was described and assigned to a morphotype. Representative samples were removed for photography and extraction of DNA. DNA was extracted using a modified 2X CTAB/chloroform extraction protocol.

Samples were amplified using ITS-1f, ITS-4 Primers (23). Successful PCR amplicons were cleaned using Concert PCR Clean spin columns. Purified PCR amplicons were sequenced with ITS-4 as the primer on an ABI 377 automated sequencer at the Davis Sequencing, Inc., CA. DNA from the fungal symbiont was compared via BLAST to the genetic library maintained by the National Center for Biotechnology Information Nucleotide database (CGBI, GenBank) to identify the fungal partner.

Contribution of bacteria was quantified indirectly by reduction in residual oil. No direct measures of bacteria were performed as a large body of research already exists concerning the role of bacteria in oil degradation.

Thus, the experimental design allowed for a test of each effect (plants, bacteria or fungi) singly on oil degradation as well as all interactions among these biological components in an analysis of variance (JMP v4.0.2, SAS Institute, Inc.). In this analysis, microcosms without oil or mixed plant culture were excluded. Mahalanobis distances were used to exclude outliers. The effect of monoculture relative to mixed plant culture on plant performance and residual petroleum was analyzed separately with analyses of variance. The effects of plants and bacteria on fungal colonization were also analyzed separately. Plant performance analyses reported here are preliminary. Tukey's HSD was used in conjunction with one-way analyses of variance to identify differences between levels within a treatment. The block effect was not significant and is not presented. Mortality was examined with a  $-loglikelihood$  ratio test.

## Results

### Degradation of petroleum

The concentration of total petroleum hydrocarbons (diesel range) across all microcosms was reduced on average by 47.2% with a range from 0.2 to 73.6% reduction in 36 weeks under greenhouse conditions.

Plants had a negative effect on reduction in total petroleum hydrocarbons (diesel range) when present alone in microcosms and in the presence of fungi and bacteria (Table 3, Fig. 1). The greatest reduction occurred in microcosms without plants, but possessing bacteria (73.6%), fungi (69.8%) and bacteria with fungi (66.0%). The smallest reduction in TPH occurred in microcosms containing plants and bacteria (25.6%) and plants with fungi (0.2%). Microcosms with plants, bacteria and fungi yielded intermediate reductions in TPH (44%). The greater quantity of residual petroleum in microcosms possessing plants was not affected by whether plants were grown in monoculture or mixed culture (Fig. 2). Microcosms possessing plants and no oil exhibited TPH values near 0 (mean  $\pm$  SE =  $4.5 \pm 0.9$  mg kg<sup>-1</sup>) indicating that the quantities of TPH observed in microcosms with plants were not the result of the chemical analysis detecting plant-derived organic compounds.

### Plant performance

Exposure to oil-contaminated soils affected survival and performance of plants and fungi. After 36 weeks, mortality was greater in microcosms receiving oil relative to controls (15.6% and 5.6%, respectively;  $p < 0.0001$ ). The presence of an additional biotic component in oil-contaminated soils, however, reduced seedling mortality.

Seedling mortality in the presence of fungi (11%) or bacteria (10.9%) was less than in the absence of fungi (19.1%;  $p < 0.0001$ ) or bacteria (19.4%;  $p < 0.0001$ ). While mortality varied significantly among species from 2.8% in *Panicum* to 38.5% in *Amaranthus*, no oil x species interaction occurred ( $p = 0.72$ ).

Twenty-two weeks after exposure to oil, the presence of fungi and bacteria also tended to improve plant performance of woody species as indicated by height x stem diameter ( $p = 0.06$ ; Fig. 3). Fungi did not contribute equally to performance of herbaceous species ( $p = 0.80$ ). Bacteria, however, significantly decreased plant growth in terms of height ( $p = 0.03$ ). In both cases responses to fungi and bacteria tended to be species specific ( $p = 0.09$  and  $0.07$ , respectively). Herbaceous species responded differentially to bacteria as well ( $p = 0.0006$ ; Fig. 4).

