



Technology Demonstration Final Report

Field Demonstration of Rhizosphere-Enhanced Treatment of Organics-Contaminated Soils on Native American Lands with Application to Northern FUD Sites

**USA Engineer Research and Development Center
Cold Regions Research and Engineering Laboratory**

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Acronyms

ADEC	Alaska Department of Environmental Conservation
BTEX	benzene, toluene, ethylbenzene, and xylene
DoD	Department of Defense
DRO	diesel-range organic
EPA	Environmental Protection Agency
FAA	Federal Aviation Administration
FSH	fraction specific hydrocarbon
FUD	formerly used defense site
GC	gas chromatography
GDD	growing degree-days
GRO	gasoline-range organics
HRGC/FID	high-resolution gas chromatography flame ionizing detection
HRGC/MS	high-resolution gas chromatography mass spectrometry
MPN	most probable number
NARL	Naval Arctic Research Laboratory
NOAA	National Oceanographic and Atmospheric Administration
PAHs	polycyclic aromatic hydrocarbons
PCBs	polychlorinated biphenyls
PCE	perchloroethylene
POLs	petroleum, oils, and lubricants
RTDF	Remediation Technologies Development Forum
TCE	trichloroethylene
TPH	total petroleum hydrocarbon
TPHCWG	Total Petroleum Hydrocarbons Criteria Working Group
UIC	Ukpeagvik Inupiat Corporation
VOCs	volatile organic compounds

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Executive Summary

The problem that these ESTCP demonstrations addressed is surface soil contaminated with petroleum, located at remote sites, covering large areas, and in cold climates. There are many such Department of Defense (DoD) sites in Alaska. These sites are generally not easily accessible, thus increasing the costs of mobilization and demobilization, have limited infrastructure to support traditional cleanup, are subject to harsh winters, causing equipment failures. Conventional cleanup strategies are sufficiently costly to limit their use, yet there are few alternatives.

These results are the first cold-regions data, and some of the few field data available, that use scientifically defensible techniques to confirm that plants have a positive effect on petroleum depletion relative to either nutrients alone or control treatments. The data all show that plant-associated effects do occur, but not uniformly for all petroleum fractions, and that the effects are greatest for more recalcitrant petroleum fractions. Rhizosphere-enhanced treatment of surface soils is a long-term strategy, and using standard analysis techniques to monitor sites may lead users to conclude that rhizosphere treatment is not working. Significantly, these field data support both theory and laboratory data.

We evaluated rhizosphere-enhanced remediation as a possible effective solution. Rhizosphere-enhanced remediation is a subset of phytoremediation, which includes techniques for many contaminants and many remediation mechanisms. These demonstrations were specific for petroleum in surface soils. The mechanisms active in rhizosphere-enhanced remediation are based on enhanced microbial activity at the root-soil interface. For petroleum remediation, plant uptake is not thought to be significant. Rather, the process is believed driven by analogue enrichment from carbon compounds released by the root or during periods of plant senescence, possibly by greater contaminant solubility due to biosurfactants or pH changes near the root surface, and by “pseudo-mixing” of soil due to root exploration.

The benefits of rhizosphere-enhanced remediation include low costs, applicability to large acreages, minimal or no infrastructure needs, self-repairing nature of plant growth, generally high public acceptance, and reduced dust and runoff. It may be an option for surface-soil treatment for situations where no other options exist, such as remote sites and ranges, or at other sites where costs limit options, such as Brownfields.

Limitations of rhizosphere-enhanced remediation are that it only addresses shallow depths influenced by root penetration. The contaminants in these situations tend to be widely dispersed and concentrations highly variable. Surface soils are not well mixed, as is the case for groundwater systems. Additionally, surface soils have highly variable conditions, such as temperature, water availability, and alternative carbon sources. These all combine to affect biological activity. Consequently, rhizosphere-enhanced remediation is not fast. Combining surface soil variability and the heterogeneous nature of contaminant distribution, it is difficult to characterize concentrations or changes in concentrations. Accordingly, monitoring may require a change in approach and a thorough understanding of how the system works. Monitoring may be thought of as

asking, “Is it working?” rather than “Are concentrations within regulatory guidance?” Treatment times probably will be years rather than weeks, months, or seasons.

Variable results for using rhizosphere-enhanced remediation are reported in the literature. Possible causes for this include: 1.) Its use is not always appropriate and it may not have an effect. This could occur when the soil that is “healthy” to begin with and adding plants does not address a limitation. In fact, plants could compete for resources and inhibit contaminant loss. 2.) Studies may have been conducted for too short a duration to observe changes among treatments or relative to controls. 3.) Inappropriate or ineffective monitoring was used. 4.) Inappropriate or ineffective sampling was used. 5.) There was insufficient replication to yield the precision needed to measure effects obtained within the duration of the experiment. 5.) An insensitive or inappropriate monitoring variable was used.

The objective of these demonstrations was to provide data to confirm or refute rhizosphere-effects on surface soil petroleum contamination. We used statistically defensible, replicated designs based on current and emerging science. Each field demonstration included control treatments, and demonstrations were designed to separate plant effects from fertilizer effects. Sites were selected to incorporate a wide range of climatic conditions representative of a variety of remote sites. We used and evaluated monitoring techniques for later application to field use.

Our approach employed a replicated, full factorial design conducted at field demonstration sites at Barrow, Galena-Campion, and Annette Island, Alaska. Composite samples and premixed “soil sock” composite samples were used to reduce variability. We monitored not just TPH, but also fraction specific hydrocarbons (FSH), and summed polynuclear aromatic compounds (PAHs). Resulting concentration data were normalized to a recalcitrant biomarker to reduce concentration and spatial variability. Biomarker-normalized data were then normalized to local climate using a growing degree-day (GDD) concept to reduce temperature effects on biological processes. Microbial monitoring, using culturable phospholipid fatty acids were conducted to relate changes on microbial communities to the status of contaminant degradation.

Our results confirm that: 1.) Plants have a positive effect on petroleum depletion relative to either nutrients alone or control treatments. 2.) The effect is not uniform across all petroleum fractions. 3.) The effect is not seen by standard monitoring techniques. 4.) Nutrients alone can have an inhibitory effect on depletion of some petroleum fractions. 5.) There are measurable microbial changes that support, and probably drive, the contaminant changes.

Lessons learned during these field demonstrations are applicable to field application practices. Although implementation is relatively straightforward, unfortunately, so are ineffective or incorrect implementation steps. 1.) The system must be limited by something that the plants will address. Soils with high fertility and high organic matter may not respond to plant effects. 2.) Excessive fertilizer can limit seed germination, plant growth, and microbial activity. 3.) Plants that look poor based on above ground observation may have effective root systems for remediation. Rhizosphere-enhanced remediation is based on stimulating microbial activity at the root-soil interface rather

than above ground growth. 4.) Surface soil remediation probably is not linear. Remediation varies with the recalcitrance of the compounds, the status of the remediation processes, and the conditions in the soil. Monitoring should consider these factors. 5.) Consideration should be given to altering the monitoring strategy to fit the technology being used. Suggestions include sampling strategies, such as “when” to sample with respect to the status of the system rather than our calendars; “how” to sample with respect to the variable we are using to monitor; and selection of a variable to monitor that is changing at the stage of remediation. The appropriate variable may vary with the degree of “completeness” of the remediation process.

Interpretation of the data is critical. We used comparisons to other treatments and controls to evaluate rhizosphere enhancement. These comparisons will not be available in routine implementation. Databases with effective metadata, such as the EPA-RTDF and the University of Saskatchewan's *PhytoPet* for plant information, may prevent users from improper implementation.

Rhizosphere-enhanced remediation may have applications in other situations, such as overseas installations or US training ranges, where the constraints, although driven by different issues, results in similar remediation limitations, or for remediating Brownfields.

Field Demonstration of Rhizosphere-Enhanced Treatment of Organics-Contaminated Soils on Native American Lands with Application to Northern FUD Sites

USA Engineer Research and Development Center Cold Regions Research and Engineering Laboratory

April 2004

Purpose of this Document

This report was prepared to document the results of ESTCP project #1011, "Field Demonstration of Rhizosphere-Enhanced Treatment of Organics-Contaminated Soils on Native American Lands with Application to Northern FUD Sites". Rhizosphere-enhanced remediation may be a feasible remediation technology useful as an alternative to more traditional remediation technologies and applicable at many remote locations. This document describes rhizosphere-enhanced remediation of petroleum hydrocarbons in northern climates. In these areas, petroleum releases are often the most prevalent contaminant issue due to widespread petroleum use during the cold war era and the quantities that were accidentally released during this period.

The document includes a brief review of remediation alternatives that could be used at remote sites, with a more thorough review and description of rhizosphere-enhanced remediation. The steps used in implementing rhizosphere-enhanced remediation are presented along with data from three field demonstration sites.

Rhizosphere-enhanced remediation is a developing technology. It is a subset of phytoremediation, a term that is often used in a broad sense and sometimes used inappropriately or too generally because phytoremediation encompasses a wide range of processes. The operative process in phytoremediation depends largely on the contaminant and can include:

- plant uptake and accumulation
- plant uptake and biological transformations in the plant

- plant uptake and possibly transpiration into the atmosphere
- hydrologic control of contaminated groundwater caused by high levels of plant-driven transpiration

The Environmental Protection Agency¹ and Interstate Technology and Regulatory Cooperation² web sites provide recent reviews of phytoremediation.

For the situation that we addressed—petroleum compounds in near-surface soils—the generally accepted mechanism is microbial degradation that is enhanced in the rhizosphere—the soil immediately adjacent to and affected by plant roots.

1. Introduction

1.1 Background Information

This project included field demonstrations of rhizosphere-enhanced bioremediation of petroleum, oils, and lubricants (POLs) at three cold-region locations. The demonstrations evaluated the use of rhizosphere-enhanced remediation for treating POL-contaminated soils in northern regions where low temperatures, site inaccessibility, permafrost, and freeze-thaw cycles limit or, in many cases, prevent cost-effective application of traditional technologies and a number of emerging innovative technologies. Rhizosphere-enhanced remediation may have application and benefits at cold-region sites and Native American lands where former Department of Defense (DoD) activities have impacted the soil.

1.1.1 The Environmental Problem. Petroleum, oils, and lubricants (POLs) are widespread contaminants at many northern facilities owned, formerly owned, or formerly used by the DoD. In cold regions, POLs and especially the polynuclear aromatic hydrocarbon fraction (PAHs) are persistent in soils due to the low mean annual soil temperatures and the brevity of the summer season. Some constituents in POLs are known human carcinogens.

Cleanup problems are compounded for sites that are in remote, inaccessible areas. The Department of Defense has numerous sites in Alaska that were constructed during World War II and expanded in the ensuing cold-war era where fuel was often transported and stored in 55-gallon drums, resulting in POL releases. At many of these sites, mobilization and demobilization costs are excessive. In some cases, ground transportation is possible only in winter, when the soil is frozen. During the summer, when biotreatment would be feasible, air transportation must be used, but landing sites

¹ http://www.epa.gov/ada/download/issue/epa_540_s01_500.pdf

² <http://www.itrcweb.org/common/content.asp?en=TA863827&sea=Yes&set=Both&sca=Yes&sct=Long>

cannot support larger aircraft. Construction supplies at many facilities were delivered by air during the winter using packed-snow runways.

Many contaminated DoD sites are co-located with Native American population centers and serve as hubs for transportation and communication. In some cases, DoD-related contamination is located on Native American Lands and DoD has responsibilities to clean many of these sites. At other cold-region sites, DoD lands are in caretaker status, awaiting turnover to Native American ownership. Low-cost, effective, and applicable treatment technologies are needed for all of these situations.

1.1.2 The Technology. Phytoremediation is an umbrella term that describes varied uses of plants for the purpose of remediating soil or groundwater. Phytoremediation has shown potential for several applications, all based on plant-driven processes but mechanistically different. These include, but are not limited to, enhancing microbially driven degradation in the rhizosphere, exudation of contaminant analogs by roots and stimulation of specific microbial degradation pathways, and using trees either to control the hydrology, or to take up and degrade trichloroethylene (TCE), or both.

Rhizosphere-enhanced remediation is based on root exudation of excess plant-produced carbon compounds, which stimulate the soil microbial population near root surfaces, which in turn stimulates bioremediation. The technology consists primarily of adding appropriate seeds and nutrients to the contaminated soil to grow plants that, in turn, stimulate rhizosphere activity. It thus requires minimal equipment and costs for setup, operation and maintenance, or shut down. Our demonstrations include seeding and fertilization of cold-tolerant grasses and legumes in POL-contaminated soils at three locations in Alaska.

1.1.3 The Benefits. The expected benefits of implementing rhizosphere-enhanced bioremediation are:

1. Costs may be reduced dramatically in treating sites that are remote from infrastructure such as roads, power, and transportation.
2. Rhizosphere-enhanced treatment can be used at active installations, releasing scarce cleanup resources for more urgent contaminated sites.
3. The technology avoids the mechanical problems caused by freezing temperatures.
4. Human and environmental risks related to POL-contaminated soils will be reduced at these sites.

The ultimate application is to be able to add appropriate nutrients and seed to a contaminated site and have reasonable assurance, based on defensible data, of the

treatment rates and endpoints. For sites in cold regions, implementing rhizosphere-enhanced treatment may significantly increase treatment rates, thereby reducing treatment times. The degree of improvement likely depends on the growing season length and the recalcitrance of the compound. Although we have demonstrated relatively short treatment times of one to three summers in some situations, in other situations the benefit may be that significant treatment is accomplished in five to ten years rather than not at all.

1.2 Objectives of the Demonstration

The objective of rhizosphere-enhanced remediation was to treat POL-contaminated soils in northern regions where low temperatures, site inaccessibility, and freeze-thaw cycles limit or prevent cost-effective application of either traditional technologies or emerging innovative technologies. In this study, we demonstrated and validated the ability of cold-tolerant plants, nutrient additions, and their combination to remediate POL-contaminated soils in cold regions. Data from the field demonstration sites have been evaluated to determine the effect of these factors on soil concentrations of POLs at three geographically diverse sites in Alaska: Annette Island (southern), Galena-Campion (interior), and Barrow (North Slope). We documented seeding, monitoring, and site-specific conditions for each location under which the technology was applied. We evaluated the technology in terms of its overall cost, regulatory acceptance, and the practicality of implementation.

1.3 Regulatory Drivers

This project addressed cleanup and restoration of contaminated soils resulting from DoD activities on Native American lands. It also addressed cleanup requirements developed by user groups within DoD for (1.3.b) On-Site Treatment of Organics Contaminated Soils and (1.3.m) Soil Bioremediation. Native American Communities and a Native American owned small businesses, ClearWater Environmental Services, Incorporated, were partners in the demonstrations at the Annette Island and Campion sites. At Annette Island, we coordinated closely with the Metlakatla Indian Community, and they were active partners in site selection. We sought assistance from Ilisagvik College in Barrow, Alaska, but were unable to develop an active partnership.

The state regulations that apply to this technology are those for petroleum-contaminated soils. These regulations generally address sampling frequency and protocols, but were developed to address more aggressive remediation technologies where treatments effects are more readily measured. Regulations for low-cost remediation strategies are still evolving in Alaska as well as many locations in the US.

1.4 Stakeholder / End-User Issues

An important issue for users is scientifically defensible data showing that rhizosphere-enhanced treatment provides a benefit relative to natural attenuation. Lack of these

data has limited acceptance of rhizosphere-enhanced remediation. These data are useful in showing that rhizosphere-enhanced remediation has a measurable and significant impact on treating petroleum-contaminated surface soils using low-cost methods that require minimal maintenance and can be used over large areas. Importantly, they also demonstrate that commonly employed monitoring methods will be insufficient for detecting changes in the contaminant concentrations in surface soils undergoing plant-based treatment. The benefits of these findings are that this plant-based approach does have a positive effect for treating surface soils, and that monitoring methods will need to be adjusted to successfully observe these changes.

2. Technology Description

2.1 Technology Development and Application

Phytoremediation is an umbrella term covering a number of different plant-based methods that can lead to contaminant degradation, removal (through accumulation or dissipation), or immobilization. Terminology is still evolving, yet some uses and terms are now becoming more commonly used. Pivetz (2001) reviewed phytoremediation and defined various phytoremediation methods to include:

1. Degradation (for destruction or alteration of organic contaminants).
 - A. Rhizodegradation: enhancement of biodegradation in the below-ground root zone by microorganisms.
 - B. Phytodegradation: contaminant uptake and metabolism above or below ground, within the root, stem, or leaves.
2. Accumulation (for containment or removal of organic and/ or metal contaminants).
 - A. Phytoextraction: contaminant uptake and accumulation for removal.
 - B. Rhizofiltration: contaminant adsorption on roots for containment and/or removal.
3. Dissipation (for removal of organic and/or inorganic contaminants into the atmosphere).
 - A. Phytovolatilization: contaminant uptake and volatilization.
4. Immobilization (for containment of organic and/or inorganic contaminants).
 - A. Hydraulic Control: control of ground-water flow by plant uptake of water.
 - B. Phytostabilization: contaminant immobilization in the soil.

Rhizosphere-enhanced remediation (or rhizodegradation in 1A above) is a form of phytoremediation based on root exudation of excess plant-produced carbon compounds. The rhizosphere is the zone of soil surrounding a plant root and influenced by the plant root. Typically, the root releases excess carbon molecules produced by the plant and the excess carbon stimulates the nearby soil microbial ecology. Researchers

generally agree that the stimulated microbial activity near the root in turn results in enhanced biotreatment.

Field implementation of rhizosphere-enhanced remediation includes selecting and adding appropriate seeds and nutrients to the contaminated soil to stimulate rhizosphere activity. It requires minimal equipment and costs for set up, operation and maintenance, or shut down. Demonstration plots at the Campion Air Force Station are shown in Figures 1 and 2.

The constraint to application is having defensible field data. Our approach to obtain defensible field data was to conduct replicated, statistically balanced field demonstrations and to obtain meaningful soil samples from the field. Although choosing the appropriate sample analysis is important, research overwhelmingly and clearly demonstrates that, due to the spatial variability of contaminants in the soil, a much greater error arises from field sampling. In brief, the success of representing the situation in the field is limited by obtaining a representative sample from the field rather than the sample analysis.

Our design to demonstrate the efficiency of rhizosphere-enhanced remediation and obtain meaningful samples from the field is described in Section 3 of this document.

2.2 Previous Testing of the Technology

Our earlier laboratory and field studies in Alaska suggested that the rhizosphere effect increases in importance as the recalcitrance of the compound in question increases (Reynolds et al., 1999; Reynolds et al., 2001). Recent carefully conducted and replicated field experiment have shown that significantly greater petroleum reductions can be verified in vegetated plots relative to non-vegetated plots. On many remediation sites, total petroleum hydrocarbon (TPH_{gc}) commonly is used as a dependent or response variable. TPH_{gc} analyses are relatively inexpensive and readily available. TPH_{gc} provides a single value that integrates all peaks and unresolved portions of a chromatogram. The compromise is that TPH_{gc} is not as sensitive as some other measurements. Nevertheless, TPH_{gc} data are useful.

In earlier Alaska field research using soil recently contaminated with diesel, we measured significant TPH_{gc} decreases during a three-year study from plots that had been both vegetated and fertilized. TPH_{gc} losses were greater than the plots receiving only fertilizer or vegetation, and greater than losses from the control treatments. The effects were similar but less dramatic for crude-oil contamination (Reynolds et al., 1997). There is some evidence that the major benefits from the rhizosphere effect, relative to non-vegetated soil, are likely greatest for heavier, more recalcitrant compounds (Reynolds et al., 2001). Resistance to degradation of heavier PAH compounds may result in longer treatment times being required before rhizosphere effects can be measured. Measuring changes in the soil microbiology, although not a

direct measure of contaminant concentration changes, may be a more direct measurement of the underlying mechanisms.

One approach to measuring treatment effects would be to conduct a two-dimensional contaminant spatial characterization at initial and subsequent sampling times. In our prior research at a one-acre landfarm site, we measured contaminant concentrations on a 25-node grid and developed spatial (two-dimensional) concentration profiles at four separate sampling times (Reynolds, 1993). Even though the soil was mechanically tilled approximately every two weeks, half-lives calculated from the concentration data varied by a factor of seven. We have concluded that costs for developing two-dimensional profiles would be prohibitive and the resulting data may not be sufficiently precise to observe changes in concentration.

2.3 Factors Affecting Cost and Performance

The greatest cost for rhizosphere-enhanced bioremediation typically is in sampling and monitoring, and that is specific to the frequency of sampling, the type of analysis done, and cost of analysis per sample. The transport, spreading, seeding, and fertilizing are essentially one-time costs, although some re-seeding may be needed annually, and even some watering may be beneficial during seedling establishment. Annual fertilizer can be added but may not be necessary. Again, this is specific to the site and the goals. We have found that in year two (and even the first season), many volunteer plants established themselves. This is usually beneficial and, in our experience, the vegetation will shift with time to resemble the local vegetation.

Typical sampling and monitoring techniques used for tracking more aggressive treatments are of little use for monitoring rhizosphere-enhanced remediation of contaminated surface soils. Data are too heterogeneous for firm conclusions to be made. Useful tools for obtaining more meaningful data and reducing variability include composite samples, fraction specific hydrocarbon analysis (FSH), biomarker normalization, and temperature normalization. Using these tools for a longer time but with greater intervals between sampling times emerged as a reasonable monitoring plan.

2.4 Advantages and Limitations of the Technology

2.4.1 Advantages. The primary advantages of this technology are cost, ease of implementation, and applicability where others technologies cannot be used, such as surface-contaminated soils covering large areas. Essentially, the costs for implementing rhizosphere-based treatment include:

1. Initial site characterization
2. Permitting
3. Transportation to the site

4. Seed and nutrient procurement and application
5. Monitoring

Because this is a new technology, the frequency and intensity of monitoring are not well defined; two goals of this project were to confirm that rhizosphere-enhancement provided a benefit and to provide initial monitoring techniques and cost data. Cost and performance data are provided in this Final Report and in the Cost and Performance Report.

Cost advantages are valid only in context of comparison to the cost of other alternatives (such as bioreactors, landfarms, and biosparging), which typically involve mobilizing heavy equipment, excavation and handling, on-site operation and maintenance, and demobilization. In extreme winter conditions, mechanical hardware systems must be protected from freezing or operations must be suspended during winter. For remote sites in cold regions, these operational costs can be prohibitive.

Natural attenuation is perhaps the definitive low-cost, passive treatment alternative. It has been successfully demonstrated for BTEX-contaminated groundwater systems where an electron acceptor is present to drive contaminant oxidation. Such systems are relatively well mixed, thereby minimizing enzyme-substrate contact and related mass-transport limitations. However, observations demonstrate that substantial contamination still remains although many World War II era sites in cold-regions have been “naturally attenuating” for over 50 years. Several conditions that occur frequently at older sites in cold regions affect this persistence and may be overcome by rhizosphere-enhanced treatment.

Firstly, for surface spills and the subsequent contamination that characterizes cold regions sites, less soluble—and therefore less mobile—compounds such as PAHs tend to predominate. Volatile fractions frequently have either volatilized or leached. If they have leached, either they are in the groundwater and subject to natural attenuation in a mixed system or they have been retarded by permafrost. The remaining PAHs would not mix within the upper soil to a great extent because there is no driving force for mixing. Recent data have shown that one of the benefits of rhizosphere-enhancement is more effective treatment of heavier compounds, such as PAHs (Reynolds et al., 1999; Reynolds et al., 2001). These data support the hypothesis put forth by Donnelly and Fletcher (1994) that suggests that root exudates may beneficially influence degradation of recalcitrant compounds.

Secondly, if the mean annual temperature is below 0 °C, as is the case in much of Alaska, sites may be underlain by either continuous or discontinuous permafrost, which serves as a natural barrier to leaching. A permafrost barrier may be an advantage for successful rhizosphere treatment as the barrier serves to keep contaminants near the surface of the soil where roots can penetrate.

Thirdly, during the spring and fall transitions between the more constant winter and summer temperatures in Alaska, soil temperatures are much more variable, fluctuating on several scales including diurnal. We have measured significant diurnal fluctuations in root-zone soil temperatures at Fairbanks. The effect of frequent temperature fluctuations on soil microbial activity is not fully understood, but there appears to be a lag time of diminished microbial activity following reduced temperatures. Our working hypothesis is that microbial stimulation from root exudates reduces the lag time following any temporary period of diminished microbial activity. We think that the lag phase in microbial activity, and hence the benefits gained from reducing the lag time, occur frequently, perhaps daily, for a portion of the year. If so, seemingly small benefits resulting from reduced lag times associated with rhizosphere treatment could be multiplied by daily temperature cycles.

2.4.2 Weaknesses. Obtaining regulatory approvals and developing suitable monitoring plans are perhaps the most difficult problem associated with using rhizosphere-enhanced biotreatment. The technical risks associated with demonstrating this technology are primarily difficulties in getting sufficiently precise data to show treatment effects in a relatively short period. We used replicated, statistically valid, field studies and multiple sampling and analyses methods (described in Section 3) to address these issues. Each site included appropriate replicated treatment controls.

Another limitation is the relatively longer treatment times compared to more aggressive treatments (Figure 3). Longer treatment times are offset by the reduced costs associated with rhizosphere-treatment.

Also unknown are the final concentrations that can be attained using rhizosphere remediation. The tendencies for concentrations to become asymptotic to a concentration greater than desired are well documented. At present, we do not know the final attainable contaminant concentration in soils for various soils types and contaminants. Moreover, we do not know how rates vary in different climates, different soils, different contaminants, or for different plants.

Because this is a root-interface phenomenon, the root must explore the soil being treated. Depth of rooting is obviously important and is an aspect we addressed in the demonstration. In laboratory studies, we can readily grow the roots of annual ryegrass to 4 ft within approximately two months. The optimum plants for site remediation are, to some degree, those plants with prolific root growth. Permafrost barriers and the sorption capacity of soils for many PAH compounds help to keep these compounds near the surface where root penetration is likely. In our research site at Fairbanks, within each treatment, we observed little difference in the petroleum concentrations at lower depths relative to petroleum concentrations at the more shallow depths, suggesting that rhizosphere treatment was reasonably effective in the lower portion of the root zone (Reynolds et al., 1997).

Wet or saturated soils may be difficult to remediate using this method. There are older sites that have been vegetated for some time and yet are still contaminated. In poor quality, well-drained soils, the carbon provided by root exudations apparently satisfies the carbon limitation to the system. We believe that carbon additions are a major part of the success of rhizosphere treatments in such soils. In wet, somewhat anaerobic soils, carbon accumulates and is probably not limiting. Therefore, root additions of carbon may not result in increased biotreatment rates.

2.5 Available Treatability Guidance

Although efforts to provide treatability guidance have been developed and are being updated, there are few examples of well-documented field studies published. Below are some documents that provide overviews of phytoremediation.

Brownfields Technology Primer: Selecting and Using Phytoremediation for Site Cleanup. Published: 2001. <http://www.clu-in.org/download/remed/phytorempriemer.pdf>.

This primer explains the phytoremediation process, discusses the potential advantages and considerations in selecting phytoremediation to clean up brownfield sites, and provides information on additional resources about phytoremediation. This document is not limited to rhizosphere remediation of petroleum in surface soils. Although treatability studies are suggested, specific information on treatability studies is not provided. A general overview of the many mechanisms potentially involved in phytoremediation is included and useful information on plant selection based on rooting depth.

Phytoremediation Decision Tree, Published: 1999.
<http://www.clu-in.org/download/partner/phytotree.pdf>.

This document was produced by the Interstate Technology Regulatory Cooperation (ITRC) workgroup. The intent of this document is to provide a tool that can be used to determine if phytoremediation has the ability to be effective at a given site. It is designed to compliment existing phytoremediation documents. It allows the user to take basic information from a specific site and, through a flow chart layout, decide if phytoremediation is feasible at that site. In its discussion of phytoremediation of organics, rather than specifically petroleum, the ITRC Phytoremediation Decision Tree document recommends first using the decision tree to assess if phytoremediation is a viable option, and then conducting treatability studies. These studies are described as growing a variety of plants proposed for use in a range of concentrations, to assess the fate of the contaminant, especially for transpiration losses, and to evaluate if desired results are achieved. The ITRC document is useful guidance for many organics. For petroleum specifically, a great deal is known about microbial degradation pathways, the generally accepted operative mechanism for rhizosphere-enhanced remediation.

