**PHYTOREMEDIATION OF WEATHERED PETROLEUM IN GROUNDWATER
BY ARROYO WILLOWS IN NUTRIENT AMENDED ON-SITE MESOCOSMS**

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California Polytechnic State University
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In partial fulfillment of
the requirements for the degree of
Master of Science in
Civil and Environmental Engineering

By

Sarah Bragg-Flavan

March 2009

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ABSTRACT

PHYTOREMEDIATION OF WEATHERED PETROLEUM IN GROUNDWATER BY ARROYO WILLOWS IN NUTRIENT AMENDED ON-SITE MESOCOSMS

SARAH BRAGG-FLAVAN

A large-scale mesocosm study was conducted to determine if vegetation with willow trees enhances biodegradation and to evaluate the mechanisms of natural biodegradation of weathered petroleum compounds under field conditions. The mesocosms were designed to model conditions at a former oil field where mid-range petroleum distillates were used as a diluent for pumping crude oil contaminated the soil and groundwater at the site with petroleum compounds. Ten mesocosms were constructed at the field site using un-impacted soil and diluent-impacted groundwater from the site. Five of the mesocosms were planted with Arroyo Willow trees native to the field site and the other five served as controls without trees. Since these willow trees are phreatophytes, their roots are capable of consuming water from the water table. A previous study was conducted using these mesocosms, however the willow trees then were in poor condition. In this study, fertilizer was added to the mesocosms to promote healthy growth of the willows. Fertilizer was added equally to mesocosms with and without willow trees to avoid introducing bias. Groundwater was circulated through the mesocosms for two 109 to 126 days runs, while the total petroleum hydrocarbon (TPH) concentrations of the groundwater were measured periodically. Dissolved oxygen concentrations were also monitored in each of the mesocosms to determine if the willow trees had any impact on oxygen transfer to the subsurface.

In the first run without nutrient amendments the trees did not enhance biodegradation. All the mesocosms started with an average TPH concentration of 6.3 mg/L and ended

with a concentration of 1.0 mg/L. After this first run, nutrient amendments were added to all the mesocosms, resulting in healthy trees with robust growth. With healthy willow trees, the planted mesocosms resulted in a statistically significant increase in long-term biodegradation of dissolved-phase petroleum compounds. The planted mesocosms resulted in 29 percent more degradation. These results agree with prior lab studies using bench-scale microcosms with media from the former oil field which indicated that TPH concentrations after 100 days were lower in containers with willows or lupines compared to controls without plants. Microtox[®] toxicity decreased for both planted and control mesocosms, showing no toxic root exudates or by-products.

There are several potential mechanisms of the observed phytoremediation. Terminal restriction fragment analyses showed that the planted mesocosms had different microbial communities than the unplanted mesocosms. Thus, a possible mechanism of the phytoremediation is stimulation of a rhizobial microbial community that biodegrades petroleum compounds. The dissolved oxygen (DO) concentrations were actually lower in the planted mesocosms, possibly due to consumption of oxygen during biodegradation of root exudates. The reduced DO concentrations in the planted mesocosm discounts the possibility that the plants stimulated biodegradation by increasing oxygen transfer to the subsurface. It is not known from these experiments if the petroleum compounds were taken up by the plants or if the plants stimulated bacterial biodegradation. Since it is difficult for plants to uptake non-polar compounds with a high octanol-water coefficient (K_{ow}), it is usually unlikely that plants could uptake petroleum compounds which usually have a $K_{ow} > 3$. However, the log K_{ow} of the dissolved phase diluent determined in this

research was only 0.14. Although the mechanism by which the willow trees increased biodegradation was not elucidated, this study demonstrated that phytoremediation of the polar and hydrophilic weathered petroleum compounds was successful.

Column chromatography was used to fractionate petroleum compounds extracted from the groundwater into aliphatic, aromatic and polar components so that biodegradation of each of these fractions could be determined independently. The first mesocosm experiments showed that regardless of the presence of trees, there was a decrease in TPH concentration for all three fractions. The overall unfractionated biodegradation rates averaged 41 ug/L/day over this experiment, and the biodegradation rate of the polar fraction was similar at 40 ug/L/day. In comparison, the biodegradation rates of the aliphatic and aromatic fractions were considerably lower at 1.2 and 2.6 ug/L/day, respectively.

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CHAPTER 1 INTRODUCTION

The purpose of this research was to determine if healthy willow trees enhance the biodegradation of petroleum groundwater contaminants at a former oil field of the Guadalupe Restoration Project (GRP). Previous laboratory studies suggested that native *Salix lasiolepis* would be successful at improving the long-term rate of biodegradation over natural attenuation through phytoremediation (Hoffman, 2003). Crossley (2006) attempted to resolve this question using on-site mesocosms (very large microcosms): Five with Arroyo Willow (*Salix lasiolepis*) and five without trees. The mesocosms allowed for control of experimental parameters under conditions closely matched to field conditions. However, the willow trees during the earlier study were not healthy, which may have caused a lack of root growth which has been strongly correlated with phytoremediation (Huang *et al.*, 2005; Kaimi *et al.*, 2006; Kechavarzi *et al.*, 2006). In the current study fertilizer was added to all the mesocosms, which greatly improved the health of the willow trees, resulting in robust growth. Once healthy trees were established in five of the mesocosms, ground water was recirculated through the mesocosms for 126 days and total petroleum hydrocarbon (TPH) concentrations were monitored.

Phytoremediation is a remediation strategy that uses plants to clean up contamination. Phytoremediation can be conducted as an *in situ* treatment of contaminated soil and/or groundwater. It is an attractive option in sensitive and inaccessible areas when compared to invasive conventional treatment options (EPA, 2000). *In situ* techniques have multiple advantages at a site: reduced ecological effects, lower disturbance of the environment, limited transport or phase change of the contaminant, possible increased safety of personnel involved in

the remediation, and lower cost of treatment (Frick *et al.*, 1999). Successful phytoremediation of many different contaminants is well documented in the literature (Aprill and Sims, 1990; Reilley *et al.*, 1996; Bient *et al.*, 2000; Dominiguez-Rosado and Pichtel, 2004; Gao *et al.*, 2006; Liste and Prutz, 2006; Mueller and Shann, 2006) as well as in previous laboratory studies on the GRP site (Hoffman, 2003; Martin, 2003).

The Guadalupe Restoration Project is located on the central coast of California. From the 1950's to 1990 a mid-range petroleum distillate, called diluent was used as a viscosity reducing agent to facilitate pumping of the viscous Monterrey Formation crude oil from the former oil field. The diluent continually leaked from piping and holding containers on this site resulting in approximately 8 million gallons being released into the environment (Lundegard and Garcia, 2001). There are numerous protected species, both plant and animal (<http://www.guaddunes.com/about>), and sensitive areas over the 2700 acres, like wetlands. Traditional remediation techniques may do more harm than good in many of these areas. Therefore, many different remediation techniques, including phytoremediation, are actively being evaluated for use in such areas.

There are currently two on-site field studies of phytoremediation at GRP. One test plot is planted with rows of Arroyo Willows and Black Poplars. The other plot is planted with a mixture of native plants including willows, lupines and poplars. The second test plot has been termed "ecoremediation" for using a mixture of native plants to restore a healthy, natural ecosystem. The field studies successfully revegetated previously excavated areas, but determining the effectiveness of the plants in these test plots is difficult due to a lack of control

of experimental parameters. Parameters such as uniformity of the plots, plume location, and depth of ground water are uncontrollable; therefore, it is difficult to conclusively prove that the plants positively influence the degradation of dissolved diluent. For this reason, laboratory microcosm experiments were conducted to examine phytoremediation under controlled conditions. These laboratory experiments showed that willows and lupines enhanced long-term biodegradation. However, these earlier laboratory studies did not match site conditions. To address this short coming, large mesocosms were created and operated as an intermediate step between the laboratory and the field test plots.

The mesocosms were constructed by Crossley (2006) out of 4'x4'x4' polypropylene vegetable containers. These containers were filled with clean soil and contaminated groundwater from the GRP. Site hydrology was simulated by circulating groundwater through the containers to match the onsite groundwater velocity of 1 ft/day. There were two experimental runs: the first run was operated for 109 days during the growing season, but with the willows in poor health. Then followed a fertilization phase for eight months to increase the health of the willow trees, and all mesocosms were treated in an identical manner. After establishing healthy, actively growing willow trees the second experiment was run for 126 days. During this run all containers were fertilized in the same manner as in the conditioning phase. Groundwater samples were collected periodically for both runs and analyzed for total petroleum hydrocarbon (TPH) concentrations to determine efficacy of the willows for phytoremediation. The purpose of this experiment was to find out if more realistic field conditions replicated the positive results achieved in the laboratory. There were potential differences in conditions especially in dissolved oxygen levels,

because the lab water was exposed to air and may have accentuated or suppressed the difference between planted and non-planted microcosms.

Potential mechanisms of phytoremediation were explored in a number of ways. Terminal restriction fragment (TRF) analysis was used to compare the microbial communities with and without plants. Nutrient concentrations were also measured to test for potential nutrient limitations. Dissolved oxygen (DO) concentrations in the mesocosms were measured to see if the plants had any impact on oxygen transfer. Microtox[®] toxicity was monitored to test for the production of any potentially toxic compound in root exudates or in biodegradation products and also to observe any reduction in toxicity during biodegradation.

The potential for direct plant uptake of diluent petroleum compounds was investigated by experimentally determining the log K_{ow} of free phase diluent and dissolved phase diluent. Compounds with log K_{ow} values greater than three are not expected to be taken up by plants (Cunningham and Berti, 1993; Schnoor *et al.*, 1995; Alkorta and Garbisu, 2001). Over the many years of research at GRP, no lab study has determined the log K_{ow} of either the free phase diluent or dissolved phase diluent. Petroleum compounds typically exhibit log K_{ow} values greater than 3 (Cunningham and Berti, 1993), but since the diluent at GRP is highly weathered it may have a very different K_{ow} . The experimental determination of the log K_{ow} of diluent could thus shed some light on the likelihood of plant uptake of diluent.

In addition to the primary purpose of testing the efficacy of willows in degrading dissolved-phase diluent (DPD), the biodegradation of different fractions (aliphatic, aromatic and polar)

during natural attenuation was also investigated using the mesocosm experiments. This was a continuation of the work of Mick (2006) who used soil-only mesocosms to investigate the degradation of different fractions of the petroleum mixture. However, in Mick's (2006) experiment, the DPD only contained the polar fraction; it did not contain any aliphatic or aromatic fractions. Mick (2006) proved that the polar fraction did biodegrade, which in the literature is often cited as the recalcitrant fraction that resist degradation (Wang and Fingus, 2003). In the current study, groundwater from a different source was used that contained some DPD in the aliphatic and aromatic fractions in addition to the polar fraction. Silica gel column fractionation was completed on the initial and final sampling days to evaluate the presence and possible degradation of the aliphatic, aromatic, and polar fractions of diluent. This part of the project is intended to increase our understanding of DPD biodegradation in conjunction with natural attenuation.

CHAPTER 2 BACKGROUND

2.1 Guadalupe Restoration Project Site

Located on the central coast of California, near the town of Guadalupe, about 45 miles south of San Luis Obispo and 15 miles west of Santa Maria, is the former Guadalupe Oil Field, now called the Guadalupe Restoration Project (GRP). The GRP is a 2700-acre property (Figure 2.1). Once an active oil field, the site is part of the Nipomo Dunes Complex (a National Natural Landmark) which is one of the largest coastal dune ecosystems on earth (Dunes Center, 2005).

Many plant and animal species of concern have been identified on site, including more than 40 threatened or endangered species, and several archeological sites have been discovered (Lundegard and Garcia, 2001). The site is partially surrounded by water as the Santa Maria River is the southern border of the site and the Pacific Ocean is the western border. The other boundaries of the site are agricultural fields to the east and the Mobil Coastal Reserve to the north. In addition to the Santa Maria River and the Pacific Ocean there are numerous wetlands within the GRP lands and these wetlands are home to many of the threatened or endangered species. These bodies of water, along with the groundwater at varying depths across the site, drive water quality concerns. Throughout the nearly seventy years of activity on the GRP, public access has been restricted, first due to oil production and currently due to remediation activities.

The GRP is a highly characterized site with over 800 active ground water monitoring wells and 3000 soil borings logged from the site. Even with the activities that have occurred, the GRP site is one of the most intact coastal sand and dune ecosystems in California (Dunes Center, 2005).

Oil exploration began at the site in 1948 by the Sand Dune Oil Company. Ownership changed hands a few times before Chevron became the current owner of the oil field in 2005. The field was in operation until 1994 under Unocal, with peak production of 4,500 barrels per day from 215 oil wells in 1988 (www.Guaddunes.com). The crude oil is very viscous at Guadalupe, which is characteristic of Santa Maria Valley oils derived from the Monterey Formation source rock (Lundegard and Garcia, 2001). To ease the difficulty of pumping this viscous product a semi-refined product was pumped to individual wells at the GRP and mixed with the crude oil to reduce the overall viscosity. This semi-refined product was distilled at a neighboring refinery and was distilled from the same crude oil. This semi-refined product was called diluent and it is the most abundant contaminant on-site. In petroleum refining terms this diluent is a straight-run gas oil (Lundegard and Garcia, 2003). The pure diluent has some similarity to diesel fuel with an equivalent carbon range of C_{10} to C_{40} (Lundegard and Garcia, 2001). To facilitate the distribution of diluent to all the wells, a large system of tanks and pipelines was constructed. As a result of this diluent distribution system, diluent was released into the environment through spills and leaks. It is estimated that 8 million gallons was released, creating over 90 identified separate source zones, and contaminating the surface, subsurface soil, and groundwater (Lundegard and Garcia, 2001).

Use of diluent was discontinued in 1990, and oil production ceased in 1994 (<http://www.guaddunes.com/about>). In 1998, a Natural Resource Damage lawsuit was settled for \$43.8 million in a civil settlement which included funding for restoration, replacement and rehabilitation of the damaged natural resources (Lundegard and Garcia, 2001). To meet the demands of the lawsuit, Unocal, and now Chevron, have investigated various remediation

technologies including excavation, biosparging, soil vapor extraction, pump and treat, land farming, steam injection, and phytoremediation. Of these excavation has been most extensively used at GRP. The extensive excavations have prevented further infiltration of diluent into the nearby Pacific Ocean and the Santa Maria River. However, the other technologies of phytoremediation and natural attenuation are being examined as polishing steps and as remediation solutions to areas where excavation is not a remediation option.

2.2 Characterization of Diluent

It has been estimated that weathered petroleum is composed of 250,000 different compounds (Group, 1998). Similarly, GRP diluent is an unresolved complex mixture of petroleum compounds and after weathering likely becomes even more complex (Group 1998). It is well documented that once petroleum mixtures are released into the environment, compositional changes occur due to processes of biodegradation, dissolution into water, and volatilization (Thorn and Aiken, 1998; Wang and Fingas, 2003). It is these processes in the environment which “weather” a petroleum mixture and add additional difficulty to the specific analyses of a mixture. Since it is difficult to identify individual compounds in weathered diluent, the diluent is characterized by broad characteristics such as equivalent carbon range and polarity. The diluent product of GRP delivered to the site was derived from a crude oil whose composition likely varied with time over the course of 40 years. Garcia and Lundegard (2001) describe the diluent as less dense than water, with a specific gravity of 0.9 at 60°F and with an apparent solubility of approximately 20 mg/L. The dissolved diluent consisted mostly of polar compounds rather than a true hydrocarbon (Garcia and Lundegard, 2001).

The chemistry of diluent has been described by Lundegard and Garcia (2001) as summarized in Table 2.1. The equivalent carbon range of diluent is similar to that of diesel fuel or kerosene. However, because of weathering and fractionation into groundwater the chemical composition of DPD is very different than that of diesel (Mick, 2006). Due to having an equivalent carbon range, diluent has been stated to be similar to diesel fuel or kerosene. The similarity of the carbon ranges between diluent and diesel can be seen in the table of properties of diluent, Table 2.1.

Table 2.1 Average Properties of Diluent
(Lundegard and Garcia, 2001)

Property	Average Value
Mono-Aromatic Content	
Benzene	9.5 mg/kg
Toluene	15 mg/kg
Ethyl Benzene	45 mg/kg
Xylenes	124 mg/kg
Equivalent Boiling Point Distribution	
<n-C ₁₁	<1%
nC ₁₁ -nC ₁₄	9%
nC ₁₄ -nC ₂₂	65%
nC ₂₂ -nC ₃₀	20%
>nC ₃₀	5%
Compound Class Composition	
Saturated Hydrocarbons	41%
Aromatic Hydrocarbons	29%
Polar + Asphaltenes (NOS)	30%
Total Poly Aromatic Hydrocarbons	12900 mg/kg
Total Naphthalene	7760 mg/kg
Specific Gravity	0.907 (60° F)
Viscosity	14 cp (70° F)
Apparent Solubility	20 mg/L

(Source: <http://torkelsongeochemistry.com/chromatogramexamples.html>)

Figure 2.3 Typical Chromatogram of Dissolved Diluent from Mesocosm Experiment at GRP

In addition to the lack of specific peaks as seen in the diesel chromatogram, the diluent has been found to have polycyclic aromatic hydrocarbons (PAH). The most abundant PAH identified was naphthalene accounting for the majority of the PAHs as seen in Table 2.1 (Lundegard and Garcia, 2001).

Typically, short chain hydrocarbons equivalent to (C10 to C18) are more bioavailable which directly correlates to their ease of biodegradation (Nocentini et al., 2000; Siddiqui and Adams, 2001). The carbon chain lengths of the diluent at the GRP site reach C30 which may retard

biodegradation. At most sites rapid first-order biodegradation occurs near the source zone (Lundegard and Johnson, 2003; Yerushalmi et al., 2003). However, at farther distances from the source zone biodegradation slowed considerably, and eventually over time the petroleum plume was considered “weathered” by both microorganisms and physical processes because the alkanes were degraded (Lundegard and Johnson, 2003). In addition to the longer chain hydrocarbons of diluent, the naphthalene and other PAH compounds may be problematic to biodegrade, due to the difficulty of breaking the multiple ring structures (Wang and Bartha, 1990). In addition to the PAH’s longevity in the environment, many PAHs are toxic which may increase the toxicity of the hydrocarbon mixture (Kropp and Fedorak, 1998). In addition, toxicity may increase during biodegradation (Belkin *et al.*, 1994).

2.3 Phytoremediation

Phytoremediation uses plants to enhance natural attenuation of contaminated sites by employing natural synergistic relationships between plant, microorganisms and the environment. Phytoremediation is a process that lies somewhere between intensive engineering techniques and natural attenuation. Phytoremediation encompasses all biological, chemical, and physical processes using plants, including the rhizosphere. These processes may be beneficial in either *in situ* or *ex situ* treatment. Phytoremediation may removal, transfer, stabilization, or destruction of contaminants, in any media: soils, sludges, sediments, other solids, or groundwater. The term “phytoremediation” (phyto = plant) and (remediation = correct evil) was coined in 1991 (EPA 2000). Plants can mineralize some toxic organic compounds and accumulate heavy metals and other inorganic compounds from soil into aboveground shoots (EPA 2000). The mechanisms which fall under the term phytoremediation are numerous. The mechanisms include rhizosphere

biodegradation which takes place in soil or groundwater immediately surrounding plant roots, and phytoextraction which is also known as phytoaccumulation, and accounts for the uptake of contaminants by plant roots and the translocation and/or accumulation of contaminants into plant shoots and leaves. Phytodegradation and phytostabilization are other mechanisms of phytoremediation; phytodegradation is the metabolism of contaminants within plant tissues and phytostabilization is the production of chemical compounds by plants to immobilize contaminants at the interface of roots and soil. While each of these processes may be defined individually, they may be interrelated. For example, a compound may be cometabolized by microbes in the soil using the root exudates as an energy source. This is followed by degradation of the subsequent compounds by plant enzymes, then ending with degradation by microbes to carbon dioxide and water. Phytoremediation continues to be researched as a cost-effective method of hydrocarbon and PAH remediation (EPA, 2000). However, phytoremediation may have to gain more public approval, which could come with proof of effectiveness (Kocher *et al.*, 2002).

Plants have been used at various sites for different types of contaminants. *Typha latifolia*, a wetland plant, was able to uptake and transport heavy metals (Mn, Cu, Zn, Cr, Ni and Pb), removing them from a wastewater stream (Bose *et al.*, 2008). Alfalfa, a legume which supports nitrogen fixing bacteria on root nodules, has been researched for phytoremediation of many types of compounds and has been successful in remediating metals (copper and lead), and organic compounds (Fan *et al.*, 2008). Common vegetables have also been evaluated for phytoremediation, zucchini and pumpkin accumulate large quantities of the pesticide 2,2-bis(chlorophenyl)-1,1,1-trichloroethane (DDT) (Lunney *et al.*, 2004). Phytoremediation of poly

aromatic hydrocarbons has a well-documented success history (Aprill and Sims, 1990; Reilley et al., 1996; Bient et al., 2000; Dominiguez-Rosado and Pichtel, 2004; Gao et al., 2006; Liste and Prutz, 2006; Mueller and Shann, 2006).

2.4 Phytoremediation of Hydrocarbons

Generally, phytoremediation of petroleum hydrocarbons is slower and less expensive than most engineering techniques or traditional bioremediation methods, but conversely, it is quicker and more expensive than natural attenuation (Frick *et al.*, 1999). For phytoremediation of petroleum hydrocarbon various plants and their associated rhizosphere micro-flora, such as *Pseudomonas*, *Arthrobacter*, and *Achromobacter*, have demonstrated an increased removal of petroleum hydrocarbons (Frick et al, 1999; Cunningham *et al.*, 1996). Of the proposed mechanisms for phytoremediation, there are three demonstrated primary mechanisms by which plants and microorganisms remediate petroleum-contaminated soil and groundwater: rhizodegradation, phytovolatilization, and phytodegradation (Cunningham *et al.*, 1996).

Figure 2.4 Mechanisms of Petroleum Hydrocarbon Phytoremediation

(based on Frick et al., 1999)

The predominant mechanism for phytoremediation for petroleum hydrocarbons is rhizodegradation which is contaminant biodegradation by microorganisms residing in the rhizosphere of plants (EPA 2000, Frick *et al.*, 1999; Newman and Reynolds, 2005). The success of rhizodegradation may be based upon petroleum compounds remaining in the rhizosphere because they are unable to move past the root surface (Burken and Schnoor 1998). The ability of a plant to absorb, translocate and metabolize contaminants is generally dependent on the solubility of the contaminant, reflected by the octanol-water partition coefficient, K_{ow} , of the

contaminant (Cunningham and Berti, 1993; Alkorta and Garbisu, 2001). K_{ow} values generally fall into three groups defining the ability of a plant to absorb, translocate, and metabolize a specific contaminant (Cunningham and Berti, 1993). Plants are able to absorb, translocate and metabolize hydrophilic contaminants with $\log K_{ow} \leq 1$ (Cunningham and Berti, 1993). These contaminants are water-soluble and thus, their absorption is controlled by water influx into the plant. Plants are able to absorb, translocate and may be able to metabolize contaminants with $\log K_{ow}$ values between 1 and 4 (Cunningham and Berti, 1993). A study by Briggs et al. (1982) on the uptake of organic compounds observed the greatest contaminant concentration translocated to the shoots at a $\log K_{ow}$ of 1.8, in a bell shaped curve. Plants are generally not able to absorb, translocate or metabolize contaminants with $\log K_{ow}$ values larger than 3, very hydrophobic or lipophilic compounds because the contaminant adsorbs to lipids on the root surface (Cunningham and Berti, 1993; Schnoor *et al.*, 1995; Alkorta and Garbisu, 2001). In a study conducted by Chanieau (2000) on the effect of growing maize on fuel oil, groups of compounds were selectively degraded. The saturated and aromatic hydrocarbons were significantly degraded, while the polar fraction did not significantly change concentration (Chanieau, 2000). The polar compounds of Chaineau's study would typically have lower $\log K_{ow}$, suggesting that the primary mechanism was not dependent on plant uptake (Alkorta and Garbisu, 2001). Recalcitrant compounds remain an issue with phytoremediation, just as it is an issue for other remediation techniques (Wang and Fingas, 2003).

2.4.1 Rhizodegradation

Plants and microorganisms are involved, both directly and indirectly, in the degradation of petroleum hydrocarbons into products (e.g., alcohols, acids, carbon dioxide, and water) that are

generally less toxic and less persistent in the environment than the parent compounds (Eweis *et al.*, 1998). Although plants and microorganisms can degrade petroleum hydrocarbons independently of one another, it is the interaction between plants and microorganisms which is thought to be the primary mechanism responsible for hydrocarbon degradation in phytoremediation (Frick *et al.*, 1999). The term rhizosphere was first termed in 1904 and has been developed to mean the very narrow zone affected by the roots, approximately 1-2 mm (Darrah, 1993). The rhizosphere enlarges microbial populations and encourages metabolic activities by chemical and physical means. Roots provide elevated concentrations of labile carbon through sloughing of cells and root exudation (Leigh, 2002).

Roots can improve transport of water and air which may achieve near-ideal moisture content for microbial communities and also encourage transport of water through the soil profile (Young, 1995). Roots control hydration content of the rhizosphere and can remove excess water after heavy rainfall (Walker *et al.*, 2000). Root surfaces may act as sinks for lipophilic (soluble in lipids) hydrocarbons, such as PAHs. In one study (Schwab *et al.*, 1998) adsorption of the PAH naphthalene was quantified for tall fescue and alfalfa. In this study, root lipid content was determined to be the controlling factor of absorption of naphthalene: Alfalfa's roots have over twice the lipid content of fescue and absorbed over twice as much naphthalene as did the tall fescue (Schwab *et al.*, 1998). In that study, the age of plants also had an effect, as older plants absorbed more PAHs.

According to a reviews by Stoetz *et al.*(2000) and Walker *et al.*(2000), plants can provide a supply of root exudates that lead to the rhizosphere effect, a release of root-associated enzymes

capable of degrading organic pollutants or enhancing cometabolic degradation, and physical and chemical effects of plants and their root systems on soil conditions. Each of these effects that can enhance the rhizosphere to increase biodegradation are described below.

Root Exudates: The indirect and direct activities of plants enhanced the ability of the rhizosphere community to degrade the petroleum hydrocarbons. Root exudates are defined as any chemical or metabolite secreted by a plant's root environment (Walker *et al.*, 2003). These include sugars, carbohydrates, amino and other organic acids, lipids, enzymes, and a variety of other substances (Walker *et al.*, 2003). As much as 40% of a plant's photosynthate can be deposited in the soil as root exudates (Kumar *et al.*, 2006). A few known root exudates are similar in chemical structure to known pollutants as seen in Figure 2.5 (Siciliano and Germida, 1998).

The type and amount of root exudate is dependent on plant species and the stage of plant development (Siciliano *et al.*, 2003). A long known root exudate of plants under stress (toxicity, nutrient deficiencies, flooding) is ethylene (Glick, 2003). Ethylene is necessary for seed germination and plant development (Deikman, 1997), but when produced under stress this leads to growth inhibition and diminished biomass, especially of roots (Glick *et al.*, 2007). There has been a distinction made that root materials sloughed off during plant growth are not considered root exudates, but rather included as part of the rhizosphere effect (Walker *et al.*, 2003). The sloughed material under discussion includes for example root caps, epidermal cells mucigel, and root hairs. Root exudates are a link between plants and microbes that leads to the rhizosphere effect. Research by Stotz *et al.* (2000) suggests that some of the exudates are actually a signal compound for either a symbiotic or a defense communication.

Figure 2.5 Root exudates and structurally similar pollutants
(Siciliano and Germida, 1998)

The Rhizosphere Effect: The successful application of rhizodegradation is largely dependent on promoting plant growth and supporting microbes to efficiently colonize the rhizosphere (Lugtenberg *et al.*, 2001). This enhancement of the microbial activity that may enhance degradation of contaminants in the rhizosphere is called the *rhizosphere effect* (Frick *et al.*, 1999). The soluble plant products of alcohols, sugars, etc and root turnover accounts for 7-27% of the total plant mass produced annually and can account for 10% to 40% of plant photosynthesis output annually (Kamath *et al.*, 2004; Kumar *et al.*, 2006). The microbial population of the rhizosphere has been reported to be 4 to 100 times larger than that of the bulk soil, since the higher availability of organic matter is thought to support these high populations (Siciliano and Germida, 1998; Penrose and Glick, 2001). The composition of the microbial community is dependent on the plant species and the root exudates (Kirk *et al.*, 2005). In addition, the microbial community may change along the roots and over time (Cook *et al.*, 2007).