Growth in oil-contaminated soils reduced height across all plant species ( $p < 0.0001$ ) and reductions in height were species specific ( $p < 0.0001$ ; Fig. D). *Amaranthus* was particularly sensitive to the presence of oil while *Quercus nigra*, *Panicum*, and *Aeschynomene* appeared to be tolerant to the effects of oil. Lastly, exposure to oil reduced the number of plants to flower or fruit by 50% ( $p < 0.0001$ ). The presence of bacteria and fungi also reduced the number of plants with inflorescences or fruits (35.4%;  $p < 0.0001$ ).

The negative effect of bacteria on plant performance in oil-contaminated soils became more pronounced at the final harvest. Preliminary analysis indicated that shoot weight ( $p = 0.0004$ ), fruit weight ( $p < 0.0001$ ) and root area ( $p = 0.0386$ ) declined in the presence of bacteria (Fig. 6). Shoot area was the lowest for plants grown in the presence of bacteria and fungi ( $p = 0.0298$ ). Plant performance was also altered as a function of culture type. Analysis of oat performance indicated that oat shoot weight ( $p < 0.0001$ ), shoot area ( $p < 0.0001$ ), fruit weight ( $p < 0.0001$ ), root weight ( $p < 0.0001$ ), root area ( $p < 0.0001$ ) and root length ( $p = 0.0005$ ) were lower when grown in monoculture relative to mixed plant culture (Fig. H).

## Fungal performance

Analysis of fungal performance after 20 weeks, indicated that feeder roots of woody species in microcosms that contained sterile soil exhibited little colonization by ectomycorrhizal fungi relative to plants in soils with all biotic components present ( $p = 0.04$  for *Pinus taeda* and  $0.27$  for *P. echinata*; Fig. 8). Most seedlings across all treatments were non-mycorrhizal (47 of 80 seedlings). Relative mycorrhizal abundance ranged from 0 to 50% of feed roots. As a result of large variances in controls, this difference disappeared by week 32 (Fig. 8). Large variances commonly occur in research involving ectomycorrhizal fungi (24). Ectomycorrhizal fungal abundance, however, remained low in sterile soils. Thus, fungal contamination via the environment was limited. Percent of feeder roots colonized tended to be lower initially in oil-contaminated soils relative to controls, particularly on *P. taeda* grown with fungi ( $p = 0.10$ ), bacteria ( $p = 0.06$ ) and fungi and bacteria ( $p = 0.08$ ; Fig. 8). Within 32 weeks, fungal colonization was equivalent to controls in these treatments (Fig. 8). Due to extensive root damage (epicormic lesions in tap roots and the appearance of phosphate deficiency), oak data was excluded. The only fungus on the oaks identified by sequencing is closely related to *Armellaria* (Basidiomycete, Tricolomataceae), a mild to severe pathogen of woody plants. Two mycorrhizal morphotypes predominated on *Pinus* roots. Type 1, the most frequently encountered, was preliminarily identified as an *Alurea* or *Otedia* (Ascomycota, Discomycetales). These are “cup” fungi expected to form ectendomycorrhizae. Type 2,

the other dominant morphotype, possessed 97% sequence homology with *Wilcoxina*. This fungus forms ectendomycorrhizal interaction with many conifers.

## Bioassay

Initial oil concentrations tended to reduce germination of *Lactuca* seeds after 10 days of exposure (by 45.4%;  $p = 0.09$ ). Germination of *Avena* seeds was not affected. The toxicity of oil declined by the end of the experiment when no difference in germination of *Lactuca* seeds between treatments was detected ( $p = 0.27$ ).

The results of the bioassay at harvest support the chemical analysis of residual petroleum (Table 4). Oil-contaminated soils that had possessed plants, reduced germination of *Lactuca* seeds by 42.4% and was unaffected by bacteria or fungi ( $p < 0.05$ ). Germination was not affected by whether plants had been grown in monoculture or mixed culture ( $p > 0.05$ ). These bioassay results differed from the initial bioassay performed within 2 weeks of the experiment's start. At that time, germination of lettuce was improved by fungi (293%;  $p = 0.004$ ), bacteria (210%;  $p = 0.03$ ), and fungi and bacteria (350%;  $p = 0.002$ ).

## DISCUSSION

The lack of basic research on bioremediation currently hinders its effective application to restoration of oil spill sites. In particular, research has tended to examine the effects that components of the biotic environment have on remediation rather than complete systems. Yet, in order to accelerate and more completely remediate terrestrial oil spills, knowledge of interactions between the biological components in the environment is required. This project specifically researches the role of plants, bacteria and fungi singly and together in oil bioremediation of an oak-hickory-pine community. These biological components are directly or indirectly responsible for degradation of oil in soils.

