MYCOREMEDIATION OF AGED PETROLEUM HYDROCARBON CONTAMINANTS IN SOIL

WA-RD 464.1

Research Report
November 1998

Washington State Department of Transportation

Washington State Transportation Commission
Planning and Programming Service Center
in cooperation with the U.S. Department of Transportation
Federal Highway Administration

PNNL-12056

FINAL REPORT

Mycoremediation of Aged Petroleum Hydrocarbon Contaminants in Soil

S. Thomas
P. Becker
M.R. Pinza
J.Q. Word

November 1998

Prepared for the
Washington State Department of Transportation
Olympia, Washington 98504

Battelle Marine Sciences Laboratory
Pacific Northwest National Laboratory
Sequim, Washington 98382

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the U.S. Government. Neither the U.S. Government nor any agency thereof, nor Battelle Memorial Institute, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the U.S. Government or any agency thereof, or Battelle Memorial Institute. The views and opinions of authors expressed herein do not necessarily state or reflect those of the U.S. Government or any agency thereof.

PACIFIC NORTHWEST NATIONAL LABORATORY
operated by
BATTELLE
for the
UNITED STATES DEPARTMENT OF ENERGY
under Contract DE-AC06-76RLO 1830

Printed in the United States of America

This document was printed on recycled paper

Acknowledgements

The authors appreciate the cooperation and funding from the Washington State Department of Transportation (WSDOT) for the field-scale testing that was the main part of this study. In particular, we thank Jim Schafer for his continued interest and support; Doug Pierce for providing the study site, test soils, and comparative bacterial and bioremediation treatments; Siv Balachandran for his assistance in project organization and in the field, and Brian Bernot and other WSDOT field staff who participated on site. We would also like to thank Paul Stamets *(Fungi Perfecti)*, who teamed with Battelle to identify and provide tissue-cultured inoculant of appropriate fungal species and to develop a bulk method of spawn production. Finally, we would like to acknowledge the help of Battelle staff: Paul Farley of the Marine Sciences Laboratory (MSL) in the field; and Ron Thom, Lyle Hibler, and Rob Cuello at the MSL and Alan Uhler at the Battelle Ocean Sciences Laboratory for their review of data and text.

Contents

Introduction 1
Background 1
Experimental Design and Implementation 2
Results. 5
Discussion 9
References 11
Appendices
A. Field Remediation Experimental Design and Workplan 18
B. WSDOT Protocol for Bioremediation 26
C. PSCI Enhanced Bacteria Experimental Design 28
D. Mesocosm and Toxicity Experimental Design 30
E. Field Remediation Results 34
F. Mesocosm and Toxicity Results 40

Figures

1. Experiment schematic showing 12 test mounds and approximate spacing to minimize cross-contamination on relatively flat test site 13
2. Setup: a) mixing source soil; b) washing bucket to avoid cross-contamination 14
3. Diesel-contaminated example: a) Time 0 control, and b) Time 0 inoculated mycoremediation 14
4. Time 0 bacterial (foreground), mycoremediation (65% green shadecloth), and control (background, with black polyfilm) mounds and their covering 15
5. Time 0: a) WSDOT bioremediation treatment, application of nitrogen fertilizer; b) PSCI application of liquid fertilizer and bacterial inoculum 15
6. Week 5: a) fruiting on mycoremediated diesel-contaminated soil; b) detail of fruit 15
7. Week 5: detail of mycelial treatment of diesel-contaminated soil 16
8. Week 17 community development: a) vascular plants on mycoremediated truck-bay soil; b) secondary decomposer fungi and vascular plants on mycoremediated gasoline-contaminated soil.. 16
9. Week 9: enhanced bacteria treatment, visible petroleum hydrocarbon pockets still present 16
10. Week 17 comparison of controls and all treatments of gasoline-contaminated soil 17

Tables

1. Chemical analysis of truck bay soils 6
2. Chemical analysis of diesel-contaminated soils 6
3. Chemical analysis of gasoline-contaminated soils 7

Mycoremediation of Aged Petroleum Hydrocarbon Contaminants in Soil

S.A. Thomas, P. Becker, M.R. Pinza, and J.Q. Word

Introduction

There are several treatments available for the remediation of petroleum hydrocarbon-contaminated soil. Some use chemical or mechanical methods, others make use of incineration, and still others use biological materials, either native or introduced, to remove or degrade the contaminants. Battelle Marine Sciences Laboratory (MSL) and the Washington State Department of Transportation (WSDOT) conducted a 4-month experiment to compare the efficacy of three different biological approaches—mycoremediation, bioremediation, and enhanced bacterial remediation—used under open environmental exposure to treat three excavated, aged-oil-contaminated soils stored at the WSDOT Maintenance Yard, Bellingham, Washington. The MSL designed the experiment in collaboration with WSDOT, and the project was jointly funded.

At the end of the experiment, the results were not conclusive in distinguishing the outcome of the various treatments; none appeared to meet the prescribed criterion for success, namely attainment of the Method A Cleanup Level of total petroleum hydrocarbons (TPH) prescribed by the Washington State Department of Ecology (WSDOE) in Washington Administrative Code (WAC) 173-340 (WAC 1996) and Ecology Publication No. ECY-97-600 (WSDOE 1997) during the allotted time period. However, much useful information was gained by the exercise, and progress was made in understanding the variables that challenge the transition from mesocosm to large-scale deployment of remediation biotechnology. This study also showed that toxicity testing of treated and control soils using native plants and invertebrates can offer valuable information on the suitability of treated substrate for beneficial uses.

The inconclusive nature of the chemical results is largely attributable to the unexpected heterogeneity of the test soils; that is, there was extremely patchy distribution of contaminant within test mounds, and extreme variability of initial contaminant level among test mounds within each soil type. Consequently, the sampling schedule for this experiment, which was designed in advance of the Time 0 sampling that revealed the variability, likely was not sufficiently intensive to determine first, the precise nature of the original contamination, and second, the degree to which it was remediated by treatment, and consequently, the relative effectiveness of the three treatments. Another important factor was that the petroleum hydrocarbons in the soils under study were found to be very weathered; oils in this condition could require a longer time period for remediation than that required for fresh, unweathered oils.

Background

The MSL biotechnology group in collaboration with Paul Stamets of Fungi Perfecti is studying *mycoremediation* of petroleum hydrocarbons and other contaminants in soil and other substrata (Pinza et al. 1998; Word et al. 1998; Becker et al. in press; Thomas et al. in press). Mycoremediation employs selected, cultured fungal mycelia to remove/degrade environmental contaminants. In laboratory and mesocosm-scale experiments, it has been demonstrated to operate on a time-scale of weeks to months, and it is particularly effective in addressing the recalcitrant and toxic, higher-molecular-weight aromatic components of fresh petroleum products (the ≥4-ring polynuclear aromatic hydrocarbons [PAHs]) that are less readily degraded by microbial systems. The MSL's application of a selected, cultured mycelial system in combination with native microbiota achieved 97% removal of PAHs from 2% Bunker C/diesel oil-

contaminated soil over an 8-week period in a laboratory bench-scale study (Pinza et al. 1998). In a followup, outdoor mesocosm study, an applied mycelial system accomplished 93.5% removal of PAHs from 2% Bunker C/diesel oil-contaminated soil over a 12-week period (Pinza et al. 1998). The system requires no maintenance after initial setup. In contrast, bioremediation, which involves the *in situ* stimulation of natural microbial systems by periodic fertilization and tilling, and bacterial remediation, which has similarly high maintenance requirements, typically require from 1 to 3 years and generally do not address the ≥4-ring PAHs.

A successful remediation of soil that is removed from its original location for remediation could be broadly applicable for federal, state, county, city, and private sites nationwide from which oil-contaminated soils or vactor wastes from catch-basins must be removed and treated. It was anticipated that mycoremediation would reduce the treatment time from the years required for bioremediation to months at equivalent or lower cost. Added benefit of mycoremediation could be the specific removal of higher-molecular-weight PAHs and the resulting production of a composted material useful for landscaping or other purposes.

Experimental Design and Implementation

Field Remediation Experiments

The overall experimental design and work plan (Appendix A) were prepared by the MSL and submitted for approval to WSDOT prior to the starting date. The study was a pilot-scale remediation of three aged oil-contaminated soils that were excavated or scraped from contaminated sites and subsequently stored at the WSDOT maintenance yard at Bellingham. The experiment compared the effectiveness of the MSL's mycoremediation approach with that of bioremediation and enhanced bacterial remediation of a 10-cubic-yard mound of each of the following test soils: a) the floor of a vehicle maintenance building located on the Bellingham Maintenance Yard (truck bay) that had operated for 30-40 years; b) a diesel-contaminated soil; and c) a soil described as gasoline-contaminated. On the basis of Time 0 chemical analysis, it was found that the "gasoline-contaminated" soil was actually high in content of diesel and heavy oil, rather than of gasoline.

Prior to and independent of negotiations for the present project, WSDOT selected the test soils and carried out initial contaminant characterization to determine whether or not remediation would be required. Documentation of this process is available from WSDOT.

The experiment took place in winter/early spring conditions, starting 2 March 1998, for a 4-month duration. Two primary concerns were addressed in the MSL's work plan: homogeneity and cross contamination. Specific instructions for the construction of the test mounds were included in the work plan to attempt to compensate for the expected lack of homogeneity of the soils in the maintenance yard. In the field, the instructions were carried out in the most practical manner possible: the truck-bay soil was excavated, mixed thoroughly by use of WSDOT's front-loader, then distributed equally to form the required four 10-cu yd mounds. A similar method was followed for the remaining two stockpiled soils: that is, a 40-cu-yd subset of each source soil was established and mixed by means of the WSDOT front-loader, and then subdivided to create four 10-cu-yd mounds of each soil type.

Specific instructions were also given in the MSL's work plan to prevent cross-contamination of the individual test mounds. Isolation of the mounds from the ground was ensured by the use of 6-mil polyetheylene underlayment. All but the bioremediated mounds were also covered by either polyfilm or shadecloth tarps, which were enfolded at the edges with the underlayment and secured with sand bags to prevent the escape of water. The test mounds were arranged at the

Bellingham maintenance yard such that the distance between each pair was no less than 20 ft; the two sets of bacterial treatments were separated completely from the mycelial treatments and controls by a distance of 40 ft (Figure 1). Although all of the bacterial and bioremediation mounds (Numbers 3, 4, 7, 8, 11, and 12, Figure 1) were moved at Week 12 due to conflicting use requirements at the Bellingham maintenance yard, the relative spacing of mounds was preserved. WSDOT's heavy equipment operators were designated to move and mix soils for initial setup of the experiment and as necessary for maintenance. The front-loader added loads of soil to the mounds without making contact with soil or inoculation materials already deposited, to avoid carrying such materials from one pile to another. When it was used for mixing the existing mounds, the bucket was carefully washed with water and brush prior to pickup of additional soils (Figure 2).

A control mound of each soil was left untreated (Figure 3a). For the mycoremediation treatment, fungal mycelium was transferred to the mounds using alder sawdust fully grown out with mycelium at an inoculation rate of 25% to 30% by volume (Figure 3b). The inoculum was layered with the test soil. Both mycoremediated and control mounds were set on polyethylene underlayment; control mounds were also covered by polyethylene tarps, and mycoremediated mounds were covered by 65% green shadecloth (Figure 4). After the initial application and documentation of moisture content, there was no further maintenance required for the mycoremediation treatments or the controls, although for the experimental purposes, periodic sampling and observation were conducted.

WSDOT established the bioremediation regime, applying 12 lb nitrogen fertilizer (30-0-0) per 50 cu yd soil contaminated at the level of 2000 mg/kg TPH (Appendix B). The mounds were set onto polyethylene tarps and were left uncovered to encourage maximum volatilization. Maintenance of bioremediated mounds consisted of monthly turning and addition of fertilizer. WSDOT contracted with PSCI Tank Services to conduct the enhanced bacterial treatment, which required initial and biweekly or monthly application of liquid fertilizer and bacterial inoculum, and biweekly or monthly turning of the soil (Appendix C). These test mounds were set onto and covered by polyethylene tarps (Figure 5). PSCI ceased applying its treatments just prior to Week 9 of the test due to closure of the company that supplied the proprietary bacteria. The three test mounds for the enhanced bacterial remediation trials were turned and mixed according to the maintenance schedule for the WSDOT test mounds for the remainder of the study period, but without further addition of bacteria or fertilizer.

WSDOT supplied access to the Bellingham maintenance yard for monitoring, maintenance, and sampling, as necessary. The assumption was that if the appropriate steps were taken to ensure relative homogeneity of the test mounds, as indicated by the experimental design and work plan, a standard, stratified random sampling approach would be satisfactory (Huesemann 1994). All test and control mounds were sampled by the MSL at 0, 9, and 17 weeks. The original schedule called for sampling on Weeks 0, 8, and 16, but the latter two sessions, along with the Week 4 observation and photodocumentation, were each postponed by one week due to WSDOT's schedule for the earth-moving equipment operation at Bellingham. Three random samples were taken from each test and control mound, each of which was about 4 ft in height, at every sampling period—one from the upper third, one from middle third, and one from bottom third—and sealed in chemically clean containers for transport (~4°C) and analysis by Analytical Resources Inc. (Seattle, Washington). Each set of samples was analyzed by the Northwest TPH-Diesel Extended (NWTPH-DX) method, which was selected by WSDOT to meet WSDOE requirements.

In addition to the sampling specified in the original experimental design, a second set of samples was taken at Week 17, using a more intensive sampling and sample-preparation method

(Huesemann 1994). From each of the 12 control/treatment mounds, multiple, stratified subsamples were taken from each of four quadrants for a total of 5 gallons of soil. The 5 gallons of soil from each mound was hand grated to a fine consistency and hand mixed in precleaned stainless-steel bowls. The composited and mixed soil was again subsampled, and the subsamples were composited to a total of 200 g to represent each mound. Battelle chemists at the MSL and at Battelle Ocean Sciences (BOS) at Duxbury, Massachusetts, analyzed these Week 16 subsamples for PAHs and alkanes by an alternative method. Once each month, the Bellingham site was visited for observation; fungal/bacterial growth, fungal fruiting, vascular plant and secondary fungal or other growth on any of the test or control mounds were recorded.