A positive correlation between increased microbial activity and petroleum hydrocarbon remediation was found in a study by Kaimi *et al.* (2006) with a time-course experiment with ryegrass grown on soil experimentally contaminated with diesel oil. The rhizosphere and root-free soil degradation rates were nearly the same until the 90-day sampling, then from the 90-day sampling to the 152-day sampling the rhizosphere soil showed a statistically significant decrease in TPH. From 90 days onward, the non-planted soils ceased degrading at around 70 % TPH reduction while the planted pots continued to almost 90 % TPH reduction by 121 days. In the final sampling, the TPH concentration in the rhizosphere was 55% lower than in the corresponding root-free soil. In the rhizosphere, the number of aerobic bacteria and the amount of soil dehydrogenase activity were higher from the 90-day sampling onwards than in the root-

free soil. At each sampling interval, the planted soils supported more aerobic colony forming units than did non-planted units. The biodegradation rate of diesel oil showed a correlation with soil dehydrogenase activity in both the rhizosphere and the root-free soil. Thus, results from Kaimi *et al.* (2006) indicate that the growth of roots enhanced the microbial activity which in turn appeared to contribute to the biodegradation of diesel oil. This is the same biodegradation behavior observed in earlier EBI studies by Hoffman (2003) and Martin (2003) described below in Section 2.5.2.

Cometabolism: Cometabolism is the process of modifying or degrading a compound that cannot support microbial growth on its own without another compound present such as a growth-supporting substrate or co-enzyme (Juhasz and Naidu 2000). Organic molecules produced from the plant, including plant exudates, can provide energy to support populations of microbes that co-metabolize petroleum hydrocarbons (Juhasz and Naidu 2000). For example, benzo[a]pyrene, a high molecular weight PAH was removed from solution by the *Sphingomonas yanoikuyae* JAR02 bacteria while growing on root products as a primary carbon and energy source (Vervaeke *et al.*, 2003; Rentz *et al.*, 2005). Plant root extracts of osage orange (*Maclura pomifera*), hybrid willow (*Salix alba x matsudana*), kou (*Cordia subcordata*), or plant root exudates of white mulberry (*Morus alba*) supported a 15-20% removal of benzo[a]pyrene over 24 hours. No differences in the removal of benzo[a]pyrene were observed between the different roots extracts tested. According to Rentz *et al.*, (2005) this research suggests that the initial degradation of the hydroxylated ring, by hydroxylating microorganisms was solely fed by root products.

Plant Enzymes Involved in Phytoremediation: Another indirect role that plants play in the degradation of petroleum hydrocarbons involves the release of enzymes from roots (Gianfreda and Rao, 2004). The plant enzymatic systems involved in phytoremediation include nitroreductases, glycosyl and glutathione transferases, oxidases, and phosphatases (Gianfreda and Rao, 2004). These enzymes have been implicated in the transformation of recalcitrant compounds (Gonzalez *et al.*, 2006). Additionally, plants and many microorganisms contain and release several oxidases, such as laccases and peroxidases, which are involved in the removal of different pollutants (Gonzalez *et al.*, 2006). These enzymes are capable of transforming organic contaminants by catalyzing chemical reactions in soil (Gonzalez *et al.*, 2006). Gianfreda and Rao (2004) identified plant enzymes as the causative agents in the transformation of multiple compounds including phenol, PAH, and PCB mixed with sediment and soil. Isolated enzyme systems included dehalogenase, nitroreductase, peroxidase, laccase, and nitrilase. It has been found that plant enzymes may have significant effects extending spatially beyond the plant itself and temporal effects continuing after the plant has died (Cunningham *et al.*, 1996).

Effect of Plants on Physical/Chemical Soil Conditions: Plants and their roots can indirectly influence biodegradation by altering the physical and chemical condition of the soil. Soil exploration by roots helps bring plants, microbes, nutrients and contaminants into contact with each other (Cunningham *et al.*, 1996). Plants also provide organic matter to the soil, either after they die or as living plants through the loss of root cap cells and the excretion of mucigel, which is a gelatinous substance that is a lubricant for root penetration through the soil (Cunningham *et al.*, 1996). In addition to providing substrates to degradative bacteria, root turnover also provides oxygen essential for the activity of dioxygenase and monooxygenase enzymes that catalyze the

first step in aerobic degradation of aromatic contaminants (Leigh *et al.*, 2002). Root turnover is considered a major contributor to soil aeration through formation of air channels created when roots die and decay (Leigh *et al.*, 2002). Leigh *et al.* (2002) demonstrated that seasonal fine root death releases several flavones which act as substrates for polychlorinated biphenyl (PCB) degrading bacteria.

Even though the root material may provide energy sources, they may be easier to consume than the petroleum hydrocarbons as an energy source, therefore delaying degradation until the rhizosphere is carbon nutrient limited (Gerhardt *et al.*, 2009). In addition, organic matter can reduce the bioavailability of some petroleum hydrocarbons, particularly those that are lipophilic and bind to organic matter (Gerhardt *et al.*, 2009).

2.4.2 Phytovolatilization

The volatilization of petroleum compounds has not been researched as extensively as rhizodegradation. There are compounds that are known to be volatilized by plants, such as 1,1,1-trichloroethene (TCE) (Doucette *et al.*, 2003). Watkins *et al.* (1994) found that the *volatilization* of naphthalene was enhanced in planted sandy loam soil compared to unplanted soil. The results of this study suggested that naphthalene was taken up by the roots of the grass, translocated within the plant, and transpired through the stems and leaves. Phytovolatilization of contaminants would reduce soil or groundwater concentrations, but there may be air quality concerns, leading to increased health risk and regulatory implications.

An EBI study completed by Elliot (2002), discussed in Section 2.4, on the phytovolatilization of diluent by Arroyo Willows found that diluent from GRP was not phytovolatilized by willow trees.

2.4.3 Phytodegradation

Phytodegradation is defined as plant uptake and metabolism (EPA 2000). Phytodegradation is different from rhizodegradation in that it is the breakdown of contaminants directly by the plant, whereas rhizodegradation, although enhanced by plants, is performed by the rhizobial microflora. Phytodegradation requires that the contaminant is biologically available for uptake and metabolism by the plant (Cunningham *et al.*, 1996). Frick *et al.*, (1999) states that the ability to assimilate *n*-alkanes and liberate $^{14}\text{CO}_2$ was identified in leaves and roots of both whole and cut plants and that the general pathway of conversion for alkanes in plants was generalized as:

n-alkane → primary alcohols → fatty acids → acetyl-CoA → various compounds (Frick *et al.*, 1999).

However, due to the low bioavailability of petroleum hydrocarbons for uptake into plants, phytodegradation is not listed as a major mechanism in the phytoremediation of petroleum hydrocarbons (Newman and Reynolds, 2005). According to Gustafson *et al.* (1997) petroleum hydrocarbons typically have a $\log K_{ow} > 3$. The findings by Newman and Reynolds (2005) are similar to the findings of Cunningham and Berti (1993) that high $\log K_{ow}$ compounds, as typical for petroleum hydrocarbons, are not absorbed into plants. Cunningham and Berti (1993) found that such compounds adsorb to the root surfaces. In the current study, the $\log K_{ow}$ of diluent was determined experimentally so that phytoremediation mechanisms of diluent could be examined in this context.

2.4.4 Willow Phytoremediation Potential

Native to the GRP, Arroyo Willows (*Salix lasiolepis*) have several qualities which make them attractive for use in phytoremediation. Willows are phreatophytes (their roots seek out the saturated zone at the top of the water table), making them good candidates for groundwater remediation (Huang *et al.*, 2005). As a result of their high evapotranspiration rates, willows have been proven to reduce percolation of nutrients and accumulate metals (Kuzovkina and Quigley, 2005). Use of native plants in phytoremediation provides advantages over other species and helps bring back the heritage of flora lost through human activity that provides for wildlife habitat enhancement and conservation (EPA, 2000). Unlike many introduced species, once established, native plants do not require fertilizer, pesticides, or watering (Frick *et al.*, 1999). Care should be taken not to introduce invasive species that may cause greater damage than the expected benefits from their use.

Willow species have successfully colonized open habitats, man-altered habitats and disturbed habitats because they are able to survive in nutrient limited sites (Kuzovkina and Quigley, 2005). Willows grow well in full sunlight but are not adapted to shade; they have a high growth rate with a relatively short life expectancy (Kuzovkina *et al.*, 2004). These characteristics can pioneer a community, accelerating the recovery of a damaged ecosystem and re-establishing the natural ecological complex of organisms (Kuzovkina and Quigley, 2005). Initial colonies of willow have produced microclimates which include shade, leaf debris, root action and formation of humus, all of which improves the soil structure and nutrient status for future plants (Kuzovkina and Quigley, 2005). The roots of willow trees are noted for their tolerance of

flooded or saturated soils and oxygen shortage in the root zone (Kuzovkina *et al.*, 2004; Jackson and Attwood, 1996). Willows' ability to transport oxygen down to the root zone through aerenchyma formation may contribute to providing better conditions for bacterial growth. (Kuzovkina *et al.*, 2004).

Willow is an effective genus for ecological restoration of wetlands; installed in riparian restoration they act as an anchor for the establishment of larger and longer-lived woody species (Kuzovkina *et al.*, 2004). Willows have a high wildlife value, providing habitat and food for diverse organisms (Kuzovkina and Quigley, 2005).

Little is known about the effect of willows on organic pollutants (Vervaeke *et al.*, 2003). In a phytoremediation experiment by Corseuil and Moreno (2001) on the remediation of shallow aquifer sites contaminated by an ethanol-blended gasoline spill using cuttings of the willow tree (*Salix babylonica*) demonstrated, under lab conditions that the willow cuttings were able to reduce ethanol and benzene concentrations by more than 99% in less than a week. In a study by Vervaeke *et al* (2003), willows (*Salix viminaliss*) were established on plots of dredged sediments containing mineral oil and PAH's. After 1.5 years the mineral oil concentration had decreased 57% in the planted plots and only 15% in the fallow plots; however, PAH decreased only 32% in the fallow plot and 23% in the planted plots. The willows provided more amenable conditions for the microorganisms degrading mineral oil, while the fallow plots favored PAH degradation (Vervaeke *et al.*, 2003).

2.4.5 Phytoremediation with Fertilization

It has been found that most soils contain the native microbe types and populations that are able to degrade many petroleum hydrocarbon compounds, but it is the nutrients of the soil that are often lacking to support these populations (Gerhardt *et al.*, 2009). Generally, nitrogen is the growth-limiting nutrient and, therefore, is needed in the highest concentration (Alexander, 1995). White *et al.* (2003) investigated soil amendments with different C:N ratios for their effects on both seed germination and plant growth. Organic and inorganic N amendments resulted in increased plant biomass production and greater reductions of TPH (White *et al.*, 2003). Addition of boiler litter, which had a C:N ratio of approximately eight to one resulted in lower soil total petroleum hydrocarbons (TPHs) compared to amendments with higher C:N ratios. In the research conducted by White *et al.* (2005) on phytoremediation of PAHs, they found that with nutrient addition of a commercially available fertilizer (nitrogen, phosphorous and potassium ratios of 13:13:13) to the phytoremediation system there was more degradation of the multiple-ring PAH structures which are typically more recalcitrant.

2.5 Studies of the Diluent of GRP

2.5.1 Phytoremediation Field Studies of the Diluent of GRP

There are two on-site field studies that were initiated at GRP prior to many of the laboratory studies. In such studies results may take years to achieve, as there may be a long lag-phase in establishing an operating field planting. Field experimental results can be different from the laboratory results due to less than optimal physical, chemical, and biological conditions as well as spatial variability of the contaminants (Gerhardt *et al.*, 2009). Microcosm experiments in the

laboratory may show unrealistically effective results for phytoremediation because conditions are optimal and the contaminants are generally distributed evenly in the soil (Qiu et al., 1997).

The O13 Test Plot is located in the southeast corner of the GRP, and up gradient of the Santa Maria River. A phytoremediation system was installed in the O13 area following excavation activities in that area during the second quarter of 1999. The phytoremediation system in the O13 area consists of a dense tree planting with alternating rows of Arroyo Willows and Black Cottonwoods in a valley. Arroyo Willows were initially planted in the O13 area. Rows of Black Cottonwood were subsequently planted during the first quarter of 2002 (Schroeder 2006).

The C8 Test Plot is located in the northwest corner of the GRP, and up-gradient of the Pacific Ocean. An ecoremediation approach to phytoremediation was implemented in the C8 area following the partial excavation of the C8 North and C8 South separate-phase diluent source areas in the third quarter of 2000 (Schroeder 2006). As of 2002, the groundwater still contained between 5 to 30 mg TPH/L. The phytoremediation system in the C8 area by EBI consists of three phytoremediation planting areas (high, moderate, and low diversity of native tree, shrub, and herb species) and a control area (Coastal Dune Scrub). Planting within the C8 test plot primarily consisted of Arroyo Willow, Black Cottonwood (also referred to as California Poplars), Coastal Dune Scrub, and Pacific Wax Myrtle. The site is being actively monitored and robust vegetation has been observed. At each of these field sites, groundwater concentrations of TPH have been extensively monitored. However, fluctuating groundwater tables, changing flow direction, changing TPH concentrations, moving plumes and other field variabilities have made it very difficult to examine the effects of phytoremediation at the site.

2.5.1.1 Mesocosm Studies

Two separate studies were completed in large mesocosms operated on-site at GRP prior to the current experiment. One study investigated phytoremediation (Crossley, 2006), while the other examined chemical changes in DPD during biodegradation. Both studies used the same two experimental runs in the mesocosms at GRP. There were ten mesocosms on site at GRP, the odd numbered boxes: 1,3,5,7 and 9 were soil-only mesocosms. The even boxes contained four Arroyo Willow trees each. Crossley's (2006) experiment researched phytoremediation by using all ten mesocosms as the soil-only mesocosms acted as natural attenuation controls. The current experiment of this paper was operated by the methods established in Crossley's research. In his research there was statistically no significant difference between the mesocosms with willows and the soil-only controls. In fact it appeared that the trees may have been inhibiting the degradation of the diluent. The willow trees in Crossley's experiment were in poor health, and this was believed to be a factor in why the trees did not enhance the biodegradation of DPD.

The research by Mick (2006) on the soil-only mesocosms attempted to observe the degradation of different fractions (aliphatic, aromatic, and polar) of dissolved phase diluent by natural attenuation. However, the dissolved phase diluent used in these two experimental runs of the mesocosms contained no detectable amounts of aliphatic or aromatic fractions. Mick was able to find that the polar phase of the DPD did biodegrade, which was thought to be recalcitrant (Mick 2006). Terminal Restriction Fragment (TRF) analysis (explained in Section 3.8.4) was completed on the initial and the final samples of each experimental run. The microbial populations were different between the two sampling times of each run.

2.5.2 EBI Laboratory Studies of Phytoremediation at GRP

Laboratory studies of the potential of willows for phytoremediation were initiated at Cal Poly by Elliot (2002) and Hoffman (2003). In Elliot's laboratory study microcosms were set up to determine if Arroyo Willows volatilized DPD. In his study gas samples were collected for 24 hours and it was determined that the Arroyo Willows did not volatilize organic compounds in parts per million concentrations when exposed to dissolved phase diluent. The willow trees did produce a compound with five carbon atoms, which was similar to findings in the literature (Elliot, 2002). In Hoffman's laboratory study, microcosms were set up in triplicate with sodium azide-inhibited soil, soil with active bacteria, and soil with active bacteria and Arroyo willows (*Salix lasiolepis*). Diluent-contaminated groundwater was recirculated through the root zone during two experiments lasting 105 days and 133 days. Biodegradation rates were estimated by measuring total petroleum hydrocarbon (TPH) concentration over time in all microcosms. TPH concentrations decreased in all chambers, the smallest decrease was for the sodium azide control chambers and the largest was in the willow chambers (Hoffman 2003) (Figure 2.6). At the end of 133 day run the azide control dropped to 2.30 ± 0.20 mg/L, the soil only chambers dropped to 0.67 ± 0.145 mg/L, and the soil and willow chambers dropped to 0.33 ± 0.212 mg/L indicating again that willow trees enhanced long-term biodegradation. Although the observed effects were not large, these results show a statistically significant effect of the willow trees compared to soil alone, suggesting the trees slowed the bioremediation for the first 20 days and then increased the biodegradation rate for the last 113 days under these conditions. Biodegradation occurred in the microcosms either directly via phytodegradation or indirectly via stimulation of bacterial biodegradation, rhizodegradation

Figure 2.6 Averaged TPH results for laboratory microcosms of willows, soil, and azided killed controls
(Hoffman, 2003)

In a companion EBI study, Martin (2003) investigated lupines (*Lupinus chamissonis*) as a potential species to be used for phytoremediation at GRP. Using the same experimental setup as Hoffman 2003, lupines were substituted with willow plantings. After 24 days the *L. chamissonis* and soil-only chambers were not significantly different from each other. However, after 105 days, the final TPH concentrations were 0.95 ± 0.22 for the azide inhibited, 0.67 ± 0.085 for the soil only, and 0.33 ± 0.12 mg/L for the *L. chamissonis* chambers. Thus, the *L. chamissonis* increased the biodegradation of the TPH significantly after 105 days relative to the soil-only chambers. From Martin's and Hoffman's studies, it was indicated that the native *L. chamissonis* and *Salix lasiolepis* are suitable species for remediation at the Guadalupe site.

Several other EBI studies have examined the sustainability of natural attenuation and bioremediation at Guadalupe, and the results are useful in understanding the current study. Marie Dreyer (2005) explored the effects of weathering on hydrocarbon biodegradation and toxicity. Groundwater samples of varying TPH concentrations were taken from 34 locations across the GRP site. The samples containing the lowest TPH were considered to be more weathered than were those with higher concentrations. Biodegradation and respiration rates were measured in the lab to determine biodegradability of each sample. Gas chromatography and simulated distillation were used to examine hydrocarbon composition changes with weathering. Dreyer found the biodegradation rates were directly proportional to initial TPH concentration ($r^2 = 0.92$) which suggested first order kinetics. The average first order rate constant was 0.023 day^{-1} . However, the rate constants decreased by 5 to 46% for down-gradient samples along four selected plumes. Biodegradability of diluent decreased with weathering, but biodegradation continued at a reduced rate (Dreyer 2005). Dreyer also compared the biodegradation of DPD to changes in toxicity using Microtox[®]. It was found that toxicity decreased rapidly with decreasing TPH concentrations.

In another EBI study, Drew Lassen (2005) investigated diluent biodegradability along vertical profiles using ground water samples taken from three nested wells along an isolated plume at GRP. Lassen found significant biodegradation rates for samples containing TPH concentrations at or above $500 \text{ } \mu\text{g/L}$ with a first order rate constant of 0.0128 day^{-1} . Cunningham (2005) used laboratory soil columns plus microcosms without soil to study potential effects of soil on biodegradation of TPH in diluent-affected groundwater from GRP. Over the 150-day study, TPH concentrations decreased by 60 – 67%. Cunningham found the presence of soil greatly enhanced

TPH biodegradation in the short term (30 days), but in the next 120 days soil only slightly enhanced TPH biodegradation. Cunningham showed that sodium azide killed controls inhibited the degradation of all fractions of the TPH. The controls were inhibited with sodium azide so that biodegradation and/or adsorption of the TPH could be evaluated, and this confirmed that biological activity was responsible for the observed decrease in TPH. Biodegradation rate constants showed a high initial average first order rate constant of 0.0329 day^{-1} for the first 30 days in the soil columns, compared to an average of 0.0196 day^{-1} in the microcosms without soil. TPH concentrations in both sets of experimental units decreased rapidly for the first 30 days compared to Days 30 – 150. Simulated distillation results showed the short-chain hydrocarbons were being degraded preferentially to the longer-chain hydrocarbons. After 50 days little or no biodegradation was observed and it was determined that the reduced degradation was not due to limited nutrient availability (Cunningham 2005).

CHAPTER 3 MATERIALS AND METHODS

3.1 Mesocosm Construction

Ten containers, referred to as mesocosms (very large microcosms), were previously constructed for experiments completed by Crossley (2006) and Mick (2006) in 2005. All ten containers were filled with clean and sterile site sand and contaminated site groundwater. Five containers were planted with four Arroyo Willow poles each, while the other five containers served as the non-planted controls. To more accurately simulate field conditions (temperature, wind, moisture, and dissolved oxygen levels) than the previous laboratory studies had done, this experiment was conducted on site at GRP, open to site environmental conditions. Monitoring wells provided groundwater which was circulated horizontally through the rhizosphere at rates estimated to match typical site groundwater velocities of approximately 1 ft/day. Water samples taken from each mesocosm for gas chromatography (GC), Microtox[®], and terminal restriction fragmentation (TRF) analyses were collected periodically over the two studies.

The mesocosms were previously used for two experiments (Runs 1 and 2) by Crossley (2006) and Mick (2006). Runs 3 and 5 were done for the current research. Run 4 was aborted because of leakage. The construction described below was undertaken by Kevin Crossley with major assistance from GRP staff, including Shane Clement, and was included here for completeness of experimental equipment design.

Figure 3.1 shows a diagram of a mesocosm. The term mesocosm is used to refer to the entire system present in Figure 3.1. There were 4 circulatory wells in each mesocosm, two for injection and two for extraction, to circulate the contaminated groundwater contained in each mesocosm. A fifth well in the center of each mesocosm was used for monitoring.

Figure 3.1 Diagram of a mesocosm
(Crossley, 2006)

The ten containers were arranged into two parallel rows of five containers each shown as in Figure 3.2. The two-row configuration had the smallest profile to the strong daily west to east wind. Secondly, the two-row arrangement limited any bias from sun exposure as it presented the most balanced orientation to the sun. The mesocosms were placed in a recessed asphalt pad for secondary containment at the former steam pad site at GRP. The containers were leveled with wooden shims.

Figure 3.2 Mesocosm orientation
(Crossley, 2006 and Mick, 2006)

The mesocosms were numbered 1-10, containers 1-5 were in a row, abutted against each other, and containers 6-10 made the second row (Figure 3.2). Odd numbered containers were non-planted controls and four willows were planted in each even numbered container. For simplicity, the mesocosms are referred to individually as “box + the number” in the results section.

3.1.1 Mesocosm Containers

Polypropylene containers measuring 4’x4’x4’ (Macro Plastic, Fairfield, CA), were the main structural component of the mesocosms. Each container’s estimated volume was 1500 liters (400

gallons) due to the rounded corners and other protruding structural supports as seen in Figure 3.3. Prior to filling, the containers were washed with a mild detergent and double rinsed to remove any residue which could interfere with future experiments.

Figure 3.3 Mesocosm container before modification

(Crossley, 2006)

3.1.2 Circulation System

Wells: Each mesocosm contained five vertical wells: two for injection, two for extraction and one well of a similar design placed in the center of the mesocosm for depth to water (DTW) and other measurements. In addition to the five vertical wells, there was one horizontal well along the bottom of each mesocosm for draining purposes. All wells and stainless steel tubing were washed with a detergent and double rinsed prior to installation. Stainless steel (SS) was chosen

for the components of the circulation system as it is resistant to corrosion and is chemically inert. The wells were made of 1¼ inch schedule 80 PVC five feet in length, custom manufactured (Dean Bennet Supply Company, Denver, CO). The slotting, which measured 0.010 inches, (250 µm) ran from one half inch above the bottom of the well, for eighteen inches. The wells used for the circulation system were modified by Crossley from their original design. The threads of both ends were cut and replaced with Schedule 80 slip-fitting caps. These cuts reduced the well lengths to 46 inches from their original sixty inch total length. The top caps were threaded with a ¼” SS bulkhead fitting. A SS tube was fitted inside of the cap, and then the cap was secured to the top of the PVC wells.

The 0.01-inch slotting of the circulation wells was used to prevent sand infiltration. A stainless steel (SS) mesh (Aquatic Eco-Systems, INC., Apopka, FL) slotted at 0.010 inches was folded and tied around each well and vertically along the interior of the containers to protect the wells from invasive roots (Figure 3.4).

Figure 3.4 Mesocosm wells with the protective SS mesh

(Crossley, 2006)

The wells were screened by the 0.010-inch SS mesh only over the bottom 18 inches to minimize circulation of the upper (more oxygenated regions) of the saturated zone. The actual suction and injection took place at the tip of the SS tube which ran 44 inches inside interior length of the PVC well. By not screening the circulation wells over the entire saturated thickness, it was hoped that a relatively stagnant layer of oxygenated water would “cap” the water circulating in the screened zone. The vadose zone in the containers is thinner than most site conditions as groundwater is rarely as close to the surface as it was in the mesocosms. Oxygen diffusion through the vadose zone of the mesocosms was a concern because diffusion to the upper region of the saturated zone would cause unrealistically high dissolved oxygen (DO) levels.

The circulation wells were secured to the inside of the containers with U-bolts. The space around the wells was filled with clean, kiln-dried, Monterey Luster sand (RMC Materials, Monterey, CA). The other end of the bulkhead was connected with a Swagelok® fitting to a 90 degree bent tubing which connected to a “T” fitting placed in-between the two wells. The two extraction and injection wells were reduced from two channels to one SS injection and extraction circulation line.

After filling the mesocosms with site sand, a hole was augured through sand in the middle of each mesocosm and the center monitoring well was pushed into the sand until it hit the bottom of the container. No modifications were made to the monitoring wells; their height was still 60 inches.

Plumbing: The water circulation system consisted of Swagelok® brand quarter-inch SS tubing and fittings (Swagelok Fluid System Technologies, Solon, OH). The only non-SS tubing was short sections of flexible fuel-grade Tygon® tubing (Cole Parmer, Vernon Hills, IL), which connected the pump drives to the rigid SS tubing. The Tygon® tubing was a flexible joint required to protect the pump heads. A rotameter (flow meter), inline dissolved oxygen sampling port, and water sample port were connected into one assembly by PVC pipes and valves to control the water path (

[Figure 3.5](#)

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[Figure 3.5](#)~~Figure 3.5~~). Rotameters were installed to provide an indicator of flow and were operated in Run 3. The rotameters were covered with black plastic tubing to reduce algae growth. Due to clogging problems they were removed in Run 5. During Run 5, the flow for each mesocosm was checked weekly with the rotameter put in line temporarily, with the Tygon® tubing at the pump.

Figure 3.5 Mesocosm dissolved oxygen port and in-line rotameter
(Crossley, 2006)

Pumps: The initial experimental run (Run 3) used Masterflex® PTFE (Teflon®) diaphragm pumps (Cole Parmer, Vernon Hills, IL) driven by Masterflex® variable-speed console drives were used to circulate the water in each mesocosm. The only wettable parts in the pump heads were Teflon®. The unit could run dry, as loss of fluid would not harm the pump.

Due to leakage problems the Masterflex® PTFE diaphragm pumps were replaced for the second experimental run (Run 5) with Masterflex® Easy Load L/S model 751870 peristaltic pumps (Cole Parmer, Vernon Hills, IL). The same length of Tygon® tubing was required for their operation. The pumps were housed in large plastic bins to protect them from the weather. For Run 5, the pumps were raised on tables above the water level in the mesocosms so that in case of leaks the contents of the mesocosms would not be siphoned out.

3.1.3 Mesocosm Soil

Sand was collected on the GRP site from the Q-4 borrow site, an uncontaminated active dune with very little organic matter. A front-end loader was used to fill the mesocosms. Initially the first six inches of sand was added to each mesocosm, and then gravel was poured in around the wells and screen. Approximately four cubic feet of clean, kiln-dried, Monterey Lapis Luster sand (RMC Materials, Monterey, CA) was poured around the wells, into the open column formed between the screen separating the clean site sand, and interior wall of the container (Figure 3.4). The fine gravel provided a porous region around the wells. Increasing the porosity of the material around the well was intended to offset the wells' limited draw, as the wells only draw and inject at one point. In this way the wells would draw from a larger range of depths and radius,

increasing the mixing in the water. After the gravel was poured, the screen was folded over the gravel and pinned to the interior wall of the container with self-boring screws. Then the containers were filled to 6 inches below the top with site sand, and two inches of the Monterey Lapis Luster sand was added as a protective cover to the sand to minimize sand loss by wind.

3.1.4 Groundwater

The groundwater used in both experiments was obtained from Well D2-1, as it was tested to have consistent TPH levels of 12 mg/L; it had been shown in the past to contain as well some non-polar fractions of diluent.

Water Circulation Rate: Groundwater velocities at GRP are commonly referenced at one foot per day, but they do vary throughout the site. The groundwater velocity of one foot per day was the rate used in the previous laboratory and on-site experiments as well as in the current study, to allow comparison of results. The porosity of the dune-sand aquifer at the GRP site was reported to be 0.4 by GRP staff. Below is the calculation, using Darcy's Law, used to determine the circulation rate needed in the mesocosm to match a groundwater velocity of one foot per day.

$$Q = \eta \times V \times A \times w \times t \text{ (Darcy's Law)}$$

$$\text{Where } Q = \text{Flow Rate } \left(\frac{mL}{Day} \right),$$

$$\eta = 0.4$$

$$V = 1 \left(\frac{ft}{day} \right),$$

$$\begin{aligned}
 w &= 4 \text{ ft (the saturated zone width),} \\
 \text{and } t &= 2 \text{ ft (the saturated zone depth),} \\
 \text{thus } Q &= 0.4 \times 1 \left(\frac{\text{ft}}{\text{day}} \right) \times 4 \text{ ft} \times 2 \text{ ft} = 3.2 \left(\frac{\text{ft}^3}{\text{day}} \right) = 63 \left(\frac{\text{mL}}{\text{min}} \right)
 \end{aligned}$$

3.1.5 Arroyo Willow Tree Establishment

Arroyo Willows (*Salix lasiolepis*) were proven to be successful in phytoremediation in a previous laboratory study (Hoffman, 2003). This species of willow is native to the Guadalupe site, which is important for ecological considerations. The willow trees poles were collected from an onsite existing field phytoremediation test site. The willow trees were established in the mesocosms on May 13, 2005. No growth aids or antibiotics were added to assist the poles (until Run 5). The willow poles were planted in 1.5-foot deep holes in the mesocosms.