The direct contact between plants, bacteria and fungi in the rhizosphere through evolutionary time suggests that positive interactions should occur. The positive interactions may, in turn, enhance bioremediation of a soil contaminant. Results of previous studies support this notion. Lee and Banks (25), Schwab and Banks (26) and Qiu *et al.* (27) demonstrated that spilled oil degrades more rapidly on vegetated soils than on soils lacking vegetation. Plants may enhance bioremediation of oil by providing limiting resources to bacteria and by improving soils. Fungi also may support plant and bacterial performance. Mycorrhizae of Scots pine, for example, supported a microbial biofilm in oil-contaminated soil (11). Vesicular-arbuscular fungi and *Frankia* improved survivorship and growth of *Elaeagnus commutata* and *Shepherdia canadensis* growing in oil sand tailings (28).

The results of this study suggest, however, that interactions among plants, bacteria and fungi in oil-contaminated soils may also be antagonistic. Although the bacteria native to oak-hickory-pine forests were able to reduce total petroleum hydrocarbons (diesel range) by 74% in 32 weeks, the addition of plants or plants and fungi decreased the quantity of petroleum that was degraded. Plant performance was also reduced in the presence of bacteria. One explanation for the reduction in degradation and performance is that plants, bacteria and fungi competed for limiting resources. Resources taken up by plants or non-oil degrading microbial species could, reduce performance

(metabolism and population growth) of oil degrading bacteria and fungi. Hodge *et al.*, (29) demonstrated that N capture was related to the competitive ability of plants and soil microbes. Finn (30) suggested that optimum bioremediation on oil occurs at C:N:P ratios of 100:20:10. Therefore, an alteration in these ratios by plant uptake may reduce the amount of oil degraded. In addition, the Sacul soils of the Louisiana Tech University Arboretum are low in phosphorus, potassium, calcium and magnesium (31) intensifying competition. Subsampled soils after completion of the experiment indicated that 15 of the 26 samples contained no detectable nitrate. Of the remainder, residual TPH tended to be lower in those soils with greater nitrate (mean = 1.2 mg kg<sup>-1</sup>; P = 0.06; R<sup>2</sup> = 0.36). The soil analysis also indicated that phosphorus was low (mean = 90.1 mg kg<sup>-1</sup>), which was further supported by symptoms of phosphorus deficiency in the oak seedlings. Insufficient concentration of these elements may have a limited degradation of oil.

In addition to, or in combination with, competition as an explanation for reduced degradation of oil in the presence of plants, oil may have altered community composition and relative abundances of fungi and bacteria. These changes will disturb normal interactions between biotic components in the soil. Pfender *et al.* (32) suggested that altered species composition of bacteria and fungi reduced *Rhizobium* performance in pentachlorophenol-contaminated soil (detected as fewer large nodules). The plant and vesicular-arbuscular mycorrhizal fungi relationship in that study was also affected. Further, the microbial community may exhibit selectivity in substrate use, preferentially selecting root exudates as a carbon source rather than hydrocarbons. A final hypothesis to explain the reduction in oil degradation with plants may be that intermediate compounds were formed during the biodegradation oil. Intermediate compounds may have formed as a result of degradation in the presence of plant exudates and cometabolism. These intermediate compounds were not toxic as indicated by the bioassay, but may have slowed reduction in total petroleum compounds. Siciliano and Germida (33) also observed a reduction in toxicity of 2,3-dichlorobenzoic acid and 3-CBA following phytoremediation with no reduction in contaminant concentration. Analysis of microbial community composition and GC/MS analysis of hydrocarbons will be required in future research to eliminate these alternate hypotheses.

Regardless of the underlying mechanism, the altered interactions among the biotic components may have consequences for restoration of oil spill sites. Revegetation required in most cases for complete restoration may retard biodegradation of oil particularly if resources such as soil nutrients become limiting or microbes selectively choose plant exudates. Lin and Mendelsohn (34) documented enhanced plant performance and degradation of residual petroleum with the addition of fertilizer. Recovery plans must weigh the benefits and costs of beginning revegetation immediately or delaying planting until TPH levels have been reduced satisfactorily. In addition, restoration must be a continuing process requiring monitoring of the soil environment and plant performance, and perhaps repeated applications of fertilizer.