Phytoremediation Technical and Regulatory Guidance Document, Interstate Technology and Regulatory Cooperation Work Group (ITRC), Phytotechnologies Work Team Document No: PHYTO-2. 124 pp, Apr 2001. <http://www.itrcweb.org/PHYTO2.pdf>.

This document covers a wide range of phytoremediation applications and is not limited to rhizosphere remediation of petroleum in surface soils. It provides useful background and descriptions of different mechanisms involved in phytoremediation of organics and metals. It discusses regulatory and permitting processes, leaching and contaminant mobilization concerns. The document provides an extensive list of possible monitoring parameters, all of which are based on changes in the contaminant chemistry. The document recommends treatability studies, both for evaluating plant survival and beneficial effects of the plants. Suggestions that are made for treatability studies include plant selection, contaminant fate and transport studies, mass balance studies, and microbial screening studies. The point is made that regulators are likely to require treatability studies prior to use of phytoremediation. The importance of plant selection is stressed. Again, this document covers a wide range of contaminant and is not limited to, or focused on, petroleum in surface soils.

Phytoremediation of Petroleum Hydrocarbons in Soil. Remediation Technologies Development Forum, Phytoremediation Action Team, Field Study Protocol, July 1999. <http://www.rtdf.org/public/phyto/protocol/protocol99.htm>.

This is the guidance document developed by the EPA-RTDF Phytoremediation Action Team. Rather than a treatability protocol, it is guidance for a series of field demonstrations for using phytoremediation for petroleum-contaminated soil. The three cold-region ESTCP sites were part of this effort.

3. Demonstration Design

3.1 Performance Objectives

The objective of this effort was to demonstrate rhizosphere-enhanced bioremediation of petroleum-contaminated soils located in cold, remote sites. We measured success by examining changes in the composition as well as concentration of petroleum in the soils.

Due to variability inherent in field data and the relatively slow treatment rates in cold regions, obtaining sufficiently precise field data to measure treatment effects on contaminant concentration is exceedingly difficult. Those involved in petroleum phytoremediation generally agree that the primary mechanism for phytoremediation of petroleum compounds is increased microbial activity in the rhizosphere rather than plant uptake, as is often erroneously assumed. Even for compounds that could be transported by mass flow of water into roots, such as relatively water soluble compounds with $\log K_{ow} < 1$ and slightly lipophilic compounds with $\log K_{ow}$ between 1

and 4, transport into the plant must occur through the rhizosphere, the zone of enhanced microbial activity.

Our laboratory studies suggest that the rhizosphere effect is increasingly important as the recalcitrance of the compound in question increases (Reynolds et al., 1999). Recent field studies have shown that increased rhizosphere degradation can be seen using an integrated measurement of diesel contamination, but within a three-year study, the effects were less dramatic for crude-oil contamination (Reynolds et al., 1997). Although the enhancement due to a rhizosphere effect, relative to non-vegetated soil, is likely greatest for heavier, more recalcitrant compounds, the resistance to degradation of these heavier compounds may result in longer treatment times being required before rhizosphere effects can be measured.

One approach is to monitor petroleum concentration changes in each treatment. At present, the final measure of performance is reduction of contaminant concentrations in the soil. We did not expect to attain concentrations that were asymptotic to a field endpoint at the end of this demonstration. To help address this, we used biomarker techniques to evaluate changes in the *composition* of petroleum. In brief, this approach compares relatively degradable fractions of petroleum to those that are recalcitrant. Highly weathered petroleum will have a high percentage of recalcitrant compounds compared to fresh or moderately weathered petroleum product. We monitored changes in fraction specific hydrocarbons (FSH)—an approach that attempts to classify hydrocarbons by grouping them into functionally similar fractions. Because of their functional similarity, the fractions can be separated by extraction and clean-up procedures. The fractions were also delineated so that there is toxicity data on at least one compound in each fraction. The assumption is that the toxicities of compounds within a fraction are more similar than across fractions, and therefore within-fraction toxicity data is the best estimate to use for extrapolating to compounds lacking toxicity data.

Table 1 summarizes our performance objectives and how they were met.

Table 1. Performance objectives.

Type of Performance Objective	Primary Performance Criteria	Expected Performance (Metric)	Actual Performance Objective Met?
Qualitative	Vegetation established on plots	Visual inspection of plots following seeding and fertilizing	Yes
Quantitative	Relate success of bioremediation to contaminant composition	Use statistically valid time-series samples to develop equations to describe degradation kinetics	Yes. Using biomarker and growing degree-day normalized data, statistical significance was shown for planted plots relative to un-planted plots.
	Relate microbial changes to degradation processes	Measure degrader numbers via MPN methods	Yes – at Annette Island site. Significant effects at one of three sites. Microbial data support chemical data results.
	Evaluate microbial population levels and composition	Use selective media techniques to compare fungal and bacterial populations	Yes – at Annette Island site, significant changes in fungal and microbial populations were related to plant and fertilizer treatments, respectively. At Barrow, plants increased the amount of fungal biomarkers relative to non-planted treatments.
	Reduce contaminant concentration	Rate of degradation	Contaminant depletion rates, biomarker and growing-degree day normalized – show greater depletion of specific petroleum fractions relative to unplanted plots.
	Remediate site	Endpoint concentrations not expected to become asymptotic	Partial. Data show significantly greater rates for planted treatments relative to un-planted treatments. Rhizosphere-enhance treatment is a long-term treatment strategy useful to remote sites, large areas, and locations/situations where other alternatives do not exist.

3.2 Selecting Test Sites

To include a climatic gradation evaluation of rhizosphere-enhanced bioremediation, we chose three sites on a south to north gradient of climatic conditions. Sites were selected to maximize the potential for successful demonstrations and to meet DoD requirements associated with ESTCP. We based our selection on the following criteria:

1. For maximizing future application and to gain the most information from the demonstrations, we sought three sites, each in a different climatic zone in Alaska.

2. To appropriately address the DoD requirement and the objectives of ESTCP, each site was required to have a Native American association and to have been contaminated by DoD activities.
3. The SERDP- and Army EQT-funded research leading to this demonstration had been conducted in well-drained (not saturated) soils. Accordingly, the sites chosen are not wetlands and the demonstrations were on well-drained areas.
4. Each site needed to have an agreeable owner or Primary Responsible Party.
5. Sites needed to have a realistic chance of success achievable within our budget. This eliminated some of the more distant formerly used defense (FUD) sites, such as the NE Cape site on St. Lawrence Island and Manning Point on the North Slope, which are typical of the proposed application for this technology because they are too remote for regular travel. Their remote locations made them too expensive for us to successfully conduct periodic monitoring required for a field demonstration.
6. We received these funds in May 1997 and were required to obligate them in FY97. To accomplish this, we selected sites where our site partners had a contracting mechanism already in place. This allowed us to modify existing contracts rather than negotiate a new contract for each site. Establishing new contracts was not feasible within the FY97 obligation requirement.
7. Additional criteria for site selection were the requirements, interest, investment in time, and likelihood of teamwork with potential partners. At each of the sites we chose, our partners demonstrated a willingness to cooperate, an eagerness to assist, and an appreciation of the potential savings to be realized pending acceptance and successful technology transfer. Consequently, we have a technology-transfer mechanism in place through our current partners.

3.3 Test Site Description

The three sites were all former DoD sites and the contaminants were mainly the result of fuel storage and use on the facilities; a dry-cleaning facility also contributed to contamination at Barrow.

3.3.1 Annette Island. The Annette Island site, on the Metlakatla peninsula of the island, is in the southern panhandle of Alaska below Juneau and Ketchikan (Figures 4 through 8). The U.S. Army Air Force Annette Island Landing Field was established in 1940 under a use permit granted by the Department of the Interior. The War Department, along with the Army Corps of Engineers, the Civil Aeronautics Administration (CAA, the predecessor to the Federal Aviation Administration), and the National Weather Bureau, constructed and operated the airfield and supporting

facilities. During construction, approximately 35 fuel tanks with a combined capacity of one million gallons were installed at various places on the island. Many government agencies and private businesses conducted operations at the airfield throughout its history

The Metlakatla Indian Community currently owns the Annette Island site. Soil samples in 1988 indicated that substantial contamination of the surrounding soil existed near the tank farm. Of 12 samples taken, seven indicated benzene concentrations above Alaska Department of Environmental Conservation (ADEC) Level A standards or 0.1 mg/kg. One-third of the benzene, toluene, ethylbenzene, and xylene (BTEX) samples showed concentrations exceeding the ADEC level A standard of 10 mg/kg, with the highest reading of 44.6 mg/kg. TPH levels were also elevated in all but three samples, with the highest reading being 2130 mg/kg. Our partner on Annette Island was the Federal Aviation Administration (FAA) and we worked with ClearWater Environmental, Inc.

The climate is wet and relatively mild by cold-regions standards. The area receives a high annual precipitation averaging 155 inches a year, with an average temperature of 45.9 °F. The site is near the old tank farm and is a relatively flat area on the east side of Tangas Harbor; the site is accessible by road. Access to Annette Island is by air or barge from Ketchikan.

3.3.2 Campion / Galena. Campion Air Force Station (AFS) is a former long-range radar site located approximately six miles east of the interior town of Galena, Alaska (Figures 4, 9, and 10). Operational from 1952 to 1984, Campion served as a communications facility supporting a high-frequency radio system, WACS, and a satellite communication system at various times during its operation. The facility was replaced by a Minimally Attended Radar installed at Galena Air Force Base and deactivated in 1984. The facility was demolished in 1986, and the ground surface was graded smooth.

For storage of heating oil fuels, Campion AFS operated a tank farm that was serviced by underground fuel pipelines from a barge-accessible fuel transfer facility on the Yukon River. Based on the findings of the 1995 Remedial Investigation, the bulk of the site hydrocarbon impacts to soil and groundwater at this site were diesel-range organic (DRO) compounds. Low levels of benzene, toluene, ethylbenzene, and xylene (BTEX) compounds were also observed in site soil. Soil samples taken in the tank farm area during the 1995 investigation revealed DRO concentrations ranging from 36 mg/kg to 75,000 mg/kg and gasoline-range organics (GRO) concentrations ranging from 59 mg/kg to 7,500 mg/kg, respectively. Soil BTEX levels ranged from 0.2 mg/kg to 33.9 mg/kg. The hydrocarbon distribution and GRO/DRO ratios indicated possible prior storage of gasoline fuel or arctic-grade heating oil or both. Our partners at Campion were ClearWater Environmental, Inc.; the Loudon Tribal Council; and the Air Force 611th CES.

The Campion site is about 250 miles west-northwest of Fairbanks, about 6 miles east of Galena, and 350 miles northwest of Anchorage. This site is interior Alaska and is cold and somewhat dry. Precipitation and surface winds are generally light with a mean annual precipitation of about 12 inches. Temperature variations between winter and summer can be extreme with a mean annual temperature of 27 °F. It is accessible by road from Galena, by river, and by air. Galena is accessible by air or by river.

3.3.3 Barrow. The Barrow site is the Naval Arctic Research Laboratory (NARL) facility, which is four miles northeast of the village of Barrow and six miles southwest of Point Barrow, the northernmost point of Alaska (Figures 4, 11, and 12). It is bordered by the Chukchi Sea to the west, the Arctic Ocean to the north, and the Beaufort Sea to the east. The NARL facility is on land governed by the North Slope Borough Regional Municipality. The facility was established in 1947 as a logistic supply center for petroleum exploration. The site was also used as a basic and applied research center, contributing to Navy operations in the Arctic. In 1987, the Navy signed a land-exchange agreement to transfer ownership of NARL to the Ukepeagvik Inupiat Corporation (UIC), a Barrow native village corporation. The complex is currently operated by the UIC. It houses a local college and provides office space for various borough departments and contractors performing projects for the North Slope Borough.

Two major contaminated sites at Barrow are a former dry-cleaning facility and a former bulk fuel tank farm. The dry-cleaning facility, located approximately 400 ft from the shore of the Chukchi Sea, was operated at NARL from 1948 through 1978. For most of the years of operation, the dry-cleaning solvent used was Stoddard solvent (a petroleum distillate containing trimethylbenzene, isopropyl benzene, nonane, decane, and undecane), and it was disposed directly onto the ground beneath the building until 1972 when a solvent purification system was installed. In 1974, the solvent was changed to the halogenated organic compound, tetrachloroethene, also called perchloroethylene (PCE).

Investigations at the dry-cleaning site after 1987 found Stoddard solvent, halogenated organic compounds, and TPH in the soils, along with alkylbenzenes, chloroform, methylene chloride, and PCE. TPH was the most abundant chemical found, exceeding 100 mg/kg throughout most of the site. The total volume of petroleum-contaminated soil was estimated at 7000 cubic yards (cy). In 1994, approximately 500 cy of soil was excavated to a maximum depth of 8.5 ft and was treated by venting for PCE contamination. The excavation was treated again in 1995 to comply with new standards for PCE contamination (the "Land Disposal Restrictions Phase II", RCRA-59 CFR 47982, lowered the risk-based standard for PCE from 18 mg/kg to 6 mg/kg). Confirmation samples after treatment showed PCE ranging from below detection limits to 4.5 mg/kg and averaging 0.93 mg/kg. Residual DRO concentrations in the treated soil ranged from 230 to 810 mg/kg and averaged 504 mg/kg. Final GRO concentrations ranged from below detection limit to 85 mg/kg and averaged 18.2 mg/kg. The treated soil was spread over the former area of contamination in October 1995.

The bulk fuel tank farm at Barrow was about two miles northeast of the main NARL complex, near the northeast end of the airstrip (no longer used) and between the North Salt Lagoon to the west and the Elson Lagoon and a large freshwater melt pond to the east. The bulk tank farm consisted of six aboveground tanks that stored diesel fuel, gasoline, Mogas, and JP-5 aviation fuel. The tanks were connected to other parts of the facility by three fuel lines that ran along the north edge of the North Salt Lagoon. The tanks and pipes were removed in 1990. Two of the tanks are known to have leaked. Investigations in 1990 and 1991 found gasoline and diesel in 5 to 20% of the samples with levels up to 2840 mg/kg. Benzene, toluene, ethylbenzene, xylenes, halogenated aliphatic hydrocarbons, solvents, phenolic, polycyclic aromatic hydrocarbons, and inorganic chemicals were also found in soil and active-zone water. TPH concentrations ranged from 47 to 9400 mg/kg and averaged 1278 mg/kg. Lead was also detected in all soil samples, ranging from 8.1 to 365 mg/kg. In 1994, no GRO was detected in six shallow soil samples, but concentrations of 838 mg/kg were found 3 ft below ground. DRO and total residual petroleum (TRP) ranged from 200 to 260 mg/kg and 230 to 250 mg/kg. Our partner was the Navy, and we worked with Battelle.

The Barrow climate is very cold and dry; temperatures range from -19 °F in February to 40 °F in July. The average annual precipitation is 14.6 inches. High relative humidity (90 to 95%) in the summer leads to foggy conditions about 25% of the time. Ground-based inversions are common in the winter and can concentrate airborne pollutants in low-lying areas when not dissipated by wind. Barrow's location between the Aleutian low-pressure system and the polar high-pressure system creates continual surface winds, predominately easterly and generally strongest in the fall and early winter.

Barrow is on the northwest edge of an extensive coastal plain. Soils are dominated by marine beach deposits consisting of coarse sand and gravel. Some finer deposits of silt, clay, and peat occur in drained lake basins and in places along beach ridges where wave action has not caused reworking. Soils are likely to be siltier in vegetated locations. In the Barrow area, a blue-black clay has been reported at depths of 10 to 60 ft.

Seasonal freeze-thaw and permafrost processes dominate the site surface hydrology and hydrogeology. The combination of permafrost and low-elevation terrain leads to the formation of thaw lakes and polygons (cracked, patterned ground characteristic of the Arctic far north). A few small streams form from surface runoff immediately after ice breakup, typically mid-to-late July. Soils at the surface are frozen through most of the year, reaching a maximum thawed depth of 22 to 55 in. by August or September. This "active zone" usually refreezes by late October, but heated buildings or the removal of the upper layers of soil disturbs it. Also, fine vegetated soils will thaw more slowly and to lesser depths than coarse, non-vegetated soils. Groundwater is confined to the active zone above the impermeable permafrost, and active-zone water movement is considered to be insignificant at NARL.

3.4 Pre-Demonstration Testing and Analysis

To initially characterize the general contaminant distribution at the site and to find the best location for the demonstration plots, we analyzed an initial set of samples in a grid pattern by organic vapor analysis. This helped us locate our demonstration plots. Additionally, an initial set of samples was taken at each site to provide t_0 data.

3.5 Testing and Evaluation Plan

3.5.1 Demonstration Installation and Start-Up. Figures 5 through 12 show maps of each of our three locations in Alaska. At each site, we are comparing the treatment effects of nutrient additions on a mix of three plant species and of the interactions of plants with nutrients, with controls for each. This resulted in four treatments: 1) a control, 2) added nutrients, 3) plants without nutrients, and 4) plants plus nutrients.

We used a mixture of annual ryegrass (*Lolium multiflorum*, Lam.), Arctared red fescue (*Festuca rubra*, L.), and white clover (*Trifolium repens*, L.) at each of the three sites in this demonstration. Low-maintenance grasses and a legume were chosen to avoid the need for intensive agricultural practices. The initial nutrient addition to the soil and watering are all that is usually required to create a viable stand of these grasses in these climates. We followed the RTDF-developed guidelines for seeding mixtures, which by weight were approximately 8 lb/1000 ft² tall fescue, 2 lb/1000 ft² annual ryegrass, 1 lb/1000 ft² legume (such as white clover, yellow sweet clover, or birdsfoot trefoil). These mixes, in general, provided a seed mix that had 10 to 15% ryegrass (annual or perennial), 20 to 25% legume (alfalfa, clover, birds-foot trefoil), and 60 to 70% fescue (varieties chosen for local conditions) on a seed quantity basis.

Minimal soil preparation was done prior to seeding. Seeds were surface applied by hand or by hand-held seeders and pressed into the soil surface to promote reasonable seed-soil contact and water imbibition. Nutrients were applied by hand or by hand-held seeders. Neither seeds nor nutrients were mixed into the soil, eliminating the need for heavy equipment mobilization to remote sites. Plot size varied at each site due to the constraints imposed by the local conditions. Figures 8 and 13 show how plots were arranged in blocks on Annette Island; Figures 1 and 2 show grass growth in the plots at Campion; and Figure 14 shows plots at Barrow.

Fertilizer requirements for bioremediation are controversial. For bioremediation without plants, different ranges of C:N ratios have been proposed. A potential issue with using C:N approaches is that for highly contaminated soils—which necessarily have high C levels—the amount of fertilizer N that is needed to maintain many C:N ratios become quite high. This can lead to osmotic stress on both microorganisms and plants. In theory, as microbial metabolism occurs, much of the contaminant C is lost from the system via CO₂ evolution. Nitrogen, however, cycles within the soil-plant-microbial

system. We have found that, in a number of Alaska soils, approximately 2000 mg N/kg *soil water* is the maximum N addition that can be applied without limiting soil microbial activity (Walworth et al., 1997). Note that this value is based on *soil water*, rather than soil. Because soil water varies with rainfall, evaporation, and transpiration, a value that relates to the soil must be used. We have used the gravimetric soil-water content that corresponds to -33 kPa soil-water potential as a basis for calculating nutrient amendments. To do this, at least a minimal soil-water response curve must be generated for each soil texture. This calculation can have a dramatic impact on nutrient additions. The gravimetric soil-water content that corresponded to -33 kPa soil-water potential for the Campion site soil was ~26%, but only ~1.6% for the Barrow soil. Additional fertilizer can be added to account for plant-uptake requirements and this can be based on agronomic requirements for the plants used. If excessive salt is in the soil from an earlier event, this should be taken into account as well. Within each site, both vegetated-plus-nutrients and nutrients-alone treatments were fertilized at the same rates.

3.5.2 Period of Operation. Figure 15 shows both actual treatment times (in days) and growing degree-day (GDD) normalized data are shown for each of the three sites.

3.5.3 Amount /Treatment Rate of Material to be Treated. Surface areas ranged from a series of 16 relatively small test plots at each site, ranging from approximately 20 ft by 10 ft at Annette Island to 20 ft by 75 ft at Campion/Galena. Treatment depth was through the root zone, or approximately two feet. Cost estimates are based on 10,000 ft² areas treated to 2 ft, or a total of 20,000 ft³.

3.5.4. Residuals Handling. Investigation Derived Waste (IDW) was minimal. Typically, this was generated only from decontamination of trowels and shovels used by the contractors. All IDW water produced during sampling was collected and put into 55-gallon drums and sampled as outlined in the Demonstration Plan. At the end of the initial sampling effort, all drummed IDW was removed from the site and kept in a secured area pending receipt of laboratory analyses. Upon receipt of analytical results, the IDW was disposed of in an appropriate manner following all applicable local, state and federal regulations. This water was found to have no appreciable levels of petroleum in it. For site monitoring, CRREL personnel merely used soil adjacent to the sampling nodes, but within the test plots, to remove any residual petroleum contamination on the sample tools. This method works well, if not better, and is significantly more practical.

3.5.5 Operating Parameters for the Technology. Site setup included initial site delineation; obtaining time-zero samples; collecting, compositing, preparing and installing soil socks for later sampling; data-logger setup; and seeding and nutrient additions. Site installation was conducted during the summer of 1998. At the Barrow

site, seeding and fertilizing were not done until the summer of 1999 due to the brevity of the summer season at Barrow.

One of the concepts associated with using rhizosphere-enhanced treatment is freedom from utilities and infrastructure. We had either electrical power or battery power at the sites, but this was merely to operate temperature data loggers; electric power is not required for the operative processes to proceed. During the demonstrations, a CRREL representative visited the sites periodically during the growing season to change data storage cans and check on the status of the sites. We were unable to keep the data loggers, batteries, and associated equipment secure at the sites.

3.5.6 Experimental Design. Our demonstrations included seeding and fertilizing cold-tolerant grasses in POL-contaminated soils. At three locations in Alaska, we compared the treatment effects of nutrient additions on a mix of three plant species and of the interactions of plants with nutrients, with controls for each. This resulted in four treatments: 1) a control, 2) added nutrients, 3) plants without nutrients, and 4) plants plus nutrients.

At each site, a factorial experiment with the above four treatments were arranged as Randomized Complete Block (RCB) with four replications. This allowed the data to be statistically analyzed for effects due to plants, nutrients, their interactions, or block effects that may have been caused by spatial trends at the sites.

3.5.7 Sampling Plan

Soil Sampling Methods. To obtain meaningful soil samples we used three sampling methods:

1. Grab samples as typically used for Alaska Department of Environmental Conservation (ADEC) regulatory purposes. These samples will only be taken at the beginning and end of the treatment demonstration.
2. Composite samples. Six to eight grab samples are taken on each plot and thoroughly mixed together.
3. Soil-sock samples. This procedure is a derivative of that used in litter decomposition studies. Approximately 200 samples were randomly taken prior to seeding or fertilization and mixed by rotary mixer. These large mixed samples, generally 10 to 20 ft³ of soil, were then apportioned into fine mesh, cylindrical, open-topped bags (soil socks) that were buried vertically in the plots from which we had taken the samples. Sufficient bags were buried so that a soil sock could be removed from each plot at each sampling time and sacrificed for analysis.

Where the field conditions suggested that there were areas that were different, based on initial chemical measurements, visual clues, or landscape position, we attempted to

use statistical blocking, so that each “distinct” area included one replication of each of the four treatments. At each site, the samples taken for the soil socks were obtained from and returned to the same block.

Sample Collection. To initially characterize the general contaminant distribution at the site and to find the best location for the demonstration plots, we analyzed an initial set of samples in a grid pattern by organic vapor analysis. To monitor the bioremediation process, we used three types of soil samples: 1) grab samples as typically used for ADEC regulatory purposes, 2) composite samples in which six to eight grab samples are taken on each plot and thoroughly mixed together, and 3) soil-sock samples to reduce variability. Each sample type is summarized below. Details are given in Section 9 “Quality Assurance Plan” of our Demonstration Plan.

Grab samples were taken from four locations of each treatment plot at the start of the demonstration and at the fall of the subsequent two growing seasons. Each of the four locations was sampled at a shallow and a deeper depth. These samples were sent, using chain of custody procedures prior to analyses for GRO, DRO, BTEX, and residual oil using ADEC-approved methods. These data provided little utility for monitoring the processes.

Composite samples were taken from each treatment plot at the start of the demonstration and at the spring and fall of the subsequent two growing seasons. The rationale for using a composite sampling technique is to account for sampling spatial variability by taking sufficient samples in each treatment plot so that their “mean value” (the composite) better represents the “population”, i.e., the soil in the treatment plot. A total of eight composite samples were obtained from each treatment plot at each sample time. Each of the eight composite samples were composed of ten random samples, taken from either a shallow or deeper depth in a treatment plot, and thoroughly mixed together. These samples were analyzed at CRREL for analysis.

For research-demonstration sites, we used **soil-sock samples** in an effort to reduce variability. This approach is not amenable to typical site implementation. The soil sock procedure is a derivative of that used in litter decomposition studies. Approximately 200 samples were randomly taken prior to seeding or fertilization and mixed by rotary mixer. These large mixed samples, generally 10 to 20 ft³ of soil, were then apportioned into fine mesh, cylindrical, open-topped bags (soil socks) that were buried vertically in the plots from which we had taken the samples. Sufficient bags were buried so that a soil sock could be removed from each plot at each sampling time and sacrificed for analysis.

Where the field conditions suggested that there were areas that were different, based on initial chemical measurements, visual clues, or landscape position, we attempted to use statistical blocking (Figure 8), so that each “distinct” area included one replication of

each of the four treatments. At each site, the samples taken for the soil socks were obtained from and returned to the same block.

Soil samples were collected using hand tools, which were decontaminated between samples. The samples were packaged in sealed bags and placed immediately into pre-cooled coolers with blue ice. Samples were collected in the spring and fall; the actual sampling dates were subject to weather conditions at each site.

Sample Analysis. Composited samples taken from the soil socks were analyzed for petroleum by several approaches to characterize the petroleum fractions in the soil. Total petroleum hydrocarbon (TPH) data are expressed as a concentration of mass of petroleum per mass of soil. Although this approach measures an integrated value of the total amount of petroleum products present, you cannot distinguish among specific compounds, degree of weathering, or degradation in the form in which TPH is usually expressed. We therefore used TPH in conjunction with more specific methods to determine contaminant degradation and the time-related depletion of specific fractions. The approaches are described below. Details of analytical methods are given in Appendix A of this document and in our Demonstration Plan in section 5.4 “Sampling Plan” and Appendix D “Sampling and Analysis Plan for Annette Island and Campion.”

For semi-volatile TPH and FSH analyses, soil samples are extracted in *n*-pentane, passed through an open silica column, and fractionated into aliphatic hydrocarbons (F₁ fraction) and aromatic hydrocarbons (F₂ fraction) using open tubular silica gel chromatography techniques. The resulting extracts are analyzed for TPH and FSH and, for selected samples, for PAHs.

Total Petroleum Hydrocarbons (TPH). High-resolution gas chromatography using flame ionization detection (HRGC/FID) yields a chromatogram such as those shown in Figures 16 through 20 (see Appendix A for a description of the HRGC/FID technique). These chromatograms show relative amounts of petroleum compounds as they differentially elute from a chromatographic column. Integrating the area under the curve and between two defined retention times provides a measure of TPH. TPH data are generally provided as a single, numeric concentration value, such as mg/kg or ppm; thus, much of the data contained in the chromatogram is lost because a numeric TPH value gives no qualitative information about the distribution of fractions. Nonetheless, when monitored over time, TPH data can show, in general, if concentrations of petroleum products are decreasing. To rely mainly on TPH as a monitoring tool, you must assume homogeneity of initial concentrations or have large concentration changes.