3.1.6 Protective covers

Run 3 was operating throughout the growing season (March to July 2007), and during the run the mesocosms were not protected from the elements. In between Run 3 and Run 5 the mesocosms were protected from the rain during winter to prevent overfilling and flooding of the mesocosms. A rain cover was built and installed for the rainy months. A PVC hoop spine was built to support visqueen, a 6 mm polyethylene film, as shown in [Figure 3.6](#)~~Figure 3.6~~~~Figure 3.6~~. A clear rain cover was used to maximize the amount of sunlight in an attempt to minimize the stress on the trees.

Figure 3.6 Mesocosm rain cover

Run 5 was also operated during the growing season. There was windburn seen on the leaves and branches in Run 3 and exposed plant material in Run 5. Since wind appeared to be stressing the health of the trees, a windscreen was placed along the edges of the mesocosms for protection from strong onshore wind from the ocean. The windscreen was two feet tall above the top of the boxes and made of shade cloth, as seen in Figure 3.7. The placement of the windscreens was justified as natural because willows in a natural stand would be protected from the strong onsite wind by the surrounding willows.

Figure 3.7 Run 5 showing the windscreens and the raised pump boxes

3.2 Experiment Timeline

The experiment was completed in three phases. The first phase was the non-fertilized operation of the mesocosms (Run 3) which was conducted from March to July 2007 after the completion of Crossley's (2006) and Mick's (2006) studies. Run 3 was operated identically to their established protocol, for a total of 109 days. The second phase was the fertilized acclimation period from July 2007 to February 2008. During this phase, fertilizer was added. Doggett 30-10-10 Foliar Tree Fertilizer, was diluted at 2 lbs per 100 gallons of water and this mixture was applied at one gallon every two weeks to each mesocosm including the soil only controls. The second study began in February of 2008 (Run 4), but it was aborted due to leakage problems with the Masterflex® peristaltic pumps heads. Throughout Run 4, which lasted from February 2008 to April 2008, fertilizer applications remained constant. The fertilized study, Run 5, began in May of 2008 and was operated for 126 days. Throughout Run 5, fertilization of all mesocosms was

continued at one gallon of a 2 lb/ 100 gallon mixture applied equally every two weeks to all containers. Nutrient analysis was done at each sampling in Run 3 and Run 5.

For Run 3 fresh groundwater from the on-site well D2-1 was added to the mesocosms and continuously circulated for 109 days. Distilled water was used to maintain water levels in all mesocosms, making up for the evaporated water. There were five samplings taken from the mesocosms during Run 3.

At the end of Run 3 the mesocosms were not emptied, but topped off with distilled water for the fertilization acclimation. For the eight-month long fertilization acclimation phase, the only samples taken were for nutrient analysis. This phase covered the dormant phase of tree growth.

Run 5 operated very similarly to Run 3, except with the addition of fertilizer applications. Prior to the experimental Run 5, the boxes were emptied and fresh groundwater from the on-site well D2-1 was added to the mesocosms. The water levels were maintained between samplings with distilled water. The fertilizer applications were continued every two weeks throughout the run. There were four sampling events in Run 5. At the end of Run 5 the mesocosms were destructively dismantled for quantification of tree and root biomass.

3.3 Mesocosm Operations

3.3.1 Groundwater Collection

Groundwater for both experiments was collected at a nearby on-site well, D2-1. The well was located outside a source zone and had a historical TPH concentration of 12 mg/L. All equipment used to collect and transport the fresh ground water was cleaned. Before collecting water from the well, the well was gauged to assess depth to water and bottom of well, and then four well volumes of well water were purged to remove any stagnant water from the well prior to the collection of ground water. A 2-inch submersible pump (Grundfos INC, Bjerringbro, Denmark) pumped all the water used for the experiments. All water was transported in a trailer-mounted polypropylene tank. Each batch of water (450 gallons) was collected and driven to the experimental mesocosm site. The trailer-mounted tank was fitted with a 4-inch cam-lock coupling at its base which was connected to a 5 H.P. Honda trash pump. As soon as the initial 450 gallons of water was fed into the mesocosms, the collection process was repeated once more to obtain enough water for an experimental run. The well was not purged before the second collection.

3.3.2 Draining and refilling the boxes

Each run (Run 3, Run 4, and Run 5) of the study required a fresh batch of contaminated groundwater after removal of the previous run's batch of groundwater. The mesocosms were drained by connecting a vacuum truck to the horizontal drain well. All hoses for draining and filling were petroleum grade Thermoid[®] hoses (HBD Industries, Bellefontaine, OH). For filling, the trash pump was connected to the polypropylene tank to transfer the groundwater to the mesocosms. Approximately 70 gallons was metered (Retro-Thrust[®] Hersey[®] Products Rotary Meter, Cleveland, NC) into each mesocosm. This volume of water provided approximately a

2.5-ft water column and a 1.5-ft thick vadose zone. Measurements for depth to water were recorded for each mesocosm, and this was the height that was maintained until the first sampling. After each sampling new depth to water measurements were recorded to account for water removed for samplings.

3.3.3 Weekly Monitoring of Mesocosm

Water levels: Water levels were important because they were an indicator of the volume of water in the mesocosm and evaporation or evapotranspiration could affect measured TPH concentrations. The center well in each of the mesocosms served as a monitoring well. Water levels refer to the height of water from the bottom of the mesocosm to the top of saturated zone. If evaporation or evapotranspiration dropped water levels below their target height, distilled water was added to compensate. During all three phases of the study, water levels were monitored on a weekly to bi-monthly basis. A Solinst® Model 101 Water Level Meter (Ontario, Canada) was used to gauge the DTW in each of the monitoring wells.

General Maintenance: Weekly operating of the mesocosms included weeding of all mesocosms and maintaining the pump drives, replacing bushings when necessary. Initially, in both experimental runs dissolved oxygen was measured, a few measurements were obtained in the beginning of Run 5. The differences in operation conditions between the experimental runs are explained below.

Run 3: Two other graduate students, Laleh Rastegarzadeh and Meghann Chell, oversaw much of the operation of Run 3. The rotameters were operating during Run 3 and were cleaned weekly to remove algae growth. The pumps were turned off and the meters were unscrewed from the plumbing assembly shown in

[Figure 3.5](#)

[Figure 3.5](#) Figure 3.5. Pipe cleaners were used to clean the rotameters, and then they were rinsed with distilled water. Black plastic tubing was used as a cover to reduce algal growth.

Run 5: The Tygon® tubing used in the Masterflex peristaltic pump heads was replaced every two weeks to prevent leaks. To assure that water was flowing in each mesocosm after the rotameters were removed, a rotameter was attached temporarily to Tygon® tubing that could be placed in the peristaltic pumps heads to measure the water flow. Water flow was tested this way weekly.

3.3.4 Water volume approximations

The initial volume of water added to each mesocosm in both runs was approximately 70 gallons. However, after the initial addition there was no direct way to measure water volumes within the containers, therefore water level measurements were used to estimate the volume of water in each box. Some variations were observed between mesocosms in the depth responses to water volume changes, especially in Run 5 between the mesocosms with and without willow trees. Evaporation and sampling caused water volumes to change.

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A calibration curve was developed for the mesocosms to determine the amount of water required to maintain water volumes. Water levels were measured before and after the removal or addition of water. When sampling, the in-line sample ports were used. Since these draw from the saturated range of the boxes, an immediate corresponding drop in water levels was observed. Water added to compensate for evaporation was poured onto the top of the mesocosms and, therefore, there was a lag time in the final DTW measurement levels. The response curve was developed for the change in height of water per unit volume of water and was used to calculate the quantity of water needed to maintain water levels.

3.4 Sampling Overview

Samples were collected several times during each experimental run. For Run 3 samples were collected at Days 0, 20, 67, and 109. For Run 5, samples were collected at Days 0, 25, 57, and 126. At each sampling, there were multiple samples taken for different analyses. All samples were collected at the sampling ports of the mesocosms, which were in-between the pump and the injection wells. From each mesocosm for each sampling event 2 L of groundwater was collected for TPH analysis, 1 L for Microtox[®] analysis, and 4.3 L for TRF analysis. The method used to analyze TPH by aqueous extraction requires at least 1 liter of water sample. Duplicates were performed, thus 2 L of water were required for each TPH analysis. The TRF analyses were performed at the microbiology EBI lab at California Polytechnic State University. TRF is a qualitative analysis of the abundance and variety of microbial populations within a sample and is described in the analytical method Section 3.8.4. In addition, samples were collected from two

planted mesocosms and two unplanted mesocosms at each sampling event for TPH and nutrient analyses by an outside lab. These samples were sent to BC Labs (Bakersfield, CA) for duplicate TPH and nutrient level analyses. The TPH analyses by BC Labs were used for QA/QC comparison to in house analysis.

During sampling, pump speeds were increased for adequate flow and water was collected through 1/4" Tygon® tubing into either 1-L plastic containers or amber jugs depending on the analyses. All sampling containers were rinsed by filling them a quarter full and then shaking them. The water was then poured back on to the mesocosm from which it came. The plastic containers were for BC Labs and they were immediately placed in coolers with ice packs and shipped the same day as sampling. The sampling containers destined for Cal Poly EBI labs were capped and transported to the labs. The samples for TRF analysis by EBI were transferred on the sampling day or one day after sampling. If the transfer of the samples occurred on the day following sampling, the samples were stored at 2°C until the transfer. Samples for TPH analysis were kept at 2 °C for up to a seven days until extraction could be performed.

3.5 Disassembly of the Mesocosm and Collection of Biomass

After Run 5 the mesocosms were destructively disassembled to quantify the biomass. After the final sampling, the willow trees were cut at 6 inches above the top of the sand in the mesocosm.

Figure 3.8 shows the collection of the tree biomass.

Figure 3.8 Cutting the trees down at the end of the experiments

The tree biomass from each mesocosm was rolled into a tarp and transported to Cal Poly. The tree biomass was allowed to air dry for seven days, prior to oven drying and then weighing. The water in the mesocosms was removed by vacuum truck. The next day, soil samples were taken

from each mesocosm for TRF analysis. The soil core analysis was collected across the vadose zone, capillary fringe, and into the saturated zone. The soil collection process is seen in Figure 3.9.

The collection of soil from the capillary fringe and the saturated zone required that the mesocosms were drained just prior to the soil coring, otherwise the core would fail. The cores were taken from the same location in all the mesocosms; in the boxes with trees they were six inches from the eastern edge and in the middle from north to south orientation of all the boxes.

After the soil sampling, the mesocosms were trucked to an on-site contained area for disposal of the soil. Prior to trucking, the mesocosms were stripped of any recoverable research equipment. The boxes were emptied with the assistance of a Gradall, boom lift, as seen in Figure 3.10.

Figure 3.9 Coring the mesocosms after completion of Run 5

Figure 3.10 Emptying of container number two

Root biomass was collected from the mesocosm by sifting through two screens in decreasing diameter, shown in Figure 3.11. The first screen was $\frac{1}{2}$ " screen and the second was $\frac{1}{4}$ " screen. The screening method lost most if not all of the fine roots but collected the majority of the root biomass. The roots were then air-dried for three days next to the tree tops. Then the roots were carefully shaken to remove soil, and then rinsed in distilled water to remove more soil. The roots were then allowed to air dry for another four days before being dried in an oven. After drying all root biomass was weighed.

Figure 3.11 Screens used for collection of the root mass from the mesocosms

3.5.1 Quantification of Biomass of the Arroyo Willows

Biomass of the Arroyo Willows was quantified by following a method based on ASTM Standard D2395-93. The dry weight of the biomass was calculated after the air-dried samples of both above ground tree biomass and root biomass were baked in a forced air oven (Pacific Combustion Engineer Co Model PCCE 224, Los Angeles, CA) for 12 hours at 100 °C. The samples were weighed after 12 hours and then were replaced in the oven and allowed to bake for an additional two hours. The samples were re-weighed to assure that the samples' weights did not change, ensuring that they were dry.

3.6 Analytical Methods

3.6.1 Total Petroleum Hydrocarbon Analysis

Total Petroleum Hydrocarbon (TPH) analysis required three steps after sampling: extraction, concentration, and gas chromatography (GC) analysis. TPH was extracted from the sample into the solvent dichloromethane (aka methylene chloride, MeCl). The solvent containing the sample was then concentrated to a volume of 5 mL. The sample was stored in 2-mL glass vials with Teflon[®]-lined crimp caps until analysis by GC. Samples in MeCl in 2 mL vials were stored in a freezer until GC analysis. The sections 3.6.1.1- 3.6.1.5 describes these steps in more detail.

3.6.1.1 Solvent Extraction of Groundwater sample (EPA method 3510c)

Duplicate 1-L samples from the mesocosm water samples were extracted for TPH following EPA method 3510c (included as Appendix A). Extraction of the samples from each sampling event was completed within seven days; the samples were stored at 4°C until extraction. The glassware necessary for extraction was washed with soap and DI water, rinsed with DI water, methanol, and finally MeCl. The glassware was allowed to dry overnight in the fume hood.

Multiple extractions could be completed simultaneously in a fume hood that had a ring stand apparatus constructed to hold five separatory funnels. The separatory funnel ring stand apparatus is shown in [Figure 3.12](#)~~Figure 3.12~~~~Figure 3.12~~. Placed below each separatory funnel was a Pyrex funnel which contained 50 mL of sodium sulfate (Fisher Scientific S415-212), as a drying agent. Sodium sulfate was used to capture any minute amount of water that was in the MeCl solvent phase. The sodium sulfate was baked at 100 °C for two hours prior to use. Glass wool

was used as a plug in the Pyrex funnel, and the baked sodium sulfate was poured into the funnel. The funnel set up was rinsed twice with MeCl before use. A TurboVap® beaker held with a claw clamp was placed below the Pyrex funnel to collect the extract.

Figure 3.12 Solvent Extraction Apparatus in a Fume Hood

Each 1-L sample was poured into a 2-L separatory funnel. In Run 5 a 1-mL internal spike of hexacosane was added at this point of the extraction. The internal standard of hexacosane was used to determine extraction efficiency. Hexacosane was selected for its high molecular weight that eluted at the upper range of the diluent in GC analysis. Run 3 was not spiked with an internal standard so it would not interfere with infrared analysis that was originally planned,

however not completed. A Kontes (Kimble/Kontes, Vineland, New Jersey) auto-pourer was used to pour 60 mL of pure MeCl into each separatory funnel. The funnels were capped and removed from the ring stand. The funnel was shaken gently four times, then inverted and the stopcock was opened to release the gas pressure. The funnel was vigorously shaken for one minute. After replacing the funnel to the ring stand and uncapping it, the funnel was left to settle into separate phases for ten minutes. MeCl is denser than water and therefore was the bottom layer in the separatory funnel. The solvent layer was slowly drained into the Pyrex funnel with sodium sulfate and collected in the TurboVap® (Caliper Life Sciences, Hopkinton, MA) beakers. The solvent addition, agitation, settling, and collection cycle was repeated two more times using a total of 180 mL of MeCl. After the final MeCl draining from the separatory funnel, the sodium sulfate was rinsed three times with 20 mL of MeCl for a total of an additional 60 mL of MeCl to insure there was no residual TPH left in the sodium sulfate.

3.6.1.2 Concentrating the Extract

Each extraction procedure generated approximately 240 mL of MeCl. Given the small amount of TPH present, the samples were concentrated to increase the relative TPH concentration in solvent and therefore increase the detection. Concentration of the MeCl containing the extracted TPH was done with an automated Zymark TurboVap® unit (Caliper Life Sciences, Hopkinton, MA). The TurboVap® concentrated the samples by evaporating the solvent. The beakers were placed in a water bath held at a constant temperature of 35°C and nitrogen gas flushed over the beakers in a closed chamber. Each extract was reduced to a final volume of 1.0 mL. After evaporation, the beakers were placed in a holder in the fume hood. The remaining 1.0 mL of

extract was transferred to a 10 mL graduated cylinder using a 2-mL glass pipette with a silicon bulb. The TurboVap® beaker was rinsed with MeCl to capture any residual TPH. The rinse solvent was also transferred to the graduated cylinder. New MeCl was added into the graduated cylinder to dilute the extract to a final volume of 5 mL. The 5 mL of extract was transferred into two 2 mL crimp-top vials for storage and analysis.

3.6.1.3 Total Petroleum Hydrocarbon Gas Chromatography Analysis (Based on EPA Method 8015c)

TPH was analyzed by gas chromatography using a method largely based on US EPA Method 8015c. The GC used was an Agilent 6890 equipped with a mass spectrometer (MS), Agilent 5978B, and an Agilent 7683B series auto sampler. An Agilent capillary column (catalog 190991s-433) 30 m by 0.250 mm was used in the GC. [Table 3.1](#) summarizes the GC operation methods. A standard and a blank were run after every four samples for quality control and to assess the operation of the GC. Section 3.6.1.4 describes the methods for standards and calibration curves.

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Table 3.1 GC/ MS operating conditions for TPH analysis

INLET			
Mode	Splitless	Purge Time	0.50 min
Initial Temperature	200°C	Total Flow	65.0 mL/min
Pressure	12.27 psi	Gas	Helium
Purge Flow	60.7 mL/min		

COLUMN			
Capillary Column	Agilent	Model	Constant Flow
Model	catalog 19091S-433	Initial Flow	1.5 mL/min
Length	30 m	Initial Pressure	12.27 psi
Diameter	250.0 um	Average velocity	44 cm/sec
Film thickness	0.25 um	Outlet pressure	Vacuum

DETECTOR			
Mass Spectrometer			
Solvent Delay	3.00 min	MS Source	230°C, max 250°C
Low Mass	45.0	MS Quad	150°C, max 200°C
High Mass	450.0		

OVEN		
Initial Temperature	45 °C	
Maxium Temperature	325 °C	
Oven Ramp		
Rate (°C/min)	Final Temperature	Final Time (min)
0	45 °C	3.00
12	275 °C	19.17
0	275 °C	12.00

Routine maintenance was performed on the GC throughout the experiment. The injection septum was changed approximately every 100 injections. When the septum was changed, the pre-column glass liner also was inspected and changed if needed.

An example gas chromatogram from Run 5, Box 4 at Day 0 is shown below in [Figure 3.13](#). The peak at 22.3 minutes was the hexacosane standard.

Figure 3.13 Chromatogram from Box 4 baseline (Day 0) for Run 5

3.6.1.4 Standards and Calibration Curves

Standards used to calibrate the samples for the GRP diluent were made from pure GRP diluent source material collected from the source zone near Well D2-1. The stock solution was made gravimetrically, with 0.9918 g of pure diluent added to 100 mL of MeCl in a volumetric flask. This created a stock with 9,918 mg diluent/L MeCl. In addition to diluent, 0.2505 g of hexacosane was added to the stock, creating a concentration of 2,505 mg hexacosane/ L MeCl.

This allowed calibration curves to be made for both the diluent and hexacosane. Eight dilutions were made volumetrically from the stock, as seen in Table 3.2.

Table 3.2 GC Diluent Calibration Standards for TPH and Hexacosane Analysis

TPH Standard Concentration (mg/L)	Hexacosane Standard Concentration (mg/L)	Concentration of TPH standard used as Stock (mg/L)	Concentration of hexacosane standard used as Stock (mg/L)	Volume of Stock (mL)	Volume of MeCl	Total Volume
1984	501.0	9918	2505	20.00	80.00	100.00
991.8	250.5	9918	2505	5.00	45.00	50.00
793.6	200.4	1984	501.0	20.00	30.00	50.00
396.7	100.2	1984	501.0	10.00	40.00	50.00
198.4	50.10	1984	501.0	5.000	45.00	50.00
103.1	26.05	793.4	200.4	6.500	43.50	50.00
49.59	12.53	793.4	200.4	3.125	46.88	50.00
24.80	6.265	396.7	100.2	1.563	23.44	25.00

A calibration curve was created from the standard dilutions. The maximum concentration analyzed was 1984 mg diluent/L MeCl. The stock was not analyzed as it could have contaminated the column when the GC was operated in split-less injection mode. A linear regression was used to correlate the TPH concentrations to the peak areas from GC analysis. The raw data for the calibration is in [Table 3.3](#)~~Table 3.3~~~~Table 3.3~~. This resulted in a linear equation ($R^2 = 1.00$) that was used to calculate the concentration of the samples, seen in Figure 3.13.

Table 3.3 Diluent and Hexacosane Calibration Data for TPH from GC Analysis

TPH concentration (mg/L)	Hexacosane concentration (mg/L)	TPH Peak Area	Hexacosane Peak Area
24.80	6.26	4.16E+07	1.25E+07
49.59	12.53	1.30E+08	2.49E+07
103.1	26.05	5.22E+08	7.62E+07
198.4	50.1	1.27E+09	1.58E+08
396.7	100.2	2.68E+09	2.87E+08
793.6	200.4	5.54E+09	5.41E+08
991.8	250.5	7.09E+09	6.86E+08
1984	501	1.48E+10	1.25E+09

Figure 3.14 Diluent Calibration Curve

The hexacosane calibration curve was made by integrating the hexacosane peaks separately from the integration of the TPH hump and correlating these peak areas to the standards concentrations. Another linear equation resulted ($r^2=0.997$) and was used to calculate the efficiency of the extraction, as seen in Figure 3.15.

Figure 3.15 Hexacosane Calibration Curve

A lower detection limit for TPH of 0.17 mg/L water was established by finding the average peak area resulting from ten MeCl blanks. The average blank peak area was converted to a TPH concentration using the linear relationship constructed in the TPH calibration curve.

The results were converted from concentration of TPH in MeCl to concentration of TPH in the groundwater, by dividing by the final volume of the concentrated extract, typically 5 mL, and multiplying by the volume of the original water sample, usually 1000 mL. The same approach was used to determine the amount of hexacosane spike that was extracted from the 1-L groundwater samples. The percent recovery was computed by finding the difference between the measured and expected amounts of hexacosane, and dividing by the expected amount then multiplying by 100.

3.6.1.5 Statistical Analysis on TPH data

A t-test was used to determine if there was a statistically significant difference between the averaged TPH concentrations of the two groups (willows and non-planted controls) and between the fertilized and unfertilized groups. The t-test is used to test the significance of data based on a 95% probability. If the P-Value is less than 0.05 then there is a 95% chance the two data sets are statistically distinguishable.

3.6.2 Silica Gel Column Fractionation

Silica gel column fractionation was completed on all samples of Run 3 and on two of the initial and final mesocosm samples without trees of Run 5. Column fractionation separates compounds from a mixture into fractions based on polarity. The fractionation was used to determine the amount of DPD in the aliphatic, aromatic and polar fractions and the percent degradation of each of these different components of diluent.

The method for silica gel column fractionation of diluent was based on those of ARCADIS-JSA as adapted by Mick (2006). The materials required for fractionation are listed in Table 3.4. To elute the aliphatic, aromatic, and polar fractions different solvents or solvent mixtures were used, as summarized in Table 3.5. The first solvent hexane elutes the non-polar compounds from the diluent and this class is the aliphatic fraction. The more polar mixture of hexane and MeCl in a ratio of 3 parts hexane to 2 parts MeCl elutes the aromatic fraction. The polar fraction is eluted by a 1 to 1 mixture of MeCl and methanol.

Table 3.4 Materials used in silica gel column fractionation (Mick, 2006)

- 10 in x 10.5 mm ID chromatographic column (Ace Glass, Inc 5906-05)
- Chromatographic silica gel (100-100 mesh, Fisher Scientific S817-1)
- Hexane (Fisher Scientific H302)
- Methanol (Fisher Scientific A452)
- Dichloromethane (Fisher Scientific D151)
- Glass Wool
- Ring stands
- Column clamps
- 50 mL beakers
- Volumetric glass pipettes and bulb
- Glass stir rod
- Disposable glass Pasteur pipettes and bulbs
- GC, vials, crimps, and crimper

Table 3.5 Solvents used for Column Fractionation

Compounds	Fraction	Eluting Solvent
Aliphatic	F1	hexane
Aromatic	F2	3:2 mixture Hexane: dichloromethane
Polar	F3	1:1 mixture dichloromethane: methanol

3.6.2.1 Fractionation Method

MeCl extracts stored in 2 mL vials in a freezer, were used as the starting place for fractionation. A solvent exchange was done which changed the solvent from MeCl to hexane by serial evaporation. The samples were transferred from the 2-mL vials to Turbovap[®] tubes with a glass pipette. Using the Turbovap[®] nitrogen evaporation system with the water bath set to 25°C the MeCl was concentrated to a volume of 1 mL. After reaching 1 mL in volume, 5 mL of hexane was added to the Turbovap[®] tube. This process was repeated twice more to evaporate the MeCl completely, changing the solvent to hexane with a final volume of 1 mL.

Silica gel was baked overnight at and stored until use at 130 °C. All glassware was triple rinsed with MeCl prior to use. The column was 10 inches x 10.5 mm inside diameter chromatographic column (Ace Glass, Inc 5906-05). The column had a plug of glass wool pushed to the bottom of the column with a glass rod. The column was partially filled with MeCl and the plug of glass wool was tapped down to remove any air bubbles. The glass wool was rinsed twice more with

MeCl. The column was packed with activated silica gel as slurry. The slurry was created mixing 11.0 g of dried silica gel to 10 mL of MeCl. The slurry was transferred into the column with rinsing with MeCl and tapping with a rubber bulb to settle the silica. The solvent level of MeCl was drained so that it was just above the level of the silica gel in the column and 25 mL of hexane was added. The hexane was allowed to drain so that it was just above the level of the silica column. The 1 mL solvent exchange sample was then carefully added to the column, so as not to disturb the silica. The sample was drawn into the column by allowing the solvent level to fall to just above the top of the silica column. Turbovap[®] tubes were put in place to begin collecting the non-polar fraction (fraction 1). Two 20-mL volumes of hexane were used to elute Fraction 1. The solvent level was never allowed to fall below the top of the silica column. After the second 20-mL volume of hexane drained to the top of the silica column, the stopcock was closed. The Turbovap[®] tube containing Fraction 1 was placed to the side and a new tube was installed to collect the aromatic fraction (Fraction 2). The next two fractions were eluted in the same manner with different solvents. Fraction 2 was eluted with two 20-mL volumes of the 3 to 2 mixture of hexane and MeCl. The polar fraction (Fraction 3) was eluted with two 20-mL volumes of 1 to 1 mixture of methanol and MeCl.

All fractions were concentrated using the Turbovap[®] nitrogen evaporating system, with a water bath temperature of 25°C, to a volume of 1 mL. Solvent exchanges were done from the solvents of the different fractions into MeCl in the same manner that was explained previously. The final volumes were brought to 2 mL, the same as the original sample volume, and stored in the 2 mL

vials in a freezer until analysis by GC/MS. The standard curves and equations used in the TPH analysis were used to calculate TPH concentration of each fraction.

3.6.3 Microtox® Toxicity Analyses

Microtox® toxicity analyses were completed on all samples of Run 5. There is a possibility of toxicity increasing during biodegradation (Belkin *et al.*, 1994). Microtox® is a biosensor linked assay that uses *Vibrio fischeri*, a bioluminescent bacterium, to assess acute toxicity. Luminescent bacteria are used because bioluminescence is a byproduct of their metabolism; therefore, the intensity of light produced is directly proportional to their metabolic rate. Microtox® was chosen to assess toxicity over traditional animal-based testing procedures because it is fast, inexpensive, reliable and humane. The main limitation of Microtox® is the lack of correlation between toxicity to these microorganisms and toxicity to species of interest (Kaiser and Palabrica, 1991). The EC₅₀ of Microtox® analysis is the percent dilution of a sample that results in 50% reduction in metabolism. This is analogous to LD₅₀ used in chemical toxicity determinations and indexes. Two species' LD₅₀, the fathead minnow and the rat, were compared to the *Photobacterium phosphoreum* linear regression line for EC₅₀, for over 200 hundred individual compounds (Kaiser and Palabrica, 1991). The fathead minnow LD₅₀ to EC₅₀ of the bacterium had a moderate correlation ($r = 0.81$), however the rat did not. Chang *et al.*, (1981) stated that bioluminescent enzymes and proteins are more sensitive to the effects of toxic molecules, increasing the sensitivity of the Microtox® method. Toxicity measurements in the current research studies used a Microtox® Analyzer (Belkin *et al.*, 1994; Wang and Bartha, 1989; Wang *et al.*, 1990; Yerushalmi *et al.*, 2003). The Microtox® method has received approval as a

method accepted by the US EPA Whole Effluent Toxicity Testing under the National Primary and Secondary Drinking Water Standards (US EPA, 2004).