Mesocosm and Toxicity Experiments

Supplementary experiments were conducted offsite at the MSL (Appendix D). In a *mesocosm study* at the MSL, a sample of each of the three oil-contaminated test soils from the WSDOT Bellingham maintenance yard was used for a smaller-scale treatment. Two 10-kg samples of each contaminated soil were prepared, and each placed in an environmental chamber. One of each pair was inoculated with fungal mycelium carried on alder wood chips, and one was left untreated as a control. They were covered with hardware cloth for mild shading, and placed in an outdoor study site for 4 months, following the schedule of the Bellingham yard experiments. Natural precipitation determined the amount of water received. Samples were taken for chemical analysis at Weeks 0, 5, 9, and 17 (in parallel to the schedule for the field experiment at Bellingham) and all growth, fungal fruiting, and vascular plant and secondary-decomposer fungal growth were recorded. Analysis of Week 0 and Week 5 samples used the NW-TPH-DX method (ARI, Inc.); Week 9 samples were not analyzed due to limitation of funds, but the samples were archived. Selected Week 17 samples were analyzed for PAHs and alkanes by an alternative method at the BOS laboratory.

Toxicity tests of the control and remediated soils were conducted at the MSL (Appendix D). A 14-day toxicity test was conducted using the worm, *Eisenia foetida andrei.* In this test, the toxicity of one set of soils, the gasoline-contaminated soils (Treatments 9 through 12, Figure 1) was assessed after remediation. The experimental design followed ASTM E1676-95 guidelines (ASTM 1994, 1995). A summary of testing requirements is shown in Table D.1, Appendix D. Three testing replicates were prepared for each soil treatment. In addition, three treatments (artificial soil, potting soil, and peat moss) were prepared. Prior to testing, soils were hydrated to 45% moisture using deionized water; test organisms were depurated for 24 hours in the dark, and 280 g of test sediment was placed into each test chamber (500 mL glass jars with screens and Teflon lids). After 24 hours, the test organisms were weighed, placed into the test chambers, and transferred to the environmental chambers maintained at the proper test conditions. During testing observations of the worms and the test conditions were noted. At termination, the test organisms were removed from the container and counted as live or dead. In addition, sublethal responses, such as segment swelling, coiling, lesions, or rigidity were noted. After data collection, the organisms were depurated for 24 hours, patted dry, and weighed.

The purpose of plant toxicity tests was to compare plant growth and/or toxicity of soils to plants in the control and three treated samples for each of the three test soils (Appendix D). Standardized replicates of three species of Washington native plant, *Festuca idahonensis* (fescue grass), *Physocarpus capitatus* (Pacific ninebark), *Sambucus cerulius* (blue elderberry), selected from a list of WSDOT highway landscaping species and purchased from Shore Road Native Plant Nursery, Sequim, Washington, were planted in each soil type; an independent set of controls was established in sterilized potting soil. Measurements were taken of the leaves, stems, and roots of the plants at the start and termination of the experiment; the experiment was originally scheduled to be 3 to 4 weeks in duration, but was extended to allow further development of the plants.

Results

Field Remediation Experiment

During the study, excellent growth of the introduced fungus was observed. After 4 to 5 weeks, and continuing monthly thereafter, there was a massive fruiting at the surface of the mycoremediated mounds of all three soils (Figure 6, for example) and the mycelial growth penetrated throughout the mounds, down to the bottom at about 4 ft (Figure 7). The smell of oil was absent from these mounds, and the pockets of oil, tar, and other petroleum hydrocarbons were no longer apparent after the first few weeks. A vascular plant community began to develop on these mounds by Week 9, and continued to become more diverse and abundant over time; by Week 12, there were several secondary decomposer species of wild fungus fruiting (Figure 8).

The control, bioremediated, and bacterially remediated soils, in contrast, did not show any visible evidence of change during the study. They retained their initial character of heavy clay composition, with an oil odor, and visible pockets of oil and asphalt (Figure 9, for example). Bacteria-based treatments usually require at least 1 year to be effective; therefore, this 4-month testing schedule may have been premature for optimal results of both bioremediation and enhanced bacterial treatment. An overall comparison of the endpoint soil condition of all control and treated mounds is shown for comparison in Figure 10.

Success of the remediation was measured as the attainment of the Washington Department of Ecology criterion of reduction to 200 ppm TPH, as determined by a U.S. Environmental Protection Agency (EPA)-approved analytical method. However, in spite of the extensive sampling and chemical analysis using the NW-TPH-DX method prescribed by WSDOT along with supplementary analyses, it could not be determined with certainty that the criterion had been met in any of the treated or control soils. This was due largely to the difficulty in adequately sampling these soils, which had extremely patchy distribution of contaminant within each test mound, and great heterogeneity of contaminant distribution among the mounds within each test soil type at the start of the experiment. This did not allow a clear representation to be made for comparison of start- and endpoint characterization of petroleum hycrocarbon content.

Petroleum hydrocarbons. Results and quality control data for the extensive chemical analyses of samples from Time 0, Week 9, and Week 17 are presented in Appendix E.[1] The Time 0 results revealed that the initial mixing of the source soils in the setup process did not ensure homogeneity within each mound, nor among the four mounds established with each soil type. For example, the mean diesel-plus-heavy-oil value (which should approximate TPH) of the truck bay soils at Time 0 are shown in Table 1.

Clearly, from the starting point, there is a wide range of values that indicate high variability among the four mounds. The starting contaminant values for the mycoremediation treatment and the control are about one-third greater than those for the bacterial and bioremediation tests. Over time, the diesel-plus-heavy-oil value of the truck bay control appeared to first increase to 1453±231 ppm at Week 9, then decrease to 787±125 ppm by Week 17. The treated soils, similarly show inconsistent patterns of increase and decrease of petroleum hydrocarbons over time.

The mean diesel-plus-heavy-oil value of the diesel-contaminated soils at Time 0 ranged from 130±15 to 180 ppm±63 (Table 2), which scarcely exceeds the Model Toxics Control Act required

[1] Data from Weeks 5, 9, and 17 are referenced as Weeks 4, 8, and 16 in the appendices, based on the original work plan.

Table 1. Chemical Analysis of Truck Bay Soils[a]

Sample Period	Soil Treatment	Mean Diesel+Oil ppm	Standard Deviation (SD) ppm
Week 0	Control	1083	433
	Mycoremediation	1027	253
	Enhanced Bacterial	660	44
	Bioremediation	703	116
Week 9	Control	1453	231
	Mycoremediation	1063	268
	Enhanced Bacterial	1303	304
	Bioremediation	950	193
Week 17	Control	787	125
	Mycoremediation	1073	38
	Enhanced Bacterial	993	38
	Bioremediation	1127	61

a) Using NW-TPH-DX (ARI, Inc.); data abstracted from Table E.1, Appendix E.

cleanup level; based on these Time 0 samples, cleanup might have been deemed unnecessary. However, WSDOT's sampling of other parts of the much larger source-mound, which is estimated to consist of about 3000 cu yd of scraped, contaminated soil, indicated that at least parts of the soil were highly contaminated and that therefore the entire mound required remediation (personal communication, Siv Balachandran). The present data indicate an increase in diesel-plus-heavy-oil concentrations in all four mounds by the endpoint.

The diesel-plus-heavy-oil content of the gasoline-contaminated soils is shown in Table 3. In this series, the control mound has a starting value that is the highest measured for any soil mound at any time during the study. It is well over two times greater than those of the other three soil mounds for treatment, but the SD of the control TPH value is very high. The latter reflects the inhomogeneity of contaminant distribution within the mound. The control mound appears to decrease dramatically in its mean contamination level during the 17 weeks. The high mean figures for the mycoremediation mounds in Weeks 9 and 17 also have very high SDs, and there is again no consistency to the pattern of apparent increase and decrease in petroleum hydrocarbon content of the soils. Although WSDOT initially characterized this soil as gasoline-

Table 2. Chemical Analysis of Diesel Soils[a]

Sample Period	Soil Treatment	Mean Diesel+Oil ppm	Standard Deviation (SD) ppm
Week 0	Control	180	63
	Mycoremediation	130	15
	Enhanced Bacterial	163	28
	Bioremediation	166	38
Week 9	Control	201	4
	Mycoremediation	188	106
	Enhanced Bacterial	354	191
	Bioremediation	347	55
Week 17	Control	395	160
	Mycoremediation	260	122
	Enhanced Bacterial	318	94
	Bioremediation	245	33

a) Using NW-TPH-DX (ARI, Inc.); data abstracted from Table E.1, Appendix E.

Table 3. Chemical analysis of gasoline-contaminated soils[a]

Sample Period	Soil Treatment	Mean Diesel+Oil ppm	Standard Deviation (SD) ppm
Week 0	Control	1847	1168
	Mycoremediation	647	42
	Enhanced Bacterial	640	195
	Bioremediation	557	83
Week 9	Control	677	205
	Mycoremediation	1313	726
	Enhanced Bacterial	747	60
	Bioremediation	993	463
Week 17	Control	470	101
	Mycoremediation	1103	728
	Enhanced Bacterial	750	56
	Bioremediation	613	153

a) Using NW-TPH-DX (ARI, Inc.); data abstracted from Table E.1, Appendix E.

contaminated, based at least in part on the odor of the soil (personal communication, WSDOT), the MSL staff understood that it was to be analyzed by the same method that was applied to the other soils. This method does not detect gasoline components; it could have been useful to apply the NW TPH-Gasoline-Extended test in combination with the –DX analysis.

In all of the chemical analysis, although there were values listed for the spectral frequency range of diesel oils and heavy oils, the analysts indicated that the spectral patterns did not specifically match those of diesel or heavy oils (several personal communications, M. Harris, ARI). The MSL therefore requested individual chromatograms from each of the samples that had been analyzed by ARI from Weeks 0, 9, and 17 to supplement ARI's summary of numerical TPH data. The chromatograms were reprocessed by the original method used in ARI's standard procedure, and then reintegrated by two different methods to try to ascertain the quantitative differences among peak heights, peak areas, and patterns.[2] These voluminous data are not included in the appendices, but are available on request. However, it was determined by Battelle's petroleum hydrocarbon specialists at the MSL and BOS that there was nevertheless no way to identify and quantify individual compounds and their retention times from these data, because of the initial preparation of samples and the NW-TPH-DX method that was used for the analysis.

Based on the MSL's earlier results with mycoremediation (Pinza et al. 1998; Word et al. 1998), it was expected that the mycelial activity would have broken down the higher-molecular-weight polycyclic aromatic hydrocarbons (PAHs) most effectively, perhaps accordingly, contributing an increase to smaller molecular weight intermediate products. Because the PAHs represent a very small, though highly toxic and recalcitrant, fraction of the TPH, such an outcome could yield data that would appear to show at first no change in TPH, but that would also reveal a shift in the ratio of higher to lower-molecular-weight components present as the fungal activity degraded the larger molecules. To clarify, the final, intensively composited and processed samples that were taken and processed were analyzed at BOS for PAHs and alkanes in controls and treated soils. It was the intention to evaluate by this alternative method the loss of oil fractions compared with the relative concentrations of compounds in the oil (e.g., hopane, pristane, etc.). This method permits the use of the stable compound as a surrogate to quantify the removal of petroleum fractions relative to the total oil in the sample and remove the problem of intersample variability.

[2] The first reintegration identified each separate peak in the chromatogram and integrated the peak to the chromatographic baseline; the second integrated the same peaks using a valley-to-valley baseline.

Because of budget limitations, the analysis was only partially completed; the analyst's primary observation was that the petroleum hydrocarbons in soil were extremely weathered.

Metals. From the mycoremediated test mounds, fruiting bodies were sampled, freeze-dried, and tested for cadmium and lead. None was accumulated in the internal mushroom tissue. These analyses were carried out by the MSL's Trace Metals Laboratory, which is recognized as a leader in ultra-trace-level analysis, and which designs and implements field and analytical programs for EPA and other clients.

Mesocosm and Toxicity Experiments

TPH was measured in all of the mesocosm samples by the NW TPH-DX method. By that measure, the data do not show that significant remediation occurred in any of the mesocosms (Appendix F, Table F.1). However, it was difficult to evaluate the results because of the extreme inhomogeneity of the soil samples, and because the type of chemical analysis that was conducted did not provide concentration values for individual compounds of interest. Fungal fruiting bodies and a vascular plant community were observed to develop on several of the mycoremediated soils. Further, the treated soils were lightened in texture, and had the appearance of rich potting soil with no trace of oil odor or of visible oil pockets or color by the end of the 17-week period. In contrast, the untreated soils were unchanged from their original heavy clay texture, and there was still some odor and considerable visual evidence of persistent oil present. Fungal fruiting bodies were sampled and a subset analyzed for the presence of petroleum hydrocarbons in the tissue (Appendix F, Table F.3). Although there was evidence of organic material present in the range of diesel or motor oil, the analyst recorded that the spectra did not correspond to those of petroleum products and were likely other organic compounds, and that it was not be clear whether the apparent contaminant was actually in the tissue or adhering to the fruiting body's surface that was in contact with the soil.

The results of toxicity tests of the treated WSDOT Bellingham soils using earthworms showed no statistically significant difference among the treatments or controls for worm survival, although growth was greater in the mycoremediated soils (Appendix F, Table F.4, Figures F.1, F.2, and F.3). Toxicity tests using Washington native plants (ASTM 1994; Tarradellas et al. 1997) indicated that the mycoremediated soils appear to offer a more beneficial substrate for the growth of the shrubs tested than do the soils that were bio- or bacterially remediated, as indicated by measures of plant growth and by plant death on some soils (Appendix F). The *Festuca* grass grew well in all soils, with possibly a slight advantage in soils to which fertilizer had been added in the course of remediation treatment.[3]

Discussion

The transition from bench-scale to field scale application poses some challenge. Nonetheless, the MSL team is confident in the efficacy and appropriateness of the mycoremediation method for treating petroleum hydrocarbon contaminants, and looks forward to investigating the variables that could determine the success of large-scale deployment. These could include bioavailability of aged petroleum hydrocarbons in soil, configuration of substrate for treatment, and accurate preassessment of contaminated soils, among other factors.

---

[3] *Festuca* and other grasses are sometimes used in phytoremediation of petroleum hydrocarbon contamination, in which the plants work in combination with fungi and bacteria in the rhizosphere to degrade petroleum products; the treatment is usually applied from 1 to 3 years (for example, Drake 1997).