Fingerprinting (Fuel Types and Weathering). With experience, the same chromatograms used for obtaining TPH values can be compared to typical curves of known products and provide information about types of petroleum products and degree of weathering. Figures 16 through 20 show typical curves for petroleum product types.

Fraction-specific hydrocarbons (FSH). Fraction-specific hydrocarbons (FSH) are based on the concept that petroleum consists of a very large number ($\sim 10^4$) of individual compounds. The distribution of broad classes of these compounds is reasonably representative of different types of petroleum products, such as diesel or bunker C. A combination of distillation and blending of the distillates are used to obtain petroleum products. Consequently, rather than being a set percentage of different compounds, petroleum products are combinations of various distillation fractions that are blended together to provide a product that meets performance guidelines. Chemically, various fractions of petroleum compounds behave similarly and, hence, can be grouped together. Chemical similarities influence both extraction from soil and also the potential toxicity of the compounds. The FSH approach was developed based on these properties. Specific FSH values are obtained similarly to TPH curves but, following extraction from soil and prior to GC analysis, the petroleum materials are fractionated into aliphatic and aromatic components. When quantifying the chromatogram for FSH, the ranges used to group compounds have been chosen based on correlations with potential toxicity. The initial fractionation provides quantitative measures for specific fractions of the petroleum material. Changes in FSH values can be compared through time. Because different petroleum fractions have different transport, bioavailability, and toxicity characteristics, FSH data can be more meaningful than TPH data. FSH values are obtained using the HRGC/FID technique (see Appendix A). For statistical analyses of data, TPH, summed PAHs, and aliphatic and aromatic fractions were all normalized using a recalcitrant biomarker.

Polycyclic Aromatic Hydrocarbons (PAHs) and Diagnostic Heteroatomic Compounds. Using high-resolution gas chromatography mass spectrometry (HRGC/MS; see Appendix A), mass spectra can be obtained that show peaks corresponding to the molecular fragments of specific petroleum compounds. Using this approach, we can determine the amounts of individual polycyclic aromatic hydrocarbons (PAHs). PAHs are various arrangements of fused, aromatic ring molecules. We can also identify heteroaromatic compounds, which are rings containing elements in addition to carbon. This approach can be used to specifically identify PAHs that have been listed by the US Environmental Protection Agency (EPA) as priority pollutants (see Table 6 in Appendix A). Inclusion on this list generally indicates that the compound is carcinogenic.

BTEX. Using appropriate handling, extraction, and analytical methods, we can characterize the volatile organic compounds (VOCs) benzene, toluene, ethylbenzene, and xylene (BTEX). These compounds are relatively volatile, are water soluble, and generally have low permissible levels. In field soils, BTEX compounds are generally the first to leach and to volatilize. Their levels in aged or weathered contaminated soil may be low. For these sites, BTEX was not considered an issue.

Depletion Monitoring with a Selected Biomarker. For a site that is contaminated with a relatively uniform type of contaminant, bioremediation effectiveness can be calculated

relative to a compound that is relatively non-degradable. These recalcitrant or stable compounds are often referred to as biomarkers. As different fractions of the total suite of petroleum degrade, the relative concentration of the recalcitrant fraction, or biomarker, increases. The compound α,β -hopane (hopane) is often chosen as a biomarker because it appears in many petroleum compounds and it degrades very slowly. Because it is often cited in petroleum literature, α,β -hopane is a good choice for TPH degradation normalization studies. The HRGC/MS method (see Appendix A) used for PAHs is used to quantify hopane.

Using this technique, the percent loss of TPH, FSH, and individual target benzene, toluene, ethyl benzene, and xylene (BTEX) and PAH compounds can be calculated as follows:

Percent depletion of individual target analytes

$$(1 - [(C_1/C_2) * (H_2/H_1)]) * 100$$

Percent depletion of total petroleum hydrocarbons (TPH)

$$(1 - (H_2/H_1)) * 100$$

Where:

C_1 = Concentration of analyte in the sample

C_2 = Concentration of analyte in the source (time zero)

H_1 = Hopane concentration in the sample

H_2 = Hopane concentration in the source (time zero)

Note: All depletion estimate calculations were done on an oil weight basis, which were obtained during sample preparation. Oil weights used were the TPH-oil (($\mu\text{g}/\text{gram TPH}$) * grams dry weight = $\mu\text{g oil}$) for the samples.

Importantly, any compound or group of compounds can be normalized relative to a recalcitrant biomarker. For statistical analyses of data, TPH, summed PAHs, and aliphatic and aromatic fractions were all normalized using a recalcitrant biomarker.

Normalization with Respect to Climate. By expressing changes in the composition of petroleum relative to the recalcitrant biomarker decalin, we normalized degradation rates with respect to concentration differences and thereby reduced concentration variability at each site. However, each site was treated for different lengths of time and at different conditions. To account for this, we normalized the treatment time based on temperature at the site. Due to issues common at remote field demonstration sites, we were unable to collect reliable soil field temperature data. As an alternative, we used air temperature data available from the National Oceanographic and Atmospheric Administration (www.cdc.noaa.gov). Barrow and Annette Island data were obtained

from this database, but Galena data were not available. To substitute for Galena data, we used Fairbanks temperature data for the Galena site. The latitude and air temperatures at Galena and Fairbanks are similar.

Growing degree days (GDD) were calculated as below, using 0°C as the base temperature:

$$\text{GGD} = \sum((\text{average high} + \text{average low})/2) - 0$$

3.5.8 Demobilization. This technique does not involve the use of any equipment or structures that need to be removed. The vegetation can and should be left in place to facilitate any further rehabilitation of the site and prevent erosion.

3.6 Selection of Analytical/Testing Methods

See Appendix A of this document and our Demonstration Plan in section 5.4 “Sampling Plan” and Appendix D “Sampling and Analysis Plan for Annette Island and Campion.”

3.7 Selection of Analytical/Testing Laboratory

Chemical analytical capabilities are important. The most useful data for describing the treatment effects were TPH, summed PAHs, and fraction specific hydrocarbons (FSH) normalized to a recalcitrant biomarker and to growing degree days (GDD). Few laboratories have the capabilities or expertise to do this, although the instrumentation is fairly common. For this project, useful analyses were conducted by Battelle, Duxbury Operations, Battelle Environmental Forensics Group, 397 Washington Street, Duxbury, MA 02332.

The analytical laboratory used for the regulatory samples was approved by the appropriate regulatory body, in this case the Alaska Department of Environmental Conservation (ADEC). These samples are insufficient for monitoring this type of remediation process. The architect-engineering firm (AE) provided chemical analysis of all quality assurance (QA) samples. All QA laboratory services were performed in accordance with the Quality Assurance Plan (see Section 9 of the Demonstration Plan) and conformed to ER 1110-1-263 (1 April 96). The AE used only those QA laboratories that are validated by the Corps of Engineers Missouri River Division (CEMRD) for the required analyses and had ADEC approval.

The certified analytical laboratory analyzed just the regulatory grab samples. Additional samples were analyzed by ERDC-CRREL soil microbiology laboratory.

4. Performance Assessment

4.1 Performance Criteria

We used two approaches to analyze the data. Because the demonstration at each site is a factorial experiment and designed as a balanced replicated, randomized complete block, we conducted factorial analysis of variance (ANOVA) to test for significance of the main effects (plants or nutrients) and (plant x nutrient) interactions on selected dependent variables. Additionally, we used 1-way ANOVA to compare each treatment. The latter approach loses sensitivity relative to the factorial approach because there are fewer replications of main factor, but this approach is also more likely to be used in by many in future demonstration where full factorial experiments are cost prohibitive. These two approaches to hypothesis testing were chosen to provide evidence for enhanced remediation at these sites, and also may provide new understanding that would suggest other tests, other monitoring approaches, or both.

A challenge in using rhizosphere-enhanced remediation is that successful treatment may be occurring at an enhanced rate relative to natural attenuation (controls), yet the treatment may still require a number of years before cleanup goals, or even chemically measurable treatment effects, can be observed. This is exacerbated in cold regions, where low temperatures and variable conditions slow surface biological processes. This lack of uncertainty may not be acceptable to stakeholders or regulatory communities. Multiple lines of evidence that rely on alternative protocols may lead to new monitoring strategies that convincingly demonstrate that remediation is occurring, but at present, the need for statistically valid changes in degradation rates, based on chemical concentration data, are needed.

Table 2 shows performance criteria.

Table 2. Performance criteria.

Performance Criteria	Description	Primary or Secondary
Contaminant Reduction	Petroleum compounds in surface soils	Primary
Contaminant Mobility	The technology does not affect the mobility of the contaminants.	Secondary
Hazardous Materials	No hazardous materials will remain.	Secondary
Process Waste	The only waste generated was from sampling equipment used in the initial sampling event. These wastes were collected, analyzed, and disposed of according to regulations. Subsequent sampling did not generate wastes.	Secondary
Factors Affecting Technology Performance	Temperature: Abnormally low temperatures or a shorter than normal growing season, will slow the microbial activity and hence the degradation rate. Precipitation: Insufficient or excessive rainfall can inhibit plant growth, root penetration, and soil microbial activity.	Primary
Reliability	Not applicable; there is no equipment involved	Secondary
Ease of Use	No special skills and training are needed beyond ability to operate simple equipment or devices used in seeding and fertilizing soil, and the ability to collect valid monitoring samples following designated procedures. There is no required number of technicians; the number of people depends on the size of the site and the time available to vegetate, fertilize, and collect monitoring samples. Initial supervision by someone familiar with the intent of the sampling is needed.	Secondary
Versatility	The technology could easily be adapted to other locations; the main issue would be selection of plant species and nutrients appropriate for the site.	Primary
Maintenance	No equipment maintenance is required. Seasonal/annual inspection to assure that plants are growing is useful. Sampling schedule needs to be tailored to meet regulatory and technical needs. Annually is likely the most frequent schedule that one would use. Less frequently would likely be appropriate from a technical perspective.	Primary
Scale-Up Constraints	There are no engineering limitations involved in the move from demonstration-scale to full-scale implementation of this technology. Full-scale use of the technology should be relatively easy to initiate. Seeding and fertilization of larger areas will bring increased costs for materials and labor, but the per-unit cost should go down due to economies of scale, and the techniques remain the same as for the ESTCP demonstrations. The main cost issues involve the number of monitoring samples to be taken and the types of analyses to be performed.	Primary

4.2 Performance Confirmation Methods

Table 3. Expected performance and performance confirmation methods.

Performance Criteria	Expected Performance Metric (pre demo)	Performance Confirmation Method	Actual (post demo)
Primary Criteria (performance objectives) (Qualitative)			
Ease of use	Minimal operator training required	Experience from other demonstration operations. Stand establishment in plots.	Data and techniques from other projects and understanding of the soil-microbial system provided insight for sampling and analyses.
Primary Criteria (performance objectives) (Quantitative)			
Measurable treatment benefit	Statistical analyses of concentration data or degradation rates	Statistical analyses of concentration data or degradation rates	Use of factorial analysis and biomarker-GDD normalized data to show statistical significance for plant treatments for specific petroleum fractions
Measurable treatment benefit manifested in microbial changes	Statistical analyses of microbial data	Statistical analyses of microbial data	Statistical analyses of microbial data showing fertilizer effect on bacteria and plant effect on fungi

4.3. Data Analysis, Interpretation and Evaluation

4.3.1 Data Normalization. Our data were from sites that had significantly different temperatures. Additionally, the actual treatment times at each site varied due to differences in starting and ending times for the performance evaluation period at each site. Concentrations of petroleum were also variable within each site. Although steps were taken to mix the soil prior to the study to normalize concentration differences, logistics issues caused by the remoteness of the locations hindered thorough mixing. Moreover, the amount of mixing that would be done at a typical site would be minimal or none. To help account for these, data were normalized relative to both a biomarker—to normalize concentration differences, or the spatial domain—and also on growing degree days—to normalize for differences in temperature and subsequent biological activity among the three locations, or the time domain.

The data used in the statistical analyses were from all three sites and biomarker normalized using decalin as the biomarker. Decalin was used because it is recalcitrant, and we found very low amounts of hopane at the Galena site, but acceptable amounts of decalin at all three sites. Using decalin allowed us to pool the biomarker-normalized data across all the sites. Data for the initial sampling times, t_i , and final sampling times, t_f , at each site were used in the depletion calculations

By expressing changes in the composition of petroleum relative to the recalcitrant biomarker decalin, we normalized degradation rates with respect to concentration differences and thereby reduced concentration variability at each site. However, each site was treated for different lengths of time and at different conditions. To account for this, we normalized the treatment time based on temperature at the site. Due to issues common at remote field demonstration sites, we were unable to collect reliable soil field temperature data. As an alternative, we used air temperature data available from the National Oceanographic and Atmospheric Administration (www.cdc.noaa.gov). Barrow and Annette Island data were obtained from this database, but Galena data were not available. To substitute for Galena data, we used Fairbanks temperature data for the Galena site. The latitude and air temperatures at Galena and Fairbanks are similar. Data not normalized to GDD, but with the GDD given, are shown in Appendix E.

Growing degree days (GDD) were calculated as below, using 0°C as the base temperature:

$$\{GDD = \sum((\text{average high} + \text{average low})/2) - 0\}$$

Using the above normalization techniques, decalin, GDD, normalized data for the dependent variables listed below were calculated:

- TPH
- Summed PAHs
- Aliphatic fractions
 - o C8-C10
 - o >C10-C12
 - o >C12-C16
 - o >C16-C35
 - o C8-C35 (the sum of the aliphatic fractions)
- Aromatic fractions
 - o C8-C10
 - o >C10-C12
 - o >C12-C16
 - o >C16-C21
 - o >C21-C35
 - o C8-C35 (the sum of the aromatic fractions)

4.3.2 Means and 95% Confidence Intervals - Comparison of Plot

Treatments. Means and 95% confidence intervals tabulated for each variable and expressed as (% Depletion / GDD-C), by site and by treatment, are shown in Tables 4. Negative values resulting from the calculations are shown for completion. The magnitude of the depletion values ranges up to 0.123 %/GDD-C. Variability is high. The magnitude of the derived GDD normalized depletion values is similar for all three sites.

4.3.3 Results for Normality Tests - Comparison of Plot Treatments. Data were first tested for distribution. All data were relatively normally distributed. Frequency distributions for each dependent variable grouped by TPH and summed PAHs, aliphatic fractions, and aromatic fractions are shown in Figures 21 through 23, respectively.

4.3.4 Results for One-Way ANOVA – Comparison of Plot Treatments. Using one-way ANOVA, we observed no significant effects for any of the dependent variables listed above. Probability values are listed in Table 4. In the table, P values < .20 are noted via bold type. Due to the variable nature of field data, probabilities < 20% are often considered to have practical significance and we have done so in these analyses. The implication of these findings is that a one-way ANOVA comparison of treatment effects is reasonably representative of the approach likely to be used in typical field demonstrations—three to four replications of two to several treatments. This ESTCP project provides data comparing two levels of two treatments, replicated four times at each of three locations, and normalized for concentration differences and the temperature of the locations, and the data did not uncover significant effects $P < .05$ for any of the treatments. Using a one-way ANOVA, only one fraction, the aromatic C>10-12, showed a significant treatment at $P = 0.146$, and this was a *reduction* in treatment efficacy for the fertilizer treatment relative to either the control or treatments that included vegetation (Figure 24). Our data from similar studies conducted at two locations in Korea showed an apparent reduction in treatment efficacy, relative to both the control and planted treatments, when fertilizer alone was used (Reynolds et al., 2001). These data suggest that “standard” monitoring approaches for “typical” treatment durations are unlikely to detect a rhizosphere treatment effect, and suggest that the greatest effect relative to a control treatment is in specific petroleum fractions.

4.3.5 Results for Two-Way Factorial ANOVA – Comparison of Main Effects of Fertilizer, Plants, and Their Interactions. Table 4 also lists the P values for the main effects and interactions of the factorial ANOVA, using all depletion data from the three sites, normalized to decalin and GDD-C. All means and 95% confidence intervals are also shown in Figures 28-35. Data showed no significant interactions.

Table 4. Table of P values for ANOVA of decalin – GDD normalized data for three ESTCP sites, P= .20 are bold.

	One way ANOVA	Factorial ANOVA		
		Fert X Plant	Plant	Fertilizer
TPH	.336	.934	.075	.699
Σ-PAH	.369	.316	.161	.847
Aliphatic C8-10	.734	.322	.868	.625
Aliphatic C>10-12	.773	.981	.512	.414
Aliphatic C>12-16	.640	.469	.477	.442
Aliphatic C>16-35	.343	.746	.078	.953
Aliphatic C8-35	.399	.950	.100	.746
Aromatic C8-10	.730	.949	.346	.567
Aromatic C>10-12	.146	.329	.313	.063
Aromatic C>12-16	.525	.778	.376	.242
Aromatic C>16-21	.396	.822	.095	.758
Aromatic C>21-35	.249	.855	.048	.801
Aromatic C8-35	.212	.977	.036	.901

Plant Effects on Depletion of Specific Petroleum Fractions. We observed significant ($P=0.075$) plant-treatment effects for TPH but not the summed PAHs (Table 4 and Figure 28). The heavier aliphatic fractions, C>16-35 aliphatics, and consequently, the C8-35 aliphatics were significantly different than the treatments without plants, but the other aliphatic fractions did not show an effect (Figure 29). Additionally, there were significant ($P<0.10$) plant effects for the C>16-21 and C>21-35 aromatic fractions and consequently, the C8-35 aromatic total, but lighter aromatic fractions did not show an effect (Figure 30). For clarity, only those aromatic fractions showing significant plant effects are also shown (Figure 31). Beneficial plant effects have been observed for heavier, more recalcitrant fractions in other studies on petroleum degradation (Reynolds et al., 2001) and in other recalcitrant compounds such as polychlorinated biphenyls (PCBs) (Leigh et al., 2002). The hypothesized mechanism for this is analogue enrichment provided by compounds released from the plant. These data are in agreement with results we have obtained in laboratory-growth chamber studies (Reynolds et al., 1997; Reynolds et al., 1998).

Fertilizer Effects on Depletion of Specific Petroleum Fractions. All fertilizer main effects comparisons are shown for TPH and summed PAHs, aliphatic fractions, and aromatic fractions in Figures 32 through 34, respectively. Fertilizer had no effect with $P<0.20$ (Table 4) except for the aromatic C>10-12, which showed a significant effect ($P=0.063$). Data for fertilizer effects on the aromatic C>10-12 fraction are shown in Figure 35. The variability in the fertilized treatments was large, yet fertilization resulted in lower degradation ($P=0.063$) of the aromatic C>10-12 fraction than the non-fertilized treatments.

Inhibition due to fertilizer is counter-intuitive, yet it agrees with the general observations from two demonstrations we conducted in Korea. These data suggest that fertilizer alone can inhibit the degradation on some petroleum fractions relative to control treatments (Reynolds et al., 2001). Whyte et al., 1997, found *Pseudomonas* spp., isolated from cold soils could degrade C5 to C12 aliphatics, toluene, and naphthalene at both 5 and 25 °C, and also possessed both the alkane and naphthalene degradation pathways. Their data indicated that both alkane and naphthalene degradation capabilities, which are located on separate plasmids, can naturally coexist in the same bacterium. Our earlier work at Fairbanks showed that the dominant culturable bacteria in both control and fertilized soils were *Pseudomonas* spp. (Reynolds and Wolf, 1999). The mechanisms for fertilizer inhibition of heavier fractions are not clear, but we have observed this in several field studies.

4.3.6 Microbial Characterization. Because the potential for successful remediation of petroleum-contaminated soils is determined by the number and activity of the hydrocarbon-degrader microbial population in the soil, we also assessed the influence of fertilizer addition and vegetation on culturable microbial numbers in a petroleum-contaminated soil at all three sites. Using culturable microorganisms as a monitoring variable, significant treatment effects were seen only at the Annette Island site. Soil samples were collected four times over a period of 20 months and total plate counts were used to enumerate bacteria and fungi. The bacterial numbers significantly increased as a result of fertilizer addition and fungal numbers increased following the establishment of vegetation (Figure 36). Bacteria but not fungi responded to fertilization. Fungi but not bacteria responded to plants (Figure 37). The results indicated that adding fertilizer and establishing vegetation increased microbial populations differentially and the potential for biodegradation of the petroleum contaminants at the site. Motor oil, cyclohexanol and benzoic acid degrader populations were determined using most probable number (MPN) methods. At 10 months, there was an increase in degraders for motor oil and cyclohexanol but a decrease for benzoic acid degraders (Figure 38).

Phospholipid fatty acid data for Barrow show an increase in the fungal biomarker, n18:2w6c (Figure 39). Plants increased fungal biomarkers at Barrow during the study. Non-planted treatments did not show this effect. These data, combined with the Annette Island data, also support the concept that one of the benefits of rhizosphere enhanced treatment is better degradation of more recalcitrant compounds. Fungi have been shown to typically have greater ability to degrade recalcitrant compounds (Donnelly and Fletcher, 1994) and the planted soils have greater fungal numbers (Figure 37). This finding is also supportive of the chemical analyses that showed a significant plant effect for depletion of the relatively recalcitrant compounds. Additionally, the fertilizer effect on bacteria but not fungi suggests that one of the results of fertilizer is an immediate or rapid bacterial response—which is fitting with bacterial growth rates relative to fungi—and this may be at the cost of reduced degradation of

petroleum. This may explain in part the inhibition of depletion of some petroleum fractions associated with fertilization that we have observed in our field studies.

5. Cost Assessment

5.1 Cost Reporting

Table 5 outlines the relevant costs during the demonstration and indicates how these might vary with site location for application of the technology. The main costs are related to initial site work and monitoring the site in subsequent years. There are few costs associated with operation and maintenance and there are no residues or debris requiring disposal.

Table 5. Cost reporting

COST CATEGORY	Sub Category	Costs (\$)
FIXED COSTS		
1. CAPITAL COSTS	Mobilization/demobilization	Minimal. Varies with site location relative to transportation. \$500/10,000 ft ²
	Planning/Preparation	Minimal. Varies with site location relative to transportation. \$500/10,000 ft ²
	Site Work	Minimal. Required only for seed preparation, fertilization, and sampling. Varies with site location relative to transportation. \$5000/10,000 ft ²
	Equipment Cost - Structures - Process Equipment (if purchased)	None Miscellaneous tools for spreading amendments and sampling \$500/10,000ft ²
	Start-up and Testing	Labor for sampling, seeding, fertilizing. Varies with site location relative to transportation. \$500/10,000 ft ²
	Other - Non-Process Equipment - Installation - Engineering - Management Support	None Labor for seeding, sampling, and fertilizing. Included in startup and testing None Varies with site location relative to transportation. \$250/10,000 ft ²
		Sub-Total (\$) 7,250/10,000 ft ²
VARIABLE COSTS		
2. OPERATION AND MAINTENANCE	Labor	\$150/10,000 ft ² /year
	Materials and Consumables	\$250 /10,000 ft ² /year
	Utilities and Fuel	N/A
	Equipment Cost (if rent or lease)	\$500/10,000 ft ² /year
	Performance Testing/Analysis	\$500/10,000 ft ² /year
	Other Direct Costs - Equipment Overhead	
		Sub-Total (\$) 1400/10,000 ft ²
3. OTHER TECHNOLOGY-SPECIFIC COSTS	Long-term monitoring, Reg./institutional oversight	\$500/10,000 ft ² /year \$5,000 year/site
	Compliance Testing/Analysis	\$500/10,000 ft ² /year
	Soil/Sludge/Debris Excavation, Collection and Control	N/A
	Disposal of Residues	N/A
		Sub-Total (\$) 6000/10,000 ft ²
TOTAL COSTS (assumes 10 year operation)		\$27,250
TOTAL TECHNOLOGY COST (\$)		
Quantity Treated 10,000 ft ² to root depth (2 ft)		20,000 ft ³
		Unit Cost (\$) 1.39 / ft ³

5.2 Cost Analysis

The greatest cost for rhizosphere-enhanced bioremediation typically is in sampling and monitoring, and that is specific to the frequency of sampling, the type of analysis done, and cost of analysis per sample. The transport, spreading, seeding, and fertilizing are essentially one-time costs, although some re-seeding may be needed annually, and even some watering may be beneficial during seedling establishment. Annual fertilizer can be added but may not be necessary. Again, this is specific to the site and the goals. We have found that in year two (and even the first season), many volunteer plants established themselves. This is usually beneficial and, in our experience, the vegetation will shift with time to resemble the local vegetation.

Typical sampling and monitoring techniques used for tracking more aggressive treatments are of little use for monitoring rhizosphere-enhanced remediation of contaminated surface soils. Data are too heterogeneous for firm conclusions to be made. Useful tools for obtaining more meaningful data and reducing variability include composite samples, fraction specific hydrocarbon analysis (FSH), biomarker normalization, and temperature normalization. Monitoring using these tools and for a longer time but with greater intervals between sampling times emerged as a reasonable monitoring plan.

6. Implementation Issues

6.1 Environmental Checklist

An up to date list of guidance documents and permits for oil-contaminated soil in Alaska is provided by the Alaska Department of Environmental Conservations (ADEC) at: <http://www.state.ak.us/dec/landhome.htm>

6.2 Other Regulatory Issues

To gain acceptance by the regulatory community, field data must demonstrate the effectiveness of phytoremediation under conditions that can be applied to potential full-scale treatment sites (Rock and Sayre, 1999). A primary purpose of these ESTCP demonstrations was to collect and evaluate data that is relevant to many cold-region cleanup sites. During the early phase of the demonstration, interactions with regulatory officials and RTDF members highlighted the challenges in monitoring these sites. In Alaska, regulations regarding use of low-cost remediation strategies are evolving and are, to a degree, subject to the interpretation of the front-line regulator. Earlier regulations concerning sampling frequency and protocols were developed to address more aggressive treatment technologies, such as incineration or biotreatment in a mixed bioreactor. Sampling requirements, which have typically been one grab (non-composited) sample for each 50 cubic yards (cy) of treated soil, are being modified to better describe surface soils and less aggressive treatment techniques. For more

passive systems, such as rhizosphere-enhanced treatment, where the soil is not mixed during treatment, grab samples are not as appropriate as they are for well-mixed systems. Our sampling plan addressed this issue by taking both grab and composite samples, as well as soil-sock samples, at described intervals. Recently, Alaska Department of Environmental Conservation requested information on this technology to address remediating former storage tank pads at a number of villages.

6.3 End-User Issues

End users at each site participated largely by agreeing to allow a technology demonstration to be conducted at their site. Due to more knowledgeable staff, changed attitudes, more experience, and resource constraints, regulators in some areas, including Alaska, are more open to low-cost approaches in recent years.

Although we have shown that this technology is more effective than the controls or than adding only fertilizer, we are still unable to predict the time necessary for a site to reach target concentration goals. We have shown that rhizosphere-treatment will proceed faster than non-rhizosphere and fertilizer alone treatments. Our data from these and other sites show that rhizosphere related processes are more effective than non-rhizosphere processes and fertilizer additions alone in reducing more recalcitrant petroleum compounds.

These data have been provided to the EPA-RTDF working group on Phytoremediation of Petroleum.

6.4 Specifics for Implementing Rhizosphere-Enhanced Remediation at Northern Locations

6.4.2 Planting

The plant has to grow. Although there may be exceptionally good and exceptionally poor plants for enhancing petroleum degradation, they have not all been identified. Extensive plant screening is difficult and costly, and results probably vary with many other conditions such as temperature, the nature of the petroleum, soil conditions, rainfall, and other conditions not yet understood or identified. The University of Saskatchewan has developed a database, *PhytoPet*© (<http://www.phytopet.usask.ca/mainpg.php>), to catalogue plants for petroleum phytoremediation. *PhytoPet* was originally developed as an inventory of plants that have demonstrated ability to either phytoremediate or tolerate soils contaminated with petroleum hydrocarbons. As with much phytoremediation information, the database is changing and allows for user interaction. There also are molecular-based efforts that are attempting to screen plants by looking for specific genes in plants and matching these to contaminant degradation pathways, but this research is not yet to the application stage.