Microtox® toxicity was measured using an M 500 Analyzer which was obtained along with all reagents from Strategic Diagnostic, Inc (Newark, DE). For this experiment the Basic Test was used, which required serial dilutions of known concentrations (81.90, 40.95, 20.48, 10.24, 5.12, 2.56, 1.28, 0.64 and 0.32) and a of dilution water control. The Basic Test measures light intensity of the bacteria at three time periods: before exposure, after 5 min exposure, and after 15 min exposure. The control was used to determine the change in luminescence without exposure to the sample. The change in the control luminescence over time was used to correct the experimental sample dilutions change in luminescence so that only the change in metabolic rate due to sample exposure was considered in the calculations.

The calculations were done on the change in luminescence. Luminescence was reported as a percent effect (% effect), the % effect is mathematically defined as

$$\% Effect = \frac{I_{ot} - I_t}{I_t}$$

I_{ot} was the corrected baseline light intensity from the control and I_t was the measured light intensity after t minutes of exposure to sample. I_{ot} was corrected for each testing of the samples by a comparison of the change in the control under the assumption that any change in luminescence in the control indicated a change in metabolic activity unrelated to the sample. The baseline light intensity was calculated by:

$$I_{ct} = I_o \times \frac{I_{ct}}{I_{co}}$$

I_{ct} was the luminescence of the control sample at time t and I_{co} was the measured luminescence of the control sample at time t = 0 min. The measured light intensities are used to calculate an EC_{50} for the sample. The EC_{50} indicated the concentration at which a 50 % reduction in activity was noted in bioluminescence. To calculate the EC_{50} , Microtox® Omni software introduces a parameter, gamma (Γ_t), Strategic Diagnostics mathematically defines as:

$$\Gamma_t = \left[\frac{I_{ot}}{I_t} \right] - 1$$

With algebraic manipulation, % effect was calculated in terms of Γ_t rather than light intensity:

$$\% \text{ Effect} = \frac{\Gamma_t}{\Gamma_t + 1} \times 100$$

To determine EC_{50} , Microtox® Omni software was used to plot the log 10 of Γ_t versus log 10 of concentration on a log-log plot for each sample time. The points were automatically fitted to a linear regression; the equation of the regression was:

$$\text{Log } C = m \log \Gamma_t + b (10)$$

The Γ_t is the gamma value produced by the sample concentration at t minutes exposure and m and b are the slope and y-intercept, respectively, determined by the linear regression. Once this regression is created, the EC_{50} can be extrapolated or interpolated from the graph by setting $\Gamma_t = 1$ and determining the concentration where this occurred. The EC_{50} concentration can be

determined by setting $\Gamma_t = 1$, because when light intensity was reduced by 50% the ratio $I_{0t}/I_t = 2$.

When Γ_t was set equal to 1 in the regression line equation, the equation reduced to:

$$\text{Log } EC_{50} = b$$

$$EC_{50} = 10^b$$

The C is the percent concentration, not the total concentration. Since the sample was diluted in order to perform the basic test, the C generated by the software was the percent concentration relative to the sample used. Therefore, the initial concentration of your sample must be known to report the final EC_{50} . Microtox® Omni software calculated percent effect and EC_{50} for samples after 5 and 15 minutes exposure, and the greater of the two values was recorded.

3.6.4 Terminal Restriction Fragment Analysis

Terminal restriction fragment (TRF) patterns, also known as terminal restriction fragment length polymorphisms (T-RFLP), are PCR-based tools for studying microbial community structure and dynamics (Kitts 2001). TRF can be used to sidestep the limitations of traditional culture techniques, using 16S rRNA to map and chronicle root-induced rhizosphere shifts (Kitts, 2001). Terminal restriction fragment (TRF) pattern analysis was performed by Alice Hamrick and Dr. Chris Kitts (EBI, Cal Poly) using the method described in Appendix B. TRF analysis was completed on the initial and final groundwater samples of Run 3 and Run 5. In addition, soil samples were collected from each mesocosm at the end of Run 5.

This method used DNA extraction, polymerase chain reaction (PCR), capillary and gel electrophoresis, enzymatic digestion, and fluorimetry. As TRF analysis is based on a PCR method, it has some of the same limitations; quantization is an issue with PCR because different microorganisms may contain more than one copy of a gene sequence (Fogel *et al.*, 1999). For example, it has been shown that an rRNA gene varies between 1 to 13 copies per genome depending on species (Kitts, 2001). TRF analysis detects changes in microbial population over time or spatially, but is not able to convert the finding in to number of microorganisms due to the fact that there may be different numbers of a gene sequence in the microorganisms that would skew the results.

3.6.5 Octanol-Water Coefficient of Pure Diluent and Dissolved Phase Diluent

The octanol-water coefficient (K_{ow}) was determined for both diluent from the source zone and the dissolved phase diluent. Duplicates were done on the pure diluent from the sources zone of Well D2-1 and on groundwater samples taken from Well D2-1. The method used was from Schantz *et al.*, (1988), the 'sit flask' method that establishes equilibrium without agitation. Agitation of octanol and water can cause an emulsion to form.

For determining the K_{ow} , the pH of the DI water was adjusted to a pH of 7.0 with a phosphate buffer, since pH can change the solubility of a compound in different solvents. The 1-octanol (Fisher Scientific) was allowed to equilibrate with the buffered water overnight so that both phases were pre-saturated and exact volumes of the phases could be measured. A sample was taken of the water phase as a baseline concentration of octanol in the water. One-liter separatory

funnels were used as the containers., in which the final volumes of octanol and buffered DI water were 100 mL each. Initially only 95 mL of octanol was added to each separatory funnel as the remaining 5 mL of octanol were used to transfer the respective sample. The pure diluent samples were weighed and then mixed with 2 mL of octanol and added to each separatory funnel. The mixing vials were rinsed with the remaining 3 mL of octanol, and then the rinsing octanol was added to the separatory funnels. DPD had previously been solvent extracted into MeCl, as described in Section 3.6.1. The TPH concentrations of the samples were already known. The solvent of the sample was exchanged from MeCl to 1 mL of octanol, using the Turbovap[®] evaporator with nitrogen. The remaining 4 mL of octanol require to have a total volume of 100 mL, was used to rinse the Turbovap[®] tube and was added to the separatory funnels. The all the separatory funnels were capped and placed on ring stands in an incubator set at 20°C for 11 days.

The aqueous phases (bottom layers) of all the separatory funnels were carefully removed without disturbing the separated layers. The octanol layer was not analyzed. The aqueous samples were extracted in to MeCl following the method in Section 3.6.1.1, but the volume of MeCl used in the extraction cycles was 30 mL instead of 60 mL. Solvent extraction was followed by concentration of the extract (Section 3.6.1.2), so the samples could be stored for TPH analysis (section 3.8.1.3). The samples that originated from the initial groundwater samples were adjusted to a final volume of 2 mL to match the original concentration factor, and stored in 2 mL vials. The pure diluent samples were diluted after concentration, to a final volume of 5 mL, and stored in 2 mL vials. All samples were stored in a freezer until analysis by GC.

The equation used to calculate the log K_{ow} is shown below.

$$\text{Log } K_{ow} = \log \left(\frac{(W_{original} - W_{H2O}) / V_{octanol}}{W_{H2O} / V_{H2O}} \right)$$

Where $W_{original}$ is the weight of the total TPH originally added to the octanol water system, and W_{H2O} is the weight of TPH remaining in the water phase after equilibrium is reached. The volumes, $V_{octanol}$ and V_{H2O} , are the respective volumes of solvents. In this experiment the solvents were of equal volume of 100 mL each.

3.6.6 Nutrient Analyses and External TPH analyses

At each sampling, as described in Section 3.4, samples from the experiment were collected and sent to an external lab, BC Labs (Bakersfield, Ca) for verification of TPH analyses and nutrient analyses. Water samples were analyzed for nitrate, sulfate, orthophosphate and ammonia.

3.6.7 Dissolved Oxygen

Due to problems maintaining the membrane in the DO meter, a ferrous iron test was used to evaluate the final oxygen conditions of the mesocosms. The tests were performed by GRP personnel.

CHAPTER 4 RESULTS

The results are described in four sections: Section 4.1 describes the results from the unfertilized experiment (Run 3), Section 4.2 describes the results from the fertilized experiment (Run 5), Section 4.3 presents the biomass of the willows and Section 4.4 presents the experimental log K_{ow} of free product and dissolved phase diluent.

4.1 Results of Unfertilized Experiment (Run 3)

Run 3 began on 3/30/07 after two days of equilibration and this was designated as Day 0. The run was operated until 7/17/07; samples were collected on Day 0 (3/30/07), 20 (4/19/07), 67 (6/5/07), and 109 (7/17/07). Water level remained relatively constant and only occasional additions of DI water were added to the mesocosms to maintain constant water levels

4.1.1 Total Petroleum Hydrocarbon result from Run 3

Results of duplicate TPH analyses for all samples are shown in Table 4.1 for Run 3. The table presents the averages of the planted and unplanted mesocosms and their associated standard deviations. Box 5 leaked in the middle of Run 3. Box 7 was excluded, as the initial concentrations were very different and remained low in comparison with the rest of the mesocosms.

Table 4.1 Unfertilized Run TPH Results (Run 3)

Day	Group	Box	Duplicate 1	Duplicate 2	Average	Group Ave ± STD Dev.	
			TPH mg/L				
Day 0 (Baseline)	Willows	2	5.15	5.71	5.43	5.46	0.74
		4	4.80	4.88	4.84		
		6	NA	NA	NA		
		8	5.53	4.63	5.08		
		10	6.68	6.33	6.51		
	Controls	1	5.89	5.55	5.72	5.54	0.23
		3	5.10	5.73	5.42		
		5	5.31	5.25	5.28		
		7	NA	NA	NA		
		9	6.00	5.50	5.75		
Day 20	Willows	2	3.71	3.00	3.36	3.66	0.39
		4	3.89	4.24	4.06		
		6	3.28	3.31	3.29		
		8	NA	NA	NA		
		10	4.07	3.79	3.93		
	Controls	1	2.84	2.81	2.83	3.19	0.75
		3	3.74	3.47	3.61		
		5	3.63	NA	3.63		
		7	1.85	1.80	1.82		
		9	2.78	2.66	2.72		
Day 67	Willows	2	2.64	NA	2.64	2.63	0.38
		4	3.05	2.95	3.00		
		6	2.19	2.16	2.17		
		8	2.02	2.17	2.09		
		10	2.77	2.67	2.72		
	Controls	1	1.49	1.47	1.48	1.82	0.43
		3	2.31	2.20	2.25		
		5	leaked				
		7	1.27	1.21	1.24		
		9	1.72	1.71	1.72		
Day 109	Willows	2	0.40	0.72	0.56	1.18	0.42
		4	0.83	1.61	1.22		
		6	1.21	NA	1.21		
		8	1.36	1.18	1.27		
		10	1.70	1.80	1.75		
	Controls	1	0.45	0.50	0.48	0.86	0.28
		3	0.88	0.77	0.83		
		5	leaked		NA		
		7	1.08	0.96	1.02		
		9	1.00	1.24	1.12		

Biodegradation in both the control and the mesocosms with plants was rapid in the initial 20 days and then continued at a slower rate (Figure 4.1). TPH concentrations were higher for the most of the planted mesocosms compared to the unplanted, suggesting lower biodegradation rates in the planted mesocosms. The t-test with a 95% confidence interval was used to determine if the TPH concentrations of the planted mesocosms were statistically distinguishable from the controls (Table 4.2). On Day 67 the two groups had over a 99% probability of being different. However, at the end of the experimental run the two groups did not meet the 95% confidence requirement to be qualified as statistically distinguishable.

Figure 4.1 TPH Concentrations during the unfertilized mesocosm experiment (Run 3)

Table 4.2 Run 3 t-test Comparing TPH concentrations of planted mesocosms and controls at each sampling time

Sampling	P-Value (Significant if $P < 0.05$)	Probability Statistically Distinguishable (%)
Day 0	0.42	57.79
Day 20	0.06	94.11
Day 67	0.01	99.18
Day 109	0.11	89.27

The first-order rate constant of 0.0130 day^{-1} was calculated for the planted mesocosms using a semi-log plot over the entire experimental run. The linear relationship had a high correlation ([Figure 4.3](#)[Figure 4.3](#)[Figure 4.3](#), $r^2=0.96$). The first order rate constant for the initial 20 days was 0.020 day^{-1} , and for the final 89 days the first order rate constant was 0.0126 day^{-1} ([Figure 4.3](#)[Figure 4.3](#)[Figure 4.3](#), $r^2=0.93$). The first order rate constants for the control mesocosms' were slightly higher, with the overall rate constant of 0.0150 day^{-1} ([Figure 4.3](#)[Figure 4.3](#)[Figure 4.3](#), $r^2=0.98$), a rate constant over the first 20 days of 0.0275 day^{-1} ([Figure 4.4](#)), and a final 89-day rate constant of 0.0131 day^{-1} ([Figure 4.4](#), $r^2=0.997$).

Figure 4.2 Semi log plot of TPH degradation in planted and control mesocosms to determine first-order rate constants.

Figure 4.3 Semi log plot of the planted mesocosms showing early and late first order degradation rates

Figure 4.4 Semi log plot of the control mesocosms showing early and late first order degradation rates

The degradation rate change is clearly seen in Table 4.3, as the controls more rapidly degrade the diluent during the initial period of the experimental run. However, during the final period of Run 3 the mesocosms with willows were degrading more than the control, even though the overall concentration of TPH in the two groups were indistinguishable. The overall degradation for the planted mesocosms and controls was 78.4% and 82.2% respectively.

Table 4.3 Run 3 TPH reduction and degradation rates

		Starting TPH	Ending TPH	Percent Reduction	Degradation Rate
	period	mg/L	mg/L	%	ug/L-day
willows	Day 0-20	5.46	3.66	32.97	90.00
	Day 20-67	3.66	2.36	35.52	27.65
	Day 67-109	2.36	1.18	50.00	28.10
Controls	Day 0-20	5.54	3.19	42.42	117.50
	Day 20-67	3.19	1.81	43.26	29.36
	Day 67-109	1.81	0.99	45.30	19.52

The QA/QC analysis from the external lab(BC Lab) did produce some differences in the calculated TPH values in the samples. BC Labs found the average baseline concentration of TPH for all boxes was 11.58 mg/L, compared to 5.50 mg/L. BC Labs typically found higher concentrations of TPH than did EBI (Table 4.4). The initial biodegradation in both the planted mesocosms and the control mesocosms was more pronounced in the analyses by BC Labs (Figure 4.5). The analysis by BC Labs also found that the planted and control mesocosm were not significantly different at the end of the experimental run (Table 4.5). There were not enough samples collected to perform statistical analyses on Day 20 or Day 67 samplings for BC Labs.

Table 4.4 Run 3 Comparison Between BC Labs and Cal Poly Lab TPH Results

		EBI	BC Labs	difference
Sampled	Box	mg/L	mg/L	%
Day 0	1	5.72	13	56.0
	2	5.43	12	54.8
	3	5.42	13	58.3
	4	4.84	9.9	51.1
	5	5.27	10	47.3
Day 20	6	3.29	4.5	26.9
	7	1.82	1.8	1.1
	8	NA	4.2	NA
	9	2.72	4.8	43.3
Day 67	1	1.47	1.7	13.5
	4	3	3.8	21.1
	6	2.17	2.3	5.7
	8	2.09	2.4	12.9
Day 109	1	0.46	1.3	64.6
	2	0.56	1.3	56.9
	3	0.83	1.2	30.8
	8	1.27	0.82	35.4
	9	1.12	1.1	1.8

Figure 4.5 BC Labs analysis of TPH Concentrations from during the unfertilized mesocosm experiment (Run 3)

Table 4.5 BC Labs analysis of Run 3 t-test Comparing TPH concentrations of planted mesocosms and controls at each sampling time

Sampling	P-Value (Significant if $P < 0.05$)	Probability Statistically Distinguishable (%)
Day 0	0.54	46
Day 20	NA	NA
Day 67	NA	NA
Day 109	0.52	48

4.1.2 Run 3 Silica Gel Column Fractionation Results

Silica gel column fractionation was done on the initial baseline samples (Day 0) and the final samples (Day 109). Since the TPH concentrations of the planted and control mesocosms in Run 3 were not statistically distinguishable, all the boxes were grouped together for fractionation analysis. [Figure 4.6](#)~~Figure 4.6~~~~Figure 4.6~~ shows chromatograms of the total unfractionated sample of Box 1, followed by the chromatograms of the three fractions: aliphatic, aromatic, and polar. The results of fractionation are summarized in Table 4.6. Initially there was a small aliphatic fraction (0.34 mg TPH/L), a small aromatic fraction (0.489 mg TPH/L) and a large polar fraction (4.9 mg TPH/L). After 109 days the polar fraction biodegraded the most, 89%. In comparison, the aliphatic and aromatic fractions biodegraded 39% and 58%, respectively (Table 4.6).

Figure 4.6 Chromatograms of Day 0 Box 1 of Run 3: Total Sample, Aliphatic Fraction (F1), Aromatic Fraction (F2), and Polar Fraction (F3).

Table 4.6 Run 3 Fractionation Results

Fraction	Initial (Day 0)	Final (Day 109)	Percent Reduction	Degradation Rate
	(TPH mg/L)		%	ug/L-day
Total	5.50	1.04	81.2	41.0
Aliphatic (F1)	0.344	0.210	38.6	1.23
Aromatic (F2)	0.489	0.203	58.5	2.62
Polar (F3)	4.90	0.533	89.1	40.1

The detection limit for TPH analysis was 0.17 mg/L.

4.1.3 Run 3 Nutrient Analysis

Throughout Run 3 there were undetectable amounts of nitrate or ammonia, except for the controls on Day 67 (Table 4.7). This may be an anomaly as there was only one box sampled for nutrient analysis of the control on Day 67. The detection limit for nitrate nitrogen was 0.1 mg-N/L and for ammonia as nitrogen was 0.02 mg-N/L. Orthophosphate was detected in all samples.

Table 4.7 Run 3 Nutrient Analyses

Mesocosms	Sample Day	Nitrate at N	Sulfate	Ortho-Phosphate	Ammonia as N	pH
		mg/L				
Willows	0	ND	10.0	ND	0.09	7.01
	20	ND	22.0	0.22	0.04	7.60
	67	ND	23.0	0.20	ND	7.41
	109	ND	25.3	0.25	ND	7.40
Controls	0	ND	7.1	0.09	0.16	7.19
	20	ND	26.5	0.38	0.03	7.61
	67	0.36	22.0	0.38	ND	7.59
	109	ND	23.0	0.39	0.01	7.55

4.1.4 Run 3 TRF Analysis

Initial samples taken in March 2007 (Day 0) did not show significant differences ($p = 0.68$) between mesocosms with and without willows. TRF data showed differences between the mesocosms with and without willows as early as the first 20 days, that was nearly significantly different ([Figure 4.7](#)). At Day 67 the differences were significant ($p = 0.01$). Not enough samples were collected to show a significant difference at Day 109 ($p = 0.10$), but the trend continued. In fact, when data from samplings at Day 20, Day 67, and Day 109 analyzed together the differences between boxes with and without willows were clearly significant ($p = 0.002$).

Figure 4.7 Run 3 TRF Dendrograms From Each Sampling Day

Red labeled samples (odd numbers) are from boxes without willows.

4.2 Results of Fertilized Experiment (Run 5)

For Run 5 fertilizer was added to all the mesocosms , starting 9 months before the run and continuing throughout the experiment. The experimental run began on 5/2/08 and ran through 9/8/08. There were four sampling dates: 5/5/08 (Day 0), 5/30/08 (Day 25), 7/2/08 (Day 57), and 9/8/08 (Day 109). There was a three-day equilibration period prior to the first sample, designated Day 0. Box 7 leaked after the Day 57 sampling. TPH analysis was completed on all samples. Silica gel column fractionation was completed on Boxes 1, 3 and 9 of Day 0 and Day 109.

4.2.1 Fertilizer amendments

After the end of Run 3, it was determined from the water nutrient analysis and a soil analysis that the nutrient level of the soil was not optimal for plant growth. Neither was it representative of the soil in planted locations at GRP. In addition, in replanting areas at the GRP that have been excavated and refilled with clean sand, fertilizers were used to establish the plants. Thus , all mesocosms were fertilized using Doggett 30-10-10 Foliar Tree Fertilizer, at a rate of one gallon of 2 lbs/100 gallons water mixture to each mesocosm every two weeks beginning on 7/20/07. Occasional nutrient analyses were completed on water samples by BC Labs: the results of these are presented in [Table 4.8](#) [Table 4.8](#) [Table 4.8](#).

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Table 4.8 Fertilizer Amendment Analysis

Mesocosm	Date	Nitrate at N	Sulfate	Ortho-Phosphate	Ammonia as N	pH
		mg/L				
Willows	8/18/2007	ND	X	X	ND	7.45
	9/24/2007	ND	X	0.23	ND	7.47
	2/24/2008	ND	3.7	X	ND	7.11
Controls	8/18/2007	ND	X	X	ND	7.63
	9/24/2007	3.2	X	0.45	ND	8.01
	2/24/2008	38	21.5	X	0.45	7.52

*ND= non-detect; X=not analyzed

4.2.2 Run 5 Water Levels

During Run 5, the planted mesocosms required substantial additions of distilled water to replace the water lost through evapotranspiration, beginning on Day 12 of the experimental run. The average target water table depth is shown in relation to the actual water levels in individual mesocosms for planted and control mesocosms in [Figure 4.8](#) and [Figure 4.9](#). The water levels in the control mesocosms were quite similar to the ideal water table depth, but the water levels in the planted mesocosms differ significantly from the target level near the final sampling. Scheduling problems did not allow for more frequent additions of distilled water to the mesocosms. [Table 4.9](#) shows the volumes added to each mesocosm throughout Run 5.

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Figure 4.8 Run 5 Planted Mesocosms Water Table Depth

Figure 4.9 Run 5 Control Mesocosms Water Table Depth

Table 4.9 Run 5 DI water additions to the mesocosms

Day	BOXES								
	1	3	7	9	2	4	6	8	10
	Gallons DI water added								
12	3.3	3.0	5.0	3.0	6.5	7.0	8.0	8.0	8.0
18	0.0	0.0	0.0	0.0	5.0	5.0	5.0	5.0	5.0
24	0.0	0.0	0.0	0.0	10.0	11.0	10.0	11.0	12.5
31	0.0	0.0	0.0	0.0	10.0	10.0	10.0	10.0	12.5
38	0.0	0.0	0.0	0.0	12.5	13.8	11.3	12.5	12.5
73	2.5	2.5	0.0	2.5	32.5	35.0	30.0	35.0	35.0
86	0.0	0.0	x	0.0	40.0	40.0	37.5	30.0	40.0
92	1.0	1.0	x	2.5	17.5	25.0	15.0	20.0	15.0
120	1.3	0.0	X	2.3	45.0	45.0	45.0	45.0	55.0
125	0.0	0.0	X	0.0	15.0	15.0	10.0	15.0	17.5
Totals gallons	8.0	6.5	5.0	10.3	194.0	206.8	181.8	191.5	213.0

The distance to the water table was used to calculate the difference in volume between actual and expected on the sampling days. The assumption made in these calculations was that the soil profile throughout the mesocosms' was constant and did not vary with height. The justification was that the entire mesocosms profile was sand. The estimated volume in the planted mesocosms varied significantly on all the sampling days (Table 4.10).

Table 4.10 Run 5 difference between target water volumes and actual water volumes

	Sampling Day			
	0	25	57	126
	% difference between actual and target water levels			
Box 1	0.00	2.88	0.85	4.66
Box 2	0.00	23.68	15.35	6.98
Box 3	0.00	0.40	0.84	5.44
Box 4	0.00	25.68	13.49	12.09
Box 6	0.00	22.57	11.63	10.70
Box 7	0.00	-2.86	-2.16	leaked
Box 8	0.00	25.88	-4.46	32.67
Box 9	0.00	2.89	0.00	8.55
Box 10	0.00	28.70	9.95	11.37

4.2.3 Total Petroleum Hydrocarbon Concentrations during Run 5

Table 4.11 shows the TPH concentrations for groundwater in the mesocosms for Run 5. There was an apparent increase in TPH concentration in the planted mesocosms on Day 25. However, the analysis of the water levels showed that the water volume for these mesocosms were 25% lower than expected on average. Thus, the higher TPH concentrations most likely resulted from the DPD being concentrated due to evapotranspiration that was not adequately compensated for with DI water additions.

Table 4.11 Fertilized Experiment TPH results (Run 5)

Day	Group	Box	Duplicate 1	Duplicate 2	Average	Group Ave ± STD Dev.	
			TPH mg/L				
Day 0 (Baseline)	Willows	2	6.86	6.84	6.85	6.84	0.04
		4	6.80	6.92	6.86		
		6	6.68	6.94	6.81		
		8	6.68	6.94	6.81		
		10	6.95	6.84	6.90		
	Controls	1	6.83	6.16	6.49	6.61	0.22
		3	6.56	6.48	6.52		
		7	6.40	6.55	6.47		
		9	6.90	6.98	6.94		
Day 25	Willows	2	6.86	6.86	6.86	6.97	0.18
		4	6.89	7.11	7.00		
		6	7.15	6.59	6.87		
		8	7.12	7.44	7.28		
		10	6.88	6.84	6.86		
	Controls	1	3.36	3.14	3.25	3.36	0.29
		3	3.78	3.73	3.76		
		7	3.18	3.50	3.34		
		9	3.02	3.14	3.08		
Day 57	Willows	2	3.81	4.78	4.30	4.14	1.23
		4	5.31	5.31	5.31		
		6	5.45	2.74	4.09		
		8	2.18	2.18	2.18		
		10	3.62	2.10	2.86		
	Controls	1	2.47	2.47	2.47	2.25	0.73
		3	3.05	2.91	2.98		
		7	1.47	2.25	1.86		
		9	1.32	1.27	1.29		
Day 126	Willows	2	1.12	1.12	1.12	0.92	0.13
		4	0.96	0.95	0.96		
		6	0.89	0.91	0.90		
		8	0.90	0.83	0.87		
		10	0.77	0.75	0.76		
	Controls	1	1.20	1.28	1.24	1.29	0.08
		3	1.24	1.52	1.38		
		7	leaked				
		9	1.31	1.19	1.25		

TPH concentrations for planted and control mesocosm are shown in Figure 4.10. For the controls, rapid initial biodegradation was followed by much slower biodegradation after 60 days. In contrast, this type of kinetics was not seen in the planted mesocosms which exhibited substantial biodegradation after 60 days (Figure 4.10). The overall first order rate constants calculated using semi-log plots were 0.017 day^{-1} ($R^2=0.95$) for the planted mesocosms and 0.012 day^{-1} ($R^2=0.91$) for the control mesocosms, as shown in Figure 4.11.

Figure 4.10 Unadjusted TPH concentrations during the fertilized mesocosm experiment (Run 5)

Figure 4.11 Run 5 Semi-log Plot of TPH Degradation

Although the biodegradation rates decreased over time for the control mesocosms, degradation was still observed through the last sampling phase of Day 57 to Day 126 (Figure 4.11). In comparison, the degradation rates for the planted mesocosms did not decrease as much and actually increased for two of the mesocosms at the end of the experimental run.

The overall amount of biodegradation for each time period for the planted versus control mesocosms is shown in Table 4.12. The overall reduction in TPH for the planted mesocosms was 86%, and 80% for the control mesocosms. The TPH results of the two groups from Run 5 were statistically distinguishable on sampling days 25 and day 126 (Table 4.13). Initially, on Day

20 the control mesocosms demonstrated more degradation of the diluent as TPH ($p=1.00$). In the final sampling there was a statistically significant ($p=0.99$) difference: the planted mesocosms degraded the diluent more than the control mesocosms.

Table 4.12 Run 5 TPH reduction and degradation rates

		Starting TPH	Ending TPH	Percent Reduction	Degradation Rate
	period	mg/L	mg/L	%	ug/L-day
willows	Day 0-25	6.84	6.974	NA	NA
	Day 25-57	6.97	4.14	40.7	60.3
	Day 57-126	4.14	0.92	77.7	76.6
Controls	Day 0-25	6.61	3.36	49.1	162.1
	Day 25-57	3.36	2.25	33.2	23.7
	Day 57-126	2.25	1.29	42.6	22.8

Table 4.13 Run 5 t-test comparing planted and control mesocosms

Sampling	P-Value (Significant if $P < 0.05$)	Probability Statistically Distinguishable (%)
Day 0	0.09	90.9
Day 20	0.00	100.0
Day 67	0.06	94.3
Day 126	0.00	99.5

Hexacosane spikes were used in each sample of Run 5 to evaluate the recovery of sample through the solvent extraction process. The recovery of hexacosane was very high, greater than

95%, for most samples (Hexacosane recovery data included as Appendix D). There were a few samples that had poor recovery of hexacosane; for example, Box 10 duplicate B of Day 57 had a recovery of only 44%. The results described above for Run 5 do not take into account the recovery of hexacosane spikes, or the concentration effects caused by low water volumes. When the TPH concentrations are adjusted for the water volume differences between actual and ideal, and for hexacosane spike recovery, the more realistic biodegradation curves are observed ([Figure 4.12](#)~~Figure 4.12~~) compared to Figure 4.10. The most notable difference is that TPH concentration does not increase at Day 25 for the planted mesocosms as it did for the uncorrected data.