Benefits of mycoremediation of the soil, as shown by these study results, are as follows:

Cost. Based on our experience in this study, the commercial cost of mycoremediation would be under $50/cubic yard, including bulk fungal spawn and sawdust for inoculation, materials such as shadecloth covering, and the transportation, labor, and equipment for the application.

This cost does not include the cost of chemical analysis, which would be a part of any monitoring program that could be instituted in a remediation activity, and which would depend on the sampling and analytical methods prescribed by the user and/or by regulatory agencies involved.

Effort. This study confirmed that no maintenance of the remediation was required after initial application setup. In contrast, bioremediation and enhanced bacterial remediation required at least monthly physical turning and periodic reapplication of fertilizer/inoculum.

Beneficial Product. The test soils treated by mycoremediation were improved in texture, organic content, and ability to encourage and support plant life, in comparison with the soils subjected to other treatments.

Because of the nature of the NW-TPH-DX method that was applied for chemical analysis, it was not possible to obtain detailed analysis of the individual petroleum hydrocarbon compounds to determine the specific ways in which the fungal treatment attacked the components of oil/diesel contaminants in comparison with the effect of the other treatments. This test measures specifically the TPH, rather than individual components. However, based on the MSL's prior experience with mycoremediation (see Background), and on the albeit incomplete, but suggestive PAH-analysis data from the Battelle Ocean Sciences Laboratory, it appears likely that toxic high-molecular-weight PAHs were degraded in the mycoremediated soils in the present study as well, as a particular benefit of the treatment. It would be of interest to elucidate this through detailed analysis of PAH and alkane components at the start and end points of a pilot-scale test in the near future. If, for example, the TPH content did not meet required cleanup levels after a period of mycoremediation treatment, but it could be shown that the toxic components of the oil had been nonetheless in that time period removed from the soil, the duration and degree of required treatment could possibly be decreased. Because regulatory agencies are in the process of reviewing petroleum hydrocarbon cleanup standards and recommended analytical methods (for example, WSDOE 1998), there could be opportunities to introduce a concept of perhaps more meaningful standards that reflect toxicity and bioavailability of contaminants, rather than total values that include components that are not necessarily harmful or toxic in the environment. Simple toxicity tests, such as those conducted as supplementary experiments in this study, could be a cost-effective and practical addition to prescribed soil testing programs.

The relatively short duration of treatment could have limited the effectiveness of all three treatment approaches because of the chemical nature of the extremely weathered contaminant in the soils. However, the bioremediation and bacterial treatment results could have been particularly incomplete, because these treatments normally require at least 1 year. A future study that would allow full-term treatment by all three methods perhaps could offer a more representative comparison.

A fundamental difficulty encountered in this study was that the distribution of contaminant in source soils was extremely patchy, both within each test mound, and within each set of four test soil mounds to be treated by the three methods. Based on the information provided to the MSL in advance of the study, it was anticipated that the random, stratified sampling regime employed would be adequate in combination with the extra measures taken for mixing of the soils at the experiment's start. However, in retrospect, it appears that a more intensive sampling and compositing scheme could have been included in the experimental design to accommodate the heterogeneous matrices and the variation within each set of soils to be tested, had the degree of patchiness and heterogeneity been known in advance.[4] Combined with analysis that detailed not only TPH, but individual components or ranges of petroleum hydrocarbon contaminants in the soils, such a sampling regime could have allowed a more precise quantification of start- and endpoint contaminant levels, and hence, a better comparison of the effectiveness of the three treatment methods.

In more than one letter and telephone discussion, the analysts at ARI, Inc., stated that the samples they received from the study were difficult to homogenize, and that it was necessary to highly dilute some of the samples of these thick, clay, clumpy matrices, some of which contained not only the soil, but also rock, wood, Styrofoam, metal scrap, and other large particles. These difficulties resulted in higher variation than normal among replicates, and the analysts attributed differences of up to 50% among replicate values in the motor oil range in samples from 9 of the 12 test mounds to nonhomogeneity of the sample matrix (Mark Harris, ARI, Letter dated 27 August 1998). It was also noted on several occasions by the ARI analysts that although the spectral patterns detected in many samples were similar to those of heavy diesel and/or motor oil, adequate matches could not be made for positive identification. It was therefore difficult to interpret the data.

One soil had been labeled before the project start as heavily contaminated with diesel oil; however, at Time 0, the MSL's chemical analysis indicated low levels of TPH in the test mounds of this soil, nearly meeting the WSDOE cleanup standard. Yet, the endpoint samples of the same soil were analyzed to have a higher TPH content, which consequently did not meet the required standard. WSDOT's end-of-August analysis of the source of the diesel-contaminated test-soil, which is an accumulation of about 2 acre-feet or >3000 cu yd of scraped, contaminated earth, indicated that in at least 4 out of 10 samples, the TPH level still significantly exceeded the required cleanup level of 200 ppm after the bioremediation and turning treatment applied by WSDOT (personal communication, WSDOT, based on NW-TPH-DX analysis by Sound Analytical Services, Inc.).

Recent work by Marine Oil Spill Response Corporation (Nordvik et al. 1995; Champ et al. 1998) offers a perspective on the ways in which different remediation technologies could be selected for use, and perhaps combined in some ways, to maximize the efficiency of cleanup. This group introduced the concept of oil weathering and corresponding windows of opportunity for treatment. To implement the concept as a decision-making tool for response to oil-contamination-event-response, a database was created to describe the weathering process, step-by-step changes in oil characteristics, and consequent changes in response technology effectiveness over time. This database, originally built on marine settings, could be adapted to terrestrial settings to plan for the most effective application of chemical and/or physical, along with biological treatments.

---

[4]The Time 0 sampling and analysis revealed the nonhomogeneity of the soils; by that time, the experiment was underway and the budget committed, so that no change in sampling or analytical approach could be reasonably made.

References

ASTM (American Society for Testing and Materials). 1994. Standard practice for conducting early seedling growth tests. E1598-94. American Society for Testing and Materials, Philadelphia, Pennsylvania.

ASTM (American Society for Testing and Materials). 1995. Standard guide for conducting a laboratory soil toxicity test with lumbricid earthworm *Eisenia foetida*. E1676-95. In *Annual Book of ASTM Standards*, American Society for Testing and Materials, Philadelphia, Pennsylvania.

Becker, P., M. Pinza, S. Thomas, J. Word. In press. Mesocosm and field studies, and testing of deployment technology for the mycoremediation of oil-contaminated soil. *Laboratory Directed Research and Development Annual Report, FY 1998*. Pacific Northwest National Laboratory, Richland, Washington.

Champ, M.A., A.B. Nordvik, and T.E. DeLaca. 1998. The integration of science and technology in oil spill response decision-making, pp. 162-164. IAEA SM 354/83, International Symposium on Marine Pollution, 5-9 October 1998, Monaco. International Atomic Energy Agency, Vienna.

Drake, E. 1997. Phytoremediation of aged petroleum hydrocarbons in soil. IBC's Second Annual Conference on Phytoremediation, 18-19 June 1997, Seattle, Washington.

Huesemann, M.H. 1994. Guidelines for the development of effective statistical soil sampling strategies for environmental applications, pp. 47-91. Chapter 4 *in Hydrocarbon Contaminated Soils and Groundwater,* eds. E.J. Calabrese and P.T. Kostecki. Conference proceedings of the Fourth Annual Conference on Hydrocarbon Contaminated Soils, March 1-4, 1993, Long Beach, California. Association for the Environmental Health of Soils (AEHS), Amherst, Massachusetts.

Nordvik, A.B., J.L. Simmons, and M.A. Champ. 1995. Oil spill cleanup: windows of opportunity. *Sea Technology* 36(9): 10-16.

Pinza, M., P. Becker, and S.A. Thomas. 1998. Bioremediation: Mycofiltration Study for the Cleanup of Oil-Contaminated Soil, pp. 16-19. *Laboratory Directed Research and Development Annual Report, FY 1997*. PNNL-11860, Pacific Northwest National Laboratory, Richland, Washington.

Tarradellas, J., G. Bitton, and D. Rossel, eds. 1997. *Soil Ecotoxicology*. Lewis Publishers, New York.

Thomas, S., P. Becker, M.R. Pinza, and J.Q. Word. In press. Adaptation of mycofiltration phenomena for wide-area and point-source decontamination of CW/BW agents. *Laboratory Directed Research and Development Annual Report, FY 1998*. PNNL-11860, Pacific Northwest National Laboratory, Richland, Washington.

WAC (Washington Administrative Code). 1996. Washington State Department of Ecology, Model Toxics Control Act (MTCA) Cleanup Regulation. WAC-173-340, Olympia, Washington.

Word, J.Q., S.A. Thomas, A.S. Drum, P. Becker, M.R. Pinza, and T. Divine. 1998. Adaptation of mycofiltration phenomena for wide-area and point-source decontamination of CW/BW agents, pp. 10-13. *Laboratory Directed Research and Development Annual Report, FY 1997*. PNNL-11860, Pacific Northwest National Laboratory, Richland, Washington.

WSDOE (Washington State Department of Ecology). January 1997. *Washington State Department of Ecology Toxics Cleanup Program Interim Interpretive Policy Statement: Cleanup of total petroleum hydrocarbons (TPH)*. ECY97-600. Washington State Department of Ecology, Olympia, Washington.

WSDOE (Washington State Department of Ecology). 1998. Total petroleum hydrocarbons (TPH) initiative. Available online: http://www.wa.gov/ECOLOGY/tcp/mtca_gen/tph/tph_fs.html.

FIGURES

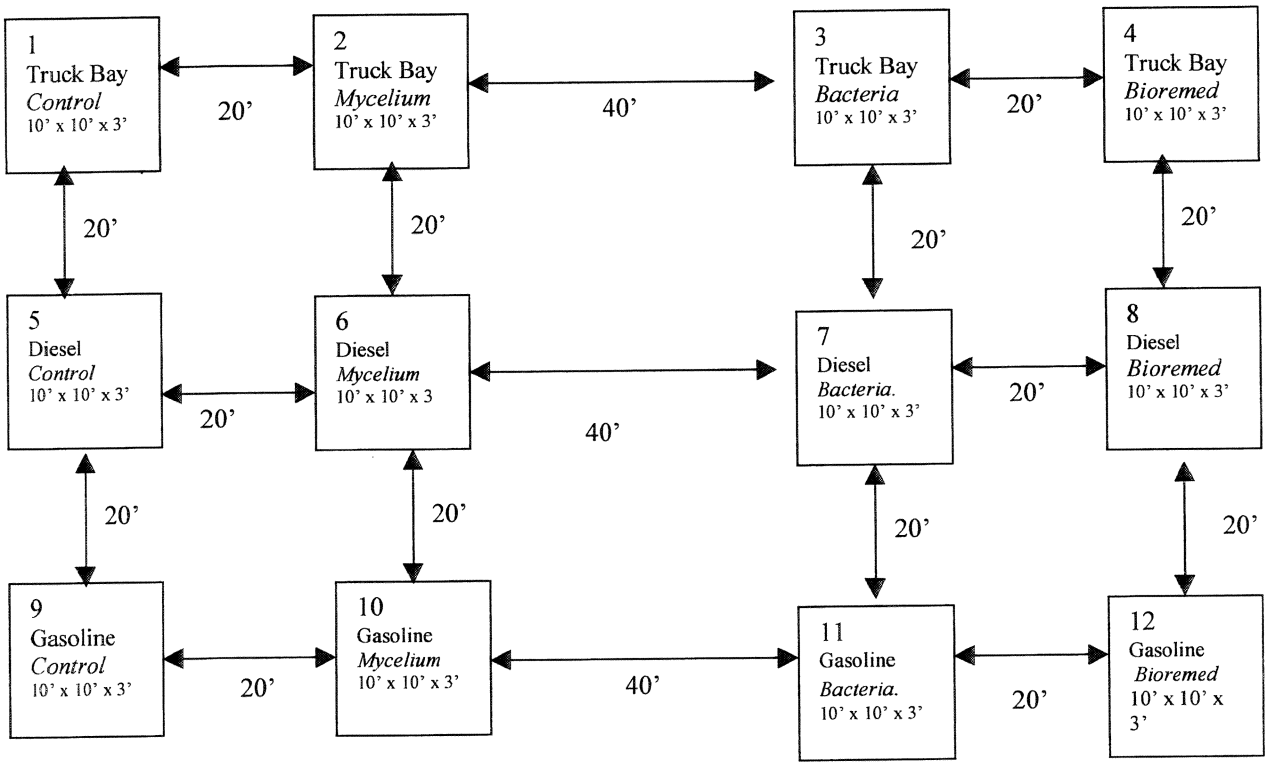


Figure 1. Experiment schematic (not to scale) showing 12 test mounds and approximate spacing to minimize cross-contamination on relatively flat test site (all of the bacterial and bioremediation mounds [3,4, 7, 8, 11, and 12] were moved at Week 12 to be located directly below the control and mycoremediated mounds instead of to the side but retained the order and spacing shown above relative to one another)

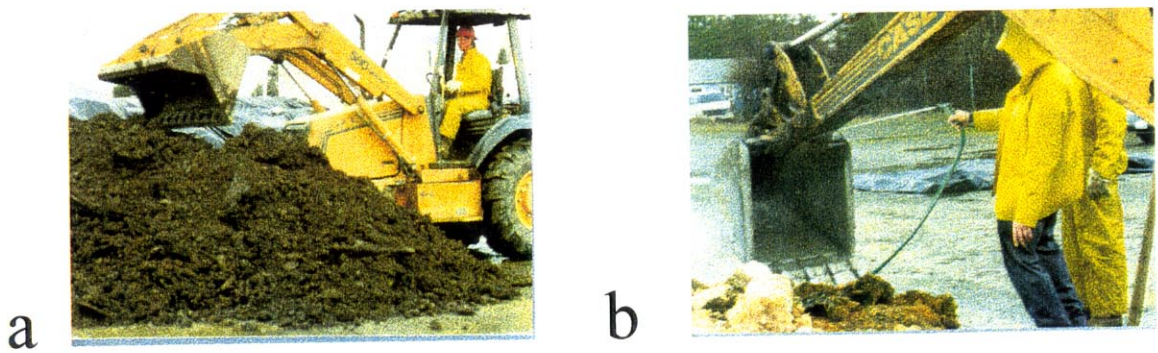


Figure 2. Setup: a) mixing source soil; b) washing bucket to avoid cross-contamination