Petroleum degradation is well characterized, and for rhizosphere-enhanced remediation the process is a root-surface phenomenon, rather than one centered in the plant. From CRREL's experience, grasses do well for petroleum. This is most likely due to their fibrous root system that explores a large volume of soil fairly completely and, in a sense, provides pseudo-mixing. In various field studies at other sites, we also have used annual ryegrass (*Lolium multiflorum*), tall fescue (*Festuca arundinaceae*), and winter rye (*Secale cereale* L.). We have seeded at rates heavier than would be used normally for establishing the grass. Extra seed is to account for losses from poor germination and seedling die-off due to petroleum contamination and poor growth conditions, such as drought. The goal is to get a good plant cover on the soil and thorough root growth and penetration in the soil.

6.4.3 Fertilizing: Rate

In many cases, there has been a tendency to add fertilizer, primarily nitrogen, to yield a final carbon:nitrogen ratio that was considered optimum—or similar to the carbon:nitrogen ratio of bacterial cells. This seems to make sense, but petroleum is mostly carbon, and petroleum-contaminated soils can have an exceedingly high carbon level. As petroleum is metabolized, carbon is eventually lost from the soil as evolved carbon dioxide (CO₂), but nitrogen remains in the system, cycling among various pools in the soil. Because nitrogen is added as a fertilizer salt, adding sufficient nitrogen to yield an optimum carbon:nitrogen ratio can cause osmotic stress to both microbes and plants. This can result in poor or no seed germination or poor plant growth simply due to the salt-effect of the fertilizer. It is similar to spilling fertilizer on your lawn or adding salt to soil. The osmotic effect is very detrimental.

Two approaches are useful. One is to add fertilizer as you would for seed establishment using the general guidance for establishing a lawn or garden. The other is to add as much nitrogen as can be added without stunting plants. The maximal level for nitrogen additions without inhibiting microbial activity is approximately 2000 mg N / kg soil water. Note that this approach is based on soil water content rather than soil. The challenge to this approach is that soil water content varies as soil wets and dries. A reasonable way to address nutrient additions is to add nutrients based on soil water concentrations of 2000 mg nitrogen / kg soil water, and use soil water content that is equivalent to a soil water matric potential of -33 KPa. We used this approach at our three demonstration locations.

The problems seem to come when you add too much nitrogen to what is already present in the soil. For many sites, there will be little available nitrogen in the soil and nitrogen applications can be made assuming that there is effectively no residual nitrogen. However, if earlier fertilizer applications have been made, they should be considered. At Galena, the soil had been fertilized earlier and some residual fertilizer remained. Our fertilizer additions inhibited seed germination until microbial processes lowered the nitrogen in the soil.

6.4.4 Fertilizing: Type of Fertilizer

There are proprietary fertilizers on the market, specifically aimed at bioremediation and phytoremediation. Data supporting the benefits of these products are quite scarce and often not critically defensible. For example, CRREL reviewed the marketing literature for a product marketed as a “petroleum remediation enhancer” that showed graphs of concentrations decreasing with time. However, the petroleum was jet fuel, the soil was sand, it was tilled every day, it was hot and windy, and there were no control treatments for comparison. Most of the petroleum almost certainly simply volatilized. Users of products need to know the test conditions in addition to the marketing data and presentations.

Because we usually are not able to identify the sequence of limiting nutrients at a site without a series of treatability studies, and the cost of conducting these studies is usually greater than the benefit gained from them, applying an appropriate level of fertilizer may be as important as using a proprietary fertilizer. Our demonstrations were successful with the use of standard agricultural fertilizer.

6.4.5 Monitoring: Sampling

Monitoring is perhaps the most difficult aspect of rhizosphere enhancement. For sampling, the goal is to determine if there is a decrease in petroleum through time. Problematically, contaminants in surface soil are not uniformly distributed, and trying to quantify the amount of contaminant in a volume of soil at any time is not trivial. The “error” or variability associated with samples is large, and estimates for the total amount of contaminant in the soil are based on the results of the samples that you take. In many instances, taking a set of random samples and using these to estimate the contaminant in the soil, and then taking samples again, exactly the same way but on the next day, would likely yield very different results for the concentration or total amount of petroleum.

At the ESTCP field demonstrations, we used both composite samples and “soil sock” samples. The soil socks consisted of a series of net tubes or socks containing premixed soil placed into each test plot. This approach was used to minimize the variability at the initiation of the study. Soil socks are useful for research, but are too labor and cost intensive and are therefore impractical for routine field use.

We have found that composite samples are helpful. Composite soil samples have been unacceptable in some areas, probably due to the fact that the regulations and guidance on sampling have been based on very aggressive (and costly) remediation methods such as incineration. The concern was that composite sampling would “dilute” possible hot spots and grab samples therefore were required. In reality, for many current remediation methods, grab samples would tend to miss the hot spots. For surface soils that are being rhizosphere-remediated, there is essentially no natural mixing, as is the case for samples from saturated or groundwater zones. For the field demonstrations in

Korea, as well as those in Alaska, we took six to eight samples in each plot and mixed them together prior to analysis. This reduced the variability significantly. Our results suggest that composite sampling provides useful data.

6.4.5 Monitoring: Analysis

For what do you analyze? This is another difficult question that these demonstrations, as well as related projects, have tried to address. Not surprisingly, the easy-to-degrade compounds will degrade readily. Although there can be a rhizosphere benefit for essentially all petroleum compounds, the benefits of rhizosphere-enhancement are most observable for recalcitrant compounds, such as PAHs. We have seen this in our laboratory studies, in the field in Alaska, and also at demonstration trials Korea.

For comparing rhizosphere-enhanced remediation to other treatments it is important to look at both the decrease in total petroleum hydrocarbons (TPH) and how the different components in the petroleum are changing—i.e., the *composition* of the contaminant. Using a biomarker approach, we have demonstrated the benefits of the rhizosphere system, and the results agree with laboratory findings

For potential DoD use in low-cost treatment, the goal may be to show that the treatment is working, but not really to compare it to other treatments. The biomarker approach is very beneficial for monitoring changes because it helps to vitiate the oddities of wildly varying contaminant concentrations caused by uneven or heterogeneous contaminant distribution. The biomarker approach looks at changes in *contaminant composition* rather than concentration. Depending on installation arrangements with the chemical laboratory that you are working with, one can obtain concentration data as well as composition data.

Again, monitoring depends on site needs, but composition or biomarker data are very informative and will better characterize the processes than the standard TPH analysis.

6.4.6 Costs

The greatest cost for rhizosphere-enhanced bioremediation typically is in sampling and monitoring, and that is specific to the frequency of sampling, the type of analysis done, and cost of analysis per sample. The transport, spreading, seeding, and fertilizing are essentially one-time costs, although some re-seeding may be needed annually, and even some watering may be beneficial during seedling establishment. Annual fertilizer can be added but is probably not necessary. Again, this is specific to the site and the goals. We have found that in year two (and even the first season); many volunteer plants tend to establish themselves. This is usually beneficial and, in our experience, the vegetation will shift with time to resemble the local vegetation.

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Appendix A. Chemical Analysis

Soil samples from the site were analyzed using three basic methods, each of which is described in detail below:

1. High-resolution gas chromatography with flame ionization detection (HRGC/FID) using modified EPA method 8015. This yields total petroleum hydrocarbons (TPH) and fraction specific hydrocarbons (FSH) for both volatile and semi-volatile constituents and it provides gas chromatography traces (GC fingerprints) that are used to characterize the sample for product type and weathering state.
2. GC Fingerprints provide information about the composition of the sample.
3. High-resolution gas chromatography with mass spectrometry (HRGC/MS) using modified EPA method 8270. This is used for selected samples to characterize polycyclic hydrocarbons (PAHs), selected heteroaromatic compounds, and the biomarkers hopane.

A.1 HRGC/FID Analyses (EPA Method 8015M): TPH, GC Fingerprints, and FSH

Soil samples were analyzed for total petroleum hydrocarbons (TPH) and fraction-specific hydrocarbons (FSH) using high-resolution gas chromatography flame ionizing detection (HRGC/FID). The analyses were performed according to Battelle Standard Operating Procedures (SOP) 5-202, *Determination of Low Level Total Petroleum Hydrocarbons and Individual Hydrocarbon Concentrations in Environmental Samples*. The procedures were modifications of existing EPA method 8015B.

Before sample analysis, a five-point response factor calibration was performed to demonstrate the linear range of the analysis and to determine the individual response factors (RF) at each calibration solution concentration. The calibration solution was composed of selected *n*-alkanes between C₈ and C₄₀, pristane, and phytane. Target analyte concentrations in the calibration standard solutions range from 0.05 ng/μL to 200.0 ng/μL. The individual target-compound response factors at each calibration concentration were determined, and the total petroleum hydrocarbon (TPH) response factor was based on the average response factors of all the target analytes in the calibration solution over the entire dynamic range.

Samples were screened based on color, and low-level (clear) samples were run before high-level (amber to brown) samples to minimize baseline drift and carry over.

The gas chromatograph (GC) operating conditions were:

Capillary column	0.32 mm x 30 m DB-5 (0.25 μm)
Initial column temperature:	35°C
Initial hold time:	5 minutes
Program rate:	6°C/minute
Final column temperature:	320°C
Final hold time:	10 minutes

Injector temperature: 275°C
 Detector temperature: 325°C
 Column flow rate: 1 mL/min (hydrogen)

Semi-volatile FSH target ranges include:

aliphatic: (F ₁ fraction)	aromatic: (F ₂ fraction)
C _{>8} -C ₁₀ , C _{>10} -C ₁₂ , C _{>12} -C ₁₆ C _{>16} -C ₃₅ , C ₈ -C ₄₀	C _{>8} -C ₁₀ , C _{>10} -C ₁₂ , C _{>12} -C ₁₆ , C _{>16} - C ₂₁ , C _{>21} -C ₃₅ , C ₈ -C ₄₀ .

These ranges correspond with the Total Petroleum Hydrocarbons Criteria Working Group (TPHCWG) criteria.

For *volatile FSH analysis*, soil samples were analyzed by purge-and-trap GC/MS. Total petroleum hydrocarbons in the C₅ to C₈ range were measured. The aromatic compounds that make up the C₆ to C₈ FSH (benzene; toluene; ethylbenzene; and *o*-, *m*-, and *p*- xylenes) were quantified and reported as the volatile aromatic FSH; the aliphatic FSH are defined and computed as the total hydrocarbons that elute between C₅ and C₈, minus the aromatic FSH that elute in this range.

Total petroleum hydrocarbons in the C₅ to C₄₀ range were defined as the sum of TPH in the C₅ to C₈ range + TPH in the C₈ to C₄₀ range F₁ + TPH in the C₈ to C₄₀ range F₂.

A.2 GC Fingerprints – TPH and PAH Degradation

Selected samples, for each treatment, can be monitored for hydrocarbon losses versus time. Using the time-zero samples as the “source” of the contamination (a conservative starting point), depletion of both TPH and PAHs can be tracked. Sample selection needs to be based primarily on those soils that contained both a “degradable” material and a recalcitrant internal marker (hopane). For this study, *degradable* was defined as material that has not undergone significant alteration (weathering) and, therefore, could be used as a time-zero starting point. Those soils containing a significantly weathered petroleum material have to some degree already been bioremediated.

We used the GC traces from the HRGC/FID analyses to help identify the fuel types and amount of degradation (weathering) present in the samples.

A.3 HRGC/MS Analyses (EPA Method 8270M): PAHs, Heteroatomic Compounds, and Biomarkers

Based on the results of the GC fingerprint identifications, a subset of samples was selected for further chemical characterization for polycyclic aromatic hydrocarbons (PAHs), diagnostic heteroatomic compounds, and selected biomarkers. These analyses were performed under a modified EPA method 8270 according to Battelle Standard Operating Procedures (SOP) 5-157, *Identification and Quantification of Polynuclear Aromatic Hydrocarbons (PAH) by Gas Chromatography/Mass Spectrometry*. Target analytes are listed in Table 6.

Table 6. List of target analytes to be scanned for standard PAH analysis.
Compounds in bold are priority pollutant PAHs.

Analyte/Analyte Groups	Abbr.	Analyte/Analyte Groups	Abbr.
Decalin	DC	Dibenzothiophene	D
C1-decalins	DC1	C1-dibenzothiophenes	D1
C2-decalins	DC2	C2-dibenzothiophenes	D2
C3-decalins	DC3	C3-dibenzothiophenes	D3
C4-decalins	DC4	C4-dibenzothiophenes	D4
Benzo(b)thiophene	BT	Fluoranthene	FL
C1-benzo(b)thiophenes	BT1	Pyrene	PY
C2-benzo(b)thiophenes	BT2	C1-fluoranthenes/pyrenes	FP1
C3-benzo(b)thiophenes	BT3	C2-fluoranthenes/pyrenes	FP2
C4-benzo(b)thiophenes	BT4	C3-fluoranthenes/pyrenes	FP3
Naphthalene	N	Benz(a)anthracene	BA
C1-naphthalenes	N1	Chrysene	C
C2-naphthalenes	N2	C1-chrysenes	C1
C3-naphthalenes	N3	C2-chrysenes	C2
C4-naphthalenes	N4	C3-chrysenes	C3
Biphenyl	BI	C4-chrysenes	C4
Acenaphthylene	ACY	Benzo(b)fluoranthene	BB
Acenaphthene	ACE	Benzo(k)fluoranthene	BK
Dibenzofuran	DI	Benzo(e)pyrene	BE
Fluorene	F	Benzo(a)pyrene	BAP
C1-fluorenes	F1	Perylene	PER
C2-fluorenes	F2	Indeno(1,2,3-c,d)pyrene	IP
C3-fluorenes	F3	Dibenz(a,h)anthracene	DA
Anthracene	A	Benzo(g,h,i)perylene	GHI
Phenanthrene	P		
C1-phenanthrenes/anthracenes	P1	17 α (H), 21 β (H) Hopane	H
C2-phenanthrenes/anthracenes	P2		
C3-phenanthrenes/anthracenes	P3		
C4-phenanthrenes/anthracenes	P4	TPAH = sum N through GHI	TPAH

Before HRGC/MS analysis, the instrument was tuned with PFTBA, and a five-point initial calibration was analyzed to determine the linear range of the analysis. The calibration solution was composed of parent and selected alkylated PAHs with concentrations ranging from 0.01 ng/ μ L to 10.0 ng/ μ L. Quantification of individual analytes was determined based on individual response factors relative to selected internal standards (for example, acenaphthene-d₁₀, fluorene-d₁₀). PAH alkyl homologues were quantified using the straight baseline integration of each level of alkylation and the relative RF of the respective parent PAH compound.

The instrument conditions for the analysis were:

Initial column temperature: 40°C
 Initial hold time: 1 minute
 Program rate: 6°/minutes

Final column temperature: 290°C
Final hold time: 10 minutes
Injector temperature: 325°C
Detector temperature: 280°C
Column flow rate: ~1 mL/min (helium)

Electronic pressure control (EPC) conditions were:

Vacuum compensation: On
Pressure at injection: 25 psi
Hold time: 1.50 min.
Pressure program ramp: 99 psi/min.
Final pressure 7.7 psi (equivalent to 1 mL/min.)

Appendix B. Plate Counts

Media for Plate Counts

Bacteria: 0.1x Tryptic Soy Agar (TSA) Medium

3.0 g Tryptic soy broth (Difco #0370-17-3)

15.0 g granulated agar

(or TSA (Difco #0369-17-6) combined TSB and agar in one product)

1.0 L distilled water

0.1 g cycloheximide (Sigma # C7698) in 1.0 mL methanol (added to medium after autoclaving)

Autoclave @ 121°C for 15 min. After cooling to approximately 45°C, add cycloheximide.

Reference Zuberer, D.A. 1994. Recovery and enumeration of viable bacteria. p. 119-144. In R.W. Weaver (ed.). Methods of soil analysis. Part 2. Microbiological and biochemical properties. SSSA Book Ser. 5. SSSA, Madison, WI.

Fungi: Martin's Medium

10.0 g glucose (dextrose)

5.0 g peptone

0.50 g KH₂PO₄

0.50 g K₂HPO₄

0.5 g MgSO₄×7H₂O

33 mg (3.3 mL) Rose Bengal*

15.0 g granulated agar

1.0 L distilled water

30.0 mg streptomycin sulfate (Sigma #S6501) (added after autoclaving)

Autoclave @ 121°C for 15 min. After cooling to approximately 45°C, add streptomycin sulfate¹.

*Rose Bengal, Dissolve 1.0 g Rose Bengal in 100.0 mL deionized water.

Reference Parkinson, D. 1994. Filamentous fungi. p. 329-350. In R.W. Weaver (ed.). Methods of soil analysis. Part 2. Microbiological and biochemical properties. SSSA Book Ser. 5. SSSA, Madison, WI.

Actinomycetes: Starch Casein Medium

10.0 g soluble starch (Fisher #S-516)

0.30 g Casein Hydrolysate (Sigma #C-9386)

2.0 g KNO₃

2.0 g NaCl

2.0 g K₂HPO₄

0.05 g MgSO₄·7H₂O

0.02 g CaCO₃

0.01 g FeSO₄·7H₂O

15.0 g granulated agar

1.0 L distilled water

0.1 g cycloheximide in 1.0 mL methanol (added after autoclaving).

Boil agar, allow to cool slightly and adjust to pH 7 with HCl or NaOH.

Autoclave @ 121°C for 15 min. After cooling to approximately 45°C, add cycloheximide.¹

Reference Wellington, E.M.H., and I.K. Toth, 1994. Actinomycetes. p. 269-290. In R.W. Weaver (ed.). Methods of soil analysis. Part 2. Microbiological and biochemical properties. SSSA Book Ser. 5. SSSA, Madison, WI.

Dilution bottles for serial dilution: MPP Buffer

0.65 g K₂HPO₄

0.35 g KH₂PO₄

0.10 g MgSO₄·7H₂O

1.0 mL Tween 80 (Baker #7-X257)²

1.0 L distilled water

² Add 2 drops (0.1 mL) Tween 80 to -1 dilution bottles only.

Reference Margesin, R., and F. Schinner. 1997. Laboratory bioremediation experiments with soil from a diesel-oil contaminated site-significant role of cold-adapted microorganisms and fertilizers. J. Chem. Tech. Biotechnol. 70:92-98.

Agar Preparation

1. Prepare the Martin's Medium using the recipe above. (You will need 2 Erlenmeyer flasks for autoclaving each liter of media.) Add 500 mL media to a 1000-mL Erlenmeyer flask. Autoclave @ 121°C for 15 min. After cooling to approximately 45°C, add 15 mg streptomycin sulfate to each 500 mL of media. Pour media (approx. 20 mL) into petri dishes. (500 mL of medium/sample.)

Prepare the 0.1 X TSA broth using the recipe on the media page. Add 500 mL of media to a 1000 mL Erlenmeyer flask. Autoclave @ 121°C for 15 min. After cooling to approx. 45°C, add 0.5 mL of 100 mg (0.1 g) cycloheximide in 1 mL MeOH solution to each 500 mL of media. Pour media into petri dishes.

Prepare Starch Casein medium using the recipe on the media page. Add 500 mL of media to a 1000 mL Erlenmeyer flask. After agar has boiled, allow to cool slightly and adjust to pH 7 with HCl or NaOH. Autoclave @ 121°C for 15 min. After cooling to approx. 45°C, add 0.5 mL of 100 mg (0.1 g) cycloheximide in 1 mL MeOH solution to each 500 mL of media. Pour media into petri dishes.

Procedure for Plating Soil

Materials:

- dilution bottles (95 mL, 90 mL, and 45 mL volumes as needed)
- top loading balance
- weighing boats
- shaker table
- 10 mL disposable glass pipets
- pipet bulb
- alcohol lamp (or gas burner)
- 100 µl Eppendorf pipet
- pipet tips
- glass spreading bars (or disposable hockey sticks (Midwest Scientific #LLS-50))
- glass bowl (2)
- inoculation turntable
- plates w/media
- matches

Preparation:

Dilution Bottles:

To allow for volume loss during autoclaving, initial dilution volumes should be measured to 97, 92, and 47 mL to achieve final volumes of 95, 90, and 45 mL of buffer, respectively. For -1 (95 mL) dilution bottles, add 3 to 5 glass beads and two drops (0.1 mL) Tween 80. For -2 (90 mL) and -3 and higher (45mL) dilution bottles use MPP Buffer without Tween 80. Cap all dilution bottles loosely and autoclave @ 121°C for 15 min.

Glass Spreading Bars:

In autoclave bags, autoclave clean glass spreading bars. Flame with alcohol and store in sterile glass bowl.

Procedure:

Weigh out 10 g of field moist soil to be plated. Place soil in -1 dilution bottle. Shake dilution bottle on horizontal shaker table for 5 minutes. Remove from shaker.

Open -2 bottle and sterilize bottle mouth and cap in flame from alcohol lamp or gas burner.

Shake -1 dilution bottle 50 times by hand (full 90-degree arc).

Pipet 10 mL from -1 bottle into -2 bottle. Cap -2 bottle. Dispose of 10 mL pipet in waste container.

Shake -2 dilution bottle on horizontal shaker table for 5 minutes. Remove from shaker.

Shake -2 dilution bottle 50 times by hand. If desired, plate -2 dilution on appropriate media plates.

Place pipet tip on 100 µl Eppendorf pipet.

Rinse pipet tip with solution from -2 bottle 3 times. Dispense 100 µl solution onto media plates. Dispose of pipet tip in waste container.

Place plate, without lid, on inoculation turntable.

Holding sterile spreading bar lightly on surface of media, spin inoculation turntable, making sure to spread suspension evenly. Place used glass spreader bar in unused glass bowl (or other suitable container).

Place lids on plates and incubate plates upside down at 25°C.

Open -3 dilution bottle and sterilize bottle mouth and lid in flame from alcohol lamp or gas burner.

Shake -2 dilution bottle 50 times by hand.

Pipet 5 mL from -2 bottle into -3 bottle (45 mL dilution bottle). Cap -2 bottle. Dispose of 5 mL pipet in waste container.

Shake -3 dilution bottle 50 times by hand.

Plate -3 dilution, or dilute to -4.

Continue in this manner, plating where appropriate to media.

Notes:

Only -1 and -2 dilution bottles get shaken on shaker table.

Only use sterile pipets or pipet tips. Do not forget to flame cap and bottle each time it is opened.

Soil Moisture Determination

1. Determine moisture content of soil by drying a known amount of soil @ 105°C to a constant weight.

Clean-up Procedures

1. All glassware and dilution bottles should be autoclaved at 121°C for 15 min prior to cleaning or disposal. After autoclaving, glass pipets can be disposed of in waste glass container. Used pipet tips and other materials may be disposed of in appropriate waste containers after autoclaving. Rinse dilution bottles in sink, making sure to catch any soil waste and glass beads in fine sieve. Rinse glass spreading bars, wash bars and dilution bottles in dishwasher. Autoclave to reuse spreading bars. Refill dilution bottles with MPP buffer and autoclave for later use.

Reading Plates

1. Incubate plates in the dark at room temperature (25°C).
Fungi: Martin's medium plates are to be read 3 and 10 days after inoculation.
Bacteria: 0.1 X TSA plates are to be read 2 and 7 days after inoculation.
Actinomycetes: Starch Casein plates are to be read 14 days after inoculation.

Values are calculated and reported as $\log_{10}$ CFU/g dry soil.

Appendix C. MPN Tubes

Media Preparation

MPP Buffer (same as described in Appendix B.)

Bushnell-Haas Negative Control (5 tubes/soil sample)

3.26 g Bushnell-Haas (BH) medium (Difco#0578-17-3)

1.0 L deionized H₂O

(each tube contains 4.5 mL of BH medium)

Dextrose Positive Control (5 tubes/soil sample)

3.26 g BH medium

10.0 g Dextrose (Fisher #D-16)

1.0 L deionized H₂O

(each tube contains 4.5 mL of Dextrose medium)

Motor Oil (40 tubes/soil sample)

3.26 g BH medium

1.0 L deionized H₂O

(one drop Coastal 30W non-detergent motor oil added to each of 40 test tube containing 4.5 mL BH medium/tube)

Reference: Walker, J.D., and R.R. Colwell. 1976. Enumeration of petroleum-degrading microorganisms. Appl. Environ. Microbiol. 31:198-207.

Vegetable Oil (40 tubes/soil sample)

3.26 g BH medium

1.0 L deionized H₂O

(one drop Wesson vegetable oil added to each of 40 test tube containing 4.5 mL BH medium/tube)

Sodium Benzoate (40 tubes/soil sample)

3.26 g BH medium

6.90 g Sodium Benzoate (Fisher#S-299)

1.0 L deionized H₂O

(each tube contains 4.5 mL of Sodium Benzoate medium)

Reference Modified from: Mesarch, M.B., and L. Nies. 1997. Modification of heterotrophic plate counts for assessing the bioremediation potential of petroleum-contaminated soils. Environ. Tech. 18:639-646.

Cyclohexanol (40 tubes/soil sample)

3.26 g BH medium
1.0 L deionized H₂O
(one drop cyclohexanol (Aldrich#10,589-9) added to each of 40 test tube containing 4.5 mL BH medium/tube)

Materials (for one soil sample)

- 170 test tubes (6-10mL)
- 5 test tube racks
- 170 test tube caps
- Aluminum foil
- 1 multi-channel pipetter
- 40 sterile tips for multi-channel pipetter
- 8 sterile multi-channel pipetter basins
- 1 aluminum weigh dish
- 25 mL BH negative control medium
- 25 mL Dextrose positive control medium
- 200 mL Motor Oil medium
- 200 mL Vegetable Oil medium
- 200 mL Sodium Benzoate medium
- 200 mL Cyclohexanol medium
- 1 95 mL MPP dilution bottle (10^{-1} dilution)
- 1 90 mL MPP dilution bottle (10^{-2} dilution)
- 7 45 mL MPP dilution bottles (10^{-3} through 10^{-9} dilutions)
(total of 9 MPP dilution bottles per sample)
- 9 stoppers for dilution bottles
- 1 wide tip 10-mL sterile pipette
- 7 5-mL sterile pipettes
- 1 pipette bulb

MPN Media Preparation per Sample

1. Prepare the MPP buffer using direction from Appendix B. You will need 9 dilution bottles and 9 stoppers per soil sample.
2. BH negative control: Prepare medium using recipe on media page, add 4.7 mL of medium to each of 5 test tubes. To allow for volume loss during autoclaving, initial tube volumes should be measured to 4.7 mL to achieve final volumes of 4.5 mL of medium. The 10^{-2} dilution will be the only dilution inoculated for BH

negative control medium. Cover test tubes with foil and autoclave @ 121°C for 15 min.

3. Dextrose positive control: Prepare medium using recipe on media page, add 4.7 mL of medium to each of 5 test tubes. The 10^{-3} dilution will be the only dilution inoculated for Dextrose positive control medium. Cover test tubes with foil and autoclave @ 121°C for 15 min.
4. Motor oil medium: Prepare medium using recipe on media page, add 4.7 mL of medium to each of 40 test tubes. After the medium has been added to the tubes, add one drop of 30W non-detergent motor oil to each test tube. Cover test tubes with foil and autoclave @ 121°C for 15 min.
5. Vegetable oil medium: Prepare medium using recipe on media page, add 4.7 mL of medium to each of 40 test tubes. After the medium has been added to the tubes, add one drop of Wesson vegetable oil to each test tube. Cover test tubes with foil and autoclave @ 121°C for 15 min.
6. Sodium Benzoate medium: Prepare medium using recipe on media page, add 4.7 mL of medium to each of 40 test tubes. Cover test tubes with foil and autoclave @ 121°C for 15 min.
7. Cyclohexanol medium: Prepare media using recipe on media page, add 4.7 mL of medium to each of 40 test tubes. After medium has been added to test tubes, add one drop of cyclohexanol to each test tube. Cover test tubes with foil and autoclave @ 121°C for 15 min.
8. Autoclave 170 test tube caps @ 121°C for 15 min.