Figure 4.12 Run 5 Adjusted TPH Concentrations for Water Volume and Hexacosane Spike

With the corrected TPH concentrations, the t-test still supports that the two groups are statistically distinguishable on sampling days Day 25, Day 57, and Day 109, as seen in Table 4.14. Also, the overall biodegradation rates did not change appreciably for either group with the inclusion of the water volume and hexacosane spike effects. The overall first order rate for the planted mesocosms was 0.017 day^{-1} and for the control mesocosms was 0.013 day^{-1} (Figure 4.13).

Table 4.14 The T-test of Run 5 with adjusted TPH concentrations for water volumes and hexacosane spikes

Sampling	Significant if $P < 0.05$	Probability Statistically Distinguishable
Day 0	0.16	84.3
Day 20	0.00	99.9
Day 67	0.01	99.2
Day 109	0.01	99.3

Figure 4.13 Run 5 Semi Log Plot of TPH Adjusted for Water Levels and Hexacosane Recovery

The analysis from the external lab (BC Labs) did produce large but consistent variance in the calculated TPH values in the samples. TPH concentrations reported by BC Labs were consistently 50% less, except for the final sampling, than the concentrations determined internally by EBI ([Table 4.15](#)[Table 4.15](#)[Table 4.15](#)). There was one data point, Day 56 Box 6, as noted earlier, in which the hexacosane spike showed Cal Poly with 44% recovery on that sample.

Table 4.15 Run 5 Comparison Between Cal Poly Unadjusted TPH Results and BC Labs TPH Results

Sampled	Box	Cal Poly	BC Labs	difference
		mg/L	mg/L	%
Day 0	2	6.85	4.6	-48.9
	4	6.86	4.2	-63.3
	3	6.52	4.2	-55.3
	7	6.47	3.7	-74.9
Day 25	4	7.00	4.6	-52.2
	10	6.86	4.5	-52.5
	1	3.25	2.1	-54.7
	7	3.34	2.3	-45.3
Day 57	2	4.30	3.0	-43.2
	6	2.18	4.7	53.5
	3	2.98	2.0	-48.8
	7	1.86	1.6	-16.3
Day 126	2	1.10	1.0	-10.0
	8	0.97	1.1	12.1
	3	1.38	1.3	-6.0
	9	1.34	1.7	21.1

4.2.4 Run 5 Silica Gel Column Fractionation Results

Silica gel column fractionation was done on three control mesocosms for the initial baseline samples (Day 0) and the final sampling (Day 126). The results of fractionation are shown in Table 4.16. For the initial sample there was a very small aliphatic fraction (0.19 mg TPH/L) and a small aromatic fraction (0.20 mg TPH/L), just above the detection limit of 0.17 mg/L. After 126 days, the final aliphatic and aromatic fractions were unchanged suggesting that neither degraded. In contrast the largest fraction, the polar fraction (2.62 mg TPH/L), biodegraded 66%.

Table 4.16 Silica Gel Column Fractionation Results

Fraction	Initial (Day 0)	Final (Day 126)	Percent Reduction	Degradation Rate
	(TPH mg/L)		%	ug/L-day
Total	6.61	1.29	80.48	48.78
Aliphatic (F1)	0.19	0.20	NA	NA
Aromatic (F2)	0.20	0.21	NA	NA
Polar (F3)	2.62	0.98	62.52	15.03

4.2.5 Run 5 Nutrient Concentrations

Throughout Run 5 there was fertilizer added to all mesocosms every two weeks. The control mesocosms had high concentrations of nitrate (25-59 mg-N/L), and orthophosphate concentrations above 0.4 mg/L (Table 4.17), indicating that adequate nutrients were available in the unplanted mesocosms.. In contrast, the willow planted mesocosms had no detectable nitrate except for a very low concentration on Day 25, and ammonia concentrations were very low (Table 4.17). The detection limit for nitrate was 0.1 mg-N/L and for ammonia was 0.02 mg-N/L. In addition, the willow-planted mesocosms had no detectable orthophosphate on Day 0 and Day 126, and orthophosphate was less than half that of the control mesocosms on Day 57. Sulfate was also limited in the willow-planted mesocosms compared to the controls (Table 4.17). These results show that the groundwater in the willow-planted mesocosms was severely nutrient limited.

Table 4.17 Run 5 Nutrient Analyses

Mesocosms	Sample Day	Nitrate as N	Nitrite as N	Sulfate	Ortho-Phosphate	Ammonia as N	pH
Willows		mg/L					
	0	ND	ND	20	ND	0.06	7.08
	25	0.31	ND	ND	X	0.0265	7.155
	57	ND	ND	1.75	0.1975	0.0455	6.99
	126	ND	ND	ND	ND	ND	7.02
Controls	0	35	0.16	31.5	0.44	0.1695	7.41
	25	25	ND	32.5	X	ND	7.63
	57	49	0.0355	32.5	0.405	0.0275	7.47
	126	59	ND	30.5	0.46	0.0305	7.58

ND=non-detect; X= Analysis not done

4.2.6 Terminal Restriction Fragmentation Analysis of Run 5

TRF patterns were compared for mesocosms with and without willows to observe differences in microbial communities within the different mesocosms over time. Dendrograms were produced using Bray-Curtis similarity grouped by group average. The dendrograms highlighted the similarities and differences observed between the mesocosms. The Day 0 and Day 25 dendrograms, seen in [Figure 4.14](#) and [Figure 4.15](#), show that the microbial communities were statistically different for the planted and control mesocosms. There was a change in the Day 57 dendrogram, such that the two groups were not statistically distinguishable ([Figure 4.16](#)). However, for final samplings the microbial communities of the planted and control mesocosms were statically distinguishable ($p=0.001$) (Figure 4.17). This shows that the willows had a dramatic influence on the microbial community in the mesocosms groundwater.

TRF analysis was also done for soil samples from each mesocosm at the conclusion of Run 5. The microbial community of the rhizosphere in the planted willows was markedly different than the soil community in the control mesocosm (Figure 4.17, $p=0.008$). For the control mesocosms, the soil microbial community was also very different than the ground water microbial community (Figure 4.17). The soil microbial community also mapped out differently than the groundwater microbial community for the planted mesocosm, although not as distinctly as for the controls.

Figure 4.14 Run 5, Day 0 TRF Dendrogram

Figure 4.15 Run 5, Day 25 TRF Dendrogram

Figure 4.16 Run 5, Day 57 TRF Dendrogram

Figure 4.17 Run 5 Day 126 Dendrograms for Groundwater, Soil, and Combined Soil and Groundwater.

4.2.7 Microtox® Analysis of Run 5

Microtox® toxicity was determined for all the samples from Run 5 to investigate potential changes in toxicity of the dissolved diluent during biodegradation. The Microtox® toxicity is reported as EC_{50} , which is the concentration at which a sample induces a 50% reduction in bioluminescence. Thus, a high EC_{50} value represents a low toxicity because it indicates that a high concentration of sample is required to cause 50% inhibition of the indicator bacteria. Microtox® toxicity decreased significantly during Run 5 for both planted and control mesocosms (Table 4.18). Toxicity decreased more rapidly initially in the control mesocosms, which is consistent with TPH concentrations.

Table 4.18 Run 5 Microtox® Results

Day	Group	Box	Effect %	EC ₅₀	EC ₅₀ Group Ave ± STD Dev.	
Day 0	Willows	2	99.55	17.03	16.8	2.2
		4	99.90	20.21		
		6	99.86	13.19		
		8	X	X		
		10	X	X		
	Controls	1	99.76	24.44	24.8	6.0
		3	99.52	31.58		
		7	99.83	26.09		
		9	96.81	17.03		
Day 25	Willows	2	91.54	26.70	23.1	8.8
		4	99.86	25.12		
		6	88.36	34.63		
		8	99.01	17.22		
		10	85.91	11.93		
	Controls	1	25.74 ¹	*	69.5	NA
		3	55.42	74.04		
		7	99.90	64.88		
		9	26.40 ¹	*		
Day 57	Willows	2	34.20	121.10	92.5	27.2
		4	48.84	83.20		
		6	63.17	52.32		
		8	45.79	92.27		
		10	38.41	113.30		
	Controls	1	Hormesis	*	284.3	*
		3	8.88	*		
		7	18.88	284.30		
		9	6.29	*		
Day 126	Willows	2	36.56	104.76	105.5	4.4
		4	5.154 ¹	*		
		6	48.45	101.60		
		8	42.36	110.26		
		10	Hormesis	*		
	Controls	1	37.95 ¹	*	ND*	ND*
		3	10.03 ¹	*		
		7	X	X		
		9	28.14	*		

X=not analyzed, *= unable to calculate, ¹= effect % at 41% concentration

4.2.8 Dissolved Oxygen Results

Dissolved oxygen concentrations in the mesocosms were measured with a DO meter on the Day 0 and Day 25 samplings dates. DO was not measured with a probe on Day 57 or Day 126 due to problems with the DO probe. On Days 0 and 25 the DO concentration was slightly higher for the willows (1.38 ± 0.33 mg/L and 2.73 ± 0.26 mg/L) than the controls (2.02 ± 0.37 mg/L and 3.13 ± 0.62 mg/L) (Table 4.19). A t-test was completed on the DO results from Day 0 and Day 25; the results are seen in Table 4.20. The two groups are significantly different ($p < 0.05$) in the DO levels.

Table 4.19 Run 5 DO Results for Day 0 and 25

	Box	Day 0	Day 25
		DO mg/L	
Willows	2	1.29	2.4
	4	1.32	3.02
	6	1.8	2.68
	8	1.57	X
	10	0.93	2.8
Controls	1	1.84	3.85
	3	1.61	2.58
	7	2.44	3.44
	9	2.2	2.66

Table 4.20 Run 5 T-test on DO Results from Day 0 and Day 25

Sampling	Mesocosm	Average	Standard Deviation	P-Value Significant if P<0.05	Probability Statistically Distinguishable (%)
Day 0	Willows	1.38	0.33	0.03	97.2
	Controls	2.02	0.37		
Day 25	Willows	2.73	0.26	0.04	96.2
	Controls	3.13	0.62		

On the final sampling (Day 126), an additional water sample was taken from each box and DO was analyzed by the ferrous iron test which determines if there is enough oxygen to convert iron from Fe^{2+} to Fe^{3+} . Thus, dissolved ferrous iron is an indicator of anaerobic conditions. In the planted mesocosms there was not enough oxygen present to convert all the iron, and therefore the boxes contained low DO levels. In contrast, the water in the control mesocosms contained enough oxygen to convert all the ferrous iron. Table 4.21 presents the Ferrous Iron results. The detection limit was $0.1 \text{ Fe}^{2+} \text{ mg/L}$. This confirms that the control boxes were operating in aerobic conditions.

Table 4.21 Run 5 Day 126 Ferrous Iron Analysis

	Box	Fe ²⁺ mg/L
willows	2	5.12
	4	4.96
	6	5.6
	8	1.49
	10	2.89
Controls	1	ND
	3	ND
	9	ND

ND= non-detect

4.3 Final Willow Biomass in the Mesocosms

The average weight of the oven-dried foliage and root biomass from the planted mesocosms was 4.45 kg and 7.83 kg, respectively (Table 4.22). The control mesocosms were devoid of any plant biomass.

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Table 4.22 Final root and foliage biomass of the willow trees

Box	weight foliage (kg)	weight roots (kg)
2	4.45	8.05
4	4.54	NA
6	4.68	7.56
8	3.75	7.24
10	4.81	8.45
average	4.45	7.83

4.4 Octanol-Water Coefficient (K_{ow}) of Diluent and Dissolved Phase Diluent

The dissolved phase diluent had a significant amount of TPH in the water phase, shown in

[Table 4.23](#)~~Table 4.23~~~~Table 4.23~~. The log K_{ow} was found to be 1.42 for diluent from the source zone and only 0.14 for DPD ([Table 4.23](#)~~Table 4.23~~~~Table 4.23~~). The resulting K_{ow}

values for the dissolved phase diluent versus the diluent were different by a factor of 20.

These values were much lower than the values reported for typical petroleum hydrocarbons

(see discussion chapter). These results show that weathered DPD is highly polar as

confirmed by column fractionation described above.

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Table 4.23 Log K_{ow} Result for Diluent and Dissolved Phase Diluent

Product	Samples	Total TPH (mg)	TPH (mg) in water phase	TPH (mg) in octanol phase	Log K	Average Log K
Pure Diluent	Diluent 1	102.3	3.73	98.57	1.421	1.42
	Diluent 2	100.0	3.62	96.38	1.425	
Dissolved Phase Diluent	initial 4-30-08	14.25	6.07	8.18	0.129	0.143
	initial 5-1-08	16.55	6.79	9.76	0.157	

CHAPTER 5 DISCUSSION

Run 5 was the first experimental operation of the mesocosms in which the trees were actively growing with significant amounts of biomass. The average final dry-weight biomass of the willow trees was 4.4 kg of foliage and 7.8 kg roots per planted mesocosm. Thus, the dry mass of roots weighed 43% more than the foliage, not including the fine roots which were not collectable. The soil in the planted mesocosms was inundated with roots, providing increased evidence that the plants were healthy. Before fertilizing, the willow trees in the planted mesocosms had less than 150 leaves per willow. The change in the health of the Arroyo Willows corresponded with the first time the planted mesocosms statistically degraded more diluent than the soil-only controls over the long run. The soil-only controls demonstrated a quicker initial biodegradation of the petroleum compounds than the planted mesocosms. However, for longer times, the biodegradation was faster with the willow trees. The long-term improvement of biodegradation was statistically significant with 95% confidence. Thus planted mesocosms had an initially slower but more complete biodegradation of the petroleum compounds. These results are similar to the results reported in the literature by Kaimi et al. (2006) and Chanieau (2000). Also, similar results were observed in earlier laboratory studies with GRP diluent (Hoffman, 2003; Martin, 2003).

A separate signal of the increased vitality of the willow trees in the planted mesocosms was the significant increase in water required to maintain the simulated water table of the mesocosms. In Crossley's (2006) experimental operation in Run 2 of the mesocosms, the only water addition was prior to the final sampling at 105 days to the planted mesocosms.

The volume added was up to 10 L (2.3 gallons) per mesocosm. In comparison, in the fertilized run (Run 5) the planted mesocosms required distilled water additions starting on the 12th day of the experimental run. Each planted mesocosm received an average of 197 gallons of distilled water, in comparison with the control mesocosms which each received only 8 gallons of distilled water over the entire 126 day run.

For Run 3 with unhealthy willow trees, the first-order biodegradation rate constant was actually slightly lower for the planted mesocosms compared to the controls (Table 5.1). However, in Run 5 with healthy willows as described above, the first-order biodegradation rate constant was higher (0.017 day^{-1}) for the planted mesocosms than the controls (0.013 day^{-1}) (Table 5.1).

Table 5.1 First-Order Biodegradation Rate Constants For Run 3 and Run 5

	First-Order Rate Constant (Day^{-1})	
	Controls	Willows
Run 3 (unhealthy willows)	0.015	0.013
Run 5 (healthy willows)	0.013	0.017

The first-order rate constants observed in these experiments were lower than those in previous laboratory microcosms experiment, which were typically 0.023 day^{-1} (Dryer, 2005; Cunningham, 2003). However, most of the earlier microcosms were observed for shorter time periods when biodegradation rates are greater. Previous field mesocosm

studies by Crossley (2006) found an average first-order biodegradation rate of 0.015 day^{-1} which is similar to the controls without willow trees in this study.

In the unfertilized run (Run 3) the roots of the willow trees may have been unable to enhance the microbial population or select for petroleum hydrocarbon degraders in the rhizosphere, due to the conditions under which these plants were growing. Insufficient nutrients probably led to stress in the plants, visible by the lack of growth. Many plants produce ethylene, which is detrimental after germination, because it inhibits root growth (Glick et al., 1998; Gerhardt et al., 2008). Ethylene would not have shown up in the GC/MS analyses, so it is not known if any ethylene was produced.

The TRF patterns in the unfertilized run (Run 3) did show that the control and the planted mesocosms were different after the Day 20 samples and Day 67 samples. This trend appeared to continue to Day 109, but lack of sufficient samples resulted in limited statistical analysis. The TRF supported the conclusion that the trees altered the microbial populations in Run 3, but the effect may not have been substantial or beneficial enough to alter long-term biodegradation rates.

In the fertilized experiment (Run 5) the TRF patterns were statistically different for planted versus unplanted mesocosms on the first sampling at Day 0. This may have been due to the effect that the healthy trees had on the microbial population before the start of Run 5. In the TRF results for Run 5 of the Day 57 sampling, the TRF patterns showed similarity between the two groups of mesocosms. In a different study of microbial

communities in a phytoremediation experiment with planted versus soil-only microcosms, the microbial populations were the most similar at 90 days (Kaimi *et al.*, 2006). The differences between the microbial communities increased from then until the end of the study, with the planted microcosms having a larger and more active community (Kaimi *et al.*, 2006). The mesocosm's microbial populations at the end of the experiment were statistically different ($p=0.008$) between the planted mesocosms and the soil-controls. The effect of plants on the microbial community suggests the possibility that plants enhanced the rhizodial biodegradation.

The dissolved oxygen concentrations were lower in the planted mesocosms than the soil-only controls, similar to the few results of dissolved oxygen reported in Crossley's (2006) previous experimental run. In Run 5 the DO levels were significantly lower in the planted mesocosms. There is a recent body of literature suggesting that plants produce and provide oxygen to the rhizosphere (Soda *et al.*, 2007; Nikolausz *et al.*, 2008). However, this does not seem to be the case in the present study, unless the plants are providing more oxygen but the microbial population is consuming it. Even though the planted mesocosms DO levels were lower than the controls in Run 5, they degraded more dissolved phase diluent over time. It would have been valuable to have 24-hour time course readings of DO levels to determine if the low DO concentrations measured were due to the diurnal demand from the trees. It may have been that during the morning when samples were taken, the DO levels were low.

The nutrient analysis in the unfertilized experiment (Run 3) suggests that there was limited availability of nitrogen in any of the mesocosms as either nitrate or ammonia. There were low but consistent amounts of sulfate and ortho-phosphate. Thus nitrogen was the limiting nutrient in Run 3. The nutrient results for Run 5 differed in that the control mesocosms had increasing amounts of nitrate. The controls also had consistent amounts of sulfate and orthophosphate throughout Run 5. However, the planted mesocosms in Run 5 had non-detectable concentrations of nitrate and non-detectable or very low concentrations of all other nutrients. The fertilizer additions were being consumed by either plant or microbial activity in the planted mesocosms. The disappearance of the sulfate, not seen in Run 3, may suggest activity of facultative anaerobic microbes. It would require more microbial analysis to determine if the planted mesocosms were supporting a facultative microbial population. Such facultative microbial populations would be more apt to survive in changing DO concentrations (Nikolausz et al., 2008).

Even though nutrient additions had a dramatic effect on the health of the trees, the plants still may have been nutrient limited, as there was no build up of nutrients in the planted mesocosms. In most stands of trees, a nitrogen cycle is created within the stand due to the detritus of the leaves, however the mesocosms were not established long enough to have such a cycle. However, the nutrient accumulation in the controls in Run 5 did not enhance the biodegradation and did not appear to hinder it in comparison with other natural attenuation operations of the mesocosms. Although, the first order rate constant was lower in the nutrient amended control mesocosms in Run 5 than the unamended soil

conditions of the control mesocosms of Run 3, but the percent degradation of DPD was similar in these runs.

The octanol-water coefficient of the diluent from the source zone of Well D2-1 and the dissolved phase diluent from Well D2-1 was different than that of typical petroleum compounds, which are reported to have $\log K_{ow} > 3$ (Gustafson et al., 1997). Contaminants with a $\log K_{ow} > 3$ are generally not absorbed, translocated or metabolized by plants, instead they generally adsorb to the root surfaces (Schoor et al., 1995; Alkorta and Garbisu, 2001). Contaminants with a $\log K_{ow}$ in the range of from 1 to 3 were found to be readily absorbed by trees (Briggs et al., 1982). Hydrophilic contaminants with a $\log K_{ow} < 1$ are absorbed, translocated, or metabolized by plants, and their absorption by trees is controlled by water influx into the plant (Cunningham and Berti, 1993). The experimentally determined $\log K_{ow}$ of the diluent and dissolved phase diluent were $\log K_{ow} = 1.42$ and $K_{ow} = 0.14$, respectively. The dissolved phase diluent is largely composed of polar compounds as confirmed by column fractionation and is hydrophilic in nature, and therefore, it has a very different $\log K_{ow}$ than the hydrophobic pure product. Thus another possible mechanism for the planted mesocosms, increased degradation of dissolved phase diluent may have been that plants were able to absorb the DPD.

There are several possible mechanisms of the observed enhanced biodegradation seen in Run 5. It was shown by TRF analysis that the plants altered the microbial populations, which suggests that a possible mechanism was rhizodegradation by altering the rhizodial community. The low $\log K_{ow}$ of the DPD suggests the possibility of plant uptake of

DPD. Elliot's (2002) research showed limited phytovolatilization of DPD by willows. Thus plant uptake may have resulted in degradation within the plant, rather than evapotranspiration. This suggests that a possible mechanism was phytodegradation. Neither of the proposed mechanisms could be confirmed by the analyses of this experiment. Further research is needed to analyze for root exudates that would point to a mechanism for enhanced rhizodegradation.

Column fractionation of TPH in the control mesocosms allowed for the comparison of biodegradation rates of the polar, aliphatic, and aromatic constituents in diluent for the first time. All previous attempts in EBI studies were thwarted by a lack of sufficient TPH in the aliphatic and aromatic fractions of the starting groundwater (Mick, 2006, Rastegarzadeh, 2007). In Run 3 of this experiment, the groundwater had measurable amounts of the aliphatic, aromatic and polar fractions while for Run 5 the aliphatic and aromatic fractions were very small. In Run 3 all fractions degraded, however the polar fraction of DPD biodegraded more readily than the aliphatic or aromatic fractions. Although this suggests some recalcitrance of the dissolved aliphatic and aromatic compounds these fractions typically have not been detected in down-gradient monitoring wells. The lack of the aromatic and aliphatic fractions were seen in this experiment in Run 5 and in Mick's (2006) study. The finding of the biodegradability of the polar fraction was a positive finding for the GRP, as previous work and the present study have found that DPD is mostly polar in nature.

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

Healthy willow trees diminished initial DPD biodegradation rates (0-57 days), but enhanced final biodegradation, resulting in lower final TPH concentrations compared to controls without trees. Microtox[®] toxicity of the diluent-contaminated groundwater decreased as TPH concentrations decreased for both the planted and unplanted mesocosms. The implications of this study are that healthy willow trees do enhance biodegradation of DPD over time under conditions at the GRP as compared to biodegradation by natural attenuation. Although the effect of the trees was not dramatic, there is probably no harm in planting trees at the site since they did not contribute to increased Microtox[®] toxicity. The planting of willow trees at the site would best be done in strategic locations. The most practical locations to plant trees would be in sensitive locations where excavation and other invasive techniques have serious drawbacks or as a polishing step after excavation. In fact, revegetation would be important after excavation for ecological restoration, so it would be advantageous to plant native willow trees regardless of their enhancement of diluent biodegradation. For any plantings on-site, it would be necessary to maintain adequate nutrition of the planted trees. Additional locations for the planting of willow trees on the GRP would possibly be down gradient from source zones where the water table is near the surface. It may be possible to show an effect of the trees with monitoring at these locations.

Another important finding of this research was that the polar compounds in dissolved-phase diluent biodegraded faster than the compounds in the aliphatic or aromatic

fractions. In some cases the biodegradation of these aliphatic and aromatic compounds was very slow, but their concentrations were very low. Thus recalcitrant aliphatic and aromatic compounds are not likely to be a problem because of their low concentrations and the fact that they are typically not observed far down gradient from source zones..

Comment [YN1]: This paragraph should be combined with the third paragraph

The mechanisms behind the enhanced final biodegradation of the planted mesocosms can not be identified definitively from this study, but chemical analyses, microbial assays as well as DO and nutrient monitoring shed some light on potential mechanisms. It is likely that multiple concurrent effects of plants contribute to phytoremediation. The log K_{ow} determined for free phase diluent and DPD were 1.42 and 0.14, respectively. These log K_{ow} values are considerably lower than expected for most petroleum compounds which typically exhibit log K_{ow} values greater than three (Gustafson *et al.*, 1997). If the log K_{ow} was greater than three, then the DPD compounds would be unlikely to be transported across the root membrane. However, the very low K_{ow} values determined in the current work suggest that direct plant uptake of polar DPD compounds is a possibility.

The work that Elliot (2002) completed on the phytovolatilization of diluent by willows reduces the likelihood that the healthy trees are removing the diluent from the groundwater through phytovolatilization. Phytodegradation and rhizodegradation are thus the two most probable mechanisms by which diluent biodegradation is enhanced by the willows. Additional research would be needed to define the operative mechanisms for plant-enhanced biodegradation under these conditions. Finding the mechanisms by which the Arroyo Willows reduced the petroleum hydrocarbons from the groundwater

may allow the information gathered from the numerous research projects on the GRP diluent to be applied to other sites with weathered petroleum.

Another important finding of this research was that the polar fraction of DPD biodegrades much more readily than the aliphatic or aromatic fractions. Previous EBI attempts to measure this were unsuccessful because the starting groundwater always had very low concentrations of the aliphatic and aromatic fractions. The consistent biodegradation of the polar fraction of DPD is an important finding because most of the DPD is thought to be polar in nature. This work confirms that the polar compounds are amenable to natural attenuation under GRP site conditions.

It is significant to note that biodegradation in the unplanted controls followed typical rapid initial degradation followed by a slower degradation rate. This phenomenon has been referred to as “hockey-stick” kinetics. There are two commonly listed explanations for the diminishing biodegradation rates over time: one is sequestration of contaminants in the soil structure which reduces their bioavailability to microorganisms (Huesemann, 1997), and the second is that the easily biodegraded compounds are consumed initially, leaving the more recalcitrant compounds (Noncentini et al., 2000). The healthy planted mesocosms biodegradation kinetics followed first-order kinetics, without any reduction in the first-order rate constant over time as previously observed “hockey stick” kinetics. The plants thus appear to help in the biodegradation of the more recalcitrant compounds possibly by supplying microorganisms nutrients that facilitate this biodegradation.

The results of the TRF analyses showed that the microbial communities in the planted mesocosms were statistically different than that in the unplanted controls. This suggests a role of the willows in affecting the bacteria in the rhizosphere, which could possibly have contributed to the enhanced biodegradation observed over the long-term.

Future Recommendations: It would be valuable to continue monitoring the field experiments to observe the plumes and determine if any effect of phytoremediation on the DPD concentrations can be observed under field conditions. It may take time and be difficult to observe the result if the plume continues to shift.

To determine the mechanism(s) responsible for the biodegradation of the DPD more definitively, lab experiments using radiotracer study could be carried out. ^{14}C labeled compounds could be used to study the effect of willows on specific petroleum compounds and determine if these compounds were taken up by the plants, adsorbed to roots or mineralized into carbon dioxide. However, this would not necessarily apply to DPD, which is a complex mixture of weathered compounds since C^{14} works on only individually labeled compounds. A radical possibility would be to use neutron-activation to prepare radiolabelled diluent. This labeled diluent could be dissolved into water and used in microcosm experiments to observe the fate of the constituent compounds of DPD.

It would be useful in a future study to determine the adsorption coefficient of DPD to the GRP site sand. This would allow for the determination of the retardation, or velocity of DPD, in groundwater. It would be valuable to learn the adsorption coefficients for both

the initial DPD and the DPD after phytoremediation, as well as of each of the aliphatic, aromatic and polar fractions. Knowing these coefficients would allow for more specific and accurate predictions of contaminant spread, before and after phytoremediation.

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Appendix A EPA METHOD 3510C

SEPARATORY FUNNEL LIQUID-LIQUID EXTRACTION

1.0 SCOPE AND APPLICATION

1.1 This method describes a procedure for isolating organic compounds from aqueous samples. The method also describes concentration techniques suitable for preparing the extract for the appropriate determinative methods described in Section 4.3 of Chapter Four.

1.2 This method is applicable to the isolation and concentration of water-insoluble and slightly water-soluble organics in preparation for a variety of chromatographic procedures.

1.3 This method is restricted to use by or under the supervision of trained analysts. Each analyst must demonstrate the ability to generate acceptable results with this method.

2.0 SUMMARY OF METHOD

2.1 A measured volume of sample, usually 1 liter, at a specified pH (see Table 1), is serially extracted with methylene chloride using a separatory funnel.