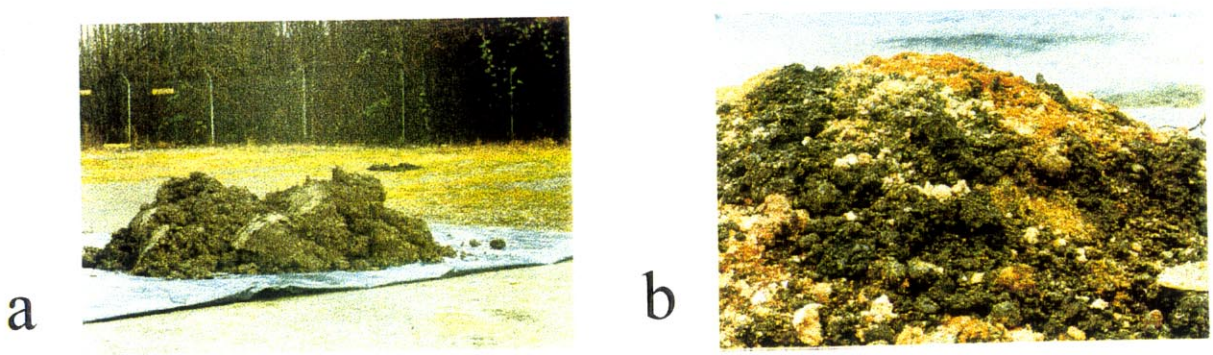


Figure 3. Diesel-contaminated example: a) Time 0 control, and b) Time 0 inoculated mycoremediation

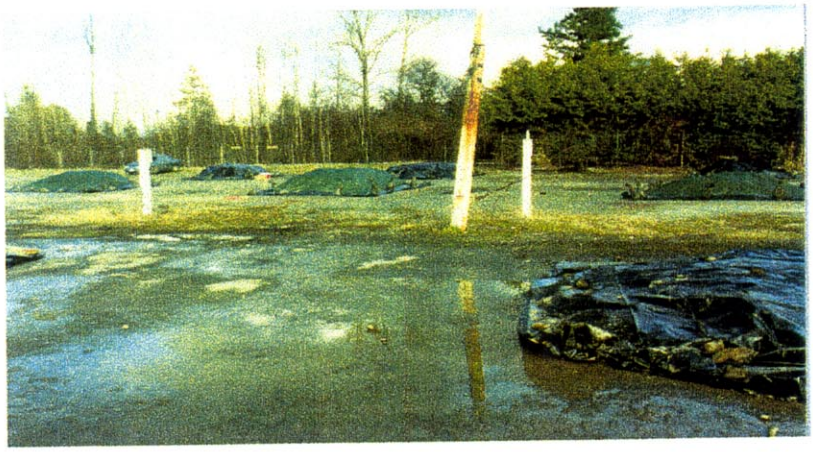
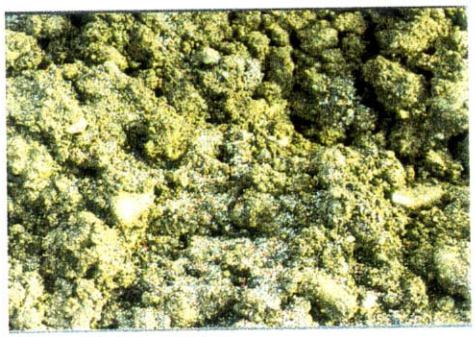


Figure 4. Time 0 bacterial (foreground), mycoremediation (65% green shadecloth), and control (background, with black polyfilm) mounds and their covering

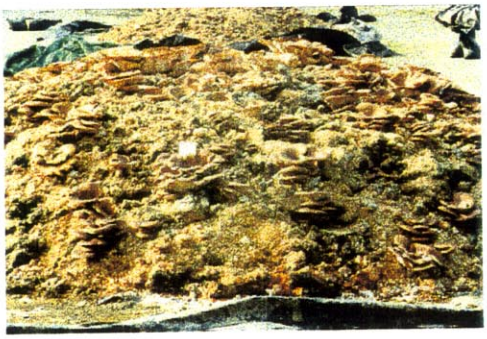
a



b

Figure 5. Time 0: a) WSDOT bioremediation treatment, application of nitrogen fertilizer; b) PSCI application of liquid fertilizer and bacterial inoculum

a



b

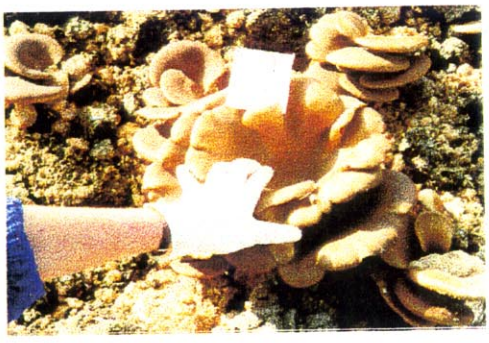


Figure 6. Week 5: a) fruiting on mycoremediated diesel-contaminated soil; b) detail of fruit

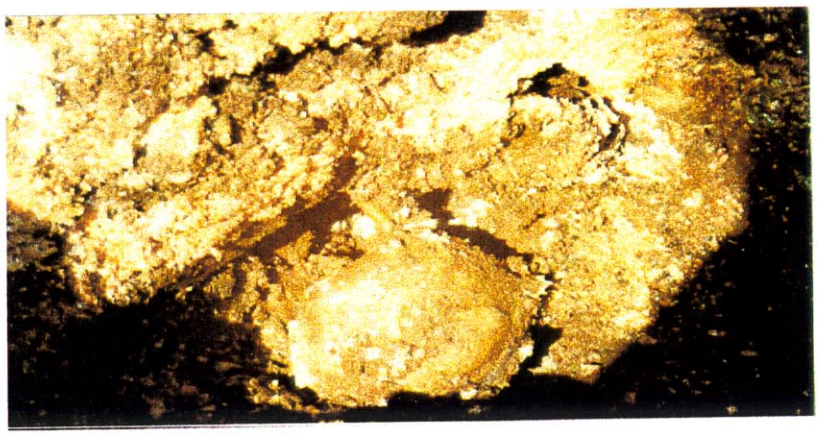


Figure 7. Week 5: detail of mycelial treatment of diesel-contaminated soil (>20" deep mycelial growth, note dense growth around a rock at lower center)

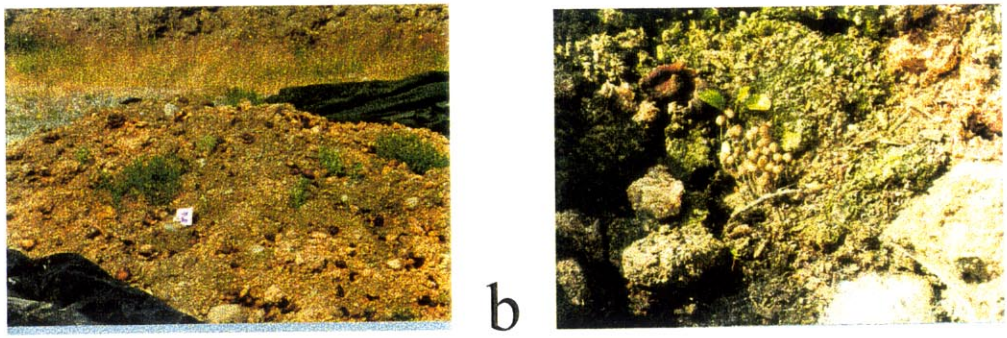


Figure 8. Week 17 community development: a) vascular plants on mycoremediated truck-bay soil; b) secondary decomposer fungi and vascular plants on mycoremediated gasoline-contaminated soil

Figure 9. Week 9: enhanced bacteria treatment of truck-bay soil, visible petroleum hydrocarbon pockets still present

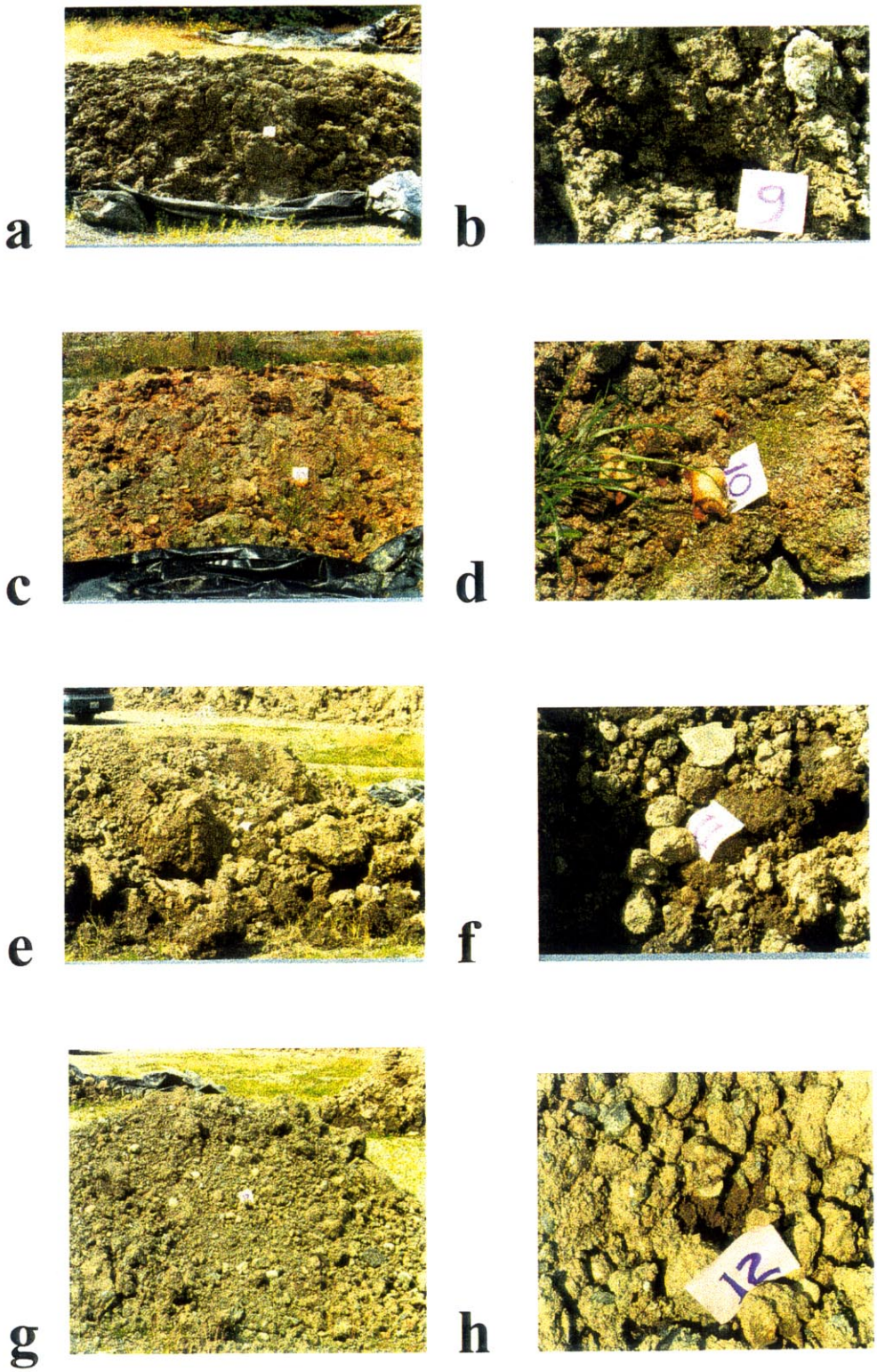


Figure 10. Week 17 comparison of controls and all treatments of gasoline-contaminated soil: a) control no. 9; b) control detail; c) mycoremediated no. 10; d) mycoremediated detail; e) enhanced bacterial no. 11; f) enhanced bacterial detail; g) bioremediated no. 12; g) bioremediated detail

APPENDIX A
Field Remediation
Experimental Design and Workplan

Appendix A

Field Remediation Experimental Design and Workplan

Introduction

In the proposed study, we will focus on mycoremediation of oil-contaminated soil that is removed from its original location for remediation. "Success" of the proposed mycoremediation will be measured as attainment of the required cleanup levels, within the test period of 4 months.

Objectives

Our objective is to measure the effectiveness of our oil-conditioned, proprietary mycelial system to reduce the level of total petroleum hydrocarbons (TPH) to the Method A Cleanup Levels prescribed by the Washington State Department of Ecology (WSDOE) in Washington Administrative Code (WAC) 173-340 (WAC 1996) and Ecology Publication No. ECY-97-600 (WSDOE 1997) in two aged oil-contaminated soils and one gasoline-contaminated soil under open environmental exposure. We will compare our results with those of an alternative biotechnology (bioremediation) and enhanced bacterial remediation employed by the Washington State Department of Transportation (WSDOT) to remediate soils from the same sources at the same site. One test soil will be excavated from an interior stall used for 30 to 40 years as a truck bay at the WSDOT Bellingham maintenance yard. The other two test soils will each consist of a subset of contaminated soils scraped from another site, presently stacked and stored at the WSDOT Bellingham yard: a) diesel and other oil-contaminated soil, and b) gasoline-contaminated soil. Our goal is to demonstrate this biotechnology at a scale that is appropriate to commercial application, and to determine a projected cost of commercial application by carefully monitoring the labor hours and the costs for equipment and material. If the demonstration is successful in its efficiency, rapidity, and cost of remediating excavated, petroleum-hydrocarbon-contaminated soils, Battelle could likely recommend a contracting vehicle to WSDOT for the large-scale treatment of soils of this type. Battelle's Pacific Northwest Division, of which the MSL is a part, is currently negotiating patent rights for the technology proposed for application at the Bellingham maintenance yard. Battelle may license its technology, once it is reduced to an engineering practice.

Experimental Design

Experiment 1 will address the truck-bay soil. This compacted substrate will be removed from the building interior by backhoe and placed outdoors on the WSDOT Bellingham yard. To allow a comparison of our mycoremediation and WSDOT's two bioremediation treatments, soil from the two contaminated truck-bay stalls will be homogenized and subdivided to four approximately equal parts, each approximately 300 cubic feet (11 cubic yards), for the following plots: a) control;[5] b) MSL's mycoremediation treatment; c) WSDOT's bioremediation treatment; and d) WSDOT's enhanced bacterial bioremediation treatment. The MSL staff should be present on the day of homogenization and mound formation to document and participate in the process. The four parts will be physically distant from one another to ensure the distinction of effects from the two treatments. Moisture content of the soil must be determined before the application of the

[5]Untreated only for the duration of the MSL experiment; it will subsequently be available for remediation by WSDOT's preferred method.

mycelial treatment; it requires 24-h drying of a 100-g sample of substrate at 100°C, recording weight before and after drying. This will be carried out by MSL staff. The MSL's mycoremediation treatment will consist of adjusting soil moisture; transferring the fungal mycelium to the soil by way of alder wood chips; and mulching or covering the system with a shade cloth.