Procedure

Preparation of MPN Dilutions

See Appendix B

Inoculation of Media

1. Motor oil, Vegetable oil, Sodium Benzoate, and Cyclohexanol media: 0.5 mL of each dilution (10^{-2} - 10^{-9}) will be added respectively to each of 5 test tubes. Begin by shaking the dilution bottle to ensure even dispersion. Then pour an appropriate amount of the dilution into a sterile pipetter basin. Add 0.5 mL of the dilution to the media. (It is best to add, for example, the 10^{-2} dilution to all of the media and then add the 10^{-3} dilution and so on. It is quicker and uses fewer pipette tips). Cover tubes with sterile caps after adding inoculant.
2. BH: 0.5 mL of the 10^{-2} dilution will be added to each of five test tubes. To

save space, you can put the neg. control tubes in the same test tube rack with the dextrose samples. Cover tubes with sterile caps after adding inoculant.

3. Dextrose: 0.5 mL of the 10^{-3} dilution will be added to each of five test tubes. Cover tubes with sterile caps after adding inoculant.

Reading Tubes

1. Incubate in the dark at room temperature (25°C).
2. Read the MPN tubes on the following schedule:

<u>Medium</u>	<u>Weeks of incubation</u>
BH -	5
Negative	5
Dextrose	5
Cyclohexanol	6
Benzoate	8
Motor Oil	8
Vegetable Oil	

3. Microbial growth or a positive reading is indicated by turbidity in the tube(s). Vortexing the tubes is helpful to discern microbial growth. When positive tubes are determined, the MPN value can be determined using appropriate MPN tables (Woomer, 1994).

Reference: Woomer, P.L. 1994. Most probable number counts. p. 59-79. In R.W. Weaver (ed.). Methods of soil analysis. Part 2. Microbiological and biochemical properties. SSSA Book Ser. 5. SSSA, Madison, WI.

Clean-up Procedures

See Appendix B.

Note: The MPN determination method appears suitable for adaptation to microtiter plate methodology. References:

Haines, J.R., B.A. Wrenn, E.L. Holder, K.L. Strohmeier, R.T. Herrington, and A.D. Venosa. 1996. Measurement of hydrocarbon-degrading microbial populations by a 96-well plate most probable number procedure. J. Indust. Microbiol. 16:36-41.

Wrenn, B.A., and A.D. Venosa. 1996. Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a most-probable-number procedure. Can. J. Microbiol. 42:252-258.

¹ To determine temperature of media after autoclaving, use stick on thermometers. DO NOT autoclave thermometer.

Appendix D. List of Participants

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Appendix E. Data

Table 7. C₃ Decalin-normalized depletions at Annette Site; 680 treatment days from t_{initial} to t_{final}; 5114.2 growing degree days from t_{initial} to t_{final} (0 °C base temp).

Treat-ment	Re p	C ₃ -Decalins (ug/g oil wt)		C ₃ Decalin-normalized depletions t _{initial} to t _{final} (%)												
		t _{initial}	t _{final}	TPH	Sum PAH	Aliphatic TPH					Aromatic TPH					
						C _{>8} -C ₁₀	C _{>10} -C ₁₂	C _{>12} -C ₁₆	C _{>16} -C ₃₅	C ₈ -C ₄₀	C _{>8} -C ₁₀	C _{>10} -C ₁₂	C _{>12} -C ₁₆	C _{>16} -C ₂₁	C _{>21} -C ₃₅	C ₈ -C ₄₀
C	1	1119.509	1113.245	-12.3	23.3	24.5	11.2	4.5	-24.0	-19.0	100.0	34.7	18.2	15.3	-0.8	10.4
F	1	1802.862	2272.672	27.3	36.0	-29.1	-3.5	-1.3	37.4	28.5	-251.6	22.1	13.5	15.2	37.8	22.9
P	1	882.687	625.873	-64.5	6.0	-55.8	-3.4	-5.4	-73.1	-65.2	-178.3	-0.4	-13.4	-24.7	150.1	-60.6
F+P	1	1349.289	1088.828	-72.2	32.1	-83.1	59.5	-37.2	-103.3	-92.3	69.6	12.8	17.9	8.2	-43.9	-3.5
P	2	2559.822	2077.563	-14.0	76.7	52.2	45.9	19.8	-56.3	-26.8	-77.3	98.1	83.8	30.4	-52.5	37.4
F	2	2027.914	817.427	-57.2	89.7	78.0	77.4	21.4	-80.5	-44.4	100.0	73.3	56.1	-93.3	495.3	-104.9
C	2	3332.552	2575.674	17.4	-412.4	10.0	39.8	29.5	33.9	33.4	-2384.5	-815.4	174.3	-84.7	-47.5	-95.1
F+P	2	2020.708	1128.776	-15.2	72.8	47.1	58.4	-4.9	1.9	0.0	100.0	-125.2	14.8	-80.1	135.9	-76.3
F	3	339.392	78.823	-179.5	87.7	76.7	36.5	-21.6	-131.6	-133.1	100.0	-446.4	-6.3	157.5	687.1	-515.9
F+P	3	210.166	216.153	23.7	90.3	-37.6	34.8	22.5	33.9	31.5	100.0	-10.4	29.8	-3.2	-23.0	-18.2
P	3	194.954	85.669	-148.1	65.7	-436.7	5.6	-8.8	-151.3	-150.5	100.0	-84.2	24.1	-62.1	151.6	-137.7
C	3	77.008	59.754	-22.8	71.3	-217.9	15.3	6.8	-18.5	-16.8	100.0	-27.7	21.9	-23.9	-65.4	-60.0
C	4	63.688	59.705	10.2	87.2	NV	22.6	23.7	15.6	13.8	NV	NV	46.3	20.6	-19.2	-14.6
F+P	4	86.338	0.000	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV
P	4	120.827	118.081	-5.1	96.2	-36.8	27.7	12.1	-3.1	-3.0	100.0	70.9	33.1	9.4	-23.9	-17.4
F	4	74.660	0.000	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV

C = no fertilizer, no plants

F = fertilizer, no plants

P = no fertilizer, plants

F+P = fertilizer, plants

NV = no value

Table 8. 17B(H),21a(H)-Hopane-normalized depletions at Barrow Site; 448 treatment days from t_{initial} to t_{final} ; 573.1 growing degree days from t_{initial} to t_{final} (0 °C base temp).

Treat-ment	Re p	17B(H),21a(H)-Hopane (ug/g oil wt)		Hopane-normalized depletions t_{initial} to t_{final} (%)												
		t_{initial}	t_{final}	TPH	Sum PAH	Aliphatic TPH					Aromatic TPH					
						C _{>8-} C ₁₀	C _{>10-} C ₁₂	C _{>12-} C ₁₆	C _{>16-} C ₃₅	C _{8-C40}	C _{>8-} C ₁₀	C _{>10-} C ₁₂	C _{>12-} C ₁₆	C _{>16-} C ₂₁	C _{>21-} C ₃₅	C _{8-C40}
F+P	1	518.650	755.200	29.72	21.51	34.17	42.34	28.76	19.77	22.11	71.20	50.90	47.46	58.46	48.97	54.23
C	1	559.273	655.268	35.19	62.41	73.40	76.67	66.48	13.16	31.83	59.02	76.49	78.26	59.39	28.39	48.71
P	1	574.003	568.472	-0.69	47.88	7.76	19.17	17.76	-9.71	-3.46	27.59	24.13	24.77	6.34	6.38	12.34
F	1	608.895	746.397	27.74	62.99	20.19	48.85	69.95	11.42	26.47	6.36	13.72	63.28	75.10	8.96	33.71
P	2	418.846	439.470	22.44	45.07	33.32	49.83	30.60	10.28	21.05	38.00	36.22	29.16	22.33	18.66	27.23
C	2	492.809	426.521	1.72	-13.36	-15.19	-29.63	-16.29	13.84	2.49	43.77	-29.43	30.42	16.79	14.68	-1.34
F+P	2	514.849	389.600	-3.41	-20.94	-4.42	-17.02	-11.09	-2.57	-7.69	19.45	-21.54	9.64	11.67	25.03	12.04
F	2	470.019	515.268	22.49	32.02	50.09	53.21	48.51	5.12	21.50	58.78	28.14	51.00	17.87	12.20	26.42
F+P	3	77.539	87.347	6.19	40.46	-40.09	0.68	2.78	-2.41	1.02	24.13	35.97	35.33	35.75	27.77	34.48
C	3	98.440	70.481	-16.99	19.43	7.90	-2.33	-29.72	2.60	-19.59	-32.47	24.62	-8.26	7.87	8.80	-1.00
F	3	91.415	112.645	26.58	54.74	35.20	34.66	24.60	7.57	24.44	73.63	52.86	40.23	33.87	25.53	39.74
P	3	138.826	90.866	10.25	53.62	42.98	37.27	-0.28	4.74	7.69	NV	67.35	27.05	16.99	14.43	26.88
F	4	312.813	174.435	39.23	66.82	41.04	57.59	36.59	25.88	38.81	NV	75.73	41.99	33.44	25.65	42.14
P	4	143.141	146.955	9.78	45.38	-29.22	16.54	4.38	10.17	6.38	NV	64.26	28.60	35.96	30.86	31.91
C	4	103.600	248.447	47.37	71.55	76.42	69.82	46.19	19.64	46.93	72.02	83.81	55.60	33.66	6.79	50.21
F+P	4	128.155	190.493	17.12	56.31	29.45	35.01	12/26	-0.90	14.21	100.00	74.45	32.88	23.05	26.20	34.28

C = no fertilizer, no plants

F = fertilizer, no plants

P = no fertilizer, plants

F+P = fertilizer, plants

NV = no value

Table 9. C₃ Decalin-normalized depletions at Galena Site; 68 treatment days from t_{initial} to t_{final}; 813.7 growing degree days from t_{initial} to t_{final} (0 °C base temp).

Treatment	Rep	C ₃ -Decalins (ug/g oil wt)		C ₃ -Decalin-normalized depletions t _{initial} to t _{final} (%)												
		t _{initial}	t _{final}	Aliphatic TPH						Aromatic TPH						
				TPH	Sum PAH	C _{>8} -C ₁₀	C _{>10} -C ₁₂	C _{>12} -C ₁₆	C _{>16} -C ₃₅	C ₈ -C ₄₀	C _{>8} -C ₁₀	C _{>10} -C ₁₂	C _{>12} -C ₁₆	C _{>16} -C ₂₁	C _{>21} -C ₃₅	C ₈ -C ₄₀
C	1	3561.0	4274.7													
		0	9	17.60	-36.96	-198.06	-39.38	32.91	14.06	18.93	NV	100.00	-92.94	-43.82	14.41	5.84
P	1	1169.7	1640.1													
		5	7	42.72	49.17	-422.38	27.23	43.15	47.66	44.90	99.44	40.61	-48.44	-58.05	36.30	31.29
F	1	7324.2	5986.7								100.0					
		7	8	13.23	-9.35	58.04	40.05	29.90	-26.27	18.58	0	-46.62	-56.84	-102.59	-73.00	-62.02
F+P	1	5173.9	6491.5								100.0					
		1	3	32.35	14.35	71.94	50.93	41.13	23.43	37.10	0	-310.85	-213.92	-101.14	2.54	-26.40
F	2	5812.0	4323.2								100.0					
		8	3	-11.99	-88.41	-189.06	3.48	8.77	-33.38	-6.92	0	-827.42	-236.93	-163.44	-47.30	-67.35
P	2	819.66	1592.7													
		8	8	38.58	46.32	83.33	45.43	33.53	39.45	38.97	NV	NV	1.60	-31.30	37.25	36.80
C	2	2045.3	2045.3								100.0					
		1	1	61.49	78.97	-4.67	32.19	43.74	66.72	59.95	0	95.16	42.48	34.66	68.88	67.53
F+P	2	2516.2	5454.5													
		7	8	51.23	37.01	61.91	44.16	37.30	59.82	49.36	31.50	72.77	40.55	19.09	65.33	61.85
P	3	1347.5	2954.5													
		4	2	33.40	36.65	2.34	5.03	28.32	33.05	30.10	NV	100.00	53.96	30.56	41.30	46.98
F+P	3	3609.1	6714.0													
		6	3	46.48	50.36	34.32	35.27	39.25	53.39	44.41	NV	71.24	48.45	39.58	60.35	59.71
F	3	5148.6	8623.4								100.0					
		6	2	45.96	60.17	36.17	33.45	38.44	56.29	44.02	0	69.04	43.32	33.20	64.02	59.14
C	3	2564.5	2567.3													
		8	9	16.04	75.03	-122.43	7.19	25.30	0.60	11.95	NV	99.28	66.38	16.24	19.17	34.21
C	4	6427.1	1285.8								100.0					
		1	5	-249.97	-669.86	98.86	27.09	-65.24	-675.11	-223.93	0	100.00	-6.06	-324.81	-956.88	-477.23
F+P	4	4175.5	4033.1								100.0					
		0	1	-4.07	-6.02	53.08	31.93	8.19	-37.69	-8.15	0	100.00	53.79	-12.68	-3.58	18.36
F	4	4370.8	3187.4								100.0					
		9	4	-0.90	6.01	72.42	33.33	6.08	-20.34	-2.55	0	100.00	49.28	-25.92	-10.47	9.30
P	4	561.80	1273.7								100.0	100.00	74.12	47.08	62.30	65.45

1

0

C = no fertilizer, no plants

F = fertilizer, no plants

P = no fertilizer, plants

F+P = fertilizer, plants

NV = no value

Table 10. C₃ Decalin- and growing-degree-day (GDD)-normalized depletions at Annette Site; 680 treatment days from t_{initial} to t_{final}; 5114.2 growing degree days from t_{initial} to t_{final} (0 °C base temp).

		C ₃ -Decalins (ug/g oil wt)		C ₃ Decalin- and GDD-normalized depletions t _{initial} to t _{final} (%)												
		t _{initial}	t _{final}	Aliphatic TPH						Aromatic TPH						
Treat- ment	Re- p			TPH	Sum PAH	C _{>8} -C ₁₀	C _{>10} -C ₁₂	C _{>12} -C ₁₆	C _{>16} -C ₃₅	C ₈ -C ₄₀	C _{>8} - C ₁₀	C _{>10} -C ₁₂	C _{>12} -C ₁₆	C _{>16} -C ₂₁	C _{>21} -C ₃₅	C ₈ -C ₄₀
C	1	1119.50	1113.24													
		9	5	-2.40E-03	4.56E-03	4.80E-03	2.18E-03	8.78E-04	-4.68E-03	-3.72E-03	1.96E-02	6.78E-03	3.56E-03	2.99E-03	-1.47E-04	2.04E-03
F	1	1802.86	2272.67													
		2	2	5.34E-03	7.04E-03	-5.68E-03	-6.91E-04	-2.62E-04	7.32E-03	5.57E-03	-4.92E-02	4.33E-03	2.64E-03	2.98E-03	7.40E-03	4.47E-03
P	1	882.687	625.873													
		1349.28	1088.82	-1.26E-02	1.18E-03	-1.09E-02	-6.59E-04	-1.06E-03	-1.43E-02	-1.27E-02	-3.49E-02	-8.21E-05	-2.62E-03	-4.82E-03	-2.93E-02	-1.18E-02
F+P	1	2559.82	2077.56													
		9	8	-1.41E-02	6.29E-03	-1.63E-02	-1.16E-02	-7.27E-03	-2.02E-02	-1.80E-02	1.36E-02	2.50E-03	3.50E-03	1.61E-03	-8.58E-03	-6.81E-04
P	2	2559.82	2077.56													
		2	3	-2.73E-03	1.50E-02	1.02E-02	8.98E-03	3.87E-03	-1.10E-02	-5.24E-03	-1.51E-02	1.92E-02	1.64E-02	5.94E-03	-1.03E-02	7.31E-03
F	2	2027.91														
		4	817.427	-1.12E-02	1.75E-02	1.53E-02	1.51E-02	4.18E-03	-1.58E-02	-8.69E-03	1.96E-02	1.43E-02	1.10E-02	-1.82E-02	-9.69E-02	-2.05E-02
C	2	3332.55	2575.67													
		2	4	3.40E-03	-8.06E-02	1.95E-03	7.79E-03	5.77E-03	6.62E-03	6.53E-03	-4.66E-01	-1.59E-01	-3.41E-02	-1.66E-02	-9.28E-03	-1.86E-02
F+P	2	2020.70	1128.77													
		8	6	-2.97E-03	1.42E-02	9.20E-03	1.14E-02	-9.64E-04	3.77E-04	8.80E-06	1.96E-02	-2.45E-02	2.89E-03	-1.57E-02	-2.66E-02	-1.49E-02
F	3	339.392	78.823													
				-3.51E-02	1.72E-02	1.50E-02	7.14E-03	-4.23E-03	-2.57E-02	-2.60E-02	1.96E-02	-8.73E-02	-1.24E-03	-3.08E-02	-1.34E-01	-1.01E-01
F+P	3	210.166	216.153													
				4.64E-03	1.77E-02	-7.35E-03	6.81E-03	4.40E-03	6.63E-03	6.15E-03	1.96E-02	-2.04E-03	5.82E-03	-6.35E-04	-4.50E-03	-3.56E-03
P	3	194.954	85.669													
				-2.90E-02	1.29E-02	-8.54E-02	1.09E-03	-1.72E-03	-2.96E-02	-2.94E-02	1.96E-02	-1.65E-02		-1.21E-02	-2.96E-02	-2.69E-02
C	3	77.008	59.754													
				-4.46E-03	1.39E-02	-4.26E-02	2.99E-03	1.32E-03	-3.62E-03	-3.28E-03	1.96E-02	-5.42E-03	4.28E-03	-4.67E-03	-1.28E-02	-1.17E-02
C	4	63.688	59.705													
				2.00E-03	1.70E-02	NV	4.41E-03	4.63E-03	3.05E-03	2.70E-03	NV	NV	9.05E-03	4.02E-03	-3.76E-03	-2.86E-03
F+P	4	86.338	0.000													
				NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV
P	4	120.827	118.081													
				-1.00E-03	1.88E-02	-7.20E-03	5.42E-03	2.37E-03	-6.15E-04	-5.79E-04	1.96E-02	1.39E-02	6.48E-03	1.85E-03	-4.68E-03	-3.39E-03
F	4	74.660	0.000													
				NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV

C = no fertilizer, no plants

F = fertilizer, no plants

P = no fertilizer, plants

F+P = fertilizer, plants
GDD = growing degree day
NV = no value

Table 11. 17B(H),21a(H)-Hopane- and growing-degree-day(GDD)-normalized depletions at Barrow Site; 448 treatment days from t_{initial} to t_{final} ; 573.1 growing degree days from t_{initial} to t_{final} (0 °C base temp).

		17B(H),21a(H)-Hopane (ug/g oil wt)		Hopane- and GDD-normalized depletions t _{initial} to t _{final} (%)												
Treatment	Rep	t _{initial}	t _{final}	TPH	Sum PAH	Aliphatic TPH					Aromatic TPH					
						C _{>8-} C ₁₀	C _{>10-} C ₁₂	C _{>12-} C ₁₆	C _{>16-} C ₃₅	C ₈₋ C ₄₀	C _{>8-} C ₁₀	C _{>10-} C ₁₂	C _{>12-} C ₁₆	C _{>16-} C ₂₁	C _{>21-} C ₃₅	C ₈₋ C ₄₀
F+P	1	518.650	755.200	5.19E-02	0.04	0.06	0.07	0.05	0.03	0.04	0.12	0.09	0.08	0.10	0.09	0.09
C	1	559.273	655.268	6.14E-02	0.11	0.13	0.13	0.12	0.02	0.06	0.10	0.13	0.14	0.10	0.05	0.09
P	1	574.003	568.472	-1.20E-03	0.08	0.01	0.03	0.03	-0.02	-0.01	0.05	0.04	0.04	0.01	0.01	0.02
F	1	608.895	746.397	4.84E-02	0.11	0.04	0.09	0.12	0.02	0.05	0.01	0.02	0.11	0.13	0.02	0.06
P	2	418.846	439.470	3.92E-02	0.08	0.06	0.09	0.05	0.02	0.04	0.07	0.06	0.05	0.04	0.03	0.05
C	2	492.809	426.521	3.00E-03	-0.02	-0.03	-0.05	-0.03	0.02	0.00	0.08	-0.05	-0.05	0.03	0.03	0.00
F+P	2	514.849	389.600	-5.94E-03	-0.04	-0.01	-0.03	-0.02	0.00	-0.01	0.03	-0.04	0.02	0.02	0.04	0.02
F	2	470.019	515.268	3.93E-02	0.06	0.09	0.09	0.08	0.01	0.04	0.10	0.05	0.09	0.03	0.02	0.05
F+P	3	77.539	87.347	1.08E-02	0.07	-0.07	0.00	0.00	0.00	0.00	0.04	0.06	0.06	0.06	0.05	0.06
C	3	98.440	70.481	-2.96E-02	0.03	0.01	0.00	-0.05	0.00	-0.03	-0.06	0.04	-0.01	0.01	0.02	0.00
F	3	91.415	112.645	4.64E-02	0.10	0.06	0.06	0.04	0.01	0.04	0.13	0.09	0.07	0.06	0.04	0.07
P	3	138.826	90.866	1.79E-02	0.09	0.07	0.07	0.00	0.01	0.01	NV	0.12	0.05	0.03	0.03	0.05
F	4	312.813	174.435	6.85E-02	0.12	0.07	0.10	0.06	0.05	0.07	NV	0.13	0.07	0.06	0.04	0.07
P	4	143.141	146.955	1.71E-02	0.08	-0.05	0.03	0.01	0.02	0.01	NV	0.11	0.05	0.06	0.05	0.06
C	4	103.600	248.447	8.27E-02	0.12	0.13	0.12	0.08	0.03	0.08	0.13	0.15	0.10	0.06	0.01	0.09
F+P	4	128.155	190.493	2.99E-02	0.10	0.05	0.06	0.02	0.00	0.02	0.17	0.13	0.06	0.04	0.05	0.06

C = no fertilizer, no plants

F = fertilizer, no plants

P = no fertilizer, plants

F+P = fertilizer, plants

GDD = growing degree day

NV = no value

Table 12. C₃ Decalin- and growing-degree-day (GDD)-normalized depletions at Galena Site; 68 treatment days from t_{initial} to t_{final}; 813.7 growing degree days from t_{initial} to t_{final} (0 °C base temp).

		C ₃ -Decalins (ug/g oil wt)		C ₃ -Decalin- and GDD-normalized depletions t _{initial} to t _{final} (%)													
Trea tme nt	Re p	t _{initial}	t _{final}	TPH	Sum PAH	Aliphatic TPH					Aromatic TPH						
						C _{>8-} C ₁₀	C _{>10-} C ₁₂	C _{>12-} C ₁₆	C _{>16-} C ₃₅	C ₈₋ C ₄₀	C _{>8-} C ₁₀	C _{>10-} C ₁₂	C _{>12-} C ₁₆	C _{>16-} C ₂₁	C _{>21-} C ₃₅	C ₈₋ C ₄₀	
C	1	3561.0	4274.7														
		0	9	0.02	-0.05	-0.24	-0.05	0.04	0.02	0.02	NV	0.12	-0.11	-0.05	0.02	0.01	
P	1	1169.7	1640.1														
		5	7	0.05	0.06	-0.52	0.03	0.05	0.06	0.06	0.12	0.05	-0.06	-0.07	0.04	0.04	
F	1	7324.2	5986.7														
		7	8	0.02	-0.01	0.07	0.05	0.04	-0.03	0.02	0.12	-0.06	-0.07	-0.13	-0.09	-0.08	
F+P	1	5173.9	6491.5														
		1	3	0.04	0.02	0.09	0.06	0.05	0.03	0.05	0.12	-0.38	-0.26	-0.12	0.00	-0.03	
F	2	5812.0	4323.2														
		8	3	-0.01	-0.11	-0.23	0.00	0.01	-0.04	-0.01	0.12	-1.02	-0.29	-0.20	-0.06	-0.08	
P	2	1592.7															
		8	8	0.05	0.06	0.10	0.06	0.04	0.05	0.05	NV	NV	0.00	-0.04	0.05	0.05	
C	2	2045.3															
		822.04	1	0.08	0.10	-0.01	0.04	0.05	0.08	0.07	0.12	0.12	0.05	0.04	0.08	0.08	
F+P	2	2516.2	5454.5														
		7	8	0.06	0.05	0.08	0.05	0.05	0.07	0.06	0.04	0.09	0.05	0.02	0.08	0.08	
P	3	1347.5	2954.5														
		4	2	0.04	0.05	0.00	0.01	0.03	0.04	0.04	NV	0.12	0.07	0.04	0.05	0.06	
F+P	3	3609.1	6714.0														
		6	3	0.06	0.06	0.04	0.04	0.05	0.07	0.05	NV	0.09	0.06	0.05	0.07	0.07	
F	3	5148.6	8623.4														
		6	2	0.06	0.07	0.04	0.04	0.05	0.07	0.05	0.12	0.08	0.05	0.04	0.08	0.07	
C	3	2564.5	2567.3														
		8	9	0.02	0.09	-0.15	0.01	0.03	0.00	0.01	NV	0.12	0.08	0.02	0.02	0.04	
C	4	6427.1	1285.8														
		1	5	-0.31	-0.82	0.12	0.03	-0.08	-0.83	-0.28	0.12	0.12	-0.01	-0.40	-1.18	-0.59	
F+P	4	4175.5	4033.1														
		0	1	-0.01	-0.01	0.07	0.04	0.01	-0.05	-0.01	0.12	0.12	0.07	-0.02	0.00	0.02	
F	4	4370.8	3187.4														
		9	4	0.00	0.01	0.09	0.04	0.01	-0.02	0.00	0.12	0.12	0.06	-0.03	-0.01	0.01	
P	4	561.80	1273.7														
				0.07	0.08	-0.04	0.03	0.04	0.07	0.06	0.12	0.12	0.09	0.06	0.08	0.08	

1

C = no fertilizer, no plants
F = fertilizer, no plants
P = no fertilizer, plants
F+P = fertilizer, plants
GDD = growing degree day
NV = no value

Figures



Figure 1. Block of sample plots at Campion Air Force Station in August 1999.



Figure 2. Plant growth on Campion plots by late September 1999.

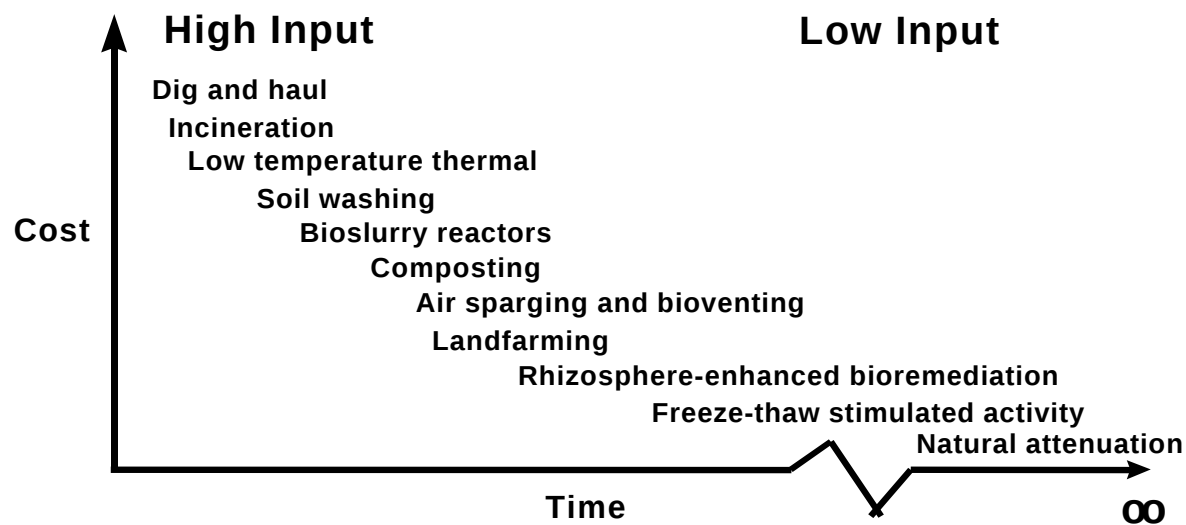


Figure 3. Cost versus time trade-off for remediation techniques.