2.2 The extract is dried, concentrated (if necessary), and, as necessary, exchanged into a solvent compatible with the cleanup or determinative method to be used (see Table 1 for appropriate exchange solvents).

3.0 INTERFERENCES

3.1 Refer to Method 3500.

3.2 The decomposition of some analytes has been demonstrated under basic extraction conditions. Organochlorine pesticides may dechlorinate, phthalate esters may exchange, and phenols may react to form tannates. These reactions increase with increasing pH, and are decreased by the shorter reaction times available in Method 3510. Method 3510 is preferred over Method 3520 for the analysis of these classes of compounds. However, the recovery of phenols may be optimized by using Method 3520, and performing the initial extraction at the acid pH.

4.0 APPARATUS AND MATERIALS

4.1 Separatory funnel - 2-liter, with polytetrafluoroethylene (PTFE) stopcock.

4.2 Drying column - 20 mm ID Pyrex® chromatographic column with Pyrex® glass wool at bottom and a PTFE stopcock.

NOTE: Fritted glass discs are difficult to decontaminate after highly contaminated extracts have been passed through. Columns without frits may be purchased.

Use a small pad of Pyrex® glass wool to retain the adsorbent. Prewash the glass wool pad with 50 mL of acetone followed by 50 mL of elution solvent prior to packing the column with adsorbent.

4.3 Kuderna-Danish (K-D) apparatus.

4.3.1 Concentrator tube - 10-mL, graduated (Kontes K-570050-1025 or equivalent). A ground-glass stopper is used to prevent evaporation of extracts.

4.3.2 Evaporation flask - 500-mL (Kontes K-570001-500 or equivalent). Attach to concentrator tube with springs, clamps, or equivalent.

4.3.3 Snyder column - Three-ball macro (Kontes K-503000-0121 or equivalent).

4.3.4 Snyder column - Two-ball micro (Kontes K-569001-0219 or equivalent).

4.3.5 Springs - 1/2 inch (Kontes K-662750 or equivalent).

NOTE: The following glassware is recommended for the purpose of solvent recovery during the concentration procedures requiring the use of Kuderna-Danish evaporative concentrators. Incorporation of this apparatus may be required by State or local municipality regulations that govern air emissions of volatile organics. EPA recommends the incorporation of this type of reclamation system as a method to implement an emissions reduction program. Solvent recovery is a means to conform with waste minimization and pollution prevention initiatives.

4.4 Solvent vapor recovery system (Kontes K-545000-1006 or K-547300-0000, Ace Glass 6614-30, or equivalent).

4.5 Boiling chips - Solvent-extracted, approximately 10/40 mesh (silicon carbide or equivalent).

4.6 Water bath - Heated, with concentric ring cover, capable of temperature control ($\pm$ 5EC). The bath should be used in a hood.

4.7 Vials - 2-mL, glass with PTFE-lined screw-caps or crimp tops.

4.8 pH indicator paper - pH range including the desired extraction pH.

4.9 Erlenmeyer flask - 250-mL.

4.10 Syringe - 5-mL.

4.11 Graduated cylinder - 1-liter.

5.0 REAGENTS

5.1 Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination. Reagents should be stored in glass to prevent the leaching of contaminants from plastic containers.

5.2 Organic-free reagent water - All references to water in this method refer to organic-free reagent water, as defined in Chapter One.

5.3 Sodium hydroxide solution (10 N), NaOH. Dissolve 40 g NaOH in organic-free reagent water and dilute to 100 mL. Other concentrations of hydroxide solutions may be used to adjust sample pH, provided that the volume added does not appreciably change (e.g., <1%) the total sample volume.

5.4 Sodium sulfate (granular, anhydrous), Na₂SO₄. Purify by heating to 400°C for 4 hours in a shallow tray, or by precleaning the sodium sulfate with methylene chloride. If the sodium sulfate is precleaned with methylene chloride, a method blank must be analyzed, demonstrating that there is no interference from the sodium sulfate. Other concentrations of acid solutions may be used to adjust sample pH, provided that the volume added does not appreciably change (e.g., <1%) the total sample volume.

5.5 Sulfuric acid solution (1:1 v/v), H₂SO₄. Slowly add 50 mL of H₂SO₄ (sp. gr. 1.84) to 50 mL of organic-free reagent water.

5.6 Extraction/exchange solvents - All solvents must be pesticide quality or equivalent.

5.6.1 Methylene chloride, CH₂Cl₂, boiling point 39.6°C.

5.6.2 Hexane, C₆H₁₄, boiling point 68.7°C.

5.6.3 2-Propanol, CH₃CH(OH)CH₃, boiling point 82.3°C.

5.6.4 Cyclohexane, C₆H₁₂, boiling point 80.7°C.

5.6.5 Acetonitrile, CH₃CN, boiling point 81.6°C.

6.0 SAMPLE COLLECTION, PRESERVATION, AND HANDLING

See the introductory material to this chapter, Organic Analytes, Sect. 4.1.

7.0 PROCEDURE

7.1 Using a 1-liter graduated cylinder, measure 1 liter (nominal) of sample. Alternatively, if the entire contents of the sample bottle are to be extracted, mark the level of sample on the outside of the bottle. If high analyte concentrations are anticipated, a smaller sample volume may be taken and diluted to 1-L with organic-free reagent water, or samples may be collected in smaller sample bottles and the whole sample used.

7.2 Pipet 1.0 mL of the surrogate spiking solution into each sample in the graduated cylinder (or sample bottle) and mix well. (See Method 3500 and the determinative method to be used for details on the surrogate standard solution and matrix spiking solution).

7.2.1 For the sample in each batch (see Chapter One) selected for use as a matrix spike sample, add 1.0 mL of the matrix spiking standard.

7.2.2 If Method 3640, Gel-Permeation Cleanup, is to be employed, add twice the volume of the surrogate spiking solution and the matrix spiking standard, since half of the extract is not recovered from the GPC apparatus. (Alternatively, use 1.0 mL of the spiking solutions and concentrate the final extract to half the normal volume, e.g., 0.5 mL instead of 1.0 mL).

7.3 Check the pH of the sample with wide-range pH paper and adjust the pH, if necessary, to the pH indicated in Table 1, using 1:1 (v/v) sulfuric acid or 10 N sodium hydroxide. Lesser strengths of acid or base solution may be employed, provided that they do not result in a significant change (<1%) in the volume of sample extracted (see Secs. 5.3 and 5.5).

7.4 Quantitatively transfer the sample from the graduated cylinder (or sample bottle) to the separatory funnel. Use 60 mL of methylene chloride to rinse the cylinder (or bottle) and transfer this rinse solvent to the separatory funnel. If the sample was transferred directly from the sample bottle, refill the bottle to the mark made in Sec. 7.1 with water and then measure the volume of sample that was in the bottle.

7.5 Seal and shake the separatory funnel vigorously for 1 - 2 minutes with periodic venting to release excess pressure. Alternatively, pour the exchange solvent into the top of the Snyder column while the concentrator remains on the water bath in Sec. 7.11.4. NOTE: Methylene chloride creates excessive pressure very rapidly; therefore, initial venting should be done immediately after the separatory funnel has been sealed and shaken once. The separatory funnel should be vented into a hood to avoid exposure of the analyst to solvent vapors.

7.6 Allow the organic layer to separate from the water phase for a minimum of 10 minutes. If the emulsion interface between layers is more than one-third the size of the solvent layer, the analyst must employ mechanical techniques to complete the phase separation. The optimum technique depends upon the sample and may include stirring, filtration of the emulsion through glass wool, centrifugation, or other physical methods. Collect the solvent extract in an Erlenmeyer flask. If the emulsion cannot be broken (recovery of < 80% of the methylene chloride, corrected for the water solubility of methylene chloride), transfer the sample, solvent, and emulsion into the extraction chamber of a continuous extractor and proceed as described in Method 3520, Continuous Liquid-Liquid Extraction.

7.7 Repeat the extraction two more times using fresh portions of solvent (Secs. 7.2 through 7.5). Combine the three solvent extracts.

7.8 If further pH adjustment and extraction is required, adjust the pH of the aqueous phase to the desired pH indicated in Table 1. Serially extract three times with 60 mL of

methylene chloride, as outlined in Secs. 7.2 through 7.5. Collect and combine the extracts and label the combined extract appropriately.

7.9 If performing GC/MS analysis (Method 8270), the acid/neutral and base extracts may be combined prior to concentration. However, in some situations, separate concentration and analysis of the acid/neutral and base extracts may be preferable (e.g. if for regulatory purposes the presence or absence of specific acid/neutral or base compounds at low concentrations must be determined, separate extract analyses may be warranted).

7.10 Perform the concentration (if necessary) using the Kuderna-Danish Technique (Secs.

7.11.1 through 7.11.6).

7.11 K-D technique

7.11.1 Assemble a Kuderna-Danish (K-D) concentrator (Sec. 4.3) by attaching a 10-mL concentrator tube to a 500-mL evaporation flask.

7.11.2 Attach the solvent vapor recovery glassware (condenser and collection device) (Sec. 4.4) to the Snyder column of the K-D apparatus following manufacturer's instructions.

7.11.3 Dry the extract by passing it through a drying column containing about 10 cm of anhydrous sodium sulfate. Collect the dried extract in a K-D concentrator. Rinse the Erlenmeyer flask, which contained the solvent extract, with 20 - 30 mL of methylene chloride and add it to the column to complete the quantitative transfer.

7.11.4 Add one or two clean boiling chips to the flask and attach a three-ball Snyder column. Prewet the Snyder column by adding about 1 mL of methylene chloride to the top of the column. Place the K-D apparatus on a hot water bath (15 - 20°C above the boiling point of the solvent) so that the concentrator tube is partially immersed in the hot water and the entire lower rounded surface of the flask is bathed with hot vapor. Adjust the vertical position of the apparatus and the water temperature as required to complete the concentration in 10 - 20 minutes. At the proper rate of distillation the balls of the column will actively chatter, but the chambers will not flood. When the apparent volume of liquid reaches 1 mL, remove the K-D apparatus from the water bath and allow it to drain and cool for at least 10 minutes.

7.11.5 If a solvent exchange is required (as indicated in Table 1), momentarily remove the Snyder column, add 50 mL of the exchange solvent, a new boiling chip, and reattach the Snyder column. Alternatively, pour the exchange solvent into the top of the Snyder column while the concentrator remains on the water bath in Sec. 7.11.4. Concentrate the extract, as described in Sec. 7.11.4, raising the temperature of the water bath, if necessary, to maintain proper distillation.

7.11.6 Remove the Snyder column and rinse the flask and its lower joints into the concentrator tube with 1 - 2 mL of methylene chloride or exchange solvent. If sulfur

crystals are a problem, proceed to Method 3660 for cleanup. The extract may be further concentrated by using the technique outlined in Sec. 7.12 or adjusted to 10.0 mL with the solvent last used.

7.12 If further concentration is indicated in Table 1, either the micro-Snyder column technique (7.12.1) or nitrogen blowdown technique (7.12.2) is used to adjust the extract to the final volume required.

7.12.1 Micro-Snyder column technique

If further concentration is indicated in Table 1, add another clean boiling chip to the concentrator tube and attach a two-ball micro-Snyder column. Prewet the column by adding 0.5 mL of methylene chloride or exchange solvent to the top of the column. Place the K-D apparatus in a hot water bath so that the concentrator tube is partially immersed in the hot water. Adjust the vertical position of the apparatus and the water temperature, as required, to complete the concentration in 5 - 10 minutes. At the proper rate of distillation the balls of the column will actively chatter, but the chambers will not flood. When the apparent volume of liquid reaches 0.5 mL, remove the K-D apparatus from the water bath and allow it to drain and cool for at least 10 minutes. Remove the Snyder column, rinse the flask and its lower joints into the concentrator tube with 0.2 mL of methylene chloride or the exchange solvent, and adjust the final volume as indicated in Table 1, with solvent.

7.12.2 Nitrogen blowdown technique

7.12.2.1 Place the concentrator tube in a warm bath (35EC) and evaporate the solvent to the final volume indicated in Table 1, using a gentle stream of clean, dry nitrogen (filtered through a column of activated carbon). CAUTION: New plastic tubing must not be used between the carbon trap and the sample, since it may introduce contaminants.

7.12.2.2 The internal wall of the tube must be rinsed several times with methylene chloride or appropriate solvent during the operation. During evaporation, the tube must be positioned to avoid water condensation (i.e., the solvent level should be below the level of the water bath). Under normal procedures, the extract must not be allowed to become dry.

CAUTION: When the volume of solvent is reduced below 1 mL, semivolatile analytes may be lost.

7.13 The extract may now be analyzed for the target analytes using the appropriate determinative technique(s) (see Sec. 4.3 of this Chapter). If analysis of the extract will not be performed immediately, stopper the concentrator tube and store refrigerated. If the extract will be stored longer than 2 days it should be transferred to a vial with a PTFE-lined screw-cap or crimp top, and labeled appropriately.

8.0 QUALITY CONTROL

8.1 Any reagent blanks, matrix spikes, or replicate samples should be subjected to exactly the same analytical procedures as those used on actual samples.

8.2 Refer to Chapter One for specific quality control procedures and Method 3500 for extraction and sample preparation procedures.

9.0 METHOD PERFORMANCE

Refer to the determinative methods for performance data.

10.0 REFERENCES

None.

Appendix B EPA METHOD 8015C

NONHALOGENATED ORGANICS BY GAS CHROMATOGRAPHY

SW-846 is not intended to be an analytical training manual. Therefore, method procedures are written based on the assumption that they will be performed by analysts who are formally trained in at least the basic principles of chemical analysis and in the use of the subject technology.

In addition, SW-846 methods, with the exception of required method use for the analysis of method-defined parameters, are intended to be guidance methods which contain general information on how to perform an analytical procedure or technique which a laboratory can use as a basic starting point for generating its own detailed Standard Operating Procedure (SOP), either for its own general use or for a specific project application. The performance data included in this method are for guidance purposes only, and are not intended to be and must not be used as absolute QC acceptance criteria for purposes of laboratory accreditation.

1.0 SCOPE AND APPLICATION

1.1 This method may be used to determine the concentrations of various nonhalogenated volatile organic compounds and semivolatile organic compounds by gas chromatography. The following RCRA compounds were quantitatively determined by this method, using the preparative techniques indicated.

Compound	CAS No. ^a	Appropriate Technique:				
		Purge-and-Trap ^b	Head-space ^a	Direct Aqueous Injection	Azeo. Dist. ^a	Vacuum Dist. ^a
Acetone	67-64-1	pp / ht	x	x	x	x
Acetonitrile	75-05-8	pp	ne	x	x	ne
Acrolein	107-02-8	pp	ne	x	x	x
Acrylonitrile	107-13-1	pp	ne	x	x	x
Allyl alcohol	107-18-6	ht	ne	x	x	ne
t-Amyl alcohol (TAA)	75-85-4	ht	x	ne	ne	x
t-Amyl ethyl ether (TAEE)	919-94-8	x/ ht	x	ne	ne	x
t-Amyl methyl ether (TAME)	994-05-8	x/ ht	x	ne	ne	x
Benzene	71-43-2	x	x	ne	ne	x
t-Butyl alcohol (TBA)	75-85-0	ht	x	x	x	x
Crotonaldehyde	123-73-9	pp	ne	x	x	ne
Diethyl ether	80-29-7	x	ne	x	ne	ne
Diisopropyl ether (DIPE)	108-20-3	x/ ht	x	ne	ne	x
Ethanol	64-17-5	l	x	x	x	x
Ethyl acetate	141-78-6	l	x	x	x	ne
Ethyl benzene	100-41-4	x	x	ne	ne	x
Ethylene oxide	75-21-8	l	ne	x	x	ne
Ethyl tert-butyl ether (ETBE)	637-92-3	x/ ht	x	ne	ne	x
Isopropyl alcohol (2-Propanol)	67-63-0	pp	x	x	x	ne

1.2.1 This method was applied to the analysis of triethylamine in water samples by direct aqueous injection onto a different GC column than is used for any other analytes. Descriptions of the GC column, temperature program, and performance data for triethylamine are provided in this method (see Secs. 6.2.5 and 11.2.6, and Table 6).

1.2.2 GRO corresponds to the range of alkanes from C₆ to C₁₀ and a boiling point range of approximately 60 EC - 170 EC (Reference 6). DRO corresponds to the range of alkanes from C₁₀ to C₂₈ and a boiling point range of approximately 170 EC - 430 EC (Reference 6). The quantitative analyses of these fuel types are based on the procedures described in Sec. 11.11. The identification of specific fuel types may be complicated by environmental processes such as evaporation, biodegradation, or when more than one fuel type is present. Methods from other sources may be more appropriate for GRO and DRO, since these hydrocarbons are not regulated under RCRA. Consult State and local regulatory authorities for any specific regulatory requirements.

1.2.3 This method may be applicable to classes of analytes and to fuel types and petroleum hydrocarbons other than those listed in Secs. 1.1 and 1.2. However, in order to be used for additional analytes, fuel types, or petroleum hydrocarbons, the analyst must demonstrate that the gas chromatographic conditions, including the GC column, are appropriate for the analytes of interest. The analyst must also perform the initial demonstration of proficiency described in Sec. 9.4 and Method 8000. Expansion of this method to other fuel types or petroleum hydrocarbons will also necessitate careful defining of the boiling point range or carbon number range of the material and modification of the quantitation approach to match such ranges. Analysts are advised to consult authoritative sources, such as the American Petroleum Institute (API), for relevant definitions of other fuel types or petroleum fractions.

NOTE: Mention of the analyses of other fuel types and petroleum fractions does *not* imply a regulatory requirement for such analyses, using this or any other method.

1.3 This method can also be used as a screening tool (for both volatile and semivolatile organics) to obtain semiquantitative data to prevent overloading the GC/MS system during quantitative analysis. This may be accomplished using a purge-and-trap method (e.g.,

Method 5030), an automated headspace method (e.g., Method 5021), direct aqueous injection, or by direct injection, if a solvent extraction method has been utilized for sample preparation.

Single-point calibration is acceptable in this situation. Performance data are not provided for screening.

1.4 Prior to employing this method, analysts are advised to consult the base method for each type of procedure that may be employed in the overall analysis (e.g., Methods 3500, 3600, 5000, and 8000) for additional information on quality control procedures, development of QC acceptance criteria, calculations, and general guidance. Analysts also should consult the disclaimer statement at the front of the manual and the information in Chapter Two for guidance on the intended flexibility in the choice of methods, apparatus, materials, reagents, and supplies, and on the responsibilities of the analyst for demonstrating that the techniques employed are appropriate for the analytes of interest, in the matrix of interest, and at the levels of concern. In addition, analysts and

data users are advised that, except where explicitly specified in a regulation, the use of SW-846 methods is *not* mandatory in response to Federal testing requirements. The information contained in this method is provided by EPA as guidance to be used by the analyst and the regulated community in making judgments necessary to generate results that meet the data quality objectives for the intended application.

1.5 This method is restricted for use by, or under the supervision of, analysts appropriately experienced and trained in the use of a gas chromatograph and skilled in the interpretation of gas chromatograms. In addition, if this method is to be used for the analysis of petroleum hydrocarbons, its use then should be limited to analysts experienced in the interpretation of hydrocarbon data. Each analyst must demonstrate the ability to generate acceptable results with this method.

2.0 SUMMARY OF METHOD

2.1 This method provides gas chromatographic conditions for the detection of certain nonhalogenated volatile and semivolatile organic compounds.

2.2 Depending on the analytes of interest, samples may be introduced into the GC by a variety of techniques, including:

- Purge-and-trap (Methods 5030 or 5035)
- Equilibrium headspace (Method 5021)
- Direct injection of aqueous samples
- Injection of the concentrate from azeotropic distillation (Method 5031)
- Vacuum distillation (Method 5032)
- Following solvent extraction (Methods 3510, 3520, 3535, 3540, 3541, 3545, 3546, 3550, 3560, or other appropriate technique)

2.3 Groundwater or surface water samples generally need to be analyzed in conjunction with Methods 5021, 5030, 5031, 5032, 3510, 3520, or other appropriate preparatory methods to obtain the necessary lower limits of quantitation. Method 3535 (solid-phase extraction) may also be applicable to some of the target analytes, however, this method has not been validated by EPA in conjunction with this determinative method.

2.4 Samples to be analyzed for diesel range organics may be prepared by an appropriate solvent extraction method.

2.5 Gasoline range organics may be introduced into the GC/FID by purge-and-trap (Methods 5030 and 5035), automated headspace (Method 5021), vacuum distillation (Method 5032), or other appropriate technique.

2.6 Triethylamine may be analyzed by direct injection of aqueous samples. This compound has not been found to be amenable to purge-and-trap techniques.

2.7 An appropriate column and temperature program are used in the gas chromatograph to separate the organic compounds. Detection is achieved by a flame ionization detector (FID).

2.8 This method allows the use of capillary or packed columns for the analysis and confirmation of the non-halogenated individual analytes. The GC columns and conditions listed have been demonstrated to provide separation of those target analytes. Other

columns and conditions may be employed, provided that the analyst demonstrates adequate performance for the intended application.

2.9 The quantitative analyses of GRO and DRO are based on the definitions provided in Sec. 1.2.2 and the procedures described in Sec. 11.11.

2.10 Given the large number of components to be separated, fused-silica capillary columns are necessary for the analysis of petroleum hydrocarbons, including GRO and DRO, and are recommended for all other analytes. A capillary column is also necessary for the analysis of triethylamine.

3.0 DEFINITIONS

Refer to Chapter One and the manufacturer's instructions for definitions that may be relevant to this procedure.

4.0 INTERFERENCES

4.1 Solvents, reagents, glassware, and other sample processing hardware may yield artifacts and/or interferences to sample analysis. All of these materials must be demonstrated to be free from interferences under the conditions of the analysis by analyzing method blanks.

Specific selection of reagents and purification of solvents by distillation in all-glass systems may be necessary. Refer to each method to be used for specific guidance on quality control procedures and to Chapter Four for general guidance on the cleaning of glassware.

4.2 When analyzing for volatile organics, samples can be contaminated by diffusion of volatile organics (particularly chlorofluorocarbons and methylene chloride) through the sample container septum during shipment and storage. A trip blank prepared from organic-free reagent water and carried through sampling and subsequent storage and handling will serve as a check on such contamination.

4.3 Contamination by carryover can occur whenever high-concentration and low-concentration samples are analyzed in sequence. To reduce the potential for carryover, the sample syringe or purging device needs to be rinsed out between samples with an appropriate solvent. Whenever an unusually concentrated sample is encountered, it should be followed by injection of a solvent blank to check for cross contamination.

4.3.1 Clean purging vessels with a detergent solution, rinse with distilled water, and then dry in a 105 EC oven between analyses. Clean syringes or autosamplers by flushing all surfaces that contact samples using appropriate solvents.

4.3.2 All glassware must be scrupulously cleaned. Clean all glassware as soon as possible after use by rinsing with the last solvent used. This should be followed by detergent washing with hot water, and rinses with tap water and organic-free reagent water. Drain the glassware and dry it in an oven at 130 EC for several hours or rinse it with methanol and drain. Store dry glassware in a clean environment.

4.4 The flame ionization detector (FID) is a non-selective detector. There is a potential for many non-target compounds present in samples to interfere with this analysis. There is also the potential for analytes to be resolved poorly, especially in samples that contain many

analytes. The data user should consider this and may wish to alter the target analyte list accordingly.

5.0 SAFETY

This method does not address all safety issues associated with its use. The laboratory is responsible for maintaining a safe work environment and a current awareness file of OSHA regulations regarding the safe handling of the chemicals listed in this method. A reference file of material safety data sheets (MSDSs) should be available to all personnel involved in these analyses.

6.0 EQUIPMENT AND SUPPLIES

The mention of trade names or commercial products in this manual is for illustrative purposes only, and does not constitute an EPA endorsement or exclusive recommendation for use. The products and instrument settings cited in SW-846 methods represent those products and settings used during method development or subsequently evaluated by the Agency.

Glassware, reagents, supplies, equipment, and settings other than those listed in this manual may be employed provided that method performance appropriate for the intended application has been demonstrated and documented.

This section does not list common laboratory glassware (e.g., beakers and flasks).

6.1 Gas chromatograph -- Analytical system equipped with gas chromatograph suitable for solvent injections, direct aqueous injection, headspace, vacuum distillation sample introduction, or purge-and-trap sample introduction, and equipped with all necessary accessories, including detectors, column supplies, recorder, gases, and syringes. A data system for measuring peak heights and/or peak areas is recommended.

6.2 Recommended GC columns

The choice of GC column will depend on the analytes of interest, the expected concentrations, and the intended use of the results. The packed columns listed below are generally used for screening analyses. The capillary columns are necessary for petroleum hydrocarbon analyses and for triethylamine analyses and are recommended for all other analyses.

The columns listed in this section were the columns used to develop the method. The listing of these columns in this method is not intended to exclude the use of other columns that are available or that may be developed. The laboratory may use either the columns listed in this method or other columns and columns of other dimensions, provided that the laboratory documents method performance data (e.g., chromatographic resolution, analyte breakdown, and sensitivity) that are appropriate for the intended application.

6.2.1 Column 1 -- 8-ft x 0.1-in. ID stainless steel or glass column, packed with 1% SP-1000 on Carbopak-B 60/80 mesh or equivalent.

6.2.2 Column 2 -- 6-ft x 0.1-in. ID stainless steel or glass column, packed with *n*-octane on Porasil-C 100/120 mesh (Durapak) or equivalent.

6.2.3 Column 3 -- 30-m x 0.53-mm ID fused-silica capillary column bonded with DB-Wax (or equivalent), 1- μ m film thickness.

6.2.4 Column 4 -- 30-m x 0.53-mm ID fused-silica capillary column chemically bonded with 5% methyl silicone (DB-5, SPB-5, RTx, or equivalent), 1.5- μ m film thickness.

6.2.5 Column 5 -- 30-m x 0.53-mm ID fused-silica capillary column bonded with HP Basic Wax (or equivalent), 1- μ m film thickness. This column is used for triethylamine.

6.2.6 Wide-bore columns should be installed in 1/4-inch injectors, equipped with deactivated liners designed specifically for use with these columns.

6.3 Detector -- Flame ionization (FID)

6.4 Sample introduction and preparation apparatus

6.4.1 Refer to the 5000 series sample preparation methods for the appropriate apparatus for purge-and-trap, headspace, azeotropic distillation, and vacuum distillation analyses.

6.4.2 Samples may also be introduced into the GC via injection of solvent extracts or direct injection of aqueous samples.

6.5 Syringes

6.5.1 5-mL Luer-Lok glass hypodermic and 5-mL gas-tight syringe equipped with a shutoff valve, for volatile analytes.

6.5.2 Microsyringes -- 10- and 25- μ L equipped with a 0.006-in. ID needle (Hamilton 702N or equivalent) and 100- μ L.

6.6 Volumetric flasks, Class A -- Appropriate sizes equipped with ground-glass stoppers.

6.7 Analytical balance -- 160-g capacity, capable of measuring to 0.0001 g.

7.0 REAGENTS AND STANDARDS

7.1 Reagent-grade chemicals must be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination. Reagents should be stored in glass to prevent the leaching of contaminants from plastic containers.

7.2 Organic-free reagent water -- All references to water in this method refer to organic-free reagent water, as defined in Chapter One.

7.3 Methanol, CH₃OH, pesticide quality or equivalent -- Store away from other solvents.

7.4 Fuels, e.g., gasoline or diesel -- Purchase from a commercial source. Low-boiling components in fuel evaporate quickly. As applicable and available, obtain the fuel from the leaking tank on site.

7.5 Alkane standard -- A standard containing a homologous series of *n*-alkanes for establishing retention times (e.g., C₁₀-C₂₈ for diesel).

7.6 Standard solutions The following sections describe the preparation of stock, intermediate, and working standards for the compounds of interest. This discussion is provided as an example, and other approaches and concentrations of the target compounds may be used, as appropriate for the intended application. See Method 8000 for additional information on the preparation of calibration standards.

7.7 Stock standards -- Stock solutions may be prepared from pure standard materials or purchased as certified solutions. When methanol is a target analyte or when using azeotropic distillation for sample preparation, standards should not be prepared in methanol.

Standards must be replaced after 6 months or sooner, if comparison with check standards indicates a problem.

7.8 Secondary dilution standards -- Using stock standard solutions, prepare secondary dilution standards, as needed, that contain the compounds of interest, either singly or mixed together. The secondary dilution standards should be prepared at concentrations such that the aqueous calibration standards prepared in Sec. 7.9 will bracket the working range of the analytical system. Secondary dilution standards should be stored with minimal headspace for volatiles and should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them.