Experiments 2 and 3 will address the scraped soils that are currently stored at the WSDOT maintenance yard, applying the same control and three treatments specified for Experiment 1. In Experiment 2, a test mound (~300 cubic feet) from the oil-contaminated soil will be addressed in the following plots: a) control;[1] b) MSL's mycoremediation treatment; c) WSDOT's bioremediation treatment; and d) WSDOT's enhanced bacterial bioremediation treatment; in Experiment 3, one mound (~300 cubic feet) of the gasoline-contaminated scraped soil will be treated in the following plots: a) control;[1] b) MSL's mycoremediation treatment; c) WSDOT's bioremediation treatment; and d) WSDOT's enhanced bacterial bioremediation treatment. To allow a comparison between the MSL's mycoremediation and WSDOT's two bioremediation treatments, the gasoline-contaminated soil and the oiled soil, respectively, will each be homogenized or mixed to the extent possible before they are subdivided to establish the parallel tests; the mounds for treatment will be physically located at sufficient distance to ensure distinction of the effects of the treatments. The MSL staff should be present on the day of homogenization and mound formation to document and participate in the process. Moisture content of the soils must be determined before the application of the mycelial treatment; it requires 24-h drying of a 100-g sample of each substrate at 100°C, recording weight before and after drying. This will be carried out by MSL staff. The MSL's mycoremediation treatment will consist of adjusting soil moisture; transferring the fungal mycelium to the soils by way of alder wood chips; and mulching or covering the systems with a shade cloth.

Two separate supplementary experiments will be conducted offsite by MSL-funded research. Experiment 4 will test the toxicity of the WSDOT maintenance-yard scraped, oil-contaminated soil at Time 0 and at the termination of the MSL's mycoremediation experiment, using standard terrestrial toxicity tests that could employ earthworms (ASTM 1994) and a horticultural variety such as a lettuce (ASTM 1995), or other landscaping species, such as Douglas fir, Pacific willow, or others relevant to WSDOT (Tarradellas et al. 1997).

Experiment 5 will consist of a mesocosm study using a 20-kg sample of the scraped, oil-contaminated soil transported from the WSDOT Bellingham maintenance yard to the MSL for a parallel, but smaller-scale treatment. The experimental chamber containing 10 kg of oiled soil will be inoculated with fungal mycelium carried on alder wood chips, and this chamber along with the control, which will contain 10 kg of untreated oiled soil, will be placed in an outdoor study site for 8 weeks. Samples will be taken for chemical analysis at Time 0 and at Month 2, when the experiment is terminated.

Table 1. Experimental Design

Experiment 1	MSL, mycoremediation of oiled soil	Control of oiled soil	WSDOT bioremediation of oiled soil	WSDOT enhanced bacterial bioremediation of oiled soil
Experiment 2	MSL, mycoremediation of gasoline soil	Control of gasoline soil	WSDOT bioremediation of gasoline soil	WSDOT enhanced bacterial bioremediation of gasoline soil
Experiment 3	MSL, mycoremediation of truck bay soil	Control of truck bay soil	WSDOT bioremediation of truck bay soil	WSDOT enhanced bacterial bioremediation of truck bay soil
Experiment 4	Mesocosm testing of 20 kg oiled soil, with control, at the MSL	-------	-------	-------
Experiment 5	Toxicity testing of oiled soil using standard terrestrial toxicity tests at the MSL	-------	-------	-------

Schedule

The experiments will begin according to WSDOT's schedule, targeting 27 February 1998 or the first week of March as start-date. The MSL requires a 1-month period for the expansion of the fungal spawn needed for these experiments. A schedule of at least one midpoint or possibly monthly visual examinations by the MSL staff of the experiment in progress will be established in coordination with WSDOT; there should be no maintenance necessary for the MSL's plots. We have designated a 4-month duration for the experiments.

Sampling

All treated and control plots will be sampled initially for characterization, at 2 months, and at the 4-month endpoint. A single sample from each plot will be composited from three random, stratified subsamples at each sampling date. Details of the sampling plan will be developed in coordination with WSDOT.

Chemical Analysis

Samples will be analyzed for TPH by Environmental Protection Agency (EPA) Method 1664 to determine whether Method A Cleanup Levels were achieved.

Deliverable

A written report will be delivered to describe the mycoremediation method, to present results and discussion of all experiments, and to compare the mycoremediation and bioremediation results (note: the bioremediation will likely occur in a longer time-frame; for the MSL's report comparison will be made only of the results within the MSL's experimental period). The study results and a discussion of the method could be presented at a workshop following delivery and review of the report.

References

ASTM (American Society for Testing and Materials). 1994. Standard practice for conducting early seedling growth tests. E1598-94. American Society for Testing and Materials, Philadelphia, Pennsylvania.

ASTM (American Society for Testing and Materials). 1995. Standard guide for conducting a laboratory soil toxicity test with lumbricid earthworm *Eisenia foetida*. E1676-95. In *Annual Book of ASTM Standards*, American Society for Testing and Materials, Philadelphia, Pennsylvania.

Becker, P., M. Pinza, S. Thomas, J. Word. In press. Mesocosm and field studies, and testing of deployment technology for the mycoremediation of oil-contaminated soil. *Laboratory Directed Research and Development Annual Report, FY 1998*. Pacific Northwest National Laboratory, Richland, Washington.

Huesemann, M.H. 1994. Guidelines for the development of effective statistical soil sampling strategies for environmental applications, pp. 47-91. Chapter 4 *in Hydrocarbon Contaminated Soils and Groundwater,* eds. E.J. Calabrese and P.T. Kostecki. Conference proceedings of the Fourth Annual Conference on Hydrocarbon Contaminated Soils, March 1-4, 1993, Long Beach, California. Association for the Environmental Health of Soils (AEHS), Amherst, Massachusetts (see Appendix H, this report).

Pinza, M., P. Becker, and S.A. Thomas. 1998. Bioremediation: Mycofiltration Study for the Cleanup of Oil-Contaminated Soil, pp. 16-19. *Laboratory Directed Research and Development Annual Report, FY 1997*. PNNL-11860, Pacific Northwest National Laboratory, Richland, Washington.

Tarradellas, J., G. Bitton, and D. Rossel, eds. 1997. *Soil Ecotoxicology*. Lewis Publishers, New York.

Thomas, S., P. Becker, M.R. Pinza, and J.Q. Word. In press. Adaptation of mycofiltration phenomena for wide-area and point-source decontamination of CW/BW agents. *Laboratory Directed Research and Development Annual Report, FY 1998*. PNNL-11860, Pacific Northwest National Laboratory, Richland, Washington.

WAC (Washington Administrative Code). 1996. Washington State Department of Ecology, Model Toxics Control Act (MTCA) Cleanup Regulation. WAC-173-340, Olympia, Washington.

Word, J.Q., S.A. Thomas, A.S. Drum, P. Becker, M.R. Pinza, and T. Divine. 1998. Adaptation of mycofiltration phenomena for wide-area and point-source decontamination of CW/BW agents, pp. 10-13. *Laboratory Directed Research and Development Annual Report, FY 1997*. PNNL-11860, Pacific Northwest National Laboratory, Richland, Washington.

WSDOE (Washington State Department of Ecology). January 1997. *Washington State Department of Ecology Toxics Cleanup Program Interim Interpretive Policy Statement: Cleanup of total petroleum hydrocarbons (TPH)*. ECY97-600. Washington State Department of Ecology, Olympia, Washington.

Work Plan for WSDOT Project at Bellingham Yard

General Comments

1. *Homogeneity of test mounds is critical to comparability of the treatment effectiveness.*
To address this concern, test mounds should be established by serial deposition: that is, the front loader would deliver one bucket to mound A-1 (see Figure 1), then one to A-2, then one to A-3, and one to A-4, and then repeat the delivery order until 10 yards is delivered to each mound. Repeat for the other two series, B-1 through B-4, and C-1 through C-4, cleaning the front-loader bucket between soils.

For the truck-bay soils, the two stalls should be excavated, the soils combined and homogenized before serial delivery to the mounds.

2. *A primary concern is that there be no cross contamination between soils and between treatments.*

To address this concern, we have provided the following plan for layout of the experiment:

a) Distance between each mound of soil should be no less than 20 ft; if possible, the two sets of bacterial treatments should be separated completely from the mycelial treatments and controls by a distance greater than 40 ft.

b) The mycoremediation and control mounds will be protected from potential water movement by an underlayment of 6-mil polyfilm. (We do not know the setup of bioremediation and bacterial enhancement treatments; if WSDOT wishes to use underlayment, it should supply it for these treatments).

c) The front-loader will be directed to deposit soils to the mounds without making contact with soil or inoculation materials already deposited, to avoid carrying such materials from one pile to another. If bucket contacts inoculation materials, it should be washed with water and brush prior to pickup of additional soils.

March 2 Work Plan

1. 3/1/98. Peter Becker, Paul Farley load Ryder truck with MSL supplies, pick up mycelial spawn in Shelton Sunday, March 1, and drive to Bellingham; remaining MSL staff meet at the lab 4 am to catch Edmonds 5:50 ferry, meet WSDOT staff at Bellingham for 8 am start: Susan Thomas, Meg Pinza, Jack Word.

2. Hold brief safety meeting with all participants (MSL and WSDOT) staff on project site to ensure safe work practices and commonly recognized signals while working near and around heavy equipment.

3. Photodocument each step of the work of the day; document in laboratory record book.

4. Lay out plan for siting the 12 mounds, with attention to drainage conditions and appropriate distancing of mounds (see general comments above); place polyfilm at appropriate deposit sites.

5. Excavate two truck bays:

a) Homogenize soil from the two in a single pile
b) Divide to four mounds, approximately 10 cu yd each, following the serial deposition method described above.
c) Inoculate mycoremediation treatment (A-2) between deliveries of soil to the mound, and adjust moisture to 16% to 20% for mycelial growth at the same time.
d) Cover completed A-2 mound with shade cloth and weight with sandbags.
e) Cover completed control (A-1) mound with polyfilm.
f) WSDOT staff and enhanced bacterial contractor inoculate bioremediation and enhanced bacterial treatment mounds (A-3 and A-4, respectively) and add required amendments.

6. Diesel soil: Create four mounds (B-1 through B-4) and inoculate and/or amend as above (7), with the exception of (7a) homogenization in a single pile.

7. Gasoline-soil: Create four mounds (C-1 through C-4) and inoculate and/or amend as above (7), with the exception of (7a) homogenization in a single pile.

8. Collect 15 kg diesel soil (homogenized) for mesocosm; collect 20 kg for toxicity testing.

9. Sample each mound for Time 0 chemical analysis (TPH D-extended, with silica gel extraction), using containers supplied by Analytical Resources, Inc.

10. Clean up; repack any excess materials for removal.

13. Return Ryder truck.

Health and Safety

1. MSL staff will supply its own safety equipment and use it appropriately: steel-toed shoes, hard hats, gloves, eye protection, hearing protection, rain gear, first aid kit; cellular phone.

2. Note location of nearest hospital and notify emergency room in advance of the project:
St. Joseph's Hospital, 809 E. Chestnut, Bellingham, approx. 0.5 miles from work site.
Phone: 360-734-5400. Emergency room contacted 2/26/98 to give advance notice.

3. Personnel will be briefed concerning on safe procedures for working on and around heavy equipment; staff will be designated for emergency communication by cellular phone.

4 Petroleum hydrocarbon-contaminated soils will be moved with hand equipment (shovels) on site; although this is not hazardous material, all personnel will wear gloves and eye protection while working with these soils.

APPENDIX B
WSDOT Protocol for Bioremediation

Appendix B

WSDOT Protocol for Bioremediation

April 27, 1998

Susan Thomas
Pacific Northwest Laboratory
1529 West Sequim Bay Road
Sequim, WA 98382

RE: WSDOT Soil Remediation Protocol

WSDOT's soil remediation plan is based on standard operating procedures(SOPs) designed by a consultant for the Army to bioremediate petroleum contaminated soils (PCS) at Fort Lewis. The SOP's are designed to optimize levels of nutrients, moisture and pH.

Nutrient application is based on historical tests and published literature. The Fort Lewis SOP recommend the following amounts of nitrogen or fertilizer to remediate approximately 50 cubic yards of soil:

Total TPH (mg/kg)	Nitrogen	10-10-10 Fertilizer (lb)	20-20-20 Fertilizer (lb)	30-30-30 Fertilizer (lb)
1,000	6	60	30	20
2,000	12	120	60	40
3,000	18	180	90	60
4,000	24	240	120	80
6,000	37	370	185	123
8,000	49	490	245	163
10,000	61	610	305	203
15,000	91	910	455	303
20,000	122	1,220	610	407

WSDOT scales from this table and adds an appropriate amount of fertilizer to contaminated soils. Moisture is visually inspected and maintained at between 50 and 100 percent of moisture retaining capacity by adding water and/or turning. The pH is usually not measured unless there are extenuating circumstances which would warrant analysis. From literature and historical data, the pH is usually within acceptable levels. If the pH is less than 5, lime is added; if the pH is greater than 8, elemental sulfur or ammonium/aluminum sulfate is added.

If further information is needed, please feel free to call me or email back. Thank you for your time and consideration on this matter.

Sincerely,

Siv Balachandran
Environmental Specialist
Environmental Service Branch
Washington State Department of Transportation

APPENDIX C
PSCI Enhanced Bacteria Experimental Design

Appendix C

PSCI Enhanced Bacteria Experimental Design

In spite of repeated requests, Pacific Specialty construction Inc. (PSCI) did not supply a description of the experimental design for the enhanced bacterial remediation system applied at the Bellingham Maintenance Yard. Further, PSCI ceased applying the treatments just prior to Week 9 of the test due to closure of the company that supplied the proprietary bacteria. The three test mounds for the enhanced bacterial remediation trials were turned and mixed according to the maintenance schedule for the WSDOT test mounds for the remainder of the study period, but without further addition of bacteria or fertilizer.