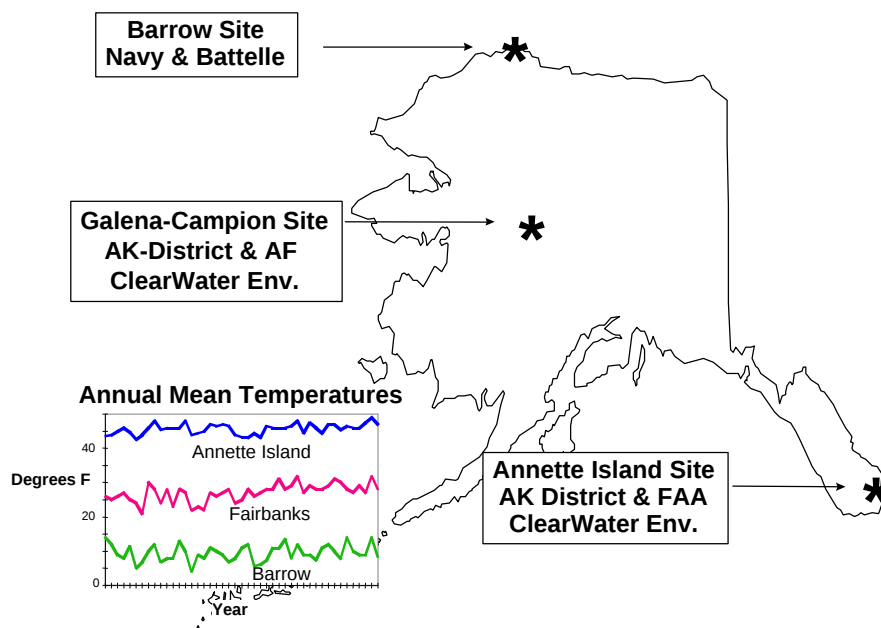


Figure 4. Location of our three sites in Alaska.

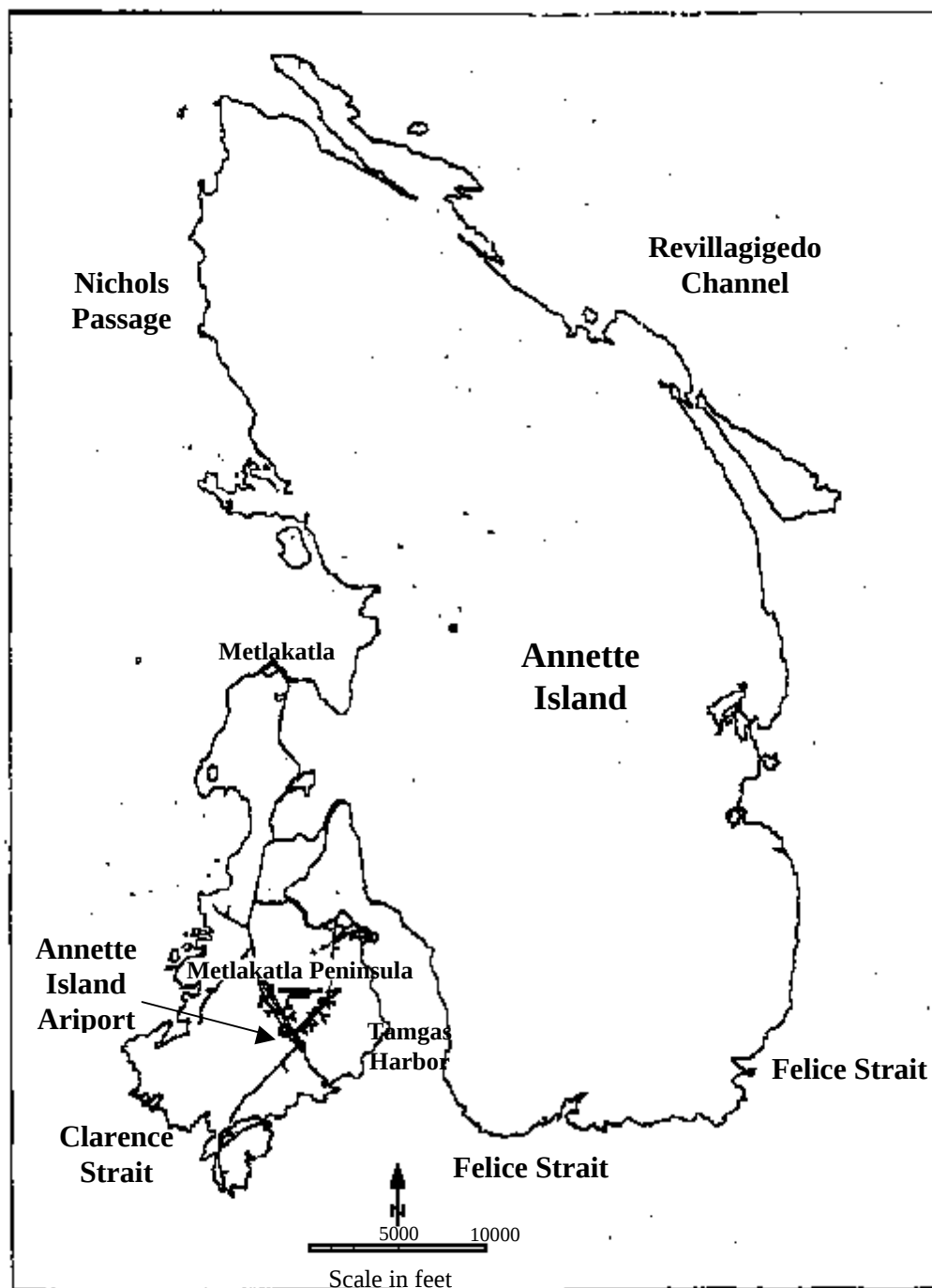


Figure 5. Map of Annette Island.

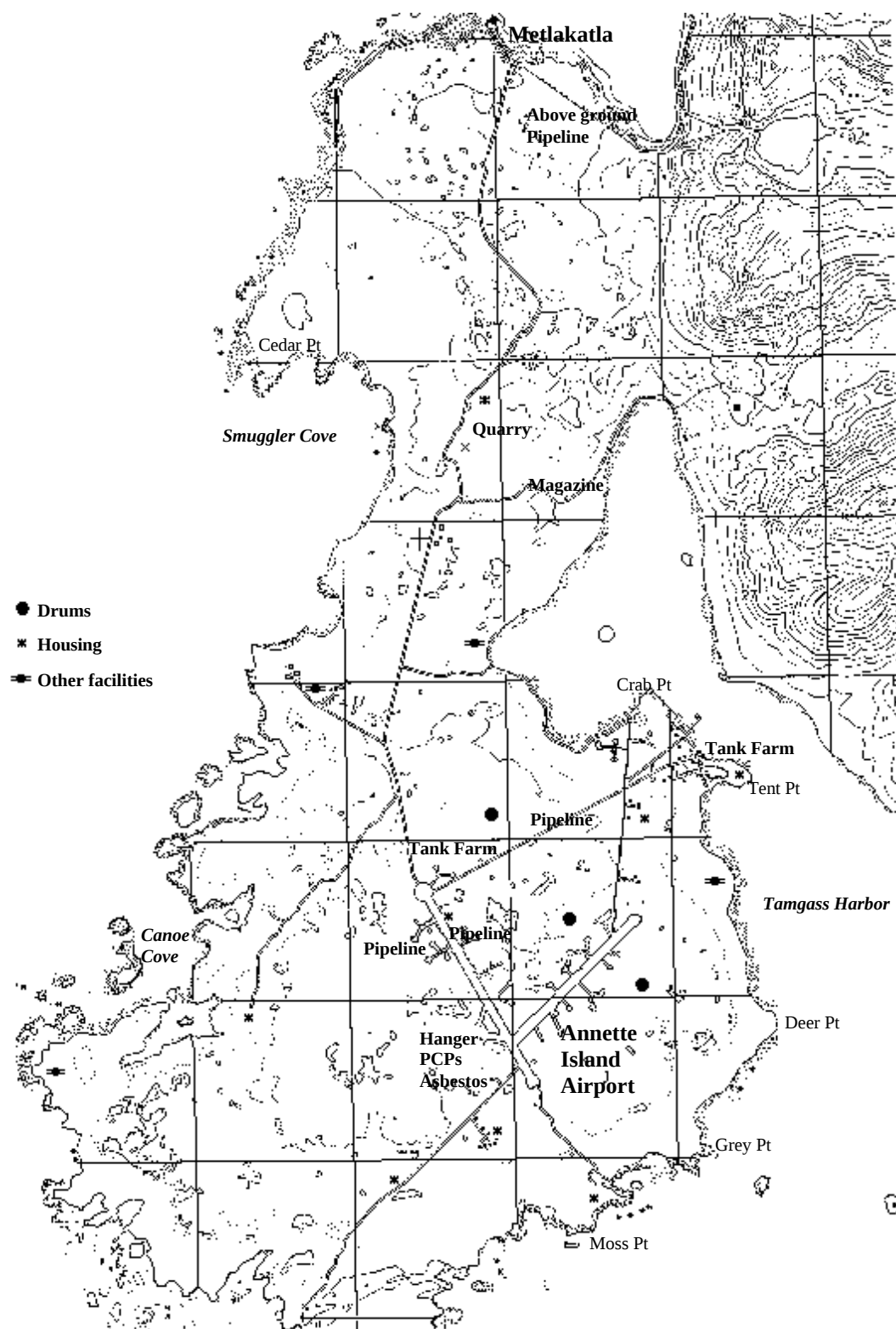


Figure 6. Metlakatla Peninsula of Annette Island showing Annette Island Airport and tank farm.

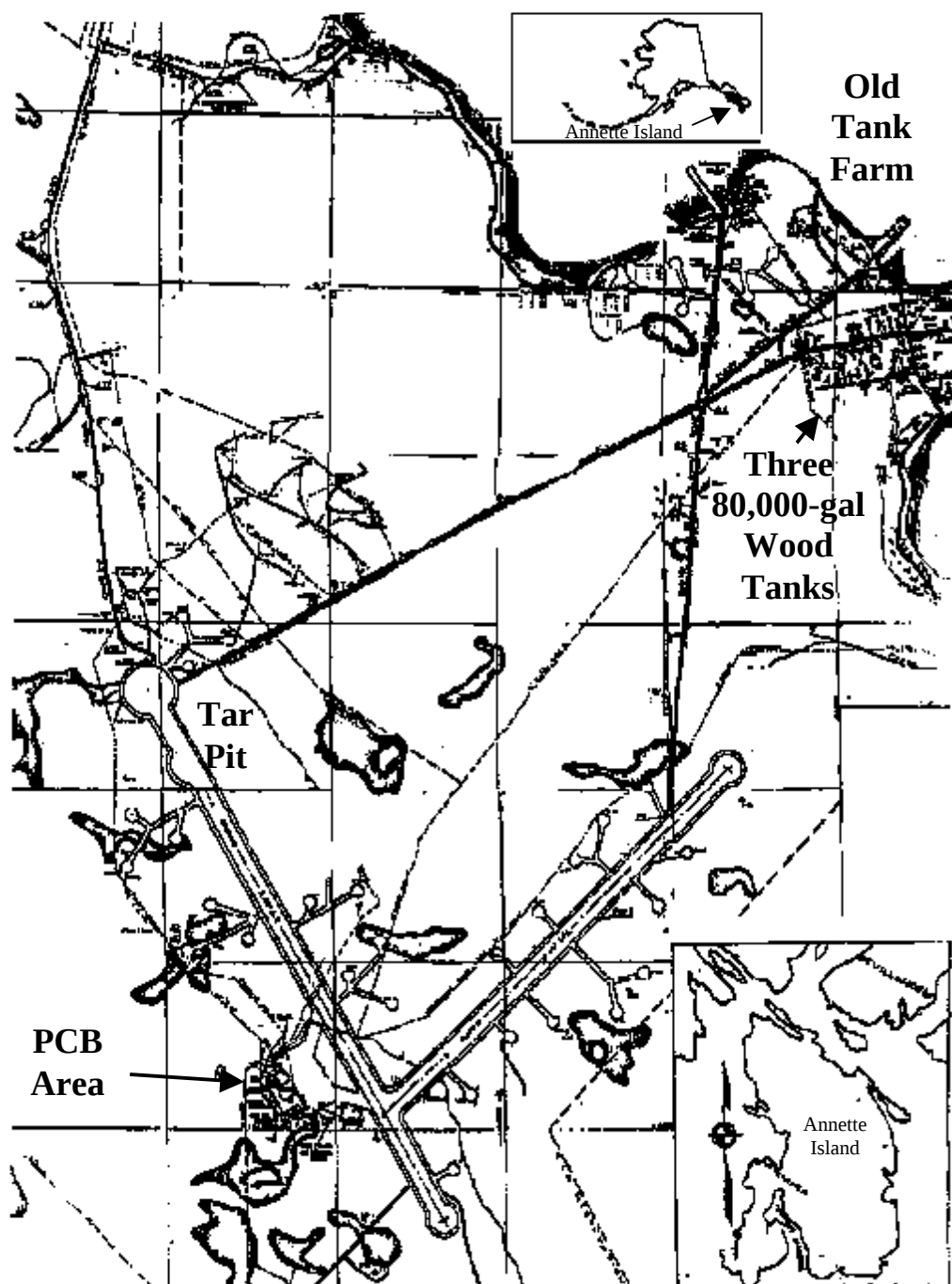


Figure 7. Annette Island Airport and tank farm.

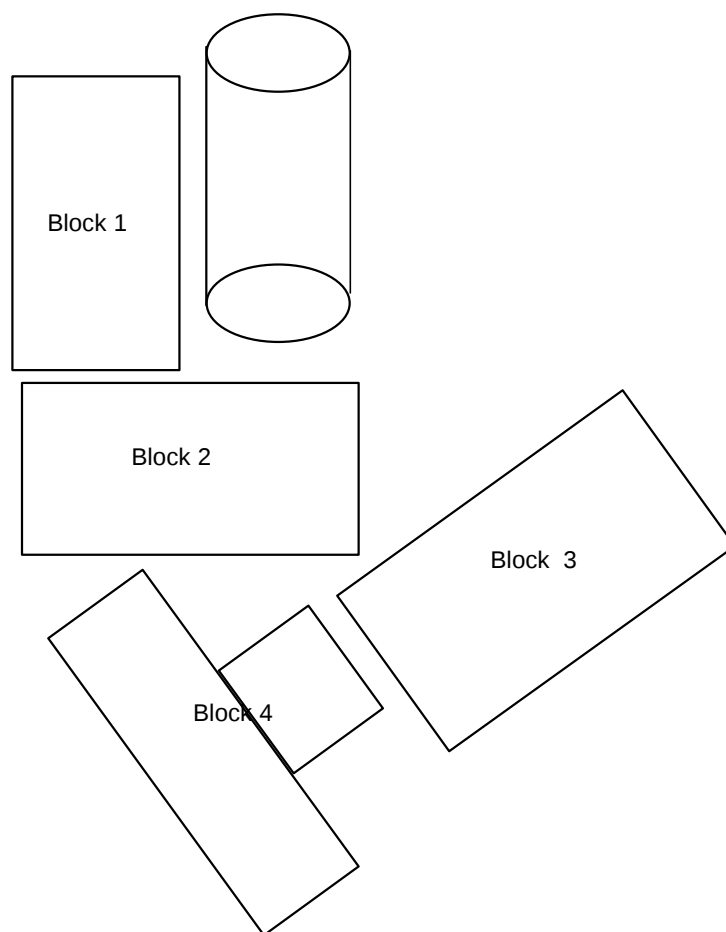


Figure 8. Annette Island site showing example of blocking approach used to reduce variability. Each block has one replication of each treatment.

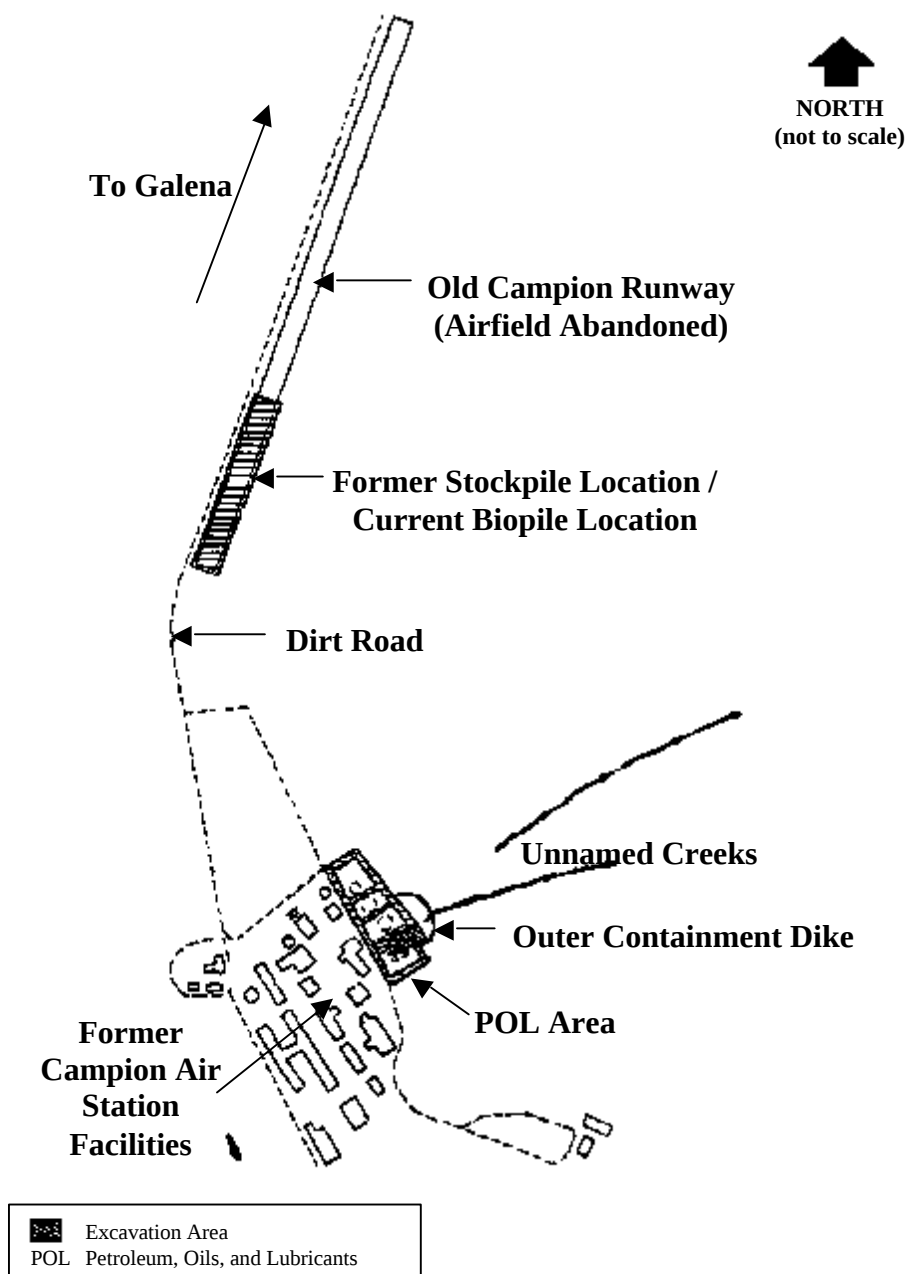


Figure 9. Campion site plan.

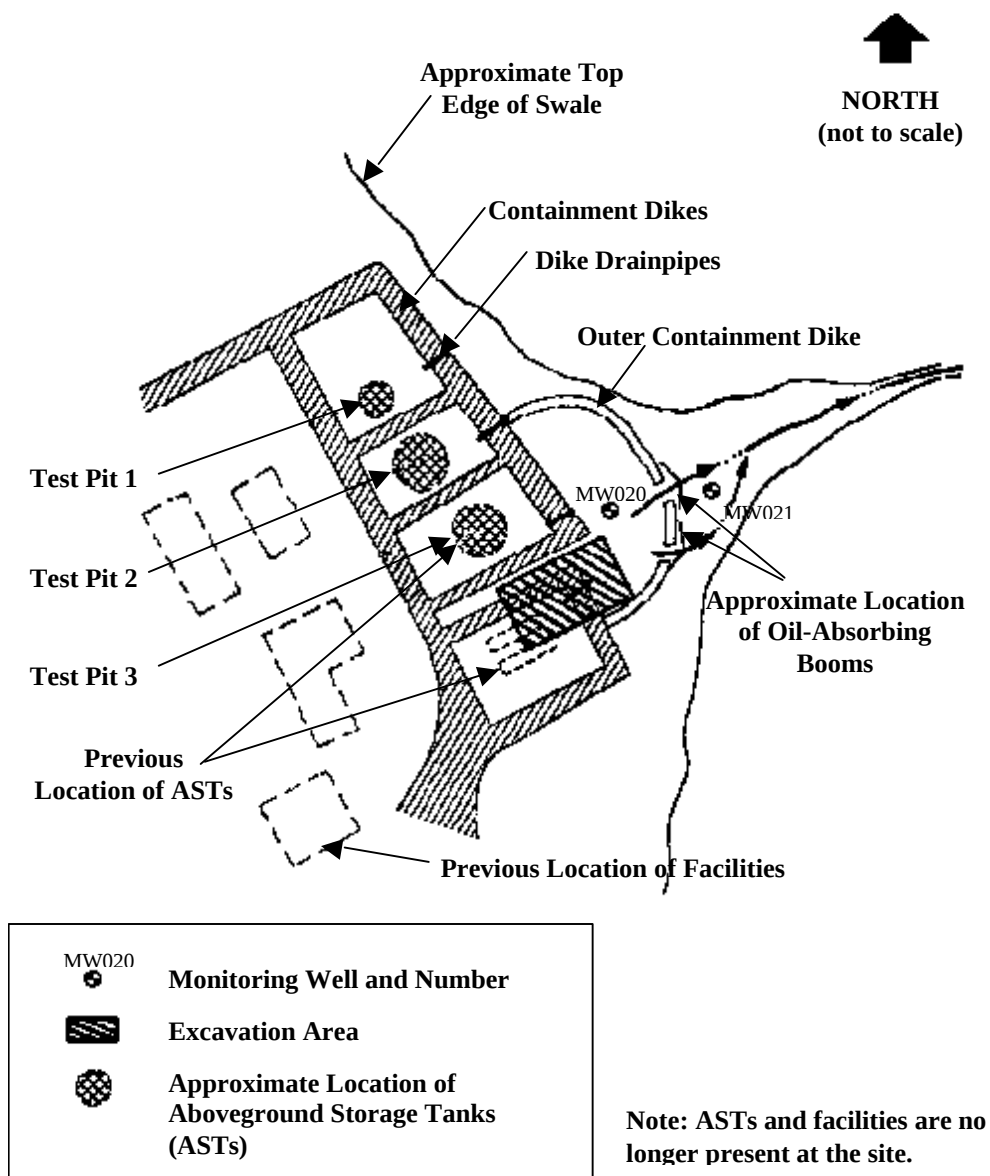


Figure 10. Detail of excavation work area at Campion.

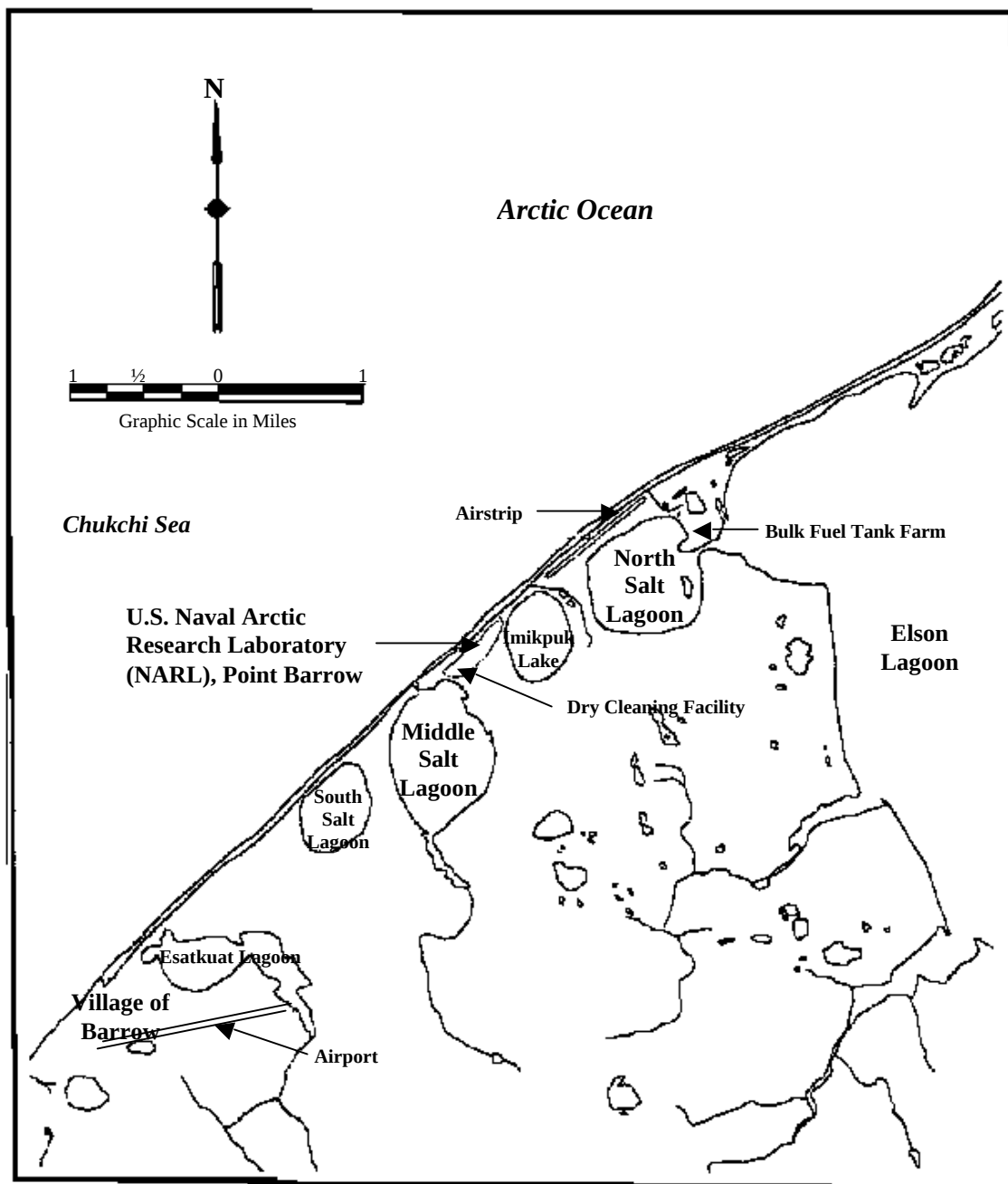


Figure 11. Regional location map for the Naval Arctic Research Laboratory (NARL), Point Barrow, Alaska.