7.9 Calibration standards -- Prepare calibration standards at a minimum of five different concentrations in organic-free reagent water (for purge-and-trap, direct aqueous injection, azeotropic distillation, or vacuum distillation) or in methylene chloride (for solvent injection) from the secondary dilution of the stock standards. For headspace, prepare the standards as directed in Method 5021. One of the standards should be at or below the concentration equivalent to the appropriate lower limit of quantitation for the project. The remaining concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the GC. Each standard should contain each analyte to be determined by this method (e.g., some or all of the compounds listed in Sec. 1.1 may be included). In order to prepare accurate aqueous standard solutions, the following precautions need to be observed:

7.9.1 Do not inject more than 20 µL of methanolic standards into 100 mL of water.

7.9.2 Use a 25-µL Hamilton 702N microsyringe or equivalent (variations in needle geometry will adversely affect the ability to deliver reproducible volumes of methanolic standards into water).

7.9.3 Rapidly inject the primary standard into the filled volumetric flask. Remove the needle as fast as possible after injection.

7.9.4 Mix diluted standards by inverting the flask three times only.

7.9.5 Fill the sample syringe from the standard solution contained in the expanded area of the flask (do not use any solution contained in the neck of the flask).

7.9.6 The negative pressure generated by pipettes makes them inappropriate for routine use in the transfer of spiked solutions. As such, use of pipettes to dilute or transfer aqueous standards should be avoided. When sample transfer is absolutely necessary, (such as in the performance of headspace sample preparation for water samples) only high quality, automatic pipettes should be used, and then with extreme care.

7.9.7 Aqueous standards used for purge-and-trap analyses (Method 5030) are not stable and should be discarded after 1 hr, unless held in sealed vials with zero headspace. If so stored, they may be held for up to 24 hrs. Aqueous standards used for azeotropic distillation (Method 5031) may be stored for up to a month in polytetrafluoroethylene (PTFE)-sealed screw-cap bottles with minimal headspace, at #6 EC, and protected from light.

7.9.8 Standards for direct aqueous injection of triethylamine are prepared by dissolving an appropriate weight of neat triethylamine in organic-free reagent water and diluting to volume in a volumetric flask.

7.10 Internal standards (if internal standard calibration is used) -- To use this approach, the analyst needs to select one or more internal standards that are similar in analytical behavior to the compounds of interest. The analyst needs to further demonstrate that the measurement of the internal standard is not affected by method or matrix interferences. Because of these limitations, no internal standard can be suggested that is applicable to all samples. The following internal standards are recommended when preparing samples by azeotropic distillation (Method 5031): 2-chloroacrylonitrile, hexafluoro-2-propanol, and hexafluoro-2-methyl-2-propanol.

7.11 Surrogate standards -- Whenever possible, the analyst should monitor both the performance of the analytical system and the effectiveness of the method in dealing with each sample matrix, by spiking each sample, standard, and blank with one or two surrogate compounds which are not affected by method interferences.

8.0 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

8.1 See the introductory material to Chapter Four, "Organic Analytes," and Method 5035.

8.2 If the headspace technique is used, also see Method 5021.

9.0 QUALITY CONTROL

9.1 Refer to Chapter One for guidance on quality assurance (QA) and quality control (QC) protocols. When inconsistencies exist between QC guidelines, method-specific QC criteria take precedence over both technique-specific criteria and those criteria given in Chapter

One, and technique-specific QC criteria take precedence over the criteria in Chapter One. Any effort involving the collection of analytical data should include development of a structured and systematic planning document, such as a Quality Assurance Project Plan (QAPP) or a Sampling and Analysis Plan (SAP), which translates project objectives and specifications into directions for those that will implement the project and assess the results. Each laboratory should maintain a formal quality assurance program. The laboratory should also maintain records to document the quality of the data generated. All data sheets and quality control data should be maintained for reference or inspection.

9.2 Refer to Method 8000 for specific determinative method QC procedures. Refer to Methods 3500 and 5000 for QC procedures to ensure the proper operation of the various sample preparation and/or sample introduction techniques. If an extract cleanup procedure I is performed, refer to Method 3600 for the appropriate QC procedures. Any more specific QC procedures provided in this method will supersede those noted in Methods 8000, 3500, 5000, or 3600.

9.3 Quality control procedures necessary to evaluate the GC system operation are found in Method 8000 and include evaluation of retention time windows, calibration verification and chromatographic analysis of samples.

9.4 Initial demonstration of proficiency

Each laboratory must demonstrate initial proficiency with each sample preparation and determinative method combination it utilizes, by generating data of acceptable accuracy and precision for target analytes in a clean matrix. If an autosampler is used to perform sample dilutions, before using the autosampler to dilute samples, the laboratory should satisfy itself that those dilutions are of equivalent or better accuracy than is achieved by an experienced analyst performing manual dilutions. The laboratory must also repeat the demonstration of proficiency whenever new staff members are trained or significant changes in instrumentation are made.

See Methods 5000 and 8000 for information on how to accomplish a demonstration of proficiency.

9.5 Initially, before processing any samples, the analyst should demonstrate that all parts of the equipment in contact with the sample and reagents are interference-free. This is accomplished through the analysis of a method blank. As a continuing check, each time samples are extracted, cleaned up, and analyzed, and when there is a change in reagents, a method blank should be prepared and analyzed for the compounds of interest as a safeguard against chronic laboratory contamination. If a peak is observed within the retention time window of any analyte that would prevent the determination of that analyte, determine the source and eliminate it, if possible, before processing the samples. The blanks should be carried through all stages of sample preparation and analysis. When new reagents or chemicals are received, the laboratory should monitor the preparation and/or analysis blanks associated with samples for any signs of contamination. It is not necessary to test every new batch of reagents or chemicals prior to sample preparation if the source shows no prior problems. However, if reagents are changed during a preparation batch, separate blanks need to be prepared for each set of reagents.

9.6 Sample quality control for preparation and analysis

The laboratory must also have procedures for documenting the effect of the matrix on method performance (precision, accuracy, method sensitivity). At a minimum, this should include the analysis of QC samples including a method blank, a matrix spike, a duplicate, and a laboratory control sample (LCS) in each analytical batch and the addition of surrogates to each field sample and QC sample when surrogates are used. Any method blanks, matrix spike samples, and replicate samples should be subjected to the same analytical procedures (Sec.11.0) as those used on actual samples.

9.6.1 Documenting the effect of the matrix should include the analysis of at least one matrix spike and one duplicate unspiked sample or one matrix spike/matrix spike duplicate pair. The decision on whether to prepare and analyze duplicate samples or a

matrix spike/matrix spike duplicate must be based on a knowledge of the samples in the sample batch. If samples are expected to contain target analytes, then laboratories may use one matrix spike and a duplicate analysis of an unspiked field sample. If samples are not expected to contain target analytes, laboratories should use a matrix spike and matrix spike duplicate pair. Consult Method 8000 for information on developing acceptance criteria for the MS/MSD.

9.6.2 A laboratory control sample (LCS) should be included with each analytical batch. The LCS consists of an aliquot of a clean (control) matrix similar to the sample matrix and of the same weight or volume. The LCS is spiked with the same analytes at the same concentrations as the matrix spike, when appropriate. When the results of the matrix spike analysis indicate a potential problem due to the sample matrix itself, the LCS results are used to verify that the laboratory can perform the analysis in a clean matrix. Consult Method 8000 for information on developing acceptance criteria for the LCS.

9.6.3 Also see Method 8000 for details on carrying out sample quality control procedures for preparation and analysis. In-house method performance criteria for evaluating method performance should be developed using the guidance found in Method 8000.

9.7 Surrogate recoveries If surrogates are used, the laboratory should evaluate surrogate recovery data from individual samples versus the surrogate control limits developed by the laboratory. See Method 8000 for information on evaluating surrogate data and developing and updating surrogate limits. Procedures for evaluating the recoveries of multiple surrogates and the associated corrective actions should be defined in an approved project plan.

9.8. It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Whenever possible, the laboratory should analyze standard reference materials and participate in relevant performance evaluation studies.

10.0 CALIBRATION AND STANDARDIZATION

See Secs. 11.3 through 11.5 for information on calibration and standardization.

11.0 PROCEDURE

11.1 Introduction/preparation methods

Various techniques are available for sample introduction. All internal standards, surrogates, and matrix spikes (when applicable) need to be added to samples before introduction into the GC/FID system. Consult the applicable sample introduction method regarding when to add standards. Other sample introduction techniques may be appropriate for specific applications and the techniques described here also may be appropriate for other matrices and analytes. Whatever technique is employed, *including* those specifically listed below, the analyst *must* demonstrate adequate performance for the analytes of interest. At a minimum, such a demonstration will encompass the initial demonstration of proficiency described in Sec. 9.6, using a clean reference matrix. Method 8000 describes procedures that may be used to develop performance criteria for such demonstrations as well as for matrix spike and laboratory control sample results.

11.1.1 Direct aqueous injection - This technique involves direct syringe injection of an aliquot of an aqueous sample into the GC injection port. This technique is applicable to the following groups of analytes in this method.

11.1.1.1 Volatile organics (includes GRO)

This technique may involve injection of an aqueous sample containing a very high concentration of analytes. Direct injection of aqueous samples has very limited application in the analysis of volatile organics. It is only appropriate for the determination of volatiles at the toxicity characteristic (TC) regulatory limits or at concentrations in excess of 10,000 µg/L. It may also be used in conjunction with the test for ignitability in aqueous samples (along with Methods 1010 and 1020) to determine if alcohol is present at > 24%.

11.1.1.2 Triethylamine in aqueous samples Triethylamine may be determined by injecting a portion of an aqueous sample directly into the GC injection port. This technique has been demonstrated to be appropriate for samples containing low µg/L (ppb) concentrations of triethylamine.

11.1.2 Purge-and-trap of volatile organics (includes GRO) This includes purge-and-trap for aqueous samples (Method 5030) and purge-and-trap for solid samples (Method 5035). Method 5035 also provides techniques for extraction of solid and oily waste samples by methanol (and other water-miscible solvents) with subsequent purge-and-trap from an aqueous matrix using Method 5030. Normally, purge-and-trap for aqueous samples is performed at ambient temperatures, while soil/solid samples utilize a 40 EC purge to improve extraction efficiency. Heated purge may also be used to improve the purging of compounds with high solubilities in water, particularly alcohols associated with fuel oxygenates such as TBA and TAA.

Occasionally, there may be a need to perform a heated purge for aqueous samples to lower detection limits; however, using a 25-mL sample will often provide the sensitivity needed in most situations.

11.1.3 Azeotropic distillation

This technique exploits the ability of selected water-soluble organic compounds to form binary azeotropes with water during distillation. The organic compounds are removed from the bulk water sample and concentrated in a distillate, as described in Method 5031. An aliquot of the distillate is then injected into the GC/FID.

11.1.4 Vacuum distillation of volatile organics

This technique employs a vacuum distillation apparatus to introduce volatile organics from aqueous, solid, or tissue samples into the GC/FID system, as described in Method 5032.

11.1.5 Automated static headspace

This technique employs a device that collects the volatile organics from the headspace over a sample contained in a sealed vial and introduces them into the GC/FID system, as described in Method 5021.

11.1.6 Solvent injection

This technique involves the syringe injection of solvent extracts of aqueous samples prepared by Methods 3510, 3520, 3535, or other appropriate technique, or extracts of

soil/solids prepared by Methods 3540, 3541, 3545, 3546, 3550, 3560, or other appropriate technique. It is applicable to many semivolatile organics, including DRO. CAUTION: Ultrasonic extraction (Method 3550) may not be as rigorous a method as the other extraction methods for soil/solids. This means that it is critical that the method be followed explicitly to achieve an extraction efficiency which approaches that of Soxhlet extraction. Consult Method 3550 for information on the critical aspects of this extraction procedure.

11.2 Suggested chromatographic conditions

Establish the GC operating conditions appropriate for the GC column being utilized and the target analytes specified in the project plan. Optimize the instrumental conditions for resolution of the target analytes and sensitivity. Suggested operating conditions are given below for the columns recommended in Sec. 6.2. The laboratory may use either the columns listed in this method or other columns and columns of other dimensions, provided that the laboratory documents method performance data (e.g., chromatographic resolution, analyte breakdown, and sensitivity) that are appropriate for the intended application.

11.2.1 Column 1

Carrier gas (helium) flow rate: 40 mL/min

Temperature program:

Initial temperature: 45 EC, hold for 3 min

Program: 45 EC to 220 EC, at 8 EC/min

Final temperature: 220 EC, hold for 15 min.

11.2.2 Column 2

Carrier gas (helium) flow rate: 40 mL/min

Temperature program:

Initial temperature: 50 EC, hold for 3 min

Program: 50 EC to 170 EC, at 6 EC/min

Final temperature: 170 EC, hold for 4 min.

11.2.3 Column 3

Carrier gas (helium) flow rate: 15 mL/min

Temperature program:

Initial temperature: 45 EC, hold for 4 min

Program: 45 EC to 220 EC, at 12 EC/min

Final temperature: 220 EC, hold for 3 min.

11.2.4 Column 4 (DRO)

Carrier gas (helium) flow rate: 5-7 mL/min

Makeup gas (helium) flow rate: 30 mL/min

Injector temperature: 200 EC

Detector temperature: 340 EC

Temperature program:

Initial temperature: 45 EC, hold for 3 min

Program: 45 EC to 275 EC, at 12 EC/min

Final temperature: 275 EC, hold for 12 min.

11.2.5 Column 4 (GRO)

Carrier gas (helium) flow rate: 5-7 mL/min

Makeup gas (helium) flow rate: 30 mL/min
Injector temperature: 200 EC
Detector temperature: 340 EC
Temperature program:
Initial temperature: 45 EC, hold for 1 min
1st Ramp: 45 EC to 100 EC at 5 EC/min
2nd Ramp: 100 EC to 275 EC, at 8 EC/min
Final temperature: 275 EC, hold for 5 min.

11.2.6 Column 5 (triethylamine only)
Carrier gas (helium) flow rate: 5 mL/min
Makeup gas (helium) flow rate: 30 mL/min
Injector temperature: 200 EC
Detector temperature: 250 EC
Temperature program:
Initial temperature: 110 EC
Program: 110 EC to 175 EC, at 10 EC/min
Final temperature: 175 EC, hold for 3 min.

11.3 Initial calibration

11.3.1 Set up the sample introduction system as outlined in the method of choice (see Sec. 11.1). A separate calibration is necessary for each sample introduction mode because of the differences in conditions and equipment. Establish chromatographic operating parameters that provide instrument performance appropriate for the intended application. Prepare calibration standards using the procedures described above (see Sec. 7.9). The external standard technique is described below. Analysts wishing to use the internal standard technique should refer to Sec. 7.10 and Method 8000.

11.3.2 External standard calibration procedure for single-component analytes

11.3.2.1 For each analyte and surrogate of interest, prepare calibration standards at a minimum of five different concentrations. For headspace analysis, the standards should be prepared in methanol or organic-free reagent water and then spiked into the organic-free water in the headspace vial. The spiking solutions should be at concentrations which will dilute to the desired standard concentrations when added into the organic-free water in the headspace vials.

Otherwise, standards should be made by adding volumes of one or more stock standards to a volumetric flask and diluting to volume with an appropriate solvent. One of the standards should be at a concentration at or below the lower limit of quantitation necessary for the project (based on the concentration in the final volume described in the preparation method, with no dilutions). The Calibration Factor ' Total Area within Retention Time Range Mass injected (nanograms) concentrations of the other standards should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.

11.3.2.2 Introduce each calibration standard using the technique that will be used to introduce the actual samples into the gas chromatograph. Tabulate peak height or area responses against the mass injected. Calculate the calibration factor (CF) for each single-component analyte as described in Method 8000.

11.3.3 External standard calibration procedure for DRO and GRO

The calibration of DRO and GRO is markedly different from that for single-component analytes. In particular, the response used for calibration needs to represent the entire area of the chromatogram within the retention time range for the fuel type (DRO or GRO), including the unresolved complex mixture that lies below the individual peaks. See Sec. 11.11 for information on calculating this area.

11.3.3.1 For each fuel type, prepare calibration standards at a minimum of five different concentrations by adding volumes of one or more stock standards to a volumetric flask and diluting to volume with an appropriate solvent (for headspace analysis, follow the instructions in Sec. 11.3.2.1, above). One of the standards should be at a concentration at or below the lower limit of quantitation necessary for the project (based on the concentration in the final volume described in the preparation method, with no dilutions). The concentrations of the other standards should correspond to the expected range of concentrations found in real samples or should define the working range of the detector. NOTE: Whenever possible, the calibration should be performed using the specific fuel that is contaminating the site (e.g., a sample of the fuel remaining in the tank suspected of leaking). Where such samples are not available or not known, use recently purchased commercially-available fuel. A qualitative screening injection and GC run may be performed to identify unknown fuels.

11.3.3.2 Introduce each calibration standard using the technique that will be used to introduce the actual samples into the gas chromatograph. Determine the area of the response as described in Sec. 11.10. Calculate the calibration factor (CF) for each fuel type as shown below:

11.3.4 Calibration linearity

The linearity of the calibration must be assessed. This applies to both the single component analytes and the fuel types.

11.3.4.1 If the percent relative standard deviation (%RSD) of the calibration factors is less than 20% over the working range, then linearity through the origin can be assumed, and the average calibration factor can be used in place of a calibration curve.

11.3.4.2 If the RSD is more than 20% over the working range, linearity through the origin cannot be assumed. See Method 8000 for other calibration options that may be employed, which may include a linear calibration not through the origin or a non-linear calibration model (e.g., a polynomial equation).

11.4 Retention time windows

Single-component target analytes (see Sec. 1.1) are identified on the basis of retention time windows. GRO and DRO are distinguished on the basis of the ranges of retention times for characteristic components in each type of fuel.

11.4.1 Before establishing retention time windows, make sure that the chromatographic system is functioning reliably and that the operating parameters have been optimized for the target analytes and surrogates in the sample matrix to be analyzed. Establish the retention time windows for single component target analytes using the procedure described in Method 8000.

11.4.2 The retention time range for GRO is defined during initial calibration. Two specific gasoline components are used to establish the range, 2-methylpentane and 1,2,4-

trimethylbenzene. Use the procedure described in Method 8000 to establish the retention time windows for these two components. The retention time range is then calculated based on the lower limit of the RT window for the first eluting component and the upper limit of the RT window for the last eluting component.

11.4.3 The retention time range for DRO is defined during initial calibration. The range is established from the retention times of the C₁₀ and C₂₈ alkanes. Use the procedure described in Method 8000 to establish the retention time windows for these two components. The retention time range is then calculated based on the lower limit of the RT window for the first eluting component and the upper limit of the RT window for the last eluting component.

11.4.4 If this method is expanded to address other fuel types or petroleum fractions, then the analyst needs to establish appropriate retention time ranges for the boiling point range or carbon number range used to define each additional fuel type or petroleum fraction. Use the procedure described in Method 8000 to establish the retention time windows.

11.5 Calibration verification

11.5.1 The initial calibration and retention times need to be verified at the beginning of each 12-hr work shift, at a minimum. When individual target analytes are being analyzed, verification is accomplished by the analysis of one or more calibration standards (normally mid-concentration, but a concentration at or near the action level may be more appropriate) that contain all of the target analytes and surrogates. When petroleum hydrocarbons are being analyzed, verification is accomplished by the measurement of the fuel standard and the hydrocarbon retention time standard. Additional analyses of the verification standard(s) throughout a 12-hr shift are strongly recommended, especially for samples that contain visible concentrations of oily material. See Method 8000 for more detailed information on calibration verification.

11.5.2 Calculate the % difference as detailed in Method 8000. If the response for any analyte is within $\pm 20\%$ of the response obtained during the initial calibration, then the initial calibration is considered still valid, and the analyst may continue to use the mean CF or RF values from the initial calibration to quantitate sample results. If the response for any analyte varies from the predicted response by more than $\pm 20\%$, corrective action must be taken to restore the system or a new calibration curve must be prepared for that compound.

11.5.3 All target analytes, surrogates, and/or *n*-alkanes in the calibration verification analyses need to fall within previously established retention time windows. If the retention time of any analyte does not fall within the established window, then corrective action must be taken to restore the system or a new calibration curve must be prepared for that compound.

11.5.4 Solvent blanks and any method blanks should be run with calibration verification analyses to confirm that laboratory contamination does not cause false positive results.

11.6 Gas chromatographic analysis

11.6.1 Samples are analyzed in a set referred to as an analytical sequence. The sequence begins with calibration verification followed by sample extract analyses.

Additional analyses of the verification standard(s) throughout a 12-hr shift are strongly recommended, especially for samples that contain visible concentrations of oily material. A verification standard is also necessary at the end of a set (unless internal standard calibration is used). The sequence ends when the set of samples has been injected or when retention time and/or % difference QC criteria are exceeded.

If the criteria are exceeded, inspect the gas chromatographic system to determine the cause and perform whatever maintenance is necessary before recalibrating and proceeding with sample analysis. All sample analyses performed using external standard calibration need to be bracketed with acceptable data quality analyses (e.g., calibration and retention time criteria). Therefore, all samples that fall between the standard that failed to meet the acceptance criteria and the preceding standard that met the acceptance criteria need to be reanalyzed. Samples analyzed using internal standard calibration need not be bracketed (see Method 8000).

11.6.2 Samples are analyzed with the same instrument configuration as is used during calibration. When using Method 5030 for sample introduction, analysts are cautioned that opening a sample vial or drawing an aliquot from a sealed vial (thus creating headspace) will compromise samples analyzed for volatiles. Therefore, it is recommended that analysts prepare two aliquots for purge-and-trap analysis. The second aliquot can be stored for 24 hrs to ensure that an uncompromised sample is available for analysis or dilution, if the analysis of the first aliquot is unsuccessful or if results exceed the calibration range of the instrument. Distillates from Method 5031 may be split into two aliquots and held at 4 EC prior to analysis. It is recommended that the distillate be analyzed within 24 hrs of distillation. Distillates should be analyzed within 7 days of distillation.

11.6.3 Sample concentrations are calculated by comparing the sample response with the response from the initial calibration of the system (see Sec. 11.3). Therefore, if the sample response exceeds the limits of the initial calibration range, a dilution of the sample or sample extract needs to be analyzed. For volatile organic analyses of aqueous samples, the dilution needs to be performed on a second aliquot of the sample which has been properly sealed and stored prior to use and reanalysis. Samples and/or sample extracts should be diluted so that all peaks are on scale, as overlapping peaks are not always evident when peaks are off scale. Computer reproduction of chromatograms, manipulated to ensure that all peaks are on scale over a 100-fold range, is acceptable as long as calibration limits are not exceeded. Peak height measurements are recommended over peak area integration when overlapping peaks cause errors in area integration.

11.6.4 Tentative identification of a single-component analyte occurs when a peak from a sample extract falls within the daily retention time window. Confirmation may be necessary on a second column or by GC/MS. Since the flame ionization detector is non-specific, it is highly recommended that GC/MS confirmation be performed on single-component analytes unless historical data are available to support the identification(s). See Method 8000 for additional information on confirmation.

11.6.5 Second-column confirmation is generally not necessary for petroleum hydrocarbon analysis. However, if analytical interferences are indicated, analysis using the second GC column may be necessary. Also, the analyst needs to ensure that the

sample hydrocarbons fall within the retention time range established during the initial calibration.

NOTE: The identification of fuels, especially gasoline, is complicated by their inherent volatility. The early eluting compounds in fuels are obviously the most volatile and the most likely to have weathered unless the samples were taken immediately following a spill. The most highly volatile fraction of gasoline constitutes 50% of the total peak area of a gasoline chromatogram. This fraction is the least likely to be present in an environmental sample or may be present at only very low concentration in relation to the remainder of a gasoline chromatogram.

11.6.6 The performance of the entire analytical system should be checked every 12 hrs, using data gathered from analyses of blanks, standards, and samples. Significant peak tailing needs to be corrected. Tailing problems are generally traceable to active sites on the column, cold spots in a GC, the detector operation, or leaks in the system. See Sec. 11.8 for GC/FID system maintenance. Follow manufacturer's instructions for maintenance of the introduction device.

11.7 Screening

11.7.1 This method can be used with a single-point calibration for screening samples prior to GC/MS analyses (e.g., Methods 8260 and 8270). Such screening can reduce GC/MS down-time when highly-contaminated samples are analyzed.

11.7.2 When this method is used for screening, it is recommended that the same sample introduction device (e.g., purge-and-trap versus direct injection) that is used for the subsequent GC/MS analyses also be used for the screening analysis. This will improve the correlation between the results and make the screening results more useful in predicting those samples that may overload the GC/MS system. However, other sample introduction techniques may be employed as well.

11.7.3 Establish that the system response and chromatographic retention times are stable. Analyze the high-point GC/MS calibration standard.

11.7.4 Analyze samples or sample extracts. Compare peak heights in the sample chromatograms with the high-point standard to establish that no compound with the same retention time as a target analyte exceeds the calibration range of the GC/MS system.

CAUTION: The FID is much less sensitive to halogenated compounds than the MS detector. As a result, a simple peak height comparison for such compounds in the GC/MS standard may underestimate the actual concentration of halogenated compounds. When using this method as a screening tool, such an underestimate could lead to GC/MS results over the calibration range or result in contamination of the GC/MS system. Therefore, the analyst should exercise caution when screening samples that also contain halogenated compounds.

11.7.5 There are no formal QC requirements applied to screening analyses using this method. However, it is recommended that the high-point standard be run at least once every 12 hrs to confirm the stability of the instrument response and chromatographic retention times. The analyst should consider the costs associated with making the wrong decision from the screening results (e.g., GC/MS instrument down-time and maintenance) and use appropriate judgment.

11.8 Instrument maintenance

11.8.1 Injection of sample extracts from waste sites often leaves a high boiling residue in the injection port area, splitters (when used), and the injection port end of the chromatographic column. This residue affects chromatography in many ways (i.e., peak tailing, retention time shifts, analyte degradation, etc.) and, therefore, instrument maintenance is very important. Residue buildup in a splitter may limit flow through one leg and therefore change the split ratios. If this occurs during an analytical run, the quantitative data may be incorrect. Proper cleanup techniques will minimize the problem and instrument QC will indicate when instrument maintenance is necessary.

11.8.2 Recommended chromatograph maintenance

Corrective measures may need any one or more of the following remedial actions. Also see Method 8000 for additional guidance on corrective action for capillary columns and the injection port.

11.8.2.1 Splitter connections -- For dual columns which are connected using a press-fit Y-shaped glass splitter or a Y-shaped fused-silica connector, clean and deactivate the splitter or replace with a cleaned and deactivated splitter. Break off the first few inches (up to one foot) of the injection port side of the column. Remove the columns and solvent backflush according to the manufacturer's instructions. If these procedures fail to eliminate the degradation problem, it may be necessary to deactivate the metal injector body and/or replace the columns.

11.8.2.2 Column rinsing -- Rinse the column with several column volumes of an appropriate solvent. Both polar and nonpolar solvents are recommended. Depending on the nature of the sample residues expected, the first rinse might be water, followed by methanol and acetone; methylene chloride is a satisfactory final rinse and in some cases may be the only solvent necessary. The column should then be filled with methylene chloride and allowed to remain flooded overnight to allow materials within the stationary phase to migrate into the solvent.

The column is then flushed with fresh methylene chloride, drained, and dried at room temperature with a stream of ultrapure nitrogen passing through the column.

11.9 Calculations and data handling

Results need to be reported in units commensurate with their intended use and all dilutions need to be taken into account when computing final results.

11.10 The concentration of each analyte in the sample may be determined by calculating the amount of standard purged or injected, from the peak response, using the mean CF or RF from the initial calibration, or another appropriate calibration model (see Method 8000).

11.11 While both diesel fuel and gasoline contain a large number of compounds that will produce well-resolved peaks in a GC/FID chromatogram, both fuels contain many other components that are not chromatographically resolved. This unresolved complex mixture results in the "hump" in the chromatogram that is characteristic of these fuels. In addition, although the resolved peaks are important for the identification of the specific

fuel type, the area of the unresolved complex mixture contributes a significant portion of the area of the total response.

11.11.1 For the analysis of DRO, sum the area of all peaks eluting between C₁₀ and C₂₈. This area is generated by projecting a horizontal baseline between the retention times of C₁₀ and C₂₈.

11.11.2 Because the chromatographic conditions employed for DRO analysis can result in significant column bleed and a resulting rise in the baseline, it is appropriate to perform a subtraction of the column bleed from the area of the DRO chromatogram. In order to accomplish this subtraction, analyze a methylene chloride blank during each 12-hr analytical shift during which samples are analyzed for DRO. Measure the area of this chromatogram in the same fashion as is used for samples (see Sec. 11.11.1), by projecting a horizontal baseline across the retention time range for DRO. Then subtract this area from the area measured for the sample and use the difference in areas to calculate the DRO concentration, using the equations in Method 8000.

11.11.3 For the analysis of GRO, sum the areas of all peaks eluting between 2-methylpentane and 1,2,4-trimethylbenzene. This area is used to calculate the GRO concentration, using the equations in Method 8000. Column bleed subtraction is not generally necessary for GRO analysis.

11.12 Refer to Method 8000 for the calculation formulae. The formulae cover external and internal standard calibration, aqueous and non-aqueous samples, and linear and non-linear calibrations.

12.0 DATA ANALYSIS AND CALCULATIONS

See Sec. 11.9 for information on data analysis and calculation.