APPENDIX D
Mesocosm and Toxicity Experimental Design

Appendix D

Mesocosm and Toxicity Experiments

Method for Mesocosm Tests

In a *mesocosm study* at the MSL, we used a sample of each of the three oil-contaminated test soils from the WSDOT Bellingham maintenance yard for a smaller-scale treatment. Two 10-kg samples of each contaminated soil were prepared, and each placed in an environmental chamber. One of each pair was inoculated with fungal mycelium carried on alder wood chips, and one was left untreated as a control. They were covered with hardware cloth for mild shading, and placed in an outdoor study site for 4 months, following the schedule of the Bellingham yard experiments. Natural precipitation determined the amount of water received. Samples were taken for chemical analysis at Weeks 0, 5, 9, and 17 (originally scheduled to be Weeks 0, 4, 8, and 16, but postponed by WSDOT's one-week delayed scheduling at Bellingham) and all growth, fungal fruiting, and vascular plant and secondary-decomposer fungal growth were recorded. Analysis of Week 0 and Week 5 samples used the NW-TPH-DX method (ARI, Inc.); Week 9 samples were not analyzed due to limitation of funds, but the samples were archived. Selected Week 17 samples were analyzed for PAHs and alkanes by an alternative method at Battelle Ocean Sciences Laboratory.

Method for the Toxicity Test with *Eisenia foetida andrei*

To assess the toxicity of gasoline-contaminated soils (Treatments 9 through 12) after remediation, a 14-day toxicity was conducted using *Eisenia foetida andrei.* The experimental design followed ASTM E1676-95 guidelines. A summary of testing requirements is shown in Table D.1.

Briefly, three testing replicates were prepared for each soil treatment. In addition, three treatments (artificial soil, potting soil, and peat moss) were prepared. The ingredients of the artificial soil were peat moss, kaolin clay, and silica sand. The artificial soil was used to validate test results, and the test was considered acceptable if survival in the negative control was ≥90%. The peat moss and the potting soil were used as reference sediments.

Prior to testing, soils were hydrated to 45% moisture using deionized water; test organisms were depurated for 24 hours in the dark, and 280 g of test sediment was placed into each test chamber (500 mL glass jars with screens and Teflon lids). After 24 hours, the test organisms were weighed, placed into the test chambers, and transferred to the environmental chambers maintained at the proper test conditions. During testing observations of the worms and the test conditions were noted.

At termination, the test organisms were removed from the container and counted as live or dead. In addition, sub-lethal responses such as segment swelling, coiling, lesions, or rigidity were noted. After data collection the organisms were depurated for 24 hours. Depuration procedures included the following: rinsing in deionized water, transferring survivors to filter paper in petri dishes, and storing at test conditions in the dark. After depuration, the test organisms were patted dry and weighed.

Table D1. Summary of Test Requirements

Test Duration	14 days
Temperature	23 ± 2°C
Light	Continuous
Humidity	>85%
Test Container	500 mL glass with screen and Teflon lid
Test Volume	200 g per replicate
Soil moisture content	35% to 45%
Test Organism	*E. foetida*
Age of organism	>300 mg per individual, clitellid adult
Number of organisms per container	10
Number of replicates	3
Soil pH	5.0 to 9.0
Endpoint measurement	Survival and growth
Negative control	Artificial soil
Test acceptability	≥90%

Method for Plant Toxicity Test

Background

WSDOT Bellingham yard—comparative test with mycoremediation, bioremediation, bacterial remediation, and control. Three contaminated soils were used: diesel, truck-bay (heavy oil), and so-called gasoline. At week 16(+), we took a 5-gal sample of each soil for toxicity testing.

Purpose

The purpose is to compare plant growth and/or toxicity of soils to plants in the control and three treated samples for each of the three test soils. Most important is a demonstration to be presented at a workshop for WSDOT and FHA. Second, a set of standardized measurements will distinguish the relative growth of plants in each of the controls and test soils.

Approach

Plant standardized replicates of three species of native plant, and a set of controls, which are established in sterilized potting soil. Plants were selected from list of WSDOT highway landscaping species, all of which are native Washington plants:

Festuca idahonensis (FES) fescue grass
Physocarpus capitatus (PHY) Pacific ninebark
Sambucus cerulius (SAM) blue elderberry

A. Control soil—sterilized potting soil
Festuca idahonensis (CONTROL-FES-A, B, C) fescue grass
Physocarpus capitatus (CONTROL-PHYS-A,B,C) Pacific ninebark
Sambucus cerulius (CONTROL-SAM-A,B,C) blue elderberry

B. All 12 soils (3 plant replicates on 4 each of 3 contaminated soils):
Festuca idahonensis (1 through 12-FES-A,B,C)
Physocarpus capitatus (1 through 12-PHY-A,B,C)

C. Truck Bay soil (3 replicates on 4 treatments)
Sambucus cerulius (1 through 4-SAM-A,B,C)

Method

1. Label and prepare containers with layer of 1" clean gravel

2. Control: prepare pots with 1" potting soil over gravel layer; prepare 3 each of the following
   -- *Festuca*
   Cut from bottom of root mass two 1-in. perpendicular cuts
   Rinse roots in bucket of clean water
   Gently shake excess water off of roots
   Flatten and measure maximum length and width of root mass
   Cut grass evenly at 3"
   Carefully spread roots and pot in the appropriately labeled container
   -- *Physocarpus*
   Cut from bottom of root mass two 1-in. perpendicular cuts
   Rinse roots in bucket of clean water
   Gently shake excess water off of roots
   Flatten and measure maximum length and width of root mass
   Trim to a single shoot, record length, number of leaves, average leaf width X length
   Carefully spread roots and pot in the appropriately labeled container

   -- *Sambucus*
   Rinse roots in bucket of clean water
   Count and record number of shoots (distinguish between shoots and leaves: leaves are pinnate with large spatulate to wide-lanceolate leaflets); separate multiple plants, if possible, and retain only one
   Gently shake excess water off of roots
   Flatten and measure maximum length and width of root mass
   Select longest shoot and record length, number of leaves, average leaf length X width
   Carefully spread roots and pot in the appropriately labeled container

3. Truck Bay: prepare pots with 1" appropriate truck-bay soil (1-4) over gravel layer; prepare three of each of the three plant species, as described above.

4. Diesel: prepare pots with 1" appropriate diesel soil (5-8) over gravel layer; prepare 3 each of the following:
   -- *Festuca* (see method above)
   -- *Physocarpus* (see method above)

5. Gasoline: prepare pots with 1" appropriate truck-bay soil (9-12) over gravel layer; prepare 3 each of the following:
   -- *Festuca* (see method above)
   -- *Physocarpus* (see method above)

7. Establish all containers on water table in ambient outdoor conditions; water every 3 days.

8. At week 3 or 4, remeasure the parameters.

APPENDIX E
Field Remediation Results

Appendix E

Table E.1 Chemical Analysis of Soils from the WSDOT Field Study

Soil Treatment	Sample Number	Rep.	Time Interval (weeks)	Diesel Range ppm	Motor Oil Range ppm	Diesel+Oil ppm	Mean Diesel+Oil	Standard Deviation	Surrogate % Recovery
Truck Bay	1	A	0	240	350	590			124
Control	1	B	0	290	970	1260			D
	1	C	0	300	1100	1400	1083	433	D
	1	A	9	320	1000	1320			D
	1	B	9	420	1300	1720			50
	1	C	9	330	990	1320	1453	231	D
	1	A	16	180	610	790			90
	1	B	16	220	690	910			80
	1	C	16	160	500	660	787	125	65
Truck Bay	2	A	0	300	1000	1300			D
Myco-	2	B	0	210	710	920			D
remediated	2	C	0	300	1100	1400	1207	253	D
	2	A	9	340	930	1270			D
	2	B	9	340	820	1160			90
	2	C	9	200	560	760	1063	268	75
	2	A	16	300	800	1100			90
	2	B	16	230	860	1090			90
	2	C	16	290	740	1030	1073	38	80
Truck Bay	3	A	0	250	440	690			112
Enhanced	3	B	0	200	480	680			113
Bacteria	3	C	0	220	390	610	660	44	110
	3	A	9	330	750	1080			85
	3	B	9	450	1200	1650			D
	3	C	9	340	840	1180	1303	304	D
	3	A	16	220	730	950			NR
	3	B	16	190	820	1010			NR
	3	C	16	210	810	1020	993	38	NR
Truck Bay	4	A	0	300	510	810			113
WSDOT	4	B	0	290	430	720			124
Remediation	4	C	0	240	340	580	703	116	120
	4	A	9	320	800	1120			D
	4	B	9	230	510	740			50
	4	C	9	320	670	990	950	193	80
	4	A	16	250	890	1140			NR
	4	B	16	280	780	1060			NR
	4	C	16	240	940	1180	1127	61	NR

Table E.1 Contd

Soil Treatment	Sample Number	Rep.	Time Interval (weeks)	Diesel Range ppm	Motor Oil Range ppm	Diesel+Oil ppm	Mean Diesel+Oil	Standard Deviation	Surrogate % Recovery
Diesel	5	A	0	120	70	190			119
Control	5	B	0	150	87	237			109
	5	C	0	74	38	112	180	63	115
	5	A	9	76	130	206			92
	5	B	9	150	48	198			86
	5	C	9	100	100	200	201	4	85
	5	A	16	66	190	256			127
	5	B	16	130	440	570			72
	5	C	16	140	220	360	395	160	137
Diesel	6	A	0	98	140	238			107
Mycoremedia	6	B	0	81	38	119			129
	6	C	0	110	30	140	130	15	108
	6	A	9	89	180	269			108
	6	B	9	16	45	61			74
	6	C	9	150	130	280	188	106	109
	6	A	16	73	56	129			100
	6	A	16	110	170	280			134
	6	B	16	130	240	370	260	122	131
Diesel	7	A	0	88	43	131			108
Enhanced	7	B	0	82	97	179			104
Bacteria	7	C	0	160	19	179	163	28	114
	7	A	9	94	89	183			62
	7	B	9	120	200	320			98
	7	C	9	200	360	560	354	191	96
	7	A	16	85	130	215			110
	7	B	16	140	200	340			122
	7	C	16	160	240	400	318	94	149
Diesel	8	A	0	88	120	208			103
WSDOT	8	B	0	82	51	133			99
Remediation	8	C	0	100	58	158	166	38	121
	8	A	9	170	150	320			100
	8	B	9	180	230	410			102
	8	C	9	120	190	310	347	55	98
	8	A	16	87	190	277			138
	8	B	16	87	160	247			153
	8	C	16	71	140	211	245	33	98.2

Table E.1 Contd

Soil Treatment	Sample Number	Rep.	Time Interval (weeks)	Diesel Range ppm	Motor Oil Range ppm	Diesel+Oil ppm	Mean Diesel+Oil	Standard Deviation	Surrogate % Recovery
Gasoline	9	A	0	320	270	590			108
Control	9	B	0	1100	1800	2900			D
	9	C	0	1800	250	2050	1847	1168	D
	9	A	9	410	390	800			100
	9	B	9	190	250	440			93
	9	C	9	380	410	790	677	205	96
	9	A	16	120	260	380			113
	9	B	16	170	280	450			NR
	9	C	16	220	360	580	470	101	126
Gasoline	10	A	0	300	360	660			130
Myco-	10	B	0	270	410	680			125
remediated	10	C	0	270	330	600	647	42	110
	10	A	9	460	710	1170			D
	10	B	9	700	1400	2100			D
	10	C	9	220	450	670	1313	726	125
	10	A	16	440	1500	1940			NR
	10	B	16	220	540	760			126
	10	C	16	190	420	610	1103	728	NR
Gasoline	11	A	0	200	240	440			101
Enhanced	11	B	0	280	370	650			126
Bacteria	11	C	0	440	390	830	640	195	117
	11	A	9	290	520	810			124
	11	B	9	280	460	740			112
	11	C	9	260	430	690	747	60	115
	11	A	16	240	560	800			75.2
	11	B	16	200	560	760			60.8
	11	C	16	180	510	690	750	56	72
Gasoline	12	A	0	250	240	490			116
WSDOT	12	B	0	270	260	530			120
Remediation	12	C	0	370	280	650	557	83	114
	12	A	9	310	500	810			116
	12	B	9	520	1000	1520			150
	12	C	9	250	400	650	993	463	115
	12	A	16	160	420	580			76.8
	12	B	16	180	600	780			70.4
	12	C	16	160	320	480	613	153	90.8

Table E.2 Quality Assurance Data for the WSDOT Field Study

Soil Treatment	Sample Number	Rep.	Time Interval (weeks)	Diesel Range ppm		Motor Oil Range ppm		Diesel+Oil ppm	RPD	Surrogate % Recovery
	Blank	A	0	5	U	10	U			112
	Blank	B	0	5	U	10	U			121
	Blank	A	9	5	U	10	U			76
	Blank	A	16	5	U	10	U			107
	Blank	B	16	5	U	10	U			95
Duplicates										
	6	C	0	110		30		140		108
	6	C	0	140		110		250	56%	126
	11	A	0	200		220		420		101
	11	A	0	180		240		420	0%	135
	4	A	9	320		800		1120		D
	4	A	9	350		830		1180	5%	D
	10	C	9	220		450		670		125
	10	C	9	210		490		700	4%	130
	3	B	16	190		820		1010		NR
	3	B	16	210		580		790	24%	NR
	4	B	16	280		780		1060		NR
	4	B	16	220		790		1010	5%	NR
	8	B	16	87		160		247		153
	8	B	16	73		110		183	30%	139
	12	A	16	160		420		580		76.8
	12	A	16	160		310		470	21%	136

Table E.2 Contd

Soil Treatment	Sample Number	Rep.	Time Interval (weeks)	Sample Conc.	Amount Spiked	Sample Conc. + Amount Spiked	Percent Recovery	RPD
Matrix Spike								
	1	B	0	290	125	605	252	
	1	B	0	290	125	474	147	53
	12	A	0	248	126	391	113	
	12	A	0	248	126	391	113	0
	1	A	9	319	115	451	115	
	1	A	9	319	115	410	79	37
	7	C	9	199	127	264	51	
	7	C	9	199	128	344	113	76
	4	A	16	247	112	383	121	
	4	A	16	247	112	365	105	14.5
	8	C	16	70.8	112	191	107	
	8	C	16	70.8	112	198	114	6