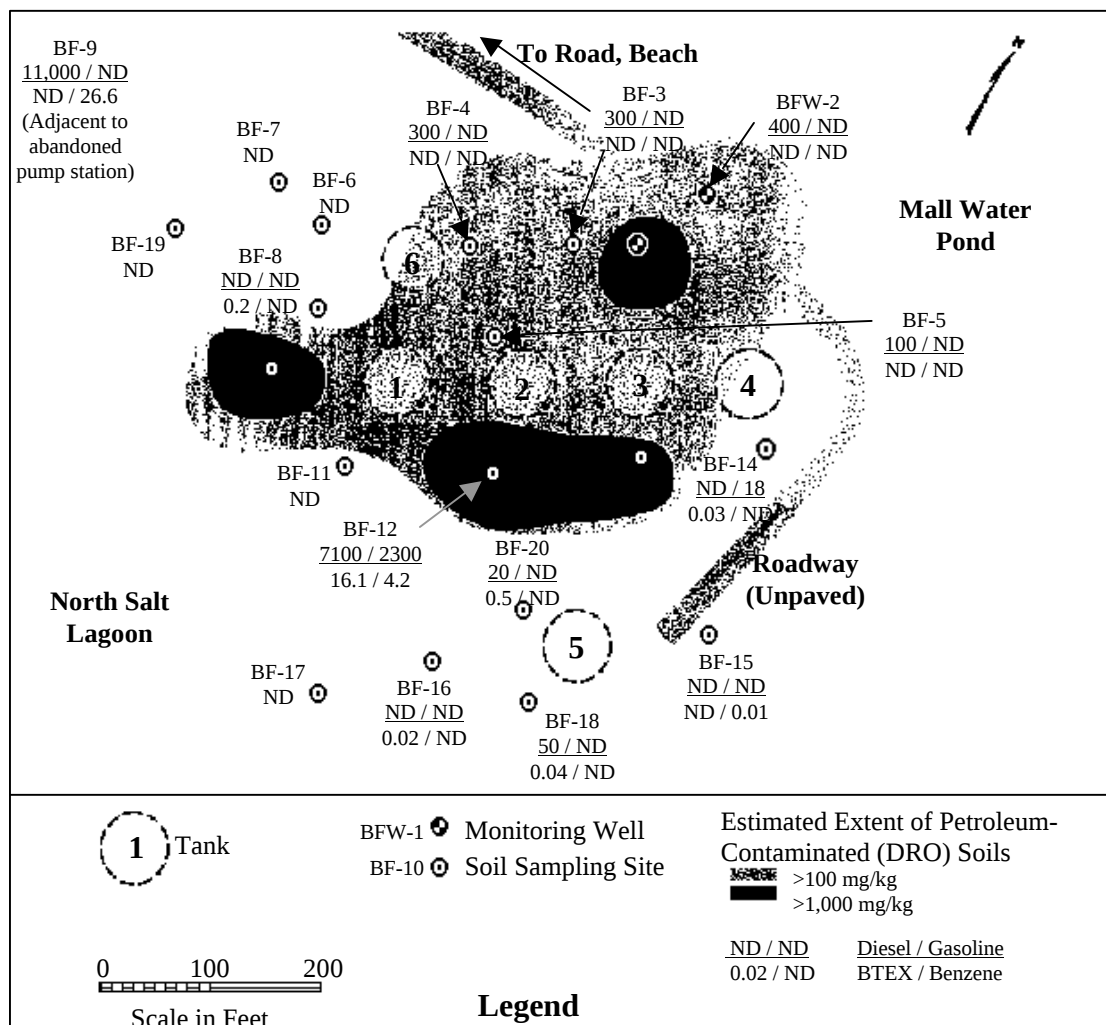


Figure 12. Site features and extent of contamination at former bulk fuel tank farm at NARL, Point Barrow.



Figure 13. Overview of Block 1 plots on Annette Island in May 2000.



Figure 14. Block 1 plots at Barrow in September 2000.

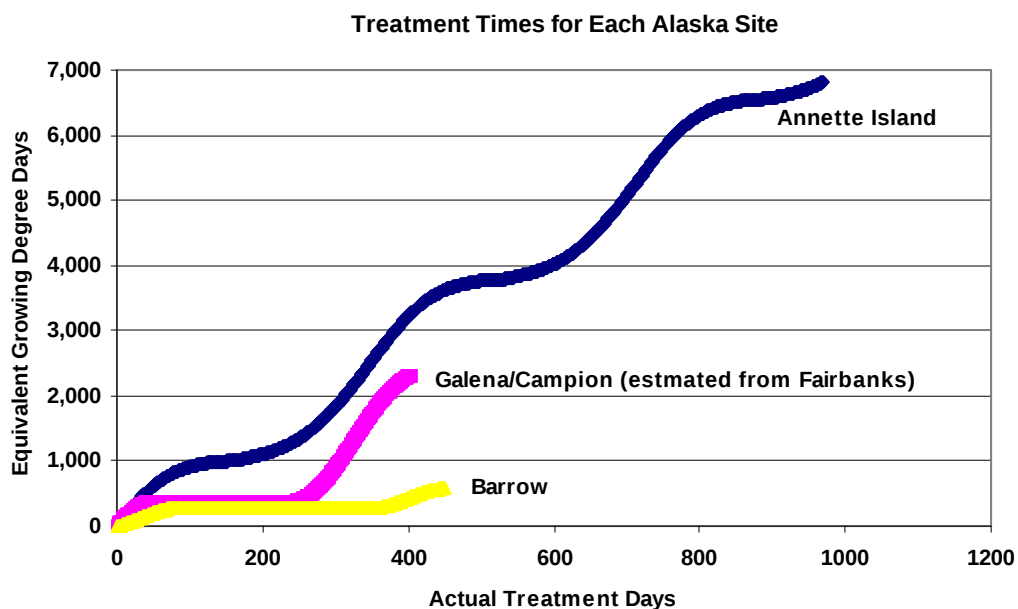


Figure 15. Treatment times for the three sites in Alaska.

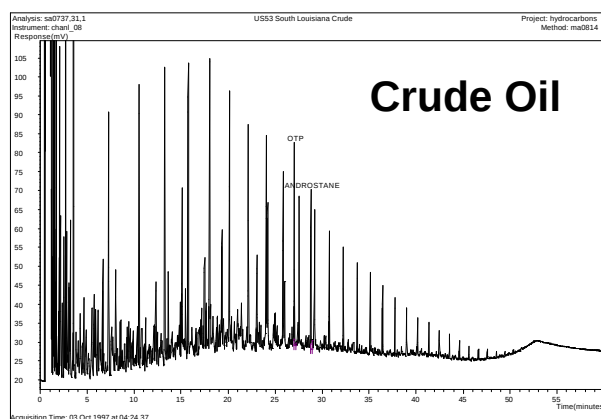


Figure 16. Typical GC fingerprint for crude oil.

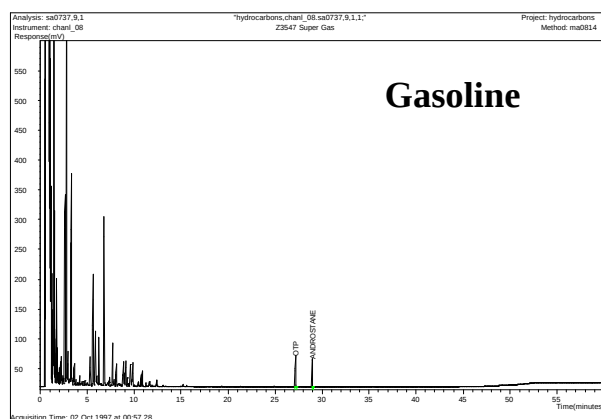


Figure 17. Typical GC fingerprint for gasoline.

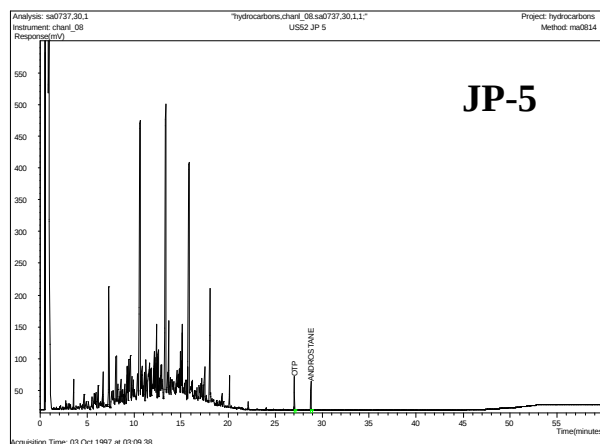


Figure 18. Typical GC fingerprint for the light mid-range distillate, JP-5.

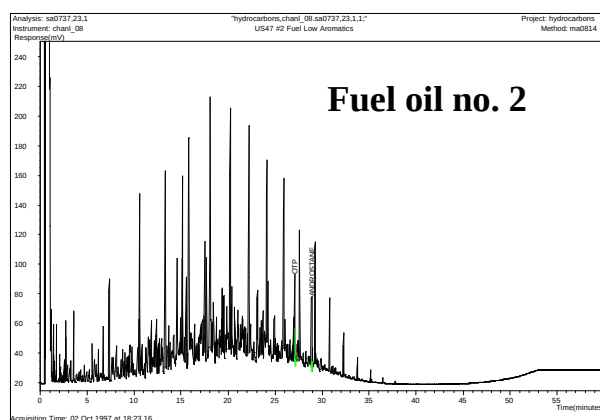


Figure 19. Typical GC fingerprint for the heavier mid-range distillate, fuel oil no. 2.

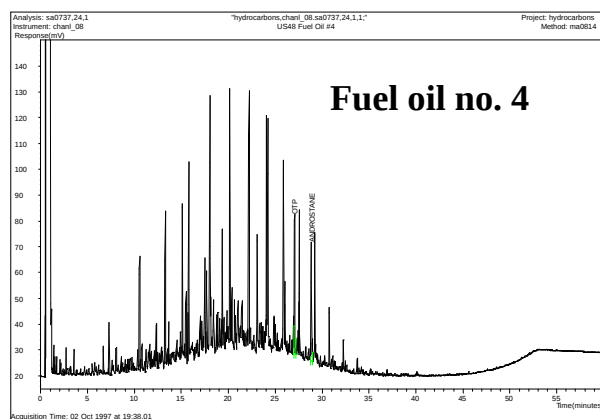


Figure 20. Typical GC fingerprint for the heavy fuel, fuel oil no. 4.

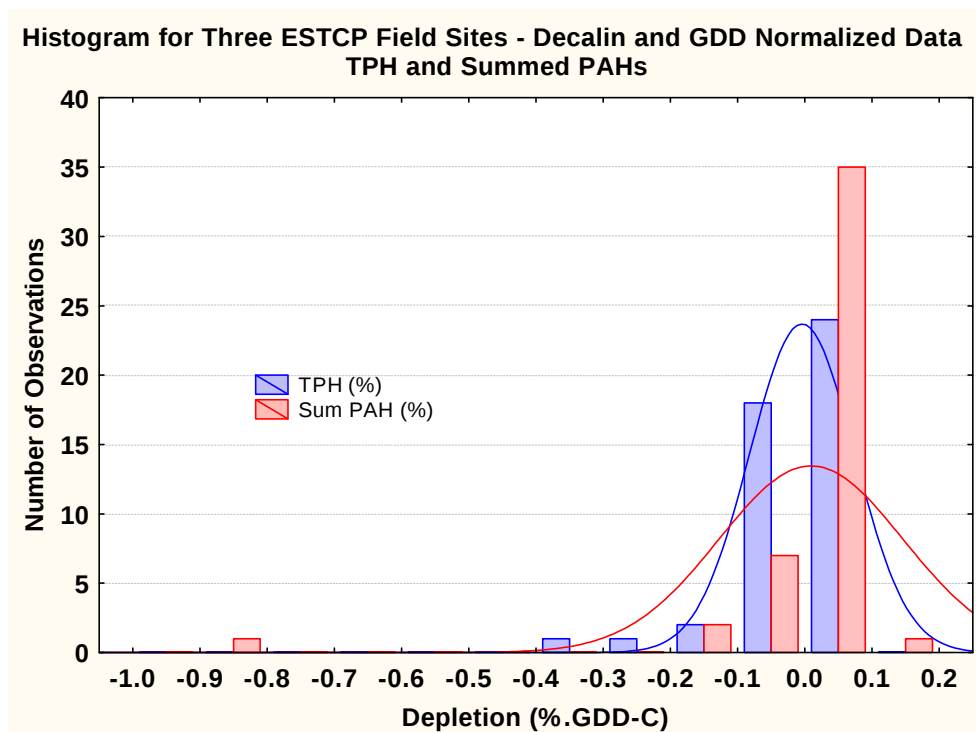


Figure 21. Histogram for three ESTCP field sites - decalin and GDD normalized data – TPH and summed PAHs.

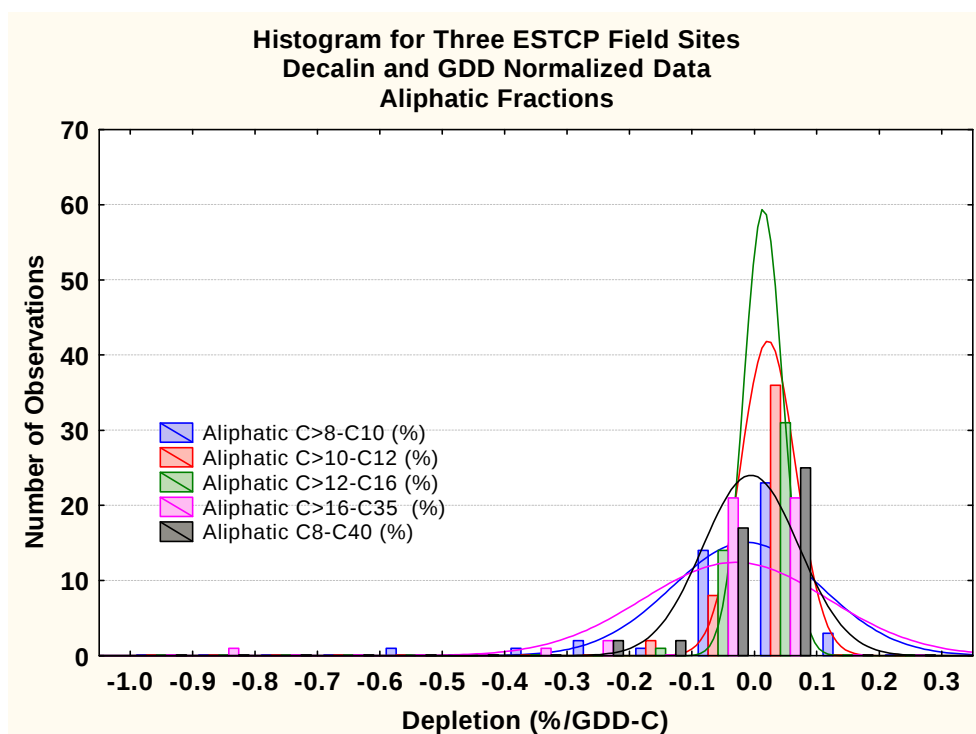


Figure 22. Histogram for three ESTCP Field sites - decalin and GDD normalized data – aliphatic fractions.

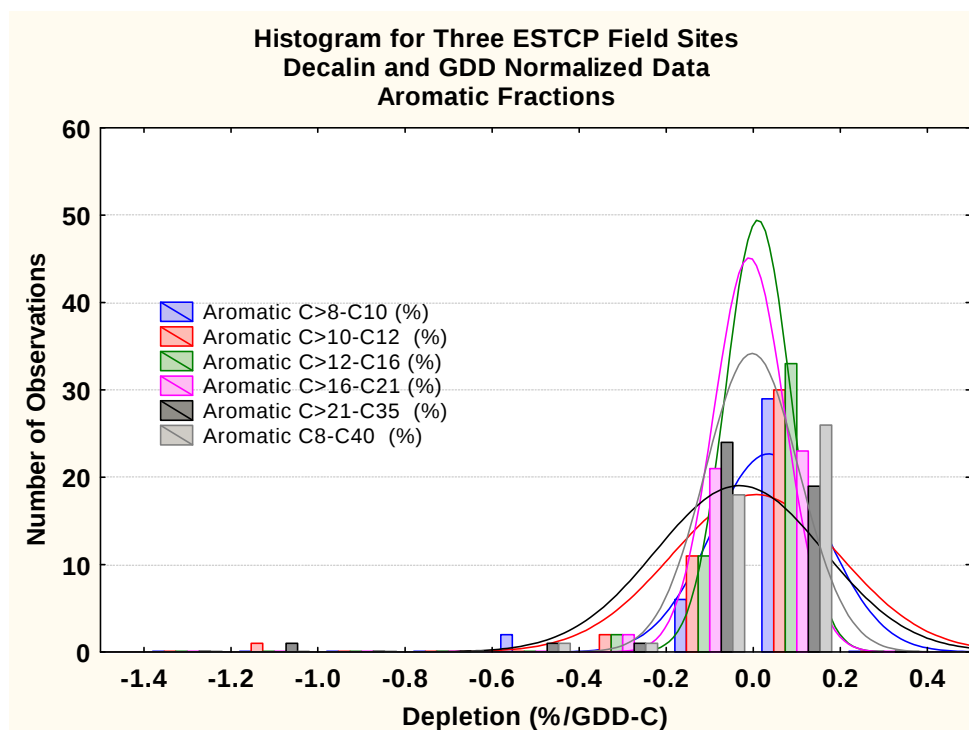


Figure 23. Histogram for three ESTCP field sites - decalin and GDD normalized data – aromatic fractions.

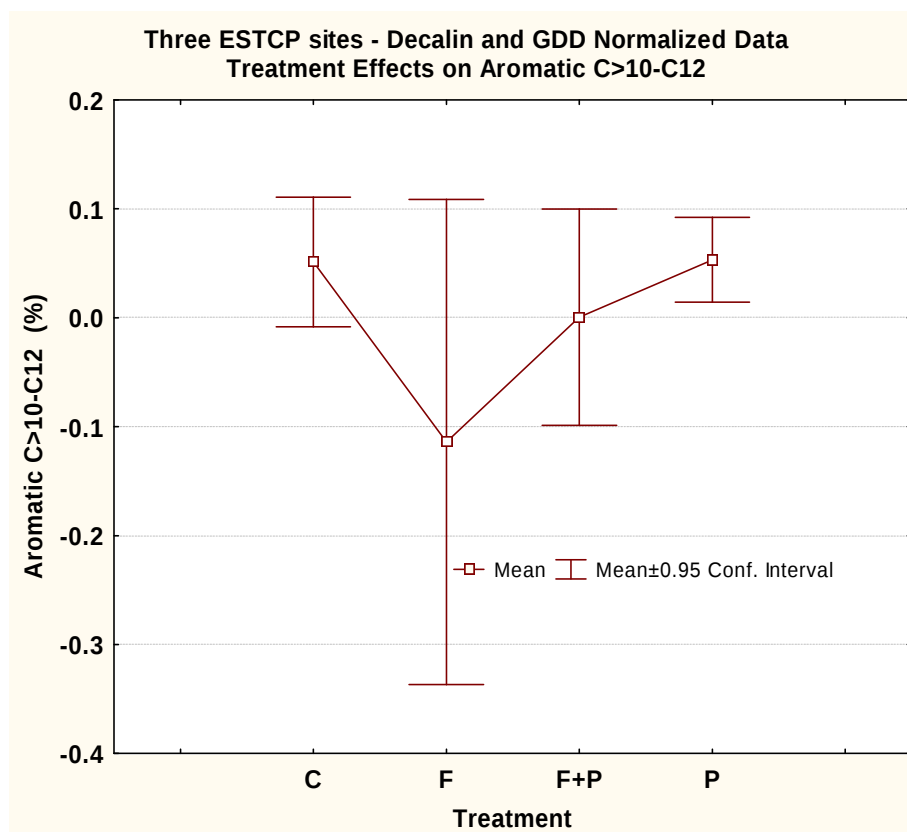


Figure 24. Three ESTCP field sites - decalin and GDD normalized data – treatment effects on aromatic C>10-C12.

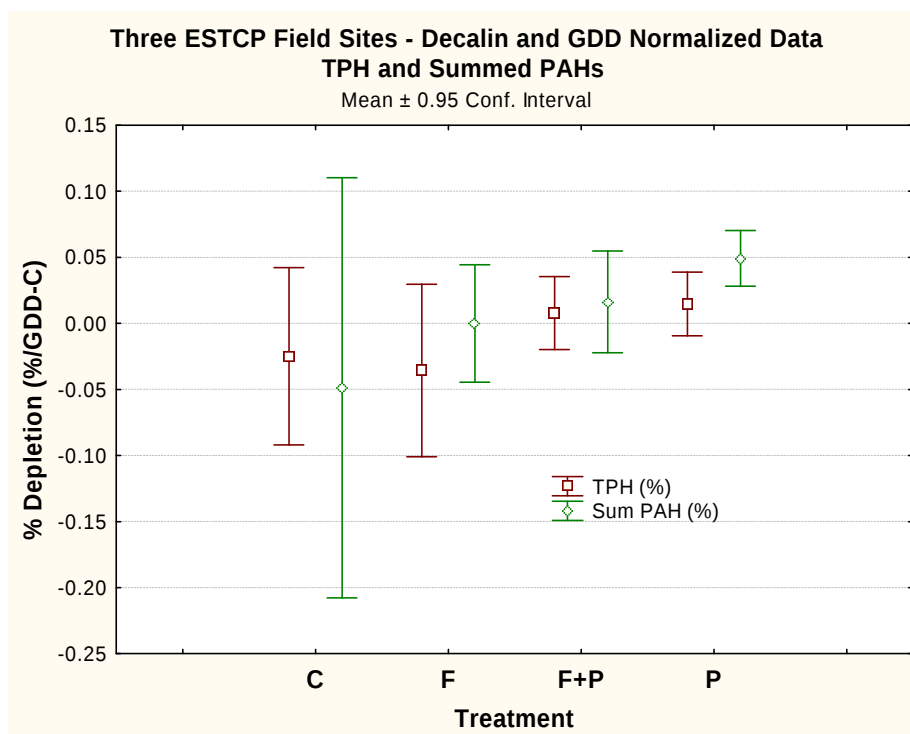


Figure 25. Three ESTCP field sites - decalin and GDD normalized data – TPH and summed PAHs.

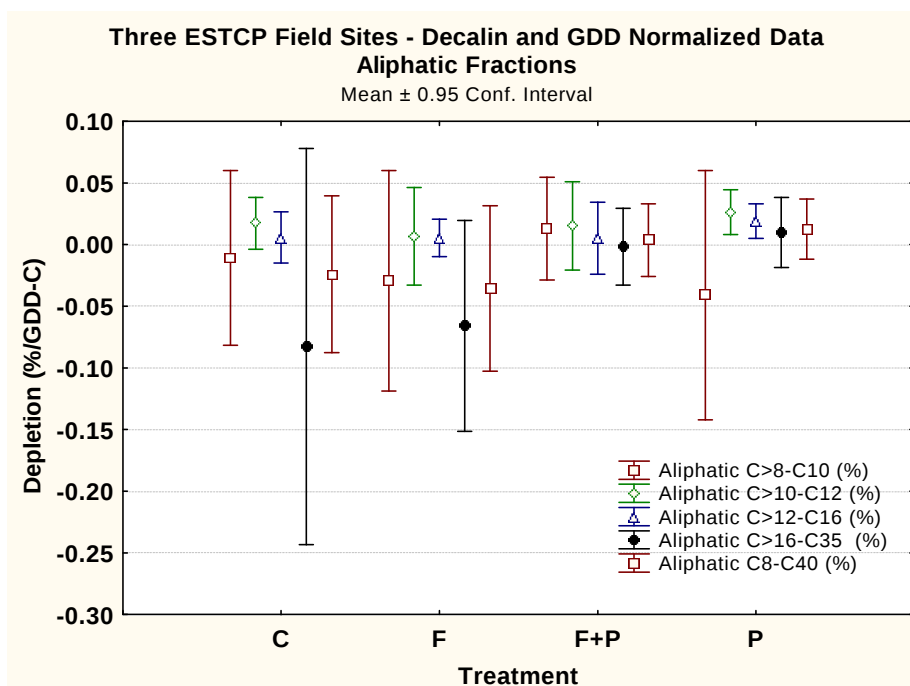


Figure 26. Three ESTCP field sites - decalin and GDD normalized data – aliphatic fractions.

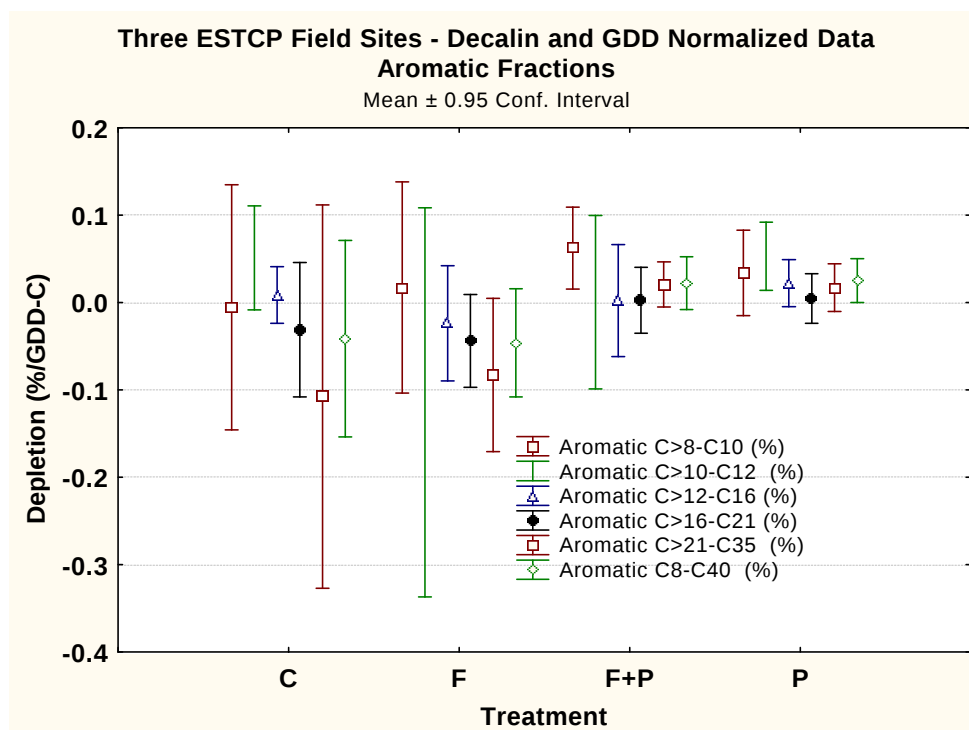


Figure 27. Three ESTCP field sites - decalin and GDD normalized data – aromatic fractions.

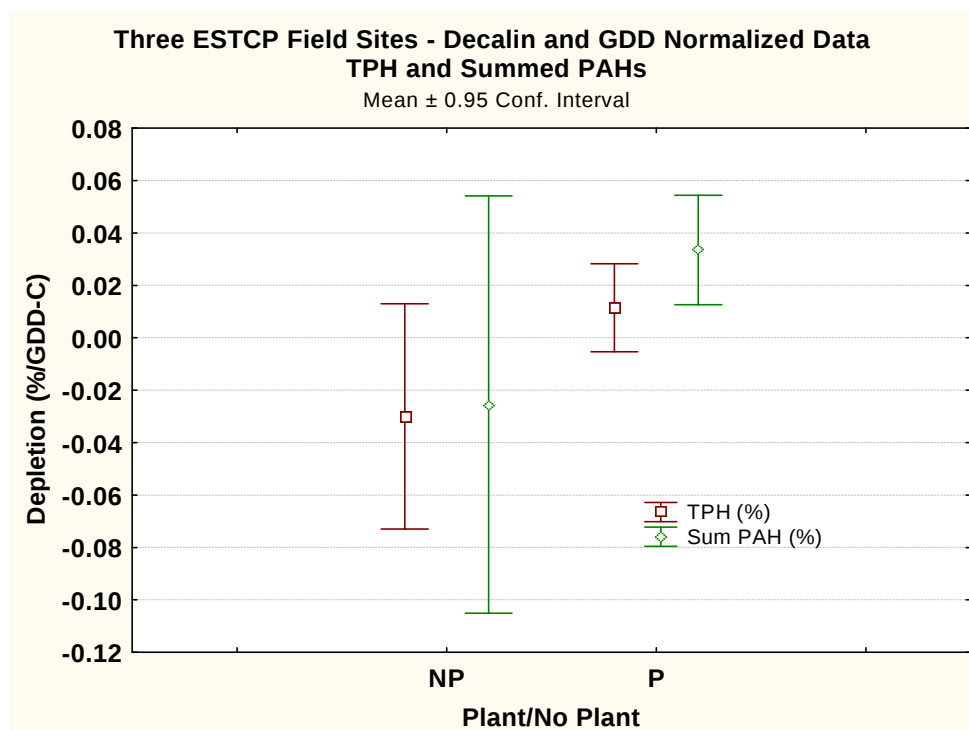


Figure 28. Three ESTCP field sites - decalin and GDD normalized data – plant effects on TPH and summed PAHs.