Using a suite of plant species possessing C<sub>3</sub> and C<sub>4</sub> photosynthetic pathways, large root systems, symbiosis with nitrogen-fixing bacteria, and tolerance to oil did not enhance degradation of hydrocarbons relative to the use of monocultures. Application of fertilizer may have yielded a different result. Plant performance, however, was improved by growing plants in mixed culture indicating revegetation of spill sites may benefit by planting a mix of species. The better performance of plants in mixed culture is most likely the result of reduced competition. Intra-specific competition tends to be stronger than inter-specific competition because of less niche separation. Further, greater

diversity reduces plant community sensitivity to environmental stress (35) and is more likely to support ecosystem function.

This project specifically addressed oil contamination of oak-hickory-pine communities. Temperate deciduous forests occupy *ca.* 30% of the area of Louisiana. Oak-hickory-pine communities occupy approximately 57% of the forested area (36). These forests are at risk of experiencing an oil spill as oil and gas wells are distributed across the state. Re-establishing woody vegetation in these communities after a spill is essential to complete remediation after a spill and may rely on ectomycorrhizal fungi. Ectomycorrhizal fungi persisting in oil-contaminated soil were detected and are being identified. Preliminary analysis suggests that these fungi are ectendomycorrhizal; little is known about their ecological role in nature. Their role in degradation of oil and plant performance is also not clear under the experimental conditions. The large quantity of residual oil in microcosms with plants and fungi may suggest, however, that plants were able to take up greater quantities of nutrients with the assistance of fungi, intensifying competition between plants and bacteria. The lack of mycorrhizal colonization on *Quercus* in addition to the low percent colonization in the first 20 weeks of the experiment strongly suggest that inoculation prior to out-planting may be required for successful establishment of woody species in the field. While identification of fungal species will apply specifically to oak-hickory-pine forests in northern Louisiana, the knowledge of the importance of the role of fungi, and the role of the interaction between plants, bacteria and fungi is applicable to all spill sites.

The conclusion from this project is that understanding the interactions among the biotic components at a spill site is essential for enhanced restoration, particularly as these interactions may be negative. Additional research is required including: quantifying soil fertility and adding fertilizer as a treatment; repeating these treatments in the field; and analyzing intermediate compounds with GC mass spectrometry. Ultimately, monitoring and perhaps amending the soil environment may be necessary to ensure effective restoration.

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**Table 1.** Treatments used to examine role of biotic community in remediation of oil in oak-hickory-pine communities (n=10).

Plant community	Soil bacteria	Soil fungi	Oil
Mixed culture	Yes	Yes	No
Mixed culture	Yes	Yes	Yes
Mixed culture	No	Yes	Yes
Mixed culture	Yes	No	Yes
Mixed culture	No	No	Yes
Monoculture	Yes	Yes	Yes
No	Yes	Yes	Yes
No	No	Yes	Yes
No	Yes	No	Yes
No	No	No	Yes

**Table 2.** Seedlings transplanted into microcosms to test the contribution of plants, bacteria, and fungi in bioremediation of crude oil.

Plant species	Family	Trait
<i>Avena sativa</i> 'Bob' (Oat)	Poaceae	C <sub>3</sub> ; shallow dense roots
<i>Lolium perenne</i> (Ryegrass)	Poaceae	C <sub>3</sub> ; shallow dense roots
<i>Panicum virgatum</i> (Switchgrass)	Poaceae	C <sub>4</sub> ; shallow dense roots
<i>Amaranthus retroflexus</i> (Redroot)	Amaranthaceae	C <sub>4</sub> ; deep roots
<i>Aeschynomene Americana</i> (Joint vetch)	Fabaceae	C <sub>4</sub> ; biological nitrogen fixation