13.0 METHOD PERFORMANCE

13.1 Performance data and related information are provided in SW-846 methods only as examples and guidance. The data do not represent required performance criteria for users of the methods. Instead, performance criteria should be developed on a project-specific basis, and the laboratory should establish in-house QC performance criteria for the application of this method. These performance data are not intended to be and must not be used as absolute QC acceptance criteria for purposes of laboratory accreditation.

13.2 Example method performance data for non-purgeable volatiles prepared using the azeotropic microdistillation technique from Method 5031 are included in Table 1 and 2 for aqueous matrices and in Table 3 for solid matrices. Typical chromatograms are included in Figs. 4 and 5. These data are for guidance purposes only.

13.3 Example method performance information are provided in Tables 4 and 5 for diesel fuel spiked into soil as are chromatograms of GRO and DRO standards in Figures 1 to 3. These data are for guidance purposes only.

13.4 Table 6 contains example precision and bias data for the analysis of triethylamine. Reagent water was spiked with triethylamine at 1.0 µg/L and analyzed by direct aqueous injection in a GC/FID equipped with an HP Basic Wax column (30-m x 0.53-mm ID). These data are for guidance purposes only.

13.5 Table 7 contains example single-laboratory data on the pressurized fluid extraction of diesel range organics (DRO) from three types of soil (sand, loam, and clay). The soils were spiked at two levels of DRO, approximately 5 mg/kg each and approximately 2000 mg/kg.

Seven replicates of each level and soil type were extracted using pressurized fluid extraction (Method 3545), using a mixture of methylene chloride and acetone (1:1). The data are taken from Reference 8 and are for guidance purposes only. This extraction technique may be applicable to other analyte classes, fuel types, or petroleum fractions (see Sec. 1.2.3).

13.6 This method was the determinative technique used by one of the three laboratories participating in the study of headspace analysis of oxygenated gasoline contaminated groundwater samples. Refer to the new version of Method 5021, which can be found at http://www.epa.gov/epaoswer/hazwaste/test/pdfs/5021a_r1.pdf, for a discussion and the results of that study. These data are provided for guidance purposes only.

13.7 This method was used in combination with Method 5021 to analyze a standard with several gasoline components, including MTBE and 2-methylpentane. As can be seen from the chromatogram in Figure 6, the two analytes were resolved quite well (column: Restek 502.2

105m x 0.53 um, 3 df). See

http://www.epa.gov/epaoswer/hazwaste/test/pdfs/5021a_r1.pdf for more information.

These data are provided for guidance purposes only.

14.0 POLLUTION PREVENTION

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity and/or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operations. The EPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation. When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions consult *Less is Better: Laboratory Chemical Management for Waste*

Reduction available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th St., N.W. Washington, D.C. 20036, <http://www.acs.org>.

15.0 WASTE MANAGEMENT

The Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management, consult *The Waste*

Management Manual for Laboratory Personnel available from the American Chemical Society at the address listed in Sec. 14.2.

16.0 REFERENCES

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Appendix C TRF Protocol

Extraction of DNA from Soil Samples

1. Weigh out sample (1 g of soil) and transfer it into a Mo Bio Bead Solution tube (Mo Bio, Carlsbad, CA).
2. Add 60 μ L of S1 solution (Mo Bio, Carlsbad, CA) to Bead Solution tube. Invert to mix.
3. Add 200 μ L IRS solution (Mo Bio, Carlsbad, CA) to Bead Solution tube. Vortex briefly to mix.
4. Place Bead Solution tube in the Bio 101 Fast Prep machine and run at: 5.0 m/s for 45 sec.
5. Centrifuge Bead Solution tubes at 10,000 x g for 2 min.
6. Remove 450 μ L of supernatant and place in a clean 2.0 mL Mo Bio tube along with 250 μ L of S2 solution (Mo Bio, Carlsbad, CA).
7. Incubate in freezer for 5 min.
8. Centrifuge tubes at 14,000 x g for 2 min.
9. Remove 450 μ L of supernatant and place in a clean 2.0 mL Mo Bio tube along with 900 μ L of S3 solution (Mo Bio, Carlsbad, CA). Invert to mix S3/DNA.
10. Transfer 750 μ L of S3/DNA to a Spin Filter tube (Mo Bio, Carlsbad, CA).
11. Centrifuge at 10,000 x g for 30 sec.
12. Discard eluate into a Mo Bio waste tube (50 mL Falcon tube).
13. Repeat the step 10-12 until all of the S3/DNA is gone.
14. Add 300 μ L of S4 solution (Mo Bio, Carlsbad, CA) on the filter and centrifuge at 10,000 x g for 1 min.
15. Discard eluate into a Mo Bio waste tube (50 mL Falcon tube).
16. Centrifuge the Spin Filter at 14,000 x g for 2 min. to dry Spin Filter.
17. Place the Spin Filter into a clean 2.0 mL Mo Bio tube.
18. Add 50 μ L of PCR water to center of Spin Filter taking care not to touch the filter with the pipette tip.
19. Incubate at room temperature for at least 10 min. (Critical step)
20. Centrifuge Spin Filter at 10 x kg for 1 minute.
21. The DNA is in the eluate. Store at -20°C .

Gel Electrophoresis

1. Prepare a 1.5 % (w/v) agarose gel in an Erlenmeyer flask. Heat in microwave to melt. Pour gel into gel box mold and allow cooling for 30 min.
2. Use 5 μ L of DNA for each well and 1.0 μ L of 5X loading buffer. Leave one lane for 1 Kb ladder per row.
3. Run the gel at ~ 100 V for ~ 35 minutes.
4. Place the gel in the ethidium bromide for 10-15 min. Be sure to wear gloves, as ethidium bromide is a potent mutagen.
5. Place the gel in the DI water (de-stain) for 10-30 minutes (optional).
6. Take a picture of the gel.
7. Discard gel in hazardous waste container. Wipe off gel illuminator surface with a Kim-wipe.

8. Combine replicate extractions discarding any extractions that appear abnormal.

A260 DNA Quantitation using SPECTRAMax UV spectrophotometer.

1. **Turn on the spec 30 minutes before making measurements.**
2. Obtain an UV spectrophotometer plate from the drawer below the spectrophotometer.
3. A 200 μ L blank should be used for each row that will have DNA. If samples were eluted with S5 solution (Mo Bio, Carlsbad, CA) or TE buffer (Sigma, St. Louis, MO) add 10 μ L of S5 or TE, whichever was used, to the blank with 190 μ L of PCR water.
4. Make a 1/20 dilution in the plate wells for each sample by adding 190 μ L of PCR water and 10 μ L of DNA for quantitation. Triturate samples to mix. The spectrophotometer will mix samples before each read.
5. Open the Spectramax analysis software alias on the desktop and choose the '**200 μ L DNA Corrected**' from the **Assays** menu.
6. Click on **Read #1** and select the **Template** button. Highlight the wells that will be used for blanks, select **Blank** from the drop menu.
7. Repeat step 6 for the DNA wells. Click the **Series** button and select the manner in which the samples are arranged. Change replicates to 1. Change the first sample name to DNA01. Select **Copy Template** from the **Template** menu. **Click OK.**
8. Open Read #2 and select **Paste Template** from the **Template** menu. Repeat for reads 3-5.
9. Click on Read #1 and Click the **READ** button. Repeat for reads 2-5.
10. Review the reads and shade any Blank or DNA wells that vary significantly.
11. Click the DNA bar and select **Print 'DNA'** from the **File** menu. It will print on the laser printer in Room 472.

Polymerase Chain Reaction (PCR) Amplification of DNA (16s labeled)

1. Using the concentrations obtained from the quantitation assay, make dilutions of the DNA to 1 ng/ μ L (or other concentration based on your PCR optimization) using PCR water.
2. Obtain a PCR worksheet and fill in the samples to be run. Three reactions should be run for each sample. Three control reactions are needed: an open negative (master mix, no DNA, open when loading DNA), a closed negative (master mix, no DNA, not opened outside PCR room), and a positive (DNA known to amplify with PCR conditions).
3. Defrost the reagents in the PCR room, leaving the Taq in the freezer until needed. The primers used are: Ba2F (GCY TAA CAC ATG CAA GTC GA) (Proligo, La Jolla, CA) and K2R (GTA TTA CCG CGG CTG CTG G) (Sigma Genosys, The Woodlands, TX). The following volumes are to be used per well for 16s PCR: template DNA (extracted from soil sample) 10 μ L, 10X Buffer - 5 μ L (Applied Biosystems, Foster City, CA), dNTPs (10 mM) - 3 μ L (Promega, Madison, WI), bovine serum albumin (BSA) (20 μ g/mL) - 2 μ L (Sigma, St. Louis, MO), $MgCl_2$ (25 mM) - 7 μ L (Applied Biosystems, Foster City, CA), Ba2f (10 mM) - 1 μ L, K2r (10 mM) - 1 μ L.
4. TX), water 20.7 μ L, Taq (5 units/ μ L) 0.3 μ L (Applied Biosystems, Foster City, CA).

5. Fill in the number of samples adding 1 to the number to adjust for pipetting errors. Multiply the reagent volumes by the sample number and this is the amount needing to be added to the master mix.
6. Prepare the master mix in an Eppendorf tube located in the PCR room. Put an M on the tube to show it is the master mix. Vortex the mix and centrifuge (all in the PCR room).
7. Obtain a PCR rack and add the number of tubes needed. Add 40 μL of the master mix to each tube. Cap the tubes lightly and put all reagents away. Bring the DNA to the DNA hood in Room 388.
8. Using the razor in the hood, cut the bands connecting the closed negative control cap to the others allowing it to remain closed during addition of DNA. Remove a row of caps and place it in the pipette tip box in the back of the hood. Using the lid to this box, cover the rack (have someone show you how this is done).
9. Add 10 μL of the DNA dilutions to the designated wells. Re-cap the wells when the row is finished.
10. Tighten the caps and check to assure all of the liquid is at the bottom of the tube. Place the rack in the Applied Biosystems 9700 thermocycler and close the lid.
11. For 9700 or 2400, Hit “run,” then “user,” then “Chris,” then “46.5 TRFP;” this is the program to use. Press “accept,” enter “50” μL at the reaction volume prompt and then press “accept” to start run.
12. When the run is finished, place the rack in a freezer.

“46.5 TRFP” cycle

Gel Electrophoresis

Same as described above for gel electrophoresis after extraction, except only load 2.5 μL of each PCR reaction.

Combination of PCR reactions

1. Combine all PCR reactions in a 1.5 mL Eppendorf tube.
2. Add 5 volumes SpinBind solution (Mo Bio, Carlsbad, CA) and vortex briefly to mix
3. Transfer 750 μL to the spin filter unit. Centrifuge for 30 sec at 10 x kg. Discard eluate.
4. Repeat step 3 until all PCR/SpinBind mixture is filtered.
5. Add 300 μL of SpinClean (Mo Bio, Carlsbad, CA) buffer to spin filter and centrifuge for 30 sec. At 10 x kg. Discard eluate.
6. Centrifuge spin filter for 120 sec. At 10 x kg to remove any remaining fluid.
7. Transfer spin filter to clean 2.0 ml collection tube.
8. Add 50 μL of PCR water to spin filter and incubate 10 min.
9. Centrifuge for 60 sec. At 10 x kg.
10. Discard spin filter and store at $-20\text{ }^{\circ}\text{C}$.

Labeled PCR Quantitation by A₂₆₀ UV spectrophotometer

Use same procedure as used for extracted DNA as described previously.

Enzyme Digests

(Amount of DNA digested varies depending on the samples being prepared. Ask for instructions before proceeding further.)

1. Place the enzyme, buffer, and the PCR water on ice to thaw. Always keep tubes with enzyme present on ice.
2. Calculate the amount of each reagent needed for digest master mix. 20 μ L reactions or 40 μ L reactions will be run using 50 ng of DNA for soil and feces, or 10 ng for pure culture bacteria.
3. For **DpnII** (10,000 U/mL) use 0.4 μ L enzyme per reaction, 2 μ L buffer for 20 μ L reaction or 4 μ L buffer for 40 μ L reaction. The enzyme amount remains the same. Add DNA and then water to bring the volume to 20 or 40 μ L depending on reaction volume.
- ♦ For **HaeIII** (10,000 U/mL) use 0.1 μ L enzyme per reaction, 2 μ L buffer for 20 μ L reaction or 4 μ L buffer for 40 μ L reaction. The enzyme amount remains the same. Add DNA and then water to bring the volume to 20 or 40 μ L depending on reaction volume.
- ♦ For **HhaI** (20,000 U/mL) use 0.05 μ L enzyme per reaction, 0.2 μ L BSA, 2 μ L buffer for 20 μ L reaction or 0.4 μ L BSA and 4 μ L buffer for 40 μ L reaction. The enzyme amount remains the same. Add DNA and then water to bring the volume to 20 or 40 μ L depending on reaction volume.
4. Calculate the volume of DNA solution to add to digest.
5. ***Add the DNA, and water to the reaction tubes first.
6. Add Digestion master mix to each sample.
7. Place tubes in PCR machine for 4 hours @ 37 °C then cycle to either **65 °C** for **DpnII**, **65 °C** for **HhaI**, or **80 °C** for **HaeIII** for 20 minutes to deactivate the enzyme and finally to 4°C for infinity.
8. Store the digests in the -20 °C freezer until ready for ethanol precipitation.

Ethanol Precipitation

(Note: prompt removal of samples from centrifuge will ensure minimal loss of sample.)

1. To the digest, add 50 μ L (2.5 x digest volume) of cold 95% ethanol and 1 μ L 3 M sodium acetate pH 4.6 (5 % digest volume) and 1 μ L glycogen (20 mg/mL)
2. Invert five times making sure the lids are securely on the sample.
3. Place the tubes in the -4 °C freezer for 30 minutes.
4. Centrifuge the tubes for 30 minutes at 3490 RPM to pellet DNA.
5. Remove ethanol by inverting the PCR tray on a paper towel.
6. Add 100 μ L of cold 70 % ethanol.
7. Centrifuge the tubes for 15 minutes at 3490 RPM.
8. Remove ethanol by inverting the PCR tray on a paper towel.
9. Centrifuge rack in inverted position on top of a paper towel for 1 min. @ 700 RPM to dry the pellet.
10. Store the DNA in the -20°C freezer until ready to proceed to CEQ 8000 preparation.

CEQ 8000 (capillary gel sequencer) sample preparation

1. Make a master mix of 20 μ L formamide and 0.25 μ L 600 base pair (bp) standard per reaction. Add 20 μ L of the master mix to each tube.
2. Add one drop of mineral oil to the top of each well to prevent sample evaporation.
3. Run in the CEQ 8000

Appendix D Hexacosane Recovery Data of the Fertilized Experiment

Table Appendix D.1 Initial water sample TPH and Hexacosane Recovery

Box	Total peak area	Hexacosane peak area	Area-hexacosane peak	Calculated TPH mg/L in analyzed sample	TPH initial GW (mg/L)	Hexacosane in Sample	Recovery efficiency of hexacosane	Recovery efficiency (%)
Initial GW 4-30-08 D2-1 (A)	2.23E+10	7.80E+08	2.15E+10	2901.60	14.51	303.02	1.52	99.68
Initial GW 4-30-08 D2-1 (B)	2.15E+10	7.77E+08	2.08E+10	2799.37	14.00	301.65	1.51	99.23
Initial GW 5-1-08 D2-1 (A)	2.67E+10	9.02E+08	2.58E+10	3471.91	17.36	351.83	1.76	115.73
Initial GW 5-1-08 D2-1 (B)	2.42E+10	8.14E+08	2.34E+10	3147.99	15.74	316.43	1.58	104.09

Table Appendix D.2 Day 0 water sample TPH and Hexacosane Recovery

Box	Total peak area	Hexacosane peak area	Area-hexacosane peak	Calculated TPH (mg/L) in analyzed sample	TPH in GW (mg/L)	Hexacosane in sample (mg/L)	Hexacosane (mg/L)	Recovery efficiency (%)
Box 1 (A)	1.00E+10	7.76E+08	9.22E+09	1365.64	6.83	301.44	1.51	99.16
Box 1 (B)	8.99E+09	7.86E+08	8.20E+09	1231.66	6.16	305.58	1.53	100.52
Box 2 (A)	1.00E+10	8.05E+08	9.20E+09	1367.45	6.84	312.93	1.56	102.94
Box 2 (B)	1.00E+10	8.14E+08	9.20E+09	1367.45	6.84	316.43	1.58	104.09
Box 3 (A)	9.60E+09	7.38E+08	8.86E+09	1312.86	6.56	286.31	1.43	94.18
Box 3 (B)	9.47E+09	6.52E+08	8.82E+09	1295.75	6.48	252.03	1.26	82.90
Box 4 (A)	9.95E+09	7.40E+08	9.21E+09	1359.23	6.80	287.09	1.44	94.44
Box 4 (B)	1.01E+10	7.40E+08	9.39E+09	1384.12	6.92	287.09	1.44	94.44
Box 6 (A)	9.77E+09	6.89E+08	9.08E+09	1335.40	6.68	266.84	1.33	87.78
Box 6 (B)	1.02E+10	7.82E+08	9.38E+09	1387.78	6.94	303.90	1.52	99.97
Box 7 (A)	9.35E+09	7.42E+08	8.60E+09	1279.14	6.40	287.96	1.44	94.73
Box 7 (B)	9.57E+09	8.05E+08	8.77E+09	1309.07	6.55	312.81	1.56	102.90
Box 8 (A)	9.77E+09	6.89E+08	9.08E+09	1335.40	6.68	266.84	1.33	87.78
Box 8 (B)	1.02E+10	7.92E+08	9.37E+09	1387.78	6.94	307.88	1.54	101.28
Box 9 (A)	1.01E+10	8.08E+08	9.30E+09	1380.59	6.90	314.32	1.57	103.39
Box 9 (B)	1.02E+10	8.08E+08	9.41E+09	1395.23	6.98	314.32	1.57	103.39
Box 10 (A)	1.09E+10	7.41E+08	1.02E+10	1389.50	6.95	287.53	1.44	94.58
Box 10(B)	1.02E+10	7.15E+08	9.49E+09	1297.87	6.49	277.26	1.39	91.20

Table Appendix D.3 Day 25 water sample TPH and Hexacosane Recovery

Box	Total peak area	Hexacosane peak area	Area-hexacosane peak	Calculated TPH (mg/L) in analyzed sample	TPH in GW (mg/L)	Hexacosane (mg/L)	hexacosane in mg/L	Recovery efficiency
Box 1 (A)	4.91E+09	8.43E+08	4.07E+09	688.48	3.44	328.06	1.64	0.97
Box 1 (A)2	4.66E+09	7.26E+08	3.93E+09	654.89	3.27	281.42	1.41	0.83
Box 1 (B)	4.96E+09	7.17E+08	4.24E+09	694.73	3.47	278.06	1.39	0.82
Box 1 (B)2	3.96E+09	4.24E+08	3.53E+09	561.57	2.81	161.07	0.81	0.48
Box 2 (A)	1.00E+10	8.17E+08	9.23E+09	1372.08	6.86	317.90	1.59	0.94
Box 2 (A)2	1.00E+10	8.27E+08	9.21E+09	1371.30	6.86	321.70	1.61	0.95
Box 2 (B)	1.05E+10	8.98E+08	9.60E+09	1432.42	7.16	349.96	1.75	1.04
Box 2 (B)2	1.04E+10	9.18E+08	9.48E+09	1419.10	7.10	357.93	1.79	1.06
Box 3 (A)	5.17E+09	9.04E+08	4.27E+09	723.60	3.62	352.39	1.76	1.04
Box 3 (A)2	5.66E+09	7.54E+08	4.91E+09	788.63	3.94	292.60	1.46	0.87
Box 3 (B)	5.34E+09	7.32E+08	4.61E+09	746.31	3.73	283.84	1.42	0.84
Box 3 (B)2	5.35E+09	7.96E+08	4.55E+09	746.40	3.73	309.58	1.55	0.92
Box 4 (A)	1.01E+10	9.26E+08	9.21E+09	1383.64	6.92	361.19	1.81	1.07
Box 4 (A)2	1.01E+10	8.05E+08	9.25E+09	1373.54	6.87	312.81	1.56	0.93
Box 4 (B)	1.01E+10	9.15E+08	9.18E+09	1378.98	6.89	356.76	1.78	1.06
Box 4 (B)2	1.07E+10	9.13E+08	9.83E+09	1465.20	7.33	355.92	1.78	1.05
Box 6 (A)	1.05E+10	9.19E+08	9.61E+09	1436.66	7.18	358.46	1.79	1.06
Box 6 (A)2	1.04E+10	9.19E+08	9.51E+09	1423.34	7.12	358.46	1.79	1.06
Box 6 (B)	1.06E+10	8.71E+08	9.75E+09	1332.90	6.66	339.39	1.70	1.00
Box 6 (B)2	1.04E+10	8.75E+08	9.53E+09	1303.20	6.52	341.00	1.71	1.01
Box 7 (A)	5.48E+09	9.10E+08	4.57E+09	643.68	3.22	354.84	1.77	1.05
Box 7 (A)2	5.36E+09	8.87E+08	4.47E+09	630.31	3.15	345.69	1.73	1.02
Box 7 (B)	5.58E+09	9.10E+08	4.67E+09	656.93	3.28	354.84	1.77	1.05
Box 7 (B)2	5.58E+09	2.72E+08	5.31E+09	741.94	3.71	100.48	0.50	0.30
Box 8 (A)	1.12E+10	8.91E+08	1.03E+10	1411.13	7.06	347.45	1.74	1.03
Box 8 (A)2	1.14E+10	8.99E+08	1.05E+10	1436.63	7.18	350.56	1.75	1.04
Box 8 (B)	1.18E+10	9.51E+08	1.09E+10	1483.06	7.42	370.98	1.85	1.10
Box 8 (B)2	1.19E+10	9.08E+08	1.09E+10	1491.87	7.46	353.89	1.77	1.05
Box 9 (A)	4.51E+09	7.86E+08	3.72E+09	529.98	2.65	305.50	1.53	0.90
Box 9 (A)2	5.77E+09	9.26E+08	4.84E+09	679.33	3.40	361.35	1.81	1.07
Box 9 (B)	4.97E+09	9.06E+08	4.07E+09	576.07	2.88	353.34	1.77	1.05
Box 9 (B)2	5.77E+09	9.16E+08	4.85E+09	680.66	3.40	357.37	1.79	1.06
Box 10 (A)	1.09E+10	9.33E+08	9.96E+09	1361.48	6.81	363.87	1.82	1.08
Box 10 (A)2	1.11E+10	9.24E+08	1.02E+10	1391.39	6.96	360.45	1.80	1.07
Box 10 (B)	1.12E+10	9.16E+08	1.03E+10	1405.46	7.03	357.38	1.79	1.06
Box 10 (B)2	1.07E+10	9.11E+08	9.74E+09	1331.98	6.66	355.05	1.78	1.05

Table Appendix D. 4 Day 57 water samples TPH and Hexacosane Recovery

Box	Total peak area	Hexacosane peak area	Area-hexacosane peak	Calculated TPH (mg/L MeCl) sample	TPH in GW (mg/L)	Hexacosane (mg/L MeCl)	Hexacosane (mg/L)	Recovery efficiency (%)
Box 1 (A)	3.68E+09	7.79E+08	2.90E+09	420.58	2.10	302.49	1.51	90.57
Box 1 (A)2	4.86E+09	8.48E+08	4.01E+09	569.12	2.85	330.20	1.65	98.86
Box 2 (A)	6.71E+09	8.51E+08	5.86E+09	814.85	4.07	331.47	1.66	99.24
Box 2 (A)2	5.81E+09	7.52E+08	5.06E+09	708.27	3.54	291.78	1.46	87.36
Box 2 (B)	7.71E+09	7.71E+08	6.94E+09	959.11	4.80	299.56	1.50	89.69
Box 2 (B)2	7.71E+09	8.02E+08	6.91E+09	954.28	4.77	311.89	1.56	93.38
Box 3 (A)	5.17E+09	8.14E+08	4.36E+09	615.24	3.08	316.50	1.58	94.76
Box 3 (A)2	5.13E+09	8.59E+08	4.27E+09	603.15	3.02	334.61	1.67	100.18
Box 3 (B)	4.98E+09	8.67E+08	4.11E+09	582.23	2.91	337.64	1.69	101.09
Box 3 (B)2	4.97E+09	8.69E+08	4.10E+09	580.39	2.90	338.66	1.69	101.39
Box 4 (A)	8.50E+09	8.62E+08	7.64E+09	1052.33	5.26	335.67	1.68	100.50
Box 4 (A)2	8.69E+09	9.08E+08	7.78E+09	1070.76	5.35	353.87	1.77	105.95
Box 6 (A)	8.79E+09	8.80E+08	7.91E+09	1088.26	5.44	342.67	1.71	102.60
Box 6 (A)2	8.84E+09	8.93E+08	7.94E+09	1092.30	5.46	348.04	1.74	104.20
Box 6 (B)	4.19E+09	3.63E+08	3.83E+09	544.27	2.72	136.83	0.68	40.97
Box 6 (B)2	4.24E+09	3.65E+08	3.88E+09	551.04	2.76	137.79	0.69	41.25
Box 7 (A)	2.35E+09	3.71E+08	1.98E+09	297.74	1.49	140.12	0.70	41.95
Box 7 (A)2	2.30E+09	3.68E+08	1.93E+09	291.97	1.46	138.93	0.69	41.60
Box 7 (B)2	5.29E+09	8.68E+08	4.43E+09	623.94	3.12	338.05	1.69	101.21
Box 8 (B)	3.44E+09	3.98E+08	3.04E+09	439.06	2.20	150.75	0.75	45.13
Box 8 (B)2	3.46E+09	4.57E+08	3.00E+09	434.45	2.17	174.37	0.87	52.21
Box 9 (A)	2.20E+09	3.91E+08	1.81E+09	275.07	1.38	148.01	0.74	44.31
Box 9 (A)2	1.96E+09	3.30E+08	1.63E+09	251.54	1.26	123.64	0.62	37.02
Box 9 (B)	1.97E+09	3.14E+08	1.65E+09	254.91	1.27	117.53	0.59	35.19
Box 9 (B)2	1.94E+09	3.16E+08	1.63E+09	251.43	1.26	118.34	0.59	35.43
Box 10 (A)	6.11E+09	9.26E+08	5.18E+09	724.27	3.62	361.36	1.81	108.19
Box 10 (A)2	6.09E+09	9.25E+08	5.16E+09	721.87	3.61	360.99	1.80	108.08
Box 10 (B)	3.28E+09	3.93E+08	2.89E+09	419.14	2.10	148.83	0.74	44.56
Box 10 (B)2	3.30E+09	3.94E+08	2.90E+09	421.20	2.11	149.26	0.75	44.69

Table Appendix D. 5 Day 126 Water samples TPH and Hexacosane Recovery

Box	Total peak area	Hexacosane peak area	Area-hexacosane peak	Calculated TPH (mg/L MeCl) in sample	TPH in GW (mg/L)	Hexacosane in sample (mg/L MeCl)	Hexacosane (mg/L)	Recovery efficiency (%)
Box 1 (A)	1.55E+09	3.63E+08	1.19E+09	240.87	1.20	136.75	0.68	99.09
Box 1 (B)	1.66E+09	3.54E+08	1.31E+09	255.94	1.28	133.25	0.67	96.56
Box 2 (A)	1.42E+09	3.68E+08	1.05E+09	223.63	1.12	139.05	0.70	100.76
Box 2 (B)	1.42E+09	3.68E+08	1.05E+09	223.50	1.12	139.05	0.70	100.76
Box 3 (A)	1.60E+09	3.41E+08	1.26E+09	248.00	1.24	128.02	0.64	92.77
Box 3 (B)	2.02E+09	3.68E+08	1.65E+09	303.13	1.52	138.84	0.69	100.61
Box 4 (A)	1.19E+09	3.21E+08	8.65E+08	192.53	0.96	120.11	0.60	87.04
Box 4 (B)	1.17E+09	3.23E+08	8.47E+08	190.37	0.95	120.97	0.60	87.66
Box 6 (A)	1.07E+09	3.12E+08	7.58E+08	177.19	0.89	116.73	0.58	84.59
Box 6 (B)	1.11E+09	3.22E+08	7.88E+08	182.52	0.91	120.72	0.60	87.47
Box 8 (A)	1.09E+09	3.30E+08	7.61E+08	179.94	0.90	123.75	0.62	89.67
Box 8 (B)	9.91E+08	3.30E+08	6.61E+08	166.63	0.83	123.75	0.62	89.67
Box 9 (A)	1.70E+09	3.48E+08	1.36E+09	261.59	1.31	130.98	0.65	94.91
Box 9 (B)	1.52E+09	3.40E+08	1.18E+09	237.67	1.19	127.91	0.64	92.69
Box 10 (A)	9.02E+08	3.24E+08	5.78E+08	154.70	0.77	121.30	0.61	87.90
Box 10(B)	8.61E+08	3.25E+08	5.36E+08	149.29	0.75	121.87	0.61	88.31