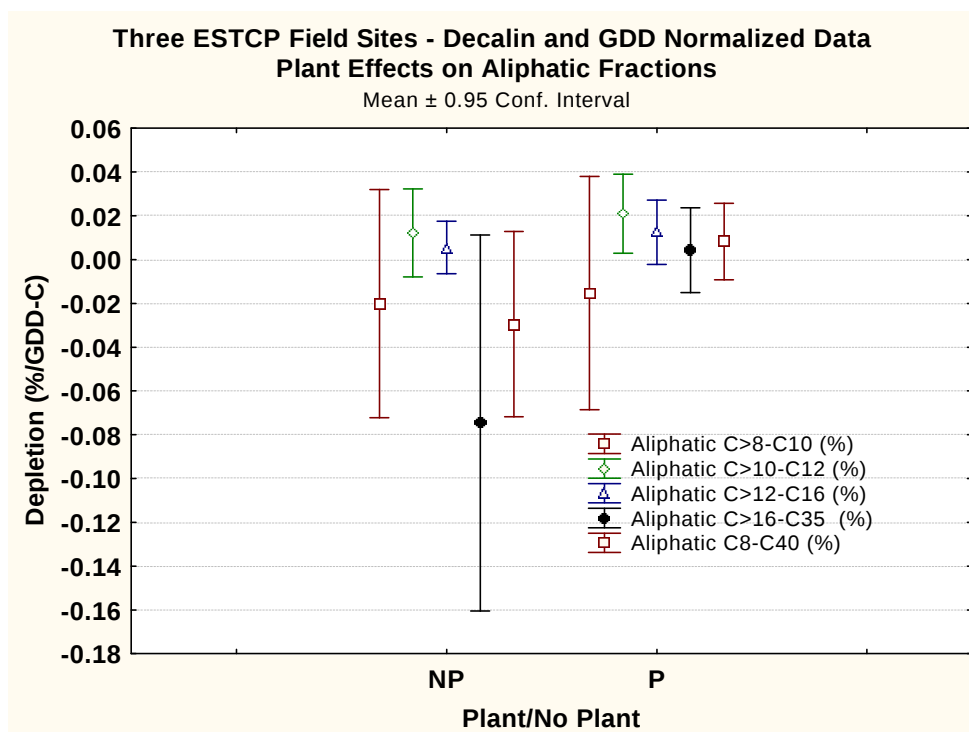


Figure 29. Three ESTCP field sites - decalin and GDD normalized data – plant effects on aliphatic fractions.

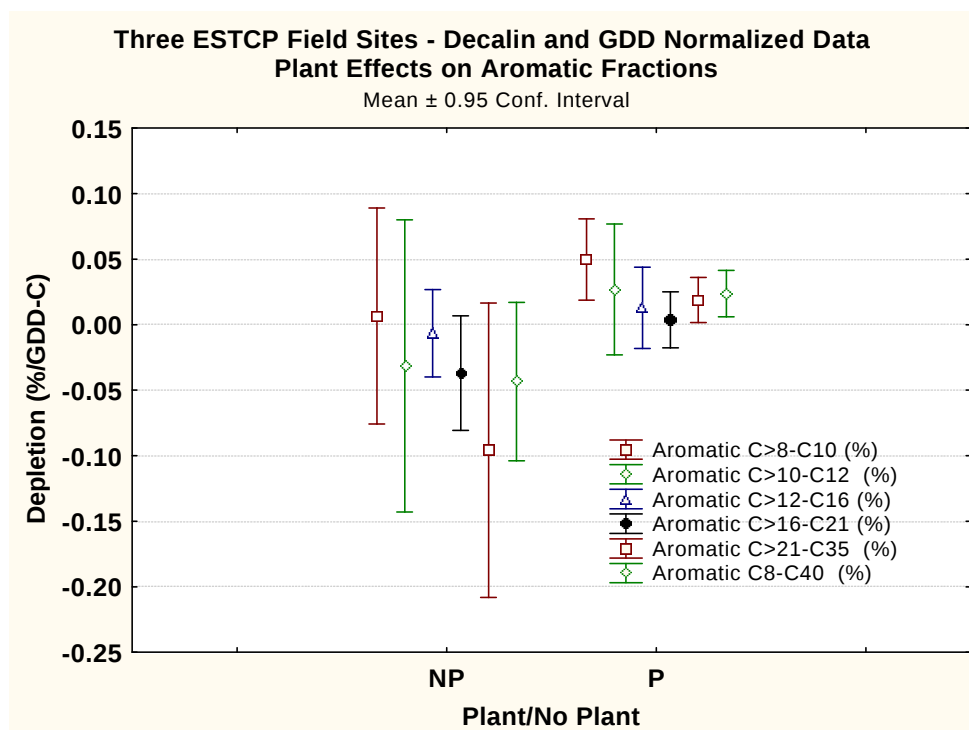


Figure 30. Three ESTCP field sites - decalin and GDD normalized data – plant effects on aromatic fractions.

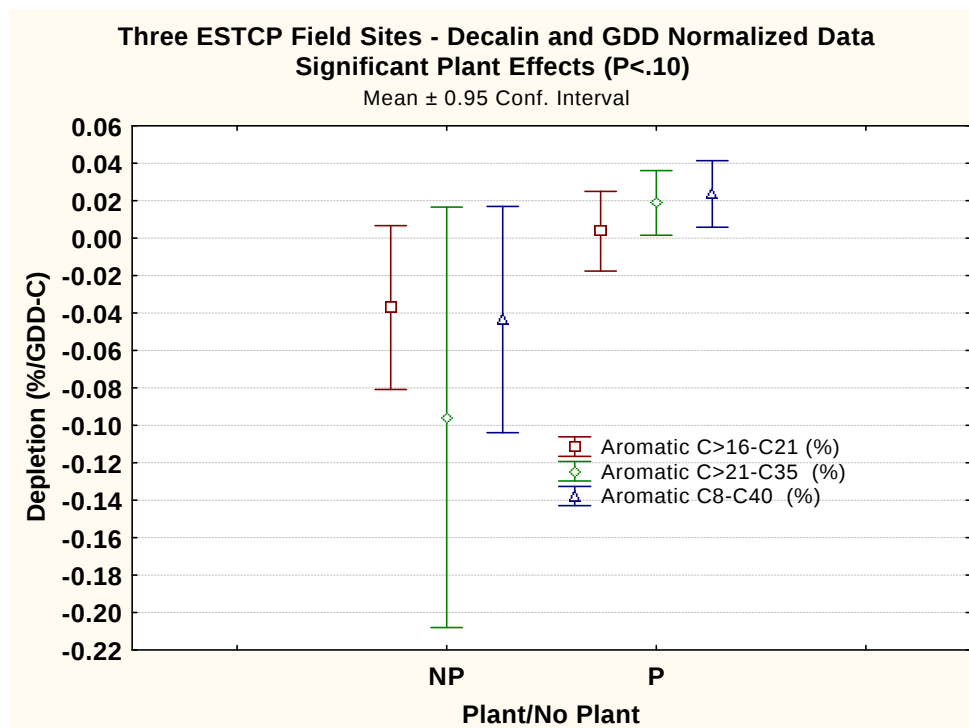


Figure 31. Three ESTCP field sites - decalin and GDD normalized data – significant ($P < 0.10$) plant effects on aromatic fractions.

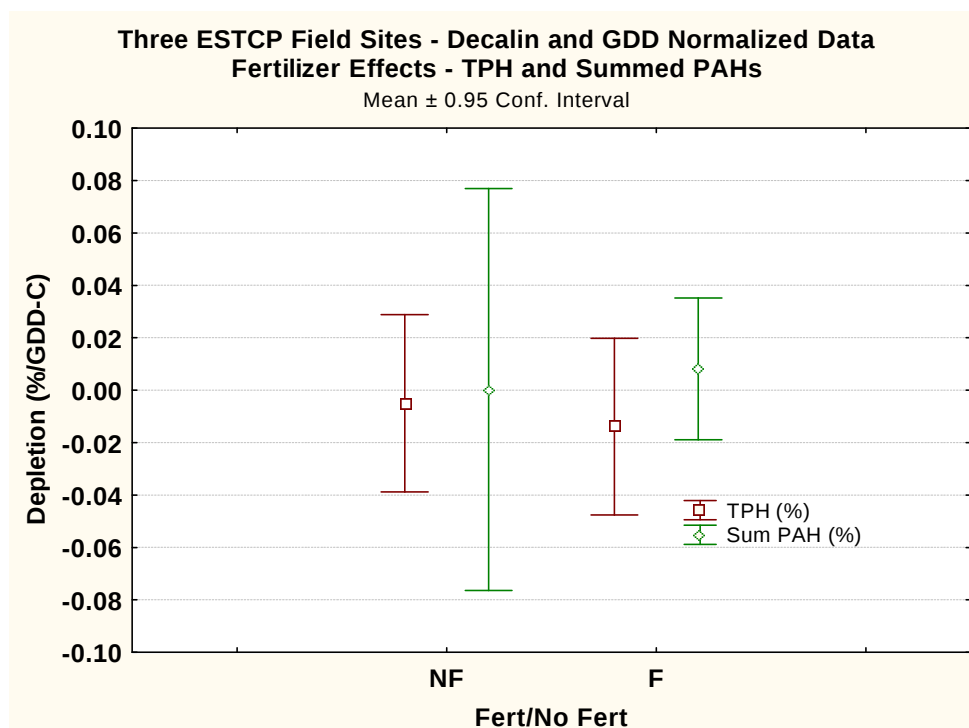


Figure 32. Three ESTCP field sites - decalin and GDD normalized data – fertilizer effects on TPH and summed PAHs.

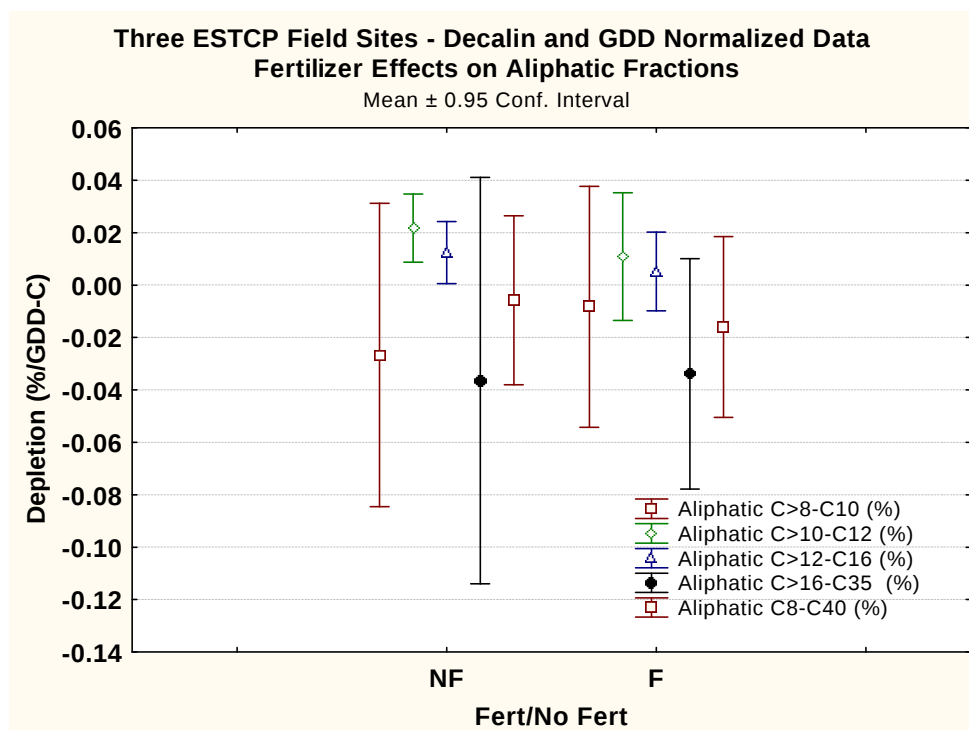


Figure 33. Three ESTCP field sites - decalin and GDD normalized data – fertilizer effects on aliphatic fractions.

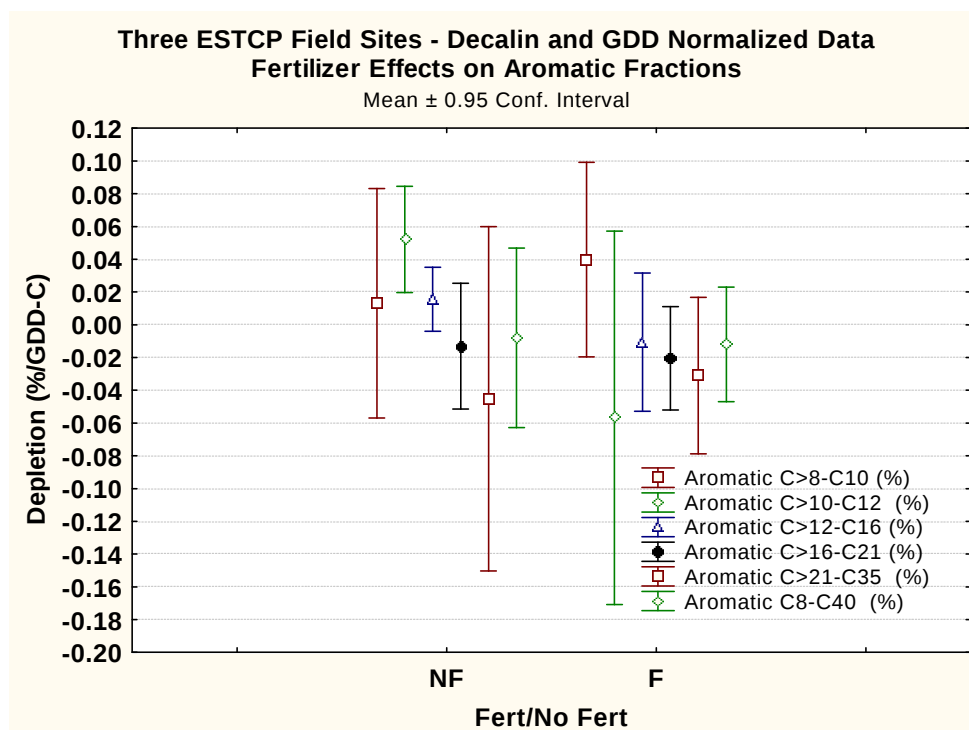


Figure 34. Three ESTCP field sites - decalin and GDD normalized data – fertilizer effects on aromatic fractions.

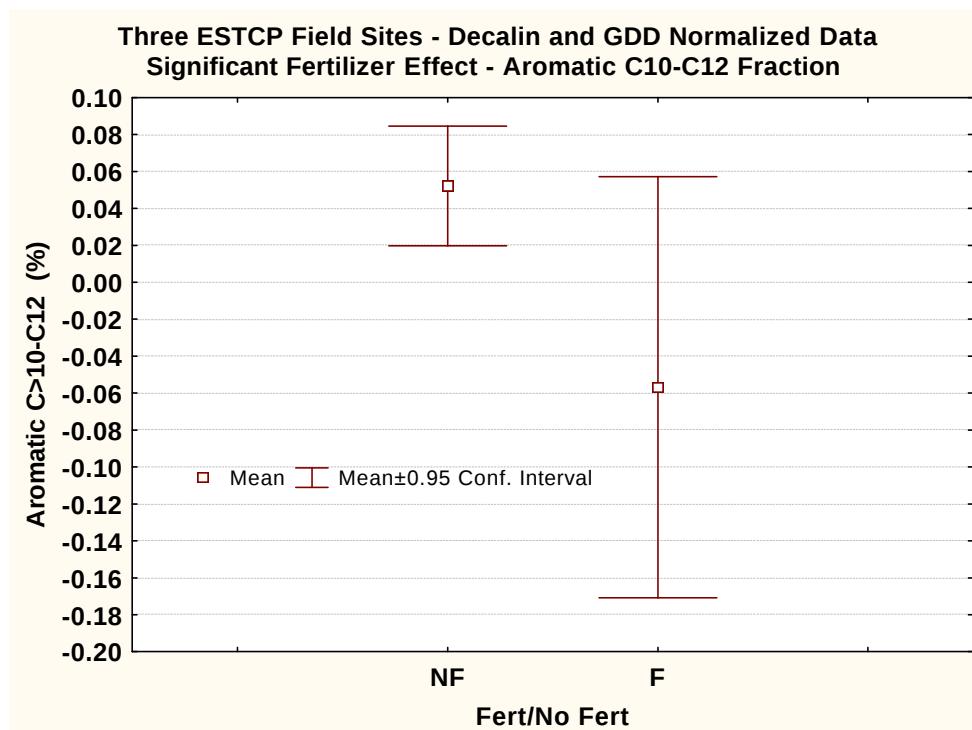


Figure 35. Three ESTCP field sites - decalin and GDD normalized data – significant fertilizer effects (inhibition) on depletion of aromatic C>10-12 fraction.

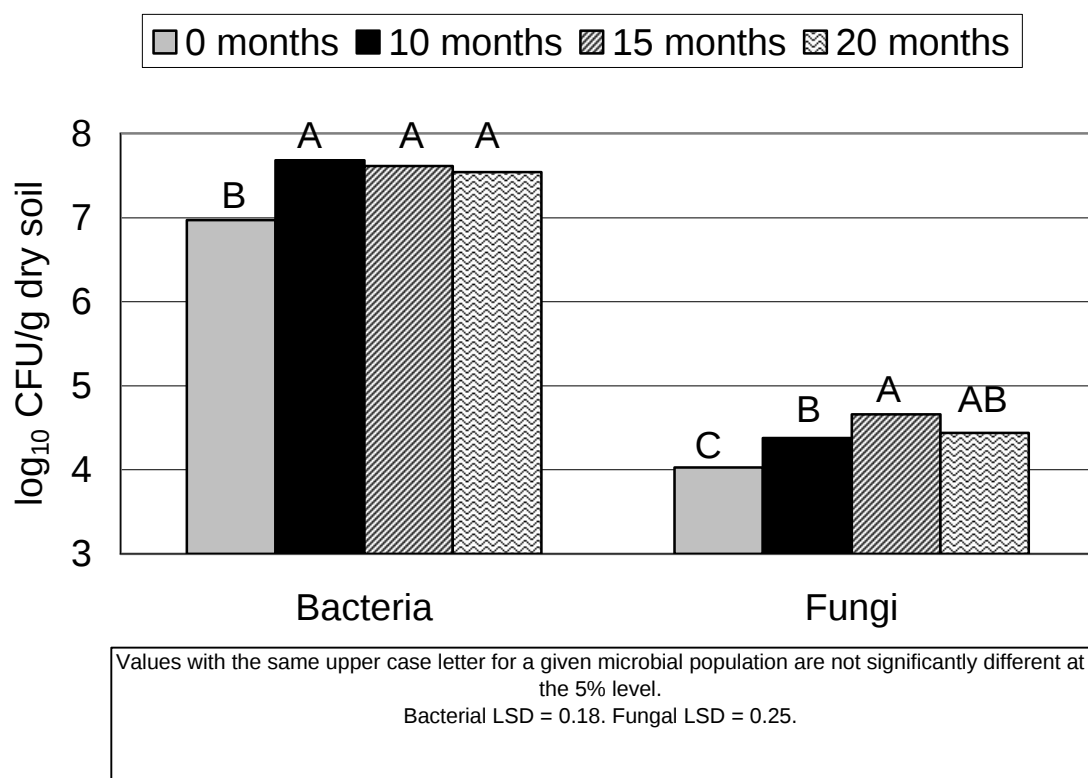


Figure 36. Bacterial and fungal population changes over time at the Annette Island site.

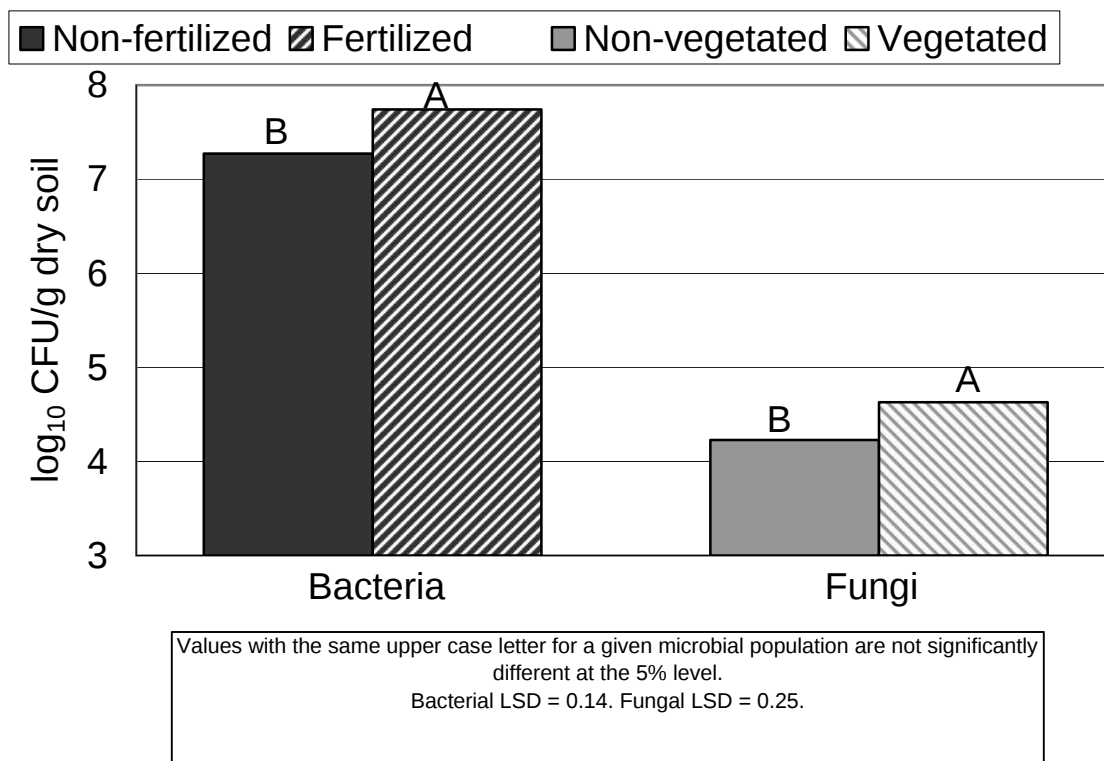


Figure 37. Bacterial populations in the non-fertilized and fertilized plots, and fungal populations in the non-vegetated and vegetated plots at the Annette Island site.

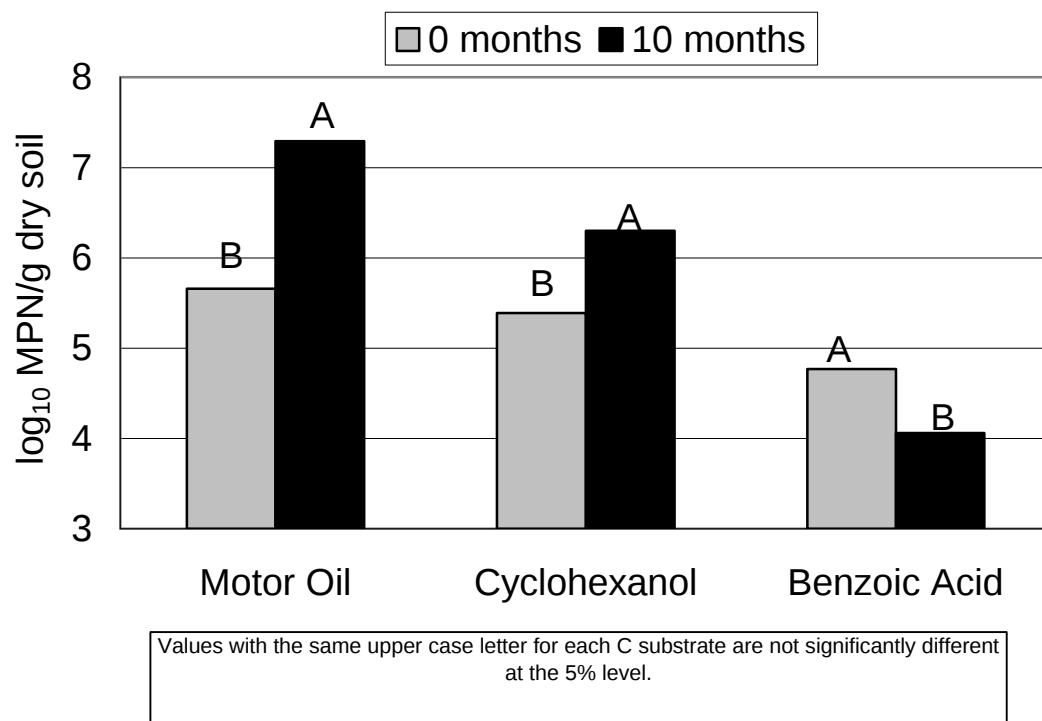


Figure 38. Motor oil, cyclohexanol, and benzoic acid degrader numbers before and 10 months after treatments were implemented at the Annette Island site.

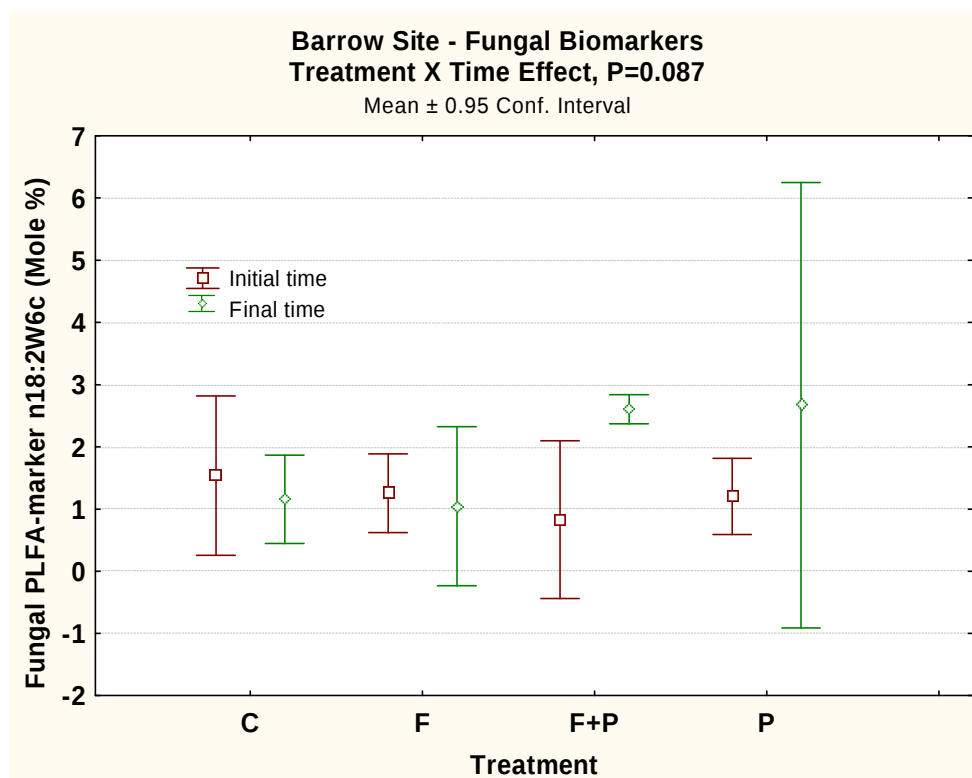


Figure 39. Changes in fungal biomarkers at the Barrow site. Plants increased fungal biomarkers during the study. Non-planted treatments did not show this effect.