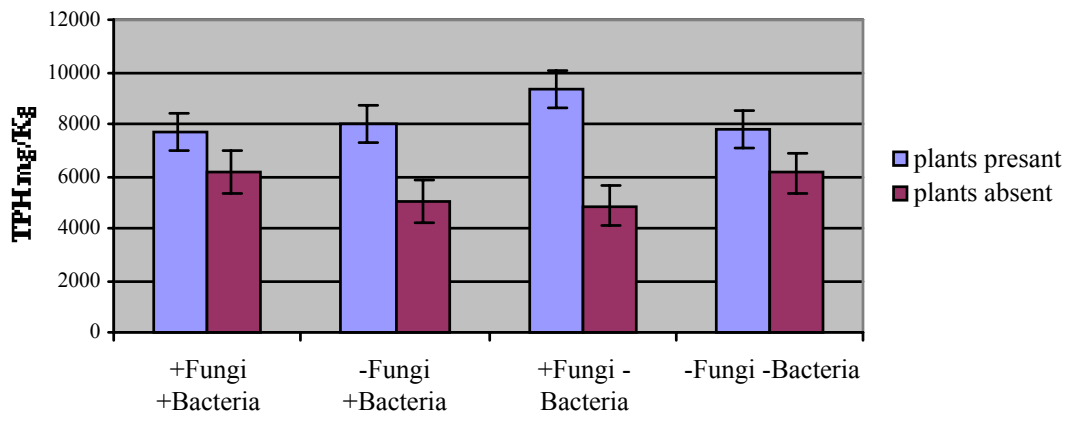
**Table 3.** Results of analysis of variance examining the effects of the biotic community on residual total petroleum hydrocarbons in a greenhouse experiment.

Effect	df	Sum of Squares	F	P
Plants	1	129820447	27.93	<0.0001
Fungi	1	1268374	0.27	0.6032
Bacteria	1	1399060	0.30	0.5851
Plants x fungi	1	2041856	0.44	0.5098
Plants x bacteria	1	2651230	0.57	0.4528
Fungi x bacteria	1	230681	0.05	0.8255
Plants x fungi x bacteria	1	20893846	4.49	0.0378
Error	66	306808540		

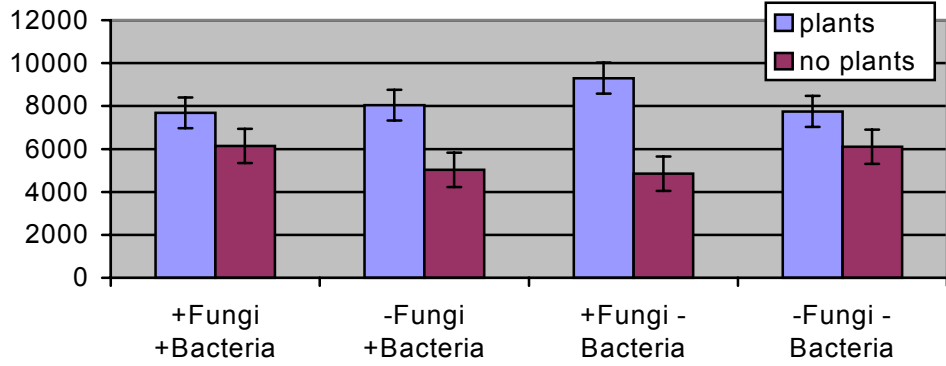
**Table 4.** Analysis of variance table indicating the effects of biotic components present during remediation on oil-contaminated soils on germination of *Lactuca* seeds.

Effect	df	Sum of Squares	F	P
Plants	1	43.51	7.291	0.0086
Fungi	1	0.01	0.002	0.9636
Bacteria	1	12.01	2.013	0.1603
Plants x fungi	1	2.11	0.354	0.5537
Plants x bacteria	1	5.51	0.924	0.3397
Fungi x bacteria	1	0.11	0.019	0.8912
Plants x fungi x bacteria	1	10.51	1.761	0.1886
Error	72	429.70		

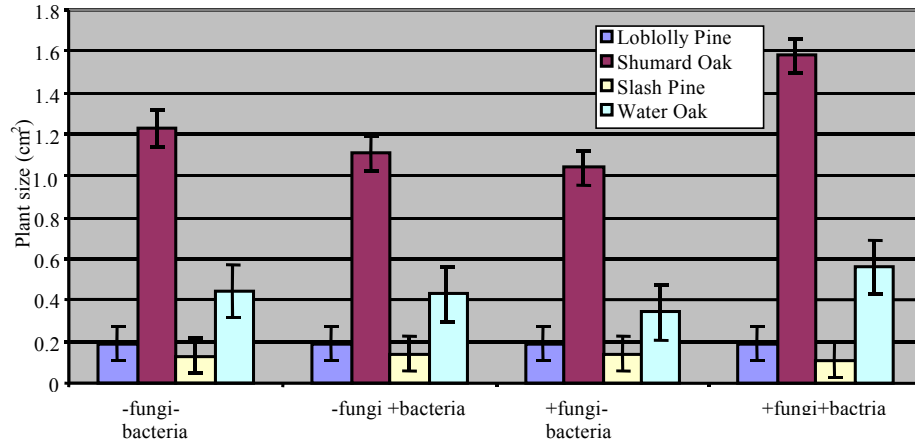
**Figure 1.** Total petroleum hydrocarbon present in microcosm soils after 36 weeks in