			Spike Found	Amount Spiked		Percent Recovery	
LCS							
		0	112	100		112	
		0	100	100		100	
		9	93.8	100		93.8	
		16	109	100		109	

APPENDIX F
Mesocosm and Toxicity Results

Appendix F

Table F.1 Chemistry Results from the Mesocosm Study using the NW-TPH D Extended Analysis, Weeks 0 and 4

Soil Treatment	Sample Number	Rep.	Time Interval (Weeks)	Diesel Range ppm		Motor Oil Range ppm		Diesel+Oil ppm		Mean Diesel+Oil	Standard Deviation	Surrogate Recovery (Percent_
W058 MB	Blank			5	U	10	U	15	U			95
Truck bay control	9	a	0	1900		3800		5700				170
	9	b	0	2400		4200		6600				150
	9	c	0	2100		3600		5700		**6000**	**520**	130
Truck bay mycoremediated	11	a	4	1900		4800		6700				160
	11	b	4	2000		5800		7800				130
	11	c	4	2200		4400		6600		**7033**	**666**	180
Truck bay control	9	a	16	2200		6200		8400				NR
	9	b	16	2200		7000		9200				NR
	9	c	16	2200		6400		8600		**8733**	**416**	NR
Truck bay mycoremediated	11	a	16	1800		5100		6900				NR
	11	b	16	1600		4900		6500				NR
	11	c	16	1700		4700		6400		**6600**	**265**	NR
Gasoline control	12	a	0	520		540		1060				1002
	12	b	0	470		570		1040				104
	12	c	0	510		710		1220		**1107**	**99**	100
Gasoline mycoremediated	10	a	4	690		1200		1890				190
	10	b	4	540		830		1370				170
	10	c	4	530		930		1460		**1573**	**278**	170
Gasoline control	12	a	16	290		640		930				NR
	12	b	16	320		700		1020				NR
	12	c	16	340		720		1060		**1003**	**67**	NR
Gasoline mycoremediated	10	a	16	94		190		284				76
	10	b	16	95		150		245				65.2
	10	c	16	73		140		213		**247**	**36**	71.6
Diesel control	2	a	0	150		230		380				120
	2	b	0	110		120		230				89
	2	c	0	310		320		630		**413**	**202**	155
Diesel mycoremediated	13	a	4	120		200		320				97
	13	b	4	110		170		280				96
	13	c	4	68		98		166		**255**	**80**	94
Diesel control	2	a	16	160		420		580				72
	2	b	16	86		270		356				42
	2	c	16	90		260		350		**429**	**131**	41
Diesel mycoremediated	13	a	16	120		160		280				61.6
	13	b	16	79		110		189				61
	13	c	16	120		140		260		243	48	55

Table F.2 **Quality Assurance Data: mesocosms**

	Sample No		Time	Diesel	Motor Oil			
blank				5 U	10U			
duplicates	2	b		86	270			
	2	b		90	260			
	11	b		1600	4900			
	11	b		1700	5900			
				sample conc.	**amount spiked**	**sample conc+spike**	**percent recovery**	**rpd**
matrix spike	2	a		163	102	249	84.3	
	2	a		163	102	239	74.5	12.4
				spike found	**amount spiked**		**percent recovery**	
LCS				99.2	100		99.2	

Table F.3 **Chemical Analysis of Fruiting Bodies from Selected Samples**

	Sample #	Rep.	Time Interval	Diesel Range ppm	Motor Oil Range ppm	Diesel+Oil ppm	Mean Diesel+Oil	Standard Deviation
Mushrooms								
Truck Bay-Myco	2		4	170	170	Although these samples are within the range		
Gasoline-Myco	6		4	300	230	of diesel and motor oil, the spectra do not		
Diesel-Myco	10		4	200	190	correspond to those of a petroleum product		
Diesel-Myco	10 dup		4	190	160	likely other organic products		

Table F.4. Summary of the *Eisenia foetida* 14-Day Test

		Test Initiation (Day 0, August 6, 1998)				Test Termination (Day 14, August 20, 1998; weights Aug 21)						Summary of Results			
Treatment	Replicate	Number Worms Initiated	Initial Weight (g)	Mean Individual Weight (mg/worm)	Treatment mean g/worm	Number Worms Surviving	Final Weight (g)	Mean Individual Weight (mg/worm)	Treatmentmean g/worm	Percent Survival	Weight Loss per worm per Rep (mg)	Mean Percent Survival	Survival sd	Mean Weight Change per worm per Treatment (mg)	Weight Loss sd
Study Site Control	1	10	3.85	385		10	3.00	300		100%	85				
Study Site Control	2	10	3.96	396	399	10	2.77	277	291	100%	119	100%	0%	108	19.6
Study Site Control	3	10	4.15	415		10	2.96	296		100%	119				
Mycoremediated	1	10	4.17	417		11	3.22	293		110%	124				
Mycoremediated	2	10	3.75	375	404	10	3.10	310	300	100%	65	103%	6%	104	34.1
Mycoremediated	3	10	4.21	421		10	2.97	297		100%	124				
Enhanced Bacteria	1	10	3.91	391		10	2.52	252		100%	139				
Enhanced Bacteria	2	10	3.83	383	381	10	2.64	264	251	100%	119	77%	40%	130	10.3
Enhanced Bacteria	3	10	3.70	370		3	0.71	237		30%	133				
WSDOT Bioremediation	1	10	3.93	393		10	2.65	265		100%	128				
WSDOT Bioremediation	2	10	3.88	388	378	9	2.22	247	252	90%	141	93%	6%	126	16.4
WSDOT Bioremediation	3	10	3.52	352		9	2.19	243		90%	109				
Plant Control-Potting Soil	1	10	3.58	358		10	2.71	271		100%	87				
Plant Control-Potting Soil	2	10	4.18	418	392	10	2.87	287	277	100%	131	100%	0%	115	24.6
Plant Control-Potting Soil	3	10	4.01	401		10	2.73	273		100%	128				
Control: Artificial Soil	1	10	3.33	333		12	2.97	248		120%	86				
Control: Artificial Soil	2	10	4.42	442	383	11	3.28	298	274	110%	144	110%	10%	109	30.5
Control: Artificial Soil	3	10	3.75	375		10	2.76	276		100%	99				
Peat Moss	1	10	4.05	405		10	2.73	273		100%	132				
Peat Moss	2	10	3.88	388	385	10	2.67	267	267	100%	121	100%	0%	118	15.2
Peat Moss	3	10	3.63	363		10	2.61	261		100%	102				

Figure F.1. *Eisenia foetida* Weight Loss

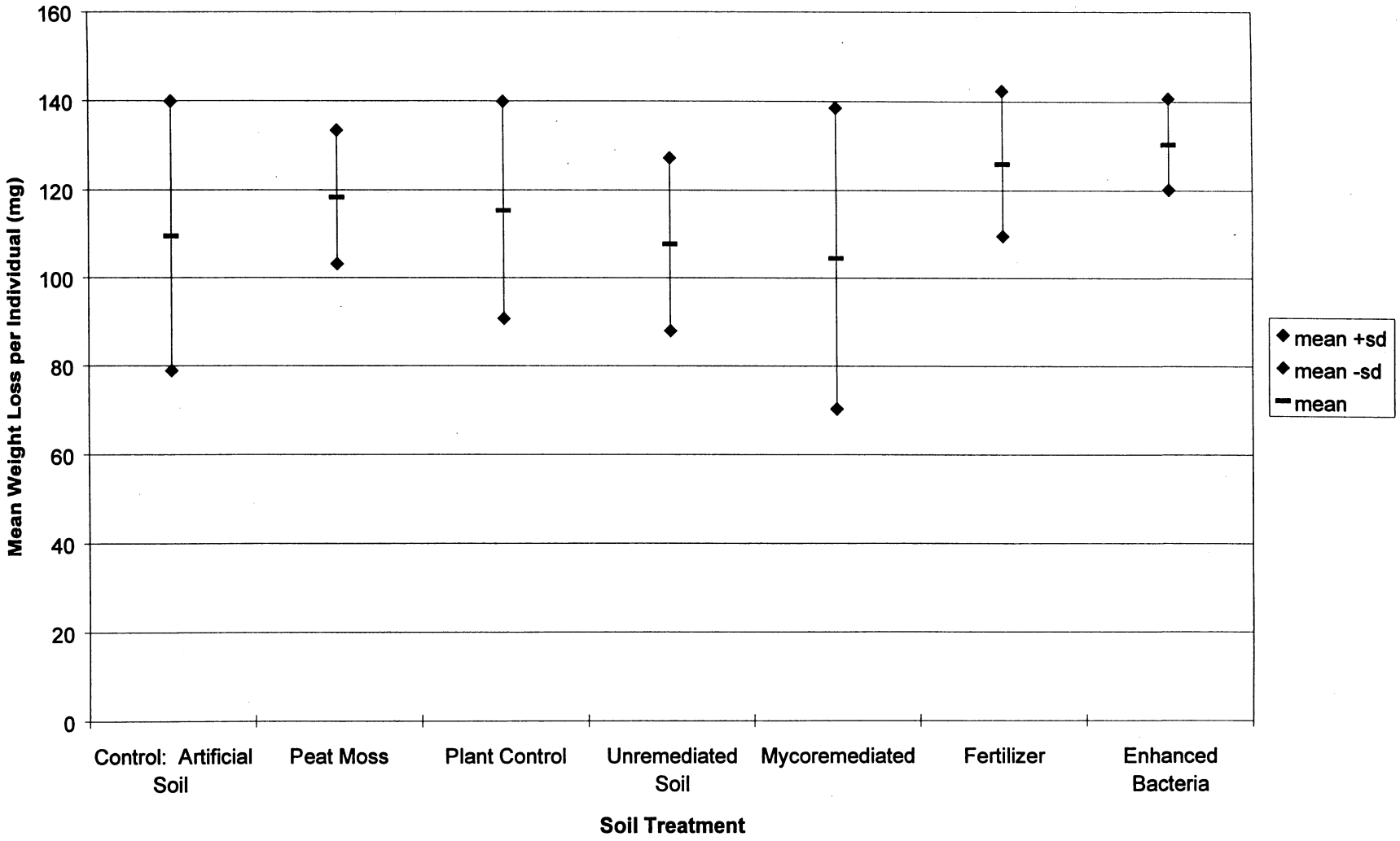


Figure F.2. *Eisenia foetida* Survival

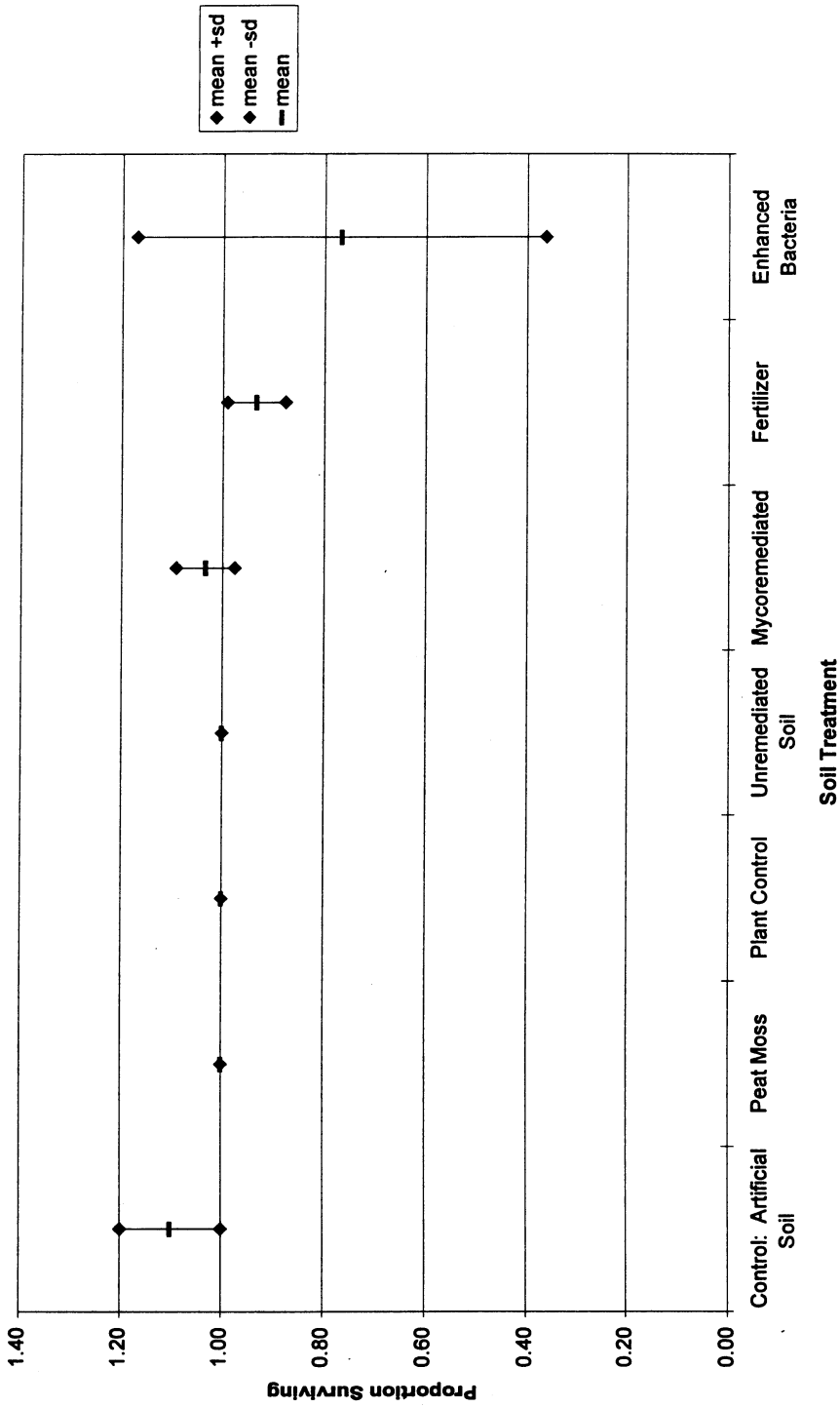
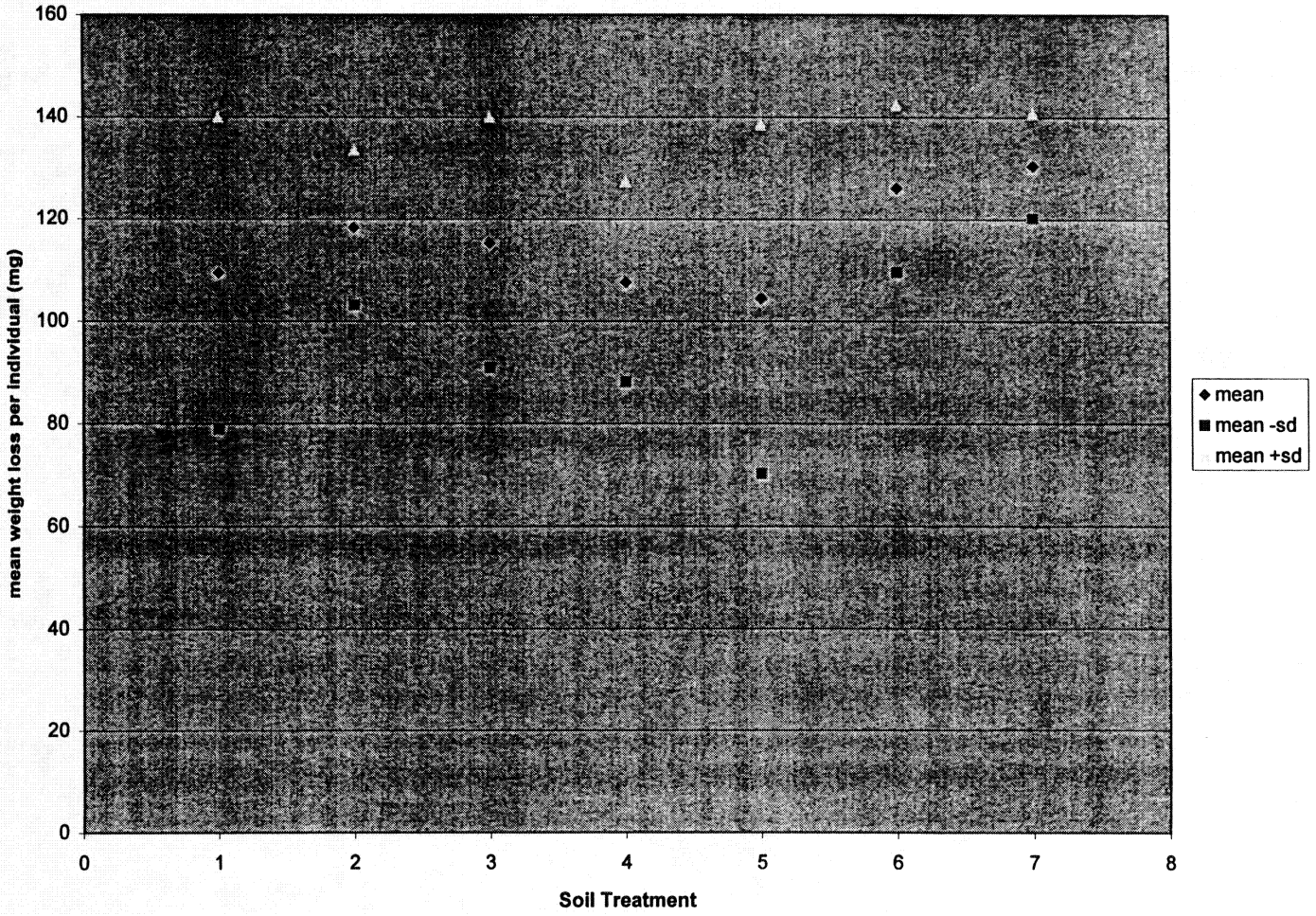