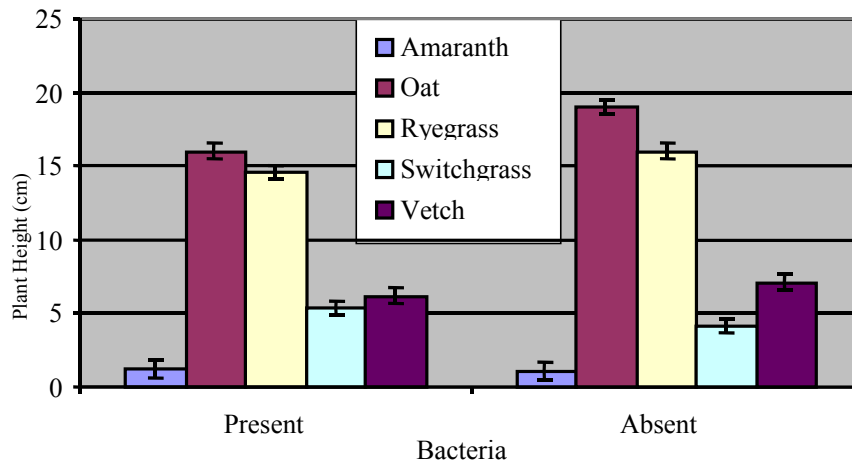
greenhouse conditions. Bars equal  $\pm$  standard error of the mean.



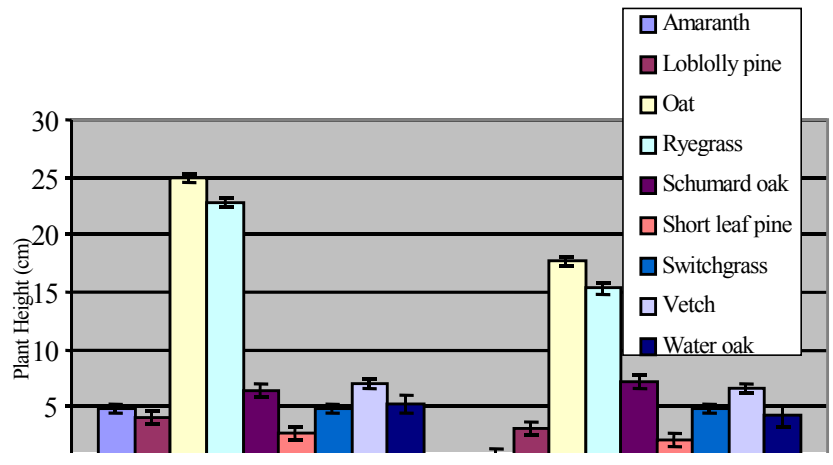
**Figure 2.** Total petroleum hydrocarbon present in soils after 36 weeks in the presence of plants, and combinations of bacteria and fungi. Bars equal  $\pm$  standard error of the mean.



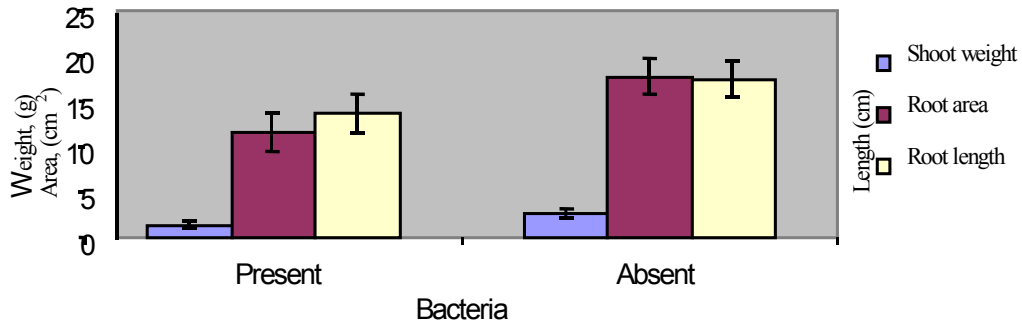
**Figure 3.** Effect of soil community on the size (height x stem diameter) of woody plants in microcosms with oil. Bars equal  $\pm$  standard error of the mean.



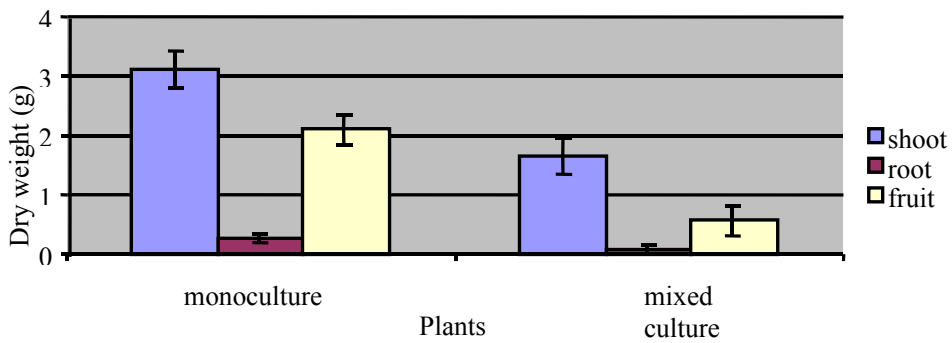
**Figure 4.** Response of herbaceous plant height to presence and absence of bacteria in microcosms treated with oil. Bars equal  $\pm$  standard error of the mean.



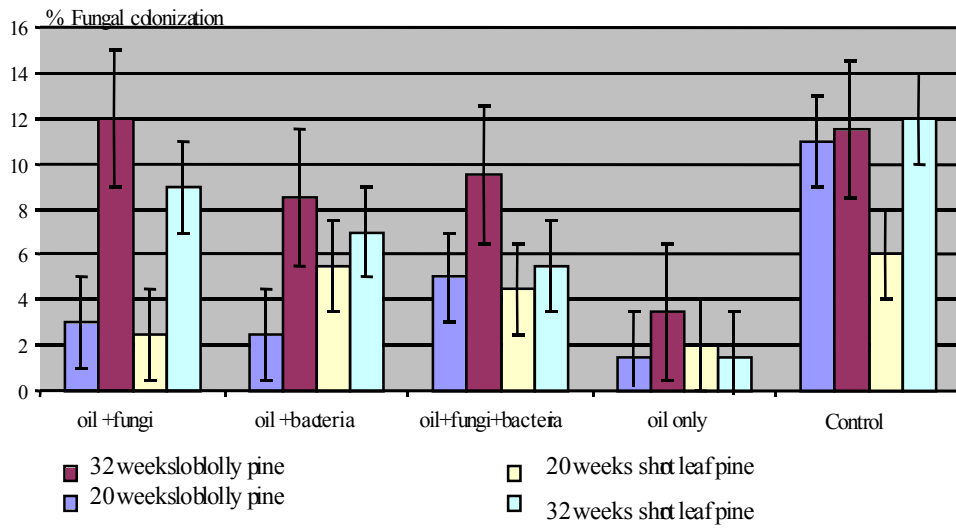
**Figure 5.** Effect of crude oil on plant height in microcosms after 22 weeks in greenhouse conditions. Bars equal  $\pm$  standard error of the mean.



**Figure 6.** Indicators of plant performance in the presence and absence of bacteria in microcosms contaminated with oil: shoot weight (g), root area (cm<sup>2</sup>), root length (cm). Bars equal  $\pm$  standard error of the mean.



**Figure 7.** Plant performance indicators for oat plants grown in monoculture and mixed culture, shoot, root, and fruit weight. Bars equal  $\pm$  standard error of the mean.



**Figure 8.** Effect of oil, bacteria and fungal inoculation on percentage of feeder roots colonized by mycorrhizal fungi. Bars equal  $\pm$  standard error of the mean.