Figure F.3. *Eisenia foetida* Weight Loss



Results of Plant Toxicity Tests

The results from the plant toxicity tests described in Appendix D are presented in this section. Photographs and tables are used to describe and quantify the results. The physical experimental setup is shown in Figures F.4 and F.5. The three, selected Washington native plants (two shrubs and one grass) were grown in commercial potting soil as a reference control; the same three species were set into the control and remediated samples of each test soil from the field study at Bellingham maintenance yard.

Plant mortality was examined at intervals during the experiment and at the termination of the experiment on 5 October 1998. The experiment was continued past the 4 weeks planned in the experimental design to allow observation of a more complete cycle of growth. The experiment was terminated when the shrubs were beginning to exhibit autumn leaf-drop, and the grass, *Festuca idahoensis,* showed evidence of becoming root-bound in the 6-inch test pots.

Table F.4. *Festuca idahoensis* Toxicity Results (difference from control plant growth)

	Truck Bay	Diesel	Gasoline
		Growth (cm)/SD (cm)	
Control	4.1/0.7	1.1/0.7	1.7/0.5
Mycoremediation	3.0/0.5	1.9/0.4	1.4/0.1
Enhanced Bacteria	4.1/1.1	3.1/1/3	2.1/0.3
Bioremediation	4.9/0.6	3.2/0.7	3.1/0.8

Note: all *Festuca* plants had reached the maximum growth allowed by the space for roots in their containers.

It appeared that there was slightly better growth in the *Festuca idahoensis* in the bioremediated and enhanced bacteria-treated diesel- and gasoline-contaminated soils, possibly due to the addition of fertilizers in the course of these treatments. In the truck bay soil, there was no significant difference between the control, and the bioremediation and bacterial treatments. Figures F.6 through F.8 show *F. idahoensis* at termination of the experiment. *F. idahoensis* at 4 weeks into the experiment did not show as much contrast in size range as it did at the termination of the experiment on 5 October 1998. Grasses such as *Festuca* and *Poa* have been used in phytoremediation applications to address petroleum hydrocarbons (for example, Drake 1997).[6]

At the 4-week observation, the elderberry shrub, *Sambucus cerulius,* in potting soil (Figure F.9), and two out of three of the untreated (control) truck bay soil plants (Figure F.10) were alive, and all of the plants in mycoremediated truck bay soil were growing well (Figure F.11). At termination of the experiment at Week 11, many of the *S. cerulius* had died back. Although there had been no frost, we might attribute it to the seasonal cycle of the plant, if not to mortality. The plants in the bioremediated truck bay soil (Figure F.12) and in enhanced bacterial-treated truck bay soil (F.13) had all died after 11 weeks. *S. cerulius* appeared to be the most sensitive plant in these soils and treatments; this suggests that it could be a good indicator species for toxicity.

At termination of the experiment, the *Physocarpus capitatus* exhibited mortality or autumn dieback to the extent that insufficient data were available for statistical comparison among soil treatments (Figures F.14 - F.16). All of the plants on mycoremediated soils survived. For the

[6] Drake (1997) has shown these grasses to grow adequately in petroleum-contaminated soils, and to work in combination with the fungi and bacteria of the rhizosphere (zone around the plant roots) to degrade some of the petroleum components; treatment requires 1 to 3 years.

enhanced bacteria treatment, all of the plants in truck bay soil died, but there was complete survival in the gasoline-contaminated soil, and one plant died in the diesel-contaminated soil. All the plants in the bioremediated truck bay soil died, but in the diesel-contaminated soil, all survived, and in the gasoline-contaminated soil, one plant died. In general, for the surviving plants, there was little change in growth, leaf size, and number of leaves from the beginning of the experiment.

At Week 4, however, the differences in *P. capitatus* survival were much more pronounced (Figures F.17-F.20). *P. capitatus* in potting soil (Figure F.17) and *P. capitatus* in mycoremediated truck bay soil (Figure F.18) are comparable in health and growth. Here, there was no mortality and only slight chlorosis. In the enhanced bacteria treatment of truck bay and untreated control gasoline-contaminated soil, most plants were in poor health, or already dead (Figures F.19 - F.20). Those on enhanced bacteria treated gasoline-contaminated soil suffered mild to severe chlorosis at that time, but those in bioremediated gasoline-contaminated soil showed leaf browning and loss, and dieback of stems (Figure F.22). All of the other *P. capitatus* at this time appeared to be healthy.

In conclusion, these plant tests captured details of the toxicity of the contamination in the soils with and without remediation treatments to supplement information gained in other portions of the project. The test demonstrated that it is possible to enhance the availability, and hence the toxicity, of components of the petroleum hydrocarbons in the soil through a remediation treatment. This may have created the conditions that resulted in the mortality in the enhanced bacterial remediation treatment of the truck bay soil. Since the ultimate goal of the remediation is to produce a usable product for roadside landscaping or fill, plant toxicity tests that can be run economically over 3-4 weeks might offer a means of suitability testing of the remediated soils before they are returned to use in the open environment.

Reference

Drake, E. 1997. Phytoremediation of aged petroleum hydrocarbons in soil. IBC's Second Annual Conference on Phytoremediation, 18-19 June 1997, Seattle, Washington.

Figure F.4. Reference plants in potting soil at Time 0

Figure F.5. Experiment set up at Time 0

Figure F.6. Week 11 *Festuca idahoensis* on potting soil and truck bay test soils (1 untreated control, 2 myco-remediation, 3 bioremediation, and 4 enhanced bacterial) from left to right

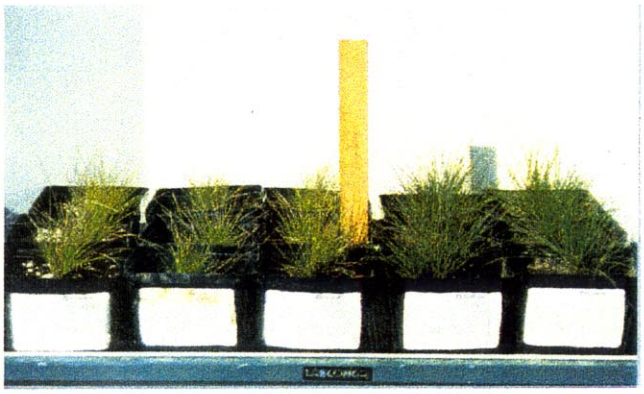


Figure F.7. Week 11 *F. idahoensis* on potting soil and diesel-contaminated test soils (5 untreated control, 6 mycoremediation, 7 bioremediation, and 8 enhanced bacteria treatment) from left to right

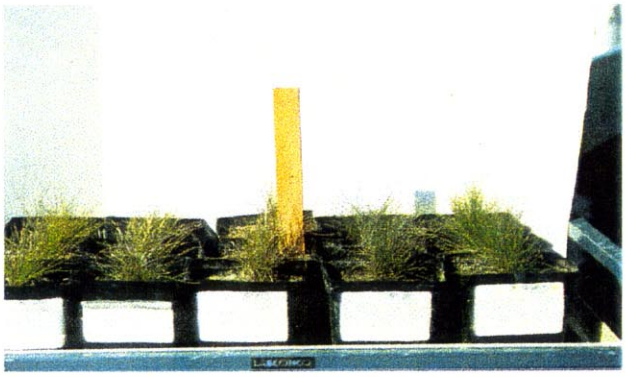


Figure F.8. Week 11 *F. idahoensis* on potting soil and gasoline-contaminated test soils (9 untreated control, 10 mycoremediation, 11 bioremediation, and 12 enhanced bacteria treatment) from left to right

Figure F.9. *Sambucus cerulius* plants in potting soil at 4 weeks

Figure F.10. *S. cerulius* on untreated truck bay control soil at 4 weeks

Figure F.11. *S. cerulius* on mycoremediated truck bay soil at 4 weeks

Figure F.12. *Sambucus cerulius* on bioremediated truck bay soil at 11 weeks

Figure F.13. *S. cerulius* on enhanced bacteria treated truck bay soil at 11 weeks

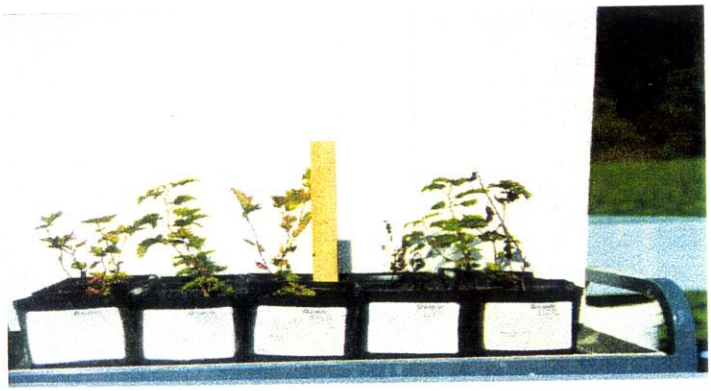


Figure F.14. Week 11 *Physocarpus capitatus* in potting soil and truck bay test soils (1 untreated control, 2 mycoremediation treatment, 3 bioremediation, and 4 enhanced bacterial treatment) from left to right

Figure F.15. Week 11 *Physocarpus capitatus* in potting soil and diesel-contaminated test soils 5-8 (5 untreated control, 2 mycoremediation, 3 bioremediation, and 4 enhanced bacterial treatment) from left to right

Figure. F.16. Week 11 *P. capitatus* in potting soil and gasoline-contaminated test soils 9-12 (9 untreated control, 10 mycoremediation, 11 bioremediation, and 12 enhanced bacterial treatment) from left to right

Figure F.17. Week 4 *P. capitatus* in potting soil

Figure F.18. *P. capitatus* in mycoremediated truck bay soil

Figure F.19. *P. capitatus* in enhanced bacterial treated truck bay soil

Figure F.20. *P. capitatus* in untreated gasoline-contaminated soil.

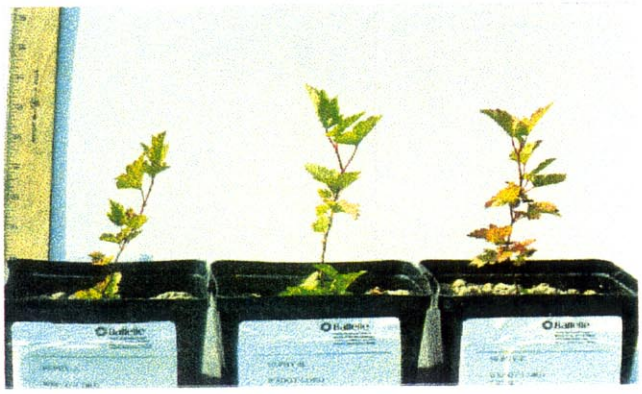


Figure F.21. *P. capitatus* in enhanced bacteria treated gasoline-contaminated soil

Figure F.22. *P. capitatus* in bioremediated gasoline-contaminated soil